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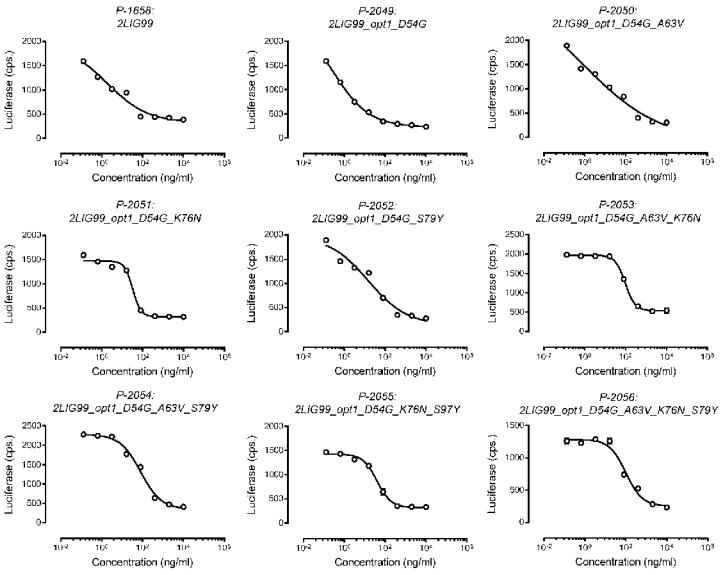
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(54) Title: PD-L1 TARGETED CHIMERIC PROTEINS AND USES THEREOF

FIG. 5



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

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PD-L1 TARGETED CHIMERIC PROTEINS AND USES THEREOF

FIELD

The present invention relates, in part, to targeting moieties that recognize and bind PD-L1 and their use as diagnostic and therapeutic agents. The present invention further relates to pharmaceutical compositions comprising chimeric proteins having a PD-L1 targeting moiety and their use in the treatment of various diseases, including cancer.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/906,447, filed September 26, 2019, the entire disclosure of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 23, 2020, is named "ORN-068PC_ST25" and is 182,668 bytes in size.

BACKGROUND

Immunotherapies have been developed to 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 immune responses against tumor cells.

However, despite impressive patient responses to agents targeting the checkpoint molecules, including, for example, the successes 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 aspects, the present invention provides a PD-L1 targeting moiety comprising a recognition domain comprising: (i) three complementarity determining regions (CDR1, CDR2, and CDR3), where (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5; (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or (ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and where (i) or (ii) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

In embodiments, the mutation is a substitution, optionally where the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In embodiments, the mutation is selected from one or more of a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

In embodiments, the mutation is selected from one or more of a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S, a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N, a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y, an arginine (R) at position K86, being

K86R, and a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

In embodiments, the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

In some aspects, the present invention is related to a PD-L1 targeting moiety including a recognition domain including (i) 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 or 5; (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and wherein (i) or (ii) further comprises one or more mutations at positions D54, G55, K76, and S79, numbering relative to SEQ ID NO: 1. In some embodiments, the PD-L1 targeting moiety includes one or more mutations at positions T74, K86, and Q110.

In some aspects, the present invention is related to a PD-L1 targeting moiety including a recognition domain comprising: (i) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein: (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30; (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or (ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26; and wherein (i) or (ii) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, S79Y, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, M97V (relative to SEQ ID NO: 26),

In another aspect, the present invention relates to chimeric proteins or chimeric protein complexes having at least one targeting moiety that specifically binds to PD-L1. In various embodiments, the chimeric proteins or chimeric protein complexes further comprise a signaling agent, *e.g.*, without limitation, an interferon, an interleukin, and a tumor necrosis factor, that may be modified to attenuate activity.

In some aspects, the present invention is related to a Fc-based chimeric protein complex including (A) a targeting moiety comprising: (a) three complementarity determining regions (CDR1, CDR2, and CDR3), where (i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5; (ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or (b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and where (a) or (b) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1 and (B) a signaling agent, wherein the signaling agent is: a) a wild type signaling agent; or b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and (C) a Fc domain, the Fc domain optionally having one or more mutations that

reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

In embodiments, the mutation is a substitution, optionally where the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In embodiments, the mutation is selected from one or more of a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

In embodiments, the mutation is selected from one or more of a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S, a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N, a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y, an arginine (R) at position K86, being K86R, and a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L. In embodiments, the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

In some aspects, the present invention is related to a Fc-based chimeric protein complex including (A) a targeting moiety comprising: (a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein: (i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5; (ii) CDR2 comprises an amino

acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or (b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1 and wherein (a) or (b) further comprises one or more mutations at positions D54, G55, K76, and S79, numbering relative to SEQ ID NO: 1; and (B) a signaling agent, wherein the signaling agent is: a) a wild type signaling agent; or b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and (C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In some aspects, the present invention is also related to a Fc-based chimeric protein complex comprising: (A) a targeting moiety comprising: (a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein: (i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30; (ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and (iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or (b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26 and wherein (a) or (b) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26; and (B) a signaling agent, wherein the signaling agent is: a) a wild type signaling agent; or b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and (C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain. In some aspects, the present invention also includes a recombinant nucleic acid encoding the PD-L1 targeting moiety or the chimeric protein or chimeric protein complexes of the present invention. In other aspects, the present invention includes a host cell that includes the recombinant nucleic acid encoding the PD-L1 targeting moiety or the chimeric protein or chimeric protein complexes of the present invention. In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, S79Y, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, M97V (relative to SEQ ID NO: 26),

In various embodiments, the chimeric protein or chimeric protein complexes 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 chimeric protein or chimeric protein complexes may directly or indirectly recruit an immune cell to a site of action (such as, by way of non-limiting example, the tumor microenvironment). In some embodiments, the present chimeric protein or chimeric protein complexes facilitates the phagocytosis of a target cell (*e.g.*, a tumor cell).

In various embodiments, the present chimeric proteins or chimeric protein complexes 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.

In some embodiments, the present invention relates to chimeric protein complexes where the chimeric protein complex includes one or more signaling agents, one or more targeting agents, and one or more fragment crystallizable domains (Fc domains). These Fc-based chimeric protein complexes of the present invention are highly target selective, enable conditional and/or regulated modulation of receptor signaling, and are highly active and/or long-acting active and/or long-acting while eliciting minimal side effects.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows the wild-type sequence for 2LIG99 VHH. The highlighted portion of the sequence shows the CDRs in ABM format and the underlined portion of the sequence shows the CDRs in Kabat format.

FIG. 2 shows the wild-type sequence for 2LIG189 VHH. The highlighted portion of the sequence shows the CDRs in ABM format and the underlined portion of the sequence shows the CDRs in Kabat format.

FIG. 3 is a table showing the affinity of 2LIG99 humanization and isomerization variants. For SEQ ID NO: 14, no dissociation could be measured in the 5 minute time interval of the assay.

FIG. 4 is a table showing the affinity of the second wave of 2LIG99 humanization and isomerization variants.

FIG. 5 shows neutralization of the PD-L1/PD-1 interaction by 2LIG99 variants of the PD-L1/PD-1 interaction on HL116 cells.

FIG. 6 is a table showing the affinity of 2LIG189 humanization, deamidation and oxidation variants.

FIG. 7 is a table showing affinity of the second wave 2LIG189 humanization, deamidation and oxidation variants.

FIG. 8 shows neutralization by 2LIG189 variants of the PD-L1/PD-1 interaction on HL116 cells.

FIGs. 9A-F, **10A-H**, **11A-H**, **12A-D**, **13A-F**, **14A-J**, **15A-D**, **16A-F**, **17A-J**, **18A-F**, **19A-L**, **20A-L**, **21A-F**, **22A-L**, **23A-L**, **24A-J**, **25A-J**, **26A-F**, and **27A-F** show various non-limiting illustrative schematics of the Fc-based chimeric protein complexes of the present invention. In embodiments, each schematic is a composition of the present invention. Where applicable in the figures, "TM" refers to a "targeting moiety" as described herein, "SA" refers to a "signaling agent" as described herein, "⌐¬" is an optional "linker" as described herein, the two long parallel rectangles are human Fc domains, *e.g.* from IgG1, from IgG2, or from IgG4, as described herein and optionally with effector knock-out and/or stabilization mutations as also described herein, and the two long parallel rectangles with one having a protrusion and the other having an indentation are human Fc domains, *e.g.* from IgG1, from IgG2, or from IgG4 as described herein, with knob-in-hole and/or ionic pair (a/k/a charged pairs, ionic bond, or charged residue pair) mutations as described herein and optionally with effector knock-out and/or stabilization mutations as also described herein.

FIGs. 9A-F show illustrative homodimeric 2-chain complexes. These figures show illustrative configurations for the homodimeric 2-chain complexes.

FIGs. 10A-H show illustrative homodimeric 2-chain complexes with two targeting moieties (TM) (as described herein, more targeting moieties may be present in some embodiments). In embodiments, the position of TM1 and

TM2 are interchangeable. In embodiments, the constructs shown in the box (*i.e.*, Figs. 10G and 10H) have signaling agent (SA) between TM1 and TM2 or between TM1 and Fc.

FIGs. 11A-H show illustrative homodimeric 2-chain complexes with two signaling agents (as described herein, more signaling agents may be present in some embodiments). In embodiments, the position of SA1 and SA2 are interchangeable. In embodiments, the constructs shown in the box (*i.e.*, Figures 11G and 11H) have TM between SA1 and SA2 or TM at N- or C-terminus).

FIGs.12A-D show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely the TM on the knob chain of the Fc and the SA on hole chain of the Fc.

FIGs.13A-F show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely with both TMs on the knob chain of the Fc and with SA on hole chain of the Fc, with two targeting moieties (as described herein, more targeting moieties may be present in some embodiments). In embodiments, the position of TM1 and TM2 are interchangeable. In some embodiments, TM1 and TM2 can be identical.

FIGs.14A-J show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely with TM on the knob chain of the Fc and with a SA on the hole chain of the Fc, with two signaling agents (as described herein, more signaling agents may be present in some embodiments). In these orientations and/or configurations, one SA is on the knob chain and one SA is on the hole chain. In embodiments, the position of SA1 and SA2 are interchangeable.

FIGs.15A-D show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely the SA on the knob chain of the Fc and the TM on hole chain of the Fc.

FIGs.16A-F show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely with SA on the knob chain of the Fc and both TMs on hole chain of the Fc, with two targeting moieties (as described herein, more targeting moieties may be present in some embodiments). In embodiments, the position of TM1 and TM2 are interchangeable. In some embodiments, TM1 and TM2 can be identical.

FIGs.17A-J show illustrative heterodimeric 2-chain complexes with split TM and SA chains, namely with SA on the knob chain of the Fc and TM on hole chain of the Fc, with two signaling agents (as described herein, more signaling agents may be present in some embodiments). In these orientations and/or configurations, one SA is on the knob chain and one SA is on the hole chain. In embodiments, the position of SA1 and SA2 are interchangeable.

FIGs.18A-F show illustrative heterodimeric 2-chain complexes with TM and SA on the same chain, namely the SA and TM both on the knob chain of the Fc.

FIGs.19A-L show illustrative heterodimeric 2-chain complexes with a TM and a SA on the same chain, namely with SA and with TM both on the knob chain of the Fc, with two targeting moieties (as described herein, more targeting moieties may be present in some embodiments). In embodiments, the position of TM1 and TM2 are interchangeable. In some embodiments, TM1 and TM2 can be identical.

FIGs.20A-L show illustrative heterodimeric 2-chain complexes with a TM and a SA on the same chain, namely with SA and with TM both on the knob chain of the Fc, with two signaling agents (as described herein, more

signaling agents may be present in some embodiments). In embodiments, the position of SA1 and SA2 are interchangeable.

FIGs. 21A-F show illustrative heterodimeric 2-chain complexes with TM and SA on the same chain, namely the SA and TM both on the hole chain of the Fc.

FIGs. 22A-L show illustrative heterodimeric 2-chain complexes with a TM and a SA on the same chain, namely with SA and with TM both on the hole chain of the Fc, with two targeting moieties (as described herein, more targeting moieties are present in some embodiments). In embodiments, the position of TM1 and TM2 are interchangeable. In embodiments, TM1 and TM2 can be identical.

FIGs. 23A-L show illustrative heterodimeric 2-chain complexes with a TM and a SA on the same chain, namely with SA and with TM both on the hole chain of the Fc, with two signaling agents (as described herein, more signaling agents may be present in some embodiments). In embodiments, the position of SA1 and SA2 are interchangeable.

FIGs. 24A-J show illustrative heterodimeric 2-chain complexes with two targeting moieties (as described herein, more targeting moieties may be present in some embodiments) and with SA on knob Fc and TM on each chain. In embodiments, TM1 and TM2 can be identical.

FIGs. 25A-J show illustrative heterodimeric 2-chain complexes with two targeting moieties (as described herein, more targeting moieties may be present in some embodiments) and with SA on hole Fc and TM on each chain. In embodiments, TM1 and TM2 can be identical.

FIGs. 26A-F show illustrative heterodimeric 2-chain complexes with two signaling agents (as described herein, more signaling agents may be present in some embodiments) and with split SA and TM chains: SA on knob and TM on hole Fc.

FIGs. 27A-F show illustrative heterodimeric 2-chain complexes with two signaling agents (as described herein, more signaling agents may be present in some embodiments) and with split SA and TM chains: TM on knob and SA on hole Fc.

FIG. 28 depicts the biological activity of PD-L1 targeted IFNa2_R149A (top), IFNa1 (middle) and IFNa2_A145G (bottom) AFNs, shown as average luciferase activities (± STDEV) in HL116 cells that were stimulated for 6 hours with serial dilution wild type IFNa2 or IFNa1 AFNs.

FIG. 29 shows inhibition of PD-1/PD-L1 interaction by PD-L1 targeted AFNs in an AlphaLisa set-up, where the PD-L1 acceptor beads were pre-incubated with a serial dilution PD-L1 AFN or Atezoluzimab before adding the donor beads. Average AlphaLisa counts of duplicate measurements were plotted ± STDEV.

FIG. 30 shows inhibition of CD80/PD-L1 interaction by PD-L1 targeted AFNs in a plate-binding assay, where the PD-L1 coated plates were pre-incubated with a serial dilution PD-L1 AFN before adding biotinylated CD80. Binding was measured using HRP-coupled streptavidin and a colorimetric peroxidase substrate.

FIGs. 31A-C depict affinities of PD-L1 VHH AFNs for human (**FIG. 31A**) and cyno (**FIG. 31B**) PD-L1 in bio-layer interferometry (BLI). In each set of graphs, at the 300 second timepoint, the points on the graph from top to bottom equate to the dose depicted from left to right. **FIG. 31C** is a table depicting kinetic parameters of said affinities.

FIGs. 32A-B show 2LIG99 and 2LIG189 VHH epitope-binning in bio-layer interferometry (BLI).

FIGs. 33A-H depict stability of PD-L1 AFN variants after freeze-thaw cycles, where samples were analyzed on analytical sizing (SEC). **FIG. 33A**: 2LIG99-IFNa2_R149A; **FIG. 33B**: 2LIG189-IFNa2_R149A; **FIG. 33C**: (2LIG99)2-IFNa2_R149A; **FIG. 33D**: (2LIG189)2-IFNa2_R149A; **FIG. 33E**: 2LIG99-IFNa1; **FIG. 33F**: 2LIG189-IFNa1; **FIG. 33G**: (2LIG99)2-IFNa1; **FIG. 33H**: (2LIG189)2-IFNa1.

FIG. 34 exhibits tumor growth in humanized mice upon treatment with PD-L1 VHH AFNs, wehre the median values (in mm3) of 5-6 animals per time point are plotted.

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-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-L1. In some embodiments, these binding agents bind to, but do not functionally modulate PD-L1. Surprisingly, the present inventors have discovered various mutations to a parental VHH against PD-L1 can have beneficial properties as demonstrated herein.

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-L1 Binding Agents/Targeting Moieties

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

In various embodiments, the present invention 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 present invention relates to mutations of a parental PD-L1 targeting moiety comprising a recognition domain to generate surprisingly beneficial properties. For instance, in various embodiments, the present PD-L1 targeting moieties have improved affinity relative to a parental PD-L1 targeting moiety. In some embodiments, the PD-L1 targeting moieties have about a 2-fold, about a 3-fold, about a 4-fold, about a 5-fold, about a 6-fold, about a 7-fold, about a 8-fold, about a 9-fold, about a 10-fold, about a 15-fold, or about a 20-fold increased affinity relative to a parental PD-L1 targeting moiety. In some embodiments, the present PD-L1 targeting moieties have about a 2-fold, about a 3-fold, about a 4-fold, about a 5-fold, about a 6-fold, about a 7-fold, about a 8-fold, about a 9-fold, about a 10-fold, about a 15-fold, or about a 20-fold reduced dissociation rate relative to a parental PD-L1 targeting moiety.

In aspects, the present invention provides a PD-L1 targeting moiety comprising a recognition domain comprising: (i) three complementarity determining regions (CDR1, CDR2, and CDR3), where (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5; (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or (ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and where (i) or (ii) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

In embodiments, the mutation is a substitution, optionally where the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In embodiments, the mutation is selected from one or more of a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

In embodiments, the mutation is selected from one or more of a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D; a hydrophobic, aliphatic amino

acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S, a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N, a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y, an arginine (R) at position K86, being K86R, and a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

In embodiments, the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

In some aspects, the present invention is related to an PD-L1 targeting moiety comprising a recognition domain comprising:

(i) 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 or 5;

(b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and

(c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or

(ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and wherein (i) or (ii) further comprises one or more mutations at positions D54, G55, K76, and S79, numbering relative to SEQ ID NO: 1.

In some embodiments, the PD-L1 targeting moiety, further comprises one or more mutations at positions T74, K86, and Q110 with respect to SEQ ID NO: 1. In some embodiments, the PD-L1 targeting moiety has a mutation that is a substitution, optionally, wherein the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In some embodiments, the mutation is selected from one or more of:

- a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T;
- a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R;
- a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S;
- a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N;
- a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y;
- an arginine (R) at position K86, being K86R; and
- a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

In various embodiments, the aforementioned mutant PD-L1 targeting moieties (i.e. those disclosed relative to SEQ ID NO: 1) have improved affinity relative to a parental PD-L1 targeting moiety of SEQ ID NO: 1.

In some aspects, the PD-L1 targeting moiety of the present invention includes a recognition domain comprising: (i) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30;

(b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and

(c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or

(ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26; and wherein (i) or (ii) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26. In some embodiments, the PD-L1 targeting moiety has a mutation that is a substitution relative to SEQ ID NO: 26. In some embodiments, the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) or an aromatic, polar and positively charged hydrophilic residue including histidine (H). In some embodiments, the substitution is a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C). In some embodiments, the substitution is a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E). In some embodiments, the substitution is a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In some embodiments, the PD-L1 targeting moiety has a substitution at position N32 that is a positive hydrophilic residue is selected from arginine (R) and lysine (K). In some embodiments, the substitution at position N32 is polar and neutral hydrophilic residue that is selected from glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C). In some embodiments, the substitution at position N32 is N32Q or N32R relative to SEQ ID NO: 26.

In some embodiments, the PD-L1 targeting moiety has a substitution at position D33 is D33H relative to SEQ ID NO: 26. In some embodiments, the PD-L1 targeting moiety has a substitution at position M97 that is aliphatic hydrophobic residues are selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) relative to SEQ ID NO: 26. In some embodiments, the PD-L1 targeting moiety has a substitution at position M97 relative to SEQ ID NO: 26 that is M97I, M97L, or M97V.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, S79Y, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, M97V (relative to SEQ ID NO: 26),

In various embodiments, the aforementioned mutant PD-L1 targeting moieties (i.e. those disclosed relative to SEQ ID NO: 26) have improved affinity relative to a parental PD-L1 targeting moiety of SEQ ID NO: 26.

In some aspects, the PD-L1 targeting moiety of the present invention comprises an amino acid sequence having at least 90% sequence identity with any one of amino acid sequences selected from SEQ ID NO: 1, 8-26, and 33-74.

In some embodiments, the PD-L1 targeting moiety of the present invention includes one or more additional recognition domains. In some embodiments, these additional recognition domains bind to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

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 some embodiments, the PD-L1 targeting moiety recognizes and optionally functionally modulates a tumor antigen. In various embodiments, the PD-L1 targeting moiety recognizes and optionally functionally modulates an antigen on an immune cell. The immune cell is selected from a T cell, B cell, dendritic cell, macrophage, neutrophil, NK cell and NKT cell. In some embodiments, the PD-L1 targeting moiety of the present invention recruits cytotoxic T cells to tumor cells or to the tumor environment.

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:

MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAA
LIVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDV
KLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVVDPVTSEHELTC
QAEGYPKAEVIWTSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIF
YCTFRRLDPEENHTAELVIPELPLAHPPNERTHLVILGAILLCLGVALTFIFRL
RKGRMMDVKKCGIQDTNSKKQSDTHLEET (SEQ ID NO: 75);

Isoform 2:

MRIFAVFIFMTYWHLLNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIW
TSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENH
TAELVIPELPLAHPPNERTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKCGI
QDTNSKKQSDTHLEET (SEQ ID NO: 76); or

Isoform 3:

MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAAL
IVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVK
LQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQA
EGYPKAEVIWTSSDHQVLSGD (SEQ ID NO: 77).

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.*, CH_1, CH_2 and CH_3), 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 binding agent’s CDR1 sequence is selected from: GTIFSINRMD (SEQ ID NO: 2); GTIFS (SEQ ID NO: 5); GKIFSGNDMG (SEQ ID NO: 27); or GKIFS (SEQ ID NO: 30).

In some embodiments, the PD-L1 binding agent’s CDR2 sequence is selected from: LITSDGTPA (SEQ ID NO: 3); LITSDGTPAYADSAKG (SEQ ID NO: 6); IITSGGITD (SEQ ID NO: 28); or IITSGGITDYADAVKG (SEQ ID NO: 31).

In some embodiments, the PD-L1 binding agent’s CDR3 sequence is selected from: SSGVYNY (SEQ ID NO: 4); SSGVYNY (SEQ ID NO: 7); RDRTIW (SEQ ID NO: 29); or RDRTIW (SEQ ID NO: 32).

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

- SEQ ID NO: 8 - P-1659: 2LIG99_OPT1
(Q1D_Q5V_A14P_T74S_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISR
DNSKKTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 9 - P-1660: 2LIG99_OPT2
(Q1D_Q5V_A14P_T23A_T74S_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCAASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISR
DNSKKTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 10 - P-1661: 2LIG99_OPT3
(Q1D_Q5V_A14P_A63V_T74S_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSVKGRFTISR
DNSKKTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 11 - P-1662: 2LIG99_OPT4
(Q1D_Q5V_A14P_T74S_K76N_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISR
DNSKNTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 12 - P-1663: 2LIG99_OPT5
(Q1D_Q5V_A14P_T74S_S79Y_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISR
DNSKKTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 13 - P-1664: 2LIG99_OPT6
(Q1D_Q5V_A14P_T23A_A63V_T74S_K76N_S79Y_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISR
DNSKKTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 14 - P-1665: 2LIG99_D54G
QVQLQESGGGLVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADSAKGRFTISR
DNTKKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSS

- SEQ ID NO: 15 - P-1666: 2LIG99_D54K
QVQLQESGGGLVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**K**GTPAYADSAKGRFTISR
DNTKKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSS

- SEQ ID NO: 16 - P-1667: 2LIG99_D54T
QVQLQESGGGLVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**T**GTPAYADSAKGRFTISR
DNTKKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSS

- SEQ ID NO: 17 - P-1668: 2LIG99_G55R
QVQLQESGGGLVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSD**R**TPAYADSAKGRFTISR
DNTKKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSS

- SEQ ID NO: 18 - P-2049: 2LIG99_OPT_D54G
(Q1D_Q5V_A14P_D54G_T74S_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADSAKGRFTISR
DNSKKTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 19 - P-2050: 2LIG99_OPT_D54G_A63V
(Q1D_Q5V_A14P_D54G_A63V_T74S_K86R_Q110L)

DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADS**V**KGRFTISR
DNSKKTVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 20 - P-2051: 2LIG99_OPT_D54G_K76N
(Q1D_Q5V_A14P_D54G_T74S_K76N_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADSAKGRFTISR
DNSK**N**TVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 21 - P-2052: 2LIG99_OPT_D54G_S79Y
(Q1D_Q5V_A14P_D54G_T74S_S79Y_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADSAKGRFTISR
DNSKKTV**Y**LQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 22 - P-2053: 2LIG99_OPT_D54G_A63V_K76N
(Q1D_Q5V_A14P_D54G_A63V_T74S_K76N_K86R_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADS**V**KGRFTISR
DNSK**N**TVSLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 23 - P-2054: 2LIG99_OPT_D54G_A63V_S79Y
(Q1D_Q5V_A14P_D54G_A63V_T74S_K86R_S97Y_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADS**V**KGRFTISR
DNSKKTV**Y**LQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 24 - P-2055: 2LIG99_OPT_D54G_K76N_S79Y
(Q1D_Q5V_A14P_D54G_T74S_K76N_K86R_S79Y_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADSAKGRFTISR
DNSK**N**TV**Y**LQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

- SEQ ID NO: 25 - P-2056: 2LIG99_OPT_D54G_A63V_K76N_S79Y
(Q1D_Q5V_A14P_D54G_A63V_T74S_K76N_K86R_S79Y_Q110L)
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITS**G**GTPAYADS**V**KGRFTISR
DNSK**N**TV**Y**LQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

In various exemplary embodiments, the PD-L1 binding agent comprises an amino acid sequence with or without the terminal histidine tag sequence (*i.e.*, HHHHHH; SEQ ID NO: 78).

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence with or without the HA tag (*i.e.*, YPYDVPDYGS; SEQ ID NO: 79).

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence with or without the AAA linker.

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence with or without the AAA linker, HA tag, and terminal histidine tag sequence (*i.e.*, AAAYPYDVPDYGSHHHHHH; SEQ ID NO: 80).

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-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-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-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-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-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 seen, 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, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general.

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

In various embodiments, the binding affinity of the 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-L1 may be described by the equilibrium dissociation constant (K_D). In various embodiments, the 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-L1 with a K_D of less than about 1 uM, 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-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-L1. For instance, in various embodiments, the targeting moiety of the 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-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-L1.

Therapeutic Agents Comprising the PD-L1 targeting moiety

In various embodiments, the PD-L1 targeting moiety of the present invention is part of a chimera or fusion with one or more targeting agents or signaling agents. Accordingly, the present invention provides for chimeric or fusion proteins that include, for example, a targeting moiety against PD-L1 and one or more signaling agents. In some embodiments, the present invention provides for one or more targeting moieties where at least one targeting moiety is against 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 mutations. In various embodiments, the modifications (*e.g.* 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 unmodified or 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 or 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).

In some embodiments, the targeting moiety of the present invention restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

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 or chimeric protein complex 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 more 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-L1 or any other targeting moiety described herein). 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 or chimeric protein complexes 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 or chimeric protein complexes 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-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 or chimeric protein complexes away from the site of therapeutic action. For instance, in some embodiments, this obviates the need of high doses of the present chimeric proteins or chimeric protein complexes 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 signaling 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, the chimeric protein or chimeric protein complex comprises a modified signaling agent having 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 the 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 the 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, any of which are optionally modified or mutated. In some embodiments, the modified signaling agent is selected from human: IFNα2, IFNα1, IFNβ, IFNγ, consensus interferon, TNF, TNFR, TGF-α, TGF-β, VEGF, EGF, PDGF, FGF, TRAIL, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IL-33, IGF-1, or EPO.

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 Interferons: 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 ϖ.

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 signaling agents, 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: CD 120a (TNFRSFIA), CD 120b (TNFRSFIB), Lymphotoxin beta receptor (LTBR, TNFRSF3), CD 134 (TNFRSF4), CD40 (CD40, TNFRSF5), FAS (FAS, TNFRSF6), TNFRSF6B (TNFRSF6B), CD27 (CD27, TNFRSF7), CD30 (TNFRSF8), CD137 (TNFRSF9), TNFRSFIOA (TNFRSFIOA), TNFRSFIOB, (TNFRSFIOB), TNFRSFIOC (TNFRSFIOC), TNFRSFIOD (TNFRSFIOD), RANK (TNFRSFI IA), Osteoprotegerin (TNFRSFI IB), 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 (TNFRSFIB) 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 some embodiments, 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-α2a having the amino acid sequence of SEQ ID NO: 81.

In an illustrative embodiment, the modified signaling agent is the allelic form IFN-α2b having the amino acid sequence of SEQ ID NO: 82 (which differs from IFN-α2a at amino acid position 23).

In some embodiments, said IFN-α2 mutant (IFN-α2a or IFN-α2b) is mutated at one or more amino acids at positions 144-154, such as amino acid positions 145, 148, 149 and/or 153. In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from L153A, R149A, M148A and A145G. 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-α2 mutants have reduced affinity and/or activity for IFNAR1. In some embodiments, the IFN-α2 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-α2 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-α2 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-α2 mutant antagonizes wildtype IFN-α2 activity. In such embodiments, said mutant IFN-α2 has reduced affinity and/or activity for IFNAR1 while affinity and/or activity of IFNR2 is retained.

In some embodiments, the human IFN-α2 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-α2 mutant comprises R120E and L153A.

In some embodiments, the human IFN-α2 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-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L30A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or R33A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or M148A as disclosed in WO 2013/059885. In some embodiments, the human

IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L153A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A, and/or Y89A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A, Y89A, and/or D114A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises one or more mutations selected from R144X_1, A145X_2, and R33A, wherein X_1 is selected from A, S, T, Y, L, and I, and wherein X_2 is selected from G, H, Y, K, and D. In some embodiments, the signaling agent is a modified IFNα2, optionally with a R149A mutation with respect to the amino acid sequence of SEQ ID NO: 81 or 82.

In some embodiments, the human IFN-α2 mutant comprises a mutation at T106. In some embodiments, T106 is substituted with A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y.

In some embodiments, the modified signaling agent is interferon α1. In an embodiment, the IFN-α1 comprises an amino acid sequence of SEQ ID NO: 83 or variants thereof. In some embodiments, the IFN-α1 is modified, *i.e.*, is a variant and comprises one or more mutations. In some embodiments, the one or more mutations reduce the biological activity of the IFN-α1. For example, the one or more mutations may reduce the affinity of the IFN-α1 interferon for a therapeutic receptor. In an embodiment, the therapeutic receptor is the interferon-α/β receptor (IFNAR), which is composed of the IFNAR1 and IFNAR2 subunits. In an embodiment, the modified IFN-α1 comprises one or more mutations that reduce its affinity for IFNAR1. In another embodiment, the modified IFN-α1 comprises one or more mutations that reduce its affinity for IFNAR2. In an embodiment, the modified IFN-α1 comprises one or more mutations that reduce its affinity for IFNAR1 and comprises one or more mutations that reduce its affinity for IFNAR2. In some embodiments, the chimeric proteins or Fc-based chimeric protein complexes comprises one or more additional signaling agents, *e.g.*, without limitation, an interferon, an interleukin, and a tumor necrosis factor, that may be modified. In various embodiments, the chimeric proteins or Fc-based chimeric protein complexes of the invention provides improved safety and/or therapeutic activity and/or pharmacokinetic profiles (*e.g.*, increased serum half-life) compared to an untargeted IFN-α1 or an unmodified, wild type IFN-α, such as, IFN-α1.

In various embodiments, the wild-type IFN-α1 comprises the following amino acid sequence:

CDLPETHSLDNRRTLMLLAQMSRISPSSCLMDRHDFGFPQEEFDGNQFQKAPAISVLHELIQQ
IFNLFTTKDSSAAWDEDLLDKFCTELYQQLNDLEACVMQEERVGETPLMNADSILAVKKYFRRI
TLYLTEKKYSPCAWEVVRAEIMRSLSLSTNLQERLRRKE (SEQ ID NO: 83).

In various embodiments, the chimeric protein or Fc-based chimeric protein complexes of the invention comprises a modified version of IFN-α1, *i.e.*, a IFN-α1 variant including a IFN-α1 mutant, as a signaling agent. In various embodiments, the IFN-α1 variant encompasses mutants, functional derivatives, analogs, precursors, isoforms, splice variants, or fragments of the interferon.

Additional IFN-α1 variant sequences are known in the art. In various embodiments the modified IFN-α1 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 known amino acid sequences of a IFN-α1 interferon variant (*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 IFN-α1 interferon is modified to have a mutation at one or more amino acids at positions L15, A19, R23, S25, L30, D32, R33, H34, Q40, C86, D115, L118, K121, R126, E133, K134, K135, R145, A146, M149, R150, S153, L154, and N157 with reference to SEQ ID NO: 83. The mutations can optionally be a hydrophobic mutation and can be, *e.g.*, selected from alanine, valine, leucine, and isoleucine. In some embodiments, the IFN-α1 interferon is modified to have a one or more mutations selected from L15A, A19W, R23A, S25A, L30A, L30V, D32A, R33K, R33A, R33Q, H34A, Q40A, C86S, C86A, C86Y, D115R, L118A, K121A, K121E, R126A, R126E, E133A, K134A, K135A, R145A, R145D, R145E, R145G, R145H, R145I, R145K, R145L, R145N, R145Q, R145S, R145T, R145V, R145Y, A146D, A146E, A146G, A146H, A146I, A146K, A146L, A146M, A146N, A146Q, A146R, A146S, A146T, A146V, A146Y, M149A, M149V, R150A, S153A, L154A, and N157A with reference to SEQ ID NO: 83. In some embodiments, the IFN-α1 mutant comprises one or more multiple mutations selected from L30A/H58Y/E59N_Q62S, R33A/H58Y/E59N/Q62S, M149A/H58Y/E59N/Q62S, L154A/H58Y/E59N/Q62S, R145A/H58Y/E59N/Q62S, D115A/R121A, L118A/R121A, L118A/R121A/K122A, R121A/K122A, and R121E/K122E with reference to SEQ ID NO: 83.

In an embodiment, the IFN-α1 interferon is modified to have a mutation at amino acid position C86 with reference to SEQ ID NO: 83. The mutation at position C86 can be, *e.g.*, C86S or C86A. These C86 mutants of IFN-α1 are called reduced cysteine based aggregation mutants.

In some embodiments, 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 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 or the chimeric protein complex comprises a modified version of mouse IFN-β. In another embodiment, the chimeric protein or the chimeric protein complex 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: 84.

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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO:84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 IFNβ comprises SEQ ID NO: 84 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 or a or chimeric protein complex comprising: (a) a modified IFN-β, having the amino acid sequence of SEQ ID NO: 84 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.*, Clec9A), 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 IFNβ mutants are provided in PCT/EP2017/061544, the entire disclosure of which is incorporated by reference herein.

In some embodiments, 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-γR) 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 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: 85, in which the signal peptide comprises the first 23 amino acids.

MKYTSYILAFQLCIVLGSLGCYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFY FKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKR KRSQMLFRGRRASQ (SEQ ID NO: 85; N-terminal signal peptide underlined).

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:

QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKED MNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ (SEQ ID NO: 86).

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: 85 (*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: 86 (*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, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

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 or the chimeric protein complexes 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: 86, 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: 86). 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 or chimeric protein complexes 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 or chimeric protein complexes 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: 86 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: 86 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: 86 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: 86 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: 86 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: 86 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-α2b 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 following amino acid sequence of SEQ ID NO: 87.

In some embodiments, the consensus interferon comprises the amino acid sequence of SEQ ID NO: 88, which differs from the amino acid sequence of SEQ ID NO: 87 by one amino acid, *i.e.*, SEQ ID NO: 88 lacks the initial methionine residue of SEQ ID NO: 87.

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 form 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-CON_2 or IFN-CON_3 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-CON_2 (SEQ ID NO: 89).

In an embodiment, the consensus interferon variant comprises the amino acid sequence of IFN-CON_3 (SEQ ID NO: 90).

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: 91.

In another embodiment, the consensus interferon variant may comprise the amino acid sequence of SEQ ID NO: 92.

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: 92.

In some embodiments, the engineered interferon is a variant of human IFN-α2a, with an insertion of Asp at approximately position 41 in the sequence Glu-Glu-Phe-Gly-Asn-Gln (SEQ ID NO: 93) to yield Glu-Glu-Phe-Asp-Gly-Asn-Gln (SEQ ID NO: 94) (which resulted in a renumbering of the sequence relative to IFN-α2a 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, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

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: 87 or 88). 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 (*e.g.*, PD-L1). 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 chimeric proteins or chimeric protein complexes 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.* K_D) and/or activation (measurable as, for example, K_A and/or EC_{50}) 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, chimeric proteins or chimeric protein complexes 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.*, PD-L1).

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, chimeric proteins or chimeric protein complexes 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.*, PD-L1).

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, chimeric proteins or chimeric protein complexes with such consensus interferon variant can provide target-selective IFNAR1 and/or IFNAR2 activity (*e.g.* IFNAR1 and/IFNAR2 activity is restorable via targeting through the targeting moiety, *e.g.*, PD-L1).

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: 88. 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: 88, 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: 88.

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: 88. In an embodiment, the consensus interferon comprises a K121E mutation, with reference to SEQ ID NO: 88.

In various embodiments, the modified signaling agent is selected from modified versions of cytokines, growth factors, and hormones. Illustrative examples of such cytokines, growth factors, and hormones include, but are not limited to, lymphokines, monokines, traditional polypeptide hormones, such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and tumor necrosis factor-β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-α; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and –II ; osteo inductive factors; interferons such as, for example, interferon-α, interferon-β and interferon-γ (and interferon type I, II, and III), colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as, for example, IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, and IL-18; a tumor necrosis factor such as, for example, TNF-α or TNF-β; and other polypeptide factors including, for example, LIF and kit ligand (KL). As used herein, cytokines, growth factors, and hormones include proteins obtained from natural sources or produced from recombinant bacterial, eukaryotic or mammalian cell culture systems and biologically active equivalents of the native sequence cytokines.

In some embodiments, the modified signaling agent is a modified version of a growth factor selected from, but not limited to, transforming growth factors (TGFs) such as TGF-α and TGF-β (and subtypes thereof including the various subtypes of TGF-β including TGFβ1, TGFβ2, and TGFβ3), epidermal growth factor (EGF), insulin-like growth factor such as insulin-like growth factor-I and -II, fibroblast growth factor (FGF), heregulin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF).

In an embodiment, the growth factor is a modified version of a fibroblast growth factor (FGF). Illustrative FGFs include, but are not limited to, FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, murine FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, and FGF23.

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, VEFG-C, VEGF-D, or VEGF-E and isoforms thereof including the various isoforms of VEGF-A such as $VEGF_{121}$, $VEGF_{121}b$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}b$, $VEGF_{189}$, and $VEGF_{206}$. 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 VEFGR-1 can cause stimulation of migration of inflammatory cells (including, for example, macrophages) and lead to inflammation associated hypervascular permeability. Activation of VEFGR-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 VEGR-1.

In an illustrative embodiment, the modified signaling agent is $VEGF_{165}$, which has the amino acid sequence of SEQ ID NO: 95).

In another illustrative embodiment, the modified signaling agent is $VEGF_{165b}$, which has the amino acid sequence of SEQ ID NO: 96.

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 some embodiments, the modified signaling agent is a modified version of a hormone selected from, but not limited to, human chorionic gonadotropin, gonadotropin releasing hormone, an androgen, an estrogen, thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, prolactin, growth hormone, adrenocorticotropic hormone, antidiuretic hormone, oxytocin, thyrotropin-releasing hormone, growth hormone releasing hormone, corticotropin-releasing hormone, somatostatin, dopamine, melatonin, thyroxine, calcitonin, parathyroid hormone, glucocorticoids, mineralocorticoids, adrenaline, noradrenaline, progesterone, insulin, glucagon, amylin, calcitriol, calciferol, atrial-natriuretic peptide, gastrin, secretin, cholecystokinin, neuropeptide Y, ghrelin, PYY3-36, insulin-like growth factor (IGF), leptin, thrombopoietin, erythropoietin (EPO), and angiotensinogen.

In some embodiments, 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 NFkB 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 or chimeric protein complexes 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 or chimeric protein complexes, in some embodiments, comprise modified TNF-α agent that allows of favoring either death or survival signals.

In some embodiments, the chimeric protein or the chimeric protein complex 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 or the chimeric protein complexes 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 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 NFkB pathway activity/signaling alterations. In some embodiments, the chimera causes the death of autoreactive T cells having lesions or modifications in the NFκB 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 TNFR-2 based chimera has additional therapeutic applications in diseases, including autoimmune disease, various 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: 97.

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 some embodiments, 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 some embodiments, the human TNF-α moiety has an E146K mutation. In some embodiments, the human TNF-α moiety has an Y87H and an E146K mutation. In some embodiments, the human TNF-α moiety has an Y87H and an A145R mutation. In some embodiments, the human TNF-α moiety has a R32W and a S86T mutation. In some embodiments, the human TNF-α moiety has a R32W and an E146K mutation. In some embodiments, the human TNF-α moiety has a L29S and a R32W mutation. In some embodiments, the human TNF-α moiety has a D143N and an A145R mutation. In some embodiments, the human TNF-α moiety has a D143N and an A145R mutation. In some embodiments, the human TNF-α moiety has an A145T, an E146D, and a S147D mutation. In some embodiments, 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-α1β2). 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-βR.

In an embodiment, the wild type TNF-β has the amino acid sequence of SEQ ID NO: 98.

In such embodiments, the modified TNF-β 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 signaling 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 signaling 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 and PCT/IB2016/001668, 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: 99.

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 TGFα. In such embodiments, the modified TGFα agent has reduced affinity and/or activity for the epidermal growth factor receptor (EGFR). In some embodiments, the modified TGFα 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 TGFβ. 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 TGFβ 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 TGFβ signaling.

In some embodiments, the modified agent is a TGF family member (*e.g.* TGFα, TGFβ) 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.* TGFα, TGFβ) 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.* TGFα, TGFβ) 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.* TGFα, TGFβ) 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-1α or IL-1β. 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-1β has the amino acid sequence of SEQ ID NO: 100.

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 IFNγ 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-1β 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-1β 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-1β 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-1β comprises the mutations Q131G and Q148G. In an embodiment, the modified human IL-1β comprises the mutations Q148G and K208E. In an embodiment, the modified human IL-1β comprises the mutations R120G and Q131G. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146A. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146N. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146R. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146E. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146G. In an embodiment, the modified human IL-1β comprises the mutations R120G and K208E. In an embodiment, the modified human IL-1β 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-2Rα and/or IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-2Rα. Such embodiments may be relevant for treatment of cancer, for instance when the modified IL-2 is agonistic at IL-2Rβ and/or IL-2Rγ. 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-2Rβ and/or IL-2Rγ over IL-2Rα avoid IL-2 side effects such as pulmonary edema. Also, IL-2-based chimeras are useful for the treatment of diseases (*e.g.*, autoimmune disease), 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-2Rβ and/or IL-2Rγ. 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 or the chimeric protein complex 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-2Rβ and/or IL-2Rγ and/or substantially reduced or ablated affinity and/or activity for IL-2Rα. 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: 101.

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 modifid 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-2Rα, 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 or a chimeric protein complex comprising the

modified IL-2 agent with substantially reduced affnity and/or activity for IL-2Rα may find application in oncology for example.

In other embodiments, the modified IL-2 agent may have substantially reduced affnity and/or activity for IL-2Rβ, 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 or a chimeric protein complex comprising the modified IL-2 agent with substantially reduced affnity and/or activity for IL-2Rβ 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/r 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 modifid 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-4Rα subunit with a common γ chain and specifically bind IL-4. Type 2 IL-4 receptors include an IL-4Rα subunit bound to a different subunit known as IL-13Rα1. 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: 102.

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 SEQ ID NO: 103.

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-6Ralpha 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-11Rα and/or IL-11Rβ and/or gp130. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-11Rα and/or IL-11Rβ 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-12Rβ1 and/or IL-12Rβ2. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-12Rβ1 and/or IL-12Rβ2.

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-4Rα) and IL-13Rα1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-4 receptor (IL-4Rα) or IL-13Rα1.

In an embodiment, the wild type IL-13 has the amino acid sequence of SEQ ID NO: 104.

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

In an embodiment, the wild type IL-18 has the amino acid sequence of SEQ ID NO: 105.

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: 106.

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 TGFalpha, 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 would 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 some embodiments, the modified signaling agent is EPO. In various embodiments, the modified EPO agent has reduced affinity and/or activity for the EPO receptor (EPOR) receptor and/or the ephrin receptor (EphR) relative to wild type EPO or other EPO based agents described herein. In some embodiments, the modified EPO agent has substantially reduced or ablated affinity and/or activity for the EPO receptor (EPOR) receptor and/or the Eph receptor (EphR). Illustrative EPO receptors include, but are not limited to, an EPOR homodimer or an EPOR/CD131 heterodimer. Also included as an EPO receptor is beta-common receptor (βcR). Illustrative Eph receptors include, but are not limited to, EPHA1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHA8, EPHA9, EPHA10, EPHB1, EPHB2, EPHB3, EPHB4, EPHB5, and EPHB6. In some embodiments, the modified EPO protein comprises one or more mutations that cause the EPO protein to have reduced affinity for receptors that comprise one or more different EPO receptors or Eph receptors (*e.g.* heterodimer, heterotrimers, *etc.*, including by way of non-limitation: EPOR-EPHB4, EPOR- βcR-EPOR). Also provided are the receptors of EP Patent Publication No. 2492355 the entire contents of which are hereby incorporated by reference, including by way of non-limitation, NEPORs.

In some embodiments, the human EPO has the amino acid sequence of SEQ ID NO: 107 (the first 27 amino acids are the signal peptide).

In some embodiments, the human EPO protein is the mature form of EPO (with the signal peptide being cleaved off) which is a glycoprotein of 166 amino acid residues having the sequence of SEQ ID NO: 108.

The structure of the human EPO protein is predicted to comprise four-helix bundles including helices A, B, C, and D. In various embodiments, the modified EPO protein comprises one or more mutations located in four regions of the EPO protein which are important for bioactivity, *i.e.*, amino acid residues 10-20, 44-51, 96-108, and 142-156. In some embodiments, the one or more mutations are located at residues 11-15, 44-51, 100-108, and 147-151. These residues are localized to helix A (Val11, Arg14, and Tyr15), helix C (Ser100, Arg103, Ser104, and Leu108), helix D (Asn147, Arg150, Gly151, and Leu155), and the A/B connecting loop (residues 42-51). In some embodiments, the modified EPO protein comprises mutations in residues between amino acids 41-52 and amino acids 147, 150, 151, and 155. Without wishing to be bound by theory, it is believed that mutations of these residues have substantial effects on both receptor binding and in vitro biological activity. In some embodiments, the modified EPO protein comprises mutations at residues 11, 14, 15, 100, 103, 104, and 108. Without wishing to be bound by theory, it is believed that mutations of these residues have modest effects on receptor binding activity and much greater effects on in vitro biological activity. Illustrative substitutions include, but are not limited to, one or more of Val11Ser, Arg14Ala, Arg14Gln, Tyr15Ile, Pro42Asn, Thr44Ile, Lys45Asp, Val46Ala, Tyr51Phe, Ser100Glu, Ser100Thr, Arg103Ala, Ser104Ile, Ser104Ala, Leu108Lys, Asn147Lys, Arg150Ala, Gly151Ala, and Leu155Ala.

In some embodiments, the modified EPO protein comprises mutations that effect bioactivity and not binding, *e.g.* those listed in Eliot, *et al.* Mapping of the Active Site of Recombinant Human Erythropoietin January 15, 1997; *Blood*: 89 (2), the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified EPO protein comprises one or more mutations involving surface residues of the EPO protein which are involved in receptor contact. Without wishing to be bound by theory, it is believed that mutations of these surface residues are less likely to affect protein folding thereby retaining some biological activity. Illustrative surface residues that may be mutated include, but are not limited to, residues 147 and 150. In illustrative embodiments, the mutations are substitutions including, one or more of N147A, N147K, R150A and R150E.

In some embodiments, the modified EPO protein comprises one or more mutations at residues N59, E62, L67, and L70, and one or more mutations that affect disulfide bond formation. Without wishing to be bound by theory, it is believed that these mutations affect folding and/or are predicted be in buried positions and thus affects biological activity indirectly.

In an embodiment, the modified EPO protein comprises a K20E substitution which significantly reduces receptor binding. *See* Elliott, *et al.,* (1997) *Blood,* 89:493-502, the entire contents of which are hereby incorporated by reference.

Additional EPO mutations that may be incorporated into the chimeric EPO protein of the invention are disclosed in, for example, Elliott, *et al.,* (1997) *Blood,* 89:493-502, the entire contents of which are hereby incorporated by reference and Taylor *et al*., (2010) *PEDS*, 23(4): 251-260, the entire contents of which are hereby incorporated by reference.

In one embodiment, the present chimeric protein or chimeric protein complex has (i) a targeting moiety including a recognition domain against 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 an embodiment, the present chimeric protein or chimeric protein complex has a targeting moiety directed against PD-L1 and a second targeting moiety directed against another targed on tumor cells.

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 or the chimeric protein complexes 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.

Linkers and Functional Groups

In various embodiments, the present chimeric protein or the chimeric protein complex 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 (*e.g.*, PD-L1) described herein. In some embodiments, such functional groups, residues or moieties confer one or more desired

properties or functionalities to the present chimeric protein or the chimeric protein complex of the invention. Examples of such functional groups and of techniques for introducing them into the present chimeric protein or the chimeric protein complex are known in the art, for example, see *Remington's Pharmaceutical Sciences,* 16th ed., Mack Publishing Co., Easton, Pa. (1980).

In various embodiments, the present chimeric protein or the chimeric protein complex may by conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In some embodiments, the present chimeric protein or the chimeric protein complex 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 present chimeric protein or the chimeric protein complex may be fused or conjugated with an antibody or an antibody fragment such as an Fc fragment. For example, the chimeric protein or the chimeric protein complex 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 or the chimeric protein complexes 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 immunogenecity of the 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 present chimeric protein or the chimeric protein complex of the invention. In some embodiments, the present chimeric protein or the chimeric protein complex 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 present chimeric protein or the chimeric protein complex, 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, theromatic 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 chimeric protein or the chimeric protein complex. In some embodiments, the present chimeric protein or the chimeric protein complex 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 present chimeric protein or the chimeric protein complex to 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 present chimeric protein or the chimeric protein complex 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 present chimeric protein or the chimeric protein complex 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 present chimeric protein or a chimeric protein complex 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 present chimeric protein or a chimeric protein complex 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 present chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex of the invention.

In some embodiments, the present chimeric protein or the chimeric protein complex optionally comprises one or more linkers. In some embodiments, the present chimeric protein or the chimeric protein complex comprises a linker connecting the targeting moiety and the signaling agent. In some embodiments, the present chimeric protein or the chimeric protein complex 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).

In some embodiments vectors encoding the present chimeric proteins or the chimeric protein complexes linked as a single nucleotide sequence to any of the linkers described herein are provided and may be used to prepare such chimeric proteins or chimeric protein complexes.

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.

As described herein, the linker length allows for efficient binding of one of the targeting moieties and the signaling agent to receptors on the same cell, the binding being sequential, *e.g.* targeting moiety/receptor binding preceding signaling agent/receptor binding.

In some embodiments, there are two linkers in a single chimera, each connecting the signaling agent to a targeting moiety. In various embodiments, the linkers have lengths that allow for the formation of a site that has a disease cell and an effector cell without steric hindrance that would prevent modulation of the either cell.

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 chimeric protein or the chimeric protein complex.

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, 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 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 $(Gly_4Ser)_n$, where n is from about 1 to about 8, *e.g.* 1, 2, 3, 4, 5, 6, 7, or 8 (SEQ ID NO: 109 - SEQ ID NO: 116). In an embodiment, the linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 117). Additional illustrative linkers include, but are not limited to, linkers having the sequence LE, GGGGS (SEQ ID NO: 109), $(GGGGS)_n$ (n=1-4) (SEQ ID NO: 109 – SEQ ID NO: 112), $(Gly)_8$ (SEQ ID NO: 118), $(Gly)_6$ (SEQ ID NO: 119), $(EAAAK)_n$ (n=1-3) (SEQ ID NO: 120 - SEQ ID NO: 122), $A(EAAAK)_nA$ (n = 2-5) (SEQ ID NO: 123 - SEQ ID NO: 126), AEAAAKEAAAKA (SEQ ID NO: 123), $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO: 127), PAPAP (SEQ ID NO: 128), KESGSVSSEQLAQFRSLD (SEQ ID NO: 129), EGKSSGSGSESKST (SEQ ID NO: 130), GSAGSAAGSGEF (SEQ ID NO: 131), and $(XP)_n$, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu. In various embodiments, the linker is $(GGS)_n$ (n=1-20) (SEQ ID NOs: 132-151). In some embodiments, the linker is G. In some embodiments, the linker is AAA. In some embodiments, the linker is $(GGGGS)_n$ (n=5-20) (SEQ ID NOs: 113-116 and SEQ ID NOs: 152-163).

In some embodiments, the linker is one or more of GGGSE (SEQ ID NO: 164), GSESG (SEQ ID NO: 165), GSEGS (SEQ ID NO: 166), GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGS (SEQ ID NO: 167), 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 Cys-Pro-Pro-Cys (SEQ ID NO: 168) 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 chimeric protein or the chimeric protein complex 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 chimeric proteins 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 chimeric protein or the chimeric protein complex. In another example, the linker may function to target the chimeric protein or the chimeric protein complex to a particular cell type or location.

Chimeric Protein Complexes with Fc Domains

In some embodiments, the present invention relates to chimeric protein complexes where the complexes include one or more fragment crystallizable domain (Fc domain). In some embodiments, the Fc domain has one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In various embodiments, the present invention includes chimeric protein complexes comprising one or more targeting agents, one or more signaling agents and one or more Fc domains. In one embodiment, the chimeric protein complex includes at least one targeting moiety that specifically binds to PD-L1 and at least one Fc domain. In another embodiment, the chimeric protein complex includes at least one targeting moiety that specifically binding to PD-L1, at least one signaling agent that is a tumor necrosis factor (TNF), and at least one Fc domain. In various embodiments, the TNF signaling agent may be modified to attenuate activity. In some embodiments, the PD-L1-targeted chimeric protein complex may directly or indirectly recruit an immune cell to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

In some aspects, the present invention is related to a Fc-based chimeric protein complex including (A) a targeting moiety comprising: (a) three complementarity determining regions (CDR1, CDR2, and CDR3), where (i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5; (ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and (iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or (b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and where (a) or (b) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1 and (B) a signaling agent, wherein the signaling agent is: a) a wild type signaling agent; or b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and (C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

In embodiments, the mutation is a substitution, optionally where the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In embodiments, the mutation is selected from one or more of a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

In embodiments, the mutation is selected from one or more of a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P; a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V; a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S, a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N, a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y, an arginine (R) at position K86, being K86R, and a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L. In embodiments, the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

In some aspects, the present invention is related to a Fc-based chimeric protein complex comprising:

(A) a targeting moiety comprising:

(a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5;

(ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and

(iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or

(b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1 and wherein (a) or (b) further comprises one or more mutations at positions D54, G55, K76, and S79, numbering relative to SEQ ID NO: 1; and

(B) a signaling agent, wherein the signaling agent is:

a) a wild type signaling agent; or

b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and

(C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In some embodiments, the Fc-based chimeric protein complex has a targeting moiety that includes one or more mutations at positions T74, K86, and Q110 relative to SEQ ID NO: 1. In some embodiments, the Fc-based chimeric protein complex of has a mutation that is a substitution, optionally wherein the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In some embodiments, the Fc-based chimeric protein complex of has a mutation that is selected from one or more of:

- a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T
- a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R,
- a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S,

- a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N,
- a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y,
- an arginine (R) at position K86, being K86R, and
- a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

In some aspects, the Fc-based chimeric protein complex of the present invention includes:

(A) a targeting moiety comprising:

(a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30;

(ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and

(iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or

(b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26 and wherein (a) or (b) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26; and

(B) a signaling agent, wherein the signaling agent is: a) a wild type signaling agent; or b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and

(C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

In some embodiments, the Fc-based chimeric protein complex of the present invention has a mutation that is a substitution relative to SEQ ID NO: 26. In some embodiments, the Fc-based chimeric protein complex has a substitution of a hydrophilic amino acid residue that is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) or an aromatic, polar and positively charged hydrophilic residue including histidine (H). In some embodiments, the substitution is a hydrophilic amino acid residue that is a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C). In some embodiments, the substitution is a hydrophilic amino acid residue that is a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E). In some embodiments, the substitution is a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine

(M), and valine (V) or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

In some embodiments, the substitution at position N32 relative to SEQ ID NO: 26 is a positive hydrophilic residue is selected from arginine (R) and lysine (K). In some embodiments, the substitution at position N32 relative to SEQ ID NO: 26 is polar and neutral hydrophilic residue is selected from glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C). In some embodiments, the substitution at position N32 relative to SEQ ID NO: 26 is N32Q or N32R.

In some embodiments, the substitution at position D33 relative to SEQ ID NO: 26 is D33H. In other embodiments, the substitution at position M97 relative to SEQ ID NO: 26 is aliphatic hydrophobic residues selected from glycine (G), leucine (L), isoleucine (I), and valine (V). In some embodiments, the substitution at position M97 relative to SEQ ID NO: 26 is M97I, M97L, or M97V.

In embodiments, the PD-L1 targeting moiety comprising a recognition domain further comprises one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, S79Y, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, M97V (relative to SEQ ID NO: 26),

In some embodiments, the present invention relates to a PD-L1-targeted chimeric protein complex having at least one targeting moiety that specifically binds to PD-L1, at least one signaling agent that is an interferon (IFN) or a modified form thereof and at least one Fc domain. In various embodiments, the IFN signaling agent may be modified to attenuate activity. In one embodiment, the interferon is IFN-γ or a modified form thereof.

The fragment crystallizable domain (Fc domain) is the tail region of an antibody that interacts with Fc receptors located on the cell surface of cells that are involved in the immune system, *e.g.*, B lymphocytes, dendritic cells, natural killer cells, macrophages, neutrophils, eosinophils, basophils, and mast cells. In IgG, IgA and IgD antibody isotypes, the Fc domain is composed of two identical protein fragments, derived from the second and third constant domains of the antibody's two heavy chains. In IgM and IgE antibody isotypes, the Fc domain contains three heavy chain constant domains (C_H domains 2–4) in each polypeptide chain.

In some embodiments, the Fc-based chimeric protein of complex the present technology includes a Fc domain. In some embodiments, the Fc domains are from selected from IgG, IgA, IgD, IgM or IgE. In some embodiments, the Fc domains are from selected from IgG1, IgG2, IgG3, or IgG4.

In some embodiments, the Fc domains are from selected from human IgG, IgA, IgD, IgM or IgE. In some embodiments, the Fc domains are from selected from human IgG1, IgG2, IgG3, or IgG4.

In some embodiments, the Fc domains of the Fc-based chimeric protein complex comprise the CH2 and CH3 regions of IgG. In some embodiments, the IgG is human IgG. In some embodiments, the human IgG is selected from IgG1, IgG2, IgG3, or IgG4.

In some embodiments, the Fc domains comprise one or more mutations. In some embodiments, the mutation(s) to the Fc domains reduces or eliminates the effector function the Fc domains. In some embodiments, the mutated Fc domain has reduced affinity or binding to a target receptor. By way of example, in some embodiments, the

mutation to the Fc domains reduces or eliminates the binding of the Fc domains to FcγR. In some embodiments, the FcγR is selected from FcγRI; FcγRIIa, 131 R/R; FcγRIIa, 131 H/H, FcγRIIb; and FcγRIII. In some embodiments, the mutation to the Fc domains reduces or eliminated binding to complement proteins, such as, *e.g.*, C1q. In some embodiments, the mutation to the Fc domains reduces or eliminated binding to both FcγR and complement proteins, such as, *e.g.*, C1q.

In some embodiments, the Fc domains comprise the LALA mutation to reduce or eliminate the effector function of the Fc domains. By way of example, in some embodiments, the LALA mutation comprises L234A and L235A substitutions in human IgG (*e.g.*, IgG1) (wherein the numbering is based on the commonly used numbering of the CH2 residues for human IgG1 according to EU convention (Edelman *et al.*, PNAS, 1969; 63 (1) 78-85)).

In some embodiments, the Fc domains of human IgG comprise a mutation at 46 to reduce or eliminate the effector function of the Fc domains. By way of example, in some embodiments, the mutations are selected from L234A, L234F, L235A, L235E, L235Q, K322A, K322Q, D265A, P329G, P329A, P331G, and P331S.

In some embodiments, the Fc domains comprise the FALA mutation to reduce or eliminate the effector function of the Fc domains. By way of example, in some embodiments, the FALA mutation comprises F234A and L235A substitutions in human IgG4.

In some embodiments, the Fc domains of human IgG4 comprise a mutation at one or more of F234, L235, K322, D265, and P329 to reduce or eliminate the effector function of the Fc domains. By way of example, in some embodiments, the mutations are selected from F234A, L235A, L235E, L235Q, K322A, K322Q, D265A, P329G, and P329A.

In some embodiments, the mutation(s) to the Fc domain stabilize a hinge region in the Fc domain. By way of example, in some embodiments, the Fc domain comprises a mutation at S228 of human IgG to stabilize a hinge region. In some embodiments, the mutation is S228P.

In some embodiments, the mutation(s) to the Fc domain promote chain pairing in the Fc domain. In some embodiments, chain pairing is promoted by ionic pairing (a/k/a charged pairs, ionic bond, or charged residue pair).

In some embodiments, the Fc domain comprises a mutation at one more of the following amino acid residues of IgG to promote of ionic pairing: D356, E357, L368, K370, K392, D399, and K409.

By way of example, in some embodiments, the human IgG Fc domain comprise one of the mutation combinations in **Table 1** to promote of ionic pairing.

Table 1

Substitution(s) on one Fc Chain	Substitution(s) on other Fc Chain
D356K D399K	K392D K409D
E357R L368R	K370D K409D
E357R L368K	K370D K409D
E357R D399K	K370D K409D
E357R	K370D
L368R D399K	K392D K409D
L368K D399K	K392D K409D
L368R D399K	K409D

Table 1	
Substitution(s) on one Fc Chain	**Substitution(s) on other Fc Chain**
L368K D399K	K409D
L368R	K409D
L368K	K409D
K370D K409D	E357R D399K
K370D K409D	E357R L368R
K370D K409D	E357R L368K
K370D K409D	E357R D399K
K370D K409D	E357R L368R
K370D K409D	E357R L368K
K370D	E357R
K370D	E357R
K392D K409D	D356K D399K
K392D K409D	L368R D399K
K392D K409D	L368K D399K
K392D K409D	D399K
D399K	K392D K409D
D399K	K409D
K409D	L368R
K409D	L368K
K409D	L368R D399K
K409D	L368K D399K
K409D	L368R
K409D	L368K
K409D	L368R D399K
K409D	L368K D399K
K409D	D399K

In some embodiments, chain pairing is promoted by a knob-in-hole mutations. In some embodiments, the Fc domain comprises one or more mutations to allow for a knob-in-hole interaction in the Fc domain. In some embodiments, a first Fc chain is engineered to express the “knob” and a second Fc chain is engineered to express the complementary “hole.” By way of example, in some embodiments, human IgG Fc domain comprises the mutations of **Table 2** to allow for a knob-in-hole interaction.

Table 2	
Substitution(s) on one Fc Chain	**Substitution(s) on other Fc Chain**
T366Y	Y407T
T366Y/F405A	T394W/Y407T
T366W	Y407A
T366W	Y407V
T366Y	Y407A
T366Y	Y407V
T366Y	Y407T

In some embodiments, the Fc domains in the Fc-based chimeric protein complexes of the present technology comprise any combination of the above-disclosed mutations. By way of example, in some embodiments, the Fc domain comprises mutations that promote ionic pairing and/or a knob-in-hole interaction. By way of example, in

some embodiments, the Fc domain comprises mutations that have one or more of the following properties: promote ionic pairing, induce a knob-in-hole interaction, reduce or eliminate the effector function of the Fc domain, and cause Fc stabilization (*e.g.* at hinge).

By way of example, in some embodiments, a human IgG Fc domains comprise mutations disclosed in **Table 3**, which promote ionic pairing and/or promote a knob-in-hole interaction in the Fc domain.

Table 3	
Substitution(s) on one Fc Chain	**Substitution(s) on other Fc Chain**
T366W K370D	E357R Y407A
T366W K370D	E357R Y407V
T366W K409D	L368R Y407A
T366W K409D	L368R Y407V
T366W K409D	L368K Y407A
T366W K409D	L368K Y407V
T366W K409D	L368R D399K Y407A
T366W K409D	L368R D399K Y407V
T366W K409D	L368K D399K Y407A
T366W K409D	L368K D399K Y407V
T366W K409D	D399K Y407A
T366W K409D	D399K Y407V
T366W K392D K409D	D399K Y407A
T366W K392D K409D	D399K Y407V
T366W K392D K409D	D356K D399K Y407A
T366W K392D K409D	D356K D399K Y407V
T366W K370D K409D	E357R D399K Y407A
T366W K370D K409D	E357R D399K Y407V
T366W K370D K409D	E357R L368R Y407A
T366W K370D K409D	E357R L368R Y407V
T366W K370D K409D	E357R L368K Y407A
T366W K370D K409D	E357R L368K Y407V
T366W K392D K409D	L368R D399K Y407A
T366W K392D K409D	L368R D399K Y407V
T366W K392D K409D	L368K D399K Y407A
T366W K392D K409D	L368K D399K Y407V
E357R T366W	K370D Y407A
E357R T366W	K370D Y407V
T366W L368R	Y407A K409D
T366W L368R	Y407V K409D
T366W L368K	Y407A K409D
T366W L368K	Y407V K409D
T366W L368R D399K	Y407A K409D
T366W L368R D399K	Y407V K409D
T366W L368K D399K	Y407A K409D
T366W L368K D399K	Y407V K409D
T366W D399K	Y407A K409D
T366W D399K	Y407V K409D
1366W D399K	K392D Y407A K409D
T366W D399K	K392D Y407V K409D
T366W D356K D399K	K392D Y407A K409D
T366W D356K D399K	K392D Y407V K409D
E357R T366W D399K	K370D Y407A K409D

Table 3	
Substitution(s) on one Fc Chain	**Substitution(s) on other Fc Chain**
E357R T366W D399K	K370D Y407V K409D
E357R T366W L368R	K370D Y407A K409D
E357R T366W L368R	K370D Y407V K409D
E357R T366W L368K	K370D Y407A K409D
E357R T366W L368K	K370D Y407V K409D
T366W L368R D399K	K392D Y407A K409D
T366W L368R D399K	K392D Y407V K409D
T366W L368K D399K	K392D Y407A K409D

By way of example, in some embodiments, a human IgG Fc domains comprise mutations disclosed in **Table 4**, which promote ionic pairing, promote a knob-in-hole interaction, or a combination thereof in the Fc domain. In embodiments, the "Chain 1" and "Chain 2" of **Table 4** can be interchanged (*e.g.* Chain 1 can have Y407T and Chain 2 can have T366Y).

Table 4			
Chain 1 mutation	**Chain 2 mutation**	**Reference**	**IgG**
T366Y	Y407T	Ridgway *et al.*, 1996 Protein Engineering, Design and Selection, Volume 9, Issue 7, 1 July 1996, Pages 617–62	IgG1
T366Y/F405A	T394W/Y407T	Ridgway *et al.*, 1996 Protein Engineering, Design and Selection, Volume 9, Issue 7, 1 July 1996, Pages 617–62	IgG1
T366W	Y407A	Atwell *et al.*, 1997 JMB Volume 270, Issue 1, 4 July 1997, Pages 26-35	IgG1
T366W	T366S/L368V/Y407A	Atwell *et al.*, 1997 JMB Volume 270, Issue 1, 4 July 1997, Pages 26-35	IgG1
T366W	L368A/Y407A	Atwell *et al.*, 1997 JMB Volume 270, Issue 1, 4 July 1997, Pages 26-35	IgG1
T366W	T366S/L368A/Y407A	Atwell *et al.*, 1997 JMB Volume 270, Issue 1, 4 July 1997, Pages 26-35	IgG1
T366W	T366S/L368G/Y407V	Atwell *et al.*, 1997 JMB Volume 270, Issue 1, 4 July 1997, Pages 26-35	IgG1
T366W/D399C	T366S/L368A/K392C/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
T366W/K392C	T366S/L368A/D399C/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
S354C/T366W	Y349C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1

Y349C/T366W	S354C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
E356C/T366W	Y349C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
Y349C/T366W	E356C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
E357C/T366W	Y349C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
Y349C/T366W	E357C/T366S/L368A/Y407V	Merchant *et al.*, 1998 Nature Biotechnology volume 16, pages 677–681 (1998)	IgG1
D339R	K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K	K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339R	K409D	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K	K409D	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K	K360D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K	K392D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K/E356K	K392D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K/E357K	K392D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K/E356K	K409E/K439D	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K/E357K	K370D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
D339K/E356K/E357K	K370D/K392D/K409E	Gunasekaran *et al.*, 2010 The Journal of Biological Chemistry 285, 19637-19646.	IgG1
S364H/F405A	Y349T/T394F	Moore *et al.*, 2011 mAbs, 3:6, 546-557	IgG1
S364H/T394F	Y349T/F405A	Moore *et al.*, 2011 mAbs, 3:6, 546-557	IgG1
D221R/P228R/K409R	D221E/P228E/L368E	Strop *et al.*, 2012 JMB Volume 420, Issue 3, 13 July 2012, Pages 204-219	IgG1
C223R/E225R/P228R/K409R	C223E/P228E/L368E	Strop *et al.*, 2012 JMB Volume 420, Issue 3, 13 July 2012, Pages 204-219	IgG2
F405L	K409R	Labrijn *et al.*, 2013 PNAS March 26, 2013. 110 (13) 5145-5150	IgG1
F405A/Y407V	T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
F405A/Y407V	T366I/T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
F405A/Y407V	T366L/T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
F405A/Y407V	T366L/K392M/T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
L351Y/F405A/Y407V	T366L/K392M/T394W	Von Kreudenstein *et al.*, 2013 mAbs	IgG1

		Volume 5, 2013 - Issue 5, pp.644-654	
T350V/L351Y/F405A/Y407V	T350V/T366L/K392M/T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
T350V/L351Y/F405A/Y407V	T350V/T366L/K392L/T394W	Von Kreudenstein *et al.*, 2013 mAbs Volume 5, 2013 - Issue 5, pp.644-654	IgG1
K409W	D339V/F405T	Choi *et al.*, 2013 PNAS January 2, 2013. 110 (1) 270-275	IgG1
K360E	Q347R	Choi *et al.*, 2013 PNAS January 2, 2013. 110 (1) 270-275	IgG1
K360E/K409W	D339V/Q347R/F405T	Choi *et al.*, 2013 PNAS January 2, 2013. 110 (1) 270-275	IgG1
Y349C/K360E/K409W	D339V/Q347R/S354C/F405T	Choi *et al.*, 2013 PNAS January 2, 2013. 110 (1) 270-275	IgG1
K392A/K409D	E356K/D399K	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
T366W	T366S/L358A/Y407A	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
D339M/Y407A	T336V/K409V	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
D339M/K360D/Y407A	T336V/E345R/Q347R/K409V	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
Y349S/T366V/K370Y/K409V	E357D/S364Q/Y407A	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
Y349S/T366M/K370Y/K409V	E356G/E357D/S364Q/Y407A	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
Y349S/T366M/K370Y/K409V	E357D/S364R/Y407A	Leaver-Fey *et al.*, 2016 Structure Volume 24, Issue 4, 5 April 2016, Pages 641-651	IgG1
And any combination as described in Tables 1-3 of US20150284475A1			

By way of example, in some embodiments, a human IgG Fc domains comprise mutations disclosed in **Table 5**, which reduce or eliminate FcγR and/or complement binding in the Fc domain. In embodiments, the **Table 5** mutations are in both chains.

Table 5

Chain 1 mutation	Reference	IgG
L234A/L235A	Alegre *et al.*, 1994 Transplantation 57:1537–1543	IgG1
F234A/L235A	Alegre *et al.*, 1994 Transplantation 57:1537–1543	IgG4
L235E	Morgan *et al.*, 1995 Immunology. 1995 Oct; 86(2): 319–324.	IgG1
L235E	Morgan *et al.*, 1995 Immunology. 1995 Oct; 86(2): 319–324.	IgG4
L235A	Morgan *et al.*, 1995 Immunology. 1995 Oct; 86(2): 319–324.	IgG1

G237A	Morgan *et al.*, 1995 Immunology. 1995 Oct; 86(2): 319–324.	IgG1
N297H	Tao and Morrison, J. Immunol. 1989; 143:2595-2601	IgG1
N297Q	Tao and Morrison, J. Immunol. 1989; 143:2595-2601	IgG1
N297K	Tao and Morrison, J. Immunol. 1989; 143:2595-2601	IgG3
N297Q	Tao and Morrison, J. Immunol. 1989; 143:2595-2601	IgG3
D265A	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
D270A, V, K	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
K322A, L, M, D, E	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
P329A, X	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
P331A, S, G, X	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
D265A	Idusogie *et al.*, 2000 J Immunol April 15, 2000, 164 (8) 4178-4184	IgG1
L234A	Hezareh *et al.*, 2001 J. Virol. December 2001 vol. 75 no. 24 12161-12168	IgG1
L234A/L235A	Hezareh *et al.*, 2001 J. Virol. December 2001 vol. 75 no. 24 12161-12168	IgG1
L234F/L235E/P331S	Oganesyan *et al.*, 2008 Acta Cryst. (2008). D64, 700-704	IgG1
H268Q/V309L/A330S/P331S	An *et al.*, 2009 mAbs Volume 1, 2009 - Issue 6, pp. 572-579	IgG1
G236R/L328R	Moore *et al.*, 2011 mAbs Volume 3, 2011 - Issue 6, pp. 546-557	IgG1
N297G	Couch *et al.*, 2013 Sci. Transl. Med., 5 (2013) 183ra57, 1–12	IgG1
N297G/D265A	Couch *et al.*, 2013 Sci. Transl. Med., 5 (2013) 183ra57, 1–12	IgG1
V234A/G237A/P328S/H268A/V309L/A330S/P331S	Vafa *et al.*, 2014 Methods Volume 65, Issue 1, 1 January 2014, Pages 114-126	IgG2
L234A/L235A/P329G	Lo *et al.*, 2016 The Journal of Biological Chemistry 292, 3900-3908	IgG1
N297D	Schlothauer *et al.*, 2016 Protein Engineering, Design and Selection, Volume 29, Issue 10, 1 October 2016, Pages 457–466	IgG1
S228P/L235E	Schlothauer *et al.*, 2016 Protein Engineering, Design and	IgG4

	Selection, Volume 29, Issue 10, 1 October 2016, Pages 457–466	
S228P/L235E/P329G	Schlothauer *et al.*, 2016 Protein Engineering, Design and Selection, Volume 29, Issue 10, 1 October 2016, Pages 457–466	IgG4
L234F/L235A/K322Q	Borrok *et al.*, 2017 J Pharm Sci April 2017 Volume 106, Issue 4, Pages 1008–1017	IgG1
L234F/L235Q/P331G	Borrok *et al.*, 2017 J Pharm Sci April 2017 Volume 106, Issue 4, Pages 1008–1017	IgG1
L234F/L235Q/K322Q	Borrok *et al.*, 2017 J Pharm Sci April 2017 Volume 106, Issue 4, Pages 1008–1017	IgG1
L234A/L235A/G237A/P328S/H268A/A330S/P331S	Tam *et al.*, 2017 Open Access Antibodies 2017, 6(3), 12; doi:10.3390/antib6030012	IgG1
S228P/F234A/L235A	Tam *et al.*, 2017 Open Access Antibodies 2017, 6(3), 12; doi:10.3390/antib6030012	IgG4
S228P/F234A/L235A/G237A/P238S	Tam *et al.*, 2017 Open Access Antibodies 2017, 6(3), 12; doi:10.3390/antib6030012	IgG4
S228P/F234A/L235A/G236−/G237A/P238S	Tam *et al.*, 2017 Open Access Antibodies 2017, 6(3), 12; doi:10.3390/antib6030012	IgG4

In some embodiments, the Fc domains in the Fc-based chimeric protein complexes of the present technology are homodimeric, *i.e.*, the Fc region in the chimeric protein complex comprises two identical protein fragments.

In some embodiments, the Fc domains in the Fc-based chimeric protein complexes of the present technology are heterodimeric, *i.e.*, the Fc domain comprises two non-identical protein fragments.

In some embodiments, heterodimeric Fc domains are engineered using ionic pairing and/or knob-in-hole mutations described herein. In some embodiments, the heterodimeric Fc-based chimeric protein complexes have a trans orientation/configuration. In a trans orientation/configuration, the targeting moiety and signaling agent are, in embodiments, not found on the same polypeptide chain in the present Fc-based chimeric protein complexes.

In some embodiments, the Fc domains includes or starts with the core hinge region of wild-type human IgG1, which contains the sequence Cys-Pro-Pro-Cys. In some embodiments, the Fc domains also include the upper hinge, or parts thereof (*e.g.*, DKTHTCPPC; see WO 2009053368), EPKSCDKTHTCPPC, or EPKSSDKTHTCPPC; see Lo *et al.*, Protein Engineering vol.11 no.6 pp.495–500, 1998)).

Fc-based Chimeric Protein Complexes

The Fc-based chimeric protein complexes of the present technology comprise at least one Fc domain disclosed herein, at least one signaling agent and at least one targeting moiety (TM) disclosed herein.

It is understood that, the present Fc-based chimeric protein complexes may encompass a complex of two fusion proteins, each comprising an Fc domain.

In some embodiments, the Fc-based chimeric protein complex is heterodimeric. In some embodiments, the heterodimeric Fc-based chimeric protein complex has a trans orientation/configuration. In some embodiments, the heterodimeric Fc-based chimeric protein complex has a cis orientation/configuration.

In some embodiments, heterodimeric Fc domains are engineered using ionic pairing and/or knob-in-hole mutations described herein. In some embodiments, the heterodimeric Fc-based chimeric protein complexes have a trans orientation.

In a trans orientation, the targeting moiety and signaling agent are, in embodiments, not found on the same polypeptide chain in the present Fc-based chimeric protein complexes. In a trans orientation, the targeting moiety and signaling agent are, in embodiments, found on separate polypeptide chains in the Fc-based chimeric protein complexes. In a cis orientation, the targeting moiety and signaling agent are, in embodiments, found on the same polypeptide chain in the Fc-based chimeric protein complexes.

In some embodiments, where more than one targeting moiety is present in the heterodimeric protein complexes described herein, one targeting moiety may be in trans orientation (relative to the signaling agent), whereas another targeting moiety may be in cis orientation (relative to the signaling agent). In some embodiments, the signaling agent and target moiety are on the same ends/sides (N-terminal or C-terminal ends) of an Fc domain. In some embodiments, the signaling agent and targeting moiety are on different sides/ends of a Fc domain (N-terminal and C-terminal ends).

In some embodiments, where more than one targeting moiety is present in the heterodimeric protein complexes described herein, the targeting moieties may be found on the same Fc chain or on two different Fc chains in the heterodimeric protein complex (in the latter case the targeting moieties would be in trans relative to each other, as they are on different Fc chains). In some embodiments, where more than one targeting moiety is present on the same Fc chain, the targeting moieties may be on the same or different sides/ends of a Fc chain (N-terminal or/and C-terminal ends).

In some embodiments, where more than one signaling agent is present in the heterodimeric protein complexes described herein, the signaling agents may be found on the same Fc chain or on two different Fc chains in the heterodimeric protein complex (in the latter case the signaling agents would be in trans relative to each other, as they are on different Fc chains). In some embodiments, where more than one signaling agent is present on the same Fc chain, the signaling agents may be on the same or different sides/ends of a Fc chain (N-terminal or/and C-terminal ends).

In some embodiments, where more than one signaling agent is present in the heterodimeric protein complexes described herein, one signaling agent may be in trans orientation (as relates to the targeting moiety), whereas another signaling agent may be in cis orientation (as relates to the targeting moiety).

In some embodiments, the heterodimeric Fc-based chimeric protein complex does not comprise the signaling agent and targeting moiety on a single polypeptide.

In some embodiments, the Fc-based chimeric protein has an improved in vivo half-life relative to a chimeric protein lacking an Fc or a chimeric protein, which is not a heterodimeric complex. In some embodiments, the Fc-based chimeric protein has an improved solubility, stability and other pharmacological properties relative to a chimeric protein lacking an Fc or a chimeric protein, which is not a heterodimeric complex.

Heterodimeric Fc-based chimeric protein complexes are composed of two different polypeptides. In embodiments described herein, the targeting domain is on a different polypeptide than the signaling agent and accordingly, proteins that contain only one targeting domain copy, and also only one signaling agent. Further, in embodiments, one targeting domain (*e.g.* VHH) only can avoid cross-linking of the antigen on the cell surface (which could elicit undesired effects in some cases). Further, in embodiments, one signaling agent may alleviate molecular "crowding" and potential interference with avidity mediated restoration of effector function in dependence of the targeting domain. Further, in embodiments, heterodimeric Fc-based chimeric protein complexes can have two targeting moieties and these can be placed on the two different polypeptides. For instance, in embodiments, the C-terminus of both targeting moieties (*e.g.* VHHs) can be masked to avoid potential autoantibodies or pre-existing antibodies (*e.g.* VHH autoantibodies or pre-existing antibodies). Further, in embodiments, heterodimeric Fc-based chimeric protein complexes, *e.g.* with the targeting domain on a different polypeptide than the signaling agent may favor "cross-linking" of two cell types (*e.g.* a tumor cell and an immune cell). Further, in embodiments, heterodimeric Fc-based chimeric protein complexes can have two signaling agent, each on different polypeptides to allow more complex effector responses.

Further, in embodiments, heterodimeric Fc-based chimeric protein complexes, *e.g.* with the targeting domain on a different polypeptide than the signaling agent combinatorial diversity of targeting moiety and signaling agent. For instance, in embodiments, polypeptides with any of the targeting moieties described herein can be combined "off the shelf" with polypeptides with any of the signaling agents described herein to allow rapid generation of various combinations of targeting moieties and signaling agents in single Fc-based chimeric protein complexes.

In some embodiments, the Fc-based chimeric protein complex comprises one or more linkers. In some embodiments, the Fc-based chimeric protein complex includes a linker that connects the Fc domain, signaling agent and targeting moiety(ies). In some embodiments, the Fc-based chimeric protein complex includes a linker that connects each signaling agent and targeting moiety (or, if more than one targeting moiety, a signaling agent). In some embodiments, the Fc-based chimeric protein complex includes a linker that connects each signaling agent to the Fc domain. In some embodiments, the Fc-based chimeric protein complex includes a linker that connects each targeting moiety to the Fc domain. In some embodiments, the Fc-based chimeric protein complex includes a linker that connects a targeting moiety to another targeting moiety. In some embodiments, the Fc-based chimeric protein complex includes a linker that connects a signaling agent to another signaling agent.

In some embodiments, a Fc-based chimeric protein complex comprises two or more targeting moieties. In such embodiments, the targeting moieties can be the same targeting moiety or they can be different targeting moieties.

In some embodiments, a Fc-based chimeric protein complex comprises two or more signaling agents. In such embodiments, the signaling agents can be the same targeting moiety or they can be different targeting moieties.

By way of example, in some embodiments, the Fc-based chimeric protein complex comprise a Fc domain, at least two signaling agents (SA), and at least two targeting moieties (TM), wherein the Fc domain, signaling agents, and targeting moieties are selected from any of the Fc domains, signaling agents, and targeting moieties disclosed herein. In some embodiments, the Fc domain is homodimeric.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of **FIGs. 9A-F**, **10A-H**, **11A-H**, **12A-D**, **13A-F**, **14A-J**, **15A-D**, **16A-F**, **17A-J**, **18A-F**, **19A-L**, **20A-L**, **21A-F**, **22A-L**, **23A-L**, **24A-J**, **25A-J**, **26A-F**, and **27A-F**.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 9A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 10A-H.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 11A-H.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 12A-D.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 13A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 14A-J.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 15A-D.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 16A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 17A-J.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 18A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 19A-L.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 20A-L.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 21A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 22A-L.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 23A-L.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 24A-J.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 25A-J.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 26A-F.

In various embodiments, the Fc-based chimeric protein complex takes the form of any of the schematics of Figs. 27A-F.

In some embodiments, the signaling agents are linked to the targeting moieties and the targeting moieties are linked to the Fc domain on the same terminus (see FIGs. 9A-F). In some embodiments, the Fc domain is homodimeric.

In some embodiments, the signaling agents and targeting moieties are linked to the Fc domain, wherein the targeting moieties and signaling agents are linked on the same terminus (see FIGs. 9A-F). In some embodiments, the Fc domain is homodimeric.

In some embodiments, the targeting moieties are linked to signaling agents and the signaling agents are linked to the Fc domain on the same terminus (see FIGs. 9A-F). In some embodiments, the Fc domain is homodimeric.

In some embodiments, the homodimeric Fc-based chimeric protein complex has two or more targeting moieties. In some embodiments, there are four targeting moieties and two signaling agents, the targeting moieties are linked to the Fc domain and the signaling agents are linked to targeting moieties on the same terminus (see FIGS. 10A-H). In some embodiments, the Fc domain is homodimeric. In some embodiments, where there are four targeting moieties and two signaling agents, two targeting moieties are linked to the Fc domain and two targeting moieties are linked to the signaling agents, which are linked to the Fc domain on the same terminus (see FIGS. 10A-H). In some embodiments, the Fc domain is homodimeric. In some embodiments, where there are four targeting moieties

and two signaling agents, two targeting moieties are linked to each other and one of the targeting moieties of from each pair is linked to the Fc domain on the same terminus and the signaling agents are linked to the Fc domain on the same terminus (see FIGS. 10A-H). In some embodiments, the Fc domain is homodimeric. In some embodiments, where there are four targeting moieties and two signaling agents, two targeting moieties are linked to each other, wherein one of the targeting moieties of from each pair is linked to a signaling agent and the other targeting moiety of the pair is linked the Fc domain, wherein the targeting moieties linked to the Fc domain are linked on the same terminus (see FIGS. 10A-H). In some embodiments, the Fc domain is homodimeric.

In some embodiments, the homodimeric Fc-based chimeric protein complex has two or more signaling agents. In some embodiments, where there are four signaling agents and two targeting moieties, two signaling agents are linked to each other and one of the signaling agents of from pair is linked to the Fc domain on the same terminus and the targeting moieties are linked to the Fc domain on the same terminus (see FIGs. 11A-H). In some embodiments, the Fc domain is homodimeric. In some embodiments, where there are four signaling agents and two targeting moieties, two signaling agents are linked to the Fc domain one the same terminus and two of the signaling agents are each linked to a targeting moiety, wherein the targeting moieties are linked to the Fc domain at the same terminus (see FIGs. 11A-H). In some embodiments, the Fc domain is homodimeric. In some embodiments, where there are four signaling agents and two targeting moieties, two signaling agents are linked to each other and one of the signaling agents of from pair is linked to a targeting moiety and the targeting moieties are linked to the Fc domain on the same terminus (see FIGs. 11A-H). In some embodiments, the Fc domain is homodimeric.

By way of example, in some embodiments, the Fc-based chimeric protein complex comprise a Fc domain, wherein the Fc domain comprises ionic pairing mutation(s) and/or knob-in-hole mutation(s), at least one signaling agent and at least one targeting moiety, wherein the ionic pairing motif and/or a knob-in-hole motif, signaling agent and targeting moiety are selected from any of the ionic pairing motif and/or a knob-in-hole motif, signaling agents, and targeting moieties disclosed herein. In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, the signaling agent is linked to the targeting moiety, which is linked to the Fc domain (see FIGs. 18A-F and 19A-F). In some embodiments, the targeting moiety is linked to the signaling agent, which is linked to the Fc domain (see FIGs. 18A-F and 19A-F). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, the signaling agent and targeting moiety are linked to the Fc domain (see FIGs. 12A-D, 13A-D, 18A-F, and 19A-F). In some embodiments, the targeting moiety and the signaling agent are linked to different Fc chains on the same terminus (see FIGs. 12A-D and 15A-D). In some embodiments, the targeting moiety and the signaling agent are linked to different Fc chains on different termini (see FIGs. 12A-D and 15A-D). In some embodiments, the targeting moiety and the signaling agent are linked to the same Fc chain (see FIGs.

18A-F and 19A-F). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are one signaling agent and two targeting moieties, the signaling agent is linked to the Fc domain and two targeting moieties can be: 1) linked to each other with one of the targeting moieties linked to the Fc domain; or 2) each linked to the Fc domain (see FIGs. 13A-F, 16A-F, 19A-L, 22A-L, 24A-J, and 25A-J). In some embodiments, the targeting moieties are linked on one Fc chain and the signaling agent is on the other Fc chain (see FIGs. 13A-F and 16A-F). In some embodiments, the paired targeting moieties and the signaling agent are linked to the same Fc chain (see FIGs. 19A-L and 22A-L). In some embodiments, a targeting moiety is linked to the Fc domain and the other targeting moiety is linked to the signaling agent and the paired targeting moiety is linked to the Fc domain (see FIGs. 19A-L, 22A-L, 24A-J, and 25A-J). In some embodiments, the unpaired targeting moiety and paired targeting moiety are linked to the same Fc chain (see FIGs. 19A-L and 22A-L). In some embodiments, the unpaired targeting moiety and paired targeting moiety are linked to different Fc chains (see FIGs. 24A-J and 25A-J). In some embodiments, the unpaired targeting moiety and paired targeting moiety are linked on the same terminus (see FIGs. 24A-J and 25A-J). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are one signaling agent and two targeting moieties, a targeting moiety is linked to the signaling agent which is linked to the Fc domain, and the unpaired targeting moiety is linked the Fc domain (see FIGs. 19A-L, 22A-L, 24A-J, and 25A-J). In some embodiments, the paired signaling agent and unpaired targeting moiety are linked to the same Fc chain (see FIGs. 19A-L and 22A-L). In some embodiments, the paired signaling agent and unpaired targeting moiety are linked to different Fc chains (see FIGs. 24A-J and 25A-J). In some embodiments, the paired signaling agent and unpaired targeting moiety are linked on the same terminus (see FIGs. 24A-J and 25A-J). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function. In some embodiments, the Fc-based chimeric protein complex has a configuration and/or orientation as shown in any one of FIGs. 9A-F, 10A-H, 11A-H, 12A-D, 13A-F, 14A-J, 15A-D, 16A-F, 17A-J, 18A-F, 19A-L, 20A-L, 21A-F, 22A-L, 23A-L, 24A-J, 25A-J, 26A-F, and 27A-F. In some embodiments, the Fc-based chimeric protein complex has a configuration and/or orientation as shown in FIG. 15B.

In some embodiments, where there are one signaling agent and two targeting moieties, the targeting moieties are linked together and the signaling agent is linked to one of the paired targeting moieties, wherein the targeting moiety not linked to the signaling agent is linked to the Fc domain (see FIGs. 19A-L and 22A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are one signaling agent and two targeting moieties, the targeting moieties are linked together and the signaling agent is linked to one of the paired targeting moieties, wherein the signaling agent

is linked to the Fc domain (see FIGs. 19A-L and 22A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are one signaling agent and two targeting moieties, the targeting moieties are both linked to the signaling agent wherein one of the targeting moieties is linked to the Fc domain (see FIGs. 19A-L and 22A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are one signaling agent and two targeting moieties, the targeting moieties and the signaling agent are linked to the Fc domain (see FIGs. 24A-J and 25A-J). In some embodiments, the targeting moieties are linked on the terminus (see FIGs. 24A-J and 25A-J). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the signaling agents are linked to the Fc domain on the same terminus and the targeting moiety is linked to the Fc domain (see FIGs. 14A-J and 17A-J). In some embodiments, the signaling agents are linked to the Fc domain on the same Fc chain and the targeting moiety is linked on the other Fc chain (see FIGs. 26A-F and 27A-F). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, a signaling agent is linked to the targeting moiety, which is linked to the Fc domain and the other signaling agent is linked to the Fc domain (see FIGs. 14A-J, 15A-J, 20A-L, and 23A-L). In some embodiments, the targeting moiety and the unpaired signaling agent are linked to different Fc chains (see FIGs. 14A-J and 17A-J). In some embodiments, the targeting moiety and the unpaired signaling agent are linked to different Fc chains on the same terminus (see FIGs. 14A-J and 17A-J). In some embodiments, the targeting moiety and the unpaired signaling agent are linked to different Fc chains on different termini (see FIGs. 14A-J and 17A-J). In some embodiments, the targeting moiety and the unpaired signaling agent are linked to the same Fc chains (see FIGs. 20A-L and 23A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the targeting moiety is linked to a signaling agent which is linked to the Fc domain and the other signaling agent is linked to the Fc domain (see FIGs. 14A-J and 17A-J). In some embodiments, the paired signaling agent and the unpaired signaling agent are linked to different Fc chains (see FIGs. 14A-J and 17A-J). In some embodiments, the paired signaling agent and the unpaired signaling agent are linked to different Fc chains on the same terminus (see FIGs. 14A-J and 17A-J). In some embodiments, the paired signaling agent and the unpaired signaling agent are linked to different Fc chains on different termini (see FIGs. 14A-J and 17A-J). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the signaling agents are linked together and the targeting moiety is linked to one of the paired signaling agents, wherein the targeting moiety is linked to the Fc domain (see FIGs. 20A-L and 23A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the signaling agents are linked together and one of the signaling agents is linked to the Fc domain and the targeting moiety is linked to the Fc domain (see FIGs. 20A-L, 23A-L, 26A-F, and 27A-F). In some embodiments, the paired signaling agents and targeting moiety are linked to the same Fc chain (see FIGs. 20A-L and 23A-L). In some embodiments, the paired signaling agents and targeting moiety are linked to different Fc chains (see FIGs. 26A-F and 27A-F). In some embodiments, the paired signaling agents and targeting moiety are linked to different Fc chains on the same terminus (see FIGs. 26A-F and 27A-F). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the signaling agents are both linked to the targeting moiety, wherein one of the signaling agents is linked to the Fc domain (see FIGs. 20A-L and 23A-L). In some embodiments, the Fc domain is heterodimeric. In some embodiments, the Fc domain comprises a mutation that reduces or eliminates its effector function.

In some embodiments, where there are two signaling agents and one targeting moiety, the signaling agents are linked together and one of the signaling agents is linked to the targeting moiety and the other signaling agent is linked to the Fc domain (see FIGs. 20A-L and 23A-L).

In some embodiments, where there are two signaling agents and one targeting moiety, each signaling agent is linked to the Fc domain and the targeting moiety is linked to one of the signaling agents (see FIGs. 20A-L and 23A-L). In some embodiments, the signaling agents are linked to the same Fc chain (see FIGs. 20A-L and 23A-L).

In some embodiments, a targeting moiety or signaling agent is linked to the Fc domain, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region. For example, vectors encoding the targeting moiety, signaling agent, or combination thereof, linked as a single nucleotide sequence to an Fc domain can be used to prepare such polypeptides.

Multi-Specific Agents

In various embodiments, the present PD-L1 targeting moiety is part of chimeric protein or the chimeric protein complex which comprises one or more signaling agents as described herein and/or one or more additional targeting moieties (*i.e.*, in addition to the targeting moiety directed against PD-L1). Accordingly, the present invention provides for chimeric or fusion proteins that include one or more signaling agents, a targeting moiety against PD-L1, and/or one or more additional targeting moieties.

In various embodiments, the chimeric proteins or the chimeric protein complexes of the present invention have 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 various embodiments, the chimeric protein or the chimeric protein complex of the invention is multispecific, *i.e.*, the chimeric protein or the chimeric protein complex comprises two or more targeting moieties having recognition domains (*e.g.* antigen recognition domains) that recognize and bind two or more targets (*e.g.* antigens, or receptors, or epitopes). In such embodiments, the chimeric protein or the chimeric protein complex of the invention may comprise two more targeting moieties having recognition domains that recognize and bind two or more epitopes on the same antigen or on different antigens or on different receptors. In various embodiments, such multi-specific chimeric proteins or the chimeric protein complexes exhibit advantageous properties such as increased avidity and/or improved selectivity. In an embodiment, the chimeric protein or the chimeric protein complex 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 or different receptors.

In various embodiments, the multispecific chimeric protein or the chimeric protein complex 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 exemplary embodiment, the multispecific chimeric protein or the chimeric protein complex of the invention comprises at least one antibody or antibody derivative (*e.g.*, a VHH) comprising an antigen recognition domain against PD-L1 and one antibody or antibody derivative comprising a recognition domain against a tumor antigen.

In various embodiments, the present multispecific chimeric proteins or the chimeric protein complexes 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 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-μM 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 chimeric protein or the chimeric protein complex 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 chimeric protein 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 chimeric

protein 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 chimeric protein 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 antibody or antibody derivative (*e.g.*, a VHH) with an antigen recognition domain against PD-L1 and a second antibody or antibody derivative with a 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 chimeric protein or the chimeric protein complex of the invention may be constructed by using linkers. For example, the carboxy-terminus of a first antibody or antibody derivative (*e.g.*, a VHH) with an antigen recognition domain against PD-L1 may be linked to the amino-terminus of a second antibody or antibody derivative with a recognition domain against a tumor antigen (or vice versa). Illustrative linkers that may be used are described herein. In some embodiments, the components of the multispecific chimeric protein or the chimeric protein complex of the invention are directly linked to each other without the use of linkers.

In various embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention recognizes and binds to 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 chimeric protein or the chimeric protein complex specifically binds to an antigen of interest and effectively directly or indirectly recruits one of more immune cells.

In various embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention recognizes and binds to PD-L1 and one or more antigens found on tumor cells. In these embodiments, the present chimeric protein or the chimeric protein complex may directly or indirectly recruit an immune cell (*e.g.*, a macrophage) to a tumor cell or the tumor microenvironment. In such embodiments, the present chimeric protein or the chimeric protein complex enhances phagocytosis of tumor cells by macrophages.

In some embodiments, the present chimeric proteins or the chimeric protein complexes 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 chimeric protein or the chimeric protein complex 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.* anti-tumor macrophages (*e.g.* M1 macrophages), T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, B cells, and dendritic cells) 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 chimeric protein or the chimeric protein complex is capable of increasing a ratio of effector T cells to regulatory T cells.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* antigen or receptor) 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.* a macrophage) that can phagocytose, kill, and/or suppress the tumor 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these tumor antigens.

In some embodiments, the present multi-specific chimeric protein or the chimeric protein complex recognizes and binds to PD-L1 as well as an antigen on a tumor cell. In some embodiments, the multi-specific chimeric protein or the chimeric protein complex directly or indirectly recruits macrophages to the tumor cell or tumor microenvironment.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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.* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, IL-7R/$CD127^+$); $CD8^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, IL-7R/$CD127^+$); effector memory T cells (*e.g.* CD62Llow, $CD44^+$, TCR, $CD3^+$, IL-7R/$CD127^+$, IL-$15R^+$, CCR7low); central memory T cells (*e.g.* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, 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, CXCRI/IL-S RA, CCR5, CCR6, IL-10R α, CCR 7, IL-l 0 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, lutegrin α 4/CD49d, CDS, Integrin α E/CD103, CD6, Integrin α M/CD 11 b, CDS, Integrin α X/CD11c, Integrin β 2/CDIS, KIR/CD15S, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, LAG-3, CD43, LAIR1, CD45, LAIR2, CDS3, Leukotriene B4-R1, CDS4/SLAMF5, NCAM-L1, CD94, NKG2A, CD97, NKG2C, CD229/SLAMF3, NKG2D, CD2F-10/SLAMF9, NT-4, CD69, NTB-A/SLAMF6, Common 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/TNFRSFIOA, 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative T cell antigens.

By way of non-limiting example, in various embodiments, the present chimeric protein or the chimeric protein complex has a targeting moiety directed against 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 some embodiments, the multi-specific chimeric protein of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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, CD72, CD73, CD74, CDw75, CDw76, CD77, CD78, CD79a/b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD89, CD98, CD126, CD127, CDw130, CD138 and CDw150. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative B cell antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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-γ RI/CD64, MICA, Fc-γ RIIB/CD32b, MICB, Fc-γ RIIC/CD32c, MULT-1, Fc-γ RIIA/CD32a, Nectin-2/CD112, Fc-γ RIII/CD16, NKG2A, FcRH1/IRTA5, NKG2C, FcRH2/IRTA4, NKG2D, FcRH4/IRTA1, NKp30, FcRH5/IRTA2, NKp44, Fc-Receptor-like 3/CD16-2, NKp46/NCR1, NKp80/KLRF1, 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative NK cell antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or receptor) associated with macrophages/monocytes. In some embodiments, the targeting moiety directly or indirectly 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/CDIIc, CCL6/C10, Integrin β 2/CD18, CD155/PVR, Integrin β 3/CD61, CD31/PECAM-1, Latexin, CD36/SR-B3, Leukotriene B4 R1, CD40/TNFRSF5, LIMPIIISR-B2, CD43, LMIR1/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-gannna R2, TREM-I, IL-I 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/CDII b, CCR8, Integrin α X/CDIIc, CD155/PVR, Integrin β 2/CD18, CD14, Integrin β 3/CD61, CD36/SR-B3, LAIR1, CD43, LAIR2, CD45, Leukotriene B4-R1, CD68, LIMPIIISR-B2, CD84/SLAMF5, LMIR1/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 RIIIICD16, 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-I, IL-6 R, TREM-

2, CXCRI/IL-8 RA, TREM-3 and TREMLI/TLT-1. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative macrophage/monocyte antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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, LIMPIIISRB2, RP105, TLR4, TLR1, TLR5, TLR2, TLR6, TLR3, TLR9, 4-IBB 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, lutegrin 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, DCE205, 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 RIII/CD16, TREML1/TLT-1, ICAM-2/CD102 and Vanilloid R1. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative DC antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or receptor) associated with immune cells selected from, but not limited to, megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, or subsets thereof. In some embodiments, the antigen recognition domains directly or indirectly recruit megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, or subsets thereof, *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 chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative megakaryocyte and/or thrombocyte antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these illustrative erythrocyte antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or receptor) associated with mast cells. Illustrative mast cells antigens of interest include, for example, SCFR/CD117, $Fc_{\varepsilon}RI$, CD2, CD25, CD35, CD88, CD203c, C5R1, CMAI, FCERIA, FCER2, TPSABI. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these mast cell antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or receptor) associated with basophils. Illustrative basophils antigens of interest include, for example, $Fc_{\varepsilon}RI$, CD203c, CD123, CD13, CD107a, CD107b, and CD164. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these basophil antigens.

In some embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to a target (*e.g.* an antigen or 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 chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these neutrophil antigens.

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

In various embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention comprises a targeting moiety having a recognition domain that specifically binds to an appropriate antigen or cell surface marker 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, MYHIO, MYHI 1, MYH9, MYOCD; fibroblast

(stromal) cell surface markers such as ALCAM, CD34, COLIAI, COL1A2, COL3A1, FAP, PH-4; epithelial cell surface markers such as CDID, K6IRS2, KRTIO, KRT13, KRT17, KRT18, KRT19, KRT4, KRT5, KRT8, MUCI, TACSTDI; neovasculature markers such as CD13, TFNA, Alpha-v beta-3 (αVβ3), E-selectin; and adipocyte surface markers such as ADIPOQ, FABP4, and RETN. In various embodiments, the chimeric protein or the chimeric protein complex comprises a targeting moiety that binds one or more of these antigens. In various embodiments, a targeting moiety of the chimeric protein or the chimeric protein complex binds one or more of cells having these antigens.

In various embodiments, the multi-specific chimeric protein or the chimeric protein complex of the invention has one or more targeting moieties directed against 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 chimeric protein or the chimeric protein complex has a targeting moiety directed against (i) 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 (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In some embodiments, the PD-L1 targeting moiety of the present invention includes one or more additional recognition domains. In some embodiments, these additional recognition domains bind to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

Modifications and Production of Chimeric Proteins or Chimeric Protein Complexes

In various embodiments, the present chimeric protein or the chimeric protein complex comprises a targeting moiety (*e.g.*, PD-L1) 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 chimeric protein or the chimeric protein complex comprises a VHH that corresponds to the V_HH domains of naturally occurring heavy chain antibodies directed against human 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-L1 molecule, (*i.e.*, so as to raise an immune response and/or heavy chain antibodies directed against 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-L1 starting from the sample, using any suitable known techniques. In some embodiments, naturally occurring V_HH domains against PD-L1 can be obtained from naive libraries of Camelid V_HH sequences, for example, by screening such a library using 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-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-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-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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 chimeric proteins or the chimeric protein complexes of the invention are described herein. For example, DNA sequences encoding the chimeric proteins of the invention (*e.g.*, DNA sequences encoding the modified signaling agent and the targeting moiety and the linker) 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 chimeric proteins or chimeric protein complex. Accordingly, in various embodiments, the present invention provides for isolated nucleic acids comprising a nucleotide sequence encoding the chimeric protein or the chimeric protein complex of the invention.

Nucleic acids encoding the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex of the invention. Accordingly, in various embodiments, the present invention provides expression vectors comprising nucleic acids that encode the chimeric protein or the chimeric protein complex of the invention. In various embodiments, the present invention additional 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 chimeric protein or the chimeric protein complex of the invention can be produced by growing a host cell transfected with an expression vector encoding the chimeric protein or the chimeric protein complex 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 tags or by chromatography.

Accordingly, in various embodiments, the present invention provides for a nucleic acid encoding a chimeric protein or the chimeric protein complex of the present invention. In various embodiments, the present invention provides for a host cell comprising a nucleic acid encoding a chimeric protein or the chimeric protein complex of the present invention.

In various embodiments, the present PD-L1 targeting moiety or chimeric protein or the chimeric protein complex comprising the same may be expressed *in vivo*, for instance, in a patient. For example, in various embodiments, the present PD-L1 targeting moiety or chimeric protein or the chimeric protein complex comprising the same may administered in the form of nucleic acid which encodes the present PD-L1 targeting moiety or chimeric proteins or the chimeric protein complex comprising the same. In various embodiments, the nucleic acid is DNA or RNA. In some embodiments, present PD-L1 targeting moiety or chimeric protein or the chimeric protein complex 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 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 or the chimeric protein complex. 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 chimeric proteins or the chimeric protein complexes 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 chimeric proteins or the chimeric protein complexes described herein and a pharmaceutically acceptable carrier or excipient. 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, dessicated 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 chimeric protein or the chimeric protein complex 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 chimeric proteins or the chimeric protein complexes 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 polyetheylene 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex (*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 chimeric protein or the chimeric protein complex 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, 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 chimeric protein or the chimeric protein complex can be administered in unit dosage forms (*e.g.*, tablets or capsules) 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 chimeric protein or the chimeric protein complex is 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex of the present invention are administered to a subject simultaneously. The term “simultaneously” as used herein, means that the additional therapeutic agent and the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex can be by simultaneous administration of a single formulation (*e.g.*, a formulation comprising the additional therapeutic agent and the chimeric protein) or of separate formulations (*e.g.*, a first formulation including the additional therapeutic agent and a second formulation including the chimeric protein).

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 chimeric protein or the chimeric protein complex overlap in time, thereby exerting a combined therapeutic effect. For example, the additional therapeutic agent and the chimeric protein or the chimeric protein complex can be administered sequentially. The term “sequentially” as used herein means that the additional therapeutic agent and the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 apart, more than about 2 weeks apart, 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 chimeric protein or the chimeric protein complex being administered. Either the additional therapeutic agent or the chimeric protein 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 chimeric protein or the chimeric protein complex described herein acts synergistically when co-administered with another therapeutic agent. In such embodiments, the chimeric protein or the chimeric protein complex 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 chimeric proteins or the chimeric protein complexes 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 cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide 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 ranimnustine; 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, carabicin, 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, epitiostanol, mepitiostane, 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, Schaumberg, 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.

In an embodiment, the present invention relates to any agent that targets the spliceosome, including any component of the spliceosome, as additional therapeutic agents in the treatment of cancer.

In an embodiment, the present invention relates to any agent that targets Myc (*i.e.*, anti-Myc therapeutics) as additional therapeutic agents in the treatment of cancer.

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 autoimmmune 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, fluclorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene

(fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (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 immunosupressive 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 present invention pertains to various agents used for treating obesity as additional therapeutic agents. Illustrative agents used for treating obesity include, but are not limited to, orlistat (*e.g.* ALL1, XENICAL), loracaserin (*e.g.* BELVIQ), phentermine-topiramate (*e.g.* QSYMIA), sibutramme (*e.g.* REDUCTIL or MERJDIA), rimonabant (ACOMPLLA), exenatide (*e.g.* BYETTA), pramlintide (*e.g.* SYMLIN) phentermine, benzphetamine, diethylpropion, phendimetrazme, bupropion, and metformin. Agents that interfere with the body's ability to absorb specific nutrients in food are among the additional agents, *e.g.* orlistat (*e.g.* ALU, XENICAL), glucomannan, and guar gum. Agents that suppress apetite are also among the additional agents, *e.g.* catecholamines and their derivatives (such as phenteimine and other amphetamine-based drugs), various antidepressants and mood stabilizers (*e.g.* bupropion and topiramate), anorectics (*e.g.* dexedrine, digoxin). Agents that increase the body's metabolism are also among the additional agents.

In some embodiments, additional therapeutic agents may be selected from among appetite suppressants, neurotransmitter reuptake inhibitors, dopaminergic agonists, serotonergic agonists, modulators of GABAergic signaling, anticonvulsants, antidepressants, monoamine oxidase inhibitors, substance P (NK1) receptor antagonists, melanocortin receptor agonists and antagonists, lipase inhibitors, inhibitors of fat absorption, regulators of energy intake or metabolism, cannabinoid receptor modulators, agents for treating addiction, agents for treating metabolic syndrome, peroxisome proliferator-activated receptor (PPAR) modulators; dipcptidyl peptidase 4 (DPP- 4) antagonists, agents for treating cardiovascular disease, agents for treating elevated triglyceride levels, agents for treating low HDL, agents for treating hypercholesterolemia, and agents for treating hypertension. Some agents for cardiovascular disease include statins (*e.g.* lovastatin, atorvastatin, fluvastatin, rosuvastatin, simvastatin and pravastatin) and omega-3 agents (*e.g.* LOVAZA, EPANQVA, VASCEPA, esterified omega-3's in general, fish oils, krill oils, algal oils). In some embodiments, additional agents may be selected from among amphetamines, benzodiazepines, suifonyl ureas, meglitinides, thiazolidinediones, biguanides, beta-

blockers, XCE inhibitors, diuretics, nitrates, calcium channel blockers, phenlermine, sibutramine, iorcaserin, cetilistat, rimonabant, taranabant, topiramate, gabapentin, valproate, vigabatrin, bupropion, tiagabine, sertraline, fluoxetine, trazodone, zonisamide, methylphenidate, varenicline, naltrexone, diethylpropion, phendimetrazine, rcpaglini.de, nateglinide, glimepiride, metformin, pioglitazone, rosiglilazone, and sitagliptin.

In some embodiments, the present invention pertains to an agent used for treating diabetes as additional therapeutic agents. Illustrative anti-diabetic agents include, but are not limited to, sulfonylurea (*e.g.* DYMELOR (acetohexamide), DIABINESE (chlorpropamide), ORINASE (tolbutamide), and TOLINASE (tolazamide), GLUCOTROL (glipizide), GLUCOTROL XL (extended release), DIABETA (glyburide), MICRONASE (glyburide), GLYNASE PRESTAB (glyburide), and AMARYL (glimepiride)); a Biguanide (*e.g.* metformin (GLUCOPHAGE, GLUCOPHAGE XR, RIOMET, FORTAMET, and GLUMETZA)); a thiazolidinedione (*e.g.* ACTOS (pioglitazone) and AVANDIA (rosiglitazone); an alpha-glucosidase inhibitor (*e.g.*, PRECOSE (acarbose) and GLYSET (miglitol); a Meglitinide (*e.g.*, PRANDIN (repaglinide) and STARLIX (nateglinide)); a Dipeptidyl peptidase IV (DPP-IV) inhibitor (*e.g.*, JANUVIA (sitagliptin), NESINA (alogliptin), ONGLYZA (saxagliptin), and TRADJENTA (linagliptin)); Sodium-glucose co-transporter 2 (SGLT2) inhibitor (*e.g.* INVOKANA (canaglifozin)); and a combination pill (*e.g.* GLUCOVANCE, which combines glyburide (a sulfonylurea) and metformin, METAGLIP, which combines glipizide (a sulfonylurea) and metformin, and AVANDAMET, which uses both metformin and rosiglitazone (AVANDIA) in one pill, KAZANO (alogliptin and metformin), OSENI (alogliptin plus pioglitazone), METFORMIN oral, ACTOS oral, BYETTA subcutaneous, JANUVIA oral, WELCHOL oral, JANUMET oral, glipizide oral, glimepiride oral, GLUCOPHAGE oral, LANTUS subcutaneous, glyburide oral, ONGLYZA oral, AMARYI oral, LANTUS SOLOSTAR subcutaneous, BYDUREON subcutaneous, LEVEMIR FLEXPEN subcutaneous, ACTOPLUS MET oral, GLUMETZA oral, TRADJENTA oral, bromocriptine oral, KOMBIGLYZE XR oral, INVOKANA oral, PRANDIN oral, LEVEMIR subcutaneous, PARLODEL oral, pioglitazone oral, NOVOLOG subcutaneous, NOVOLOG FLEXPEN subcutaneous, VICTOZA 2-PAK subcutaneous, HUMALOG subcutaneous, STARLIX oral, FORTAMET oral, GLUCOVANCE oral, GLUCOPHAGE XR oral, NOVOLOG Mix 70-30 FLEXPEN subcutaneous, GLYBURIDE-METFORMIN oral, acarbose oral, SYMLINPEN 60 subcutaneous, GLUCOTROI XL oral, NOVOLIN R inj, GLUCOTROL oral, DUETACT oral, sitagliptin oral, SYMLINPEN 120 subcutaneous, HUMALOG KWIKPEN subcutaneous, JANUMET XR oral, GLIPIZIDE-METFORMIN oral, CYCLOSET oral, HUMALOG MIX 75-25 subcutaneous, nateglinide oral, HUMALOG Mix 75-25 KWIKPEN subcutaneous, HUMULIN 70/30 subcutaneous, PRECOSE oral, APIDRA subcutaneous, Humulin R inj, Jentadueto oral, Victoza 3-Pak subcutaneous, Novolin 70/30 subcutaneous, NOVOLIN N subcutaneous, insulin detemir subcutaneous, glyburide micronized oral, GLYNASE oral, HUMULIN N subcutaneous, insulin glargine subcutaneous, RIOMET oral, pioglitazone-metformin oral, APIDRA SOLOSTAR subcutaneous, insulin lispro subcutaneous, GLYSET oral, HUMULIN 70/30 Pen subcutaneous, colesevelam oral, sitagliptin-metformin oral, DIABETA oral, insulin regular human inj, HUMULIN N Pen subcutaneous, exenatide subcutaneous, HUMALOG Mix 50-50 KWIKPEN subcutaneous, liraglutide subcutaneous, KAZANO oral, repaglinide oral, chlorpropamide oral, insulin aspart subcutaneous, NOVOLOG Mix 70-30 subcutaneous, HUMALOG Mix 50-50 subcutaneous, saxagliptin oral, ACTOPLUS Met XR oral, miglitol oral,

NPH insulin human recomb subcutaneous, insulin NPH and regular human subcutaneous, tolazamide oral, mifepristone oral, insulin aspart protam-insulin aspart subcutaneous, repaglinide-metformin oral, saxagliptin-metformin oral, linagliptin-metformin oral, NESINA oral, OSENI oral, tolbutamide oral, insulin lispro protamine and lispro subcutaneous, pramlintide subcutaneous, insulin glulisine subcutaneous, pioglitazone-glimepiride oral, PRANDIMET oral, NOVOLOG PenFill subcutaneous, linagliptin oral, exenatide microspheres subcutaneous, KORLYM oral, alogliptin oral, alogliptin-pioglitazone oral, alogliptin-metformin oral, canagliflozin oral, Lispro (HUMALOG); Aspart (NOVOLOG); Glulisine (APIDRA); Regular (NOVOLIN R or HUMULIN R); NPH (NOVOLIN N or HUMULIN N); Glargine (LANTUS); Detemir (LEVEMIR); HUMULIN or NOVOLIN 70/30; and NOVOLOG Mix 70/30 HUMALOG Mix 75/25 or 50/50.

In some embodiments, the present invention relates to combination therapy with a blood transfusion. For instance, the present compositions may supplement a blood transfusion. In some embodiments, the present invention relates to combination therapy with iron supplements.

In some embodiments, the present invention relates to combination therapy with one or more EPO-based agents. For example, the present compositions may be used as an adjuvant to other EPO-based agents. In some embodiments, the present compositions are used as a maintenance therapy to other EPO-based agents. Other EPO-based agents include the following: epoetin alfa, including without limitation, DARBEPOETIN (ARANESP), EPOCEPT (LUPIN PHARMA), NANOKINE (NANOGEN PHARMACEUTICAL), EPOFIT (INTAS PHARMA), EPOGEN (AMGEN), EPOGIN, EPREX, (JANSSEN-CILAG), BINOCRIT (SANDOZ), PROCRIT; epoetin beta, including without limitation, NEORECORMON (HOFFMANN–LA ROCHE), RECORMON, Methoxy polyethylene glycol-epoetin beta (MIRCERA, ROCHE); epoetin delta, including without limitation, DYNEPO (erythropoiesis stimulating protein, SHIRE PLC); epoetin omega, including without limitation, EPOMAX; epoetin zeta, including without limitation, SILAPO (STADA) and RETACRIT (HOSPIRA) and other EPOs, including without limitation, EPOCEPT (LUPIN PHARMACEUTICALS), EPOTRUST (PANACEA BIOTEC LTD), ERYPRO SAFE (BIOCON LTD.), REPOITIN (SERUM INSTITUTE OF INDIA LIMITED), VINTOR (EMCURE PHARMACEUTICALS), EPOFIT (INTAS PHARMA), ERYKINE (INTAS BIOPHARMACEUTICA), WEPOX (WOCKHARDT BIOTECH), ESPOGEN (LG LIFE SCIENCES), RELIPOIETIN (RELIANCE LIFE SCIENCES), SHANPOIETIN (SHANTHA BIOTECHNICS LTD), ZYROP (CADILA HEALTHCARE LTD.), EPIAO (RHUEPO) (SHENYANG SUNSHINE PHARMACEUTICAL CO. LTD), CINNAPOIETIN (CINNAGEN).

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), MPDL328OA (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 or the chimeric protein complex 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 or the chimeric protein complex 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 chimeric protein or the chimeric protein complex acts synergistically when used in combination with Chimeric Antigen Receptor (CAR) T-cell therapy. In an illustrative embodiment, the chimeric protein or the chimeric protein complex acts synergistically when used in combination with CAR T-cell therapy in treating tumor or cancer. In an embodiment, the chimeric protein or the chimeric protein complex acts synergistically when used in combination with CAR T-cell therapy in treating blood-based tumors. In an embodiment, the chimeric protein or the chimeric protein complex acts synergistically when used in combination with CAR T-cell therapy in treating solid tumors. For example, use of the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex of the invention induces CAR T-cell division. In various embodiments, the chimeric protein or the chimeric protein complex of the invention induces CAR T-cell proliferation. In various embodiments, the chimeric protein or the chimeric protein complex 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, IL13Rα2, 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 (Cellectis), UCART123 (Cellectis), UCART38 (Cellectis), UCARTCS1 (Cellectis), OXB-302 (Oxford BioMedica, MB-101 (Mustang Bio) and CAR T-cells developed by Innovative Cellular Therapeutics.

In some embodiments, the chimeric protein or the chimeric protein complex 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-ß-2 (U. S. Patent Publication No. 2002/0025304), spirogermaniums (*e.g.*, N-(3-dimethylaminopropyl)-2-aza-8,8-dimethyl-8-germanspiro [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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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/m2).
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 therafter
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	PAR PHARMACEUTICAL, PERRIGO COMPANY ,	Oral

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
Epithelial sodium channel Na+/H+ exchanger)	SIGMAPHARM LABORATORIES	
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 S1P1 and S1P5 B cell distrubution T cell distribution)	NOVARTIS PHARMA	Oral
BGC20-0134 (targets Proinflammatory and anti-inflammatory cytokines)	BTG PLC	Oral
BIIB033 (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

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
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 c-KIT) receptor as well as select other tyrosine kinases Mast cells)	AB SCIENCE	Oral
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

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
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
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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex increases 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/m^2, or 2 to 3 years of therapy. In some embodiments, supplementation with the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex is used as an entry therapy that precedes the use of a second-line therapy. For example, the chimeric protein or the chimeric protein complex

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 chimeric protein or the chimeric protein complex finds use in patients that have received, and perhaps failed one or more DMT. For example, in some embodiments, the chimeric protein or the chimeric protein complex 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, the chimeric protein or the chimeric protein complex 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 chimeric protein or the chimeric protein complex described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The chimeric protein or the chimeric protein complex 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, cyclothosphamide, 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 antimytotic 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, tenoposide, 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, gemcytabine, 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 chimeric protein or the chimeric protein complex, 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, anemia, autoimmune diseases, cardiovascular diseases, wound healing, ischemia-related diseases, neurodegenerative diseases, metabolic diseases and many other diseases and disorders.

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 one or more of chronic granulomatous disease, osteopetrosis, idiopathic pulmonary fibrosis, Friedreich's ataxia, atopic dermatitis, Chagas disease, cancer, heart failure, autoimmune disease, sickle cell disease, thalassemia, blood loss, transfusion reaction, diabetes, vitamin B12 deficiency, collagen vascular disease, Shwachman syndrome, thrombocytopenic purpura, Celiac disease, endocrine deficiency state such as hypothyroidism or Addison's disease, autoimmune

disease such as Crohn's Disease, systemic lupus erythematosis, rheumatoid arthritis or juvenile rheumatoid arthritis, ulcerative colitis immune disorders such as eosinophilic fasciitis, hypoimmunoglobulinemia, or thymoma/thymic carcinoma, graft versus host disease, preleukemia, Nonhematologic syndrome (*e.g.,* Down's, Dubowwitz, Seckel), Felty syndrome, hemolytic uremic syndrome, myelodysplasic syndrome, nocturnal paroxysmal hemoglobinuria, osteomyelofibrosis, pancytopenia, pure red-cell aplasia, Schoenlein-Henoch purpura, malaria, protein starvation, menorrhagia, systemic sclerosis, liver cirrhosis, hypometabolic states, and congestive heart failure.

In some embodiments, the present invention relates to the treatment of, or a patient having one or more of chronic granulomatous disease, osteopetrosis, idiopathic pulmonary fibrosis, Friedreich's ataxia, atopic dermatitis, Chagas disease, mycobacterial infections, cancer, scleroderma, hepatitis, hepatitis C, septic shock, and rheumatoid arthritis.

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, 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 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); 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 relates to the treatment of Myc-driven cancers, *i.e.*, cancer cells that overexpress Myc. In some embodiments, the cancer cells overexpress any one of c-Myc, N-Myc, and/or L-Myc. In some embodiments, methods of the invention renders the cancer cells susceptible to treatment with any one of the anti-cancer therapeutic agents described herein. In some embodiments, methods of the invention reduce the transcriptional activities of the cancer cells.

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

In various embodiments, the present compositions are used to treat, control or prevent cardiovascular disease, such as a disease or condition affecting the heart and vasculature, including but not limited to, coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischaemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, vavular disease, and/or congestive heart failure.

In various embodiments, the present compositions are used to treat or prevent one or more metabolic-related disorders. In various embodiments, the present invention is useful for the treatment, controlling or prevention of diabetes, including Type 1 and Type 2 diabetes and diabetes associated with obesity. The compositions and methods of the present invention are useful for the treatment or prevention of diabetes-related disorders, including without limitation diabetic nephropathy, hyperglycemia, impaired glucose tolerance, insulin resistance, obesity, lipid disorders, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis and its sequelae, vascular restenosis, irritable bowel syndrome, inflammatory bowel disease, including Crohn's disease and ulcerative colitis, other inflammatory conditions, pancreatitis, abdominal obesity, neurodegenerative disease, retinopathy, neoplastic conditions, adipose cell tumors, adipose cell carcinomas, such as liposarcoma, prostate cancer and other cancers, including gastric, breast, bladder and colon cancers, angiogenesis, Alzheimer's disease, psoriasis, high blood pressure, Metabolic Syndrome (*e.g.* a person has three or more of the following disorders: abdominal obesity, hypertriglyceridemia, low HDL cholesterol, high blood pressure, and high fasting plasma glucose), ovarian hyperandrogenism (polycystic ovary syndrome), and other disorders where insulin resistance is a component, such as sleep apnea. The compositions and methods of the present invention are useful for the treatment, control, or prevention of obesity, including genetic or environmental, and obesity-related disorders. The obesity-related disorders herein are associated with, caused by, or result from obesity. Examples of obesity-related disorders include obesity, diabetes, overeating, binge eating, and bulimia, hypertension, elevated plasma insulin concentrations and insulin resistance, dyslipidemia, hyperlipidemia, endometrial, breast, prostate, kidney and colon cancer, osteoarthritis, obstructive sleep apnea, gallstones, heart disease, abnormal heart rhythms and arrythmias, myocardial infarction, congestive heart failure, coronary heart disease, sudden death, stroke, polycystic ovary disease, craniopharyngioma, Prader-Willi Syndrome, Frohlich's syndrome, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, e.g, children with acute lymphoblastic leukemia. Further examples of obesity-related disorders are Metabolic Syndrome, insulin resistance syndrome, reproductive hormone abnormalities, sexual and reproductive dysfunction, such as impaired fertility, infertility, hypogonadism in males and hirsutism in females, fetal defects associated with maternal obesity, gastrointestinal motility disorders, such as obesity-related gastro-esophageal reflux, respiratory disorders, such as obesity-hypoventilation syndrome (Pickwickian syndrome), breathlessness, cardiovascular disorders, inflammation, such as systemic inflammation of the vasculature, arteriosclerosis, hypercholesterolemia, lower back pain, gallbladder disease, hyperuricemia, gout, and

kidney cancer, and increased anesthetic risk. The compositions and methods of the present invention are also useful to treat Alzheimer's disease.

In various embodiments, the present compositions are used to treat or prevent one or more respiratory diseases, such as idiopathic pulmonary fibrosis (IPF), asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, allergic rhinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, Hantavirus pulmonary syndrome (HPS), Loeffler's syndrome, Goodpasture's syndrome, Pleurisy, pneumonitis, pulmonary edema, pulmonary fibrosis, Sarcoidosis, complications associated with respiratory syncitial virus infection, and other respiratory diseases.

In some embodiments, the present invention is used to treat or prevent one or more neurodegenerative disease. Illustrative neurodegenerative diseases include, but are not limited to, Friedreich's ataxia, multiple sclerosis (including without limitation, benign multiple sclerosis; relapsing-remitting multiple sclerosis (RRMS); secondary progressive multiple sclerosis (SPMS); progressive relapsing multiple sclerosis (PRMS); and primary progressive multiple sclerosis (PPMS)), 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).

In various embodiments, the present chimeric proteins or the chimeric protein complexes find use in treating wounds, *e.g.*, a non-healing wound, an ulcer, a burn, or frostbite, a chronic or acute wound, open or closed wound, internal or external wound (illustrative external wounds are penetrating and non-penetrating wound. In various embodiments, the present chimeric proteins or the chimeric protein complexes find use in treating ischemia, by way of non-limiting example, ischemia associated with acute coronary syndrome, acute lung injury (ALI), acute myocardial infarction (AMI), acute respiratory distress syndrome (ARDS), arterial occlusive disease, arteriosclerosis, articular cartilage defect, aseptic systemic inflammation, atherosclerotic cardiovascular disease, autoimmune disease, bone fracture, bone fracture, brain edema, brain hypoperfusion, Buerger's disease, burns, cancer, cardiovascular disease, cartilage damage, cerebral infarct, cerebral ischemia, cerebral stroke, cerebrovascular disease, chemotherapy-induced neuropathy, chronic infection, chronic mesenteric ischemia, claudication, congestive heart failure, connective tissue damage, contusion, coronary artery disease (CAD), critical limb ischemia (CLI), Crohn's disease, deep vein thrombosis, deep wound, delayed ulcer healing, delayed wound-healing, diabetes (type I and type II), diabetic neuropathy, diabetes induced ischemia, disseminated intravascular coagulation (DIC), embolic brain ischemia, frostbite, graft-versus-host disease, hereditary hemorrhagic telengiectasiaischemic vascular disease, hyperoxic injury, hypoxia, inflammation, inflammatory bowel disease, inflammatory disease, injured tendons, intermittent claudication, intestinal ischemia, ischemia, ischemic brain disease, ischemic heart disease, ischemic peripheral vascular disease, ischemic placenta, ischemic renal disease,

ischemic vascular disease, ischemic-reperfusion injury, laceration, left main coronary artery disease, limb ischemia, lower extremity ischemia, myocardial infarction, myocardial ischemia, organ ischemia, osteoarthritis, osteoporosis, osteosarcoma, Parkinson's disease, peripheral arterial disease (PAD), peripheral artery disease, peripheral ischemia, peripheral neuropathy, peripheral vascular disease, pre-cancer, pulmonary edema, pulmonary embolism, remodeling disorder, renal ischemia, retinal ischemia, retinopathy, sepsis, skin ulcers, solid organ transplantation, spinal cord injury, stroke, subchondral-bone cyst, thrombosis, thrombotic brain ischemia, tissue ischemia, transient ischemic attack (TIA), traumatic brain injury, ulcerative colitis, vascular disease of the kidney, vascular inflammatory conditions, von Hippel-Lindau syndrome, or wounds to tissues or organs

In various embodiments, the present invention relates to the treatment of one or more of anemia, including anemia resulting from chronic kidney disease (*e.g.* from dialysis) and/or an anti-cancer agent (*e.g.* chemotherapy and/or HIV treatment (*e.g.* Zidovudine (INN) or azidothymidine (AZT)), inflammatory bowel disease (*e.g.* Crohn's disease and ulcer colitis), anemia linked to inflammatory conditions (*e.g.* arthritis, lupus, IBD), anemia linked to diabetes, schizophrenia, cerebral malaria, as aplastic anemia, and myelodysplasia from the treatment of cancer (*e.g.* chemotherapy and/or radiation), and various myelodysplastic syndrome diseases (*e.g.* sickle cell anemia, hemoglobin SC disease, hemoglobin C disease, alpha- and beta-thalassemias, neonatal anemia after premature birth, and comparable conditions).

In some embodiments, the present invention relates to the treatment of, or a patient having anemia, *i.e.* a condition in which the number of red blood cells and/or the amount of hemoglobin found in the red blood cells is below normal. In various embodiments, the anemia may be acute or chronic. For example, the present anemias include but are not limited to iron deficiency anemia, renal anemia, anemia of chronic diseases/inflammation, pernicious anemia such as macrocytic achylic anemia, juvenile pernicious anemia and congenital pernicious anemia, cancer-related anemia, anti-cancer-related anemia (*e.g.* chemotherapy-related anemia, radiotherapy-related anemia), pure red cell aplasia, refractory anemia with excess of blasts, aplastic anemia, X-lined siderobalstic anemia, hemolytic anemia, sickle cell anemia, anemia caused by impaired production of ESA, myelodysplasia syndromes, hypochromic anemia, microcytic anemia, sideroblastic anemia, autoimmune hemolytic anemia, Cooley's anemia, Mediterranean anemia, Diamond Blackfan anemia, Fanconi's anemia and drug-induced immune hemolytic anemia. Anemia may cause serious symptoms, including hypoxia, chronic fatigue, lack of concentration, pale skin, low blood pressure, dizziness and heart failure.

In some embodiments, the present invention relates to the treatment of anemia resulting from chronic renal failure. In some embodiments, the present invention relates to the treatment of anemia resulting from the use of one or more renal replacement therapies, inclusive of dialysis, hemodialysis, peritoneal dialysis, hemofiltration, hemodiafiltration, and renal transplantation.

In some embodiments, the present invention relates to the treatment of anemia in patients with chronic kidney disease who are not on dialysis. For instance, the present invention relates to patients in stage 1 CKD, or stage 2 CKD, or stage 3 CKD, or stage 4 CKD, or stage 5 CKD. In some embodiments, the present patient is stage 4 CKD

or stage 5 CKD. In some embodiments, the present patient has undergone a kidney transplant. In some embodiments, the present invention relates to the treatment of anemia is a patient having an acute kidney injury (AKI).

In some embodiments, the anemia is induced by chemotherapy. For instance, the chemotherapy may be any myelosuppressive chemotherapy. In some embodiment, the chemotherapy is one or more of Revlimid, Thalomid, dexamethasone, Adriamycin and Doxil. In some embodiments, the chemotherapy is one or more platinum-based drugs including cisplatin (*e.g.* PLATINOL) and carboplatin (*e.g.* PARAPLATIN). In some embodiments, the chemotherapy is any one of the chemotherapeutic agents described herein. In some embodiments, the chemotherapy is any agent described in Groopman *et al.* J Natl Cancer Inst (1999) 91 (19): 1616-1634, the contents of which are hereby incorporated by reference in their entireties. In some embodiments, the present compositions and methods are used in the treatment of chemotherapy-related anemia in later stage cancer patients (*e.g.* a stage IV, or stage III, or stage II cancer). In some embodiments, the present compositions and methods are used in the treatment of chemotherapy-related anemia in cancer patients receiving dose-dense chemotherapy or other aggressive chemotherapy regimens.

In some embodiments, the present invention relates to the treatment of anemia in a patient having one or more blood-based cancers, such as leukemia, lymphoma, and multiple myeloma. Such cancers may affect the bone marrow directly. Further, the present invention relates to metastatic cancer that has spread to the bone or bone marrow. In some embodiments, the present invention relates to the treatment of anemia in a patient undergoing radiation therapy. Such radiation therapy may damage the bone marrow, lowering its ability to make red blood cells. In further embodiments, the present invention relates to the treatment of anemia in a patient having a reduction or deficiency of one or more of iron, vitamin B12, and folic acid. In further embodiments, the present invention relates to the treatment of anemia in a patient having excessive bleeding including without limitation, after surgery or from a tumor that is causing internal bleeding. In further embodiments, the present invention relates to the treatment of anemia in a patient having anemia of chronic disease.

In some embodiments, the present methods and compositions stimulate red blood cell production. In some embodiments, the present methods and compositions stimulate division and differentiation of committed erythroid progenitors in the bone marrow.

Certain embodiments of the present invention are particularly useful for treating chemotherapy-induced anemia in cancer patients. In some embodiments, the present methods and compositions allows for continued administration of the chimeric protein or the chimeric protein complex after a cancer patient's chemotherapy is finished. In some embodiments, the present methods and compositions allows for treatment of a cancer patient without dose reduction relative to a non-cancer patient. In some embodiments, the present methods and compositions allows for treatment of a cancer patient receiving chemotherapy and considered curable. In various embodiments, the cancer patient has one or more of a history of blood clots, recent surgery, prolonged periods of bed rest or limited activity, and treatment with a chemotherapeutic agent.

Kits

The invention also provides kits for the administration of any agent described herein (*e.g.* the chimeric protein with or without various 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 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.

Unless specifically stated or obvious from context, as used herein, the term “or” is understood to be inclusive and covers both “or” and “and”.

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, *e.g.*, within (plus or minus) 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. 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 terms “AFN”, “A-Kine”, “AcTa”, “AcTakine”, “AcTaferon” are occasionally used herein to reference the chimeras described herein.

In these examples, we show that mutagenesis of PD-L1 VHHs results in variants with increased affinity and neutralization potency of the PD-1/PD-L1 interaction. This mutagenesis includes humanization and removal of sequence liabilities (risk motives for isomerization, deamidation and oxidation) by site-directed mutagenesis. These were performed separately in a first step, and interesting mutations were combined in a second step. PD-L1 VHHs used here are named 2LIG99 (SEQ ID NO: 1) and 2LIG189 (SEQ ID NO: 26).

Example 1: First Round of Mutagenesis for 2LIG99 VHH

The VHH 2LIG99 (SEQ ID NO: 1) contains the sequence motif DG in CDR2 which has a high risk for isomerization. To circumvent this problem the residues D54 and G55 were individually randomly mutagenized. For each D54 and G55, 48 clones were randomly picked, sequenced, and the VHH purified from TES extracts based on the C-terminal His-tag and using HisPur Cobalt Spin Plates (ThermoFisher) according to the manufacturer’s guidelines.

Affinities of purified VHH variants were screened using the bio-layer interferometry technology on an Octet Red 96 instrument (ForteBio). In brief, recombinant PD-L1 extracellular domain fused to the mouse IgG1 Fc domain (SinoBiologicals) was immobilized on anti-murine IgG quantitation (AMQ) biosensors (ForteBio). Loaded sensors

were incubated with a single concentration of PD-L1 VHH variant, association and dissociation monitored and kinetic parameters were calculated using the Octet Data Analysis software v10 (ForteBio). Based on the analysis of the dissociation kinetics the mutations D54G, D54K, D54T and G55R were selected for further mutagenesis of the 2LIG99 VHH.

For the humanization of 2LIG99, the sequence was aligned with the human VH3-23_JH5 sequence and a construct designed with a series of mutations in the framework regions (Q1D_Q5V_A14P_T74S_K86R_Q110; 2LIG99_OPT1; SEQ ID NO: 8). An aspartic acid at position 1 was included to avoid the risk of pyroglutamate formation. An additional series of variants was designed by combining with individual mutation of T23A, A63V, K76N, S97Y, or a combination of these latter (SEQ ID NO: 9-13). In addition, for the DG isomerization site in CDR2, mutations D54G, D54K, D54T or G55R were evaluated again (SEQ ID NO: 14-17). These variants (with a C-terminal His-tag) were cloned in the pHEN6C vector for periplasmic expression in *E. coli* WK6 cells. After overnight expression upon IPTG induction, cells were pelleted, and proteins purified from the periplasmic extracts using the HisPur Cobalt Spin Plates (ThermoFisher) according to the manufacturer's guidelines.

Affinities of resulting variants were measured using the bio-layer interferometry technology on an Octet Red 96 instrument (ForteBio). In brief, recombinant PD-L1 extracellular domain fused to the mouse IgG1 Fc domain (SinoBiologicals) was immobilized on anti-murine IgG quantitation (AMQ) biosensors (ForteBio). Loaded sensors were incubated with a serial dilution of PD-L1 VHH variant, association and dissociation monitored and kinetic parameters were calculated using the Octet Data Analysis software v10 (ForteBio). Results summarized in **Fig. 3** illustrate that humanization mutations can result in a loss of affinity especially for SEQ ID NOs: 9 and 13 containing the mutation T23A.

Example 2: Combination of Mutations for 2LIG99 VHH

Based on the affinity in Octet of the first series of 2LIG99 variants, we selected the OPT1 humanization variant (with mutations Q1D_Q5V_A14P_T74S_K86R_Q110) and combined these with the variations A63V, K76N, and S79Y resulting in SEQ ID Nos: 18-25. The isomerization motif in all these variants was removed by the D54G mutation. Variants were produced as described above and affinity measured on an Octet instrument using the recombinant PD-L1-mouse Fc protein. Data in **Fig. 4** illustrate that it is possible to humanize and remove the isomerization motif in the 2LIG99 sequence without losing affinity or even improving affinity in case of SEQ ID Nos: 20, 21, and 24 indicating the surprisingly beneficial effect of the presence of the mutation K76N and/or S79Y in the framework region.

We next evaluated the effect of the sequence variations on ability to interfere with the PD-L1/PD-1 interaction. To test is, we have generated a bi-valent AFN based on the extracellular portion of PD-L1 (see SEQ ID Nos: 169 and 170. When applied on HL116 cells, a HT1080 derived clone stably transfected with the firefly luciferase gene controlled by the IFN-inducible 6-16 promoter, this AFN is more active on cells expressing PD-1 compared to parental cells. This bioassay was used to test the neutralizing capacity of the 2LIG99 variants. In brief, a sub-optimal PD-L1 AFN concentration (i.e. 1 µg/ml) was pre-incubated with a serial dilution of 2LIG99 variants. Half an

hour later, the combination of AFN and VHH was added to 20.000 HL116-PD-1 cells in a 96-well and cells were further incubated for 6 hours. Luciferase was measured and plotted in function of the VHH concentration. Due to the concentration of PD-L1 AFN needed and the relative high affinity and neutralization of the 2LIG99 VHH the sensitivity of the assay does not allow to discriminate variants with improved neutralization. Nevertheless, the data confirm that the variants containing the mutation K76N and/or S79Y (also in combination with D54G and or A63V) are potently neutralizing the PD-L1/PD-1 interaction as shown in **Fig. 5**.

Example 3: Mutagenesis for 2LIG189

For the humanization of 2LIG189 (SEQ ID NO: 26) the sequence was aligned with the human VH3-23_JH5 sequence and a construct designed with a series of mutations in the framework regions (Q1D_Q5V_A14P_A74S_K86R_Q109L; 2LIG189_OPT1; SEQ ID NO: 33). The aspartic acid at position 1 was included to avoid the risk of pyroglutamate formation. An additional series of variants was designed by combining with mutation of M77T and/or M78V (SEQ ID NOS: 34-36). The N32 and D33 residues in the deamidation motif in CDR1 were mutated separately, resulting in 22 variants (SEQ ID NOs: 37-58). M97, at the border of CDR3, possibly sensitive to oxidation was mutated to E, F, H, I, L, Q, R, V, or Y (SEQ ID NOs: 59-67). Resulting mutants were purified and affinities measured as described in Example 1. Results are summarized in **Fig. 6**. Mutations that increase affinity by twofold or more include N32Q, N32R, D33H, M97I, M97L, and M97V.

Example 4: Combination of Mutations for 2LIG189

Based on the affinity in Octet of the first series of 2LIG189 variants, we combined a humanization variant (Q1D_Q5V_A14P_A74S_M77T_M78V_K86R_Q109L) with the deamidation variants N32R or D33H and the oxidation variants M97V or M97I (SEQ ID NO: 68-73). Affinities (summarized in **Fig. 7**) and neutralization potency (**Fig. 8**) were measured as described above. Both datasets illustrate that some variants with both the deamidation and oxidation risk site removed (especially SEQ ID NO: 72 and 73) increase both affinity (40- and 10-fold respectively) and neutralization of the PD-L1/PD-1 interaction.

Example 5: Selection of Variant 2LIG99 and 2LIG189 VHH

In order to generate PD-L1 targeted AcTakines the following variants are selected.

2LIG189; SEQ ID NO: 74:
Q1D_Q5V_A14P_D33H_A62S_A74S_M77T_M78V_S79Y_K86R_M97V_Q109L
DVQLVESGGGLVQPGGSLRLSCAASGKIFSGNHMGWYRQAPGKQRELVGIITSGGITDYADSVKGRFTISRDNS
KNTVYLQMNSLRPEDTAVYYCNVRDRTIWWGQGTLVTVSS

2LIG99; SEQ ID NO: 24:
Q1D_Q5V_A14P_D54G_T74S_K76N_S79Y_K86R_Q110L
DVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSGGTPAYADSAKGRFTISRDNS
KNTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS

Example 6: PD-L1 targeted AFNs based on selected 2LIG99 and 2LIG189 VHH variants

In this example, twelve PD-L1 targeted AFNs were generated and evaluated. For targeting two sequence optimized and humanized PD-L1 VHHs (2LIG99 and 2LIG189; see earlier) were used in a mono- or bi-valent format. Warheads included both the R149 (IFNa2_R149A) and A145G (IFNa2_A145G) variants of human IFNa2 or wild type human IFNa1. Residue C86 in IFNa1 was mutated to a S for stability reasons. VHHs and warheads were cloned into a Merchant based knob-in-hole Fc format.

Constructs

The following constructs were made by gene-synthesis (GeneArt). The Fc region contains domains CH2 and CH3 of human IgG1. Mutations in the Fc sequence include: LALA: L234A_L235A; KQ: 223Q; Hole_Merchant: Y349C_T366S_L368A_Y407V; Knob_Merchant: S354C_T366W.

1. 2LIG99-Fc3 (pcDNA3.4 2LIG99_opt-5*GGS-hIgG1-LALA-KQ-Hole_Merchant; **P-2204**) (SEQ ID NO: 171)
   MEFGLSWLFLVAILKGVQCDVQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELV
   ALITSGGTPAYADSAKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS
   GGSGGSGGSGGSGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
   EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTISK
   AKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF
   LVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
2. 2LIG189-Fc3 (pcDNA3.4 2LIG189_opt-5*GGS-hIgG1-LALA-KQ-Hole_Merchant; **P-2206**) (SEQ ID NO: 172)
   MEFGLSWLFLVAILKGVQCDVQLVESGGGLVQPGGSLRLSCAASGKIFSGNHMGWYRQAPGKQREL
   VGIITSGGITDYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNVRDRTIWWGQGTLVTVSSG
   GSGGSGGSGGSGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
   VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTISKA
   KGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
   VSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
3. (2LIG99)2-Fc3 (pcDNA3.4 2LIG99_opt-20*GGS2LIG99_opt -5*GGS-hIgG1-LALA-KQ-Hole_Merchant; **P-2399**) (SEQ ID NO: 173)
   MGWSCIIFFLVATATGVHSDVQLQESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELV
   ALITSGGTPAYADSAKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSS
   GGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSD
   VQLVESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSGGTPAYADSAKGRFTI
   SRDNSKNTVYLQMNSLRPEDTAVYYCHVSSGVYNYWGQGTLVTVSSGGSGGSGGSGGSGGSDKT
   HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
   PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDE

LTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVF
SCSVMHEALHNHYTQKSLSLSPGK

4. (2LIG189)2-Fc3 (pcDNA3.4 2LIG189_opt-20*GGS-2LIG189_opt -5*GGS-hIgG1-LALA-KQ-Hole_Merchant; **P-2400**) (SEQ ID NO: 174)
MGWSCIIFFLVATATGVHSDVQLQESGGGLVQPGGSLRLSCAASGKIFSGNHMGWYRQAPGKQREL
VGIITSGGITDYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNVRDRTIWWGQGTLVTVSSG
GSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSDV
QLVESGGGLVQPGGSLRLSCAASGKIFSGNHMGWYRQAPGKQRELVGIITSGGITDYADSVKGRFTI
SRDNSKNTVYLQMNSLRPEDTAVYYCNVRDRTIWWGQGTLVTVSSGGSGGSGGSGGSGGSDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTK
NQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK
5. Fc3 (pcDNA3.4 hIgG1-LALA-KQ-Hole_Merchant; **P-1542**) (SEQ ID NO: 175)
MKLPVRLLVLMFWIPASSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTI
SKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
6. Fc4-IFNa2_R149A (pcDNA3.4 huIgG1-LALA-KQ-Knob_Merchant–10*GGS-hIFNa2_R149A; **P-1483**) (SEQ ID NO: 176)
MKLPVRLLVLMFWIPASSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGGSGGSGGSGGSGGSG
GSGGSGGSGGSGCDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKAETIP
VLHEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKEDSILAVRKYF
QRITLYLKEKKYSPCAWEVVRAEIMASFSLSTNLQESLRSKE
7. Fc4-IFNa2_A145G (pcDNA3.4 huIgG1-LALA-KQ-Knob_Merchant–10*GGS-hIFNa2_A145G; **P-2157**) (SEQ ID NO: 177)
MKLPVRLLVLMFWIPASSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGGSGGSGGSGGSGGSG
GSGGSGGSGGSGCDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKAETIP
VLHEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKEDSILAVRKYF
QRITLYLKEKKYSPCAWEVVRGEIMRSFSLSTNLQESLRSKE

8. Fc4-IFNa1 (pcDNA3.4 huIgG1-LALA-KQ-Knob_Merchant–10*GGS-hIFNa1_C86S; **P-2213**) (SEQ ID NO: 178)
MKLPVRLLVLMFWIPASSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCQVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGGSGGSGGSGGSGGSG
GSGGSGGSGGSCDLPETHSLDNRRTLMLLAQMSRISPSSCLMDRHDFGFPQEEFDGNQFQKAPAIS
VLHELIQQIFNLFTTKDSSAAWDEDLLDKFSTELYQQLNDLEACVMQEERVGETPLMNADSILAVKKYF
RRITLYLTEKKYSPCAWEVVRAEIMRSLSLSTNLQERLRRKE

Production and purification

The following combinations of plasmids were transiently transfected in ExpiCHO cells (ThermoFisher Scientific) according to the manufacturer's guidelines:

1. **P-2204** + **P-1483**: 2LIG99-Fc3 + Fc4-IFNa2_R149A
2. **P-2206** + **P-1483**: 2LIG189-Fc3 + Fc4-IFNa2_R149A
3. **P-2399** + **P-1483**: (2LIG99)2-Fc3 + Fc4-IFNa2_R149A
4. **P-2400** + **P-1483**: (2LIG189)2-Fc3 + Fc4-IFNa2_R149A
5. **P-1542** + **P-1483**: Fc3 + Fc4-IFNa2_R149A
6. **P-2204** + **P-2157**: 2LIG99-Fc3 + Fc4-IFNa2_A145G
7. **P-2206** + **P-2157**: 2LIG189-Fc3 + Fc4-IFNa2_A145G
8. **P-2399** + **P-2157**: (2LIG99)2-Fc3 + Fc4-IFNa2_A145G
9. **P-2400** + **P-2157**: (2LIG189)2-Fc3 + Fc4-IFNa2_A145G
10. **P-1542** + **P-2157**: Fc3 + Fc4-IFNa2_A145G
11. **P-2204** + **P-2213**: 2LIG99-Fc3 + Fc4-IFNa1
12. **P-2206** + **P-2213**: 2LIG189-Fc3 + Fc4-IFNa1
13. **P-2399** + **P-2213**: (2LIG99)2-Fc3 + Fc4-IFNa1
14. **P-2400** + **P-2213**: (2LIG189)2-Fc3 + Fc4-IFNa1
15. **P-1542** + **P-2213**: Fc3 + Fc4-IFNa1

One week after transfection, supernatant was collected, and cells removed by centrifugation. Recombinant proteins were purified based on protein A binding properties (Hitrap MabSelect SuRe column, GE Healthcare) and by subsequent size exclusion chromatography (Superdex 200 increase HiScale 16/40 column, GE Healthcare), both on an Äkta purifier (GE Healthcare). Concentrations were measured with a spectrophotometer (NanoDrop instrument, Thermo Scientific), purity estimated on SDS-PAGE and endotoxin-levels quantified on the EndoSafe Nexgen instrument (Charles River).

Potency in the HL116 reporter cell-line

Biological activity of resulting PD-L1 VHH AFNs was tested on 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 of the different PD-L1 VHH AFNs or the untargeted control. Luciferase activity was measured on an EnSight Multimode Plate Reader (Perkin Elmer). **Fig. 28** illustrates clearly that all PD-L1 targeted AFNs were far more active than the untargeted variants.

Neutralization of the PD-1/PD-L1 Interaction

Programmed cell death protein 1 (PD-1) is a well characterized ligand for PD-L1. Here, the ability of the PD-L1 VHH AFNs to interfere with the PD-1/PD-L1 interaction was compared in a commercial AlphaLisa set-up according to the manufacturer's instructions (cat AL356; PerkinElmer). In brief, a biotinylated PD-1 binds to the Streptavidin-coated Alpha donor beads, while His-tagged PD-L1 is captured by anti-His AlphaLISA acceptor beads. When PD-L1 binding to PD-1 happens, donor beads and acceptor beads come into close proximity. The excitation of the donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the acceptor beads, resulting in a sharp peak of light emission at 615 nm. To evaluate neutralization of the interaction, the acceptor beads were pre-incubated with a serial dilution of PD-L1 VHH AFN before adding the donor beads. Data in **Fig. 29** clearly illustrate that all tested PD-L1 AFNs inhibited the interaction to the same extent (IC50 values between 350 and 400 pM) and that this neutralization was comparable to the that of the anti-PD-L1 Ab atezoluzimab.

Neutralization of the CD80/PD-L1 Interaction

A second known ligand for PD-L1 is cluster of differentiation 80 (CD80), also called B7-1. To determine whether the PD-L1 AFNs interfere with the CD80/PD-L1 interaction a plate-binding assay was set-up. Here, MaxiSorp plates (Nunc) were coated overnight with human PD-L1-mouse Fc (SinoBiologicals; 10084-H05H; 2 µg/ml in PBS). After washing and blocking (in 0.5% Casein in PBS), plates were incubated with a serial dilution PD-L1 VHH AFNs for 30 minutes. A sub-optimal concentration (here 2 µg/ml) biotinylated human CD80-His (SinoBiologicals; 10698-H08H-B) was added and binding was quantified with streptavidin-HRP (Jackson ImmunoResearch) and the TMB microwell peroxidase substrate (KPL). Like with the PD-1/PD-L1 assay, the different VHHs (mono- or bi-valent) inhibited to a comparable extent the CD80/PD-L1 interaction (IC50 values between 650 and 1100 pM) (**Fig. 30**). The neutralizing potency was independent of the warhead (IFNa2_R149A or IFNa1).

Affinity

Affinities of the resulting PD-L1 VHH AFN variants were measured using the bio-layer interferometry (BLI) technology on an Octet Red 96 instrument (ForteBio). In brief, recombinant human or cyno PD-L1 were immobilized on a sensor. Human PD-L1 (SinoBiologicals; 10084-H05H) was fused to mouse IgG Fc and loaded on an anti-murine IgG quantitation (AMQ) biosensors (ForteBio). Cyno PD-L1 (genetically fused to the human IgG Fc; SinoBiologicals; 90251-C02H) was biotinylated using the Pierce™ Antibody Biotinylation Kit for IP (ThermoFisher Scientific) and loaded onto a Streptavidin biosensor (ForteBio). Loaded sensors were incubated with a serial dilution of PD-L1 VHH AFN variant, association and dissociation monitored and kinetic parameters were calculated

using the Octet Data Analysis software v10 (ForteBio). Results summarized in **Fig. 31A**, **Fig. 31B**, and **Fig. 31C** illustrate that (i) 2LIG99 based AFNs have a higher affinity for both human and cyno PD-L1 than their 2LIG189 counterparts, (ii) the presence of two VHHs in the AFN leads to a 3- and 10-fold increase in affinity for respectively 2LIG99 and 2LIG189 AFNs, and (iii) affinities for human and cyno PD-L1 are comparable.

Epitope Binning

Bio-layer interferometry (BLI) technology was used to investigate to what extent the PD-L1 VHHs bind to overlapping epitopes on PD-L1. In brief, 2LIG99 and 2LIG189 AFNs were biotinylated using the Pierce™ Antibody Biotinylation Kit for IP (ThermoFisher Scientific) and loaded onto a Streptavidin biosensor (ForteBio). Subsequent binding of human PD-L1 (SinoBiologicals; 10084-H05H), or PD-L1 pre-incubated with 2LIG99 AFN or 2LIG189 AFN was monitored. Data in **Fig. 32A** and **Fig. 32B** clearly shows that an excess 2LIG189 AFN inhibits binding of PD-L1 to immobilized 2LIG99 AFN and vice versa, indicating that both VHHs bind overlapping epitopes.

Stability and Manufacturability

To gain insights into the stability of eight PD-L1 VHH AFNs, proteins were concentrated up to 10 mg/ml and subjected to 5 freeze (-20°C)-thaw-cycles. After each cycle the sample was centrifuged, and protein concentrations were measured on a Nanodrop spectrophotometer and no major impact on protein concentration was observed. Before and after the freeze-thaw cycles, the samples were analyzed by size exclusion chromatography (Superdex 200 increase HiScale 16/40 column, GE Healthcare) on an Äkta purifier (**Fig. 33A-H**). The relatively stable protein concentrations and the absence of higher-order aggregates in analytical SEC, illustrate that all variants displayed a good stability towards freeze-thaw.

Stability in Human Serum

In a parallel approach, the stability of the PD-L1 VHH AFNs in serum was tested. Proteins were diluted in human serum at 10 µg/ml and incubated for 7, 4, 2 or 0 days at 37°C. Biological activity of the incubated proteins was measured using the HL116 reporter cell-line. EC_{50} values (in ng/ml) of these stimulations are summarized in Table 6 and illustrate that incubation in serum at 37°C did not result in a decrease in biological activity, illustrating that all PD-L1 VHH AFNs are similarly stable under these conditions.

Table 6. Biological activity (represented by EC50) of AFNs on HL116 reporter after incubation in serum at 37°C.

	7 days	4 days	2 days	0 days
2LIG99-IFNa2_R149A	0,85	0,76	0,68	0,89
2LIG189-IFNa2_R149A	51,2	43,7	21,55	33,8
(2LIG99)2-IFNa2_R149A	0,058	0,096	0,101	0,047
(2LIG189)2-IFNa2_R149A	0,60	1,48	0,46	0,47
2LIG99-IFNa1	0,15	0,26	0,17	0,13
2LIG189-IFNa1	42,28	40,52	44,1	40,98
(2LIG99)2-IFNa1	0,027	0,030	0,055	0,045
(2LIG189)2-IFNa1	0,14	0,10	0,13	0,16

In Vivo Efficacy

To evaluate the efficacy of the PD-L1 VHH AFNs, the molecules were tested in a tumor model in a humanized mouse. In brief, newborn NSG mice (1-2 days of age) were sublethal irradiated with 100 cGy prior to intrahepatic delivery of $1x10^5$ $CD34^+$ human stem cells (from HLA-A2 positive cord bloods). At week 13 after stem cell transfer, mice were subcutaneously inoculated with $25x10^5$ human RL follicular lymphoma cells (ATCC CRL-2261; not sensitive to the direct anti-proliferative effect of IFN). Mice were treated weekly intravenously with indicated amounts of AFN (see **Fig. 34**), at day 12 and 19 after tumor inoculation (n=5-6 mice per group). Tumor size (caliper measurements) and, body weight was assessed every 2-3 days. Data in **Fig. 34** show the tumor growth until one week after the second treatment and demonstrates that (i) PD-L1 VHH AFNs strongly inhibited tumor growth as compared to an equimolar dosing of untargeted AFN (untargeted AFN did not reduce tumor growth compared to buffer treated animals); (ii) IFNa1 and A145G AFNs have similar activity as the R149A AFNs, albeit at about 10-fold lower dosing; and (iii) 2LIG99 based AFNs have similar potency as 2LIG89 based AFNs. Data on body weight did not show any major difference between buffer and AFN treatment, supporting that all AFN treatments were well tolerated..

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 targeting moiety comprising one or more recognition domains comprising:

(i) 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 or 5;

(b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and

(c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or

(ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1; and

wherein (i) or (ii) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1.

2. The PD-L1 targeting moiety of claim 1, further comprising one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

3. The PD-L1 targeting moiety of claim 1 or 2, wherein the mutation is a substitution, optionally wherein the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

4. The PD-L1 targeting moiety of any one of claims 1-3, wherein the mutation is selected from one or more of:

a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and

a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

5. The PD-L1 targeting moiety of any one of claims 1-4, wherein the mutation is selected from one or more of:

a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D;

a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V;

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P;

a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V;

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S,

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N,

a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y,

an arginine (R) at position K86, being K86R, and a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

6. The PD-L1 targeting moiety of any one of claims 1-5, wherein the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, K76N, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

7. The PD-L1 targeting moiety of any one of claims 1-6, wherein the targeting moiety is a full-length antibody, a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody, a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule, optionally wherein the targeting moiety comprises a variable domain heavy chain antibody (V_HH) or a humanized V_HH.

8. The PD-L1 targeting moiety of any of the above claims, wherein the targeting moiety recognizes and binds PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.

9. The PD-L1 targeting moiety of any of the above claims, wherein the targeting moiety recognizes and/or binds to its target without substantially neutralizing the target's activity or wherein the targeting moiety recognizes and/or binds to its target and substantially neutralizes the target's activity.

10. The PD-L1 targeting moiety of any one of the above claims, wherein the targeting moiety comprises one or more additional recognition domains.

11. The PD-L1 targeting moiety of claim 10, wherein the one or more additional recognition domain binds to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

12. The PD-L1 targeting moiety of any one of the above claims, wherein the targeting moiety recognizes and optionally functionally modulates a tumor antigen.

13. The PD-L1 targeting moiety of any one of the above claims, wherein the targeting moiety recognizes and optionally functionally modulates an antigen on an immune cell.

14. The PD-L1 targeting moiety of claim 13, wherein the immune cell is selected from a T cell, B cell, dendritic cell, macrophage, neutrophil, NK cell and NKT cell.

15. The PD-L1 targeting moiety of any of the above claims, wherein the targeting moiety recruits cytotoxic T cells to tumor cells or to the tumor environment.

16. The PD-L1 targeting moiety of any of the above claims, further comprising one or more (a) wild type signaling agents or (b) modified signaling agents that have reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

17. The PD-L1 targeting moiety of claim 16, wherein the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

18. The PD-L1 targeting moiety of claim 16, wherein the modifications in the modified signaling agent allow for attenuation of activity.

19. The PD-L1 targeting moiety of claim 16, wherein agonistic or antagonistic activity of the modified signaling agent is attenuated.

20. The PD-L1 targeting moiety of claims 16-19, 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 or mutated.

21. The PD-L1 targeting moiety of claim 20, wherein the signaling agent is selected from human: IFNα2, IFNα1, IFNβ, IFNγ, consensus interferon, TNF, TNFR, TGF-α, TGF-β, VEGF, EGF, PDGF, FGF, TRAIL, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IL-33, IGF-1, or EPO.

22. The PD-L1 targeting moiety of claim 21, wherein the human IFNα2 comprises one or more mutations selected from R33A, T106X_3, R120E, R144X_1, A145X_2, M148A, R149A, and L153A and with respect to the amino acid sequence of SEQ ID NO: 81 or 82, wherein X_1 is selected from A, S, T, Y, L, and I, wherein X_2 is selected from G, H, Y, K, and D, and wherein X_3 is selected from A and E.

23. The PD-L1 targeting moiety of claim 21, wherein the human IFNα1 comprises one or more mutations selected from A146G, C86X_1, and M149X_2 and with respect to the amino acid sequence of SEQ ID NO: 83, wherein X_1 is selected from A, Y, and S, and wherein X_2 is selected from V and A.

24. The PD-L1 targeting moiety of claim 21, wherein the human IFNβ comprises one or more mutations selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, and R152G with respect to the amino acid sequence of SEQ ID NO: 84.

25. The PD-L1 targeting moiety of claim 21, wherein the human IL-1β comprises one or more mutations selected from A117G/P118G, R120G, R120A, L122A, T125G/L126G, R127G, Q130A, Q130W, Q131G, K132A, S137G/Q138Y, L145G, H146A, H146G, H146E, H146N, H146R, L145A/L147A, Q148E, Q148G, Q148L, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209A, K209D, K209A/K210A, K219S, K219Q, E221S, E221K, E221S/N224A, N224S/K225S, E244K, and N245Q with respect to the amino acid sequence of SEQ ID NO: 100.

26. The PD-L1 targeting moiety of claim 21, wherein the human IL-2 comprises one or more mutations selected from R38A, F42A, Y45A, E62A, N88R, N88I, N88G, D20H, Q126L, Q126F, D109, and C125 with respect to the amino acid sequence of SEQ ID NO: 101.

27. The PD-L1 targeting moiety of claim 21, wherein the human TNFα comprises one or more mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y870, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G, A145T, and Y87Q/I97A with respect to the amino acid sequence of SEQ ID NO: 97.

28. The PD-L1 targeting moiety of any of the above claims, wherein the PD-L1 targeting moiety binds PD-L1 with improved affinity as compared to PD-L1 targeting moiety of SEQ ID NO: 1.

29. A Fc-based chimeric protein complex comprising:

(A) a targeting moiety comprising:

(a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2 or 5;

(ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 3 or 6; and

(iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 4 or 7; or

(b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 1 and

wherein (a) or (b) further comprises one or more mutations at positions D54 and G55, numbering relative to SEQ ID NO: 1; and

(B) a signaling agent, wherein the signaling agent is:

a) a wild type signaling agent; or

b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and

(C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

30. The Fc-based chimeric protein complex of claim 29, wherein the targeting moiety further comprising one or more mutations at positions Q1, Q5, A14, A63, T74, K76, S79, K86, and Q110.

31. The Fc-based chimeric protein complex of claim 29 or 30, wherein the mutation is a substitution, optionally wherein the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), an aromatic, polar and positively charged hydrophilic residue including histidine (H), a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) or a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V), or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

32. The Fc-based chimeric protein complex of any one of claims 29-31, wherein the mutation is selected from one or more of:

a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position D54, optionally being D54G, or a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K), optionally being D54K, or a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C), optionally being D54T and

a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) at position G55, optionally being G55R.

33. The Fc-based chimeric protein complex of any one of claims 29-32, wherein the mutation is selected from one or more of:

a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E) at position Q1, optionally being Q1D;

a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q5, optionally being Q5V;

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position A14, optionally being A14P;

a hydrophobic, aliphatic amino acid selected from glycine (G), leucine (L), isoleucine (I), methionine (M), and valine (V) at position A63, optionally being A63V;

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), proline (P), and cysteine (C) at position T74, optionally being T74S;

a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C) at position K76, optionally being K76N;

a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y) at position S79, optionally being S79Y;

an arginine (R) position K86, being K86R;and

a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) at position Q110, optionally being Q110L.

34. The Fc-based chimeric protein complex of any one of claims 29-33, wherein the mutation is selected from one or more of Q1D, Q5V, A14P, A63V, T74S, K76N, S79Y, K86R, and Q110L, optionally all of Q1D, Q5V, A14P, D54G, T74S, K76N, S79Y, K86R, and Q110L.

35. The Fc-based chimeric protein complex of any one of claims 29-34, wherein the targeting moiety is a full-length antibody, a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody, a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule, optionally wherein the targeting moiety comprises a variable domain heavy chain antibody (V_HH), or a humanized V_HH.

36. The Fc-based chimeric protein complex of any of claims 29-35, wherein the targeting moiety recognizes and binds PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.

37. The Fc-based chimeric protein complex of any of claims 29-35, wherein the targeting moiety recognizes and/or binds to its target without substantially neutralizing the target's activity or wherein the targeting moiety recognizes and/or binds to its target and substantially neutralizes the target's activity.

38. The Fc-based chimeric protein complex of any of claims 29-37, further comprising one or more additional targeting moieties.

39. The Fc-based chimeric protein complex of claim 38, wherein the one or more additional targeting moieties bind to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

40. The Fc-based chimeric protein complex of any one of claims 29-39, wherein the targeting moiety recognizes and optionally functionally modulates a tumor antigen.

41. The Fc-based chimeric protein complex of any one of the claims 29-40, wherein the targeting moiety recognizes and optionally functionally modulates an antigen on an immune cell.

42. The Fc-based chimeric protein complex of claim 41, wherein the immune cell is selected from a T cell, B cell, dendritic cell, macrophage, neutrophil, NK cell and NKT cell.

43. The Fc-based chimeric protein complex of any of claims 29-42, wherein the targeting moiety recruits cytotoxic T cells to tumor cells or to the tumor environment.

44. The Fc-based chimeric protein complex of claim 29, further comprising one or more linkers.

45. The Fc-based chimeric protein complex of claim 44, wherein the Fc domain is selected from IgG, IgA, IgD, IgM, or IgE.

46. The Fc-based chimeric protein complex of claim 45, wherein the IgG is selected from IgG1, IgG2, IgG3, or IgG4.

47. The Fc-based chimeric protein complex of claim 46, wherein the Fc domain is selected from human IgG, IgA, IgD, IgM, or IgE.

48. The Fc-based chimeric protein complex of claim 47, wherein the human IgG is selected from human IgG1, IgG2, IgG3, or IgG4.

49. The Fc-based chimeric protein complex of any one of claims 29-48, wherein the signaling agent is a modified signaling agent and has reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

50. The Fc-based chimeric protein complex of claim 49, wherein the signaling agent is a modified signaling agent and the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

51. The Fc-based chimeric protein complex of any one of claims 29-50, wherein the Fc chain pairing is promoted by ionic pairing and/or a knob-in-hole pairing.

52. The Fc-based chimeric protein complex of any one of claims 29-51, wherein the one or more mutations to the Fc domain results in an ionic pairing between the Fc chains in the Fc domain.

53. The Fc-based chimeric protein complex of any one of claims 29-52, wherein the one or more mutations to the Fc domain results in a knob-in-hole pairing of the Fc domain.

54. The Fc-based chimeric protein complex of any one of claims 29-53, wherein the one or more mutations to the Fc domain results in the reduction or elimination of the effector function of the Fc domain.

55. The Fc-based chimeric protein complex of any one of claims 29-54, wherein the complex is a homodimer or a heterodimer.

56. The Fc-based chimeric protein complex of any one claims 29-55, wherein Fc-based chimeric protein complex has a configuration and/or orientation as shown in any one of **FIGs. 9A-F, 10A-H, 11A-H, 12A-D, 13A-F, 14A-J, 15A-D, 16A-F, 17A-J, 18A-F, 19A-L, 20A-L, 21A-F, 22A-L, 23A-L, 24A-J, 25A-J, 26A-F,** and **27A-F**.

57. The Fc-based chimeric protein complex of claim 56, wherein Fc-based chimeric protein complex has a configuration and/or orientation as shown in **FIG. 15B**.

58. The Fc-based chimeric protein complex of any one claims 29-57, wherein the Fc-based chimeric protein complex has a trans orientation/configuration, as relates to any targeting moiety and signaling agent, relative to each other, and/or any targeting moieties relative to each other, and/or any signaling agents relative to each other.

59. The Fc-based chimeric protein complex of any one claims 29-58, wherein the Fc-based chimeric protein complex has a cis orientation/configuration, as relates to any targeting moiety and signaling agent, relative to each other, and/or any targeting moieties relative to each other, and/or any signaling agents relative to each other.

60. The Fc-based chimeric protein complex of any one claims 29-59, wherein the Fc comprises L234A, L235A, and one additional mutation selected from K322A, K322Q, D265A, P329G, and P331S substitutions in human IgG1, wherein the numbering is based on the EU convention.

61. The Fc-based chimeric protein complex of any one claims 29-60, wherein the Fc comprises a S228P substitution in human IgG4, wherein the numbering is based on the EU convention.

62. The Fc-based chimeric protein complex of any of claims 29-61, wherein the modified signaling agent has a reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

63. The Fc-based chimeric protein complex of any of claims 29-61, wherein the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

64. The Fc-based chimeric protein complex of claim 63, wherein the modifications in the modified signaling agent allow for attenuation of activity.

65. The Fc-based chimeric protein complex of claim 63, wherein agonistic or antagonistic activity of the modified signaling agent is attenuated.

66. The Fc-based chimeric protein complex of claims 29-65, 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 or mutated.

67. The Fc-based chimeric protein complex of claim 66, wherein the signaling agent is selected from human: IFNα2, IFNα1, IFNβ, IFNγ, consensus interferon, TNF, TNFR, TGF-α, TGF-β, VEGF, EGF, PDGF, FGF, TRAIL, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IL-33, IGF-1, or EPO.

68. The Fc-based chimeric protein complex of claim 67, wherein the human IFNα2 comprises one or more mutations selected from R33A, T106X_3, R120E, R144X_1 A145X_2, M148A, R149A, and L153A and with respect to

the amino acid sequence of SEQ ID NO: 81 or 82, wherein X_1 is selected from A, S, T, Y, L, and I, wherein X_2 is selected from G, H, Y, K, and D, and wherein X_3 is selected from A and E.

69. The Fc-based chimeric protein complex of claim 67, wherein the human IFNα1 comprises one or more mutations selected from A146G, C86X_1, and M149X_2 and with respect to the amino acid sequence of SEQ ID NO: 83, wherein X_1 is selected from A, Y, and S, and wherein X_2 is selected from V and A.

70. The Fc-based chimeric protein complex of claim 67, wherein the human IFNβ comprises one or more mutations selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, and R152G with respect to the amino acid sequence of SEQ ID NO: 84.

71. The Fc-based chimeric protein complex of claim 67, wherein the human IL-1β comprises one or more mutations selected from A117G/P118G, R120G, R120A, L122A, T125G/L126G, R127G, Q130A, Q130W, Q131G, K132A, S137G/Q138Y, L145G, H146A, H146G, H146E, H146N, H146R, L145A/L147A, Q148E, Q148G, Q148L, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209A, K209D, K209A/K210A, K219S, K219Q, E221S, E221K, E221S/N224A, N224S/K225S, E244K, and N245Q with respect to the amino acid sequence of SEQ ID NO: 100.

72. The Fc-based chimeric protein complex of claim 67, wherein the human IL-2 comprises one or more mutations selected from R38A, F42A, Y45A, E62A, N88R, N88I, N88G, D20H, Q126L, Q126F, D109, and C125 with respect to the amino acid sequence of SEQ ID NO: 101.

73. The Fc-based chimeric protein complex of claim 67, wherein the human TNFα comprises one or more mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y870, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G, A145T, and Y87Q/I97A with respect to the amino acid sequence of SEQ ID NO: 97.

74. The Fc-based chimeric protein complex of claim 67, wherein the signaling agent is a modified IFNα2, optionally with a R149A mutation with respect to the amino acid sequence of SEQ ID NO: 81 or 82.

75. A PD-L1 targeting moiety comprising a recognition domain comprising:

(i) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30;

(b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and

(c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or

(ii) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26; and

wherein (i) or (ii) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26.

76. The PD-L1 targeting moiety of claim 75, wherein the mutation is a substitution.

77. The PD-L1 targeting moiety of claim 75, wherein the substitution is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) or an aromatic, polar and positively charged hydrophilic residue including histidine (H).

78. The PD-L1 targeting moiety of claim 75, wherein the substitution is a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C).

79. The PD-L1 targeting moiety of claim 75, wherein the substitution is a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E).

80. The PD-L1 targeting moiety of claim 75, wherein the substitution is a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

81. The PD-L1 targeting moiety of claim 75, wherein the substitution at position N32 is a positive hydrophilic residue is selected from arginine (R) and lysine (K).

82. The PD-L1 targeting moiety of claim 75, wherein the substitution at position N32 is polar and neutral hydrophilic residue is selected from glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C).

83. The PD-L1 targeting moiety of claim 81 or 82, wherein the substitution at position N32 is N32Q or N32R.

84. The PD-L1 targeting moiety of claim 75, wherein the substitution at position D33 is D33H.

85. The PD-L1 targeting moiety of claim 75, wherein the substitution at position M97 is aliphatic hydrophobic residues are selected from glycine (G), leucine (L), isoleucine (I), and valine (V).

86. The PD-L1 targeting moiety of claim 85, wherein the substitution at position M97 is M97I, M97L, or M97V.

87. The PD-L1 targeting moiety of any one of claims 75-86, further comprising one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, M97V, and Q109L.

88. The PD-L1 targeting moiety of any one of claims 75-87, wherein the targeting moiety is a full-length antibody, a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody, a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule.

89. The PD-L1 targeting moiety of claim 88, wherein the targeting moiety comprises a variable domain heavy chain antibody (V_HH) or a humanized V_HH.

90. The PD-L1 targeting moiety of any of claims 75-89, wherein the targeting moiety recognizes and binds PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.

91. The PD-L1 targeting moiety of any of claims 75-90, wherein the targeting moiety recognizes and/or binds to its target without substantially neutralizing the target's activity or wherein the targeting moiety recognizes and/or binds to its target and substantially neutralizes the target's activity.

92. The PD-L1 targeting moiety of any one of claims 75-91, wherein the targeting moiety comprises one or more additional recognition domains.

93. The PD-L1 targeting moiety of claim 92, wherein the one or more additional recognition domain binds to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

94. The PD-L1 targeting moiety of any one of the claims 75-93, wherein the recognition domains recognize and optionally functionally modulate a tumor antigen.

95. The PD-L1 targeting moiety of any one of the claims 75-94, wherein the targeting moiety recognizes and optionally functionally modulates an antigen on an immune cell.

96. The PD-L1 targeting moiety of claim 95, wherein the immune cell is selected from a T cell, B cell, dendritic cell, macrophage, neutrophil, NK cell and NKT cell.

97. The PD-L1 targeting moiety of any of the claims 75-96, wherein the targeting moiety recruits cytotoxic T cells to tumor cells or to the tumor environment.

98. The PD-L1 targeting moiety of any of the claims 75-97, further comprising one or more (a) wild type signaling agents or (b) modified signaling agents that have reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

99. The PD-L1 targeting moiety of claim 98, wherein the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

100. The PD-L1 targeting moiety of claim 98, wherein the modifications in the modified signaling agent allow for attenuation of activity.

101. The PD-L1 targeting moiety of claim 98, wherein agonistic or antagonistic activity of the modified signaling agent is attenuated.

102. The PD-L1 targeting moiety of claims 98-101, 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 or mutated.

103. The PD-L1 targeting moiety of claim 102, wherein the signaling agent is selected from human: IFNα2, IFNα1, IFNβ, IFNγ, consensus interferon, TNF, TNFR, TGF-α, TGF-β, VEGF, EGF, PDGF, FGF, TRAIL, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IL-33, IGF-1, or EPO.

104. The PD-L1 targeting moiety of claim 103, wherein the human IFNα2 comprises one or more mutations selected from R33A, T106X_3, R120E, R144X_1 A145X_2, M148A, R149A, and L153A and with respect to the amino

acid sequence of SEQ ID NO: 81 or 82, wherein X_1 is selected from A, S, T, Y, L, and I, wherein X_2 is selected from G, H, Y, K, and D, and wherein X_3 is selected from A and E.

105. The PD-L1 targeting moiety of claim 103, wherein the human IFNα1 comprises one or more mutations selected from A146G, C86X_1, and M149X_2 and with respect to the amino acid sequence of SEQ ID NO: 83, wherein X_1 is selected from A, Y, and S, and wherein X_2 is selected from V and A.

106. The PD-L1 targeting moiety of claim 103, wherein the human IFNβ comprises one or more mutations selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, and R152G with respect to the amino acid sequence of SEQ ID NO: 84.

107. The PD-L1 targeting moiety of claim 103, wherein the human IL-1β comprises one or more mutations selected from A117G/P118G, R120G, R120A, L122A, T125G/L126G, R127G, Q130A, Q130W, Q131G, K132A, S137G/Q138Y, L145G, H146A, H146G, H146E, H146N, H146R, L145A/L147A, Q148E, Q148G, Q148L, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209A, K209D, K209A/K210A, K219S, K219Q, E221S, E221K, E221S/N224A, N224S/K225S, E244K, and N245Q with respect to the amino acid sequence of SEQ ID NO: 100.

108. The PD-L1 targeting moiety of claim 103, wherein the human IL-2 comprises one or more mutations selected from R38A, F42A, Y45A, E62A, N88R, N88I, N88G, D20H, Q126L, Q126F, D109, and C125 with respect to the amino acid sequence of SEQ ID NO: 101.

109. The PD-L1 targeting moiety of claim 103, wherein the human TNFα comprises one or more mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y870, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G, A145T, and Y87Q/I97A with respect to the amino acid sequence of SEQ ID NO: 97.

110. The PD-L1 targeting moiety of any one of claims 75 to 109, wherein the PD-L1 targeting moiety binds PD-L1 with improved affinity as compared to PD-L1 targeting moiety of SEQ ID NO: 26.

111. A Fc-based chimeric protein complex comprising:

(A) a targeting moiety comprising:

(a) three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(i) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 27 or 30;

(ii) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 28 or 31; and

(iii) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 29 or 32; or

(b) an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 26 and

wherein (a) or (b) further comprises one or more mutations at positions N32, D33, and M97, numbering relative to SEQ ID NO: 26; and

(B) a signaling agent, wherein the signaling agent is:

a) a wild type signaling agent; or

b) a modified signaling agent that has one or more mutations that confer improved safety relative to the wild type signaling agent; and

(C) a Fc domain, the Fc domain optionally having one or more mutations that reduces or eliminates one or more effector functions of the Fc domain, promotes Fc chain pairing in the Fc domain, and/or stabilizes a hinge region in the Fc domain.

112. The Fc-based chimeric protein complex of claim 111, wherein the mutation is a substitution.

113. The Fc-based chimeric protein complex of claim 111, wherein the substitution is a hydrophilic amino acid residue that is a polar and positively charged hydrophilic residue selected from arginine (R) and lysine (K) or an aromatic, polar and positively charged hydrophilic residue including histidine (H).

114. The Fc-based chimeric protein complex of claim 111, wherein the substitution is a hydrophilic amino acid residue that is a polar and neutral of charge hydrophilic residue selected from asparagine (N), glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C).

115. The Fc-based chimeric protein complex of claim 111, wherein the substitution is a polar and negatively charged hydrophilic residue selected from aspartate (D) and glutamate (E).

116. The Fc-based chimeric protein complex of claim 111, wherein the substitution is a hydrophobic, aliphatic amino acid selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) or a hydrophobic, aromatic amino acid selected from phenylalanine (F), tryptophan (W), and tyrosine (Y).

117. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position N32 is a positive hydrophilic residue is selected from arginine (R) and lysine (K).

118. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position N32 is polar and neutral hydrophilic residue is selected from glutamine (Q), serine (S), threonine (T), proline (P), and cysteine (C).

119. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position N32 is N32Q or N32R.

120. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position D33 is D33H.

121. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position M97 is aliphatic hydrophobic residues are selected from glycine (G), leucine (L), isoleucine (I), and valine (V).

122. The Fc-based chimeric protein complex of claim 111, wherein the substitution at position M97 is M97I, M97L, or M97V.

123. The Fc-based chimeric protein complex of any one of claims 111-122, further comprising one or more of the following mutations Q1D, Q5V, A14P, A62S, A74S, M77T, M78V, K86R, and Q109L, optionally all of Q1D, Q5V, A14P, D33H, A62S, A74S, M77T, M78V, K86R, and M97V.

124. The Fc-based chimeric protein complex of any one of claims 111-123, wherein the targeting moiety is a full-length antibody, a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody, a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule.

125. The Fc-based chimeric protein complex of claim 124, wherein the targeting moiety comprises a variable domain heavy chain antibody (V_HH), or a humanized V_HH.

126. The Fc-based chimeric protein complex of any of claims 111-125, wherein the targeting moiety recognizes and binds PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.

127. The Fc-based chimeric protein complex of any of claims 111-126, wherein the targeting moiety recognizes and/or binds to its target without substantially neutralizing the target's activity or wherein the targeting moiety recognizes and/or binds to its target and substantially neutralizes the target's activity.

128. The Fc-based chimeric protein complex of any of claims 111-127, further comprising one or more additional targeting moieties.

129. The Fc-based chimeric protein complex of claim 128, wherein the one or more additional targeting moieties bind to CD8, CD13, CD20, NKp46, Clec9A, Clec4c, PD-1, PD-L1, PD-L2, SIRP1α, FAP, XCR1, tenascin CA1, Flt3, or an ECM protein.

130. The Fc-based chimeric protein complex of any one of claims 111-129, wherein the recognition domains recognize and optionally functionally modulate a tumor antigen.

131. The Fc-based chimeric protein complex of any one of the claims 111-129, wherein the targeting moiety recognizes and optionally functionally modulates an antigen on an immune cell.

132. The Fc-based chimeric protein complex of claim 131, wherein the immune cell is selected from a T cell, B cell, dendritic cell, macrophage, neutrophil, NK cell and NKT cell.

133. The Fc-based chimeric protein complex of any of claims 111-132, wherein the targeting moiety recruits cytotoxic T cells to tumor cells or to the tumor environment.

134. The Fc-based chimeric protein complex of claim 133, further comprising one or more linkers.

135. The Fc-based chimeric protein complex of claim 111, wherein the Fc domain is selected from IgG, IgA, IgD, IgM, or IgE.

136. The Fc-based chimeric protein complex of claim 135, wherein the IgG is selected from IgG1, IgG2, IgG3, or IgG4.

137. The Fc-based chimeric protein complex of claim 111, wherein the Fc domain is selected from human IgG, IgA, IgD, IgM, or IgE.

138. The Fc-based chimeric protein complex of claim 137, wherein the human IgG is selected from human IgG1, IgG2, IgG3, or IgG4.

139. The Fc-based chimeric protein complex of any one of claims 108-135, wherein the signaling agent is a modified signaling agent and has reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

140. The Fc-based chimeric protein complex of claim 139, wherein the signaling agent is a modified signaling agent and the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

141. The Fc-based chimeric protein complex of any one of claims 111-140, wherein the Fc chain pairing is promoted by ionic pairing and/or a knob-in-hole pairing.

142. The Fc-based chimeric protein complex of any one of claims 111-141, wherein the one or more mutations to the Fc domain results in an ionic pairing between the Fc chains in the Fc domain.

143. The Fc-based chimeric protein complex of any one of claims 111-142, wherein the one or more mutations to the Fc domain results in a knob-in-hole pairing of the Fc domain.

144. The Fc-based chimeric protein complex of any one of claims 111-143, wherein the one or more mutations to the Fc domain results in the reduction or elimination of the effector function of the Fc domain.

145. The Fc-based chimeric protein complex of any one of claims 111-144, wherein the complex is a homodimer or a heterodimer.

146. The Fc-based chimeric protein complex of any one claims 111-145, wherein Fc-based chimeric protein complex has a configuration and/or orientation as shown in any one of **FIGs. 9A-F, 10A-H, 11A-H, 12A-D, 13A-F, 14A-J, 15A-D, 16A-F, 17A-J, 18A-F, 19A-L, 20A-L, 21A-F, 22A-L, 23A-L, 24A-J, 25A-J, 26A-F,** and **27A-F**.

147. The Fc-based chimeric protein complex of claim 146, wherein Fc-based chimeric protein complex has a configuration and/or orientation as shown in **FIG. 15B**.

148. The Fc-based chimeric protein complex of any one claims 111-147, wherein the Fc-based chimeric protein complex has a trans orientation/configuration, as relates to any targeting moiety and signaling agent, relative to each other, and/or any targeting moieties relative to each other, and/or any signaling agents relative to each other.

149. The Fc-based chimeric protein complex of any one claims 111-147, wherein the Fc-based chimeric protein complex has a cis orientation/configuration, as relates to any targeting moiety and signaling agent, relative

to each other, and/or any targeting moieties relative to each other, and/or any signaling agents relative to each other.

150. The Fc-based chimeric protein complex of any one claims 111-149, wherein the Fc comprises L234A, L235A, and one additional mutation selected from K322A, K322Q, D265A, P329G, and P331S substitutions in human IgG1, wherein the numbering is based on the EU convention.

151. The Fc-based chimeric protein complex of any one claims 111-150, wherein the Fc comprises a S228P substitution in human IgG4, wherein the numbering is based on the EU convention.

152. The Fc-based chimeric protein complex of any of claims 111-151, wherein the signaling agent has a reduced affinity or activity at the signaling agent's receptor relative to a wild type signaling agent.

153. The Fc-based chimeric protein complex of any of claims 111-153, wherein the targeting moiety restores the modified signaling agent's affinity or activity at the signaling agent's receptor.

154. The Fc-based chimeric protein complex of claim 153, wherein the modifications in the modified signaling agent allow for attenuation of activity.

155. The Fc-based chimeric protein complex of claim 153, wherein agonistic or antagonistic activity of the modified signaling agent is attenuated.

156. The Fc-based chimeric protein complex of claims 111-155, 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 or mutated.

157. The Fc-based chimeric protein complex of claim 156, wherein the signaling agent is selected from human: IFNα2, IFNα1, IFNβ, IFNγ, consensus interferon, TNF, TNFR, TGF-α, TGF-β, VEGF, EGF, PDGF, FGF, TRAIL, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IL-33, IGF-1, or EPO.

158. The Fc-based chimeric protein complex of claim 156, wherein the human IFNα2 comprises one or more mutations selected from R33A, T106X_3, R120E, R144X_1 A145X_2, M148A, R149A, and L153A and with respect to the amino acid sequence of SEQ ID NO: 81 or 82, wherein X_1 is selected from A, S, T, Y, L, and I, wherein X_2 is selected from G, H, Y, K, and D, and wherein X_3 is selected from A and E.

159. The Fc-based chimeric protein complex of claim 156, wherein the human IFNα1 comprises one or more mutations selected from A146G, C86X_1, and M149X_2 and with respect to the amino acid sequence of SEQ ID NO: 83, wherein X_1 is selected from A, Y, and S, and wherein X_2 is selected from V and A.

160. The Fc-based chimeric protein complex of claim 156, wherein the human IFNβ comprises one or more mutations selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, and R152G with respect to the amino acid sequence of SEQ ID NO: 84.

161. The Fc-based chimeric protein complex of claim 156, wherein the human IL-1β comprises one or more mutations selected from A117G/P118G, R120G, R120A, L122A, T125G/L126G, R127G, Q130A, Q130W, Q131G,

K132A, S137G/Q138Y, L145G, H146A, H146G, H146E, H146N, H146R, L145A/L147A, Q148E, Q148G, Q148L, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209A, K209D, K209A/K210A, K219S, K219Q, E221S, E221K, E221S/N224A, N224S/K225S, E244K, and N245Q with respect to the amino acid sequence of SEQ ID NO: 100.

162. The Fc-based chimeric protein complex of claim 156, wherein the human IL-2 comprises one or more mutations selected from R38A, F42A, Y45A, E62A, N88R, N88I, N88G, D20H, Q126L, Q126F, D109, and C125 with respect to the amino acid sequence of SEQ ID NO: 101.

163. The Fc-based chimeric protein complex of claim 156, wherein the human TNFα comprises one or more mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y870, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G, A145T, and Y87Q/I97A with respect to the amino acid sequence of SEQ ID NO: 97.

164. The Fc-based chimeric protein complex of claim 156, wherein the signaling agent is a modified IFNα2, optionally with a R149A mutation with respect to the amino acid sequence of SEQ ID NO: 1 or 2.

165. A recombinant nucleic acid composition encoding the PD-L1 targeting moiety of any one of claims 1-28 and 75-110.

166. A host cell comprising a nucleic acid of claim 165.

167. The PD-L1 targeting moiety of any one of claims 1-28 and 75-110, wherein the targeting moiety is suitable for use in a patient having one or more of: cancer, infections, immune disorders, and/or autoimmune diseases.

168. A method for treating or preventing cancer, comprising administering to a patient in need thereof an effective amount of:

(a) the targeting moiety of any one of claims 1-28 and 75-110; or

(b) the Fc-based chimeric protein complex of any one of claims 29-74 and 111-164.

169. The method of claim 168, 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.

170. A method for treating or preventing an autoimmune and/or neurodegenerative disease, comprising administering to a patient in need thereof an effective amount of:

(a) the targeting moiety of any one of claims 1-28 and 75-110; or

(b) the Fc-based chimeric protein complex of any one of claims 29-74 and 111-164.

171. The method of claim 170, wherein the autoimmune and/or neurodegenerative disease is selected from multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (*e.g.*, prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, myasthenia gravis, Reiter's syndrome, and Grave's disease.

172. The method of claim 171, wherein the autoimmune and/or neurodegenerative disease is multiple sclerosis.

173. Use of the targeting moiety of any one of claims 1-28 and 75-110 for treating or preventing an autoimmune disease, neurodegenerative disease, metabolic disease, and/or cardiovascular disease.

174. Use of the targeting moiety of any one of claims 1-28 and 75-110 for the preparation of a medicament for the treatment of prevention of an autoimmune disease, neurodegenerative disease, metabolic disease, and/or cardiovascular disease.

175. An PD-L1 targeting moiety comprising an amino acid sequence having at least 90% sequence identity with any one of amino acid sequences selected from SEQ ID NO: 1, 8-26, and 33-74.

176. A method for treating or preventing cancer, comprising administering to a patient in need thereof an effective amount of the PD-L1 targeting moiety of claim 175.

177. A method for treating or preventing an autoimmune disease, neurodegenerative disease, metabolic disease, and/or cardiovascular disease, comprising administering to a patient in need thereof an effective amount of the PD-L1 targeting moiety of claim 175.

Fig. 1

2LIG99 VHH WILD-TYPE SEQUENCE (CDRS ANNOTATED ACCORDING TO **KABAT**)

QVQLQESGGGLVQAGGSLRLSCTAS**GTIFS**INRMDWFRQAPGKQRELVALITSDGTPA**YADSAKG**RFTISR
DNTKKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSS

Fig. 2

2LIG189 SEQUENCES (CDRS ANNOTATED ACCORDING TO **KABAT**)

QVQLQESGGGLVQAGGSLRLSCAAS**GKIFS**GNDMGWYRQAPGKQRELVGIITSGGITD**YADAVKG**RFTIS
RDNAKNMMYLQMNSLKPEDTAVYYCNMRDRTIWWGQGTQVTVSS

FIG. 3

	SEQ ID	Construct	Mutation	Affinity in Octet KD (M)	kon(1/Ms)	kdis(1/s)	Ratio KD
2LIG99 humanisation	SEQ ID 1	P-1658	2LIG99	2,82E-10	1,58E+06	4,47E-04	**1,00**
	SEQ ID 8	P-1659	2LIG99_OPT1	7,94E-10	8,86E+05	7,04E-04	**0,35**
	SEQ ID 9	P-1660	2LIG99_OPT2	1,48E-09	4,45E+05	6,58E-04	**0,19**
	SEQ ID 10	P-1661	2LIG99_OPT3	ND	ND	ND	**ND**
	SEQ ID 11	P-1662	2LIG99_OPT4	4,52E-10	6,71E+05	3,04E-04	**0,62**
	SEQ ID 12	P-1663	2LIG99_OPT5	1,24E-10	6,07E+05	7,55E-05	**2,27**
	SEQ ID 13	P-1664	2LIG99_OPT6	1,03E-08	1,21E+05	1,24E-03	**0,03**
2LIG99 isomerisation	SEQ ID 14	P-1665	2LIG99_D55G	ND	1,28E+06	ND	**ND**
	SEQ ID 15	P-1666	2LIG99_D55K	4,59E-10	1,53E+06	7,00E-04	**0,61**
	SEQ ID 16	P-1667	2LIG99_D55T	1,48E-09	2,95E+05	4,36E-04	**0,19**
	SEQ ID 17	P-1668	2LIG99_G56R	1,01E-09	7,53E+05	7,61E-04	**0,28**

FIG. 4

SEQ_ID	Construct	Mutation	Affinity in Octet					
			KD (M)	*KD Error*	ka (1/Ms)	*ka Error*	kdis (1/s)	*kdis Error*
SEQ1	P-1658	2LIG99	7.19E-10	*3.43E-12*	1.00E+06	*2.40E+03*	7.21E-04	*2.97E-06*
SEQ18	P-2049	2LIG99_OPT_D54G	5.53E-10	*2.71E-12*	1.55E+06	*4.54E+03*	8.56E-04	*3.36E-06*
SEQ19	P-2050	2LIG99_OPT_D54G_A63V	1.16E-09	*4.12E-12*	1.30E+06	*3.53E+03*	1.51E-03	*3.48E-06*
SEQ20	P-2051	2LIG99_OPT_D54G_K76N	3.58E-10	*1.87E-12*	1.67E+06	*4.21E+03*	5.99E-04	*2.74E-06*
SEQ21	P-2052	2LIG99_OPT_D54G_S79Y	1.82E-10	*1.61E-12*	1.54E+06	*3.32E+03*	2.79E-04	*2.40E-06*
SEQ22	P-2053	2LIG99_OPT_D54G_A63V_K76N	6.02E-10	*2.67E-12*	1.87E+06	*5.86E+03*	1.12E-03	*3.52E-06*
SEQ23	P-2054	2LIG99_OPT_D54G_A63V_S79Y	4.27E-10	*2.07E-12*	1.83E+06	*5.11E+03*	7.79E-04	*3.09E-06*
SEQ24	P-2055	2LIG99_OPT_D54G_K76N_S79Y	4.45E-11	*2.32E-12*	1.24E+06	*3.16E+03*	5.52E-05	*2.88E-06*
SEQ25	P-2056	2LIG99_OPT_D54G_A63V_K76N_S79Y	7.35E-10	*6.28E-12*	9.21E+05	*3.70E+03*	6.77E-04	*5.10E-06*

FIG. 5

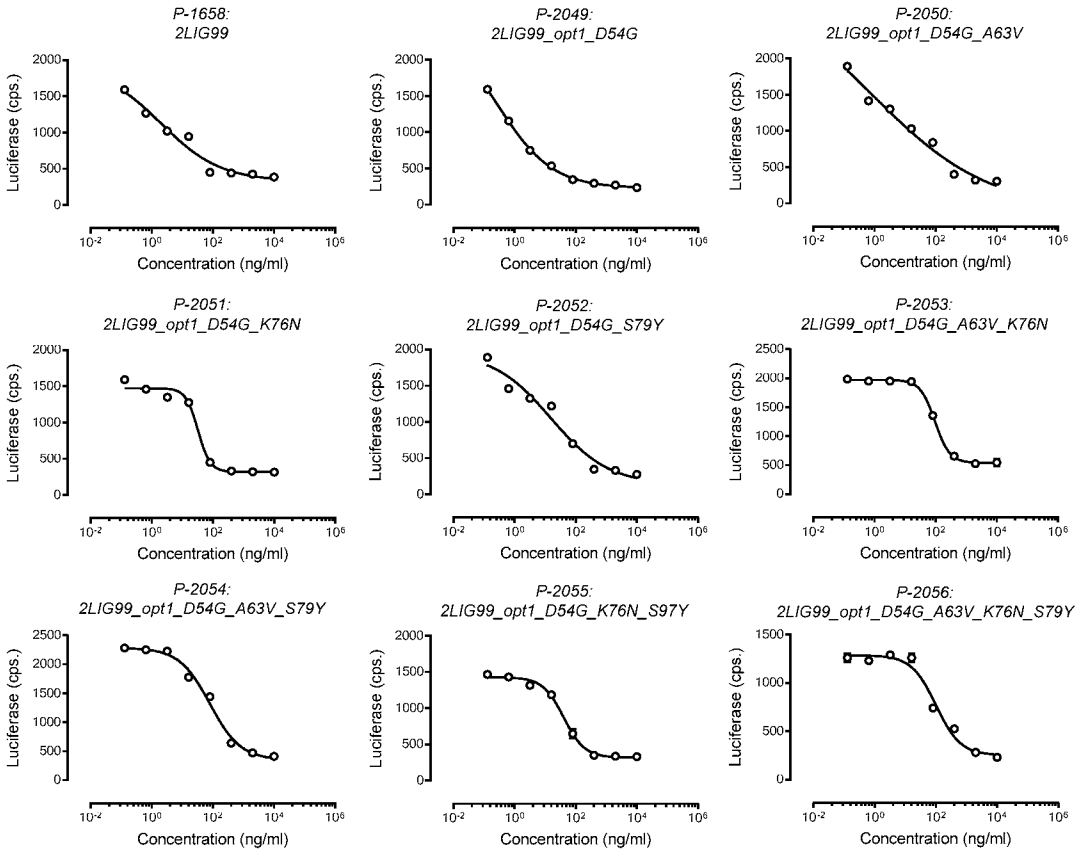

P-1658:
2LIG99
P-2049:
2LIG99_opt1_D54G
P-2050:
2LIG99_opt1_D54G_A63V
P-2051:
2LIG99_opt1_D54G_K76N
P-2052:
2LIG99_opt1_D54G_S79Y
P-2053:
2LIG99_opt1_D54G_A63V_K76N
P-2054:
2LIG99_opt1_D54G_A63V_S79Y
P-2055:
2LIG99_opt1_D54G_K76N_S97Y
P-2056:
2LIG99_opt1_D54G_A63V_K76N_S79Y
Luciferase (cps.)
Concentration (ng/ml)

FIG. 6

				Affinity in Octet			
	SEQ ID	Construct	Mutation	KD (M)	kon(1/Ms)	kdis(1/s)	Ratio KD
2UG189 humanisation	SEQ ID 26	P-1669	2UG189	7,91E-10	1,87E+05	1,48E-04	1,00
	SEQ ID 33	P-1670	2UG189_OPT1	6,92E-09	7,18E+04	4,97E-04	8,75
	SEQ ID 34	P-1671	2UG189_OPT2	1,28E-09	2,34E+05	2,99E-04	1,62
	SEQ ID 35	P-1672	2UG189_OPT3	4,89E-09	1,93E+05	9,42E-04	6,18
	SEQ ID 36	P-1673	2UG189_OPT4	1,74E-09	1,74E+05	3,01E-04	2,19
2UG189 deamidation	SEQ ID 37	P-1674	2UG189_N32A	2,70E-09	2,72E+05	7,38E-04	3,41
	SEQ ID 38	P-1675	2UG189_N32E	5,42E-10	1,82E+05	9,85E-05	0,68
	SEQ ID 39	P-1676	2UG189_N32F	5,50E-10	3,20E+05	1,76E-04	0,70
	SEQ ID 40	P-1677	2UG189_N32G	1,90E-08	1,20E+05	2,28E-03	23,98
	SEQ ID 41	P-1678	2UG189_N32H	1,81E-09	2,47E+05	4,46E-04	2,28
	SEQ ID 42	P-1679	2UG189_N32I	6,51E-09	1,34E+05	8,72E-04	8,23
	SEQ ID 43	P-1680	2UG189_N32L	1,62E-09	1,21E+05	1,95E-04	2,04
	SEQ ID 44	P-1681	2UG189_N32Q	1,25E-10	7,05E+04	8,84E-06	0,16
	SEQ ID 45	P-1682	2UG189_N32R	<1,0E-12	3,51E+05	<1,0E-07	0
	SEQ ID 46	P-1683	2UG189_N32S	1,80E-09	1,63E+05	2,92E-04	2,27
	SEQ ID 47	P-1684	2UG189_N32T	6,31E-09	1,64E+05	1,04E-03	7,97
	SEQ ID 48	P-1685	2UG189_N32V	3,02E-09	2,98E+05	9,00E-04	3,82
	SEQ ID 49	P-1686	2UG189_N32Y	2,04E-09	3,08E+05	6,29E-04	2,58
	SEQ ID 50	P-1687	2UG189_D33E	4,12E-08	2,14E+05	8,83E-03	52,09
	SEQ ID 51	P-1688	2UG189_D33F	9,78E-09	1,09E+06	1,07E-02	12,37
	SEQ ID 52	P-1689	2UG189_D33H	3,72E-10	4,21E+05	1,57E-04	0,47
	SEQ ID 53	P-1690	2UG189_D33I	no binding			
	SEQ ID 54	P-1691	2UG189_D33L	1,64E-08	2,08E+06	3,42E-02	20,72
	SEQ ID 55	P-1692	2UG189_D33Q	2,50E-08	4,74E+05	1,18E-02	31,57
	SEQ ID 56	P-1693	2UG189_D33R	no binding			
	SEQ ID 57	P-1694	2UG189_D33V	no binding			
	SEQ ID 58	P-1695	2UG189_D33Y	2,23E-09	3,75E+06	8,35E-03	2,82
2UG189 oxidation	SEQ ID 59	P-1696	2UG189_M97E	no binding			
	SEQ ID 60	P-1697	2UG189_M97F	1,51E-09	1,38E+05	2,08E-04	1,90
	SEQ ID 61	P-1698	2UG189_M97H	no binding			
	SEQ ID 62	P-1699	2UG189_M97I	7,41E-12	4,65E+04	3,45E-07	0,01
	SEQ ID 63	P-1700	2UG189_M97L	1,15E-11	4,25E+04	4,88E-07	0,01
	SEQ ID 64	P-1701	2UG189_M97Q	no binding			
	SEQ ID 65	P-1702	2UG189_M97R	no binding			
	SEQ ID 66	P-1703	2UG189_M97V	5,99E-12	6,37E+04	3,81E-07	0,01
	SEQ ID 67	P-1704	2UG189_M97Y	4,82E-09	3,03E+05	1,46E-03	6,09

FIG. 7

SEQ_ID	Construct	Mutation	Affinity in Octet					
			KD (M)	KD Error	ka (1/Ms)	ka Error	kdis (1/s)	kdis Error
SEQ26	P-1669	2LIG189	5,41E-09	3,02E-11	2,43E+05	9,91E+02	1,31E-03	5,00E-06
SEQ68	P-2043	2LIG189_OPT_N32R	1,94E-09	1,14E-11	3,77E+05	1,12E+03	7,31E-04	3,70E-06
SEQ69	P-2044	2LIG189_OPT_D33H	3,24E-10	8,81E-12	4,46E+05	1,44E+03	1,45E-04	3,90E-06
SEQ70	P-2045	2LIG189_OPT_N32R_M97V	3,38E-09	5,86E-11	1,66E+05	1,39E+03	5,60E-04	8,54E-06
SEQ71	P-2046	2LIG189_OPT_N32R_M97I	2,32E-09	2,05E-11	1,97E+05	6,90E+02	4,57E-04	3,71E-06
SEQ72	P-2047	2LIG189_OPT_D33H_M97V	1,44E-10	8,74E-12	5,36E+05	1,95E+03	7,71E-05	4,67E-06
SEQ73	P-2048	2LIG189_OPT_D33H_M97I	5,55E-10	5,41E-12	5,93E+05	1,47E+03	3,29E-04	3,10E-06

FIG. 8

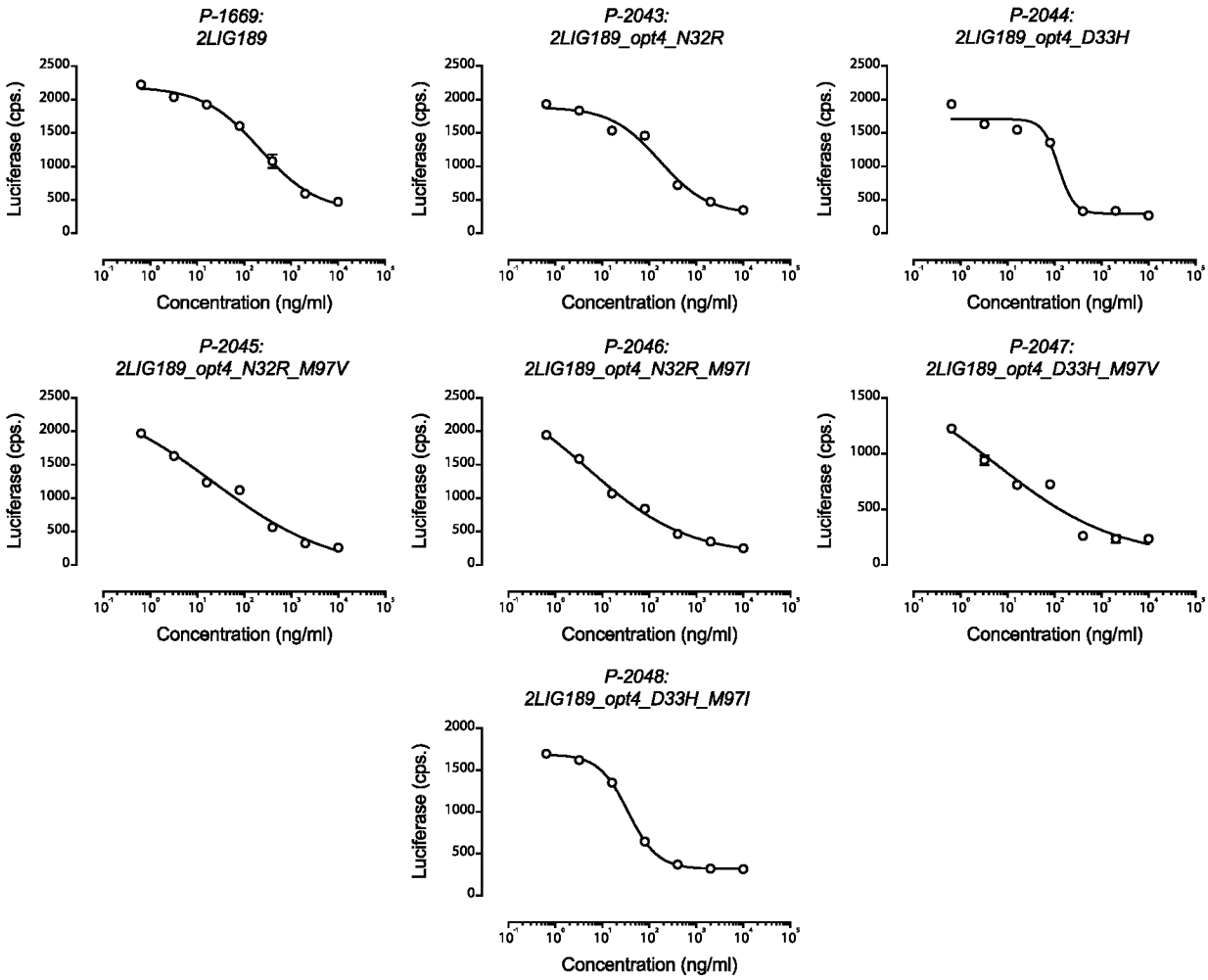

P-1669:
2LIG189
P-2043:
2LIG189_opt4_N32R
P-2044:
2LIG189_opt4_D33H
P-2045:
2LIG189_opt4_N32R_M97V
P-2046:
2LIG189_opt4_N32R_M97I
P-2047:
2LIG189_opt4_D33H_M97V
P-2048:
2LIG189_opt4_D33H_M97I
Luciferase (cps.)
Concentration (ng/ml)

FIG. 9A FIG. 9B FIG. 9C FIG. 9D FIG. 9E FIG. 9F

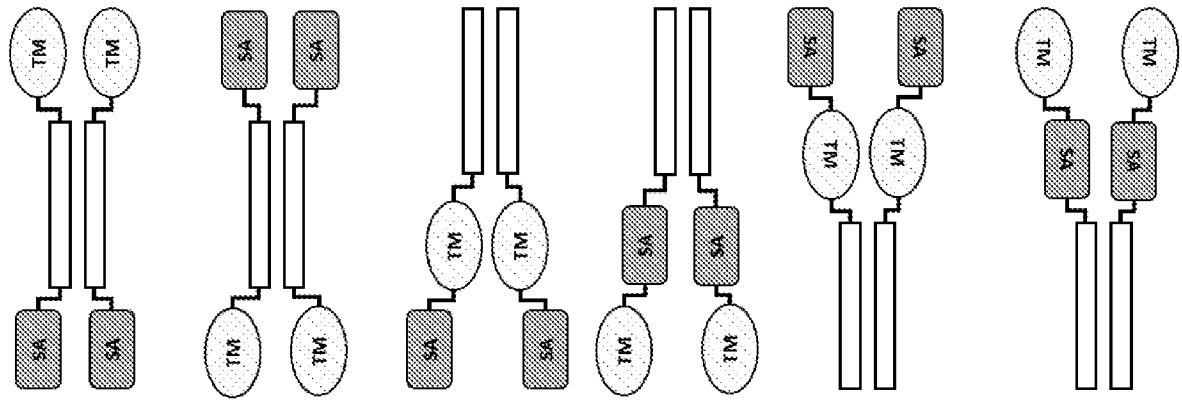


FIG. 10A FIG. 10B FIG. 10C FIG. 10D

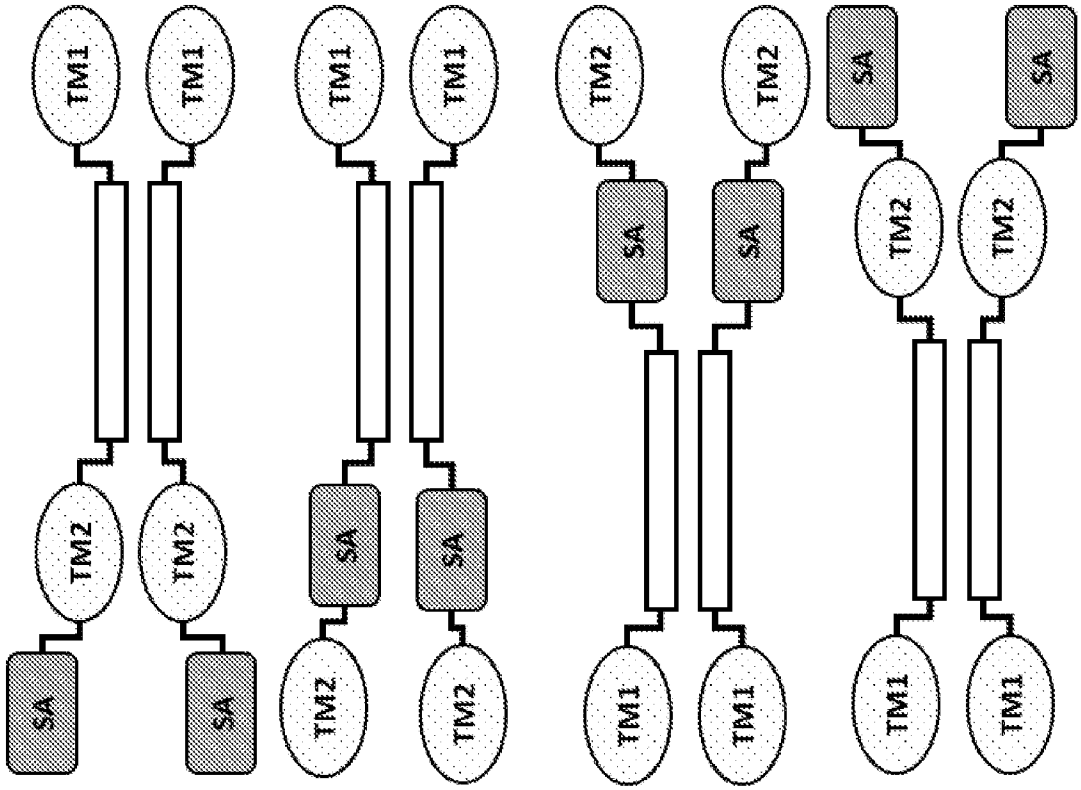


FIG. 10E

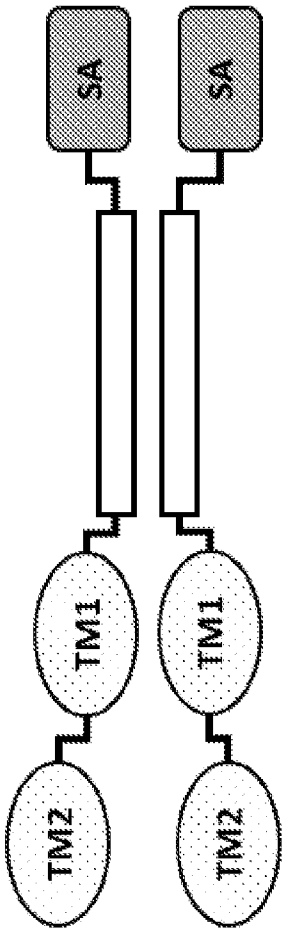


FIG. 10F

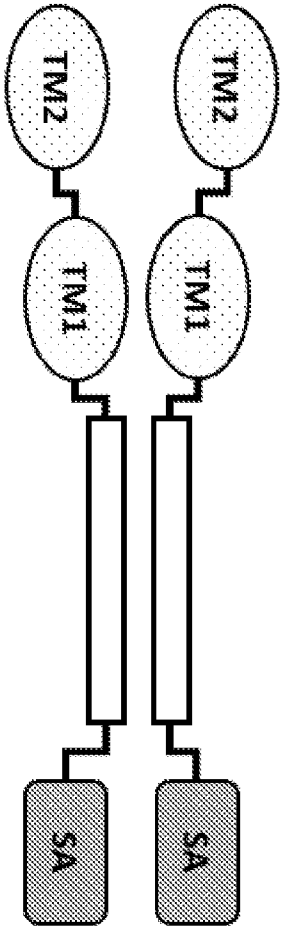


FIG. 10G

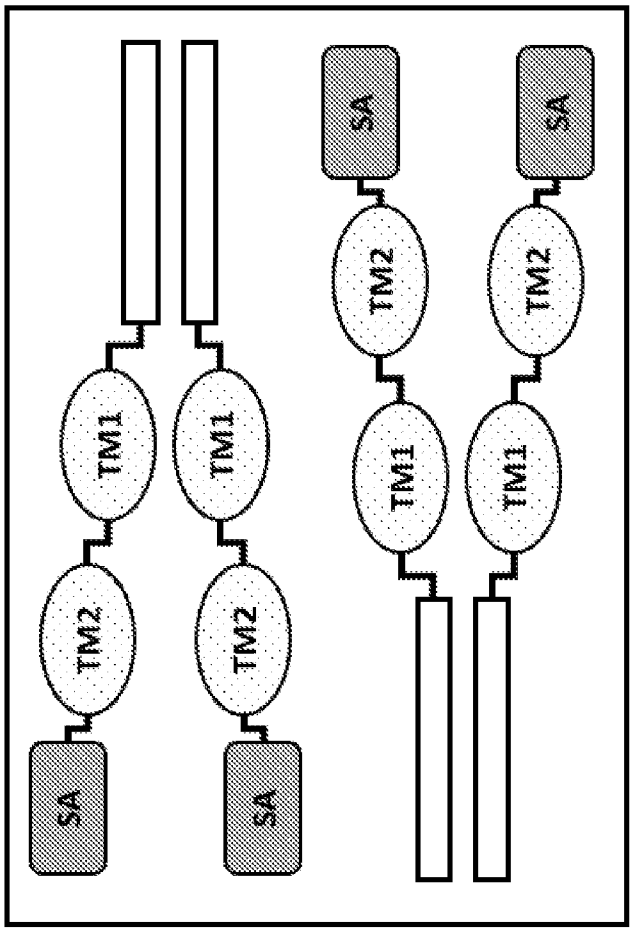


FIG. 10H

FIG. 11A FIG. 11B FIG. 11C FIG. 11D

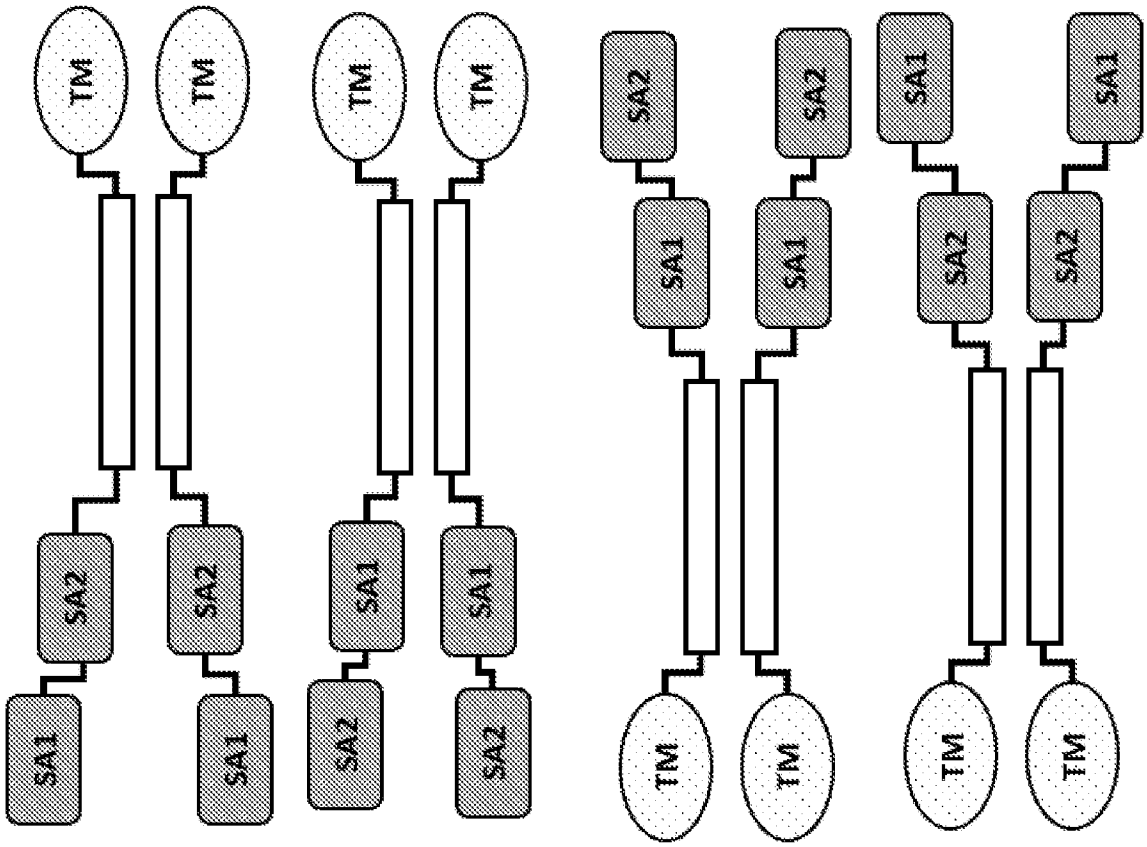


FIG. 11E

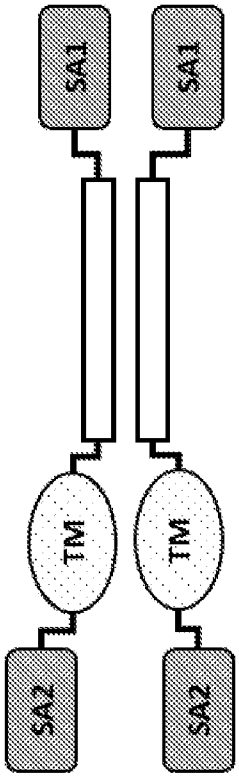


FIG. 11F

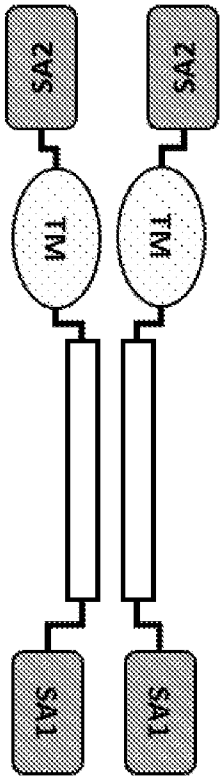


FIG. 11G

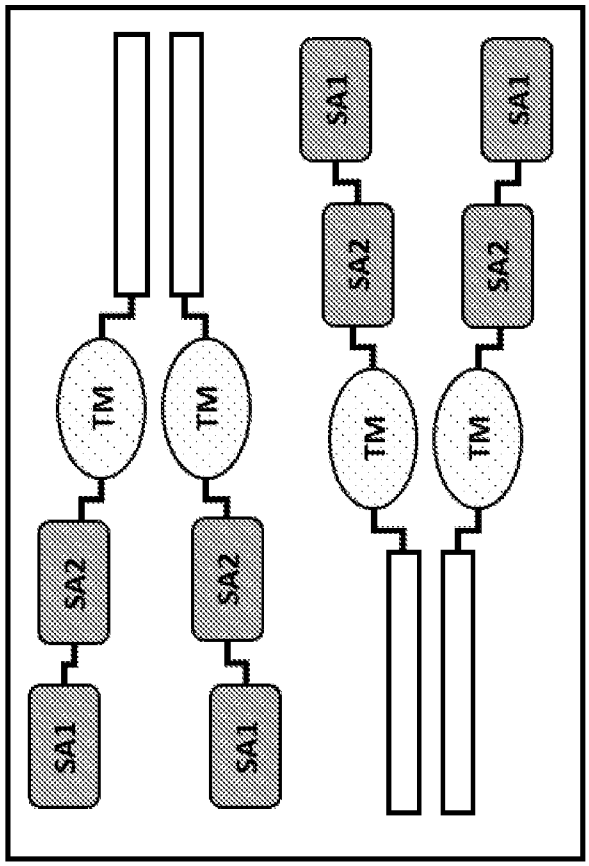


FIG. 11H

FIG. 12A FIG. 12B FIG. 12C FIG. 12D

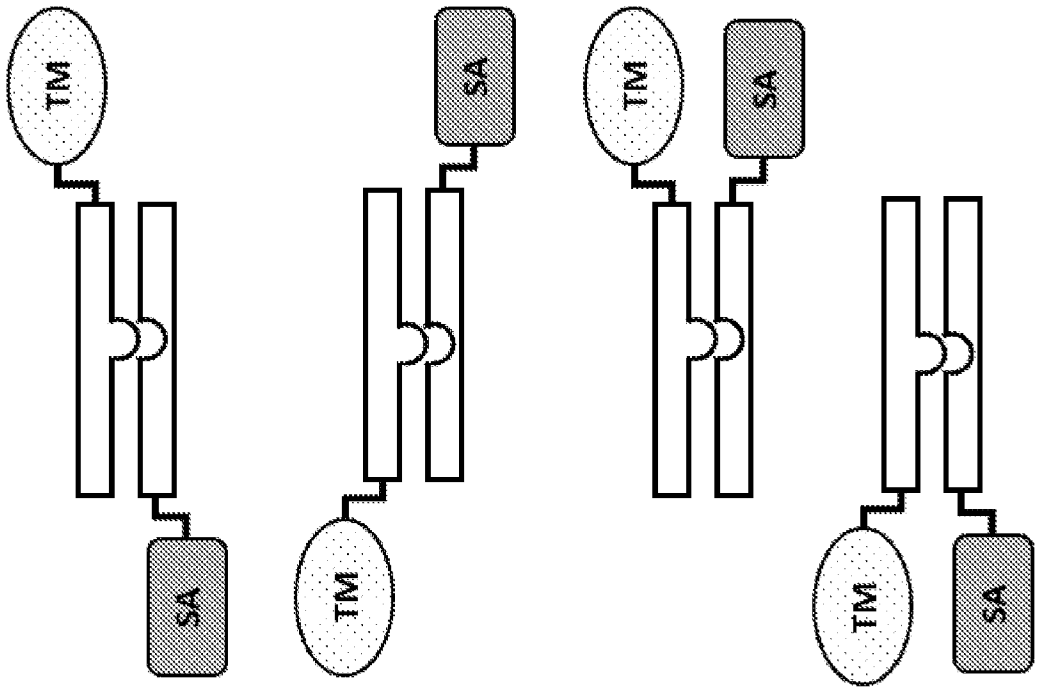


FIG. 13A FIG. 13B FIG. 13C FIG. 13D FIG. 13E FIG. 13F

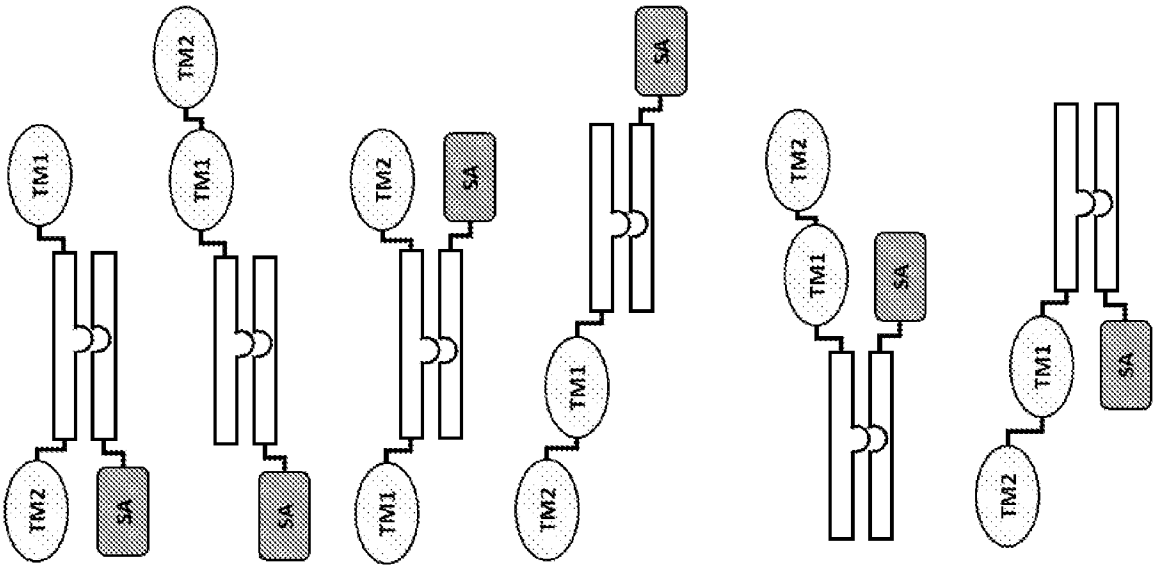


FIG. 14A FIG. 14B FIG. 14C FIG. 14D FIG. 14E FIG. 14F

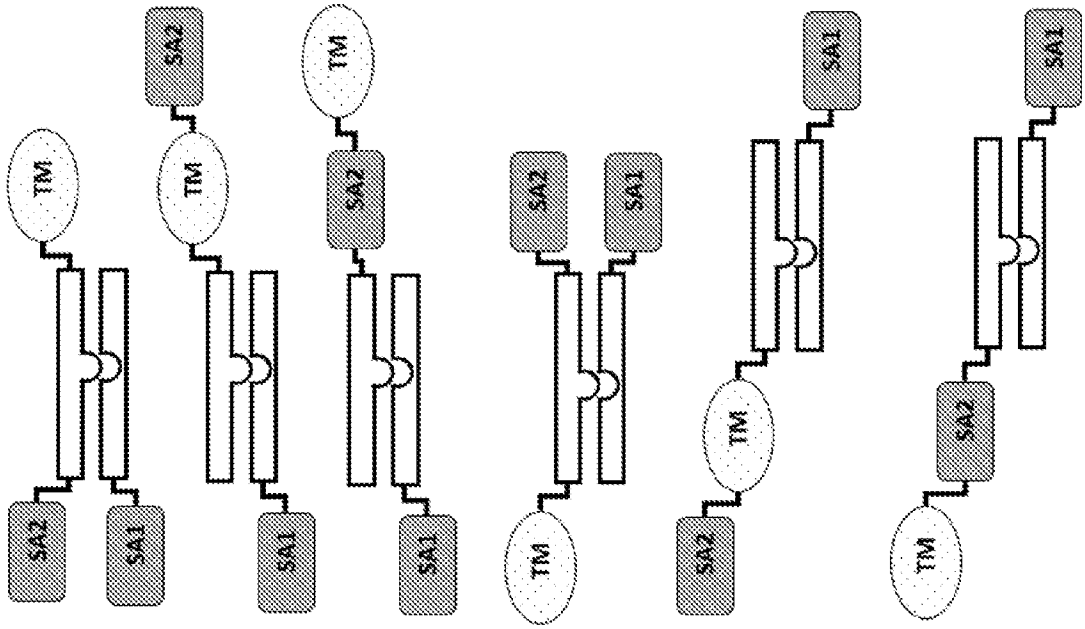


FIG. 14G

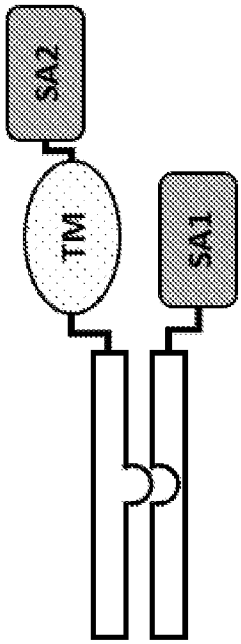


FIG. 14H

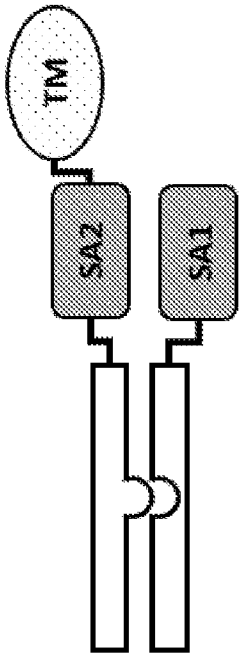


FIG. 14I

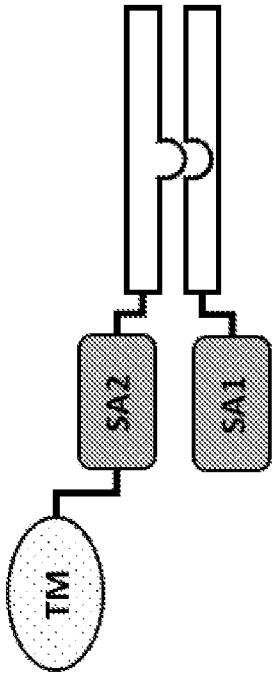


FIG. 14J

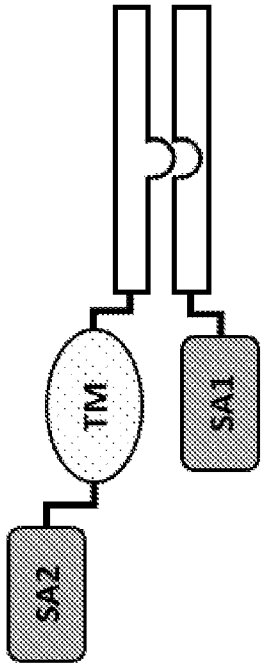


FIG. 15A FIG. 15B FIG. 15C FIG. 15D

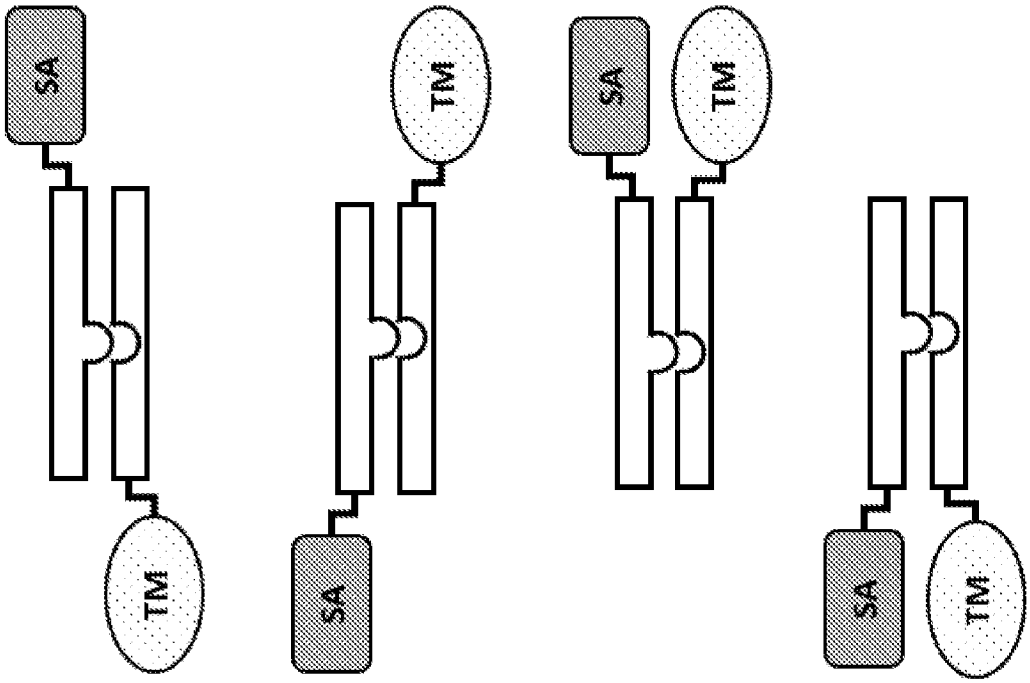


FIG. 16A FIG. 16B FIG. 16C FIG. 16D FIG. 16E FIG. 16F

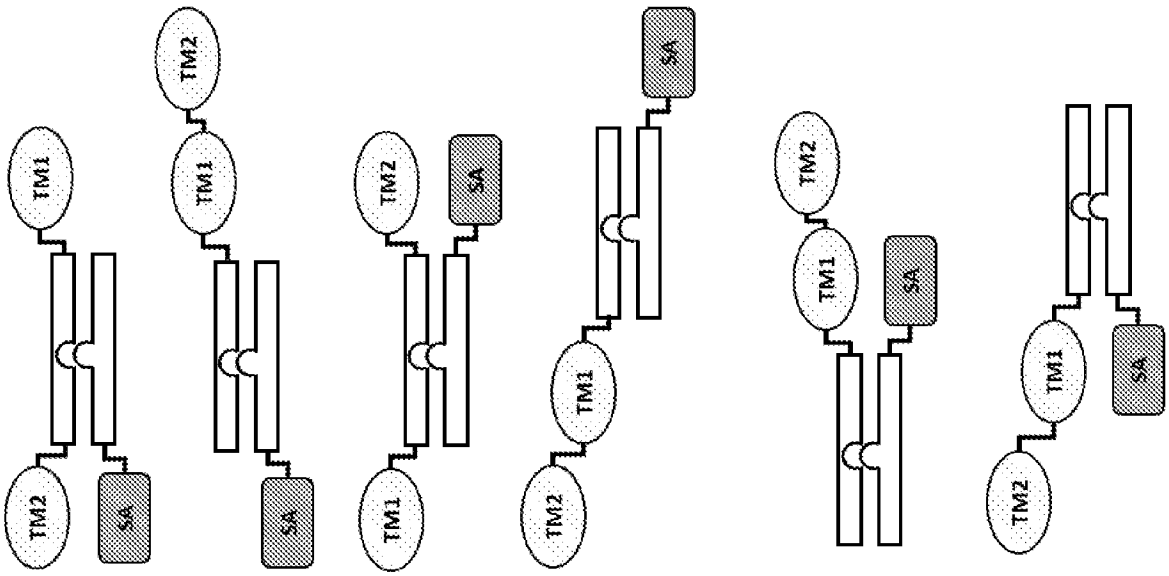


FIG. 17A FIG. 17B FIG. 17C FIG. 17D FIG. 17E

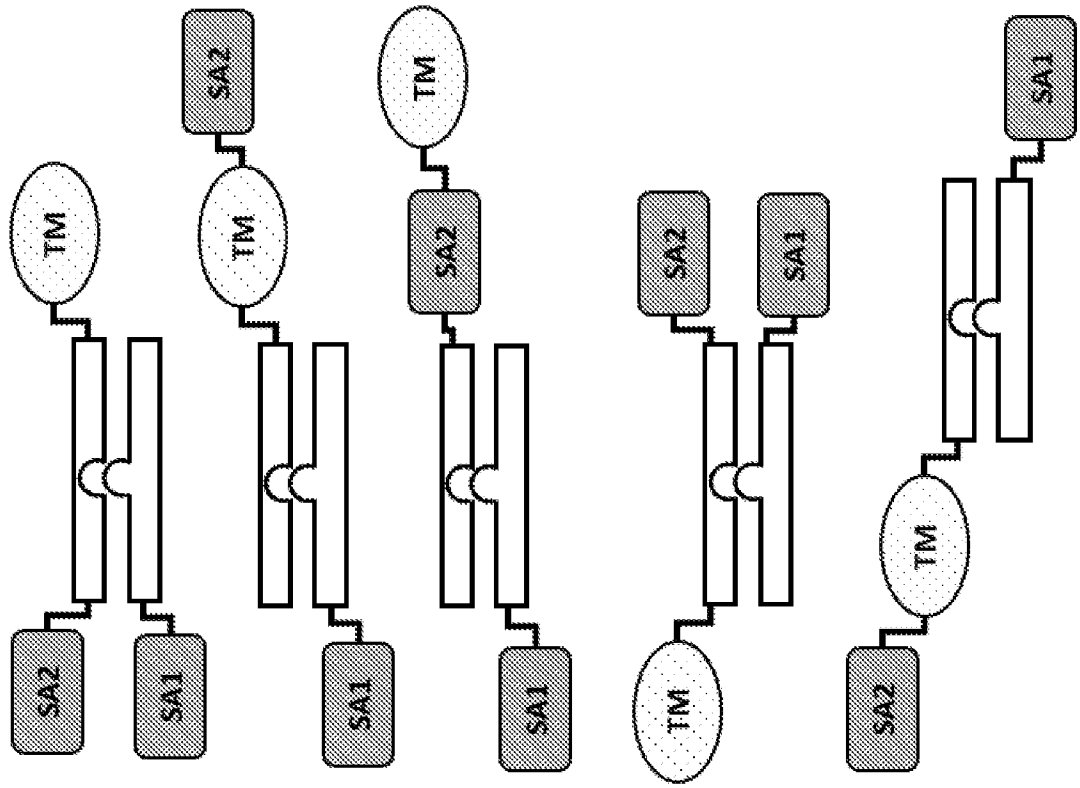


FIG. 17F FIG. 17G FIG. 17H FIG. 17I FIG. 17J

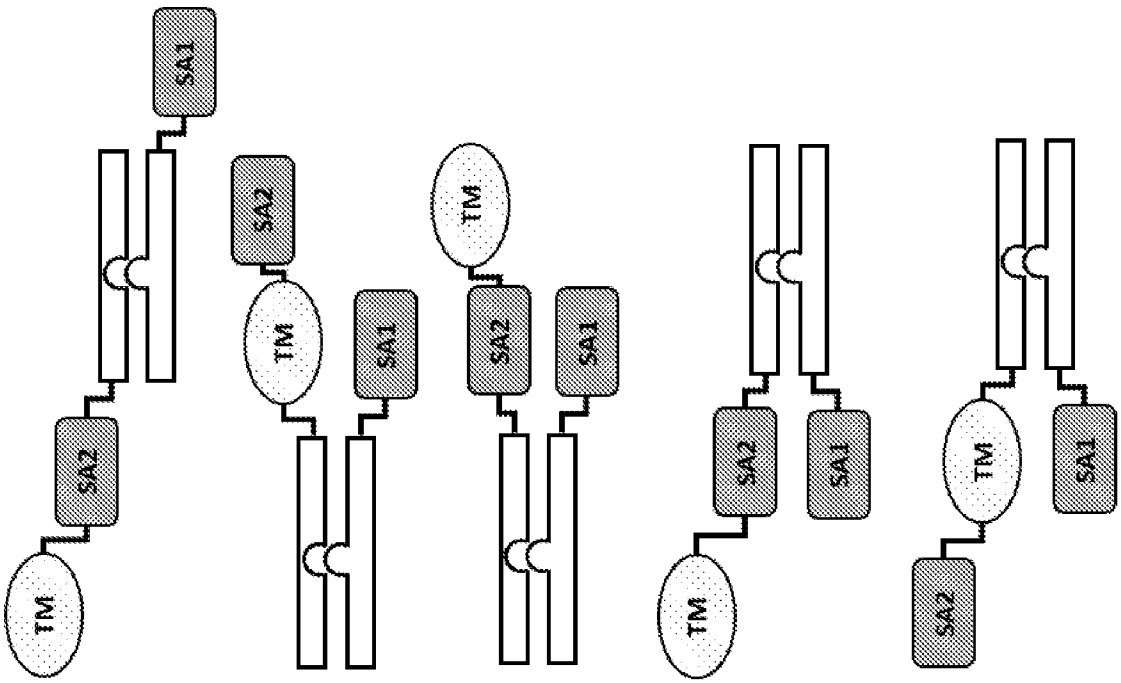


FIG. 18A FIG. 18B FIG. 18C FIG. 18D FIG. 18E FIG. 18F

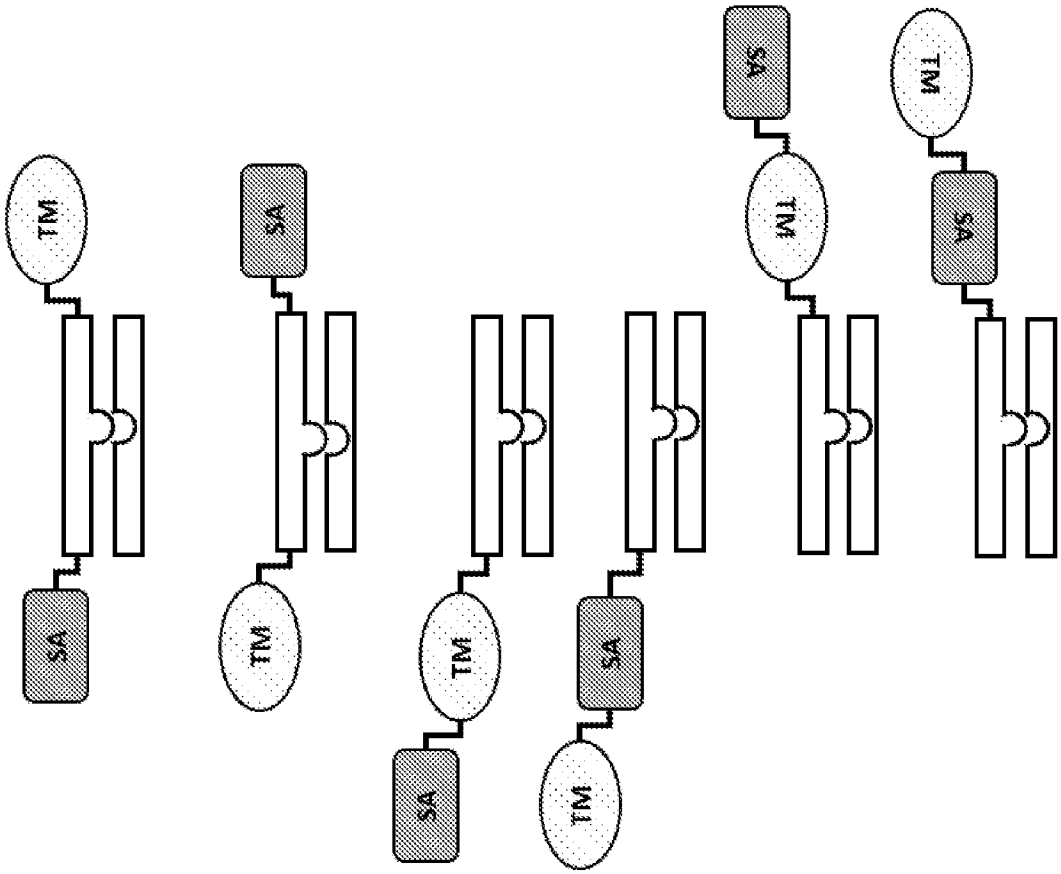


FIG. 19A FIG. 19B FIG. 19C FIG. 19D FIG. 19E

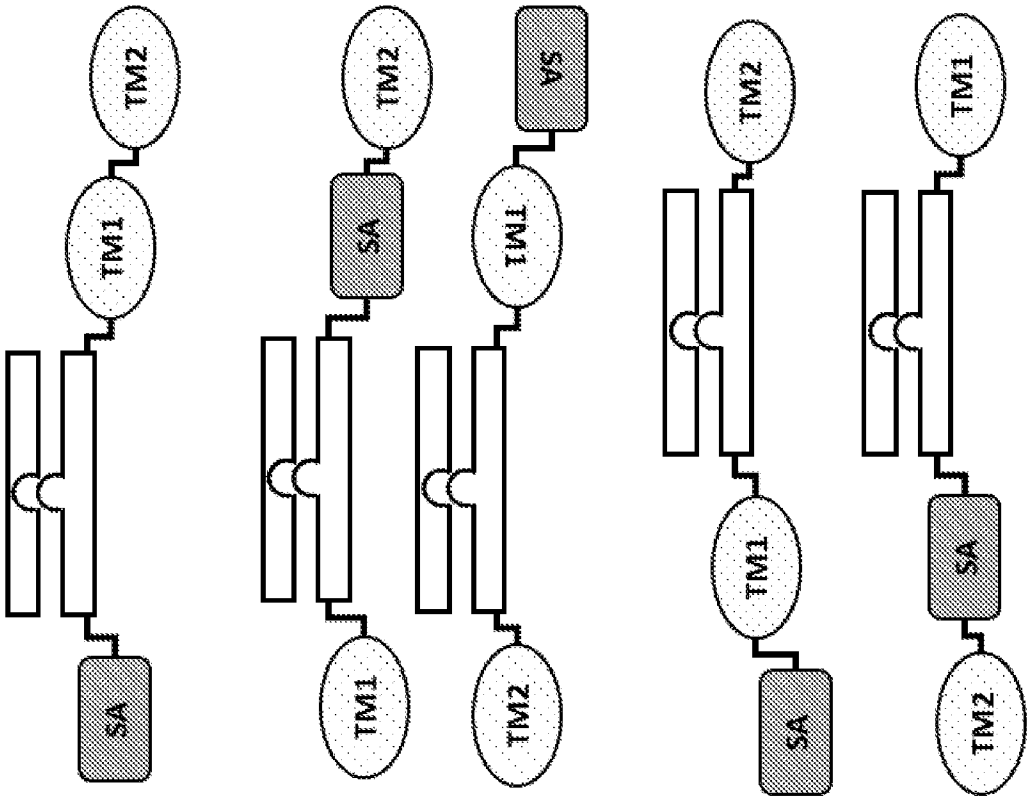


FIG. 19F FIG. 19G FIG. 19H FIG. 19I FIG. 19J FIG. 19K FIG. 19L

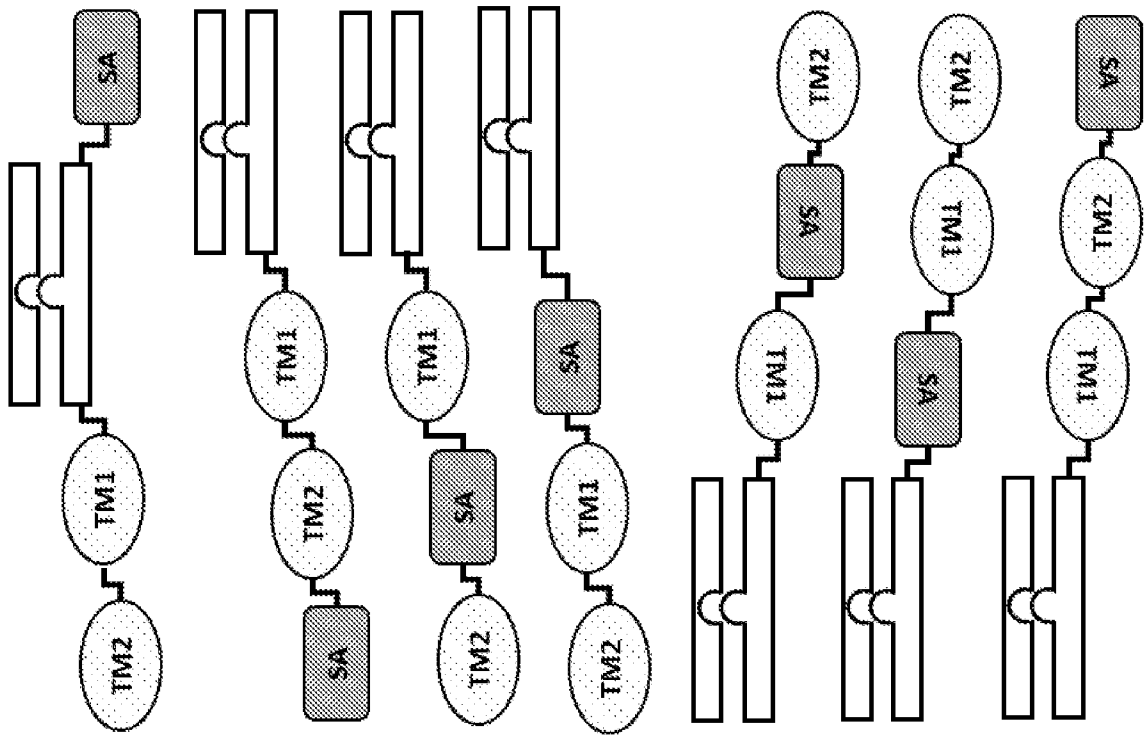


FIG. 20A FIG. 20B FIG. 20C FIG. 20D FIG. 20E FIG. 20F

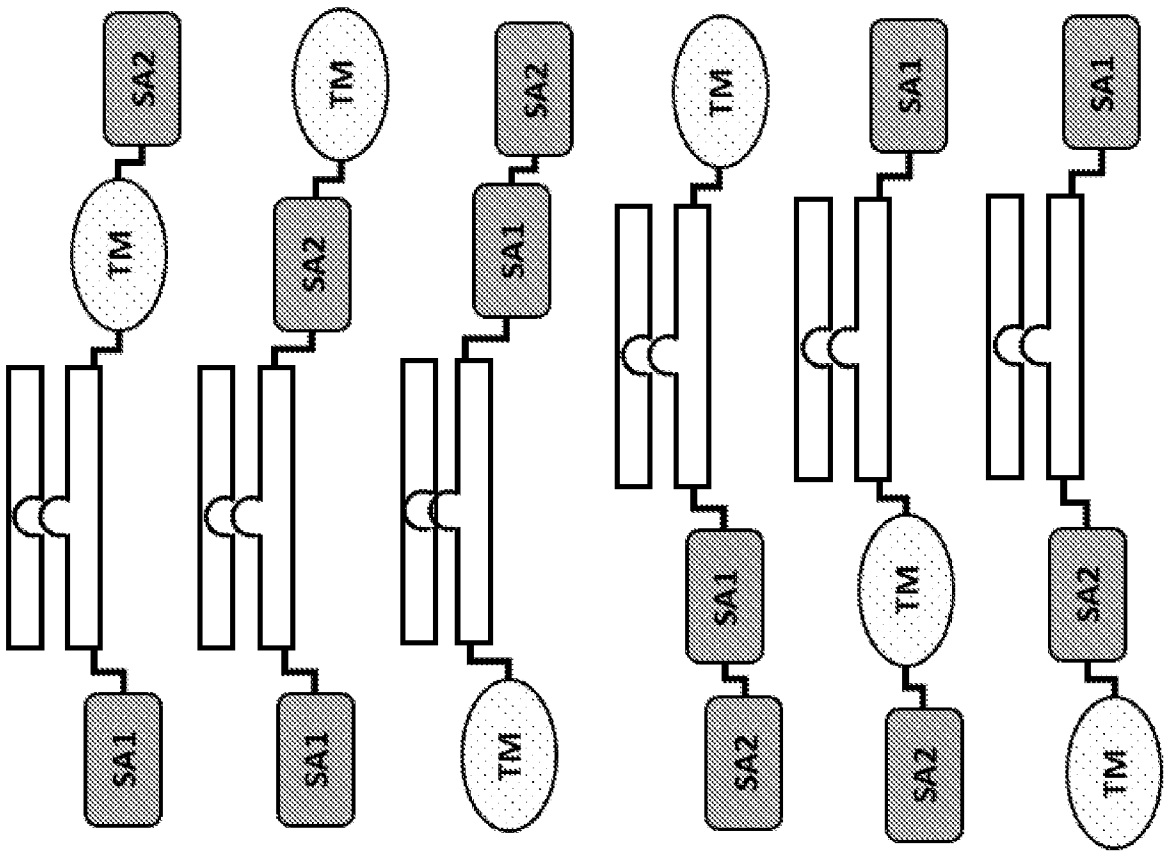


FIG. 20G FIG. 20H FIG. 20I FIG. 20J FIG. 20K FIG. 20L

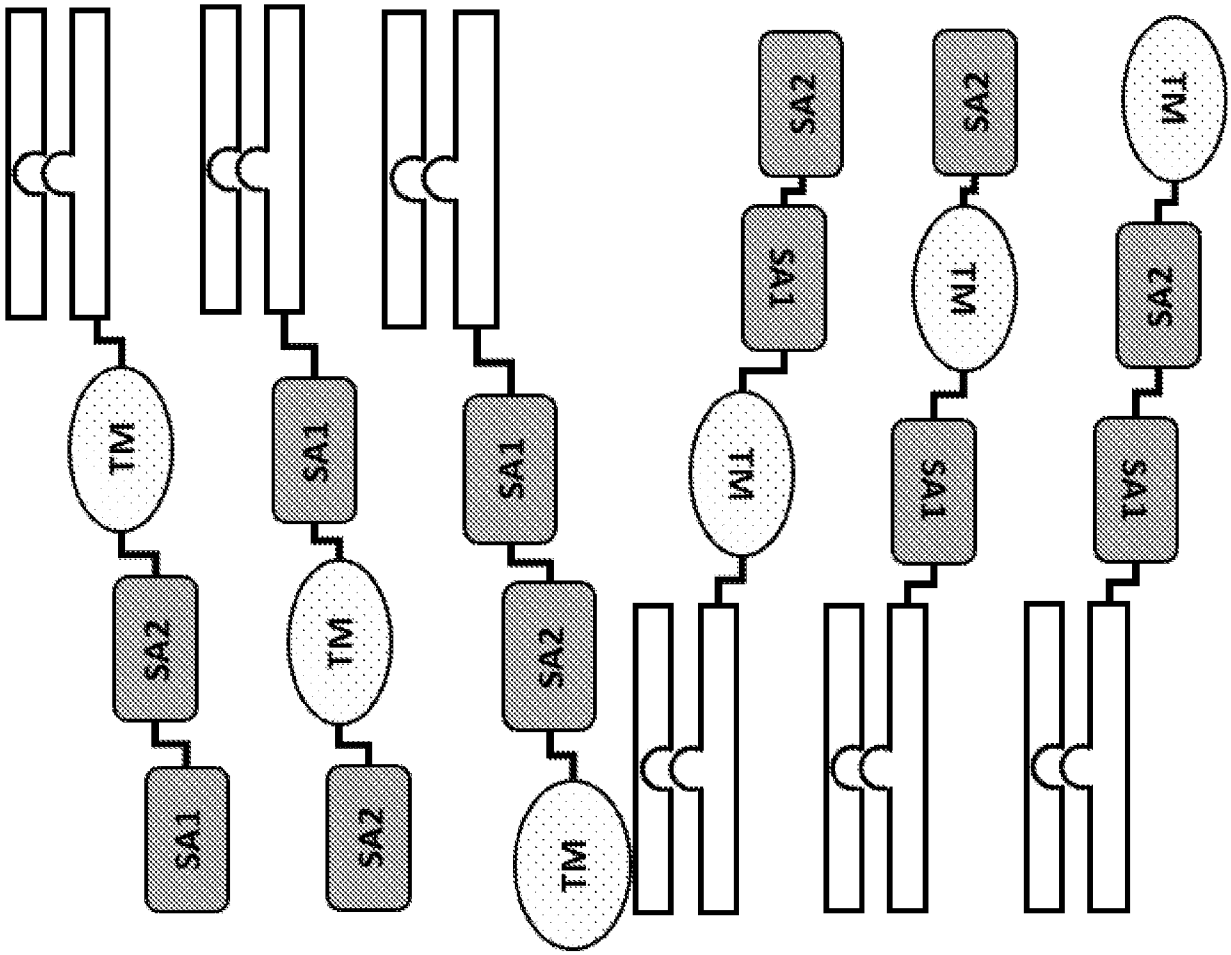


FIG. 21A FIG. 21B FIG. 21C FIG. 21D FIG. 21E FIG. 21F

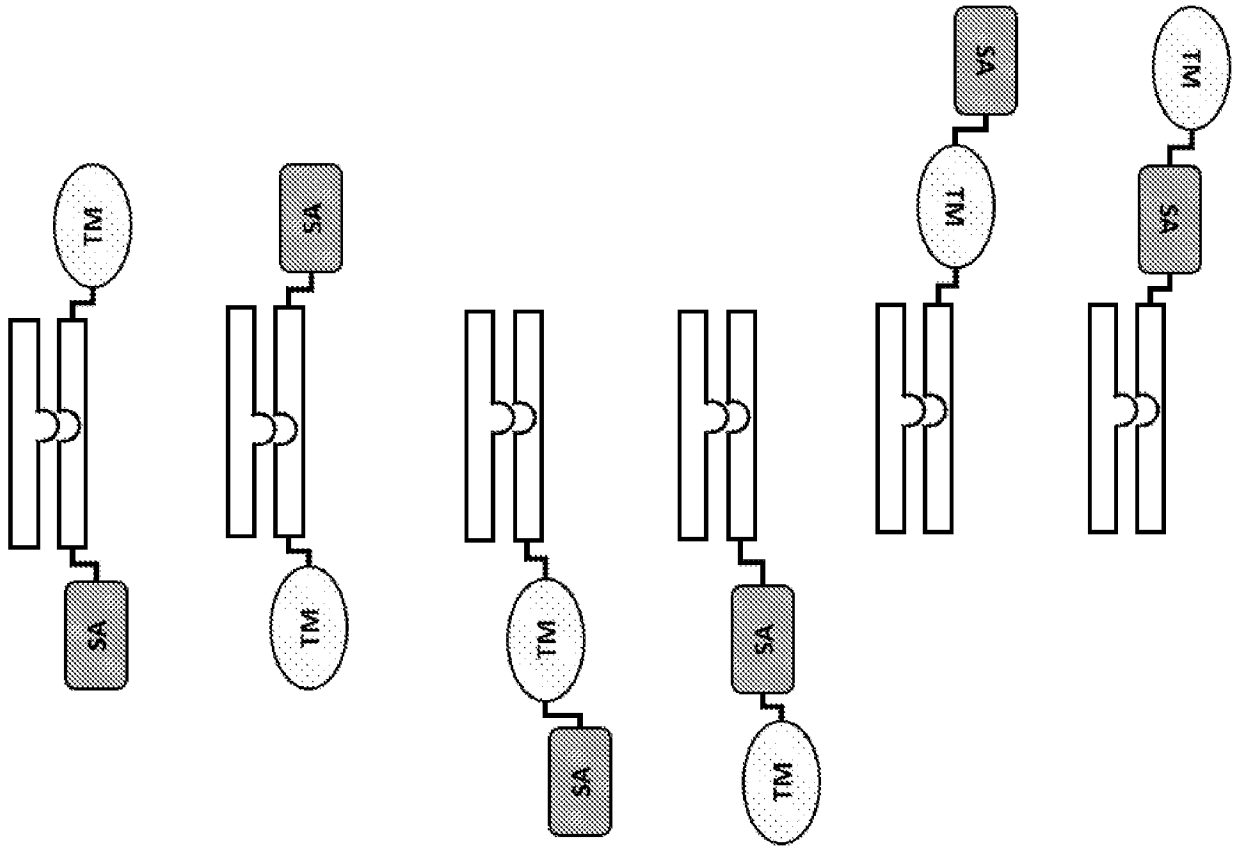


FIG. 22A FIG. 22B FIG. 22C FIG. 22D FIG. 22E FIG. 22F

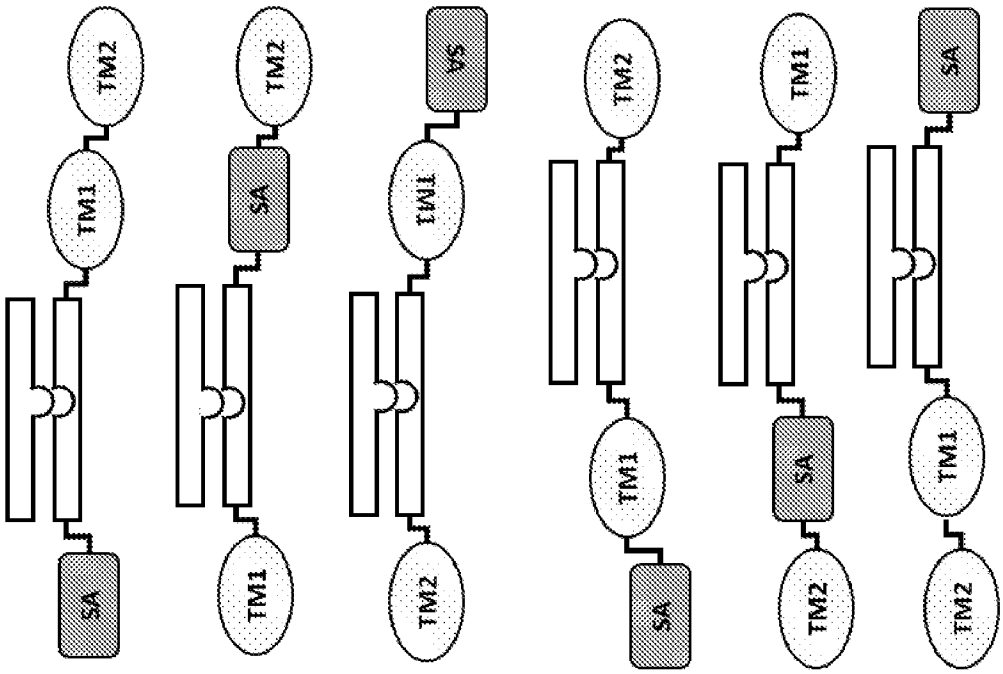


FIG. 22G FIG. 21H FIG. 22I FIG. 22J FIG. 22K FIG. 22L

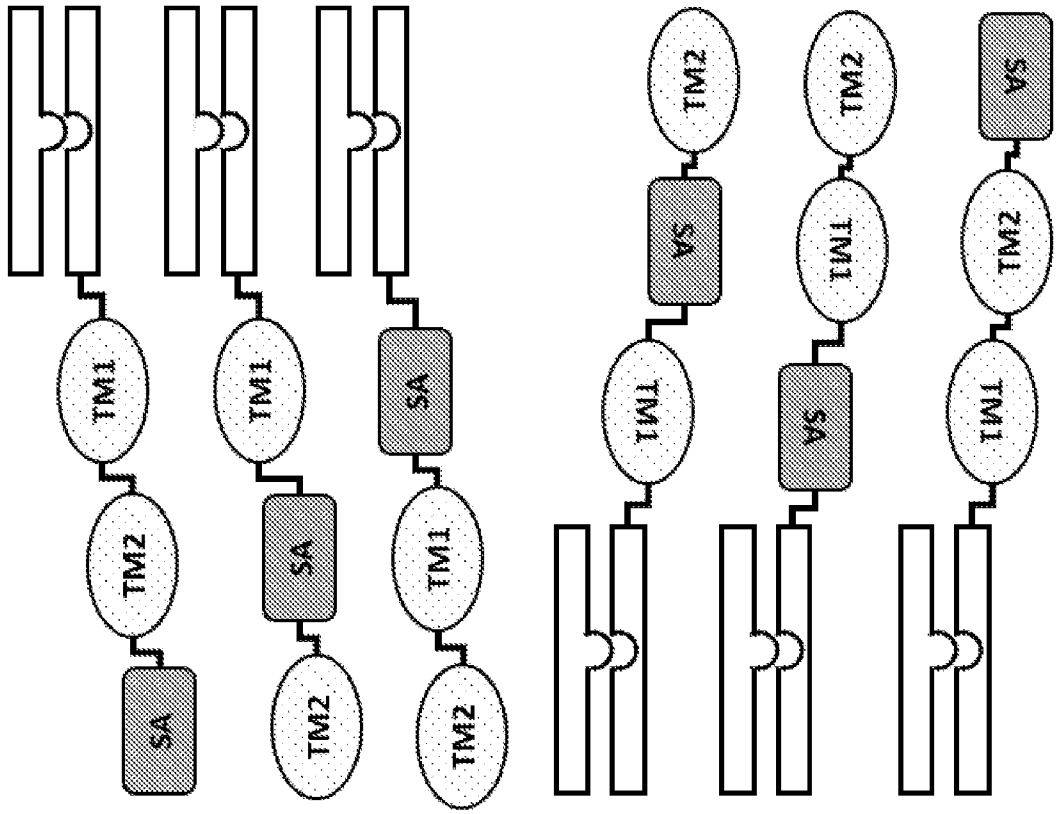


FIG. 23A FIG. 23B FIG. 23C FIG. 23D FIG. 23E FIG. 23F

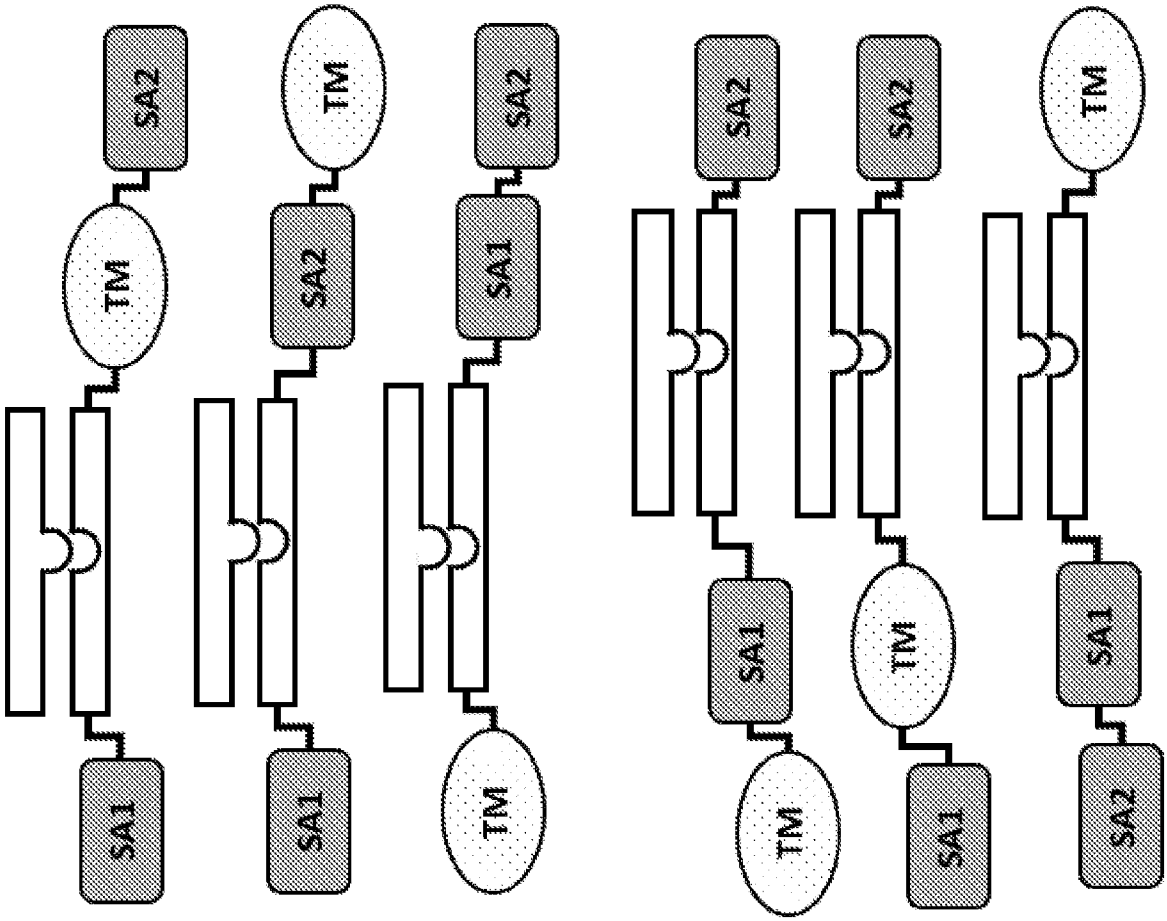


FIG. 23G FIG. 23H FIG. 23I FIG. 23J FIG. 23K FIG. 23L

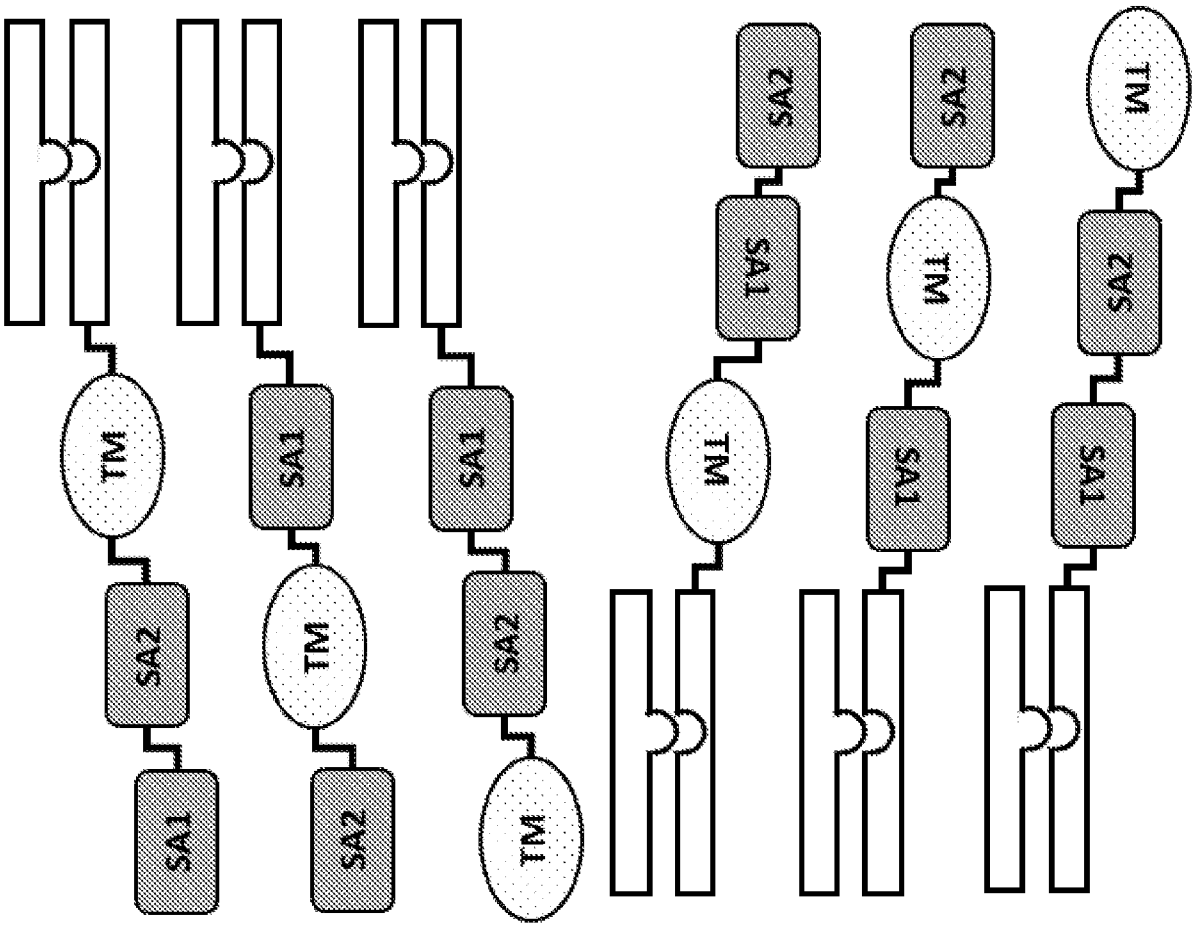


FIG. 24A FIG. 24B FIG. 24C FIG. 24D FIG. 24E

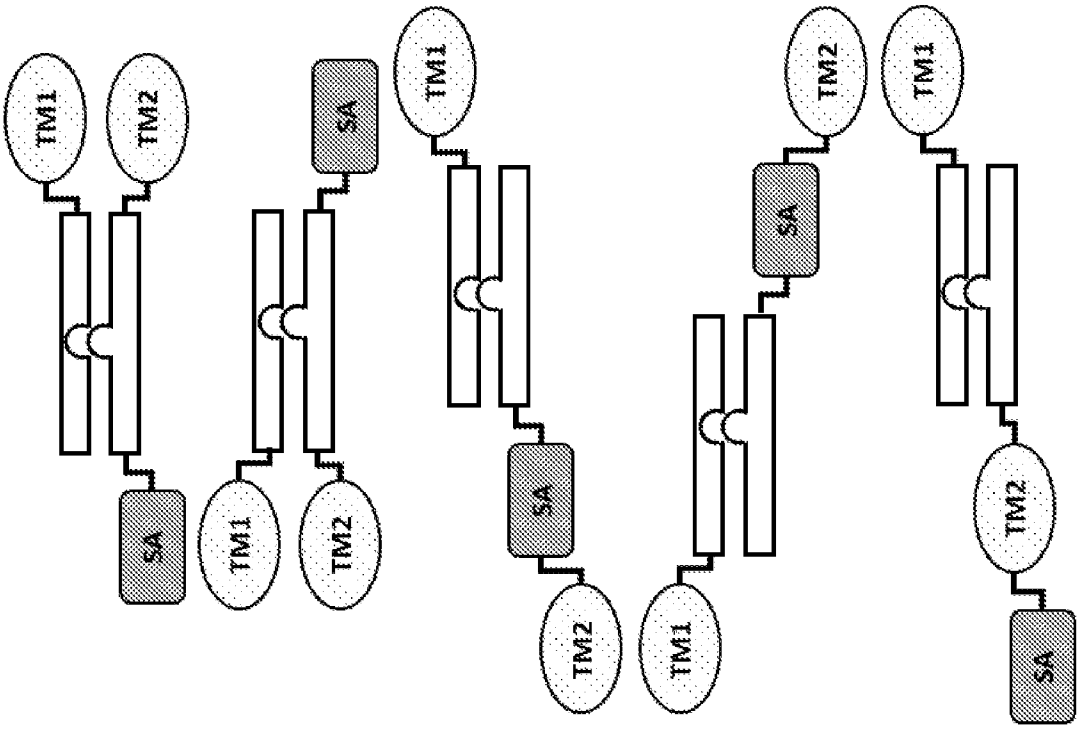


FIG. 24F FIG. 24G FIG. 24H FIG. 24I FIG. 24J

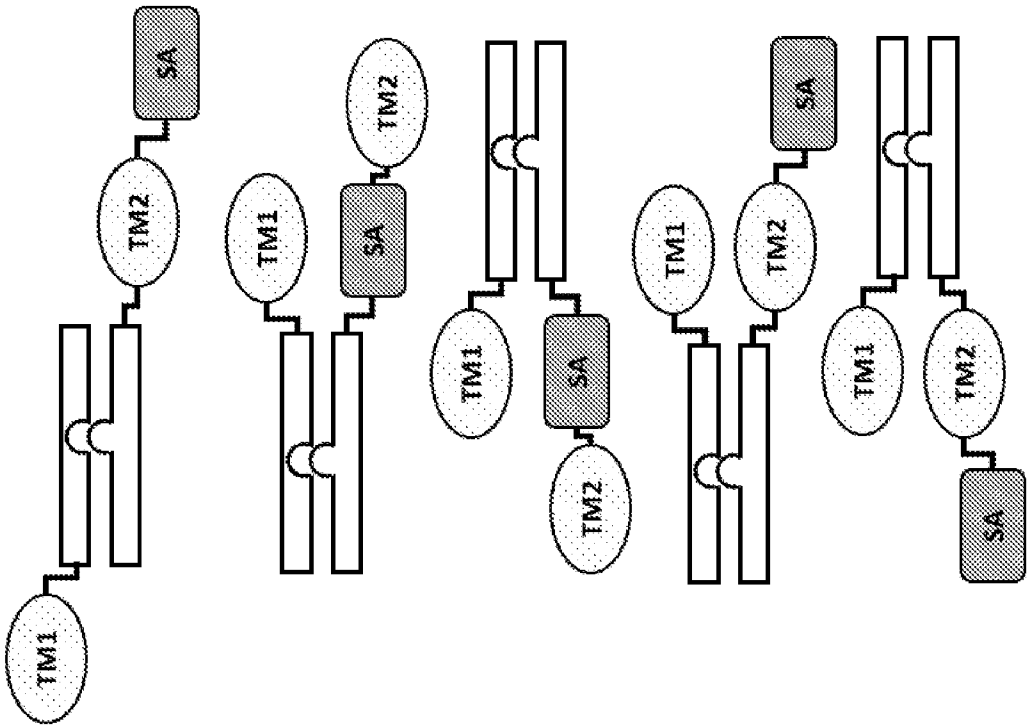


FIG. 25A FIG. 25B FIG. 25C FIG. 25D FIG. 25E

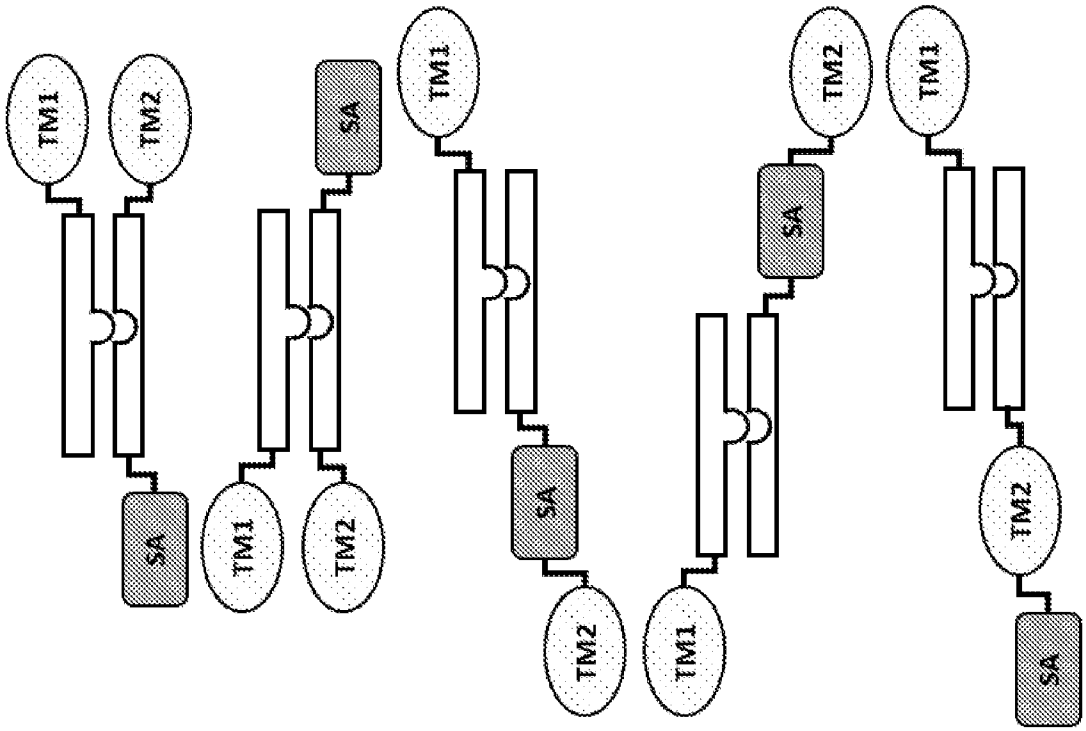


FIG. 25F FIG. 25G FIG. 25H FIG. 25I FIG. 25J

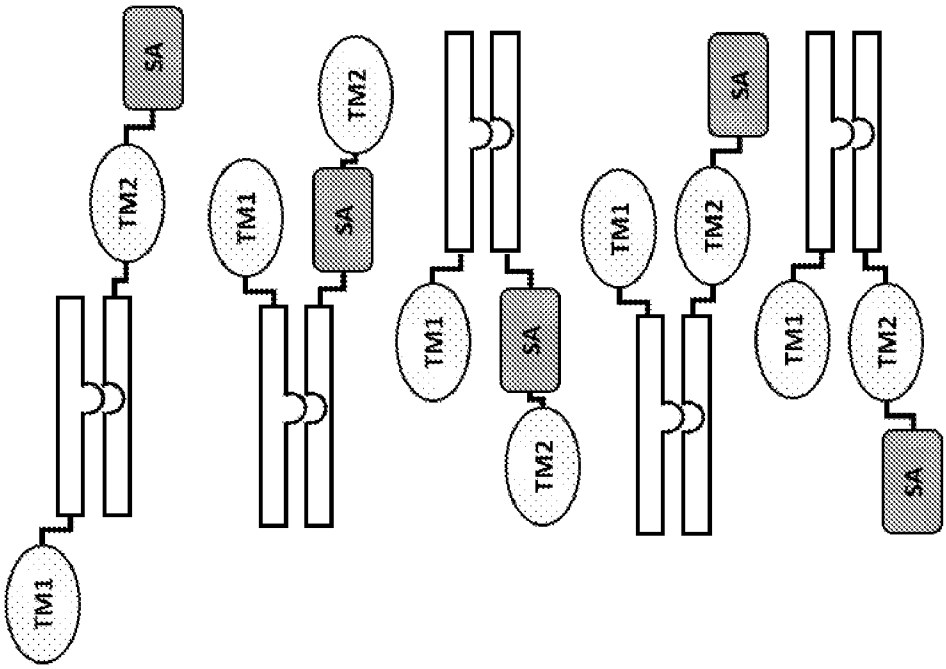


FIG. 26A FIG. 26B FIG. 26C FIG. 26D FIG. 26E FIG. 26F

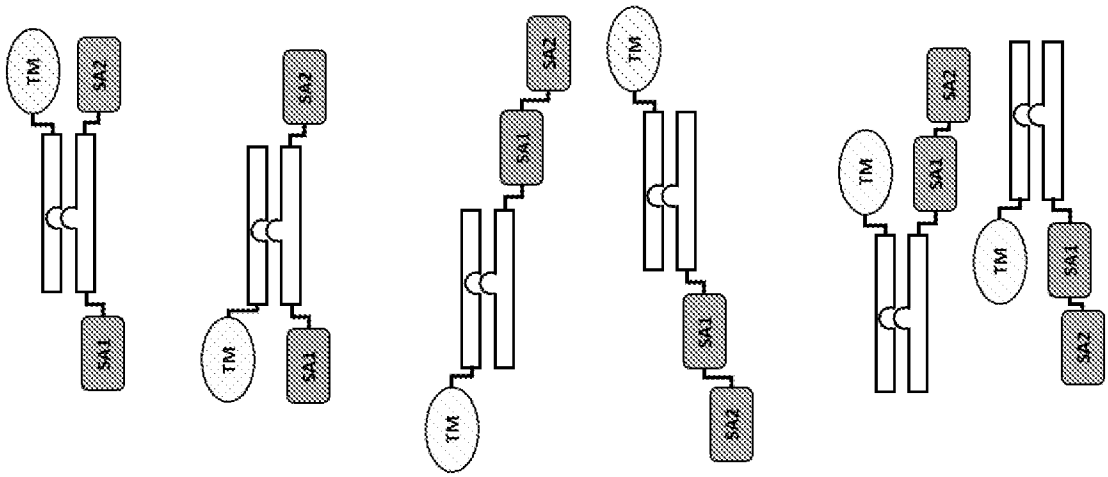


FIG. 27A FIG. 27B FIG. 27C FIG. 27D FIG. 27E FIG. 27F

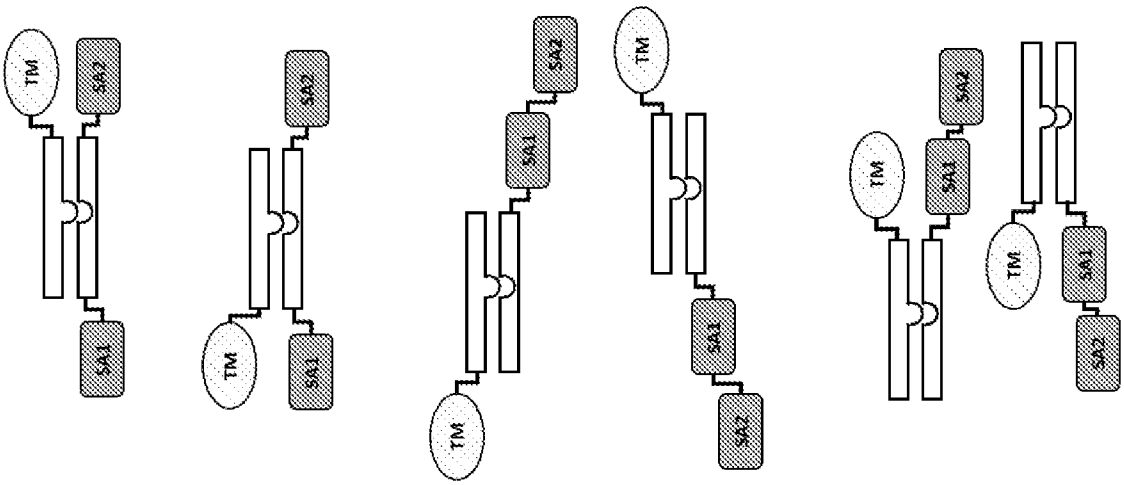
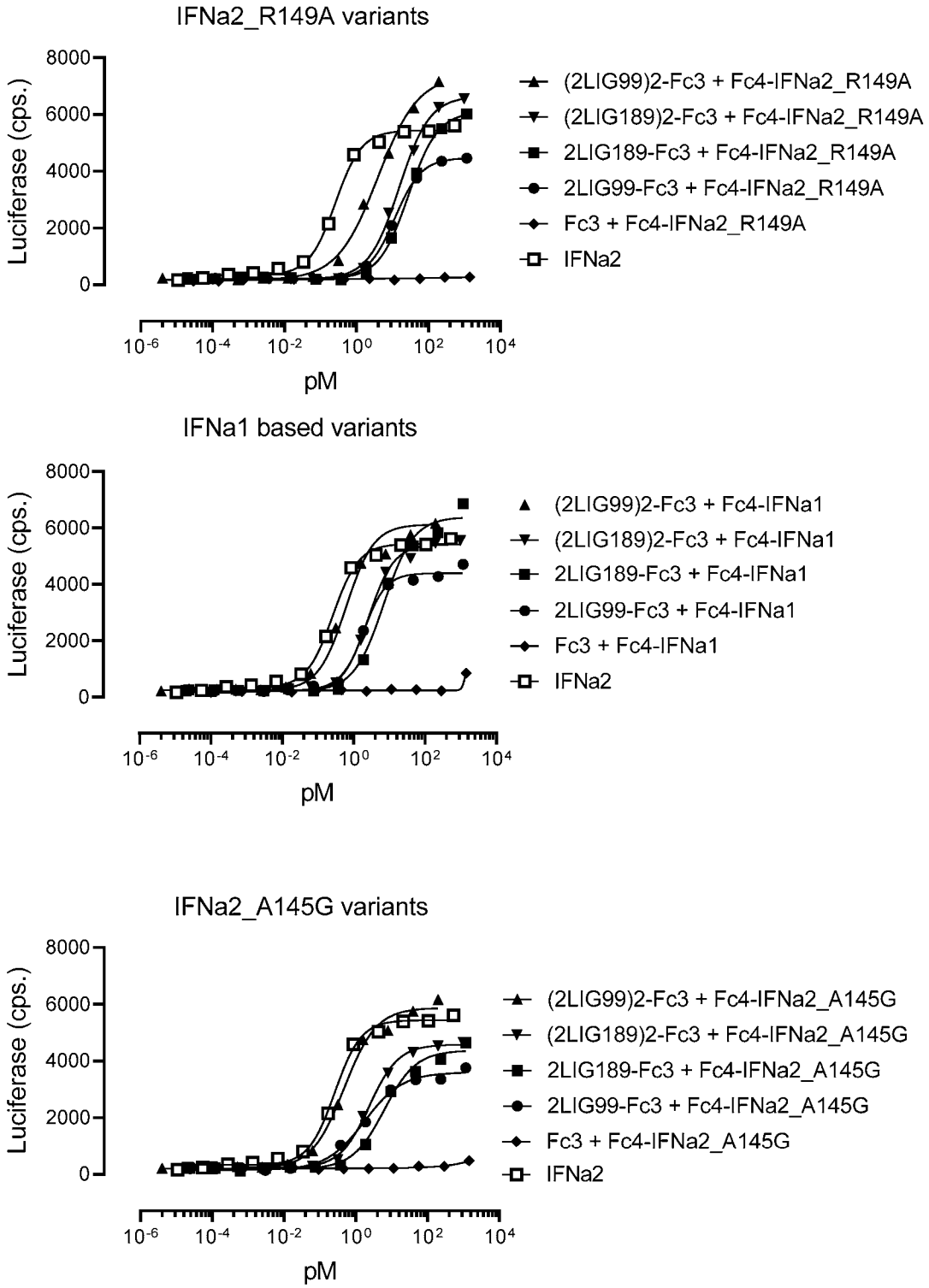


FIG. 28


IFNa2_R149A variants
Luciferase (cps.)
8000
6000
4000
2000
0
10-6
10-4
10-2
100
102
104
pM
(2LIG99)2-Fc3 + Fc4-IFNa2_R149A
(2LIG189)2-Fc3 + Fc4-IFNa2_R149A
2LIG189-Fc3 + Fc4-IFNa2_R149A
2LIG99-Fc3 + Fc4-IFNa2_R149A
Fc3 + Fc4-IFNa2_R149A
IFNa2
IFNa1 based variants
Luciferase (cps.)
8000
6000
4000
2000
0
10-6
10-4
10-2
100
102
104
pM
(2LIG99)2-Fc3 + Fc4-IFNa1
(2LIG189)2-Fc3 + Fc4-IFNa1
2LIG189-Fc3 + Fc4-IFNa1
2LIG99-Fc3 + Fc4-IFNa1
Fc3 + Fc4-IFNa1
IFNa2
IFNa2_A145G variants
Luciferase (cps.)
8000
6000
4000
2000
0
10-6
10-4
10-2
100
102
104
pM
(2LIG99)2-Fc3 + Fc4-IFNa2_A145G
(2LIG189)2-Fc3 + Fc4-IFNa2_A145G
2LIG189-Fc3 + Fc4-IFNa2_A145G
2LIG99-Fc3 + Fc4-IFNa2_A145G
Fc3 + Fc4-IFNa2_A145G
IFNa2

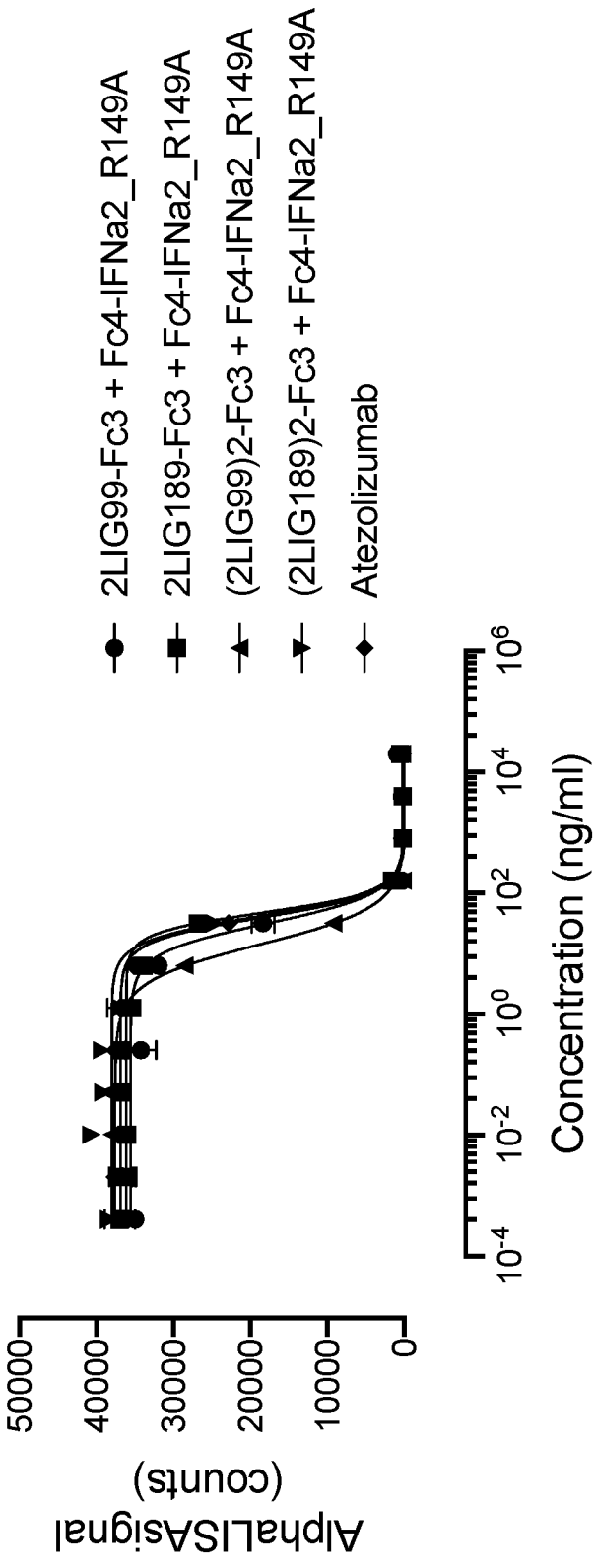

2LIG99-Fc3 + Fc4-IFNa2_R149A
2LIG189-Fc3 + Fc4-IFNa2_R149A
(2LIG99)2-Fc3 + Fc4-IFNa2_R149A
(2LIG189)2-Fc3 + Fc4-IFNa2_R149A
Atezolizumab
AlphaLISAsignal (counts)
50000
40000
30000
20000
10000
0
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
Concentration (ng/ml)

FIG. 29

FIG. 30

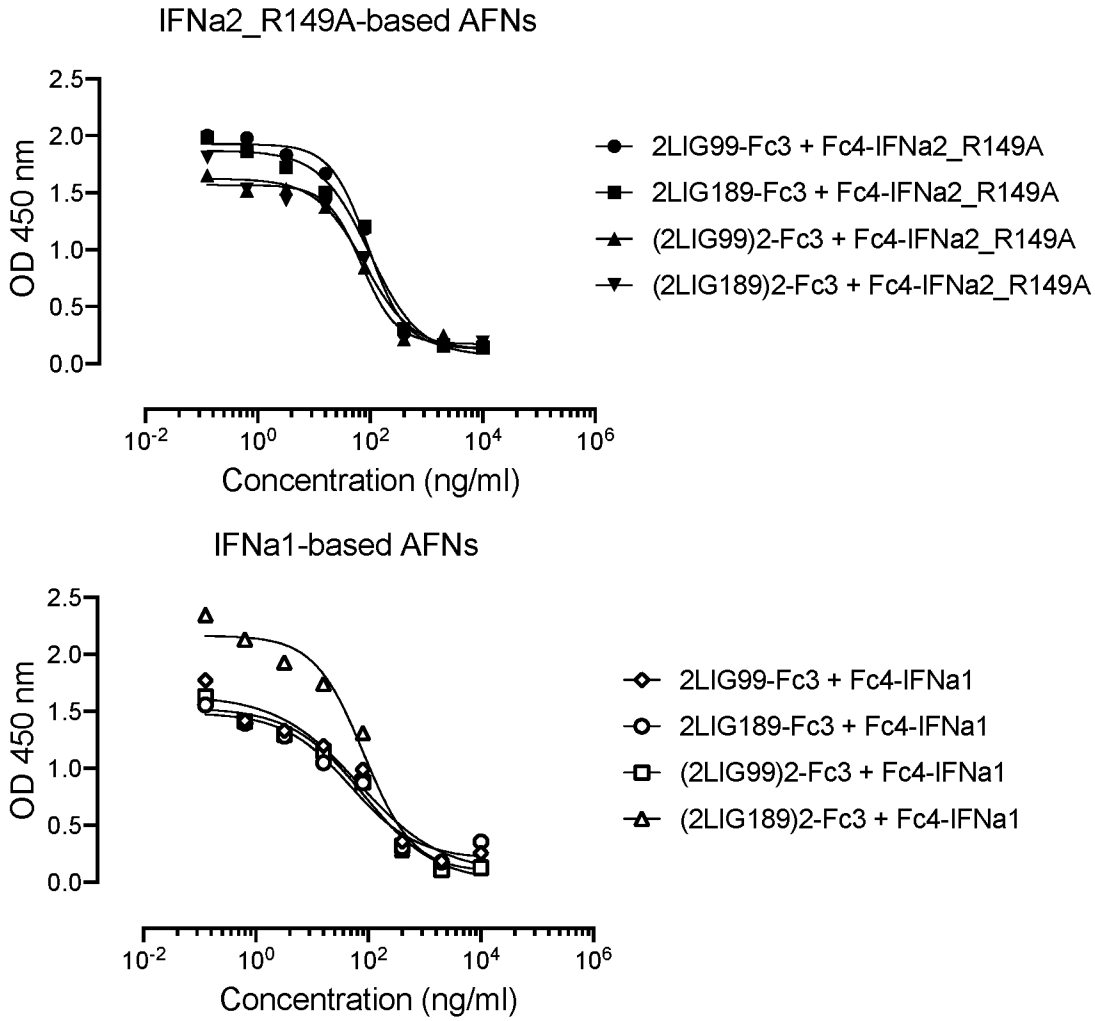

IFNa2_R149A-based AFNs
OD 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
Concentration (ng/ml)
2LIG99-Fc3 + Fc4-IFNa2_R149A
2LIG189-Fc3 + Fc4-IFNa2_R149A
(2LIG99)2-Fc3 + Fc4-IFNa2_R149A
(2LIG189)2-Fc3 + Fc4-IFNa2_R149A
IFNa1-based AFNs
OD 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
Concentration (ng/ml)
2LIG99-Fc3 + Fc4-IFNa1
2LIG189-Fc3 + Fc4-IFNa1
(2LIG99)2-Fc3 + Fc4-IFNa1
(2LIG189)2-Fc3 + Fc4-IFNa1

FIG. 31A

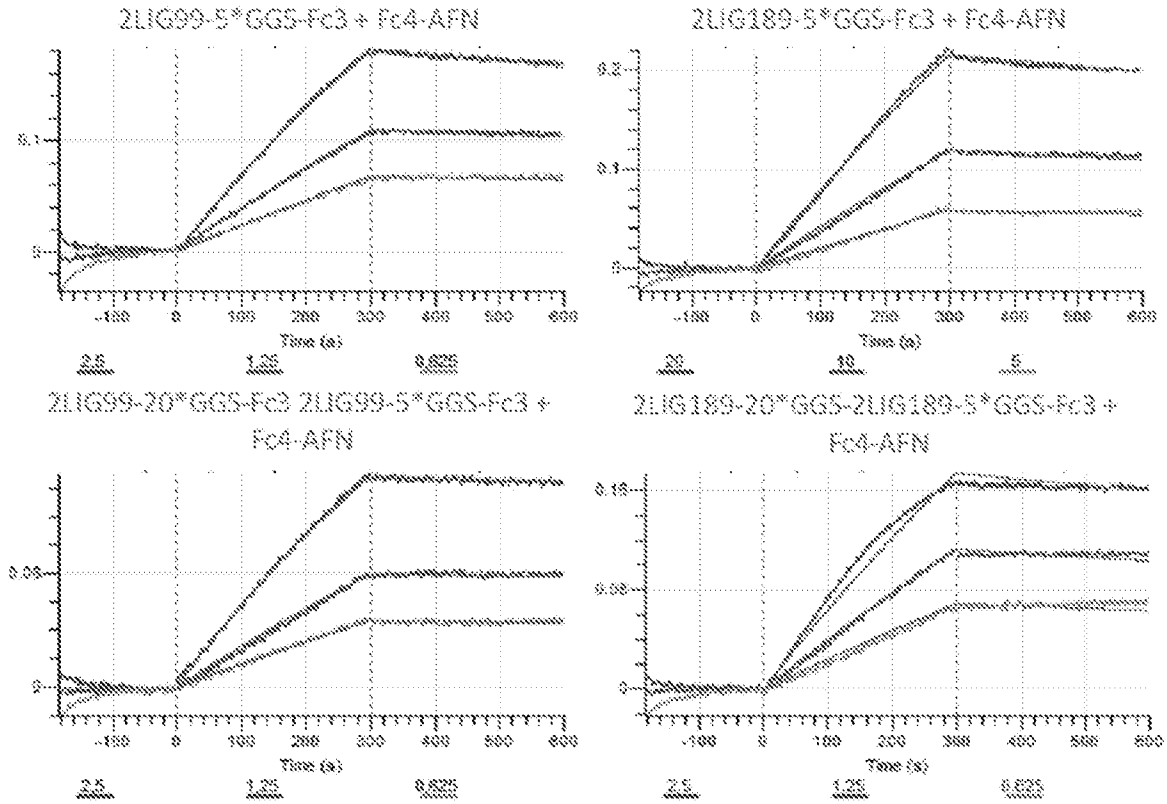


FIG. 31B

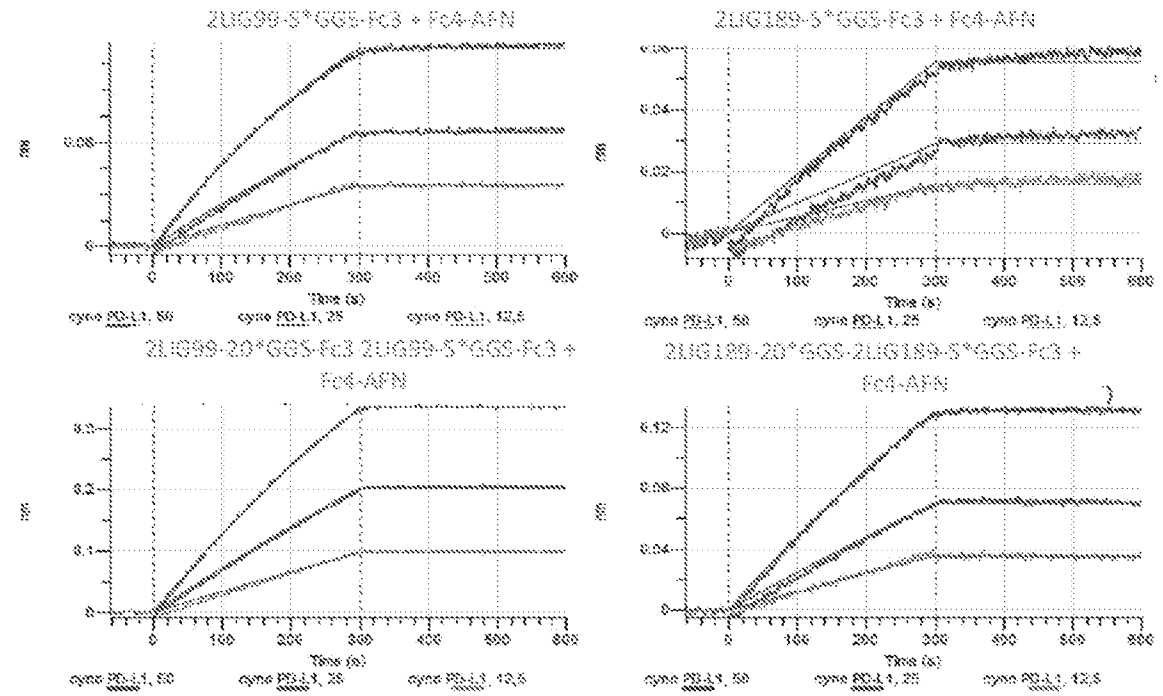


FIG. 31C

Sample ID	Affinity for human PD-L1			Affinity for cyno PD-L1		
	KD (M)	ka(1/Ms)	kdis (1/s)	KD (M)	ka(1/Ms)	kdis(1/s)
2LIG99-Fc3 + Fc4-AFN	3,06E-10	5,75E+05	1,76E-04	1,02E-11	4,57E+04	4,68E-07
2LIG189-Fc3 + Fc4-AFN	8,34E-09	3,26E+04	2,72E-04	3,68E-11	7,07E+03	2,60E-07
(2LIG99)2-Fc3 + Fc4-AFN	1,07E-10	6,75E+05	7,20E-05	9,10E-12	2,83E+04	2,62E-07
(2LIG189)2-Fc3 + Fc4-AFN	9,88E-10	2,91E+05	2,88E-04	4,92E-12	2,51E+04	1,24E-07

FIG. 32A

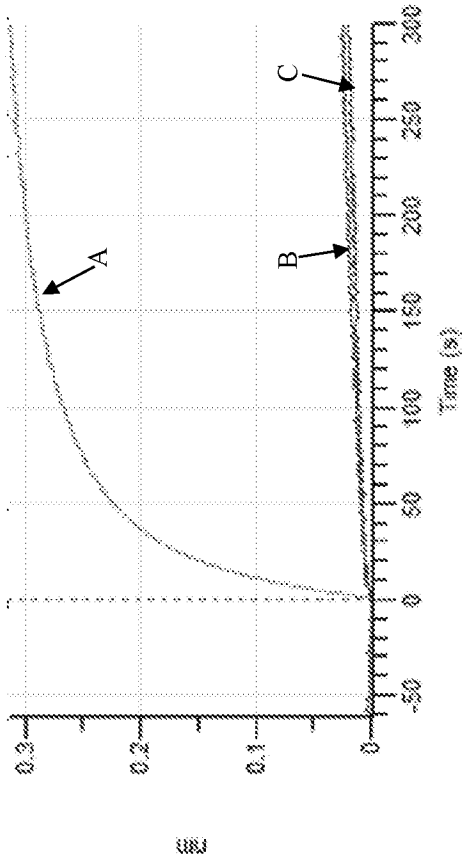


FIG. 32B

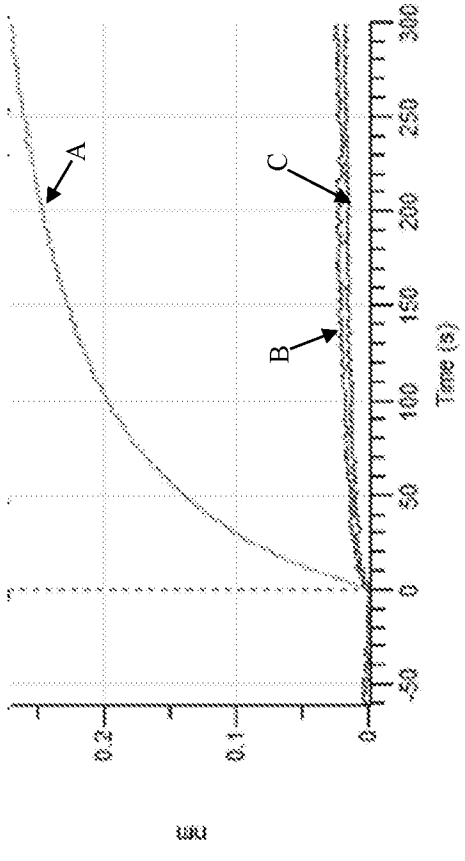


FIG. 33A

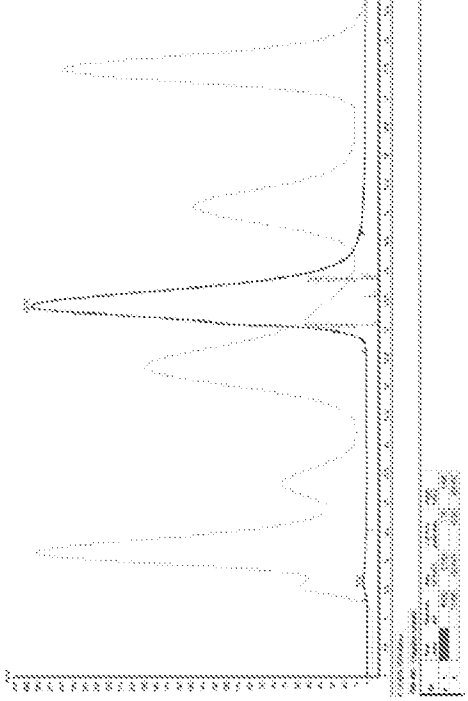


FIG. 33B

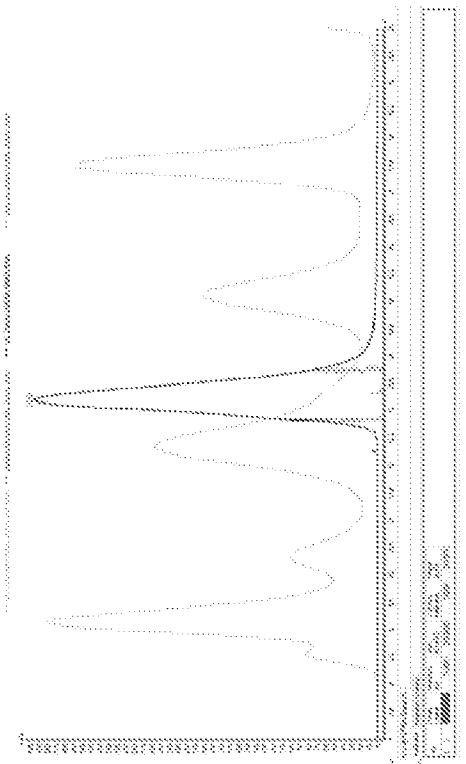


FIG. 33C

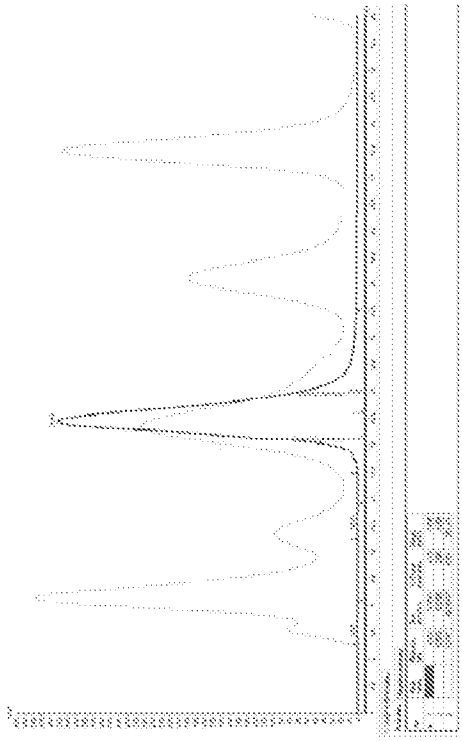


FIG. 33D

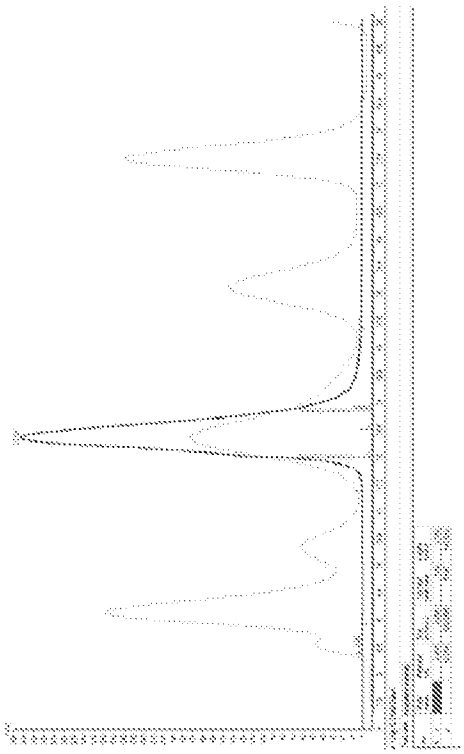


FIG. 33F

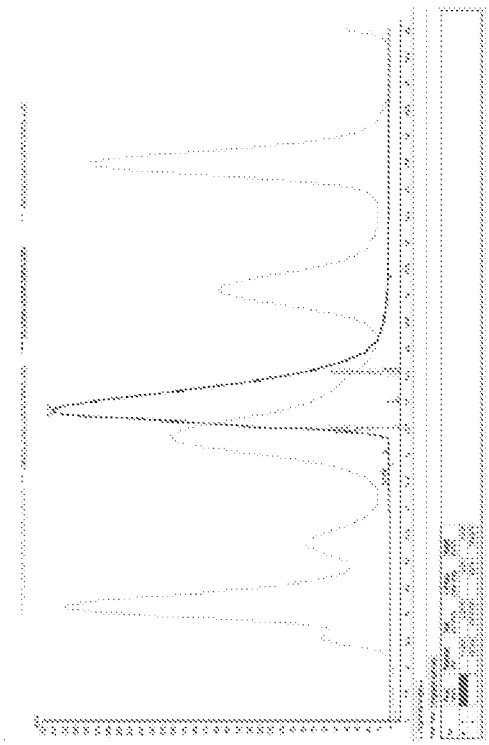


FIG. 33H

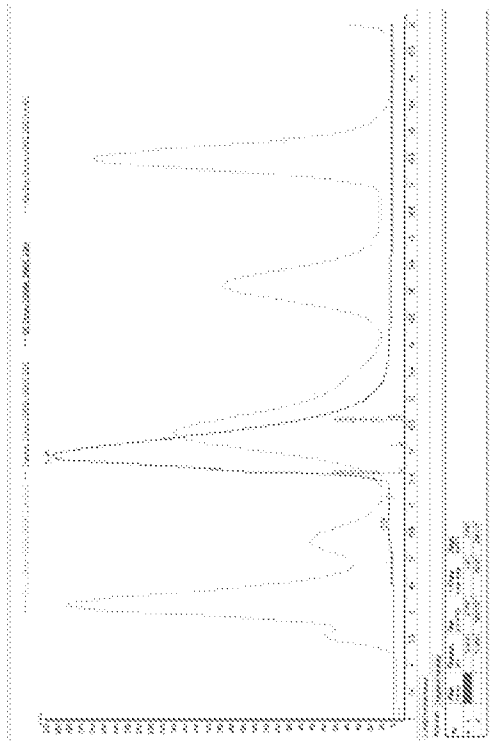


FIG. 33E

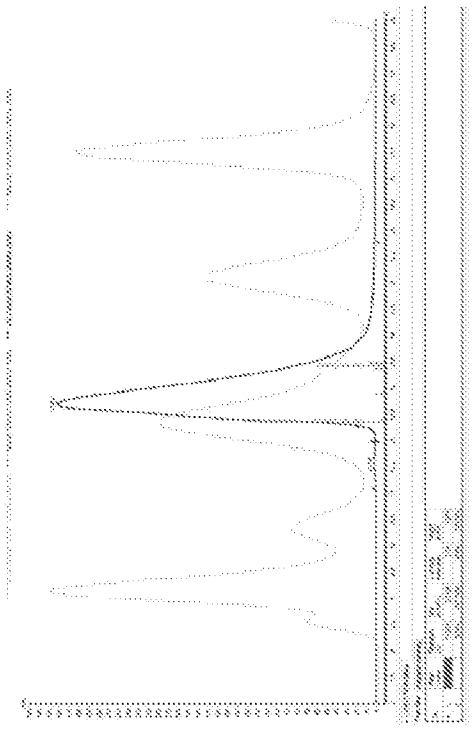


FIG. 33G

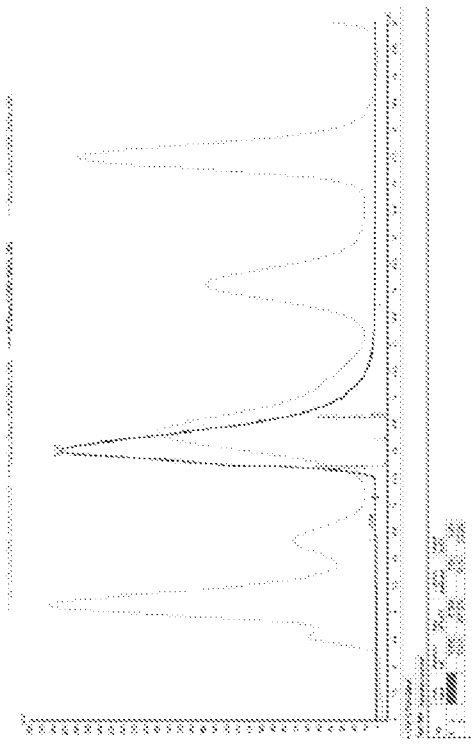
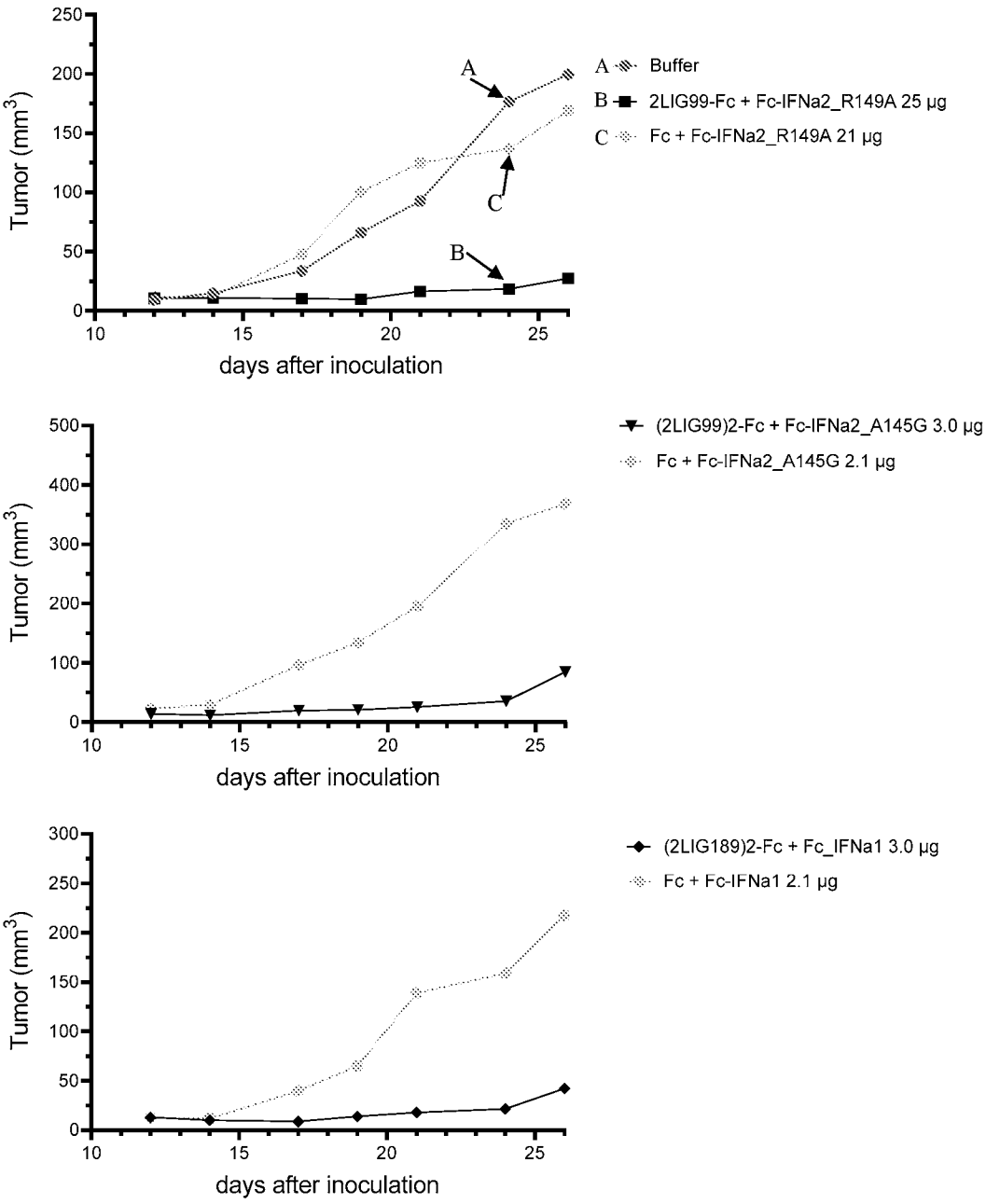


FIG. 34


Tumor (mm3)
days after inoculation
A
B
C
A Buffer
B 2LIG99-Fc + Fc-IFNa2_R149A 25 µg
C Fc + Fc-IFNa2_R149A 21 µg
(2LIG99)2-Fc + Fc-IFNa2_A145G 3.0 µg
Fc + Fc-IFNa2_A145G 2.1 µg
(2LIG189)2-Fc + Fc_IFNa1 3.0 µg
Fc + Fc-IFNa1 2.1 µg

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/052764

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 16/28; C07K 19/00 (2020.01)
CPC - C07K 16/2827; C07K 2317/565; C07K 2319/30; C07K 2319/74 (2020.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2019/032663 A1 (ORIONIS BIOSCIENCES INC. et al) 14 February 2019 (14.02.2019) entire document	175-177 --- 1-3, 29-31, 44-48, 75-86, 111-123, 135-138
Y	WO 2019/091384 A1 (YAFEI SHANGHAI BIOLOG MEDICINE SCIENCE & TECHNOLOGY CO., LTD.) 16 May 2019 (16.05.2019) entire document	1-3, 29-31, 44-48
Y	US 2014/0079691 A1 (ANAPTYSBIO, INC.) 20 March 2014 (20.03.2014) entire document	2, 30, 123
Y	US 2017/0158767 A1 (E.R. SQUIBB & SONS, L. L. C.) 08 June 2017 (08.06.2017) entire document	46-48, 136, 138
Y	WO 2018/170021 A1 (ALPINE IMMUNE SCIENCES, INC.) 20 September 2018 (20.09.2018) entire document	75-86, 111-123, 135-138
Y	WO 2019/008335 A1 (AVACTA LIFE SCIENCES LIMITED) 10 January 2019 (10.01.2019) entire document	81-83, 117-119
Y	XU et al. "Key mutations stabilize antigen-binding conformation during affinity maturation of a broadly neutralizing influenza antibody lineage," Proteins, 28 February 2015 (28.02.2015), Vol. 83, No. 4, Pgs. 771-780. entire document	84, 120

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2020	27 JAN 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/052764

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/0309250 A1 (BRISTOL-MYERS SQUIBB COMPANY) 21 November 2013 (21.11.2013) entire document	1-3, 29-31, 44-48, 75-86, 111-123, 135-138, 175-177

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/052764

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-28, 32-43, 49-74, 87-110, 124-134, 139-174
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/052764

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-45, 81-84, 97, 100, and 101 were searched.