MULTIVALENT AND MULTISPECIFIC OX40-BINDING FUSION PROTEINS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/277,027, filed January 11, 2016; the contents of each of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] This invention relates generally to molecules that specifically engage OX40, a member of the TNF receptor superfamily (TNFRSF). More specifically this invention relates to multivalent and multispecific molecules that bind at least OX40.

BACKGROUND OF THE INVENTION

[0003] The tumor necrosis factor receptor superfamily consists of several structurally related cell surface receptors. Activation by multimeric ligands is a common feature of many of these receptors. Many members of the TNFRSF have therapeutic utility in numerous pathologies if activated properly. Agonism of this receptor family often requires higher order clustering and conventional bivalent antibodies are not ideal for this. Therefore, there exists a therapeutic need for more potent agonist molecules of the TNFRSF.

SUMMARY

[0004] The disclosure provides multivalent and multispecific TNF receptor superfamily (TNFRSF) binding fusion polypeptides that bind at least OX40 (also known as tumor necrosis factor receptor superfamily, member 4 (TNFRSF4) and/or CD134)). The use of the term “OX40” is intended to cover any variation thereof, such as, by way of non-limiting example, OX-40, and all variations are used herein interchangeably. These molecules that bind at least OX40 are referred to herein as “OX40-targeting molecules” or “OX40-targeting fusions” or “OX40-targeting proteins” or “OX40-targeting fusion polypeptides” or “OX40-targeting fusion proteins.” In some examples, the OX40-

targeting molecule is a multivalent molecule, for example, a multivalent OX40-targeting fusion protein. In some examples, the OX40-targeting molecule is a multispecific molecule, for example, a multispecific OX40-targeting fusion protein. In some examples, the OX40-targeting molecule is a multivalent and multispecific molecule, for example, a multivalent and multispecific OX40-targeting fusion protein. As used herein, the term "fusion protein" or "fusion polypeptide" or "OX40-targeting fusion protein" or "OX40-targeting fusion polypeptide," unless otherwise specifically denoted, refers to any fusion protein example of the disclosure, including, but not limited to, multivalent fusion proteins, multispecific fusion proteins, or multivalent and multispecific fusion proteins.

[0004a] In one example, there is provided an isolated polypeptide that binds OX40 and comprises a plurality of Tumor Necrosis Factor receptor superfamily (TNFRSF) binding domain (TBDs), wherein at least a first TBD (TBD1) binds OX40.

[0005] The disclosure also provides multivalent and multispecific fusion polypeptides that bind at least programmed death ligand 1 (PDL1), also known as PD-L1, CD274, B7 homolog 1 and/or B7-H1. The use of the term "PDL1" is intended to cover any variation thereof, such as, by way of non-limiting example, PD-L1 and/or PDL-1, all variations are used herein interchangeably. These molecules that bind at least PDL1 are referred to herein as "PDL1-targeting molecules" or "PDL1-targeting fusions" or "PDL1-targeting proteins" or "PDL1-targeting fusion polypeptides" or "PDL1-targeting fusion proteins." In some examples, the PDL1-targeting molecule is a multivalent molecule, for example, a multivalent PDL1-targeting fusion protein. In some examples, the PDL1-targeting molecule is a multispecific molecule, for example, a multispecific PDL1-targeting fusion protein. In some examples, the PDL1-targeting molecule is a multivalent and multispecific molecule, for example, a multivalent and multispecific PDL1-targeting fusion protein. As used herein, the term "fusion protein" or "fusion polypeptide" or "PDL1-targeting fusion protein" or "PDL1-targeting fusion polypeptide," unless otherwise specifically denoted, refers to any fusion protein example of the disclosure, including, but not limited to, multivalent fusion proteins, multispecific fusion proteins, or multivalent and multispecific fusion proteins.

[0005a] In another example, there is provided an isolated polypeptide that binds PDL1 and comprises a plurality of polypeptide binding domains (BDs), wherein at least a first BD (BD1) binds PDL1.

[0006] The disclosure also provides multivalent and multispecific fusion polypeptides that bind at least PDL1 and OX40. These molecules that bind at least PDL1 are referred to herein as "PDL1xOX40-targeting molecules" or "PDL1xOX40-targeting fusions" or "PDL1xOX40-targeting proteins" or "PDL1xOX40-targeting fusion polypeptides" or "PDL1xOX40-targeting fusion proteins." In some examples, the PDL1xOX40-targeting molecule is a multivalent molecule, for example, a multivalent

PDL1xOX40-targeting fusion protein. In some examples, the PDL1xOX40-targeting molecule is a multispecific molecule, for example, a multispecific PDL1xOX40-targeting fusion protein. In some examples, the PDL1xOX40-targeting molecule is a multivalent and multispecific molecule, for example, a multivalent and multispecific PDL1-targeting fusion protein. As used herein, the term "fusion protein" or "fusion polypeptide" or "PDL1xOX40-targeting fusion protein" or "PDL1xOX40-targeting fusion polypeptide," unless otherwise specifically denoted, refers to any fusion protein example of the disclosure, including, but not limited to, multivalent fusion proteins, multispecific fusion proteins, or multivalent and multispecific fusion proteins.

[0006a] In one aspect, the present disclosure refers to an isolated polypeptide comprising at least one VHH domain that binds PDL1 and at least one VHH domain that binds OX40, wherein at least one VHH domain that binds PDL1 comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from SEQ ID NOs: 58, 61, and 64; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from SEQ ID NOs: 59, 62, 65, and 69; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from SEQ ID NOs: 60, 63, 66-68, and 70.

[0006b] In one aspect, the present disclosure refers to an isolated polynucleotide that encodes a polypeptide as described herein. In another aspect, the present disclosure refers to an isolated cell that comprises the polynucleotide as described herein. In yet another aspect, the present disclosure refers to an isolated cell that expresses the polypeptide as described herein.

[0006c] In one aspect, the present disclosure refers to a method of producing the polypeptide as described herein comprising culturing the host cell as described herein under conditions suitable for producing the polypeptide.

[0007] Conventional antibodies targeting members of the TNF receptor superfamily (TNFRSF) have been shown to require exogenous crosslinking to achieve sufficient agonist activity, as evidenced by the necessity for Fc-gamma Receptor (FcγRs) for the activity of antibodies to DR4, DR5, GITR and OX40 (Ichikawa *et al* 2001 al Nat. Med. 7, 954–960, Li *et al* 2008 Drug Dev. Res. 69, 69–82; Pukac et al 2005 Br. J. Cancer 92, 1430–1441; Yanda *et al* 2008 Ann. Oncol. 19, 1060–1067; Yang *et al* 2007 Cancer Lett. 251:146–157; Bulliard *et al* 2013 JEM 210(9): 1685; Bulliard *et al* 2014 Immunol and Cell Biol 92: 475-480). In addition to crosslinking via FcγRs other exogenous agents including addition of the oligomeric ligand or antibody binding entities (e.g. protein A and secondary antibodies) have been demonstrated to enhance anti-TNFRSF antibody clustering and downstream signaling. For example, the addition of the DR5 ligand TRAIL enhanced the apoptosis inducing ability of an anti-DR5 antibody (Graves *et al* 2014 Cancer Cell 26: 177-189). These findings suggest the need for clustering of TNFRSFs beyond a dimer.

[0008] The present disclosure provides multivalent TNFRSF binding fusion proteins, which comprise 2 or more TNFRSF binding domains (TBDs) where at least one TBD binds OX40. In some examples, the fusion proteins of the present disclosure have utility in treating neoplasms.

[0008a] In another aspect, the present disclosure refers to use of a polypeptide as described herein in the manufacture of a medicament for treating cancer.

[0009] In some examples, the fusion protein contains two or more different TBDs, where each TBD binds OX40. In some examples, the fusion protein contains multiple copies of a TBD that binds OX40. For example, in some examples, the fusion protein contains at least two copies of a TBD that binds OX40. In some examples, the fusion protein contains at least three copies of a TBD that binds OX40. In some examples, the fusion protein contains at least four copies of a TBD that binds OX40. In some examples, the fusion protein contains at least five copies of a TBD that binds

OX40. In some examples, the fusion protein contains at least six copies of a TBD that binds OX40. In some examples, the fusion protein contains six or more copies of a TBD that binds OX40.

[0010] In other examples, the fusion proteins of the present disclosure bind OX40 and a second TNFRSF member such as, for example, GITR, CD137, CD27, TNFR2, and/or CD40. In these examples, the fusion proteins of the present disclosure modulate immune cells leading to enhanced tumor destruction. In other examples, the fusion proteins of the present disclosure have utility in treating inflammatory conditions. In these examples, the fusion proteins of the present disclosure modulate immune cells leading to dampening of the inflammatory insult. For example, specifically agonizing TNFR2 can enhance Treg proliferation leading to immune suppression.

[0010a] In another aspect, the present disclosure refers to use of a polypeptide as described herein in the manufacture of a medicament for increasing T cell activation and/or proliferation. In another aspect, the present disclosure refers to an in vitro method of increasing T cell activation and/or proliferation comprising contacting T cells with a polypeptide as described herein.

[0011] The fusion proteins of the present disclosure are capable of enhanced clustering of TNFRSF members compared to non-cross-linked bivalent antibodies. The enhanced clustered of TNFRSF members mediated by the fusion proteins of the present disclosure induce enhanced TNFRSF-dependent signaling compared to non-cross-linked bivalent antibodies. In most examples, the fusion protein will incorporate more than 2 TBDs, for example, three, four, five, or six.

[0012] In some examples, the fusion proteins are multispecific containing a TBD and a binding domain directed toward a second antigen. In these, examples, the binding to the second antigen is capable of providing the additional crosslinking function and TNFRSF activation can be achieved with only one or two TBDs. In these examples, the TNFRSF signaling is enhanced and focused by the presence of the second antigen. These multispecific TBD containing fusion proteins are useful means to achieve conditional signaling of a given TNFRSF member.

[0013] The present disclosure provides isolated polypeptides that specifically bind OX40. In some examples, the isolated polypeptide is derived from antibodies or antibody fragments including scFv, Fabs, single domain antibodies (sdAb), V_{NAR}, or VHHs. In some examples, the isolated polypeptide is human or humanized sdAb. The sdAb fragments can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families. In other

examples, the isolated polypeptides are derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimers, anticalin/lipocalins, centyrins and fynomers.

[0014] In some examples, the isolated polypeptide includes an amino acid sequence selected from the group consisting of SEQ ID NO: 16-29 and 377-386.

[0015] In some examples, the isolated polypeptide includes an amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 16-29 and 377-386.

[0016] In some examples, the isolated polypeptide comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 36, and 44; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 35, and 37; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 32-34, 48-43, and 45.

[0017] The present disclosure provides multivalent fusion proteins, which comprise two or more binding domains (BDs) where at least one BD binds PDL1. In some examples, the fusion proteins of the present disclosure have utility in treating neoplasms.

[0018] In some examples, the fusion protein contains two or more different BDs, where each BD binds PDL1. In some examples, the fusion protein contains multiple copies of a BD that binds PDL1. For example, in some examples, the fusion protein contains at least two copies of a BD that binds PDL1. In some examples, the fusion protein contains at least three copies of a BD that binds PDL1. In some examples, the fusion protein contains at least four copies of a BD that binds PDL1. In some examples, the fusion protein contains at least five copies of a BD that binds PDL1. In some examples, the fusion protein contains at least six copies of a BD that binds PDL1. In some examples, the fusion protein contains six or more copies of a BD that binds PDL1.

[0019] The present disclosure provides isolated polypeptides that specifically bind OX40. In some examples, the isolated polypeptide is derived from antibodies or antibody fragments including scFv, Fabs, single domain antibodies (sdAb), V_{NAR}, or VHHs. In some examples, the isolated polypeptide is human or humanized sdAb. The sdAb

fragments can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families. In other examples, the isolated polypeptides are derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimers, anticalin/lipocalins, centyrins and fynomers.

[0020] In some examples, the isolated polypeptide includes an amino acid sequence selected from the group consisting of SEQ ID NO: 46-57. In some examples, the isolated polypeptide includes an amino acid sequence selected from the group consisting of SEQ ID NO: 52-57.

[0021] In some examples, the isolated polypeptide includes an amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 46-57. In some examples, the isolated polypeptide includes an amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 52-57.

[0022] In some examples, the isolated polypeptide comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 58, 61, and 64; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 59, 62, 65, and 69; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 60, 63, 66-68, and 70.

[0023] In some examples, the present disclosure provides isolated polypeptides that specifically bind at least OX40 and PDL1. In some examples, each binding domain (BD) in the isolated polypeptide is derived from antibodies or antibody fragments including scFv, Fabs, single domain antibodies (sdAb), V_{NAR}, or VHHs. In some examples, each BD is human or humanized sdAb. The sdAb fragments can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from

conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families. In other examples, the isolated polypeptides are derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimers, anticalin/lipocalins, centyrins and fynomers.

[0024] In some examples, the isolated polypeptide includes a first amino acid sequence that binds 4B11 selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and a second amino acid sequence that binds PDL1 selected from the group consisting of SEQ ID NO: 46-57.

[0025] In some examples, the isolated polypeptide includes a first amino acid sequence that binds 4B11 selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and a second amino acid sequence that binds PDL1 selected from the group consisting of SEQ ID NO: 52-57.

[0026] In some examples, the isolated polypeptide includes a first amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence that binds 4B11 selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and a second amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence that binds PDL1 selected from the group consisting of SEQ ID NO: 46-57.

[0027] In some examples, the isolated polypeptide includes a first amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence that binds 4B11 selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and a second amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence that binds PDL1 selected from the group consisting of SEQ ID NO: 52-57.

[0028] In some examples, the isolated polypeptide includes (i) a first amino acid sequence that binds 4B11 and comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 36, and 44; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 35, and 37; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 32-34, 48-43, and 45; and (ii) a second amino

acid sequence that binds PDL1 and comprises a CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 58, 61, and 64; a CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 59, 62, 65, and 69; and a CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 60, 63, 66-68, and 70.

[0029] In some examples, the binding domains (BDs) of the present disclosure are derived from antibodies or antibody fragments including scFv, Fabs, single domain antibodies (sdAb), V_{NAR}, or VHHs. In some examples, the BDs are human or humanized sdAb. The sdAb fragments, can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families. In other examples, the DBs are derived from non-antibody scaffold proteins for example, but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0030] Generally, the fusion proteins of the present disclosure consist of at least two or more BDs operably linked via a linker polypeptide. The utilization of sdAb fragments as the specific BD within the fusion protein of the present disclosure has the benefit of avoiding the heavy chain : light chain mis-pairing problem common to many bi/multispecific antibody approaches. In addition, the fusion proteins of the present disclosure avoid the use of long linkers necessitated by many bispecific antibodies.

[0031] In some examples, all of the TBDs of the fusion protein recognize the same epitope on the given TNFRSF member. For example, the fusion proteins of present disclosure may incorporate 2, 3, 4, 5, or 6 TBDs with identical specificity to OX40. In other examples, the fusion protein incorporates TBDs that recognize distinct epitopes on the given TNFRSF member. For example, the fusion proteins of present disclosure may incorporate 2, 3, 4, 5, or 6 TBDs with distinct recognition specificities toward various epitopes on OX40. In these examples, the fusion proteins of the present disclosure contain multiple TBDs that target distinct regions of the particular TNFRSF member. In some examples, the TBDs may recognize different epitopes on the same TNFRSF member or recognize epitopes on distinct TNFRSF members. For example, the present

disclosure provides multispecific fusion proteins incorporating TBDs that bind GITR and OX40 or CD137 and OX40.

[0032] In some examples, all of the BDs of the fusion protein recognize the same epitope on PDL1. For example, the fusion proteins of present disclosure may incorporate 2, 3, 4, 5, or 6 BDs with identical specificity to PDL1. In other examples, the fusion protein incorporates BDs that recognize distinct epitopes on PDL1. For example, the fusion proteins of present disclosure may incorporate 2, 3, 4, 5, or 6 BDs with distinct recognition specificities toward various epitopes on PDL1. In these examples, the fusion proteins of the present disclosure with contain multiple BDs that target distinct regions of the PDL1. In some examples, the BDs may recognize different epitopes on PDL1.

[0033] In some examples, the multispecific fusion protein is a bispecific molecule that targets OX40 and PDL1 and comprises an amino acid sequence that is selected from the group consisting of SEQ ID NO: 387-394.

[0033a] In another example, there is provided an isolated polypeptide that binds OX40 and PDL1, wherein the polypeptide comprises an amino acid sequence that is selected from the group consisting of SEQ ID NO: 387-394.

[0034] In some examples, the multispecific fusion protein is a bispecific molecule that targets OX-40 and PDL1 and comprises an amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 387-394.

[0035] In some examples, the fusion protein of the present disclosure is composed of a single polypeptide. In other examples, the fusion protein of the present disclosure is composed of more than one polypeptide. For example, wherein a heterodimerization domain is incorporated into the fusion protein so as the construct an asymmetric fusion protein. For example, if an immunoglobulin Fc region is incorporated into the fusion protein the CH3 domain can be used as a homodimerization domain, or the CH3 dimer interface region can be mutated so as to enable heterodimerization.

[0036] In some examples, the fusion protein contains the BDs at opposite ends. For example, the BDs are located on both the amino-terminal (N-terminal) portion of the fusion protein and the carboxy-terminal (C-terminal) portion of the fusion protein. In other examples, all the TBDs reside on the same end of the fusion protein. For example, BDs reside on either the amino- or carboxy-terminal portions of the fusion protein.

[0037] In some examples, the linker polypeptide contains an immunoglobulin Fc region. In some examples, the immunoglobulin Fc region is an IgG isotype selected from the group consisting of IgG1 isotype, IgG2 isotype, IgG3 isotype, and IgG4 isotype.

[0038] In some examples, the immunoglobulin Fc region or immunologically active fragment thereof is an IgG isotype. For example, the immunoglobulin Fc region of the fusion protein is of human IgG1 isotype, having an amino acid sequence:

PAP**ELL**GGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV
DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP
APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH
EALHNHYTQK SLSLSPGK (SEQ ID NO: 1)

[0039] In some examples, the immunoglobulin Fc region or immunologically active fragment thereof comprises a human IgG1 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 1.

[0040] In some examples, the human IgG1 Fc region is modified at amino acid Asn297 (Boxed in SEQ ID NOs: 1-4, Kabat Numbering) to prevent to glycosylation of the fusion protein, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some examples, the Fc region of the fusion protein is modified at amino acid Leu235 (Bold in SEQ ID NO: 1, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E) or Leu235Ala (L235A). In some examples, the Fc region of the fusion protein is modified at amino acid Leu234 (Bold in SEQ ID NO: 1, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, modified at amino acid Leu234 (Boxed, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E). In some examples, the Fc region of the fusion protein is Leu234Ala (L234A). In some examples, the Fc region of the fusion protein is altered at both amino acid 234 and 235, *e.g.*, Leu234Ala and Leu235Ala (L234A/L235A) or Leu234Val and Leu235Ala (L234V/L235A). In some examples, the Fc region of the fusion protein is lacking an amino acid at one or more of the following positions to reduce Fc receptor binding: Glu233 (E233, Bold in SEQ ID NO: 1), Leu234 (L234), or Leu235 (L235). In some examples, the Fc region of the fusion protein is altered at Gly235 to reduce Fc receptor binding. For example, wherein Gly235 is deleted from the fusion protein. In some examples, the human IgG1 Fc region is modified at amino acid Gly236 (Boxed in SEQ ID NO: 1) to enhance the interaction with CD32A, e.g.,

Gly236Ala (G236A). In some examples, the human IgG1 Fc region lacks Lys447 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0041] In some examples, the Fc region of the fusion protein is altered at one or more of the following positions to reduce Fc receptor binding: Leu 234 (L234), Leu235 (L235), Asp265 (D265), Asp270 (D270), Ser298 (S298), Asn297 (N297), Asn325 (N325) orAla327 (A327). For example, Leu 234Ala (L234A), Leu235Ala (L235A), Asp265Asn (D265N), Asp270Asn (D270N), Ser298Asn (S298N), Asn297Ala (N297A), Asn325Glu (N325E) orAla327Ser (A327S). In preferred examples, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while have minimal impact on binding to the neonatal Fc receptor (FcRn).

[0042] In some examples, the Fc region of the fusion protein is lacking an amino acid at one or more of the following positions to reduce Fc receptor binding: Glu233 (E233), Leu234 (L234), or Leu235 (L235). In these examples, Fc deletion of these three amino acids reduces the complement protein C1q binding. These modified Fc region polypeptides are referred to herein as "Fc deletion" polypeptides.

PAPGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV
EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI
EKTISKAKGQ PREPQVYTLP PSRDELTKNQ VSLTCLVKGF YPSDIAVEWE
SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL
HNHYTQKSLS LSPGK (SEQ ID NO: 2)

[0043] In some examples, the immunoglobulin Fc region or immunologically active fragment thereof comprises a human IgG1 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 2.

[0044] In some examples, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG2 isotype, having an amino acid sequence:

PAPPVAGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVQFNWYVD
GVEVHNAKTK PREEQFNSTF RVVSVLTVVH QDWLNGKEYK CKVSNKGLPA
PIEKTISKTK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDISVE

WESNGQPENN YKTTPPMLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 3)

[0045] In some examples, the fusion or immunologically active fragment thereof comprises a human IgG2 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 3.

[0046] In some examples, the human IgG2 Fc region is modified at amino acid Asn297 (Boxed in SEQ ID NOs: 1, 3, 4, and 5, to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A). In some examples, the human IgG2 Fc region lacks Lys447, which corresponds to residue 217 of SEQ ID NO: 3 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0047] In some examples, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG3 isotype, having an amino acid sequence: PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVQFKWYV DGVEVHNAKT KPREEQYNST FRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKT KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV EWESSGQPEN NYNTTPPMLD SDGSFFLYSK LTVDKSRWQQ GNIFSCSVMH EALHNRFTQK SLSLSPGK (SEQ ID NO: 4)

[0048] In some examples, the antibody or immunologically active fragment thereof comprises a human IgG3 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 4.

[0049] In some examples, the human IgG3 Fc region is modified at amino acid Asn297 (Boxed in SEQ ID NOs: 1-4, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A). In some examples, the human IgG3 Fc region is modified at amino acid 435 to extend the half-life, *e.g.*, Arg435His (R435H, Boxed in SEQ ID NO: 3). In some examples, the human IgG3 Fc region lacks Lys447, which corresponds to residue of 218 SEQ ID NO: 4 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0050] In some examples, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG4 isotype, having an amino acid sequence:

PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP
SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH
EALHNHYTQK SLSLSLGK (SEQ ID NO: 5)

[0051] In some examples, the antibody or immunologically active fragment thereof comprises a human IgG4 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 5.

[0052] In other examples, the human IgG4 Fc region is modified at amino acid 235 to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E). In some examples, the human IgG4 Fc region is modified at amino acid Asn297 (Boxed in SEQ ID NOs: 1-4, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A). In some examples, the human IgG4 Fc region lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 4 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0053] In some examples, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG4 isotype, having an amino acid sequence:

PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP
SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH
EALHNHYTQK SLSLSLGK (SEQ ID NO: 6)

[0054] In some examples, the antibody or immunologically active fragment thereof comprises a human IgG4 polypeptide sequence that is at least 50%, 60%, 65%,

70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 6.

[0055] In some examples, the human IgG Fc region is modified to enhance FcRn binding. Examples of Fc mutations that enhance binding to FcRn are Met252Tyr, Ser254Thr, Thr256Glu (M252Y, S254T, T256E, respectively) (Kabat numbering, Dall'Acqua *et al* 2006, *J. Biol Chem* Vol. 281(33) 23514–23524), Met428Leu and Asn434Ser (M428L, N434S) (Zalevsky *et al* 2010 *Nature Biotech*, Vol. 28(2) 157-159), or Met252Ile, Thr256Asp, Met428Leu (M252I, T256D, M428L, respectively), (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*). Met252 corresponds to residue 23 in SEQ ID NOs: 1, 4, and 5 and residue 22 in SEQ ID NO: 3. Ser254 corresponds to corresponds to residue 25 in SEQ ID NOs: 1, 4, and 5 and residue 24 in SEQ ID NO: 3. Thr256 corresponds to residue 27 in SEQ ID NOs: 1, 4, and 5 and residue 26 in SEQ ID NO: 3. Met428 corresponds to residue 199 in SEQ ID NOs: 1, 4, and 5 and residue 198 in SEQ ID NO: 3. Asn434 corresponds to residue 205 in SEQ ID NOs: 1, 4, and 5 and residue 204 in SEQ ID NO: 3. In some examples where the fusion protein of the disclosure includes an Fc polypeptide, the Fc polypeptide is mutated or modified. In these examples, the mutated or modified Fc polypeptide includes the following mutations: Met252Tyr and Met428Leu (M252Y, M428L) using the Kabat numbering system.

[0056] In some examples, the human IgG Fc region is modified to alter antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), *e.g.*, the amino acid modifications described in Natsume et al., 2008 Cancer Res, 68(10): 3863-72; Idusogie et al., 2001 J Immunol, 166(4): 2571-5; Moore et al., 2010 mAbs, 2(2): 181-189; Lazar et al., 2006 PNAS, 103(11): 4005–4010, Shields et al., 2001 JBC, 276(9): 6591–6604; Stavenhagen et al., 2007 Cancer Res, 67(18): 8882-8890; Stavenhagen et al., 2008 Advan. Enzyme Regul., 48: 152-164; Alegre et al, 1992 J Immunol, 148: 3461-3468; Reviewed in Kaneko and Niwa, 2011 Biodrugs, 25(1):1-11. Examples of mutations that enhance ADCC include modification at Ser239 and Ile332, for example Ser239Asp and Ile332Glu (S239D, I332E). Examples of mutations that enhance CDC include modifications at Lys326, which corresponds to residue 97 of SEQ ID NOs: 1, 4, and 5 and residue 96 of SEQ ID NO: 3, and Glu333, which corresponds to residue 104 of SEQ ID NOs: 1, 4, and 5 and residue 103 of SEQ ID NO: 3. In some examples, the Fc region is modified at one or both of these positions, for example, Lys326Ala and/or Glu333Ala (K326A and E333A).

[0057] In some examples, the human IgG Fc region is modified to induce heterodimerization. For example, having an amino acid modification within the CH3 domain at Thr366, which when replaced with a more bulky amino acid, *e.g.*, Trp (T366W), is able to preferentially pair with a second CH3 domain having amino acid modifications to less bulky amino acids at positions Thr366, which corresponds to residue 137 of SEQ ID NOs: 1, 4, and 5 and residue 136 of SEQ ID NO: 3, Leu368, which corresponds to residue 139 of SEQ ID NOs: 1, 4, and 5 and residue 138 of SEQ ID NO: 3, and Tyr407, which corresponds to residue 178 of SEQ ID NOs: 1, 4, and 5 and residue 177 of SEQ ID NO: 3, *e.g.*, Ser, Ala and Val, respectively (T366S/L368A/Y407V). Heterodimerization via CH3 modifications can be further stabilized by the introduction of a disulfide bond, for example, by changing Ser354, which corresponds to residue 125 of SEQ ID NOs: 1, 4, and 5 and residue 124 of SEQ ID NO: 3, to Cys (S354C) and Tyr349, which corresponds to residue 120 of SEQ ID NOs: 1, 4, and 5 and residue 119 of SEQ ID NO: 3, to Cys (Y349C) on opposite CH3 domains (Reviewed in Carter, 2001 Journal of Immunological Methods, 248: 7–15). In some of these examples, the Fc region may be modified at the protein-A binding site on one member of the heterodimer so as to prevent protein-A binding and thereby enable more efficient purification of the heterodimeric fusion protein. An exemplary modification within this binding site is Ile253, which corresponds to residue 24 of SEQ ID NOs: 1, 4, and 5 and residue 23 of SEQ ID NO: 3, for example, Ile253Arg (I253R). For example, the I253R modification maybe combined with either the T366S/L368A/Y407V modifications or with the T366W modifications. The T366S/L368A/Y407V modified Fc is capable of forming homodimers as there is no steric occlusion of the dimerization interface as there is in the case of the T336W modified Fc. Therefore, in some examples, the I253R modification is combined with the T366S/L368A/Y407V modified Fc to disallow purification any homodimeric Fc that may have formed.

[0058] In some examples, the human IgG Fc region is modified to prevent dimerization. In these examples, the fusion proteins of the present disclosure are monomeric. For example, modification at residue Thr366 to a charged residue, e.g. Thr366Lys, Thr366Arg, Thr366Asp, or Thr366Glu (T366K, T366R, T366D, or T366E, respectively), prevents CH3-CH3 dimerization.

[0059] In some examples, the Fc region of the fusion protein is altered at one or more of the following positions to reduce Fc receptor binding: Leu 234 (L234), Leu235

(L235), Asp265 (D265), Asp270 (D270), Ser298 (S298), Asn297 (N297), Asn325 (N325) orAla327 (A327). For example, Leu 234Ala (L234A), Leu235Ala (L235A), Asp265Asn (D265N), Asp270Asn (D270N), Ser298Asn (S298N), Asn297Ala (N297A), Asn325Glu (N325E) orAla327Ser (A327S). In preferred examples, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while have minimal impact on binding to the neonatal Fc receptor (FcRn).

[0060] In some examples, the fusion protein contains a polypeptide derived from an immunoglobulin hinge region. The hinge region can be selected from any of the human IgG isotypes. For example, the fusion protein may contain a modified IgG1 hinge having the sequence of EPKSSDKTHTCPPC (SEQ ID NO: 7), where in the Cys220 that forms a disulfide with the C-terminal cysteine of the light chain is mutated to serine, e.g., Cys220Ser (C220S). In other examples, the fusion protein contains a truncated hinge having a sequence DKTHTCPPC (SEQ ID NO: 8).

[0061] In some examples, the fusion protein has a modified hinge from IgG4, which is modified to prevent or reduce strand exchange, *e.g.*, Ser228Pro (S228P), having the sequence ESKYGPPCPPC (SEQ ID NO: 9). In some examples, the fusion protein contains one or more linker polypeptides. In other examples, the fusion protein contains one or more linker and hinge polypeptides.

[0062] In some examples, the fusion proteins of the present disclosure lack or have reduced Fucose attached to the N-linked glycan-chain at N297. There are numerous ways to prevent fucosylation, including but not limited to production in a FUT8 deficient cell line; addition inhibitors to the mammalian cell culture media, for example, Castanospermine, 2-deoxy-fucose, 2-flurofucose; the use of production cell lines with naturally reduced fucosylation pathways, and metabolic engineering of the production cell line.

[0063] In some examples, the single domain antibody, VHH, or humanized single domain antibody, or human single domain antibody is engineered to eliminate recognition by pre-existing antibodies found in humans. In some examples, single domain antibodies of the present disclosure are modified by mutation of position Leu11, for example, Leu11Glu (L11E) or Leu11Lys (L11K). In other examples, single domain antibodies of the present disclosure are modified by changes in carboxy-terminal region, for example, the terminal sequence consists of GQGTLVTVKPGG (SEQ ID NO: 14) or GQGTLVTVEPGG (SEQ ID NO: 15) or modification thereof. In some examples, the

single domain antibodies of the present disclosure are modified by mutation of position 11 and by changes in carboxy-terminal region.

[0064] In some examples, the TBDs of the fusion proteins of the present disclosure are operably linked via amino acid linkers. In some examples, these linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure can be of various lengths, for example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length.

[0065] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., $(GGS)_2$ (SEQ ID NO: 10); GGSGGSGGS, i.e., $(GGS)_3$ (SEQ ID NO: 11); GGSGGSGGSGGS, i.e., $(GGS)_4$ (SEQ ID NO: 12); and GGSGGSGGSGGSGGS, i.e., $(GGS)_5$ (SEQ ID NO: 13).

[0066] In some examples, the multivalent binding fusion protein is tetravalent. In some examples, the tetravalent fusion protein has the following structure: BD-Linker-BD-Linker-Hinge-Fc. In some examples, the tetravalent fusion protein has the following structure: BD-Linker-Hinge-Fc-Linker-BD.

[0067] In some examples, the BD of the tetravalent fusion protein is a single domain antibody or VHH. In some examples, each BD of the tetravalent fusion protein is a single domain antibody or VHH. In some examples, the tetravalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some examples, the tetravalent fusion protein has the following structure: VHH-Linker-Hinge-Fc-Linker-VHH, where the VHH is a humanized or fully human VHH sequence.

[0068] In some examples, the multivalent TNFRSF binding fusion protein is tetravalent. In some examples, the tetravalent TNFRSF binding fusion protein has the following structure: TBD-Linker-TBD-Linker-Hinge-Fc. In some examples, the tetravalent TNFRSF binding fusion protein has the following structure: TBD-Linker-Hinge-Fc-Linker-TBD.

[0069] In some examples, the TBD of the tetravalent TNFRSF binding fusion protein is a single domain antibody or VHH. In some examples, each TBD of the multivalent TNFRSF binding fusion protein is single domain antibody or VHH. In some examples, the multivalent TNFRSF binding fusion protein is tetravalent. In some examples, the tetravalent TNFRSF binding fusion protein has the following structure:

VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some examples, the tetravalent TNFRSF binding fusion protein has the following structure: VHH-Linker-Hinge-Fc-Linker-VHH, where the VHH is a humanized or fully human VHH sequence.

[0070] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., $(GGS)_2$ (SEQ ID NO: 10); GGSGGSGGS, i.e., $(GGS)_3$ (SEQ ID NO: 11); GGSGGSGGSGGS, i.e., $(GGS)_4$ (SEQ ID NO: 12); and GGSGGSGGSGGSGGS, i.e., $(GGS)_5$ (SEQ ID NO: 13).

[0071] In some examples, the TBD of the tetravalent TNFRSF binding fusion protein is a single domain antibody or VHH. In some examples, each TBD of the multivalent TNFRSF binding fusion protein is single domain antibody or VHH. In some examples, the multivalent fusion protein is hexavalent. In some examples, the hexavalent fusion protein has the following structure: BD-Linker-TBD-Linker-BD-Linker-Hinge-Fc. In some examples, the hexavalent fusion protein has the following structure: BD-Linker-BD-Linker-Hinge-Fc-Linker-BD, or BD-Linker-Hinge-Fc-Linker-BD-Linker-BD.

[0072] In some examples, the BD of the hexavalent fusion protein is a single domain antibody or VHH. In some examples, each BD of the hexavalent fusion protein is a single domain antibody or VHH. In some examples, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some examples, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc-Linker-VHH, or VHH-Linker-Hinge-Fc-Linker-VHH-Linker-VHH where the VHH is a humanized or fully human VHH sequence.

[0073] In some examples, the multivalent TNFRSF binding fusion protein is hexavalent. In some examples, the hexavalent TNFRSF binding fusion protein has the following structure: TBD-Linker-TBD-Linker-TBD-Linker-Hinge-Fc. In some examples, the hexavalent TNFRSF binding fusion protein has the following structure: TBD-Linker-TBD-Linker-Hinge-Fc-Linker-TBD, or TBD-Linker-Hinge-Fc-Linker-TBD-Linker-TBD.

[0074] In some examples, the multivalent TNFRSF binding fusion protein is hexavalent. In some examples, the hexavalent TNFRSF binding fusion protein has the following structure: VHH-Linker-VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a

humanized or fully human VHH sequence. In some examples, the hexavalent TNFRSF binding fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc-Linker-VHH, or VHH-Linker-Hinge-Fc-Linker-VHH-Linker-VHH where the VHH is a humanized or fully human VHH sequence.

[0075] In some examples, the multivalent fusion protein lacks an Fc region. In some of these examples, the fusion protein is tetravalent and has the following structure BD-Linker-BD-Linker-BD-Linker-BD-Linker. In some of these examples, the fusion protein is pentavalent and has the following structure BD-Linker-BD-Linker-BD-Linker-BD-Linker-BD. In some of these examples, the fusion protein is hexavalent and has the following structure BD-Linker-BD-Linker-BD-Linker-BD-Linker-BD-Linker-BD.

[0076] In some examples, the multivalent TNFRSF binding fusion protein lacks an Fc region. In some of these examples, the TNFRSF binding fusion protein is tetravalent and has the following structure TBD-Linker-TBD-Linker-TBD-Linker-TBD-Linker. In some of these examples, the TNFRSF binding fusion protein is pentavalent and has the following structure TBD-Linker-TBD-Linker-TBD-Linker-TBD-Linker-TBD. In some of these examples, the TNFRSF binding fusion protein is hexavalent and has the following structure TBD-Linker-TBD-Linker-TBD-Linker-TBD-Linker-TBD-Linker-TBD.

[0077] In some examples, the BD of a multivalent fusion protein is a single domain antibody or VHH. In some examples, the multivalent fusion protein lacks an Fc region. In some of these examples, the fusion protein is tetravalent and has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker. In some of these examples, the fusion protein is pentavalent and has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In some of these examples, the fusion protein is hexavalent and has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In any of these examples, the VHH is a humanized or fully human VHH sequence.

[0078] In some examples, the TBD of the a multivalent TNFRSF binding fusion protein is a single domain antibody or VHH. In some examples, the multivalent TNFRSF binding fusion protein lacks an Fc region. In these examples, the TNFRSF binding fusion protein is tetravalent and has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker. In these examples, the TNFRSF binding fusion protein is pentavalent and has the following structure VHH-Linker-VHH-Linker-VHH-

Linker-VHH-Linker-VHH. In these examples, the TNFRSF binding fusion protein is hexavalent and has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In these examples, the VHH is a humanized or fully human VHH sequence.

[0079] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., $(GGS)_2$ (SEQ ID NO: 10); GGSGGSGGS, i.e., $(GGS)_3$ (SEQ ID NO: 11); GGSGGSGGSGGS, i.e., $(GGS)_4$ (SEQ ID NO: 12); and GGSGGSGGSGGSGGS, i.e., $(GGS)_5$ (SEQ ID NO: 13).

[0080] In some examples, the fusion proteins are multispecific containing a TBD and a binding domain directed toward a second antigen. In these examples, the second antigen binding domain can be positioned at numerous positions within the molecule relative to the TBD. In some examples, the second antigen binding domain is located N-terminal TBD. In other examples, the second antigen binding domain is located to C-terminal to the TBD. In other examples, the second antigen binding domain is located on a distinct polypeptide that associates with a first polypeptide containing the TBD.

[0081] In some examples, the fusion proteins are multispecific containing an anti-OX40 binding domain and a binding domain directed toward a second antigen. In these examples, the second antigen binding domain can be positioned at numerous positions within the molecule relative to the an anti-OX40 binding domain. In some examples, the second antigen binding domain is located N-terminal an anti-OX40 binding domain. In other examples, the second antigen binding domain is located to C-terminal to the an anti-OX40 binding domain. In other examples, the second antigen binding domain is located on a distinct polypeptide that associates with a first polypeptide containing the an anti-OX40 binding domain.

[0082] In some examples, the fusion proteins are multispecific containing an anti-PDL1 binding domain and a binding domain directed toward a second antigen. In these examples, the second antigen binding domain can be positioned at numerous positions within the molecule relative to the an anti-PDL1 binding domain. In some examples, the second antigen binding domain is located N-terminal an anti-PDL1 binding domain. In other examples, the second antigen binding domain is located to C-terminal to the an anti-PDL1 binding domain. In other examples, the second antigen binding domain is located on a distinct polypeptide that associates with a first polypeptide containing the an anti-PDL1 binding domain.

[0083] In some examples, the TBD within the multispecific TNFRSF binding fusion protein is a single domain antibody or VHH. In some examples, the TBD within the multispecific TNFRSF binding fusion protein is a composed of antibody variable heavy (VH) chain and variable light (VL) chain region. In some examples, the VH and VL of the TBD are formatted as a single chain variable fragment (scFv) connected via a linker region. In some examples, the VH and VL of the TBD are formatted as a FAB fragment that associates via a constant heavy 1 (CH1) domain and a constant light chain (CL) domain. In some examples, non-antibody heterodimerization domains are utilized to enable the proper association of the VH and VL of the TBD. In some examples, the TBD within the multispecific TNFRSF binding fusion protein is derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0084] In some examples, the TBD within the multispecific TNFRSF binding fusion protein is a single domain antibody or VHH that binds OX40. In some examples, the anti-OX40 binding domain within the multispecific TNFRSF binding fusion protein is a composed of antibody variable heavy (VH) chain and variable light (VL) chain region. In some examples, the VH and VL of the anti-OX40 binding domain are formatted as a single chain variable fragment (scFv) connected via a linker region. In some examples, the VH and VL of the anti-OX40 binding domain are formatted as a Fab fragment that associates via a constant heavy 1 (CH1) domain and a constant light chain (CL) domain. In some examples, non-antibody heterodimerization domains are utilized to enable the proper association of the VH and VL of the anti-OX40 binding domain. In some examples, the anti-OX40 binding domain within the multispecific TNFRSF binding fusion protein is derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0085] In some examples, the binding domain within the multispecific fusion protein is a single domain antibody or VHH that binds PDL1. In some examples, the anti-PDL1 binding domain within the multispecific TNFRSF binding fusion protein is a composed of antibody variable heavy (VH) chain and variable light (VL) chain region. In some examples, the VH and VL of the anti-PDL1 binding domain are formatted as a single chain variable fragment (scFv) connected via a linker region. In some examples, the VH and VL of the anti-PDL1 binding domain are formatted as a Fab fragment that

associates via a constant heavy 1 (CH1) domain and a constant light chain (CL) domain. In some examples, non-antibody heterodimerization domains are utilized to enable the proper association of the VH and VL of the anti-PDL1 binding domain. In some examples, the anti-PDL1 binding domain within the multispecific fusion protein is derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0086] In some examples, the anti-OX40 binding domain of the multispecific TNFRSF binding fusion protein is a bispecific antibody or antigen-binding fragment thereof.

[0087] In some examples, the anti-PDL1 binding domain of the multispecific fusion protein is a bispecific antibody or antigen-binding fragment thereof.

[0088] In any of these examples, the bispecific antibody or antigen-fragment thereof can be any suitable bispecific format known in the art, including, by way of non-limiting example, formats based on antibody fragments such as, e.g., X-Link Fab, cross-linked Fab fragments; tascFv/BiTE, tandem-scFv/Bispecific T cell Engager; Db, diabody; taDb, tandem diabody; formats based on Fc-fusions such as, e.g., Db-Fc, diabody-Fc fusion; taDb-Fc fusion, tandem diabody-Fc fusion; taDb-CH3, tandem diabody-CH3 fusion; (scFv)4-Fc, tetra scFv-Fc fusion; DVD-Ig, dual variable domain immunoglobulin; IgG formats such as, e.g., knob-hole and SEED, strand exchange engineered domain; CrossMab, knob-hole combined with heavy and light chain domain exchange; bsAb, quadroma derived bispecific antibody; sdAb, single domain based antibody; and kappa-lambda bodies such as those described in PCT Publication No. WO 2012/023053.

[0089] In any of the above examples, at least one TBD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 16-29 and 377-386.

[0090] In any of the above examples, at least one TBD comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 36, and 44; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 35, and 37; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 32-34, 48-43, and 45.

[0091] In any of the above examples, at least one BD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 46-57.

[0092] In any of the above examples, at least one BD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 52-57.

[0093] In any of the above examples, at least one BD comprises a CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 58, 61, and 64; a CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 59, 62, 65, and 69; and a CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 60, 63, 66-68, and 70.

[0094] In any of the above examples, at least one TBD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and at least one BD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 46-57.

[0095] In any of the above examples, at least one TBD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 16-29 and 377-386, and at least one BD comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 52-57.

[0096] In any of the above examples, at least one TBD comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 36, and 44; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 35, and 37; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 32-34, 48-43, and 45, and at least one BD comprises a CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 58, 61, and 64; a CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 59, 62, 65, and 69; and a CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 60, 63, 66-68, and 70.

BRIEF DESCRIPTION OF THE DRAWINGS

[0097] Figure 1 is schematic of exemplary multivalent and multispecific fusion proteins of the present disclosure.

[0098] Figures 2A and 2B are graphs demonstrating the ability of OX40 single domain antibodies (VHHs) to bind cell surface OX40. Binding was assessed by flow

What is claimed is:

1. An isolated polypeptide comprising at least one VHH domain that binds PDL1 and at least one VHH domain that binds OX40, wherein at least one VHH domain that binds PDL1 comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from SEQ ID NOs: 58, 61, and 64; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from SEQ ID NOs: 59, 62, 65, and 69; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from SEQ ID NOs: 60, 63, 66-68, and 70.

2. The isolated polypeptide of claim 1, wherein at least one VHH domain that binds PDL1 comprises CDR1, CDR2, and CDR3 selected from: SEQ ID NOs: 58, 59, and 60; SEQ ID NOs: 61, 62, and 63; SEQ ID NOs: 64, 65, and 66; SEQ ID NOs: 58, 65, and 67; SEQ ID NOs 58, 65, and 68; and SEQ ID NOs: 58, 69, and 70.

3. The isolated polypeptide of claim 1 or claim 2, wherein at least one VHH domain that binds PDL1 comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 61; a CDR2 comprising the amino acid sequence of SEQ ID NO: 62; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 63.

4. The isolated polypeptide of any one of claims 1-3, wherein at least one VHH domain that binds PDL1 is humanized.

5. The isolated polypeptide of any one of claims 1-4, wherein at least one VHH domain that binds PDL1 comprises an amino acid sequence that is at least 95% identical to an amino acid sequence selected from SEQ ID NOs: 52-57.

6. The isolated polypeptide of any one of claims 1-5, wherein at least one VHH domain that binds PDL1 comprises an amino acid sequence selected from SEQ ID NOs: 52-57.

7. The isolated polypeptide of any one of claims 1-6, wherein at least one VHH domain that binds PDL1 comprises the amino acid sequence of SEQ ID NO: 57.

8. The isolated polypeptide of any one of claims 1-7, wherein the polypeptide comprises one VHH domain that binds PDL1.

9. The isolated polypeptide of any one of claims 1-7, wherein the polypeptide comprises two VHH domains that bind PDL1.

10. The isolated polypeptide of any one of claims 1-9, wherein at least one VHH domain that binds OX40 comprises a CDR1 comprising an amino acid sequence selected from SEQ ID NO: 30, 36, and 44; a CDR2 comprising an amino acid sequence selected from SEQ ID NO: 31, 35, and 37; and a CDR3 comprising an amino acid sequence selected from SEQ ID NO: 32-34, 38-43, and 45.

11. The isolated polypeptide of any one of claims 1-10, wherein at least one VHH domain that binds OX40 comprises CDR1, CDR2, and CDR3 selected from: SEQ ID NOs: 30, 31, and 32; SEQ ID NOs: 30, 31, and 33; SEQ ID NOs: 30, 31, and 34; SEQ ID NOs: 30, 35, and 32; SEQ ID NOs: 36, 37, and 38; SEQ ID NOs: 36, 37, and 39; SEQ ID NOs: 36, 37, and 40; SEQ ID NOs: 36, 37, and 42; SEQ ID NOs: 36, 37, and 43; SEQ ID NOs: 44, 37, and 39; and SEQ ID NOs: 36, 37, and 45.

12. The isolated polypeptide of any one of claims 1-11, wherein at least one VHH domain that binds OX40 comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 36, a CDR2 comprising the amino acid sequence of SEQ ID NO: 37, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 42; or comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 36, a CDR2 comprising the amino acid sequence of SEQ ID NO: 37, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 39.

13. The isolated polypeptide of any one of claim 1-12, wherein at least one VHH domain that binds OX40 is humanized.

14. The isolated polypeptide of any one of claims 1-13, wherein at least one VHH domain that binds OX40 comprises an amino acid sequence that is at least 95% identical to an amino acid sequence selected from SEQ ID NOs: 377-385 and 29.

15. The isolated polypeptide of any one of claims 1-14, wherein at least one VHH domain that binds OX40 comprises an amino acid sequence selected from SEQ ID NOs: 377-385 and 29.

16. The isolated polypeptide of any one of claims 1-15, wherein the polypeptide comprises one VHH domain that binds OX40.

17. The isolated polypeptide of any one of claims 1-15, wherein the polypeptide comprises two VHH domains that bind OX40.

18. The isolated polypeptide of any one of claims 1-17, wherein the isolated polypeptide comprises an Fc region.

19. The isolated polypeptide of claim 18, wherein the Fc region comprises an amino acid sequence that is at least 97% identical to an amino acid sequence selected from SEQ ID NOs: 1-6.

20. The isolated polypeptide of claim 18, wherein the Fc region comprises an amino acid sequence selected from SEQ ID NOs: 1-6.

21. The isolated polypeptide of any one of claims 18-20, which forms a homodimer under physiological conditions.

22. The isolated polypeptide of any one of claims 18-21, wherein the polypeptide has the structure VHH-Linker-VHH-Linker-Hinge-Fc.

23. The isolated polypeptide of claim 22, wherein at least one linker is, independently, 5-20 amino acids in length.

24. The isolated polypeptide of claim 22 or claim 23, wherein the hinge comprises a sequence selected from SEQ ID NOs: 7-9.

25. The isolated polypeptide of any one of claims 18-24, which forms a dimer at physiological conditions.

26. An isolated polynucleotide that encodes the polypeptide of any one of claims 1-25.

27. An isolated cell that comprises the polynucleotide of claim 26.

28. An isolated cell that expresses the polypeptide of any one of claims 1-25.

29. A method of producing the polypeptide of any one of claims 1-25 comprising culturing the host cell of claim 27 or claim 28 under conditions suitable for producing the polypeptide.

30. Use of a polypeptide of any one of claims 1-25 in the manufacture of a medicament for treating cancer.

31. The use of claim 30, wherein the cancer is carcinoma, sarcoma, lymphoma, or leukemia.

32. An in vitro method of increasing T cell activation and/or proliferation comprising contacting T cells with a polypeptide of any one of claims 1-25.

33. The method of claim 32, wherein the T cells are CD4+ T cells and/or CD8+ T cells.

34. Use of a polypeptide of any one of claims 1-25 in the manufacture of a medicament for increasing T cell activation and/or proliferation.

35. The use of claim 34, wherein the T cells are CD4+ T cells and/or CD8+ T cells.