

CD25 BASED LYSOSOMAL DEGRADER AND USES THEREOF**PRIORITY**

[0001] This application claims priority to European patent application 23306925.1, filed on November 8, 2023, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Over the past two decades, target protein degradation technologies have elicited great interest in expanding the landscape of druggable targets. It has also provided a unique mechanism of action for therapeutics as “event-driven” pharmacology as opposed to “occupancy-driven” associated with conventional inhibitors. For example, PROteolysis TArgeting Chimeras or PROTACs, small heterobifunctional molecules that form a ternary complex with an E3 ubiquitin ligase and a target of interest, resulting in target ubiquitination and degradation, have advanced through clinical trials. However, the therapeutic potential of PROTACs has been hampered by the poor permeability, pharmacokinetics and pharmacodynamic properties commonly seen with high molecular mass small molecules (over 1,000 Da). More recently, large molecule-based degrader technologies, such as lysosome targeting chimeras (LYTACs), have highlighted the potential of leveraging large molecules for targeted degradation of extracellular soluble and membrane-associated proteins. There is a need in the art for targeted degradation strategies, particularly those that are able to degrade targets in specific cell types such as CD25 positive cells.

[0003] CD25 is the 55 kDa α -chain subunit of the IL-2 receptor, also known as IL2-R α , CD25, p55, and Tac (T cell activation) antigen and is unique to the IL-2 receptor because the other IL-2 receptor subunits are functional components of other cytokine receptors and undergo a distinct cellular trafficking pattern once internalized by a CD25 positive cell. Monospecific CD25 target therapies can modulate the immune response in a patient suffering from *e.g.*, a neoplastic disorder or an autoimmune disease which results in suppression of regulatory T cells. As such, there is a need in the art for both monospecific and multispecific strategies to target CD25.

SUMMARY

[0004] The present disclosure provides The present disclosure provides a method for degrading a target protein, comprising:

contacting a CD25 positive cell with a multispecific binding protein, wherein the multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of the CD25 positive cell, wherein the first cell surface binding moiety comprises at least one immunoglobulin domain or an antigen binding fragment thereof; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to the target protein, wherein specific binding of the multispecific binding protein to the CD25 facilitates the internalization of the target protein into the CD25 positive cell.

[0005] In one aspect the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein. In one aspect, the CD25 and/or the multispecific binding protein is recycled back to the surface of the cell independent of the target protein. In one aspect, the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell. In one aspect, the CD25 positive cell is a neoplastic cell. In one aspect, the CD25 positive cell is a T-cell or a NK cell.

[0006] In one aspect the multispecific binding protein exhibits increased degradation of the target protein compared to a reference binding polypeptide. In one aspect, the reference binding polypeptide does not comprise the first cell surface binding moiety that specifically binds to CD25 but is otherwise identical to the multispecific binding protein. In one aspect, the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide.

[0007] In one aspect, the multispecific binding protein degrades the target protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide. In one aspect, the multispecific binding protein exhibits increased degradation of the target protein compared to the reference binding polypeptide as assessed by confocal microscopy.

[0008] In one aspect the first cell surface binding moiety binds an extracellular domain of CD25. In one aspect, the first cell surface binding moiety does not inhibit the binding of IL-2 to CD25. In one aspect, the first cell surface binding moiety does not inhibit the signaling of IL-2 *via* CD25. In one aspect, binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25. In one aspect, the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay.

[0009] In one aspect the first cell surface binding moiety comprises at least one CD25 specific variable domain. In one aspect, the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide. In one aspect, the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide *via* an amino acid linker. In one aspect, the amino acid linker is at least 90% identical to an amino acid linker sequence encoded by an amino acid linker sequence set forth in Table 10.

[0010] In one aspect the first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In one aspect, the first cell surface binding moiety comprises a fusion protein comprising a CD25 specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25:Fc fusion polypeptide or a variant thereof. In one aspect, the second binding moiety that specifically binds to the target protein comprises at least one target specific variable domain. In one aspect, the target specific variable domain is operatively linked to a second Fc domain polypeptide. In one aspect, the target specific variable domain is operatively linked to a second Fc domain polypeptide *via* an amino acid linker. In one aspect, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid linker sequence set forth in Table 10. In one aspect, the second Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In one aspect, the second binding moiety comprises a fusion protein comprising a target specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-target:Fc fusion polypeptide or a variant thereof. In one aspect, the second binding moiety that specifically binds to the target protein comprises an antibody or an antigen binding fragment thereof.

[0011] In one aspect the methods of the disclosure provide that the first and second IgG Fc domain polypeptides dimerize to form the multispecific binding protein. In one aspect, the first and second IgG Fc domain polypeptides dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof. In one aspect, the first IgG Fc domain polypeptide comprises a knob substitution, and the second IgG Fc domain polypeptide comprises a hole substitution or wherein the first IgG Fc domain polypeptide comprises a hole substitution, and the second IgG Fc domain polypeptide comprises a knob substitution. In one aspect, the knob substitution is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W). In one

aspect, the hole substitution is selected from the group consisting of alanine (A), asparagine (N), aspartic acid (D), glycine (G), serine (S), threonine (T), and valine (V).

[0012] In one aspect the first cell surface binding moiety and the second binding moieties of the multispecific binding moiety each independently comprises a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®. In one aspect, the scFV is a linear scFV or a tandem scFV. In one aspect, the multispecific binding protein further comprises a Fc domain or a variant thereof. In one aspect, the ISVD is a VHH, a VH, or a VNAR. In one aspect, the ISVD is a humanized VHH, a camelized VH, a camelized human VH, a domain antibody, a single domain antibody, or a dAb. In one aspect, the second binding moiety comprises at least one ISVD. In one aspect, the second binding moiety comprises at least two ISVDs. In one aspect, the second binding moiety comprises at least two ISVDs that bind the same target protein. In one aspect, the at least two ISVDs bind to the same or different epitopes on the same target protein. In one aspect, the target protein is a membrane-associated target protein, a soluble target protein, or both. In one aspect, the target protein is expressed on the surface of the same or a different a CD25 positive cell. In one aspect, the target protein is expressed on the surface of a neoplastic cell and/or an immune cell. In one aspect, the target protein is expressed on a T-cell. In one aspect, the T-cell is an activated T cell or a regulatory T (Treg) cell. In one aspect, the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein.

[0013] In one aspect the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF-β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein.

[0014] In one aspect, the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder. In one aspect, the multispecific binding protein exhibits pH-dependent binding to CD25 on the CD25 positive cell. In one aspect, the multispecific binding protein exhibits pH-dependent binding to the

target protein. In one aspect, the multispecific binding protein exhibits reduced binding at acidic pH.

[0015] In one aspect, the first cell surface binding moiety of the multispecific binding protein binds to CD25 on the CD25 positive cell with an affinity from about 100 pM to about 1 μ M. In one aspect, the second binding moiety of the multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M. In one aspect, the multispecific binding protein comprises one or more mutations or glycan modifications. In one aspect, to modulate Fc mediated effector function. The multispecific binding protein can comprise one or more mutations to modulate serum half-life.

[0016] The present disclosure provides a multispecific binding protein comprising:

a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell, wherein the first cell surface binding moiety comprises an immunoglobulin domain; and

b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein,

wherein specific binding of the multispecific binding protein to the CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.

[0017] In one aspect, the present disclosure provides a multispecific binding protein comprising the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein. In one aspect, the CD25 and/or the multispecific binding protein is recycled back to the surface of the cell independent of the target protein. In one aspect, the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell. In one aspect, the CD25 positive cell is a neoplastic cell. In one aspect, the CD25 positive cell is a T-cell or a NK cell.

[0018] In one aspect, the present disclosure provides a multispecific binding protein exhibiting increased degradation of the target protein compared to a reference binding polypeptide. In one aspect, the reference binding polypeptide does not comprise the first cell surface binding moiety that specifically binds to CD25 but is otherwise identical to the multispecific binding protein. In one aspect, the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide. In one aspect, the multispecific binding protein degrades the target

protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide. In one aspect, the multispecific binding protein exhibits increased degradation of the target protein compared to the reference binding polypeptide as assessed by confocal microscopy.

[0019] In one aspect, the present disclosure provides a multispecific binding protein comprising a first cell surface binding moiety that binds an extracellular domain of CD25. In one aspect, the binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25. In one aspect, the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay. In one aspect, the first cell surface binding moiety does not inhibit the binding of IL-2 to CD25. In one aspect, the first cell surface binding moiety does not inhibit the signaling of IL-2 *via* CD25.

[0020] In one aspect, the present disclosure provides a multispecific binding protein comprising a first cell surface binding moiety that comprises at least one CD25 specific variable domain. In one aspect, the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide. In one aspect, the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide *via* an amino acid linker. The amino acid linker sequence can be at least 90% identical to an amino acid linker encoded by an amino acid linker sequence set forth in Table 10. In one aspect, the first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In one aspect, the first cell surface binding moiety comprises a fusion protein comprising a CD25 specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25:Fc fusion polypeptide or a variant thereof.

[0021] In one aspect, the present disclosure provides a multispecific binding protein where the second binding moiety that specifically binds to the target protein comprises at least one target specific variable domain. In one aspect, the target specific variable domain is operatively linked to a second Fc domain polypeptide. In one aspect, the target specific variable domain is operatively linked to a second Fc domain polypeptide *via* an amino acid linker. In one aspect, the amino acid linker is at least 90% identical to an amino acid linker sequence encoded by an amino acid linker sequence set forth in Table 10. In one aspect, the second Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In one aspect, the second binding moiety comprises a fusion protein comprising a target specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-target:Fc fusion

polypeptide or a variant thereof. In one aspect, the second binding moiety that specifically binds to the target protein comprises an antibody or an antigen binding fragment thereof.

[0022] In one aspect, the present disclosure provides a multispecific binding protein comprising first and second IgG Fc domain polypeptides that dimerize to form the multispecific binding protein. In one aspect, the first and second IgG Fc domain polypeptides dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof. In one aspect, the first IgG Fc domain polypeptide comprises a knob substitution, and the second IgG Fc domain polypeptide comprises a hole substitution or wherein the first IgG Fc domain polypeptide comprises a hole substitution, and the second IgG Fc domain polypeptide comprises a knob substitution. The knob substitution can be selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W). In one aspect, the hole substitution is selected from the group consisting of alanine (A), asparagine (N), aspartic acid (D), glycine (G), serine (S), threonine (T), and valine (V).

[0023] In one aspect, the present disclosure provides a multispecific binding protein comprising the first cell surface binding moiety and the second binding moieties of the multispecific binding moiety, wherein each independently comprise a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®. In one aspect, the scFv is a linear scFv or a tandem scFv. In one aspect, the ISVD is a VHH, humanized VHH, a camelized VH, a single domain antibody, a domain antibody, a dAb, or a VNAR. In one aspect, the second binding moiety comprises at least one ISVD. In one aspect, the second binding moiety comprises at least two ISVDs. In one aspect, the second binding moiety comprises at least two ISVDs that bind the same target protein. In one aspect, the at least two ISVDs bind to the same or different epitope on the same target protein. In one aspect, the multispecific binding protein further comprises a Fc domain or a variant thereof. In one aspect, the target protein is a membrane-associated target protein, a soluble target protein, or both.

[0024] In one aspect, the present disclosure provides a multispecific binding protein comprising a target protein that is expressed on the surface of the same or a different a CD25 positive cell. In one aspect, the target protein is expressed on the surface of

a neoplastic cell and/or an immune cell. In one aspect, the target protein is expressed on a T-cell. In one aspect, the T-cell is an activated T cell or a regulatory T (Treg) cell. In one aspect, the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein.

[0025] In one aspect, the present disclosure provides a multispecific binding protein comprising the target protein, which is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein. In one aspect, the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.

[0026] In one aspect, the present disclosure provides a multispecific binding protein that exhibits pH-dependent binding to CD25 on the CD25 positive cell. In one aspect, the multispecific binding protein exhibits pH-dependent binding to the target protein. In one aspect, the multispecific binding protein exhibits reduced binding at acidic pH. In one aspect, the first cell surface binding moiety of the multispecific binding protein binds to CD25 on In one aspect, the CD25 positive cell with an affinity from about 100 pM to about 1 μ M. In one aspect, the second binding moiety of the multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M.

[0027] In one aspect, the present disclosure provides a multispecific binding protein that comprises one or more mutations or glycan modifications to modulate Fc mediated effector function. In one aspect, the multispecific binding protein comprises one or more mutations to modulate serum half-life.

[0028] In one aspect, the disclosure provides a pharmaceutical composition comprising the multispecific binding protein of any one of the preceding aspects and a pharmaceutically acceptable carrier or diluent. In another aspect, a nucleic acid molecule encodes the multispecific binding protein. In another aspect, a vector comprises the nucleic acid molecule. In another aspect, a cell comprises the nucleic acid molecule or the vector.

[0029] In one aspect, the disclosure provides a method of depleting a target protein comprising administering to a subject an effective amount of the multispecific binding

protein of any one of preceding aspects or the pharmaceutical composition comprising the same. In one aspect, the multispecific binding protein is internalized by the CD25 positive cell. In one aspect, an amount of multispecific binding protein internalized by the CD25 positive cell is greater than an amount of a reference binding polypeptide that does not comprise the first cell surface binding moiety. In one aspect, the target protein is selectively depleted from a target tissue or circulation of the subject. In one aspect, administering the multispecific binding protein results in at least about 10%, 20, 30%, 40%, 50%, 75%, or 90% depletion of the target protein from the target tissue or circulation of the subject.

[0030] In one aspect, the disclosure provides a method of treating a disease comprising administering an effective amount of a multispecific binding protein of any one of preceding aspects or the pharmaceutical composition of comprising the same to a subject. In one aspect, the disease is selected from a group consisting of: cancer, autoimmune disease, inflammatory disorder, infectious disease, and neurodegenerative disorder.

[0031] In one aspect, the disclosure provides a binding protein comprising at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell. In one aspect, the CD25 ISVD binds an extracellular domain of a CD25 protein. In one aspect, the cell comprises human or cynomolgus CD25 protein (hCD25 or cynoCD25, respectively). In one aspect, the binding protein is cross-reactive to hCD25 or cynoCD25 but not to CD25 from other species. In one aspect, the binding protein specifically binds to hCD25 and cynoCD25.

[0032] In one aspect, the binding protein of the disclosure is an antagonist of CD25 activity. In one aspect, the binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25. In one aspect, the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay. In one aspect, the CD25 ISVD binding to CD25 on the cell surface does not result in CD25 degradation. In one aspect, the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell. In one aspect, the CD25 positive cell is a neoplastic cell. In one aspect, the CD25 positive cell is a T-cell or a NK cell.

[0033] In one aspect, a binding protein comprises at least two CD25 ISVDs. In one aspect, the at least two CD25 ISVDs bind the same CD25 protein. In one aspect, the at least two CD25 ISVDs bind to the same or different epitopes on the same CD25

protein. In one aspect, the at least two CD25 ISVDs are operatively linked via an amino acid linker sequence.

[0034] In one aspect, the binding protein specifically binds to hCD25 with: (a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 2×10^{-8} , 1.7×10^{-8} , 1.5×10^{-8} , 1×10^{-8} , 5×10^{-9} , 1×10^{-9} ; (b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 3.5×10^{-3} , 3.4×10^{-3} , 1×10^{-3} , 5×10^{-4} , 10^{-4} ; or (c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.5×10^5 , 2.0×10^5 , 5×10^5 , 10^6 , as measured by surface plasmon resonance (SPR).

[0035] In one aspect, the binding protein specifically binds to cynoCD25 with: (a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 5×10^{-8} , 3.5×10^{-8} , 10^{-8} , 5×10^{-9} , 10^{-9} ; (b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 4×10^{-3} , 10^{-3} , 5×10^{-4} , 10^{-4} ; or (c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.0×10^5 , 1.2×10^5 , 5×10^5 , 1×10^6 , as measured by surface plasmon resonance (SPR).

[0036] In one aspect, the binding protein has an EC_{50} value for binding to human or cyno CD25 on HEK293-MZA cells of less than $10^{-8}M$, such as less than $5 \cdot 10^{-9}M$, such as between $5 \cdot 10^{-9}M$ and $2 \times 10^{-9} M$, as measured in a FACS binding assay.

[0037] In one aspect, the binding protein has an IC_{50} value in competition with IL-2 for binding to - human CD25 on HEK293-MZA cells of less than $10^{-8}M$, such as between $10^{-8}M$ and $10^{-9} M$, - cyno CD25 on HEK293-MZA cells of less than $10^{-7}M$, such as between $10^{-7}M$ and $10^{-8} M$, as measured in a FACS competition assay.

[0038] In one aspect, the disclosure provides a binding protein comprising a CD25 ISVD that consists essentially of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 (AbM numbering) has an amino acid sequence selected from: the amino acid sequence of SEQ ID NO: 113; amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 113; or amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 113; and CDR2 (AbM numbering) has an amino acid sequence selected from: the amino acid sequence of SEQ ID NO: 115; amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 115; or amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 115; and CDR3 (AbM numbering) has an amino

acid sequence selected from: the amino acid sequence of SEQ ID NO: 117; amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 117; or amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 117.

[0039] In one aspect, the disclosure provides a binding protein comprising CDR1 (AbM numbering) having an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 113; CDR2 (AbM numbering) having an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 115; and CDR3 (AbM numbering) having an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 117.

[0040] In one aspect, a binding protein is provided comprising a CD25 ISVD that is at least 80%, 85%, 90%, or 95% identical to an amino acid sequence set forth in SEQ ID NO: 72. In one aspect, the CD25 ISVD consists essentially of SEQ ID NO: 72. In one aspect, the CD25 ISVD consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb. In one aspect, the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation. In one aspect, the VHH sequence is a humanized VHH sequence.

[0041] In one aspect, the disclosure provides a binding protein operatively linked to a Fc domain polypeptide or variant thereof. In one aspect, the binding protein is operatively linked to a Fc domain polypeptide or variant thereof *via* an amino acid linker sequence. In one aspect, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

[0042] In one aspect, the Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide or variant thereof. In one aspect, the binding protein comprises a fusion protein comprising a CD25 ISVD and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25 ISVD:Fc fusion polypeptide or a variant thereof.

[0043] In one aspect, the disclosure provides a binding protein operatively linked to a serum albumin ISVD. The serum albumin ISVD can have an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88. In one aspect, the serum albumin ISVD consists essentially of SEQ ID NO: 88. The CD25 ISVD can be operatively linked to the serum albumin ISVD via an amino acid linker sequence.

[0044] In one aspect, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10. In one aspect, one or more other groups, residues, moieties or binding units, are optionally linked *via* one or more amino acid linker sequence(s). In one aspect, one or more other groups, residues, moieties or binding units are ISVDs. In one aspect, one or more other groups, residues, moieties or binding units are chosen from the group consisting of VHHs, humanized VHHs, camelized VHs, domain antibodies, single domain antibodies and dAbs.

[0045] In one aspect, the disclosure provides a binding protein comprising one or more mutations or glycan modifications to modulate Fc mediated effector function. In one aspect, the binding protein comprises one or more mutations to modulate serum half-life.

[0046] In one aspect, the disclosure provides a pharmaceutical composition comprising the binding protein of any one of the preceding claims and a pharmaceutically acceptable carrier or diluent. In another aspect, a nucleic acid molecule encodes the binding protein. In another aspect, a vector comprises the nucleic acid molecule. In another aspect, a cell comprises the nucleic acid molecule or the vector.

[0047] In one aspect, the disclosure provides a method of treating a neoplastic disorder in a subject comprising administering to a subject an effective amount of the binding protein of any one of the preceding aspects or a pharmaceutical composition comprising said binding protein.

[0048] In one aspect, the disclosure provides a method of treating an autoimmune disorder comprising administering to a subject an effective amount of the binding protein of any one of the preceding aspects or a pharmaceutical composition comprising said binding protein.

[0049] In one aspect, the disclosure provides a method of depleting human antigen-specific CD25+ regulatory T cells in a subject comprising administering to a subject an effective amount of the binding protein of any one of the preceding aspects or a pharmaceutical composition of said binding protein.

[0050] In one aspect, the disclosure provides a binding protein wherein the binding protein is a multispecific binding protein. In one aspect, the multispecific binding protein comprises at least one CD25 ISVD and a second binding moiety that specifically binds to a target protein. In one aspect, the binding of the multispecific

binding protein to the cell surface CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell. In one aspect, the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein.

[0051] In one aspect, the disclosure provides a binding protein wherein the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein. In one aspect, the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein. In one aspect, the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.

[0052] In one aspect, the disclosure provides a binding protein wherein the second binding moiety that specifically binds to the target protein comprises at least one antigen binding fragment. In one aspect, the target antigen binding fragment comprises at least one of a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®, a linear scFv, or a tandem scFv. The target antigen binding fragment can comprises at least one ISVD that specifically binds the target protein. The target antigen binding fragment can comprise at least two ISVDs that specifically bind the target protein. The target protein ISVD(s) can consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb. In one aspect, the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation. In one aspect, the VHH sequence is a humanized VHH sequence. In one aspect, at least two target protein ISVDs are operatively linked via an amino acid linker sequence. In one aspect, the at least two target protein ISVDs bind the same target protein. In one aspect, the at least two target protein ISVDs bind to the same or different epitopes on the same target protein.

[0053] In one aspect, the disclosure provides a binding protein wherein the binding protein is operatively linked to a serum albumin ISVD. In one aspect, the serum

albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88. In one aspect, the serum albumin ISVD consists essentially of SEQ ID NO: 88. In one aspect, the second binding moiety that specifically binds to the target protein is operatively linked to the serum albumin ISVD via an amino acid linker sequence. In one aspect, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence amino acid linker sequence encoded by an amino acid sequence set forth in Table 10. In one aspect, the binding protein is encoded by an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 119.

[0054] In one aspect, the disclosure provides a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD; b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD; and c) a third binding moiety that specifically binds to serum albumin ISVD.

[0055] In one aspect, the disclosure provides a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a second binding moiety; b) the second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a third binding moiety; and c) the third binding moiety that specifically binds to serum albumin ISVD.

[0056] In one aspect, the disclosure provides a multispecific binding protein comprising: a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a second binding moiety; b) the second binding moiety that specifically binds to a target protein comprising at least two target protein ISVDs wherein one of the at least two target protein ISVDs are linked to the N- and/or C-terminal end of a third binding moiety; and c) the third binding moiety that specifically binds to serum albumin ISVD.

[0057] In one aspect, the disclosure provides a multispecific binding protein wherein the multispecific binding protein comprises at least two target protein ISVDs and the at least two target protein ISVDs are at least two TNF ISVDs that both specifically bind

to TNF. In one aspect, the at least two TNF ISVDs bind the same or different TNF epitopes. In one aspect, the multispecific binding protein further comprises the serum albumin ISVD comprises SEQ ID NO: 88.

[0058] In one aspect, the disclosure provides a multispecific binding protein comprising an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence set forth in SEQ ID NO: 119. In one aspect, the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide. In one aspect, the multispecific binding protein degrades the target protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide.

[0059] In one aspect, the disclosure provides a multispecific binding protein wherein the CD25 ISVD(s) and the target protein ISVD(s) are operatively linked to a first and second Fc domain polypeptide, respectively. In one aspect, the CD25 ISVD(s) and the target protein ISVD(s) are operatively linked to the first and second Fc domain polypeptide *via* an amino acid linker sequence. In one aspect, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10. In one aspect, the first and second Fc domain polypeptides each comprise a first and a second IgG domain that dimerize to form the multispecific binding protein.

[0060] In one aspect, the disclosure provides a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a first IgG; and b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof.

[0061] In one aspect, the disclosure provides a pharmaceutical composition comprising the binding protein of any one of the preceding aspects and a pharmaceutically acceptable carrier or diluent. In one aspect, the disclosure provides a nucleic acid molecule encoding the multispecific binding protein of any one of the preceding aspects. In one aspect, the disclosure provides a vector comprising the nucleic acid molecule of encoding the multispecific binding protein of any one of the

preceding aspects. In one aspect, the disclosure provides a cell comprising the nucleic acid molecule or the vector encoding the multispecific binding protein of any one of the preceding aspects.

[0062] In one aspect, the disclosure provides a method of treating a disease comprising administering an effective amount of the multispecific binding protein of any one of preceding aspects or the pharmaceutical composition of said aspects to a subject. In one aspect, the disease is selected from a group consisting of: neoplastic disorder, cancer, autoimmune disease, inflammatory disorder, infectious disease, and neurodegenerative disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] The foregoing and other features and advantages of the present application will be more fully understood from the following detailed description of illustrative aspects taken in conjunction with the accompanying drawings.

[0064] FIGURE 1A is a schematic drawing of steps in CD25-mediated internalization and endocytic trafficking routes for a CD25, a multispecific binding protein of the disclosure (e.g., TNF α /CD25 bispecific antibody), and a target protein (e.g., a TNF α). The Figure 1A schematic was created with BioRender.com. **FIGURE 1B** are schematics of two different formats for the multispecific binding protein of the disclosure, i.e., on the left a bispecific antibody format and on the right a bispecific ISVD format. One skilled in the art can appreciate that the other multispecific binding protein formats, other target proteins (e.g., but not limited to TNF or FcRL3) and additional endocytic trafficking steps are possible.

[0065] FIGURE 2 shows SEC-HPLC characterization of purified TNF α /CD25 bispecific antibody 1 (Ab1). The chromatogram shows the elution profile of purified bispecific antibody obtained prior to polishing step.

[0066] FIGURE 3 shows SEC-HPLC characterization of purified anti- TNF α /CD25 bispecific antibody 2 (Ab2) by SEC. The chromatogram shows the elution profile of purified bispecific antibody obtained prior to polishing step.

[0067] FIGURE 4 shows the results of mass spectrometry of the anti- TNF α /CD25 bispecific Ab1 obtained prior to polishing step. The expected mass for bispecific binding protein is 144,482 Daltons (Da) and the observed mass was 144,479 Da.

[0068] **FIGURE 5** shows the results of mass spectrometry of the TNF α /CD25 bispecific Ab2 obtained prior to polishing step. The expected mass for bispecific binding protein is 144,409 Da and the observed mass was 144,411 Da.

[0069] **FIGURE 6** is Labchip analysis of TNF α /CD25 bispecific Ab1 (lane 2, A4) and TNF α /CD25 bispecific Ab2 (lane 3, B4) prior to the polishing step. Heterodimers were visible as a band above the ladder band of 119 kDa.

[0070] **FIGURE 7A** and **FIGURE 7B** show SEC-HPLC characterization of re-purified TNF α /CD25 bispecific Ab1 after a polishing step using an ÄKTA system. The chromatogram shows the elution profile obtained for two different lots of the bispecific binding protein construct with purity of more than 99% as monomer.

[0071] **FIGURE 8A** AND **FIGURE 8B** show SEC-HPLC characterization of re-purified TNF α /CD25 bispecific Ab2 after a polishing step using an ÄKTA system. The chromatogram shows the elution profile obtained for two different lots of bispecific binding protein construct.

[0072] **FIGURE 9** is a Labchip analysis of TNF α /CD25 bispecific Ab1 (lanes 1 and 2, A7 and B7) and TNF α /CD25 bispecific Ab2 (lanes 3 and 4, C7 and D7) bispecific binding protein constructs in the two different lots after the polishing step. Heterodimers were visible as a band above the ladder band of 119 kDa.

[0073] **FIGURE 10** shows the results of mass spectrometry of the TNF α /CD25 bispecific Ab1 obtained after the polishing step for lot 1. The expected mass for bispecific binding protein is 144,482 Da and the observed mass was 144,481 Da.

[0074] **FIGURE 11** shows the results of mass spectrometry of the TNF α /CD25 bispecific Ab1 obtained after the polishing step for lot 2. The expected mass for bispecific binding protein is 144,482 Da and the observed mass was 144,480 Da.

[0075] **FIGURE 12** shows the results of mass spectrometry of the TNF α /CD25 bispecific Ab2 obtained after the polishing step for lot 1. The expected mass and the observed mass for bispecific binding protein are the same at 144,409 Da.

[0076] **FIGURE 13** shows the results of mass spectrometry of the TNF α /CD25 bispecific Ab2 obtained after the polishing step for lot 2. The expected mass and the observed mass for bispecific binding protein are the same at 144,409 Da.

[0077] **FIGURE 14** is a schematic of the binding and internalization steps of fluorescently labeled TNF α (TNF-biotin complexed with Alexa Fluor 647 labeled streptavidin) *via* binding to a TNF α /CD25 bispecific antibody as described herein. The TNF α /CD25 bispecific antibody binds to CD25 positive cells which natively

express human IL-2 (e.g., activated T cells) or which are artificially transfected with a human CD25 such as a Human Embryonic Kidney cell line, HEK-IL2.

[0078] FIGURE 15 displays the binding of TNF α /CD25 bispecific antibodies of the disclosure to HEK wild-type (HEK-WT) cells or a CD25 expressing HEK-IL2 cell line. The amount of CD25 surface expression staining after incubation with APC-labeled anti-human IgG monoclonal antibody plus: (1) TNF α /CD25 bispecific Ab1, (2) TNF α /CD25 bispecific Ab2, (3) anti-TNF α IgG1 control, or (4) anti-TNP IgG1 control in either HEK-WT or HEK-IL2 cells.

[0079] FIGURE 16 displays the internalization of fluorescently labeled TNF α using the TNF α /CD25 bispecific antibodies of the disclosure in either HEK-WT or HEK-IL2 cells. HEK-WT or HEK-IL2 cells were incubated with TNF-biotin complexed with-Alexa Fluor 647 and either (1) TNF α /CD25 bispecific Ab1, (2) TNF α /CD25 bispecific Ab2, (3) anti-TNF IgG1 control, or (4) anti-TNF IgG1 control at a concentration of 5nM or 50nM. Subsequently, cells were analyzed using flow cytometry.

[0080] FIGURE 17 is a gel image of a western blot demonstrating the cellular degradation of TNF α by an exemplary TNF α /CD25 bispecific antibody described herein. HEK-WT or HEK-IL2 cells were incubated with either anti- TNF α /CD25 bispecific Ab2 plus TNF α or with an antibody control plus TNF α for two hours followed by a wash. HEK-WT and HEK-IL2 cells were then either treated with Bafilomycin – a potent lysosomal inhibitor or DMSO for 2-, 8-, or 22-hours post incubation at which point cell lysates were probed with an anti-TNF α antibody. The level of TNF α in the cell lysate samples was quantified and normalized to β -actin control and arbitrary unit was displayed under each sample.

[0081] FIGURE 18 is a gel image of a western blot demonstrating the cellular degradation of TNF α by a second TNF α /CD25 bispecific antibody described herein. HEK-WT or HEK-IL2 cells were incubated with either anti- TNF α /CD25 bispecific Ab1 plus TNF α or with an antibody control plus TNF α for two hours followed by a wash. HEK-WT and HEK-IL2 cells were then either treated with Bafilomycin or DMSO for 2, 8, or 22 hours post incubation at which point cell lysates were probed with an anti-TNF α antibody. The level of TNF in the cell lysate samples was quantified and normalized to β -actin control and arbitrary unit was displayed under each sample.

[0082] FIGURE 19 is a gel image of a western blot demonstrating the depletion of TNF α in the cell supernatant by two different TNF α /CD25 bispecific antibodies

described herein. HEK-WT or HEK-IL2 cells were incubated with either TNF α /CD25 bispecific Ab1 or TNF α /CD25 bispecific Ab2 plus TNF α or with an antibody control plus TNF α for 24, 48, 72 hours. The level of TNF α in the cell supernatant samples was quantified and normalized and arbitrary unit was displayed under each sample.

[0083] FIGURE 20 displays the internalization of TNF α by two different TNF α /CD25 bispecific antibodies described herein in peripheral blood mononuclear cells (PBMCs) from two different human donors (donor 1 and donor 2) analyzed using FACs.

[0084] FIGURE 21 displays the internalization of TNF α using two different TNF α /CD25 bispecific antibodies described herein in CD3⁺ CD4⁺ T cells. The experimental set-up was the same as in **FIGURE 20** but the plots represent CD3⁺CD4⁺ T cells which were gated from the total PMBCs from the two different PMBC donors.

[0085] FIGURE 22 is a competition assay showing no-competition between anti-CD25 antibody (clone M-A251) and either of the two TNF α /CD25 bispecific antibodies described herein.

[0086] FIGURE 23 displays a schematic illustration of the experimental set-up for detection and visualization of the internalization and lysosomal trafficking steps of the target protein (e.g., TNF α) by a TNF α /CD25 multispecific binding protein complex described herein in an IL-2 receptor surface expressing cell. HEK-WT or HEK-IL2 cell lines as described in Figure 14 were utilized. The multispecific binding protein complex was comprised of: a biotinylated human TNF α , streptavidin conjugated to AlexaFluor488 (streptavidin-AF488), and an anti-TNF α /CD25 bispecific antibody construct described herein (e.g., a TNF α /CD25 bispecific Ab1 or Ab2). The Figure 23 schematic was created with BioRender.com.

[0087] FIGURE 24 are representative confocal maximum intensity projection images of HEK-IL2 (left column) or HEK-WT (right column) cells incubated for two-hours with the bispecific antibody complex (TNF α /CD25 bispecific Ab2) described in Figure 23. After incubation cells were fixed and co-stained with fluorescently labeled endosome and lysosome markers, rabbit anti-EEA1 and mouse anti-LAMP1 antibodies, fluorescently-labeled goat anti-rabbit and goat anti-mouse secondary antibodies, as well as a nuclear stain (Hoechst). Images were captured using ZEISS Airyscan Joint Deconvolution for optimized resolution and channel separation. Scale bar: 15 μ m.

[0088] **FIGURE 25** are representative confocal maximum intensity projection images utilizing the same experimental set-up as Figure 24 but using TNF α /CD25 bispecific Ab1 in the bispecific antibody complex.

[0089] **FIGURE 26** are representative confocal maximum intensity projection images utilizing the same experimental set-up as Figure 24 and 25 with a TNF α /CD25 bispecific Ab2 (left panel), a TNF α /CD25 bispecific Ab1 (central panel), or a non-CD25 binding isotype control (right panel) in the bispecific antibody complex.

[0090] **FIGURE 27** are representative confocal maximum intensity projection images utilizing a TNF α /CD25 bispecific Ab2 (left panel) or a TNF α /CD25 bispecific Ab1 (right panel) in the bispecific antibody complex incubated with HEK-IL2 cells at 1-, 2-, or 4-hours. Scale bar: 15 μ m.

[0091] **FIGURE 28** is a graph of the weighted colocalization values to quantify the different levels of colocalization for EEA1 and LAMP1 fluorescent markers with the staining from the bispecific antibody complex with either TNF α /CD25 bispecific Ab2 or Ab1 at 1-, 2-, and 4- hour time course experiments described in Figure 27.

[0092] **FIGURE 29** are representative confocal maximum intensity projection images of HEK-IL2 cells incubated for one-(left column), two-(middle column), or four- (right column) hours with a bispecific antibody complex comprising TNF α /CD25 bispecific Ab2. After incubation cells were fixed and co-stained using endosome and lysosome markers as well as nuclear stain and subsequently captured as described in Figure 24. Scale bar: 10 μ m.

[0093] **FIGURE 30** is the same experimental set-up described in Figure 29 but utilizing a TNF α /CD25 bispecific Ab1 in the bispecific antibody complex.

[0094] **FIGURE 31** are representative confocal maximum intensity projection images of HEK-IL2 cells incubated for one- (top), two- (middle), or four-hours (bottom) with a bispecific antibody complex comprising TNF α /CD25 bispecific Ab2 utilizing the same experimental set-up as described in Figure 24. Overlay images in the left column panel show fluorescent labeling for the bispecific antibody complex, EEA1, LAMP1, and the nucleus while images in the middle and right column panels show fluorescent labeling for the bispecific antibody complex which colocalizes with LAMP1 and EEA1, respectively. Scale bar: 10 μ m.

[0095] **FIGURE 32** are representative confocal maximum intensity projection images utilizing the same experimental set up as Figure 31 but utilizing a TNF α /CD25 bispecific Ab1 in the bispecific antibody complex.

[0096] **FIGURE 33** Quantification of weighted colocalization between each bispecific antibody complex (designated by the fluorescence channel 488) and EEA1 or LAMP1 shown in two graphs of the weighted colocalization values to quantify the different levels of colocalization for EEA1 and LAMP1 fluorescent markers with the staining from the bispecific antibody complex with either TNF α /CD25 bispecific Ab2 (top graph) or Ab1 (bottom graph) at 1-, 2-, and 4- hour time course experiments described in Figures 31 and 32.

[0097] **FIGURE 34** shows two schematics of the bispecific antibody complex described in Figure 24 which was further modified by either conjugation of streptavidin-AF488 ("488-Sa"; top) or pHrodo-red to the bispecific antibody ("pHrodo-Sa"; bottom).

[0098] **FIGURE 35** are representative images collected during a 100-minute live imaging time-course captured at 10-, 30-, 60-, 100- minute time points generated by HEK-IL2 cell incubation with Hoechst nuclear dye and the modified bispecific antibody complex comprising Ab2 and conjugated to 488-Sa.

[0099] **FIGURE 36** are representative images utilizing the same experimental set-up as in Figure 25 but utilizing Ab1 in the modified bispecific antibody complex conjugated to 488-Sa.

[00100] **FIGURE 37** are representative images collected during a 100-minute live imaging time-course captured at 10-, 30-, and 60- minute time points generated by HEK-IL2 cell incubation with Hoechst nuclear dye and the modified bispecific antibody complex comprising Ab2 conjugated to pHrodo-Sa.

[00101] **FIGURE 38** are representative images utilizing the same experimental set-up as in Figure 37 but utilizing Ab1 in the modified bispecific antibody complex conjugated to pHrodo-Sa.

[00102] **FIGURE 39** shows two graphs displaying the mean MFI of images collected during the 100-minute time-course either utilizing the modified bispecific antibody complex conjugated to 488-Sa as described in Figures 35 and 36 (top) or to pHrodo-Sa as described in Figures 37 and 38 (bottom).

[00103] **FIGURE 40** shows two graphs displaying the MFI of images collected during a 100-minute time-course generated by either HEK-IL2 or HEK-WT cells after incubation with a modified bispecific antibody conjugated to 488-Sa utilizing either Ab2 (top graph) or Ab1 (bottom graph) in the bispecific antibody complex.

[00104] **FIGURE 41** displays a MFI graph of flow cytometry-based detection of binding of a CD25 ISVD to human and cynomolgus (cyno) CD25 stably expressed on HEK293-MZA cells (HEK293 cells engineered to express human or cyno CD25), whereas no binding was detected on parental HEK293 cells. (LCI: lower confidence interval, UCI: upper confidence interval)

[00105] **FIGURE 42** displays an MFI graph of flow cytometry-based detection of competition of a CD25 ISVD and IL-2 for ligand binding to human and cyno CD25 as measured by flow cytometry on HEK293-MZA cells expressing human or cyno CD25. Fixed concentration of biotinylated human IL-2 at 30 nM was added to the cells in combination with different concentrations of the CD25 ISVD. Binding of biotinylated IL-2 to the cells was assessed using PE-labelled streptavidin.

[00106] **FIGURE 43** displays the degradation of TNF α by an anti-TNF α /CD25 bispecific VHH ("Nb 59") or CD25 monospecific VHH control ("Nb 60") as determined by western blotting. HEK-WT or HEK-IL2 cells were incubated with TNF α (at 50 nM) and either the anti- TNF α /CD25 bispecific VHH (at 25nM) or with the control for 2 hours. Some samples were treated with Bafilomycin or DMSO control followed by two washes. After the washes, cells were cultured for another 8- or 22-hours and then subsequently lysed for sample harvest and western blot analysis.

[00107] **FIGURE 44** displays the degradation of TNF α by an anti-TNF α /CD25 bispecific VHH (Nb 59) or CD25 monospecific VHH control ("Nb 60") as determined by western blotting. HEK-WT or HEK-IL2 cells were incubated with TNF α (at 50 nM) plus either the anti-TNF α /CD25 bispecific VHH (at 25nM) or with control for 24-, 48-, or 72- hours and subsequently cell supernatants were harvested for western blot analysis.

DETAILED DESCRIPTION

[00108] The present disclosure is directed to, *inter alia*, a method for degrading a target protein, comprising:

contacting a CD25 positive cell with a multispecific binding protein, wherein the multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of the CD25 positive cell, wherein the first cell surface binding moiety comprises at least one immunoglobulin domain or an antigen binding fragment thereof; and b) a second binding moiety that is operatively

linked to the first cell surface binding moiety and that specifically binds to the target protein, wherein specific binding of the multispecific binding protein to the CD25 facilitates the internalization of the target protein into the CD25 positive cell. Methods of making the CD25 targeting multispecific binding proteins, compositions comprising the same, and methods of treatment with CD25 targeting multispecific binding proteins or compositions comprising the same are also disclosed herein.

Definitions

[00109] It is to be understood that the methods described in this disclosure are not limited to particular methods and experimental conditions such that methods and conditions can vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[00110] The experiments described herein, unless otherwise indicated, use conventional molecular and cellular biological and immunological techniques within the skill of the art. Such techniques are well known to the skilled worker and are explained fully in the literature. See, e.g., Ausubel, *et al.*, ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987-2008), including all supplements, Molecular Cloning: A Laboratory Manual (Fourth Edition) by MR Green and J. Sambrook and Harlow *et al.*, Antibodies: A Laboratory Manual, Chapter 14, Cold Spring Harbor Laboratory, Cold Spring Harbor (2013, 2nd edition). Unless otherwise defined, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including," as well as other forms, such as "includes" and "included," is not limiting. The embodiments illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are specifically or not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" can be replaced with either of the other two terms, while retaining their ordinary meanings. Any single term, single element, single

phrase, group of terms, group of phrases, or group of elements described herein can each be specifically excluded from the claims.

[00111] Generally, nomenclature used in connection with cell culture, molecular biology, immunology, microbiology, genetics, protein biology, and chemistry described herein is well-known and commonly used in the art. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art.

[00112] For the disclosure to be more readily understood, select terms are defined below.

Sequences

[00113] The term “sequence” as used herein, for example, in terms like “immunoglobulin sequence,” “antibody sequence,” “variable domain sequence,” “VHH sequence,” “protein sequence,” “amino acid sequence” or “nucleic acid sequence,” can generally be understood to include both the relevant amino acid sequence as well as nucleic acids or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

[00114] Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code. Reference is made to *e.g.*, Table A-2 of WO 2008/020079 which is incorporated by reference herein.

[00115] A nucleic acid or amino acid is considered to be “(in) (essentially) isolated (form),” *e.g.*, when compared to the reaction medium or cultivation medium from which it has been obtained when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another nucleic acid, another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. A nucleic acid or amino acid is considered “(essentially) isolated” when it has been purified at least 2-fold, in particular at least 10-fold, more in particular at least 100-fold, and up to 1000-fold or more. A nucleic acid or amino acid that is “in (essentially) isolated form” can be essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide-gel electrophoresis.

[00116] When a nucleic acid sequence (also called “a nucleotide sequence”) or amino acid sequence is said to “comprise” another nucleotide sequence or amino acid sequence, respectively, or to “consist essentially of” another nucleotide sequence or amino acid sequence, this may mean that the latter nucleotide sequence or amino acid sequence has been incorporated into the first mentioned nucleotide sequence or amino acid sequence, respectively, but more generally means that the first mentioned nucleotide sequence or amino acid sequence comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the first mentioned sequence has actually been generated or obtained (which may for example be by any suitable method described herein). By means of a non-limiting example, when a binding protein or binding polypeptide is said to comprise an acid or nucleotide sequence is said to comprise another nucleotide sequence, the first mentioned nucleic acid or nucleotide sequence is preferably such that, when it is expressed into an expression product (e.g., a polypeptide), the amino acid sequence encoded by the latter nucleotide sequence forms part of said expression product (in other words, that the latter nucleotide sequence is in the same reading frame as the first mentioned, larger nucleic acid or nucleotide sequence).

[00117] The terms “(essentially) consist of,” “consist essentially of” are used interchangeably to mean that the later nucleic acid sequence or amino acid sequence either is exactly the same as the polypeptide (e.g., the CDR region; the ISV) or corresponds to the polypeptide (e.g., the CDR region; the ISVD) which has a limited number of amino acid residues, such as 1-20 amino acid residues, for example 1-10 amino acid residues or 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, added at the amino terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of e.g., the ISVD.

[00118] For the purposes of comparing two or more amino acid sequences, the percentage of “sequence identity” between a first amino acid sequence and a second amino acid sequence may be calculated by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying

by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence compared to the first amino acid sequence is considered as a difference at a single amino acid residue (i.e., at a single position). For the purpose of determining the percentage of “sequence identity” between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the “first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence.

[00119] An “amino acid difference” or *e.g.*, an “amino acid modification” or “Fc modification” as used herein can refer to a deletion, insertion, or substitution of a single amino acid residue vis-à-vis a reference sequence. In one aspect, an “amino acid difference” is a substitution. In certain aspects, amino acid substitutions are conservative substitutions. In certain aspects, the monospecific or multispecific binding protein of the disclosure comprises a Fc domain comprising a Fc modification.

[00120] Conservative substitutions are substitutions in which one amino acid within the following groups (a) – (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp. In one aspect, conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[00121] A “VHH family” as used in the present specification refers to a group of VHH sequences that have identical lengths (i.e., they have the same number of amino acids within their sequence) and of which the amino acid sequence between position 8 and position 106 (according to Kabat numbering) have an amino acid sequence identity of more than 89%.

Polypeptide and Isolated Polypeptide

[00122] The term “polypeptide” refers to any polymeric chain of amino acids and encompasses native or artificial proteins, polypeptide analogs or variants of a protein sequence, or fragments thereof, unless otherwise contradicted by context. A polypeptide can be monomeric or polymeric. For a polypeptide (e.g., a polypeptide encoding a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell and/or a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein), a fragment of a polypeptide optionally contains at least one contiguous or nonlinear epitope of a polypeptide. In another example, the polypeptide or a fragment polypeptide encodes a monospecific CD25 binding protein (e.g., a CD25 ISVD or a target protein ISVD).

[00123] A fragment polypeptide can be about 25, 50, 75, 100, 150, 200, 250, 300, 350, 400 or more amino acids in length while retaining the capacity to bind to both CD25 and a target protein. The precise boundaries of the at least one epitope fragment can be confirmed using ordinary skill in the art. A polypeptide fragment comprises at least about 5 contiguous amino acids, at least about 10 contiguous amino acids, at least about 15 contiguous amino acids, or at least about 20 contiguous amino acids, at least about 50 contiguous amino acids, at least about 100 contiguous amino acids, at least about 150 contiguous amino acids, at least about 200 contiguous amino acids, at least about 250 contiguous amino acids, at least about 300 contiguous amino acids, at least about 400 contiguous amino acids for example.

[00124] In certain aspects, a first cell surface binding moiety and a second binding moiety polypeptides are “isolated polypeptides.” The term “isolated polypeptide” refers to a protein or polypeptide that by virtue of its origin or source of derivation is not associated with naturally associated components that accompany it in its native state; is substantially free of other proteins from the same species. An isolated recombinant polypeptide is expressed by a cell from a different species. In some aspects, an isolated polypeptide does not occur in nature. A protein or polypeptide that is chemically synthesized or synthesized in a cellular system can be different from the cell from which it naturally originates and therefore will be “isolated” from its naturally associated components. A protein or polypeptide can also be

rendered substantially free of naturally associated components by isolation using protein purification techniques.

Binding protein or binding polypeptide

[00125] As used herein, the term "binding protein" and "binding polypeptide" can embrace a "multispecific binding polypeptide or protein," or "monospecific binding polypeptide or protein." In certain aspects, the binding protein refers to a protein or polypeptide (e.g., an antibody or an antigen binding fragment thereof) that contains at least one binding site which is responsible for selectively binding to a CD25 or to a target protein or antigen of interest (e.g., a TNF α). In certain aspects, the binding protein comprises or consists essentially of a CD25 ISVD.

[00126] Binding sites include an antibody variable domain, a ligand binding site of a receptor, a receptor binding site of a ligand, an extracellular domain or a target protein or antigen. In certain aspects, the binding proteins or binding polypeptides comprise monospecific (i.e., one) or multispecific (e.g., two, three, four, or more) binding sites. In certain aspects, the binding protein or binding polypeptide is not a therapeutic enzyme.

[00127] In certain aspects, the binding protein is a monospecific binding protein that specifically targets CD25. In certain aspects, the binding protein comprises at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell. In certain aspects, the CD25 ISVD binds an extracellular domain of a CD25 protein.

[00128] In certain aspects, a multispecific binding protein of the disclosure comprises: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell, wherein the first cell surface binding moiety comprises an immunoglobulin domain; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein, wherein specific binding of the multispecific binding protein to the CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.

[00129] In certain aspects of the disclosure, a multispecific binding protein comprises a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a first IgG; and b) a second binding moiety that

specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C- terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof.

[00130] In certain aspects, a multispecific binding protein comprises a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD that is at least 80% or more identical to an amino acid sequence set forth in SEQ ID NO: 72 wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a first IgG; and b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C- terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof.

Native residue vs. modified binding polypeptide

[00131] As used herein, the term "native residue" refers to an amino acid residue that occurs naturally at a particular amino acid position of a binding polypeptide (e.g., an antibody or fragment thereof) and which has not been modified, introduced, or altered by the hand of man. As used herein, the term "altered binding protein," "altered binding polypeptide," "modified binding protein" or "modified binding polypeptide" shall refer to binding polypeptides and/or binding proteins (e.g., an antibody or fragment thereof) comprising at least one amino acid substitution, deletion and/or addition relative to the native (i.e., wild-type) amino acid sequence, and/or a mutation that results in altered glycosylation (e.g., hyperglycosylation, hypoglycosylation and/or aglycosylation) at one or more amino acid positions relative to the native (i.e., wild-type) amino acid sequence.

Ligand and Antigen

[00132] The term "ligand" refers to any substance capable of binding, or of being bound, to another substance. The term "antigen" or "target antigen" as used herein refers to a molecule or a portion of a molecule that is capable of being bound by the binding site of a binding polypeptide or protein e.g., any substance to which an antibody can be generated. A target antigen may have one or more epitopes.

[00133] Although "antigen" is commonly used in reference to an antibody binding substrate, and "ligand" is often used when referring to receptor binding

substrates, these terms are not distinguishing, one from the other, and encompass a wide range of overlapping chemical entities. For the avoidance of doubt, antigen and ligand are used interchangeably throughout herein.

[00134] Examples of antigens/ligands can be a peptide, a polypeptide, a protein, an aptamer, a polysaccharide, a sugar molecule, a carbohydrate, a lipid, an oligonucleotide, a polynucleotide, a synthetic molecule, an inorganic molecule, an organic molecule, and any combination thereof.

Immunoglobulin domain

[00135] The term immunoglobulin domain as used herein can refer to an immunoglobulin A, an immunoglobulin D, an immunoglobulin E, an immunoglobulin G, or an immunoglobulin M. The immunoglobulin domain can be an immunoglobulin heavy chain region or fragment thereof. In some instances, the immunoglobulin domain is from an antibody (e.g., a mammalian antibody, a recombinant antibody, a chimeric antibody, an engineered antibody, a human antibody, a humanized antibody) or an antigen binding fragment thereof.

[00136] In certain aspects, the monospecific binding protein or the multispecific binding protein of the disclosure comprises an IgG domain (e.g., a human IgG domain). In certain aspects, the IgG domain is IgG1, IgG2, IgG3, or IgG4.

Antibody

[00137] As used herein, the term "antibody" refers to such assemblies (e.g., intact antibody molecules, antibody fragments, or variants thereof) which have significant known specific immunoreactive activity to an antigen of interest (e.g., a CD25 associated antigen or a target protein associated antigen). Antibodies and immunoglobulins comprise light and heavy chains, with or without an interchain covalent linkage between them. Basic immunoglobulin structures in vertebrate systems are relatively well understood.

[00138] As will be discussed in more detail below, the generic term "antibody" comprises five distinct classes of antibody that can be distinguished biochemically. While all five classes of antibodies are clearly within the scope of the current disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, immunoglobulins comprise two identical light chains of molecular weight approximately 23,000 Daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined

by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[00139] Light chains of immunoglobulin are classified as either kappa or lambda (κ , λ). Each heavy chain class can be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells, or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, (γ , μ , α , δ , ϵ) with some subclasses among them (e.g., γ 1- γ 4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA, IgG, or IgE, respectively. The immunoglobulin isotype subclasses (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, etc.) confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the current disclosure.

[00140] Both the light and heavy chains are divided into regions of structural and functional homology. The term "region" refers to a part or portion of an immunoglobulin or antibody chain and includes constant region or variable regions, as well as more discrete parts or portions of said regions. For example, light chain variable regions include "complementarity determining regions" or "CDRs" interspersed among "framework regions" or "FRs," as defined herein.

Constant and variable domains

[00141] The regions of an immunoglobulin heavy or light chain can be defined as "constant" (C) region or "variable" (V) regions, based on the relative lack of sequence variation within the regions of various class members in the case of a "constant region", or the significant variation within the regions of various class members in the case of a "variable regions." The terms "constant region" and "variable region" may also be used functionally. In this regard, it will be appreciated that the variable regions of an immunoglobulin or antibody determine antigen recognition and specificity. Conversely, the constant regions of an immunoglobulin or antibody confer important effector functions such as secretion, transplacental

mobility, Fc receptor binding, complement binding, and the like. The subunit structures and three-dimensional configurations of the constant regions of the various immunoglobulin classes are well known.

[00142] The constant and variable regions of immunoglobulin heavy and light chains are folded into domains. The term "domain" refers to a globular region of a heavy or light chain comprising peptide loops (*e.g.*, comprising 3 to 4 peptide loops) stabilized, for example, by β -pleated sheet and/or intrachain disulfide bond. Constant region domains on the light chain of an immunoglobulin are referred to interchangeably as "light chain constant region domains", "CL regions" or "CL domains." Constant domains on the heavy chain (*e.g.*, hinge, CH1, CH2 or CH3 domains) are referred to interchangeably as "heavy chain constant region domains", "CH" region domains or "CH domains". Variable domains on the light chain are referred to interchangeably as "light chain variable region domains", "VL region domains" or "VL domains." Variable domains on the heavy chain are referred to interchangeably as "heavy chain variable region domains", "VH region domains" or "VH domains."

[00143] By convention the numbering of the variable constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the immunoglobulin or antibody. The N-terminus of each heavy and light immunoglobulin chain is a variable region and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively. Accordingly, the domains of a light chain immunoglobulin are arranged in a VL-CL orientation, while the domains of the heavy chain are arranged in the VH-CH1-hinge-CH2-CH3 orientation.

[00144] The assignment of amino acids to each variable region domain is in accordance with the definitions of Kabat, Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD, 1987 and 1991). Kabat also provides a widely used numbering convention (Kabat numbering) in which corresponding residues between different heavy chain variable regions or between different light chain variable regions are assigned the same number. CDRs 1, 2 and 3 of a VL domain are also referred to herein, respectively, as CDR-L1, CDR-L2 and CDR-L3. CDRs 1, 2 and 3 of a VH domain are also referred to herein, respectively, as CDR-H1, CDR-H2 and CDR-H3. If so noted, the assignment of CDRs can be in accordance with IMGT® (Lefranc et al., Developmental & Comparative Immunology

27:55-77; 2003) in lieu of Kabat. Numbering of the heavy chain constant region is via the EU index as set forth in Kabat (Kabat, Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD, 1987 and 1991).

In some aspects, a CD25 positive cell can be contacted with a multispecific binding protein, wherein the multispecific binding protein comprises: a) a first cell surface binding moiety comprising a CD25 antibody or an antigen binding fragment thereof that binds to CD25 on the surface of the CD25 positive cell; and b) a second binding moiety that binds to the target protein and is linked to the first cell surface binding moiety, wherein binding of the multispecific binding protein to the CD25⁺ cell facilitates the internalization of the target protein bound to the multispecific binding protein.

In some aspects, a multispecific binding protein comprising a first cell surface binding moiety binds to CD25 on the surface of the CD25 positive cell, binds an extracellular domain of CD25. In some aspects, a first cell surface binding moiety comprises a CD25 specific variable domain. In some aspects, a first cell binding moiety comprises a CD25-fragment crystallizable (Fc) fusion polypeptide or variant thereof. In some aspects, a first cell binding moiety that specifically binds to CD25 is a CD25 antibody or an antigen binding fragment thereof. In some aspects, a variable domain specific for CD25 is operatively linked to a first Fc domain polypeptide. In some aspects, a first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In some aspects, the first cell surface binding moiety comprises a fusion protein comprising a CD25 specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25:Fc fusion polypeptide or a variant thereof. In some aspects, a second binding moiety that binds to the target protein comprises an antibody or an antigen binding fragment thereof. In some aspects, the antibody or the antigen binding fragment thereof specific for a target protein comprises a target specific variable domain. In some aspects, a target specific variable domain for a target protein is operatively linked to a second Fc domain polypeptide. In some aspects, a second Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide. In some aspects, the second binding moiety comprises a fusion protein comprising a target specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-target:Fc fusion polypeptide or a variant thereof. In some aspects, the second

binding moiety that specifically binds to the target protein comprises an antibody or an antigen binding fragment thereof.

VH domain and VL domain

[00145] As used herein, the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain, and the term "VL domain" includes the amino terminal variable domain of an immunoglobulin light chain.

[00146] As used herein, the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain that extends, e.g., from about positions 114-223 in the Kabat numbering system (EU positions 118-215). The CH1 domain is adjacent to the VH domain and amino terminal to the hinge region of an immunoglobulin heavy chain molecule and does not form a part of the Fc region of an immunoglobulin heavy chain.

[00147] As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux et al. J. Immunol. 1998, 161 :4083).

CH2 domain

[00148] As used herein, the term "CH2 domain" includes the portion of a heavy chain immunoglobulin molecule that extends, e.g., from about positions 244-360 in the Kabat numbering system (EU positions 231-340). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. In certain aspects, a multispecific binding protein of the current disclosure comprises a CH2 domain derived from an IgG1 molecule (e.g., a human IgG1 molecule).

CH3 domain

[00149] As used herein, the term "CH3 domain" includes the portion of a heavy chain immunoglobulin molecule that extends approximately 110 residues from N-terminus of the CH2 domain, e.g., from about positions 361-476 of the Kabat numbering system (EU positions 341 -445). The CH3 domain typically forms the C-

terminal portion of the antibody. In some immunoglobulins, however, additional domains may extend from CH3 domain to form the C-terminal portion of the molecule (e.g., the CH4 domain in the μ chain of IgM and the e chain of IgE). In certain aspects, a multispecific binding protein of the current disclosure comprises a CH3 domain derived from an IgG1 molecule (e.g., a human IgG1 molecule).

CL domain

[00150] As used herein, the term "CL domain" includes the constant region domain of an immunoglobulin light chain that extends, e.g., from about Kabat position 107A-216. The CL domain is adjacent to the VL domain. In certain aspects, a multispecific binding protein of the current disclosure comprises a CL domain derived from a kappa light chain (e.g., a human kappa light chain).

[00151] As indicated above, the variable regions of an antibody allow it to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain of an antibody combine to form the variable region (Fv) that defines a three dimensional antigen binding site. This quaternary antibody structure forms the antigen binding site present at the end of each arm of the Y. More specifically, the antigen binding site is defined by three complementary determining regions (CDRs) on each of the heavy and light chain variable regions. As used herein, the term "antigen binding site" includes a site that specifically binds (immunoreacts with) an antigen (e.g., a cell surface or soluble antigen). The antigen binding site includes an immunoglobulin heavy chain and light chain variable region and the binding site formed by these variable regions determines the specificity of the antibody. An antigen binding site is formed by variable regions that vary from one antibody to another. The altered antibodies of the current disclosure comprise at least one antigen binding site.

[00152] In certain aspects, a multispecific binding protein of the current disclosure comprise at least two different antigen binding domains that provide for the association of the binding polypeptide with the selected antigen or target. In some aspects, a first cell surface binding moiety binds to CD25 on the surface of a CD25 positive cell and a second binding moiety binds to a target protein and is linked to the first binding moiety.

[00153] In certain aspects, the antigen binding domains are not derived from the same immunoglobulin molecule. In this regard, the variable region may or be

derived from any type of animal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region of a multispecific binding protein may be, for example, of mammalian origin *e.g.*, may be human, murine, rat, goat, sheep, non-human primate (such as cynomolgus monkeys, macaques, etc.), lupine, or camelid (*e.g.*, from camels, llamas and related species).

[00154] In naturally occurring antibodies, the six CDRs present on each monomeric antibody are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding site as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the heavy and light variable domains show less inter-molecular variability in amino acid sequence and are termed the framework regions. The framework regions largely adopt a β -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β -sheet structure. Thus, these framework regions act to form a scaffold that provides for positioning the six CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to the immunoreactive antigen epitope.

CDR and FR

[00155] As used herein, the term “complementarity determining region” or “CDR” refers to sequences of amino acids within antibody variable regions, which confer antigen specificity and binding affinity. In general, there are three CDRs in each heavy chain variable region (HCDR1, HCDR2, HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, LCDR3). “Framework regions” or “FR” are known in the art to refer to the non-CDR portions of the variable regions of the heavy and light chains. In general, there are four FRs in each heavy chain variable region (FR-H1, FR-H2, FR-H3, and FR-H4), and four FRs in each light chain variable region (FR-L1, FR-L2, FR-L3, and FR-L4).

[00156] The precise amino acid sequence boundaries of a given CDR or FR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), “Sequences of Proteins of Immunological Interest,” 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md.

("Kabat" numbering scheme), Al-Lazikani et al., (1997) JMB 273, 927-948 ("Chothia" numbering scheme), MacCallum et al., J. Mol. Biol. 262:732-745 (1996), "Antibody-antigen interactions: Contact analysis and binding site topography," J. Mol. Biol. 262, 732-745. ("Contact" numbering scheme), Lefranc M P et al., "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains," Dev Comp Immunol, 2003 January; 27(1):55-77 ("IMGT" numbering scheme), and Honegger A and Pluckthun A, "Yet another numbering scheme for immunoglobulin variable domains: an automatic modeling and analysis tool," J Mol Biol, 2001 Jun. 8; 309(3):657-70, (Aho numbering scheme).

[00157] The boundaries of a given CDR or FR may vary depending on the scheme used for identification. For example, the Kabat scheme is based on structural alignments, while the Chothia scheme is based on structural information. Numbering for both the Kabat and Chothia schemes is based upon the most common antibody region sequence lengths, with insertions accommodated by insertion letters, for example, "30a," and deletions appearing in some antibodies. The two schemes place certain insertions and deletions ("indels") at different positions, resulting in differential numbering. The Contact scheme is based on analysis of complex crystal structures and is similar in many respects to the Chothia numbering scheme.

[00158] A "CDR" or "complementarity determining region," or individual specified CDRs (e.g., "HCDR1," "HCDR2," "HCDR3"), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) complementarity determining region as defined by any of the known schemes. Likewise, an "FR" or "framework region," or individual specified FRs (e.g., "FR-H1," "FR-H2") of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) framework region as defined by any of the known schemes. In some instances, the scheme for identification of a particular CDR or FR is specified, such as the CDR as defined by the IMGT, Kabat, Chothia, AbM, or Contact method. In other cases, the particular amino acid sequence of a CDR or FR is given. Unless otherwise specified, all particular CDR amino acid sequences mentioned in the disclosure are IMGT CDRs. CDR sequences in ISVDs were determined according to the AbM numbering as described in Kontermann and Dübel (Eds. 2010, Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51). According to this

method, FR1 of an ISVD comprises the amino acid residues at positions 1-25, CDR1 of an ISVD comprises the amino acid residues at positions 26-35, FR2 of an ISVD comprises the amino acids at positions 36-49, CDR2 of an ISVD comprises the amino acid residues at positions 50-58, FR3 of an ISVD comprises the amino acid residues at positions 59-94, CDR3 of an ISVD comprises the amino acid residues at positions 95-102, and FR4 of an ISVD comprises the amino acid residues at positions 103-113. However, alternative CDRs defined by other schemes are also encompassed by the present disclosure, such as those determined by abYsis Key Annotation (Website: abysis.org/abysis/sequence_input/key_annotation/key_annotation.cgi).

Antibody Variant

[00159] Monospecific and multispecific binding proteins of the disclosure can comprise antibody variants and/or specific antibody binding fragments variants. As used herein, the term “antibody variant” and “antibody specific binding fragment variant” includes synthetic and engineered forms of antibodies which are altered such that they are not naturally occurring, e.g., antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as, domain deleted antibodies or minibodies) and multispecific forms of antibodies altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules and the like.

Antigen Binding Fragments

[00160] Unless specifically indicated otherwise, the term “antibody,” as used herein, shall be understood to encompass antibody molecules comprising two immunoglobulin heavy chains and two immunoglobulin light chains (i.e., “full antibody molecules”) as well as antigen binding fragments thereof.

[00161] Other engineered molecules, such as domain specific binding proteins, single domain binding proteins, domain deleted binding proteins, chimeric binding proteins, CDR grafted binding proteins, diabodies, triabodies, tetrabodies, minibodies, immunoglobulin single variable domains (ISVDs) (e.g., monovalent, bivalent, or trivalent ISVDs), small modular immunopharmaceuticals (SMIPs), shark variable IgNAR domains, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, or an AFFIBODY® are also encompassed within the expression “antigen binding fragment,” as used herein.

[00162] The term “multispecific antibody” denotes a binding fragment or derivative thereof that combines the antigen-binding sites of two or more antibodies within a single molecule. The terms “antigen binding portion”, “antigen binding fragment”, “binding protein” or “binding moiety” and the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds to at least one target antigen to form a complex.

[00163] Both the monospecific and the multispecific binding protein can comprise at one or at least two antigen binding fragments. In some aspects, the antigen binding fragment is at least one ISVD. In some aspects, the antigen binding fragments are at least two ISVDs. In some aspects, the antigen binding fragments are at least three ISVDs.

[00164] In certain aspects, a binding moiety can refer to one or more fragments of a CD25 targeting multispecific binding protein that retain the ability to specifically bind to CD25 on the surface of a CD25 positive cell and/or a second target protein or a target protein. In certain aspects, a binding moiety can refer to one or more fragments of a CD25 targeting multispecific binding protein that retain the ability to specifically bind to CD25 on the surface of a CD25 positive cell and/or a second target protein or a target protein.

[00165] In certain aspects, the term “antigen binding fragment” refers to a polypeptide fragment of a monospecific or a multispecific binding protein. Antigen binding fragments of a monospecific or multispecific binding protein, or a binding fragment or derivative thereof can be derived, e.g., from full multispecific binding protein molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding a monospecific or a multispecific binding protein, or a binding fragment or derivative thereof variable and (optionally) constant domains. Such DNA is known and/or is readily available from, e.g., commercial sources, DNA libraries (including, e.g., phage-antibody libraries), or can be synthesized. The DNA can be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add, or delete amino acids, etc.

Monospecific

[00166] The term “monospecific” in monospecific binding protein can encompass a binding protein that only binds to one protein or antigen. In the case of the present disclosure the monospecific binding protein only specifically binds to CD25 and not to a different target polypeptide or protein and is referred to as a “CD25 monospecific binding protein” or a “monospecific binding protein.”

[00167] In certain aspects, a CD25 monospecific binding protein comprises at least one antigen binding fragment wherein the antigen binding fragment binds to CD25. In certain aspects, the monospecific binding protein comprises at least one antigen binding fragment that specifically binds to a CD25 on the surface of a CD25 positive cell. In certain aspects, the monospecific binding protein of the disclosure is at least one that specifically binds to a CD25 (e.g., a CD25 ISVD) on the surface of a CD25 positive cell.

[00168] The term “CD25 monospecific binding protein” can embrace CD25 binding proteins that are cross-reactive to human CD25 (hCD25) and cynomolgus CD25 (cynoCD25) but are not cross reactive to CD25 from other species. In certain aspects, the binding protein specifically binds to hCD25 and cynoCD25.

[00169] In certain aspects, the term “CD25 monospecific binding protein” can embrace CD25 binding proteins that are multivalent (e.g., polypeptides that comprise two or more binding units such as at least two ISVDs). In certain aspects, the binding protein comprises at least two or at least three CD25 ISVDs that bind to the same CD25 protein. In certain aspects, the at least two or at least three CD25 ISVDs bind to the same or different contacts on the same CD25 protein. In certain aspects, the at least two CD25 ISVDs are operatively linked via an amino acid linker sequence.

Multispecific

[00170] As used herein, a “multispecific” binding protein is a binding protein that specifically binds two or more types of antigens. A multispecific binding protein that binds two antigens or proteins, and/or two different epitopes of different antigens or proteins, is also referred to herein as a “bispecific” binding protein. A multispecific binding protein that binds three antigens, and/or three different epitopes, is also referred to herein as a “trispecific” binding protein. Thus, the multispecific binding protein is able to bind two or more different targets simultaneously, for example, a

CD25 and a target protein of interest. Genetic engineering can be used to design, modify, and produce the multispecific binding protein, or a binding fragment or derivative thereof with a desired set of binding properties and effector functions.

[00171] A CD25 targeting multispecific binding protein of this disclosure, in some aspects, comprises a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell and a second binding moiety that is operatively linked to the first cell surface binding moiety and specifically binds to a target protein. In some aspects, a first cell surface binding moiety and a second binding moiety of the multispecific binding moiety each independently selected from a group consisting of an antibody or an antigen binding fragment thereof such as, a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a tandem di-scFv, a tandem tri-scFv, a minibody, a diabody, a triabody, a tetrabody, an immunoglobulin single variable domain (ISVD), such as, a VHH (including humanized VHH), a camelized VH, a single domain antibody, a NANOBODY[®] molecule, a domain antibody, or a dAb, or an AFFIBODY[®].

[00172] In some aspects, a multispecific binding protein of the disclosure comprises a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY[®]. In some aspects, wherein the multispecific binding protein further comprises a Fc domain polypeptide or a variant thereof. In one aspect, the ISVD is a VHH, humanized VHH, a camelized VH, a single domain antibody, a domain antibody, a dAb, a NANOBODY[®] molecule, or a VNAR. In some aspects, the scFV is a linear scFV or a tandem scFV.

[00173] Techniques for making multispecific binding proteins (e.g., multispecific antibodies) include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein, C. and Cuello, A. C., *Nature* 305 (1983) 537-540, WO 93/08829, and Traunecker, A. et al., *EMBO J.* 10 (1991) 3655-3659), and "knob-in-hole" engineering (see, e.g., U.S. Pat. No. 5,731,168). In some aspects, the bispecific antibodies are prepared using a macro-assembly technique using two half-antibodies, e.g., as described in Spiess, C., Merchant, M., Huang, A. et al. Bispecific antibodies with natural architecture produced by co-culture of bacteria expressing two distinct half-antibodies. *Nat Biotechnol* 31, 753–758 (2013).

[00174] Multispecific antibodies may also be made by engineering electrostatic steering effects for making binding protein Fc-heterodimeric molecules (WO 2009/089004); cross-linking two or more antibodies or fragments (see, e.g., U.S. Pat. No. 4,676,980, and Brennan, M. et al., Science 229 (1985) 81-83); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny, S. A. et al., J. Immunol. 148 (1992) 1547-1553; using "diabody" technology for making multispecific binding protein fragments (see, e.g., Holliger, P. et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448); and using single-chain Fv (scFv) dimers (see, e.g., Gruber, M et al., J. Immunol. 152 (1994) 5368-5374); and preparing trispecific binding proteins as described, e.g., in Tutt, A. et al., J. Immunol. 147 (1991) 60-69).

[00175] A wide variety of recombinant multispecific binding protein formats have been developed, e.g., by fusion an IgG binding protein format and single chain domains (see Kontermann RE, mAbs 4:2, (2012) 1-16). Multispecific binding proteins wherein the variable domains VL and VH or the constant domains CL and CH1 are replaced by each other are described in WO2009080251 and WO2009080252.

[00176] In one aspect, a multispecific binding protein a first and a second IgG Fc domain polypeptide of a multispecific binding protein can dimerize. In some aspects, a multispecific binding protein of the disclosure dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof. In certain aspects, the first and second IgG is a IgG1, IgG2, IgG3, IgG4. In certain aspects, the first and second IgG is a human IgG1, IgG2, IgG3, IgG4. In certain aspects, the first and second IgG is a human IgG1.

Binding moiety

[00177] The term "binding moiety" is used herein in the broadest sense to encompass any chemical entity capable of specific binding to a target antigen or protein, such as e.g., CD25. The terms "binding moiety," "binding domain," and "binding polypeptide" can be used interchangeably herein. As described herein, all the binding proteins of the disclosure comprise a binding moiety that targets CD25 on the surface of a CD25 positive cell (e.g., a monospecific CD25 ISVD).

[00178] In some aspects, the binding protein of the disclosure targets CD25 and a target protein or antigen (e.g., a multispecific CD25 binding protein). In some aspects, the disclosure describes the use of a CD25 binding moiety to shuttle target

proteins to CD25 positive cells for internalization and ultimately for degradation of the target proteins via the lysosomal pathway.

[00179] In some aspects, a method for degrading a target protein comprises: contacting a CD25 positive cell with a CD25 targeting multispecific binding protein as described herein. In some aspects, the multispecific binding protein comprises two binding moieties: 1) a first cell surface binding moiety that specifically binds to CD25 on the surface of the CD25 positive cell; and 2) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to the target protein, wherein specific binding of the multispecific binding protein to the CD25 facilitates the internalization of the target protein bound to the multispecific binding protein.

[00180] Examples of a binding moiety of the multispecific binding protein described herein can comprise an antibody, a variable domain, a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®. Other binding moiety examples include a Fab fragment, a F(ab')₂ fragment, an Fv fragment a fragment containing a complementarity determining region (CDR), an isolated CDR, or other suitable fragment.

[00181] In certain aspects, a first cell surface binding moiety and a second binding moiety of a multispecific binding protein each independently comprise an antigen binding fragment that comprises at least one variable domain of an antibody or an antigen binding fragment. At least one constant domain can optionally be covalently linked to one or both the first and second binding moieties. The variable domain can be of any size or amino acid composition and will generally comprise at least one CDR, which is adjacent to or in frame with one or more framework sequences. In antigen binding fragments having a VH domain associated with a VL domain, the VH and VL domains can be situated relative to one another in any suitable arrangement. For example, the variable region can be dimeric and contain VH VH, VH VL or VL VL dimers. Alternatively, the antigen binding fragment can contain a monomeric VH or VL domain.

[00182] Non limiting, exemplary configurations of variable and constant domains that can be found within an antigen binding fragment include: (i) VH CH1; (ii) VH CH2; (iii) VH CH3; (iv) VH CH1 CH2; (v) VH CH1 CH2 CH3; (vi) VH CH2 CH3; (vii) VH CL; (viii) VL CH1; (ix) VL CH2; (X) VL CH3; (xi) VL CH1 CH2; (xii) VL

CH1 CH2 CH3; (xiii) VL CH2 CH3; and (xiv) VL CL. In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains can be either directly linked to one another or can be linked by a full or partial hinge or linker region. A hinge region can comprise of at least 2 (e.g., 5, 10, 15, 20, 40, 60 or more) amino acids, which result in a flexible or semi flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen binding fragment of a CD25 targeting multispecific binding protein of this disclosure can comprise a homo dimer or hetero dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric VH or VL domain (e.g., by disulfide bond(s)).

Fab, F(ab')₂, Fab'

[00183] The term "Fab" denotes a binding protein or a binding fragment thereof having a molecular weight of about 50,000 Da and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating IgG with a protease, papain, are bound together through a disulfide bond.

[00184] In certain aspects, a CD25 targeting multispecific binding protein comprises a first cell surface binding moiety comprising a Fab specific for CD25. In certain aspects, a CD25 targeting multispecific binding protein comprises a second binding moiety comprising a Fab that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the CD25 targeting multispecific binding protein, is each independently a Fab.

[00185] The term F(ab')₂ refers to a binding protein or a binding fragment thereof having a molecular weight of about 100,000 Da and antigen binding activity, which is slightly larger than a Fab bound via a disulfide bond of the hinge region, among fragments obtained by treating IgG with a protease, pepsin.

[00186] In certain aspects, a CD25 targeting multispecific binding protein comprises a first cell surface binding moiety comprising a F(ab')₂ specific for CD25. In certain aspects, a CD25 multispecific binding protein comprises a second binding moiety comprising a F(ab')₂ that specifically binds to a target protein. In certain aspects, the first and the second binding moiety of the CD25 targeting multispecific binding protein, is each independently a F(ab')₂.

[00187] The term Fab' refers to a binding protein or a binding fragment having a molecular weight of about 50,000 Da and antigen binding activity, which is obtained by cutting a disulfide bond of the hinge region of the F(ab')₂.

[00188] In certain aspects, a CD25 targeting multispecific binding protein comprises a first cell surface binding moiety comprising a Fab' that specifically binds CD25. In certain aspects, a CD25 targeting multispecific binding protein comprises a second binding moiety comprising a Fab' that specifically binds to a target protein. In certain aspects, the first and the second binding moiety of the CD25 targeting multispecific binding protein is each independently a Fab'.

scFV

[00189] A single chain Fv ("scFv") polypeptide is a covalently linked VH:VL heterodimer that is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker. A human scFv fragment includes CDRs that are held in appropriate conformation by, e.g., using gene recombination techniques. Divalent and multivalent multispecific binding proteins, or binding fragments or derivatives thereof can form either spontaneously by association of monovalent scFvs, or can be generated by coupling monovalent scFvs by an amino acid linker sequence, such as divalent sc(Fv)₂. A "dsFv" is a VH:VL heterodimer stabilized by a disulfide bond. "(dsFv)₂" denotes two dsFv coupled by an amino acid linker sequence.

[00190] In certain aspects, a CD25 targeting multispecific binding protein comprises a first cell surface binding moiety comprising a scFv that specifically binds CD25. In certain aspects, a CD25 targeting multispecific binding protein comprises a second binding moiety that comprises a scFv that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the CD25 targeting multispecific binding protein is each independently a scFV. In some aspect, the scFV is a linear scFV or a tandem scFV.

Immunoglobulin single variable domains

[00191] The term "immunoglobulin single variable domain" (ISV or ISVD), interchangeably used with "single variable domain", defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from "conventional" immunoglobulins (e.g., monoclonal antibodies) or their fragments

(such as Fab, Fab', F(ab')₂, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (VH) and a light chain variable domain (VL) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both VH and VL will contribute to the antigen binding site, i.e., a total of 6 CDRs will be involved in antigen binding site formation.

[00192] In view of the above definition, the antigen binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')₂ fragment, an Fv fragment such as a disulfide linked Fv or a scFv fragment, or a diabody derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain because in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e., by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

[00193] In contrast, ISVDs are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single VH, a single VHH, or single VL domain.

[00194] As such, the single variable domain may be a light chain variable domain sequence (e.g., a VL-sequence) or a suitable fragment thereof, or a heavy chain variable domain sequence (e.g., a VH-sequence or VHH sequence) or a suitable fragment thereof as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that consists essentially of the single variable domain such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

[00195] An ISVD can for example be a heavy-chain ISVD, such as a VHH, including a humanized VHH, a VH, including a camelized VH and a human VH. In certain aspects, the ISVD is a VHH, a camelized VH, or humanized VHH. Heavy chain ISVDs can be derived from a conventional four-chain antibody or from a heavy chain antibody.

[00196] For example, the ISVD may be a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a NANOBODY® ISVD (as defined herein, and including but not limited to a VHH); other single variable domains, or any suitable fragment of any one thereof.

[00197] In particular, the ISVD may be a NANOBODY® ISVD (such as a VHH, including a humanized VHH or camelized VH) or a suitable fragment thereof. [Note: NANOBODY® and NANOBODIES® are registered trademarks of Ablynx N.V.]

[00198] "VHH domains," also known as "VHHs" or "VHH antibody fragments" have originally been described as the antigen binding immunoglobulin variable domain of "heavy chain antibodies" (i.e., of "antibodies devoid of light chains"; Hamers-Casterman et al. Nature 363: 446-448, 1993). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "VL domains"). For a further description of VHH's, reference is made to the review article by Muyldermans (Reviews in Molecular Biotechnology 74: 277-302, 2001).

[00199] The generation of immunoglobulin sequences, such as VHHs, has been described e.g., in WO 1994/004678 A1; Hamers-Casterman *et al.* (1993), Naturally occurring antibodies devoid of light chains, Nature, vol. 363: 446-448; and Muyldermans (2001), Single domain camel antibodies: current status, J. Biotechnol., vol. 74(4): 277-302 which are incorporated by reference. In these methods, camelids are immunized with the target antigen or protein to induce an immune response against said target antigen or protein. The repertoire of VHHs obtained from said immunization is further screened for VHHs that bind the target antigen or protein.

[00200] A "VHH family" as used in the present specification refers to a group of VHH sequences that have identical lengths (i.e., they have the same number of amino acids within their sequence) and of which the amino acid sequence between position 8 and position 106 (according to Kabat numbering) has an amino acid sequence identity of 89% or more.

[00201] Sharks and other cartilaginous fish also naturally produce heavy-chain antibodies that recognize antigens with their single domain variable regions called VNAR.

[00202] In certain aspects, a monospecific CD25 binding protein comprises one cell surface binding moiety comprising at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell.

[00203] In certain aspects, a CD25 targeting multispecific binding protein comprises a first cell surface binding moiety comprising an ISVD that specifically binds CD25. In certain aspects, a CD25 targeting multispecific binding protein comprises a second binding moiety that comprises an ISVD that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the multispecific binding protein is each independently an ISVD.

[00204] In certain aspects, a multispecific binding protein comprises a first cell surface binding moiety comprising a VHH that specifically binds CD25. In certain aspects, a multispecific binding protein comprises a second binding moiety comprising a VHH that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the multispecific binding protein is each independently a VHH. In some aspects, a VHH is humanized VHH or a camelized VHH.

[00205] In certain aspects, a multispecific binding protein comprises a first cell surface binding moiety comprising a VNAR that specifically binds CD25 antibody. In certain aspects, a multispecific binding protein comprises a second binding moiety that comprises a VNAR that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the multispecific binding protein is each independently a VNAR.

[00206] In certain aspects, a multispecific binding protein comprises a first cell surface binding moiety comprising at least one ISVD that specifically binds CD25. In certain aspects, a CD25 targeting multispecific binding protein comprises a second binding moiety that comprises at least one ISVD that specifically binds to the target protein. In certain aspects, the first and the second binding moiety of the CD25 targeting multispecific binding protein is each independently an ISVD. In certain aspects, each binding moiety can comprise multivalent ISVDs.

[00207] In some aspects, the antigen-binding fragments of the disclosure are immunoglobulin single variable domains, such as a domain antibody, a "dAb", a VHH (including a humanized VHH), a camelized VH, other single variable domains, or any suitable fragment of any one thereof. In particular, antigen-binding fragments of the disclosure may be a VHH or a fragment thereof.

[00208] In certain aspects, methods of the disclosure comprise contacting a CD25 positive cell with a bispecific ISVD construct, wherein the bispecific ISVD construct comprises: a) a first ISVD that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second ISVD that specifically binds to a target protein of interest, such that the bispecific ISVD construct binds to the membrane-bound CD25 on the surface of the CD25 positive cell and to the target protein.

[00209] In certain exemplary aspects, methods of the disclosure comprise contacting a CD25 positive cell with a bispecific NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH), wherein the bispecific NANOBODY® ISVD comprises: a) a first NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH) that specifically binds to CD25 on the surface of the CD25 positive cell; and b) a second NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH) that specifically binds to the target protein of interest, such that the bispecific NANOBODY® ISVD binds to the CD25 on the surface of the CD25 positive cell and to the target protein.

[00210] ISVDs of the so-called “VH3 class” (i.e., ISVDs with a high degree of sequence homology to human germline sequences of the VH3 class such as DP-47, DP-51 or DP-29), can be used herein. Furthermore, any type of ISVD directed against CD25 on the surface of a CD25 positive cell and/or target protein, including for example, ISVDs belonging to the so-called “VH4 class” (i.e., ISVs with a high degree of sequence homology to human germline sequences of the VH4 class such as DP-78), as for example described in WO 2007/118 670 A1.

[00211] ISVDs (in particular VHH sequences and partially humanized VHHs) can in particular be characterized by the presence of one or more “hallmark residues” (as described herein in Table 1 and in subsequent paragraphs describing NANOBODY®ISVD) such that the ISVD is a NANOBODY® ISVD.

[00212] Thus, generally, a NANOBODY® ISVD (in particular a VHH, including (partially or fully) humanized VHH and camelized VH) can be defined as an amino acid sequence with the (general) structure:

FR1 – CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined in Table 1.

In particular, a NANOBODY® ISVD (in particular a VHH, including (partially) humanized VHH and camelized VH) can be an amino acid sequence with the (general) structure:

FR1 – CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein. More in particular, an ISVD can be an amino acid sequence with the (general) structure:

FR1 – CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4 in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively.

[00213] ISVDs can specifically bind to (as defined herein) and/or are directed against CD25 on the surface of a CD25 positive cell and/or the target protein. Also useful are suitable fragments of these ISVDs and polypeptides that comprise or consist essentially of one or more of such ISVDs and/or suitable fragments of the ISVs. The term “immunoglobulin single variable domain (ISVD)” encompasses a NANOBODY® VHH as described in or WO 2008/020079 A1 or WO 2009/138519 A1, and thus in an aspect denotes a VHH, a humanized VHH or a camelized VH (such as a camelized human VH) or generally a sequence optimized VHH (such as *e.g.*, optimized for chemical stability and/or solubility, maximum overlap with known human framework regions and maximum expression).

[00214] In certain aspects, the monospecific binding protein of the disclosure comprises a CD25 ISVD that comprises or consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb. In certain aspects, the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation. In certain aspects, the VHH sequence is a humanized VHH sequence.

[00215] In certain aspects, the multispecific binding protein of the disclosure comprises a CD25 ISVD and a target protein ISVD wherein each independent ISVD comprises or consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb. In certain aspects, the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation. In certain aspects, the VHH sequence is a humanized VHH sequence.

[00216] Generally, NANOBODY® ISVDs (in particular VHH sequences, including (partially) humanized VHH sequences and camelized VH sequences) can be characterized by the presence of one or more “Hallmark residues” (as described herein) in one or more of the framework sequences (again as further described herein). Thus, generally, a NANOBODY® ISVD can be defined as an immunoglobulin sequence with the (general) structure:

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined herein.

[00217] In particular, a NANOBODY® ISVD can be an immunoglobulin sequence with the (general) structure:

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

[00218] More in particular, a NANOBODY® ISVD can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which: one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in **Table 1** below.

Table 1: Hallmark Residues in NANOBODY® ISVDs.

Position	Human V _H 3	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L
37	V, I, F; usually V	F ⁽¹⁾ , Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably F ⁽¹⁾ or Y
44 ⁽⁸⁾	G	E ⁽³⁾ , Q ⁽³⁾ , G ⁽²⁾ , D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably G ⁽²⁾ , E ⁽³⁾ or Q ⁽³⁾ ; most preferably G ⁽²⁾ or Q ⁽³⁾ .

45 ⁽⁸⁾	L	L ⁽²⁾ , R ⁽³⁾ , P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably L ⁽²⁾ or R ⁽³⁾
47 ⁽⁸⁾	W, Y	F ⁽¹⁾ , L ⁽¹⁾ or W ⁽²⁾ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably W ⁽²⁾ , L ⁽¹⁾ or F ⁽¹⁾
83	R or K; usually R	R, K ⁽⁵⁾ , T, E ⁽⁵⁾ , Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K
84	A, T, D; predominantly A	P ⁽⁵⁾ , S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P
103	W	W ⁽⁴⁾ , R ⁽⁶⁾ , G, S, K, A, M, Y, L, F, T, N, V, Q, P ⁽⁶⁾ , E, C; preferably W
104	G	G, A, S, T, D, P, N, E, C, L; preferably G
108	L, M or T; predominantly L	Q, L ⁽⁷⁾ , R, P, E, K, S, T, M, A, H; preferably Q or L ⁽⁷⁾

Notes:

⁽¹⁾ In particular, but not exclusively, in combination with KERE (SEQ ID NO: 9) or KQRE (SEQ ID NO: 10) at positions 43-46.

⁽²⁾ Usually as GLEW (SEQ ID NO: 11) at positions 44-47.

⁽³⁾ Usually as KERE (SEQ ID NO: 9) or KQRE (SEQ ID NO: 10) at positions 43-46, e.g., as KEREL (SEQ ID NO: 12), KERE (SEQ ID NO: 13), KQREL (SEQ ID NO: 14), KQREF (SEQ ID NO: 15), KERE (SEQ ID NO: 16), KQREW (SEQ ID NO: 17) or KQREG (SEQ ID NO: 18) at positions 43-47. Alternatively, also sequences such as TERE (SEQ ID NO: 19) (for example TEREL (SEQ ID NO: 20)), TQRE (SEQ ID NO: 21) (for example TQREL (SEQ ID NO: 22)), KECE (SEQ ID NO: 23) (for example KECEL (SEQ ID NO: 24) or KECER (SEQ ID NO: 25)), KQCE (SEQ ID NO: 26) (for example KQCEL (SEQ ID NO: 27)), RERE (SEQ ID NO: 28) (for example REREG (SEQ ID NO: 29)), RQRE (SEQ ID NO: 30) (for example RQREL (SEQ ID NO: 31), RQREF (SEQ ID NO: 32) or RQREW (SEQ ID NO: 33)), QERE (SEQ ID NO: 34) (for example QEREG (SEQ ID NO: 35)), QQRE (SEQ ID NO: 36), (for example QQREW (SEQ ID NO: 37), QQREL (SEQ ID NO: 38) or QQREF (SEQ ID NO: 39)), KGRE (SEQ ID NO: 40) (for example KGRE (SEQ ID NO: 41)), KDRE (SEQ ID NO: 42) (for example KDREV (SEQ ID NO: 43)) are possible. Some other possible, but less preferred sequences include for example DECKL (SEQ ID NO: 44) and NVCEL (SEQ ID NO: 45).

⁽⁴⁾ With both GLEW (SEQ ID NO: 11) at positions 44-47 and KERE (SEQ ID NO: 9) or KQRE (SEQ ID NO: 10) at positions 43-46.

⁽⁵⁾ Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.

⁽⁶⁾ In particular, but not exclusively, in combination with GLEW (SEQ ID NO: 11) at positions 44-47.

⁽⁷⁾ With the proviso that when positions 44-47 are GLEW (SEQ ID NO: 11), position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.

⁽⁸⁾ The GLEW (SEQ ID NO: 11) group also contains GLEW (SEQ ID NO: 11)-like sequences at positions 44-47, such as for example GVEW (SEQ ID NO: 46), EPEW (SEQ ID NO: 47), GLER (SEQ ID NO: 48), DQEW (SEQ ID NO: 49), DLEW (SEQ ID NO: 50), GIEW (SEQ ID NO: 51), ELEW (SEQ ID NO: 52), GPEW (SEQ ID NO: 53), EWLP (SEQ ID NO: 54), GPER (SEQ ID NO: 55), GLER (SEQ ID NO: 48) and ELEW (SEQ ID NO: 52).

[00219] In certain aspects, a first cell surface binding moiety and a second binding moiety of a multispecific binding protein is each independently an ISVD. Examples of ISVDs include variable domains obtained from heavy chain antibodies (VHHs), variable domains obtained from antibodies naturally devoid of light chains (VHHs), and ISVDs derived from conventional four-chain antibodies, engineered ISVDs. ISVDs may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit, bovine. ISVDs may be naturally occurring ISVDs present in a heavy chain antibody devoid of light chains. Camelidae species, for example camel, dromedary, llama, alpaca and guanaco, produce heavy chain antibodies naturally devoid of light chain. Camelid heavy chain antibodies also lack the CH1 domain.

[00220] In certain aspects, methods of the disclosure comprise contacting a CD25 positive cell with a binding protein comprising at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell.

[00221] In certain aspects, methods of the disclosure comprise contacting a CD25 positive cell with a bispecific ISVD construct, wherein the bispecific ISVD construct comprises: a) a first ISVD that specifically binds to CD25 on the surface of the CD25 positive cell; and b) a second ISVD that specifically binds to a target protein of interest, such that the bispecific ISVD construct binds to the CD25 on the surface of the CD25 positive cell and to the target protein. In certain aspects, methods of the disclosure comprise contacting a CD25 positive cell with a bispecific ISVD construct, wherein the bispecific ISVD construct comprises: a) multivalent ISVDs that specifically binds to CD25 on the surface of the CD25 positive cell; and b) multivalent ISVDs that specifically binds to a target protein of interest, such that the

bispecific ISVD construct binds to the CD25 on the surface of the CD25 positive cell and to the target protein.

[00222] In certain aspects, methods of the disclosure comprise contacting a CD25 positive cell with a bispecific NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH), wherein the bispecific NANOBODY® ISVD comprises: a) a first NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH) that specifically binds to CD25 on the surface of the CD25 positive cell; and b) a second NANOBODY® ISVD (such as a VHH, including a humanized VH or a camelized VH) that specifically binds to the target protein of interest, such that the bispecific NANOBODY® ISVD binds to the CD25 on the surface of the CD25 positive cell and to the target protein.

ISVD polypeptides and constructs

[00223] The process of designing/selecting and/or preparing a polypeptide, starting from an ISVD such as a VHH, humanized VHH, camelized VH, domain antibody or dAb, is also referred to herein as “formatting” said ISVD; and an ISVD that is made part of a polypeptide is said to be “formatted” or to be “in the format of” said polypeptide.

[00224] For example, and without limitation, one or more ISVDs may be used as a “binding unit”, “binding domain” or “building block” (these terms are used interchangeable) for the preparation of a polypeptide, which may optionally contain one or more further ISVDs that can serve as a binding unit (i.e., against the same or another epitope on CD25 and/or against one or more other target proteins (e.g., TNF α) as described herein.

[00225] The present disclosure also provides a monospecific or multispecific binding protein that comprises or consists essentially of one or more ISVDs. In one aspect, said binding protein further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers. The one or more other groups, residues, moieties or binding units can be any groups, residues, moieties or binding units known in the art. In one aspect the one or more other groups, residues, moieties or binding units are amino acid sequences, so that the resulting binding protein is a fusion (protein) or fusion (polypeptide).

[00226] The further groups, residues, moieties, binding units or amino acid sequences may or may not provide further functionality to the ISVD, polypeptide, or

construct and may or may not modify the properties of the ISVD, polypeptide, and/or construct. Such groups, residues, moieties or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the one or more ISVD and/or polypeptide so as to provide a “derivative” of the ISVD and/or polypeptide.

[00227] The one or more further amino acid sequences may be any suitable and/or desired amino acid sequences. The further amino acid sequences may or may not change, alter or otherwise influence the (biological) properties of the ISVD or polypeptide, and may or may not add further functionality to the ISVD or the polypeptide. In an aspect, the further amino acid sequence is such that it confers one or more desired properties or functionalities to the ISVD or the polypeptide. Examples of such amino acid sequences generally comprise all amino acid sequences that are used in peptide fusions based on conventional antibodies and fragments thereof (including but not limited to ScFv's and single domain antibodies). Reference is for example made to the review by Holliger and Hudson, “Engineered antibody fragments and the rise of single domains,” *Nature Biotechnology*, vol. 23, 1126-1136 (2005). For example, such an amino acid sequence may be an amino acid sequence that increases the half-life, the solubility, or the absorption, reduces the immunogenicity or the toxicity, eliminates or attenuates undesirable side effects, and/or confers other advantageous properties to and/or reduces the undesired properties of the ISVD or polypeptide, compared to the ISVD or polypeptide per se. Some non-limiting examples of such amino acid sequences are serum proteins, such as human serum albumin (see for example WO 2000/27435 A1) or haptenic molecules (for example haptens that are recognized by circulating antibodies, see for example WO 1998/22141 A2).

[00228] The further amino acid sequence may also provide a second binding site which binding site may be directed against a target protein of interest (e.g., a TNF) as described herein. In some aspects, the one or more other groups, residues, moieties or binding units are binding units that are directed against a target protein of interest described herein. In some aspects, the binding unit is an immunoglobulin sequence (e.g., an IgG). In some aspects, a binding unit is an ISVD, such as a VHH, humanized VHH, camelized VH, domain antibody, single domain antibody or dAb that specifically binds the target protein (target protein ISVD(s)).

[00229] Monovalent polypeptides comprise or consist essentially of only one binding unit (such as *e.g.*, ISVD). Polypeptides that comprise two or more binding units (such as *e.g.*, ISVDs) will also be referred to herein as “multivalent” polypeptides, and the binding units/ISVDs present in such polypeptides will also be referred to herein as being in a “multivalent format”. For example a “bivalent” polypeptide may comprise two ISVDs, optionally linked via a linker sequence, whereas a “trivalent” polypeptide may comprise three ISVDs, optionally linked via two linker sequences; whereas a “tetravalent” polypeptide may comprise four ISVDs, optionally linked via three linker sequences; whereas a “pentavalent” polypeptide may comprise five ISVDs, optionally linked via four linker sequences; whereas a “hexavalent” polypeptide may comprise six ISVDs, optionally linked via five linker sequences, etc.

[00230] An example of a monovalent polypeptide is *e.g.*, SEQ ID NO: 72 that encodes a CD25 ISVD. A bivalent polypeptide *e.g.*, can encode a multispecific binding protein of the disclosure comprising a first cell surface binding moiety that comprises a CD25 ISVD (*e.g.*, comprising SEQ ID NO: 72); and a second binding moiety comprising a target protein ISVD (*e.g.*, a TNF). An example of a trivalent polypeptide *e.g.*, can encode a multispecific binding protein of the disclosure comprising a first cell surface binding moiety that comprises a CD25 ISVD (*e.g.*, comprising SEQ ID NO: 72); and a second binding moiety comprising two target protein ISVD (*e.g.*, two ISVDs that target the same or a different epitope on TNF). As described in the example, in the multivalent polypeptide, the two or more ISVDs may be the same or different, and may be directed against the same antigen or antigenic determinant (for example against the same part(s) or epitope(s) or against different parts or epitopes) or may alternatively be directed against different antigens or antigenic determinants; or any suitable combination thereof.

[00231] Polypeptides that contain at least two binding units (*e.g.*, an ISVD) in which at least one binding unit is directed against a first cell surface binding moiety (*i.e.*, CD25) and at least one binding unit is directed against a second target protein (*i.e.*, different from CD25) will also be referred to as “multispecific” polypeptides or multispecific binding proteins, and the binding units (such as *e.g.*, ISVDs) present in such polypeptides will also be referred to herein as being in a “multispecific format”. Thus, for example, a “bispecific” polypeptide or bispecific binding protein is a polypeptide that comprises at least one ISVD directed against a CD25 and at least

one further ISVD directed against a second target protein (i.e., different from CD25), whereas a “trisppecific” polypeptide is a polypeptide that comprises at least one ISVD directed against a first antigen (i.e., CD25), at least one further ISVD directed against a second target protein (i.e., different from CD25) and at least one further ISVD directed against a third antigen or protein (i.e., different from both CD25 and the second target protein).

[00232] A multispecific binding protein of the disclosure comprising at least two ISVDs can be found at e.g., **Table 13**. A multispecific binding protein of the disclosure includes a multispecific binding protein that comprises an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence set forth in SEQ ID NO: 119.

Half-life extending units and ISVD

[00233] In one aspect, the monospecific or multispecific binding protein can further comprise one or more other groups, residues, moieties or binding units, optionally linked via one or more peptide linkers, in which said one or more other groups, residues, moieties or binding units provide the binding protein with increased *in vivo* half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. *In vivo* half-life extension means, for example, that the binding protein has an increased half-life in a mammal, such as a human subject, after administration. Half-life can be expressed for example as $t_{1/2\beta}$.

[00234] The type of groups, residues, moieties or binding units is not generally restricted and may for example be chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

[00235] More specifically, said one or more other groups, residues, moieties or binding units that provide the binding protein with increased half-life can be chosen from the group consisting of binding units that can bind to serum albumin, such as human serum albumin, or a serum immunoglobulin, such as IgG. In one aspect, said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is a binding unit that can bind to human serum albumin. In one aspect, the binding unit is an ISVD.

Serum albumin binding ISVD

[00236] In certain aspects, the binding unit is an ISVD that binds to a human serum albumin (i.e., referred to herein as a “serum albumin ISVD”) e.g., an ISVD that binds to a human serum albumin described in WO 2004/041865, WO 2006/122787, WO 2006/122787, WO2012/175400, WO 2012/175741, WO 2015/173325, WO 2017/080850, WO 2017/085172, WO 2018/104444, WO 2018/134235, or WO 2018/134234, which are incorporated by reference herein in their entirety. Examples of serum albumin binding ISVD sequences are shown in **Table 11** below.

[00237] In some aspects, the monospecific or the multispecific binding protein of the disclosure comprises an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88.

Table 11. Serum albumin binding ISVD sequences

Name	Amino acid sequence
SEQ ID NO: 73 Alb8	EVQLVESGGGLVQPGLNSLRRLSCAASGFTFSSFGMSWVRQAP GKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQM NSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
SEQ ID NO: 74 Alb23	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAP GKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQM NSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
SEQ ID NO: 75 Alb129	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQ MNSLRPEDTATYYCTIGGSLSRSSQGTLVTVSSA
SEQ ID NO: 76 Alb132	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQA PGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTATYYCTIGGSLSRSSQGTLVTVSSA
SEQ ID NO: 77 Alb11	EVQLVESGGGLVQPGLNSLRRLSCAASGFTFSSFGMSWVRQAP GKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQM NSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
SEQ ID NO: 78 Alb11 (S112K)- A	EVQLVESGGGLVQPGLNSLRRLSCAASGFTFSSFGMSWVRQAP GKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQM NSLRPEDTAVYYCTIGGSLSRSSQGTLVKVSSA
SEQ ID NO: 79 Alb82	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSS
SEQ ID NO: 80 Alb82-A	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
SEQ ID NO: 81 Alb82-AA	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSAA

Name	Amino acid sequence
SEQ ID NO: 82 Alb82-AAA	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSAAA
SEQ ID NO: 83 Alb82-G	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSG
SEQ ID NO: 84 Alb82-GG	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSGG
SEQ ID NO: 85 Alb82-GGG	EVQLVESGGGVVQPGNSLRRLSCAASGFTFSSFGMSWVRQA PGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSGGG
SEQ ID NO: 86 Alb223	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQA PGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
SEQ ID NO: 87 AlbX00001	EVQLVESGGGVVQPGGSLRLSCAASGLTFSSYAMGWFRQA PGKERERVVSSISRGGGYTYADSVKGRFTISRDNSENTVYLQ MNSLRPEDTALYYCAAARYWATGSEYEFDYWGQGTLVTVSS
SEQ ID NO: 88 Alb23002	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQA PGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSS

[00238] In certain aspects, an ISVD that binds to a human serum albumin is linked to the first binding moiety of the monospecific binding protein (e.g., a CD25 ISVD) described herein. In certain aspects, an ISVD that binds to a human serum albumin is linked to the first and/or second binding moieties of the multispecific binding protein described herein. In certain aspects, the ISVD that binds to a human serum albumin extends the half-life of the monospecific or the multispecific binding protein relative to a reference binding protein that does not have said ISVD.

[00239] In certain aspects, an ISVD binding to human serum albumin is linked to the C-terminal end of the monospecific or the multispecific binding protein so that the C-terminal end of the human serum albumin is an exposed C-terminal end. In certain aspects, the C-terminal end of the ISVD binding to human serum albumin comprises a C-terminal extender. In certain aspects, the C-terminal extender is a C-terminal alanine (A) or glycine (G) extension. In certain aspects, C-terminal alanine (A) or glycine (G) extension comprises 1-3 alanine or glycine residues, respectively.

[00240] In certain aspects, an ISVD capable of binding to human serum albumin is not linked to the C-terminal end of the monospecific or the multispecific binding protein and the C-terminal end of the human serum albumin is not an

exposed C-terminal end. In certain aspects, the C-terminal sequence "VTVSS(X)_n" (SEQ ID NO: 89) is directly followed by a linker region, e.g., a linker region of Table 10.

[00241] In certain aspects, the monospecific binding protein of the disclosure comprises at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell and at least one serum albumin ISVD. In certain aspects, the binding protein (e.g., the CD25 ISVD subunit) is operatively linked to a serum albumin ISVD. In certain aspects, the serum albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88. In certain aspects, the serum albumin ISVD consists essentially of SEQ ID NO: 74. In certain aspects, the CD25 ISVD is operatively linked to the serum albumin ISVD via an amino acid linker sequence.

[00242] In certain aspects, a multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD; b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD; and c) a third binding moiety that specifically binds to a serum albumin ISVD. In certain aspects, the serum albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88. In certain aspects, the serum albumin ISVD consists essentially of SEQ ID NO: 88. In certain aspects, the CD25 ISVD is operatively linked to the serum albumin ISVD via an amino acid linker sequence.

[00243] In certain aspects, a multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C-terminal end of a second binding moiety; b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a third binding moiety; and c) a third binding moiety that specifically binds to a serum albumin ISVD. In certain aspects, the serum albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88. In certain aspects, the serum albumin ISVD consists essentially of SEQ ID NO: 88. In certain aspects, the CD25 ISVD is operatively linked to the serum albumin ISVD via an amino acid linker sequence.

[00244] In certain aspects, a multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C-terminal end of a second binding moiety; b) the second binding moiety that specifically binds to a target protein comprising at least two target protein ISVDs wherein one of the at least two target protein ISVDs is linked to the N- and/or C-terminal end of a third binding moiety; and c) the third binding moiety that specifically binds to a serum albumin ISVD.

[00245] In certain aspects, a multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C-terminal end of a second binding moiety; b) the second binding moiety that specifically binds to a target protein comprising at least two target protein ISVDs (e.g., two TNF α ISVDs) wherein one of the at least two target protein ISVDs is linked to the N- and/or C-terminal end of a third binding moiety; and c) the third binding moiety that specifically binds to serum albumin ISVD comprising SEQ ID NO: 88.

ISVD optimization and ISVD C-terminus

[00246] The present disclosure provides sequence optimized ISVDs and polypeptides that show increased stability upon storage during stability studies. In one aspect, the sequence optimized ISVDs and polypeptides show reduced pyroglutamate post-translational modification of the N-terminus and hence have increased product stability. Pyroglutamate (pGlu) modification leads to heterogeneity of the final product and should be avoided. The possibility of pGlu post-translational modification of the N-terminus was eliminated by changing the N-terminal Glutamic acid (E) into an Aspartic acid (D) which leads to increased product stability. Accordingly, the present disclosure also relates to ISVDs and polypeptides as described above wherein the Glutamic acid at position 1 (said position determined according to Kabat numbering) is changed into an Aspartic acid (E1D).

[00247] The disclosure also provides sequence optimized ISVDs and polypeptides that are "humanized", i.e., in which one or more amino acid residues in the amino acid sequence of said naturally occurring VHH sequence (and in particular in the framework sequences) are replaced 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.

[00248] The present disclosure also provides sequence optimized ISVDs and polypeptides that exhibit reduced binding by pre-existing antibodies present in human serum. To this end, in one aspect, the polypeptide comprises a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least one ISVD. In one aspect, the polypeptide comprises a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in each ISVD. Accordingly, aspects also relate to ISVDs and polypeptides as described above that have been sequence optimized with a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least one ISVD, such as in all ISVDs.

[00249] In one aspect, the monospecific or multispecific binding protein of the disclosure comprises an ISVD or a polypeptide comprising a C-terminal end of the sequence VTVSS(X)_n (SEQ ID NO: 89), in which n is 1 to 10, such as 1 to 5, such as 1, 2, 3, 4 or 5, and in which each X is an amino acid residue that is independently chosen. In one aspect, the polypeptide comprises such an ISVD at its C-terminal end. In one aspect, n is 1 or 2, such as 1. In one aspect, X is a naturally occurring amino acid. In one aspect, X is chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I).

[00250] In another aspect monospecific or multispecific binding protein of the disclosure comprises an ISVD or a polypeptide comprising a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD. In another aspect, the ISVD comprises a lysine (K) or glutamine (Q) at position 112 (according to Kabat numbering) in at least one ISVD. In these aspects, the C-terminus of the ISVD is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an ISVD C-terminus amino acid sequence encoded in SEQ ID NO: 89-106 (see Table 15).

Table 15: ISVD C-terminus Amino Acid Sequences

SEQ ID NO:	Sequence
SEQ ID NO: 89	VTVSS(X) _n
SEQ ID NO: 90	VKVSS
SEQ ID NO: 91	VQVSS

SEQ ID NO:	Sequence
SEQ ID NO: 92	VTVKS
SEQ ID NO: 93	VTVQS
SEQ ID NO: 94	VKVKS
SEQ ID NO: 95	VKVQS
SEQ ID NO: 96	VQVKS
SEQ ID NO: 97	VQVQS
SEQ ID NO: 98	VTVSSA
SEQ ID NO: 99	VKVSSA
SEQ ID NO: 100	VQVSSA
SEQ ID NO: 101	VTVKSA
SEQ ID NO: 102	VTVQSA
SEQ ID NO: 103	VKVKSA
SEQ ID NO: 104	VKVQSA
SEQ ID NO: 105	VQVKSA
SEQ ID NO: 106	VQVQSA

[00251]

Linker Region

[00252] In certain aspects, a monospecific or a multispecific binding protein of the disclosure comprises a linker, e.g., a polypeptide linker, a peptide linker, or an amino acid linker. The terms a “polypeptide linker,” “peptide linker,” and an “amino acid linker” are sometimes referred to herein interchangeably because amino acid linker is the smallest unit (i.e., comprising at least one amino acid residue) in a peptide or polypeptide linker. In certain aspects, a polypeptide linker, a peptide linker, or an amino acid linker can comprise or consist of a glycine or glycine-serine linker or other suitable linker.

[00253] In an aspect, a linker can link a variable and constant domain in a multispecific binding protein of the disclosure. In another aspect, a linker can link a scFv polypeptide. In another aspect, linkers described herein, can be used to link together two or more ISVDs into a single bispecific binding protein.

[00254] In another aspect, linkers can be used to link together two or more multivalent ISVDs comprised in either a monospecific or a multispecific binding protein of the disclosure. In one aspect, at least two CD25 ISVDs are operatively

linked via an amino acid linker sequence. In one aspect, at least two target protein ISVDs are operatively linked via an amino acid linker sequence.

[00255] Examples of amino acid linkers are shown in Table 10. One often used class of peptidic linker are known as the “Gly-Ser” or “GS” linkers. These are linkers that consist essentially of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif. For example, a single glycine (Gly) residue; a diglycine peptide (Gly-Gly); a tripeptide (Gly-Gly-Gly); a peptide with four glycine residues (Gly-Gly-Gly-Gly) (SEQ ID NO: 1); a peptide with five glycine residues (Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 2); a peptide with six glycine residues (Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 3); a peptide with seven glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 4); a peptide with eight glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 5). Other combinations of amino acid residues can be used such as the peptide Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 6) and the peptide Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 7). Reference is for example made to Chen *et al.* (2013), “Fusion Fusion protein linkers: property, design and functionality,” *Adv. Drug Deliv. Rev.*, vol. 65(10): 1357–1369; and Klein *et al.* (2014), “Design and characterization of structured protein linkers with differing flexibilities,” *Protein Eng. Des. Sel.*, vol. 27 (10): 325-330.

[00256] An exemplary glycine serine linker comprises an amino acid sequence of the formula (Gly4Ser)_n, wherein n is a positive integer (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10). In some aspects, the glycine-serine linker is Gly4Ser (G4S). In some aspects, the glycine linker is (G4S)₂, (G4S)₃, or (G4S)₄. In some aspects, the linker region comprises a poly-Glycine-Serine (G4S) linker.

[00257] In some aspects, the linker comprises an amino acid sequence which is at least 75, 80, 85, 90, 93, 95, 99% or more identical to the amino acid sequence of SEQ ID NO: 8.

[00258] In some aspects, the linker comprises at least a portion of a hinge region (e.g., derived from an IgG1, IgG2, IgG3, or IgG4 molecule) and a series of glycine-serine amino acid residues.

[00259] In certain aspects, a monospecific or a multispecific binding protein of the disclosure comprises a linker connecting an ISVD to another ISVD via an amino acid linker sequence. In certain aspects, the monospecific binding protein of the

disclosure comprises multivalent ISVD that binds to CD25 and are linked *via* an amino acid linker sequence. In certain aspects, the multispecific binding protein of the disclosure comprises multivalent ISVDs that bind to the same antigen or protein (e.g., at least two ISVDs that bind to CD25 and/or at least two ISVDs that bind to the target protein). In certain aspects, the multispecific binding protein of the disclosure comprises a linker connecting at least one CD25 ISVD to at least one target protein ISVD.

[00260] In certain aspects, a linker also connects an ISVD to the first and/or second Fc heavy chain can be an amino acid linker sequence. In certain aspects, the amino acid linker sequence is a poly-Glycine-Serine (G4S)_n linker. In certain aspects, n equals 1, 2, 3, 4, or 5.

[00261] In certain aspects, the linker can connect a binding moiety (i.e., the CD25 and/or the target protein binding moiety) to a Fc domain polypeptide or a Fc domain variant polypeptide.

[00262] In certain aspects, a multispecific binding protein of the disclosure comprises one or more CD25 ISVD(s) and one or more target protein ISVD(s) operatively linked to a first and second Fc domain polypeptide, respectively. In certain aspects, the CD25 ISVD(s) and the target protein ISVD(s) are operatively linked to the first and second Fc domain polypeptide via an amino acid linker. In certain aspects, the amino acid linker is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10. In certain aspects, the first and second Fc domain polypeptides each comprise a first and a second IgG domain that dimerize to form the multispecific binding protein.

[00263] In certain aspects, a multispecific binding protein comprises a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C-terminal end of a first IgG; and b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof.

Table 10. Linker Table Amino Acid Sequence

SEQ ID NO / Description	Amino acid sequence
SEQ ID NO: 1	GGGG
SEQ ID NO: 2	GGGGG
SEQ ID NO: 3	GGGGGG
SEQ ID NO: 4	GGGGGGG
SEQ ID NO: 5	GGGGGGGG
SEQ ID NO: 6 5GS linker	GGGGS
SEQ ID NO: 7 10GS linker	GGGGS GGGGS
SEQ ID NO: 8	GGGGGS
SEQ ID NO: 56	(GGGGS) _N
SEQ ID NO: 57 15GS linker	GGGGS GGGGS GGGGS
SEQ ID NO: 58 20GS linker	GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 59 3A linker	AAA
SEQ ID NO: 60 7GS linker	SGGS GGS
SEQ ID NO: 61 8GS linker	GGGGS GGS
SEQ ID NO: 62 9GS linker	GGGGS GGS
SEQ ID NO: 63 18GS linker	GGGGS GGGGS GGGGS GGS
SEQ ID NO: 64 25GS linker	GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 65 30GS linker	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 66	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS

SEQ ID NO / Description	Amino acid sequence
35GS linker	
SEQ ID NO: 67 40GS linker	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 68 G1 hinge	EPKSCDKTHTCPPCP
SEQ ID NO: 69 9GS-G1 hinge	GGGGSGGGSEPKSCDKTHTCPPCP
SEQ ID NO: 70 Llama upper long hinge region	EPKTPKPQAAA
SEQ ID NO: 71 G3 hinge	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPP CPRCPEPKSCDTPPPCPRCP

[00264] In certain aspects, the amino acid linker sequence comprises an amino acid sequence which is at least 75, 80, 85, 90, 93, 95, 99% or more identical to an amino acid sequence of Table 10.

[00265] In certain aspects, the linker comprises at least a portion of a hinge region (e.g., derived from an IgG1, IgG2, IgG3, or IgG4 molecule) and a series of glycine-serine amino acid residues.

[00266] In certain aspects, a multispecific binding protein comprises a second binding moiety which is operatively linked to a first cell surface binding moiety and that specifically binds to the target protein.

[00267] In certain aspects, a multispecific binding protein further comprises one or more binding moieties operatively linked to the first cell surface binding moiety or the second binding moiety that specifically binds to the target protein. In certain aspects, the operative linker is an amino acid linker sequence. In certain aspects, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in SEQ ID NOs: 1-8 or 56-71.

[00268] In certain aspects, a compound or construct comprises the multispecific or the bispecific binding protein according to the disclosure that further

comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more amino acid linker sequence(s).

ISVD linkage

[00269] In certain aspects, the monospecific or the multispecific binding protein can be linked (optionally via a suitable linker or hinge region) to one or more constant domains (for example, 2 or 3 constant domains that can be used as part of/to form an Fc portion), to an Fc portion and/or to one or more antibody parts, fragments or domains that confer one or more effector functions to the ISVD or polypeptide and/or may confer the ability to bind to one or more Fc receptors. For example, for this purpose, and without being limited thereto, the one or more further amino acid sequences may comprise one or more CH2 and/or CH3 domains of an antibody, such as from a heavy chain antibody (as described herein), for example, from a conventional human 4-chain antibody; and/or may form (part of) and Fc region, for example from IgG (e.g. from IgG1, IgG2, IgG3 or IgG4), from IgE or from another human Ig such as IgA, IgD or IgM. For example, WO 1994/04678 A1 describes heavy chain antibodies comprising a camelid VHH domain or a humanized derivative thereof in which the camelidae CH2 and/or CH3 domain have been replaced by human CH2 and CH3 domains, so as to provide an immunoglobulin that has 2 heavy chains each comprising a VHH and human CH2 and CH3 domains (but no CH1 domain), which immunoglobulin has the effector function provided by the CH2 and CH3 domains and which immunoglobulin can function without the presence of any light chains. Other amino acid sequences that can be suitably linked to the ISVD or polypeptide of the present disclosure so as to provide an effector function will be clear to the skilled person and may be chosen on the basis of the desired effector function(s). References which are incorporated by reference in their entirety include WO 2004/058820 A1, WO 99/42077, WO 2002/056910 A1 and WO 2005/017148 A1, as well as the review by Holliger and Hudson, *supra*; and to WO 2009/068628 A1.

[00270] Coupling of ISVD or polypeptide to an Fc portion may also lead to an increased half-life, compared to the corresponding reference ISVD or polypeptide. For some applications, the use of an Fc portion and/or of constant domains (i.e. CH2 and/or CH3 domains) that confer increased half-life without any biologically significant effector function are also suitable. Other suitable constructs comprising

one or more ISVDs or polypeptides and one or more constant domains with increased half-life in vivo will be clear to the skilled person and may for example comprise ISVDs or polypeptides linked to a CH3 domain, optionally via a linker sequence. Generally, any fusion protein or derivatives with increased half-life can have a molecular weight of more than 50 kD, the cut-off value for renal absorption.

[00271] The components, e.g., the ISVDs, of the binding proteins described herein may be linked to each other by one or more suitable linkers, such as peptidic linkers. The use of linkers to connect two or more (poly)peptides is discussed above. In some aspects, ISVDs are operatively linked to another ISVD or a Fc domain polypeptide or variant thereof via an amino acid linker sequence. In some aspects, the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

Fc domain

[00272] In some aspects, a CD25 targeting monospecific or multispecific binding protein can comprise a Fc domain. As used herein, the term “Fc domain” or “Fc region” (used interchangeably) is defined as the portion of a heavy chain constant region beginning in the hinge region just upstream of the papain cleavage site (i.e., residue 216 in IgG, taking the first residue of heavy chain constant region to be 114) and ending at the C-terminus of the antibody. Accordingly, a complete Fc domain comprises at least a hinge domain, a CH2 domain, and a CH3 domain.

[00273] The terms “Fc variant,” “modified Fc,” “engineered Fc” are interchangeably used herein refer to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). Exemplary Fc variants, and their interaction with the salvage receptor, are known in the art. A modified Fc domain can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the monospecific or multispecific binding protein compositions. Thus, the term “modified Fc domain” comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has been modified, that affect or are involved in: (1) disulfide bond formation, (2) incompatibility with a selected host cell, (3) N-terminal heterogeneity

upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC).

[00274] “Effector function” as used herein is meant a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. A “functional Fc region” possesses an “effector function” of a native sequence Fc region. Exemplary “effector functions” include antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP).

[00275] The term “EU index” refers to the EU numbering convention for the constant regions of an antibody, as described in Edelman, GM. et al., Proc. Natl. Acad. USA, 63, 78-85 (1969) and Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Dept. Health and Human Services, 5th edition, 1991, each of which is herein incorporated by reference in its entirety. Unless otherwise stated, all antibody Fc region numbering employed herein corresponds to the EU numbering scheme, as described in Edelman et al. (Proc. Natl. Acad. Sci. 63(1): 78-85. 1969).

[00276] The Fc domain of a CD25 targeting monospecific or multispecific binding protein of the disclosure may be engineered to promote heterodimerization over homodimerization. For example, the heavy chain constant region of the first heavy-light chain pair may comprise a different amino acid sequence from the heavy chain constant region of the second heavy-light chain pair, wherein the different amino acid sequences are engineered to promote heterodimerization of the heavy chain constant regions. Examples include knobs-into-holes mutations or charge pair mutations. Alternatively, the heavy chain constant region of the first heavy-light chain pair may be identical to the heavy chain constant region of the second heavy-light chain pair, in which case it is expected that both homodimers and heterodimers will assemble, and these will be subsequently separated using one or more purification steps in the antibody manufacturing process to isolate the desired heterodimer comprising one anti-CD25 arm and one anti-target protein arm.

[00277] In certain aspects, a first and/or second Fc constant domain is derived from an immunoglobulin class selected from a group consisting of: IgM, IgG, IgD, IgA, IgE. Chimeric Fc domains comprising portions of Fc domains from different

species or Ig classes can also be employed. In certain aspects, a Fc constant domain is an IgG Fc domain, an IgG1 Fc domain, or an IgG4 Fc domain.

[00278] In certain aspects, methods for degrading a target protein is provided. The methods comprise contacting a CD25 positive cell with a multispecific binding protein, wherein the multispecific binding protein comprises: a) a first cell surface binding moiety comprising a CD25 antibody or an antigen binding fragment thereof comprising a CD25-fragment crystallizable (Fc) fusion polypeptide or variant thereof that binds to CD25 on the surface of the CD25 positive cell; and b) a second binding moiety that specifically binds a target protein and comprises a second Fc domain polypeptide. In certain aspects the first and/or second Fc domain can be modified.

[00279] In certain aspects, the first Fc domain can be engineered to pair or heterodimerize with the second Fc domain.

[00280] For example, a monospecific binding protein of the disclosure (e.g., a CD25 ISVD) can be operatively linked to a Fc domain polypeptide (e.g., *via* an amino acid linker sequence). In certain aspects, the Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide or variant thereof. In certain aspects, the binding protein comprises a fusion protein comprising a CD25 ISVD and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25 ISVD:Fc fusion polypeptide or a variant thereof.

[00281] For example, a multispecific binding protein of the disclosure may comprise a first polypeptide comprising a fusion of a CD25 antibody or the antigen binding fragment thereof and an Fc domain and a second polypeptide comprising a binding specificity (e.g., a VHH, Fab, or scFv) for a target protein of interest, wherein the second polypeptide comprises a second Fc domain capable of dimerizing with the first Fc domain. In certain aspects the first and/or second Fc domain can be modified.

STY Mutation

[00282] Exemplary aspects of a modified glycan linked to a Fc domain *via* an engineered glycosylation site present on the Fc domain are described in WO2014043361A1 which is incorporated by reference in its entirety.

[00283] In certain aspects, a monospecific or a multispecific binding protein of the disclosure comprising a Fc domain has an engineered N-linked glycosylation

site. In certain aspects, a multispecific binding protein of the disclosure comprises one or more mutations or glycan modifications to modulate Fc mediated effector function. In certain aspects, a multispecific binding protein can comprise one or more mutations to modulate serum half-life.

[00284] In the case of a human IgG1 Fc domain, mutation of the wild-type amino acid at Kabat position 298 to an asparagine and Kabat position 300 to a serine or threonine results in the formation of an engineered N-linked glycosylation site (i.e., the N—X-T/S sequon, where X is any amino acid except proline). However, in the case of Fc domains of other species and/or Ig classes or isotypes, the skilled artisan will appreciate that it can be necessary to mutate Kabat position 299 of the Fc domain if a proline residue is present to recreate an N—X-T/S sequon.

[00285] In certain aspects, a monospecific or a multispecific binding protein comprises a constant domain comprising a Fc domain having an asparagine residue at amino acid position 298, according to EU numbering; and a serine or threonine residue at amino acid position 300, according to EU numbering. In certain aspects, a Fc domain further comprises an alanine residue at amino acid position 299, according to the EU numbering. In certain aspects, a Fc domain further comprises a glutamine residue at amino acid position 297, according to EU numbering.

[00286] In certain aspects, the at least one modified glycan is linked to an asparagine residue at amino acid position 298, according to EU numbering. In certain aspects, the at least one modified glycan is linked through a side chain of the asparagine residue through a β -glycosylamide linkage.

[00287] In certain aspects, a monospecific or a multispecific binding protein of the disclosure comprises mutations or glycan modifications to modulate Fc mediated effector function. In certain aspects, a multispecific binding protein comprises one or more mutations or glycan modifications to modulate serum half-life.

Additional Fc mutations

[00288] In certain aspects, a monospecific or a multispecific binding protein of the disclosure comprises a modified Fc domain and can further comprise one or more, e.g., two or more, three or more, or four or more, amino acid substitutions that confers a monospecific or multispecific binding protein with one or more biochemical characteristics other than glycan modification.

[00289] Exemplary modified Fc domain amino acid substitutions that can confer additional biochemical characteristics to the monospecific or multispecific binding proteins described herein are disclosed in WO 2021/016571 A2, which is incorporated by reference in its entirety.

[00290] In certain aspects, a modified Fc region of a multispecific binding protein of the current disclosure comprises one or more mutations to modulate half-life (See *e.g.*, Dall'Acqua et al. (2006) J Biol Chem 281: 23514-24, Zalevsky et al. (2010) Nat Biotechnol 28: 157-9, Hinton et al. (2004) J Biol Chem 279: 6213-6, Hinton et al. (2006) J Immunol 176: 346-56, Shields et al. (2001) J Biol Chem 276: 6591-604, Petkova et al. (2006) Int Immunol 18: 1759-69, Datta-Mannan et al. (2007) Drug Metab Dispos 35: 86-94, Vaccaro et al. (2005) Nat Biotechnol 23: 1283-8, Yeung et al. (2010) Cancer Res 70: 3269-77 and Kim et al. (1999) Eur J Immunol 29: 2819-25. (*e.g.*, T250Q, M252Y, I253A, S254T, T256E, P257I, T307A, D376V, E380A, M428L, H433K, N434S, N434A, N434H, N434F, H435A and/or H435R).

[00291] In certain aspects, a modified Fc region of a monospecific or a multispecific binding protein of the current disclosure can have enhanced FcRn binding affinities at both an acidic pH (*e.g.*, less than about 7.0, no more than about 6.5, or no more than about 6.0) and a non-acidic pH (*e.g.*, no less than about 7.0, or no less than about 7.4), as compared to its wild-type. In another example, a monospecific or a multispecific binding protein comprising a modified Fc can comprise one or more amino acid mutations (*e.g.*, substitutions) which alter the effector functions (*e.g.*, ADCC or CDC function) of the Fc domain, as compared to a corresponding wild-type molecule, *e.g.*, a molecule having the same structure as the FcRn antagonist except that it has a wild-type Fc domain. In another example, a monospecific or a multispecific binding protein can comprise a modified Fc domain comprising one or more amino acid mutations (*e.g.*, substitutions) which alter (*e.g.*, increase or decrease) the circulating half-life (*e.g.*, serum half-life) of the FcRn antagonist, as compared to the corresponding wild-type molecule.

[00292] In certain aspects, a monospecific or a multispecific binding protein described herein can comprise a modified Fc domain that alters serum half-life compared to a multispecific binding protein comprising a wild-type Fc domain. In certain aspects, a modified Fc domain has an increased serum half-life compared to a multispecific binding protein comprising a wild-type Fc domain. In certain aspects,

a Fc domain is modified to alter FcRn binding affinity compared to a monospecific or a multispecific binding protein comprising a wild-type Fc domain. In certain aspects, a modified Fc domain has enhanced FcRn binding affinity compared to a monospecific or a multispecific binding protein comprising a wild-type Fc domain. In certain aspects, a Fc domain is modified to enhance the FcRn binding affinity at an acidic pH compared to a monospecific or a multispecific binding protein comprising a wild-type Fc domain.

[00293] In certain aspects, a monospecific or a multispecific binding protein can have a modified Fc domain. In certain aspects, a monospecific or a multispecific binding protein can have a tyrosine (Y) at amino acid position 252, according to EU numbering. In certain aspects, a multispecific binding protein can have an aspartic acid (D) or a glutamic acid (E) at amino acid position 256, according to EU numbering. In certain aspects, a multispecific binding protein can have a tryptophan (W) or a glutamine (Q) at amino acid position 307, according to EU numbering. In certain aspects, a multispecific binding protein can have a phenylalanine (F) or a tyrosine (Y) at amino acid position 434; according to EU numbering.

[00294] In certain aspects, a monospecific or a multispecific binding protein can have a modified Fc domain comprising any combination of the following four amino acid residues: a tyrosine (Y) at amino acid position 252, an aspartic acid (D) or a glutamic acid (E) at amino acid position 256, a tryptophan (W) or a glutamine (Q) at amino acid position 307, and a phenylalanine (F) or a tyrosine (Y) at amino acid position 434; according to EU numbering.

[00295] In certain aspects, a monospecific or a multispecific binding protein can comprise a modified Fc domain having a combination of amino acid residues selected from the group consisting of: a) a tyrosine (Y) at amino acid position 252, an aspartic acid (D) at amino acid position 256, a glutamine (Q) at amino acid position 307, and a tyrosine (Y) at amino acid position 434; b) a tyrosine (Y) at amino acid position 252, a glutamic acid (E) at amino acid position 256, a tryptophan (W) at amino acid position 307, and a tyrosine (Y) at amino acid position 434; c) a tyrosine (Y) at amino acid position 252, a glutamic acid (E) at amino acid position 256, a glutamine (Q) at amino acid position 307, and a tyrosine (Y) at amino acid position 434; d) a tyrosine (Y) at amino acid position 252, an aspartic acid (D) at amino acid position 256, a glutamine (Q) at amino acid position 307, and a phenylalanine (F) at

amino acid position 434; e) a tyrosine (Y) at amino acid position 252, an aspartic acid (D) at amino acid position 256, a tryptophan (W) at amino acid position 307, and a tyrosine (Y) at amino acid position 434; and f) a tyrosine (Y) at amino acid position 252, an aspartic acid (D) at amino acid position 256, a tryptophan (W) at amino acid position 307, and a phenylalanine (F) at amino acid position 434; according to EU numbering.

[00296] In certain aspects, a monospecific or a multispecific binding protein can comprise a modified Fc domain comprising a quadruple amino acid substitution selected from the group consisting of: M252Y/T256D/T307Q/N434Y, M252Y/T256E/T307W/N434Y, M252Y/T256E/T307Q/N434Y, M252Y/T256D/T307Q/N434F, M252Y/T256D/T307W/N434Y, and M252Y/T256D/T307W/N434F; according to EU numbering.

Knobs and Holes

[00297] Techniques for making multispecific binding proteins (e.g., multispecific antibodies) include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein, C. and Cuello, A. C., *Nature* 305 (1983) 537-540, WO 93/08829, and Traunecker, A. et al., *EMBO J.* 10 (1991) 3655-3659), and “knob-in-hole” engineering (see, e.g., U.S. Pat. No. 5,731,168). Multispecific binding proteins may also be made by engineering electrostatic steering effects for making binding protein Fc-heterodimeric molecules (WO 2009/089004); cross-linking two or more antibodies or fragments (see, e.g., U.S. Pat. No. 4,676,980, and Brennan, M. et al., *Science* 229 (1985) 81-83); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny, S. A. et al., *J. Immunol.* 148 (1992) 1547-1553; using “diabody” technology for making multispecific binding protein fragments (see, e.g., Holliger, P. et al., *Proc. Natl. Acad. Sci. USA* 90 (1993) 6444-6448); and using single-chain Fv (scFv) dimers (see, e.g., Gruber, M et al., *J. Immunol.* 152 (1994) 5368-5374); and preparing trispecific binding proteins as described, e.g., in Tutt, A. et al., *J. Immunol.* 147 (1991) 60-69).

[00298] A wide variety of recombinant multispecific binding protein formats have been developed, e.g., by fusion of, e.g., an IgG binding protein format and single chain domains (see Kontermann RE, *mAbs* 4:2, (2012) 1-16). Multispecific

binding proteins wherein the variable domains VL and VH or the constant domains CL and CH1 are replaced by each other are described in WO 2009/080251 A1 and WO 2009/080252 A1.

[00299] An approach to circumvent the problem of mispaired byproducts, is known as “knobs-into-holes”, aims at forcing the pairing of two different binding protein heavy chains by introducing mutations into the CH3 domains to modify the contact interface. On one chain bulky amino acids can be replaced by amino acids with short side chains to create a “hole.” Conversely, amino acids with large side chains can be introduced into the other CH3 domain, to create a “knob.” By co-expressing these two heavy chains (and two identical light chains, which have to be appropriate for both heavy chains), high yields of heterodimer formation (“knob-hole”) versus homodimer formation (“hole-hole” or “knob-knob”) was observed (Ridgway JB, Presta LG, Carter P; and WO1996027011). The percentage of heterodimer could be further increased by remodeling the interaction surfaces of the two CH3 domains using a phage display approach and the introduction of a disulfide bridge to stabilize the heterodimers (Merchant A.M, et al, Nature Biotech 16 (1998) 677-681; Atwell S, Ridgway JB, Wells JA, Carter P., J Mol Biol 270 (1997) 26-35). New approaches for the knobs-into-holes technology are described in e.g., in EP 1870459A1. Xie, Z., et al, J Immunol Methods 286 (2005) 95-101 refers to a format of multispecific binding protein using scFvs in combination with knobs-into-holes technology for the Fc part.

[00300] In certain aspects, the CH3 domains of the heavy chains of a multispecific binding protein can be altered by the “knob-into-holes” technology, which is described in detail with several examples in e.g., WO 96/027011, WO 98/050431, Ridgway J. B. et al., Protein Eng. 9 (1996) 617-621, Merchant A. M. et al., Nat Biotechnol 16 (1998) 677-681. In this method, the interaction surfaces of the two CH3 domains are altered to increase the heterodimerization of both heavy chains containing said two CH3 domains. Each of the two CH3 domains (of the two heavy chains) can be the “knob,” while the other is the “hole.” The introduction of a disulfide bridge can be utilized to stabilize the heterodimers (Merchant A. M et al., Nature Biotech 16 (1998) 677-681, Atwell, S. et al., J. Mol. Biol. 270 (1997) 26-35), as well as to increase the yield.

[00301] The Fc domain of a multispecific binding protein can be engineered to promote heterodimerization over homodimerization. For example, the heavy chain constant region of the first heavy-light chain pair can comprise a different amino acid sequence from the heavy chain constant region of the second heavy-light chain pair, wherein the different amino acid sequences are engineered to promote heterodimerization of the heavy chain constant regions. Examples include knobs-into-holes mutations or charge pair mutations. Alternatively, the heavy chain constant region of the first heavy-light chain pair may be identical to the heavy chain constant region of the second heavy-light chain pair, in which case it is expected that both homodimers and heterodimers will assemble, and these will be subsequently separated using one or more purification steps in the antibody manufacturing process to isolate the desired heterodimer comprising one anti-CD25 arm and one anti-target protein arm.

[00302] In certain aspects, a multispecific binding protein of the disclosure comprises a first and a second IgG Fc domain polypeptide that dimerize to form the bispecific binding protein. In certain aspects, the first and second IgG Fc domain polypeptides dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof. In certain aspects, the first IgG Fc domain polypeptide comprises a knob substitution, and the second IgG Fc domain polypeptide comprises a hole substitution. In certain aspects, a multispecific binding protein comprises a knob substitution that is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W). In certain aspects, the multispecific binding protein comprises a hole substitution that is selected from the group consisting of alanine (A), asparagine (N), aspartic acid (D), glycine (G), serine (S), threonine (T), and valine (V).

CD25 Binding Moieties

[00303] A monospecific and a multispecific binding protein of the disclosure can comprise a binding moiety which binds to CD25. In the monospecific and/or the multispecific binding proteins of the disclosure as described herein the CD25 binding moiety binds to a CD25 extracellular domain on a CD25 positive cell.

[00304] The CD25 subunit of the IL-2 receptor is also known as the alpha subunit (i.e., IL-2R α) and is found on T cells (both activated and regulatory T cells), activated B cells, some thymocytes, myeloid precursors and oligodendrocytes. CD25 associates with CD122 and CD132 (i.e., the IL-2R $\beta\gamma$ subunits) to form a heterotrimeric complex that acts as the high-affinity receptor for IL-2. The natural ligand for the IL-2 receptor is soluble IL-2. Soluble IL-2 (also known as the T cell growth factor) is a cytokine with pleiotropic effects on immune system.

[00305] CD25 is constitutively highly expressed on T regulatory cells. For this reason, IL-2 is essential for T regulatory cell survival and expansion, and mice deficient in IL-2 or CD25 develop T cell-mediated autoimmunity.

[00306] In certain aspects, CD25 is an endogenous cell membrane-bound surface receptor expressed on an immune cell, a white blood cell, or a hematopoietic cell. In certain aspects, CD25 is a cell membrane-bound surface receptor expressed on a neoplastic cell. In certain aspects, CD25 is an endogenous cell membrane-bound surface receptor expressed on a T-cell (e.g., a regulatory T cell) or a NK cell or a B cell.

[00307] In some aspects, a multispecific binding protein comprising a first cell surface binding moiety binds to CD25 on the surface of the CD25 positive cell, binds an extracellular domain of CD25. In some aspects, a first cell surface binding moiety does not inhibit the binding of an Interleukin (IL-2) to CD25. In some aspects, a first cell surface binding moiety does not inhibit the signaling of IL-2 *via* CD25. In some aspects, a first cell surface binding moiety comprises a CD25 specific variable domain. In some aspects, a first cell binding moiety comprises a CD25-fragment crystallizable (Fc) fusion polypeptide or variant thereof. In some aspects, a first cell binding moiety that specifically binds to CD25 is a CD25 antibody or an antigen binding fragment thereof. In some aspects, a CD25 specific variable domain is operatively linked to a first Fc domain polypeptide. In some aspects, a first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.

[00308] In certain aspects, a multispecific binding protein of the disclosure comprises a CD25 binding moiety, such as an antibody or an antigen binding fragment thereof that specifically binds a CD25 on CD25 positive cells to facilitate lysosomal targeting. In certain aspects, a CD25 antibody or an antigen binding

fragment thereof binds an extracellular domain of CD25. In certain aspects, a CD25 antibody or an antigen binding fragment thereof does not inhibit the binding of an Interleukin (IL-2) to CD25. In certain aspects, a CD25 antibody or an antigen binding fragment thereof does not inhibit the signaling of IL-2 *via* CD25. In certain aspects, a CD25 antibody or an antigen binding fragment thereof comprises a CD25-fragment crystallizable (Fc) fusion polypeptide or variant thereof. In certain aspects, a CD25 antibody or an antigen binding fragment thereof comprises a variable domain that specifically binds to CD25. In certain aspects, a CD25 antibody or an antigen binding fragment thereof comprising a variable domain is operatively linked to a first Fc domain polypeptide. In certain aspects, a first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.

[00309] Exemplary CD25 binding moieties can be derived from CD25 antibodies that are obtained by immunizing mice with native CD25 or a full length recombinant CD25 peptide. Alternatively, CD25 or a fragment thereof can be produced using biochemical techniques and modified and used as immunogen. In certain aspects, an immunogen can be a peptide from the N terminal or C terminal end of CD25. In certain aspects, an immunogen can be a recombinant CD25 peptide expressed in a prokaryote, such as *E. coli*, or in eukaryotic cells or mammalian cells such as Chinese hamster ovary (CHO) cells. In certain aspects, an extracellular domain of human CD25 is used to raise the anti-CD25 antibody. In certain aspects, the CD25 antibody can be obtained by immunizing a transgenic mouse that expresses the human immune repertoire (*e.g.*, the VELOCIMMUNE® mouse from Regeneron). The VELOCIMMUNE® mouse comprises a genome comprising human heavy and light chain variable regions operably linked to endogenous mouse constant region loci such that the mouse produces antibodies comprising a human variable region and a mouse constant region in response to antigenic stimulation. The DNA encoding the variable regions of the heavy and light chains the CD25 antibody can be isolated and incorporated into the multispecific binding proteins of the disclosure.

[00310] In certain aspects, a CD25 binding moiety comprises the variable domains of a CD25 antibody known in the art. For example, the multispecific binding protein of the disclosure can comprise a CD25 binding moiety comprising the amino acid sequences of a known, commercially available anti-CD25 binding protein. In

other aspects, the CD25 binding moiety is the bioequivalent of a known binding protein. A bioequivalent CD25 binding protein can comprise amino acid sequences that vary from those of known CD25 binding proteins, but that retain the ability to bind CD25. Such variant CD25 binding proteins comprise one or more additions, deletions, or substitutions of amino acids when compared to a parent sequence but exhibit biological activity that is essentially equivalent to that of the known CD25 binding protein. Two CD25 binding proteins are considered bioequivalent if, for example, they are pharmaceutical equivalents or pharmaceutical alternatives whose rate and extent of absorption do not show a significant difference when administered at the same molar dose under similar experimental conditions, either as a single dose or multiple doses. Some CD25 binding proteins will be considered equivalents or pharmaceutical alternatives if they are equivalent in the extent of their absorption but not in their rate of absorption and yet can be considered bioequivalent because such differences in the rate of absorption are intentional and are reflected in the labeling, are not essential to the attainment of effective body drug concentrations on, e.g., chronic use, and are considered medically insignificant for the particular drug product studied. Bioequivalent variants of known CD25 antibodies can be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues not essential for biological activity can be deleted or replaced with other amino acids to prevent formation of unnecessary or incorrect intramolecular disulfide bridges upon renaturation. In other contexts, bioequivalent CD25 binding moieties can include variants comprising amino acid changes, which modify the glycosylation characteristics of known CD25 binding proteins e.g., mutations that eliminate or remove glycosylation.

[00311] Specific examples of CD25 binding proteins are CD25 ISVDs, of which an example is given in Table 12.

Table 12: Sequences for CDRs according to AbM numbering and frameworks for the CD25 ISVD (“ID” refers to the given SEQ ID NO)

I	FR1	I	CDR1	I	FR2	I	CDR 2	I	FR3	I	CDR 3	I	FR4
D		D		D		D		D		D		D	

1	EVQLVESGGG	1	RNIF	1	WYRQA	1	SITG	1	YADSVKGRFTISRDN	1	YRN	1	WGQ
1	LVQAGGSLRL	1	SSNA	1	PGKQR	1	GGG	1	AKNTVYLMNSLKPE	1	VIPG	1	GTLV
2	TCAAS	3	MG	4	ELVA	5	TN	6	DTATYYCNI	7	RLS	8	TVSS

[00312] In certain aspects, the CD25 binding moiety comprises at least one CD25 ISVD, that consists essentially of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

CDR1 (AbM numbering) has an amino acid sequence selected from:

- a) the amino acid sequence of SEQ ID NO: 113;
- b) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 113; or
- c) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 113;

and

CDR2 (AbM numbering) has an amino acid sequence selected from:

- d) the amino acid sequence of SEQ ID NO: 115;
- e) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 115; or
- f) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 115;

and

CDR3 (AbM numbering) has an amino acid sequence selected from:

- g) the amino acid sequence of SEQ ID NO: 117;
- h) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 117; or
- i) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 117.

[00313] In certain aspects, the CD25 binding moiety comprises at least one CD25 ISVD, that consists essentially of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 113;
 - CDR2 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 115;
- and
- CDR3 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 117.

[00314] In certain aspects, the CD25 binding moiety either in a monospecific or multispecific binding protein described herein comprises at least one CD25 ISVD comprising at least 80%, 85%, 90%, or 95% identity to an amino acid sequence set forth in SEQ ID NO: 72.

[00315] In certain aspects, the CD25 binding moiety either in a monospecific or multispecific binding protein described herein is antagonistic of CD25 activity. In certain aspects, the CD25 binding moiety either in a monospecific or multispecific binding protein described herein removes the CD25 from the cell surface but does not result in CD25 degradation.

[00316] In certain aspects, the CD25 binding moiety is a competitive inhibitor of soluble human IL-2 binding to CD25. In certain aspects, the CD25 binding moiety blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay.

[00317] In general, monospecific or multispecific binding proteins as described herein can function by binding to CD25 and a target protein with high affinity or avidity. In certain aspects, the multispecific binding protein can bind CD25 and/or a target protein with a K_D of less than about 1 μ M as measured by surface plasmon resonance (e.g., at 25° C or at 37° C). In certain aspects, the multispecific binding proteins bind CD25 and/or the target protein with a K_D of less than about 40 nM, less than about 30 nM, less than about 20 nM, less than about 10 nM, less than about 5 nM, less than about 2 nM or less than about 1 nM, as measured by surface plasmon resonance.

[00318] In certain aspects, monospecific or multispecific binding proteins described herein bind CD25 with a dissociative half-life ($t_{1/2}$) of greater than about 1.1

minutes as measured by surface plasmon resonance at, e.g., about 25° C or 37° C. In certain aspects, the multispecific binding proteins bind CD25 and the soluble target protein with a $t_{1/2}$ of greater than about 5 minutes, greater than about 10 minutes, greater than about 30 minutes, greater than about 50 minutes, greater than about 60 minutes, greater than about 70 minutes, greater than about 80 minutes, greater than about 90 minutes, greater than about 100 minutes, greater than about 200 minutes, greater than about 300 minutes, greater than about 400 minutes, greater than about 500 minutes, greater than about 600 minutes, greater than about 700 minutes, greater than about 800 minutes, greater than about 900 minutes, greater than about 1000 minutes, or greater than about 1200 minutes, as measured by surface plasmon resonance at 25° C or 37° C.

[00319] In certain aspects, the monospecific or the multispecific binding proteins described herein comprise a modified binding moiety to alter binding affinity compared to a reference binding proteins comprising a wild-type binding moiety. In certain aspects, a modified binding moiety has an enhanced binding affinity compared to a reference binding proteins comprising a wild-type binding moiety.

[00320] In certain aspects, a binding moiety is modified to enhance the binding affinity at an acidic pH compared to a monospecific or a multispecific binding proteins comprising a wild-type binding moiety. In certain aspects, a binding moiety is modified to enhance the binding affinity at a basic pH compared to a monospecific or a multispecific binding proteins comprising a wild-type binding moiety. In certain aspects, a modified binding moiety has a decreased binding affinity compared to a monospecific or a multispecific binding proteins comprising a wild-type binding moiety. In certain aspects, a binding moiety is modified to decrease the binding affinity at an acidic pH compared to a monospecific or a multispecific binding proteins comprising a wild-type binding moiety. In certain aspects, a binding moiety is modified to decrease the binding affinity at a basic pH compared to a monospecific or a multispecific binding proteins comprising a wild-type binding moiety.

[00321] In certain aspects, the binding moiety of the monospecific or the multispecific binding protein binds to the CD25 on the surface of the CD25 positive cell with an affinity from about 100 pM to about 1 μ M (e.g., about 100 pM to about 1,000 pM, about 1,000 pM to about 0.01 μ M, about 0.01 μ M to about 0.1 μ M, or about 0.1 μ M to about 1.0 μ M). In certain aspects, the second binding moiety of the

multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M (e.g., about 100 pM to about 1,000 pM, about 1,000 pM to about 0.01 μ M, about 0.01 μ M to about 0.1 μ M, or about 0.1 μ M to about 1.0 μ M).

[00322] In certain aspects, a monospecific and/or multispecific binding protein of the disclosure binds to CD25 on a CD25 positive cell with an affinity from about 100 pM to about 1 μ M.

[00323] In certain aspects, a monospecific and/or a multispecific binding protein of the disclosure specifically binds to hCD25 with: (a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 2×10^{-8} , 1.7×10^{-8} , 1.5×10^{-8} , 1×10^{-8} , 5×10^{-9} , 1×10^{-9} or lower; (b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 3.5×10^{-3} , 3.4×10^{-3} , 1×10^{-3} , 5×10^{-3} ; or (c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.5×10^5 , 2.0×10^5 , 5×10^5 , or 10^6 . In certain aspects, a monospecific and/or a multispecific binding protein of the disclosure specifically binds to cynoCD25 with: (a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 5×10^{-8} , 3.5×10^{-8} , 10^{-8} , 5×10^{-9} , 10^{-9} or lower; (b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 4×10^{-3} , 10^{-3} , 5×10^{-4} , 10^{-4} ; or (c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.0×10^5 , 1.5×10^5 , 2.0×10^5 , or 10^6 . Affinities, on rates (K_a) and off rates (K_d) can be measured, e.g., by surface plasmon resonance. In certain aspects, the binding protein has an EC_{50} value for binding to human or cyno CD25 on HEK293-MZA cells of less than 10^{-8} M, such as less than $5 \cdot 10^{-9}$ M, such as between $5 \cdot 10^{-9}$ M and 2×10^{-9} M, as measured by a FACS binding assay. In certain aspects, the binding protein has an IC_{50} value in competition with IL-2 for binding to human CD25 on HEK296-MZA cells of less than 10^{-8} M, such as between 10^{-8} M and 10^{-9} M, and in competition with IL-2 for binding to cyno CD25 on HEK296-MZA cells of less than 10^{-7} M, such as between 10^{-7} M and 10^{-8} M, all as measured in a FACS competition assay.

pH Sensitive CD25 Binding

[00324] In certain aspects, a monospecific and/or multispecific binding proteins of the disclosure comprise a CD25 binding moiety which exhibits pH-sensitive binding to CD25. In certain aspects, the pH-sensitive binding moiety facilitates dissociation from CD25 within the lysosomal compartment, allowing CD25

and/or the binding protein to recycle back to the cell surface where it can bind additional target protein for degradation.

[00325] In certain aspects, methods for degrading a target protein are provided. The methods can comprise contacting a CD25 positive cell with a multispecific binding protein, wherein the multispecific binding protein comprises: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of the CD25 positive cell wherein the first cell surface binding moiety comprises at least one immunoglobulin domain or an antigen binding fragment thereof; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to the target protein, wherein specific binding of the multispecific binding protein to the CD25 positive cell facilitates the internalization of the target protein into the CD25 positive cell bound to the multispecific protein. In certain aspects, a target protein bound to a multispecific binding protein traffics to lysosomes for degradation of the target protein.

[00326] In certain aspects, a CD25 monospecific and/or a multispecific binding protein is recycled back to the surface of the cell.

[00327] After dissociation, in a pH-dependent manner the a monospecific and/or multispecific binding protein (e.g., a CD25 antibody or an antigen binding fragment thereof (e.g., a CD25 ISVD)) can be recycled back to the cell surface membrane whereby the multispecific binding protein (e.g., a CD25 antibody or antigen binding fragment thereof (e.g., a CD25 ISVD)) is released back into the circulation. In one aspect, a CD25 monospecific and/or a multispecific binding protein is recycled back to the surface of the cell and released into the circulation where it can bind additional CD25 and/or target protein.

[00328] Concomitantly, during dissociation, CD25 can be recycled back to the cell surface membrane of the CD25 positive cell. In one aspect, a CD25 is recycled back to the surface of the cell. In another aspect, a CD25 is recycled back to the surface of the cell where it can bind additional CD25 and/or target protein.

[00329] The CD25 binding moiety can comprise a Fab domain comprising one or more mutations which enhance or diminish binding to CD25 under different pH conditions e.g., at acidic pH as compared to neutral pH. For example, the CD25 binding portion of the multispecific antibody can comprise a mutation in the CH1, CL,

VH, or the VL region of the Fab domain, wherein the mutation(s) increases the affinity of the Fab domain to its antigen in an acidic environment (e.g., in tumor microenvironment where pH is about 7.2, 7.0, 6.8, 6.5, 6.3 or lower. Such mutations can result in an increase in serum half-life of the monospecific and/or multispecific binding protein when administered to a subject.

In certain aspects, the sensitivity of CD25 binding at acidic pH may be increased, whereby the CD25 binding moiety demonstrates reduced binding to CD25 at lower pH. In certain aspects, binding to CD25 is reduced at a pH that reflects the endosomal compartment. In certain aspects, binding to CD25 is reduced at pH 5.5 relative to binding at neutral pH (pH 7.0). Such reduced binding at pH 5.5 may be 50% or more of the CD25 binding observed at neutral pH. A change, such as a reduction or increase, in an anti-CD25 activity described herein, such as binding, may be in comparison to a reference or wild-type antibody. The reference antibody may be a CD25 antibody or a CD25 conjugate known in the art. The change may also be relative between two different pH levels of a particular antibody composition described herein. pH sensitive anti-CD25 antibodies may be identified by testing the interaction between plate coated CD25 and soluble CD25 antibodies over a pH range of 4.5 to 7.0, and selecting antibodies with increased pH sensitivity such that reduced binding is observed at acidic pH. Exemplary aspects of an anti-CD25 antibody with reduced binding to CD25 at acidic pH comprises replacing tyrosine with histidine within or near one or more CDR1-3 regions of at least one of a light chain and heavy chain variable region of the antibody. See WO 2020/214748 A1, incorporated by reference in its entirety. In certain aspects, a CD25 monospecific and/or a multispecific binding protein of the disclosure exhibits pH-dependent binding to CD25 on the CD25 positive cell.

Target Proteins

[00330] A multispecific binding protein of the disclosure comprise a binding moiety which binds a CD25 and a target protein of interest. As the skilled artisan will appreciate, when paired with the first CD25 binding moiety to form a multispecific binding protein of the disclosure, the second binding moiety of the multispecific binding protein can facilitate the internalization and lysosomal degradation of a target protein of interest to which it binds. By specifically binding to a target protein, the

multispecific binding protein enables its internalization by the CD25 positive cell. The internalized target protein is then transported to the lysosomal compartment, where it undergoes degradation. This mechanism provides a means to target and degrade various target proteins or target proteins.

[00331] In certain aspects, a multispecific binding protein of the disclosure comprises a second binding moiety that specifically binds a target protein comprising a target specific variable domain. In certain aspects, a second binding moiety comprises at least one antigen binding fragment. In certain aspects, a multispecific binding protein of the disclosure comprises a second binding moiety that specifically binds a target protein comprising a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®. In certain aspects, the target antigen binding fragment comprises at least one ISVD that specifically binds the target protein (target protein ISVD). In certain aspects, a second binding moiety comprises multivalent target protein ISVDs (i.e., at least two ISVDs that specifically bind the same target protein). In certain aspects, the multivalent target protein ISVDs bind to the same or different contacts on the same target protein.

[00332] As used herein a "target protein" can be a protein having a deleterious function and for which degradation can be therapeutically advantageous. In certain aspects, a target protein is a membrane-associated target protein, a soluble target protein, or both. In certain aspects, a target protein is a pathogenic protein or a peptide which causes a disease or symptom of disease. In certain aspects, a target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein. In certain aspects, a target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.

[00333] In certain aspects, a target protein is a soluble protein. In certain aspects, a target protein is a membrane target protein. In certain aspects, a target protein is expressed on the surface of the same or a different a CD25 positive cell. In other aspects, the target protein is expressed on the surface of a non- CD25 positive cell (e.g., an antigen presenting cell).

[00334] In certain aspects, a membrane-associated target protein is expressed on the surface of a neoplastic cell and/or an immune cell. In certain aspects, a membrane-associated target protein is expressed on a T-cell. In certain aspects, the T-cell is an activated T cell or a regulatory T (Treg) cell.

[00335] In other aspects, a target protein is a soluble protein. Exemplary target proteins or peptides include proteins or peptides secreted by tumors, inflammatory protein or peptides; signaling molecules including cytokines, interleukins, interferons, tumor necrosis factors, growth factors, hormones, neurotransmitters, lipid mediators, activating factors, extracellular matrix (ECM) proteins, Wnt proteins, members of the Transforming Growth Factor-beta (TGF- β) family, Notch, and Fc receptor-like protein 3 (FcRL3) ligands. In certain aspects, the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein.

[00336] In certain aspects, a target protein is an antigen. Antigens are molecules that can elicit an immune response. In certain aspects, an antigen is an autoantigen or self-antigen produced in the cell of a subject. For example, an antigen could be a surface marker expressed on specific cell types, allowing a multispecific binding protein of the disclosure to selectively target and modulate those cells. By engaging a CD25 positive cell via a CD25 binding portion, antigen-targeting multispecific binding proteins of the disclosure can enhance immune responses, facilitate cell-mediated cytotoxicity, or regulate immune cell functions in immunotherapy.

[00337] In other aspects, a target protein is an antibody (e.g., an autoantibody) or fragment thereof. An autoantibody is an antibody that specifically binds to one or more antigens made or formed by a subject's own body. Autoantibodies mistakenly recognize and target self-antigens, leading to autoimmune diseases. By binding autoantibodies as the second binding moiety, a multispecific binding protein, or a binding fragment thereof can specifically bind to the self-antigens associated with autoimmune disorders. This approach offers the

potential for targeted therapy by redirecting the immune response towards the autoreactive cells or molecules involved in the autoimmune process.

[00338] By targeting specific proteins, a multispecific binding protein of the disclosure can, *e.g.*, interfere with protein-protein interactions, disrupt signaling pathways, or block protein-mediated cellular functions. Membrane proteins are a class of proteins located within or associated with cellular membranes, playing, *e.g.*, roles in cell signaling, transport of molecules across membranes, and maintaining the structural integrity of the cell. Membrane proteins can be attached to a cell membrane. Membrane proteins can be buried in a cell membrane or can be anchored on to a cell membrane. In certain aspects, a target protein is membrane protein.

[00339] In certain aspects, the target protein is a soluble protein. Soluble proteins are a class of proteins that readily dissolve in aqueous environments or in extracellular fluid, maintaining stability and performing diverse functions within both cellular and tissue contexts. Soluble proteins can be proteins that are not membrane bound. In certain aspects, a target protein is a soluble protein.

[00340] In certain aspects, a target protein is associated with a disease or disorder where aberrant protein signaling is involved, such as certain cancers or metabolic disorders. A multispecific binding protein of the disclosure may be designed to target proteins with high affinity and selectivity enabling precise modulation of the aberrant signaling pathway.

[00341] In certain aspects, a target protein is a pathogenic protein. Pathogenic proteins are those that are associated with disease development or progression. By facilitating degradation of target pathogenic protein a multispecific binding protein of the disclosure can neutralize their activity, inhibit their binding to receptors or other molecules, or facilitate their clearance from the body. This approach is relevant in the field of, *e.g.*, infectious diseases, or chronic infectious diseases such as such as Herpes viral infection (HSV, CMV, EBV), HIV-1, and HBV infections. In some aspects, multispecific binding proteins may be used to treat chronic viral infection. In certain aspects, multispecific binding proteins of the current disclosure can be designed to target viral or bacterial proteins involved in pathogenesis. By blocking or neutralizing pathogenic proteins, a multispecific binding

protein, can help control the spread of the disease and limit its impact on the host. In certain aspects, a multispecific binding protein can comprise the variable domains of, be co-administered with, or fused to an agent targeting an infectious disease target of interest. In certain aspects, the agent can be Palivizumab (e.g., to target the fusion (F) glycoprotein).

[00342] In certain aspects, a target protein can be a protein secreted by tumors. Tumor cells can release proteins that contribute to tumor growth such as growth factors, angiogenesis, immune evasion, or metastasis. A multispecific binding protein that target tumor secreted protein can interfere with their function, inhibit tumor-promoting activities, or enhance anti-tumor immune responses. This approach offers the potential for targeted therapy against cancer by specifically neutralizing or modulating tumor-secreted proteins that play critical roles in tumorigenesis and progression. In certain aspects, a target protein secreted by tumors of the present disclosure is Vascular endothelial growth factor A (VEGFA). In certain aspects, a multispecific binding protein can comprise the variable domains of, be co-administered with or fused to an agent targeting a particular tumor. An exemplary agent can include pegaptanib, bevacizumab, ranibizumab, brolucizumab, aflibercept (e.g., to target VEGFA).

[00343] In certain aspects, a target protein can be an inflammatory protein. Inflammatory proteins are involved in the immune response and can contribute to chronic inflammation, autoimmune disorders, or tissue damage. A multispecific binding protein can be designed to target inflammatory proteins and help regulate the inflammatory cascade, suppress excessive immune responses, or modulate immune cell functions. By selectively binding and neutralizing inflammatory proteins, a multispecific binding protein of the disclosure has the potential to dampen inflammation and restore immune balance in various inflammatory conditions. Examples of inflammatory conditions include rheumatoid arthritis, dermatitis, and systemic lupus erythematosus (SLE). In certain aspects, a multispecific binding protein can comprise the variable domains of, be co-administered with, or fused to an agent targeting a proinflammatory protein of interest. An exemplary agent can include Eculizumab or Ravulizumab (e.g., to target complement component C5).

[00344] In certain aspects, a target protein can be an interleukin. In certain aspects, a multispecific binding protein can comprise the variable domain of, be co-

administered with, or fused to an agent targeting an IL of interest. Interleukins (ILs) are a specific group of signaling molecules involved in immune responses and inflammation. A multispecific binding protein of the disclosure can be engineered to target specific ILs or their receptors thereby modulating their activity and downstream signaling. This approach can be applied to various immune-related disorders, such as autoimmune diseases, allergies, or inflammatory conditions. By interfering with IL signaling, a multispecific binding protein can regulate immune cell activation, cytokine production, or immune cell trafficking, providing a potential avenue for therapeutic intervention. An exemplary agent can include Siltuximab (e.g., to target IL6), Mepolizumab or Reslizumab (e.g., to target IL5), Secukinumab or Ixekizumab (e.g., to target IL17A), Guselkumab, Tildrakizumab, or Risankizumab (e.g., to target the p19 subunit of IL23), Rilonacept (e.g., to target IL1A and IL1B), Canakinumab (e.g., to target IL1B), Ustekinumab (e.g., to target the p40 subunit of IL12 and IL23).

[00345] In certain aspects, a target protein can be a Wnt protein. Wnt proteins are a family of secreted signaling molecules that regulate cell proliferation, differentiation, and tissue development. Dysregulation of Wnt signaling is implicated in numerous diseases, including cancer, developmental disorders, and degenerative diseases. A multispecific binding protein targeting Wnt proteins can modulate their activity, block aberrant signaling pathways, or interfere with Wnt protein interactions. This approach offers potential therapeutic strategies for diseases driven by aberrant Wnt signaling. In certain aspects, a multispecific binding can comprise the variable domains, of, be co-administered with or fused to an agent targeting an Wnt protein of interest. An exemplary agent can include Vantictumab (e.g., to target FZD1/2/5/7/8).

[00346] In certain aspects, a target protein can be a cytokine. In certain aspects, the cytokine can be member of the Transforming Growth Factor-beta (TGF- β). Members of the TGF- β family are a group of multifunctional cytokines involved in various cellular processes, including cell growth, differentiation, immune regulation, and tissue repair. Dysregulation of TGF- β signaling is associated with fibrosis, cancer progression, immune disorders, and other diseases. A multispecific binding protein designed to target TGF- β family members can modulate their signaling pathways, inhibit their effects on immune cells or stromal cells, or interfere with TGF- β ligand-receptor interactions. By modulating TGF- β signaling, a multispecific binding

protein holds potential therapeutic value for a range of diseases associated with TGF- β dysregulation. In certain aspects, a TGF- β cytokine is a TGF- β 1. In other aspects, the cytokine can be a member of the insulin-like growth factors (IGF). An exemplary IGF of the present disclosure includes IGF-1 and IGF-2. Other exemplary cytokines of the current disclosure include IgE and IgA. In certain aspects, a multispecific binding protein can comprise the variable domains of, be co-administered with or fused to an agent targeting a cytokine of interest. An exemplary agent can include omalizumab (*e.g.*, to target IgE).

[00347] In certain aspects, a target protein can be a Notch ligand. Notch ligands are cell surface proteins involved in cellular communication and tissue development. Dysregulation of Notch signaling is implicated in cancer, cardiovascular diseases, and neurodegenerative disorders. A multispecific binding protein of the disclosure can disrupt Notch signaling pathways, block ligand-receptor interactions, or modulate downstream gene expression. This approach offers potential therapeutic strategies for diseases driven by aberrant Notch signaling, with the goal of restoring normal cellular processes and tissue homeostasis.

[00348] In certain aspects, a target protein can be a Fc receptor-like protein 3 (FcRL3). The FcRL3 protein is a type I transmembrane glycoprotein, having an extracellular region, a transmembrane domain and a cytoplasmic tail and contains immunoreceptor-tyrosine activation motif (ITAM) and immunoreceptor-tyrosine inhibitory motif (ITIM) in its cytoplasmic domain. FcRL3 can modulate the regulation of the immune system. Mutations in the gene encoding FcRL3 are associated with rheumatoid arthritis, autoimmune thyroid disease, and systemic lupus erythematosus

[00349] In certain aspects, a target protein is expressed on the T-cell membrane (*e.g.*, on the same CD25 positive T-cell that is expressing the CD25). In other aspects, a target protein is expressed on activated T cells and/or Treg cells. In some aspects, the target protein comprises a membrane associated protein, including immune checkpoint proteins and receptors expressed on T cell surface. In some aspects, a target protein is an immune checkpoint protein and the second binding moiety comprises an agonist or an antagonist immune checkpoint modulator (*e.g.*, an agonist or an antagonist immune checkpoint inhibitor). In some aspects, a second binding moiety comprises an agonist or an antagonistic antibody or antigen-binding fragment thereof against a receptor involved in immune modulation. In some

aspects, a second binding moiety comprises an agonist or an antagonistic ISV against a receptor involved in immune modulation.

[00350] In certain aspects, a multispecific binding protein of the disclosure exhibits pH-dependent binding to the target protein. In certain aspects, the multispecific binding protein exhibits reduced binding at acidic pH. In certain aspects, the second binding moiety of the multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M.

Cell internalization

[00351] One function of CD25 is the uptake IL-2-bearing molecules or compositions and their internalization and transport to the lysosomes. For example, CD25 can bind IL-2. CD25-mediated internalization of IL-2 requires the presence of the IL-2 receptor (i.e., requiring hetero-trimerization of CD25, CD122, and CD132 subunits) and is referred to herein as the IL-2 complex. Once IL-2 binds CD25 (in the presence of CD122 and CD132) the IL-2 complex is internalized *via* endocytosis. CD25 traffics with the IL-2 complex in early recycling compartments. Upon dissociation of the complex, CD25 is recycled back to the cell surface membrane. Cendrowski et al. (2016), Cytokine Growth Factor Rev., vol. 32: 63-73. Differently, CD122 and CD132 are found to traffic with IL-2 to the late endocytic compartments. Accordingly, along with IL-2, CD122 and CD132 are ultimately degraded in the lysosome by lysosomal degradation. Hemar *et al.* (1995), J Cell Biol, vol. 129 (1): 55-64; Su et al. (2015), Sci Transl Med., vol. 7(311): 311ra170.

[00352] Therefore, multispecific binding proteins disclosed herein, which comprise a CD25 targeting moiety, such as a CD25 antibody or an antigen binding fragment thereof (e.g., a CD25 ISVD), can deliver and release a bound target protein to the endocytic compartment whereby a CD25, a target protein, and a multispecific binding protein dissociate.

[00353] After dissociation, in a pH-dependent manner the multispecific binding protein (e.g., a CD25 antibody or an antigen binding fragment thereof (e.g., a CD25 ISVD)) can be recycled back to the cell surface membrane whereby the multispecific binding protein (e.g., a CD25 antibody or antigen binding fragment thereof (e.g., a CD25 ISVD)) is released back into the circulation. In one aspect, a multispecific

binding protein is recycled back to the surface of the cell and released into the circulation where it can bind additional target protein.

[00354] Concomitantly, during dissociation, CD25 can be recycled back to the cell surface membrane of the CD25 positive cell. In one aspect, a CD25 is recycled back to the surface of the cell. In another aspect, a CD25 is recycled back to the surface of the cell where it can bind additional multispecific binding protein bound to the target protein.

[00355] Differently, the target protein continues along the late endocytic pathway for ultimate lysosomal degradation. In this way, CD25 bound to the multispecific binding protein of the disclosure can act as a shuttling mechanism for taking target proteins to the lysosomal degradation pathway.

[00356] Accordingly, in certain aspects, a multispecific binding protein of the disclosure can be internalized by a CD25 positive cell. In certain aspects, the amount of multispecific binding protein internalized by the CD25 positive cell is greater than the amount of a reference binding protein or polypeptide lacking the targeting moiety internalized by the cell.

[00357] In certain aspects, the disclosure provides a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein, wherein specific binding of the multispecific binding protein to the CD25 positive cell facilitates internalization of the target protein bound to the multispecific binding protein.

[00358] In one aspect, the disclosure provides a multispecific binding protein comprising: a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell, wherein the first cell surface binding moiety comprises an immunoglobulin domain; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein, wherein specific binding of the multispecific binding protein to the CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.

[00359] In one aspect, the disclosure provides a multispecific binding protein comprising a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to

the N- and/or C- terminal end of a first IgG; and b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C- terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof, wherein specific binding of the multispecific binding protein to the CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.

[00360] In certain aspects, the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein. In certain aspects, the CD25 and/or the multispecific binding protein is recycled back to the surface of the cell independent of the target protein. In certain aspects, the CD25 is recycled back to the surface of the cell where it can bind additional multispecific binding protein bound to the target protein.

[00361] In certain aspects, a multispecific binding protein of the disclosure exhibits increased degradation of the target protein compared to a reference binding polypeptide. In certain aspects, the reference binding polypeptide does not comprise the first cell surface binding moiety that specifically binds to CD25 but is otherwise identical to the multispecific binding protein. In certain aspects, a multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide. In certain aspects, a multispecific binding protein degrades the target protein in at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide.

[00362] The cell internalization of a multispecific binding protein of the disclosure can deplete a target protein from the circulation and/or a specific tissue. In one aspect, the method of depleting a target protein comprises administering to a subject an effective amount of the disclosed multispecific binding protein or a pharmaceutical composition comprising the same.

[00363] In certain aspects, upon administration of a multispecific binding protein described herein the target protein is selectively depleted from a circulation or a target tissue of the subject. In some aspects, administering the multispecific binding protein results in 10%, 20, 30%, 40%, 50%, 75%, or 90% depletion of the target protein from the target tissue or circulation of the subject.

Disease Indications

[00364] The monospecific or multispecific binding proteins of the current disclosure are useful in a number of different applications. In certain aspects, a monospecific or multispecific binding protein disclosed herein is effective in reducing the concentration of or eliminating a target protein in the circulation. In certain aspects, a multispecific binding protein can reduce inflammatory symptoms. In certain aspects, a multispecific binding protein can reduce tumor size.

[00365] The monospecific CD25 binding proteins described herein can be used in methods of treating a patient with a neoplastic or autoimmune disorder or to deplete human antigen-specific CD25+ regulatory T cells in a subject. In some aspects, the disclosure provides a method of treating a neoplastic disorder in a subject comprising administering to a subject an effective amount of a binding protein comprising at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell or the pharmaceutical composition thereof. In some aspects, the disclosure provides a method of treating an autoimmune disorder comprising administering to a subject an effective amount of a binding protein comprising at least one CD25 ISVD or the pharmaceutical composition thereof. In some aspects, the disclosure provides a method of depleting human antigen-specific CD25+ regulatory T cells in a subject comprising administering to a subject an effective amount of a binding protein comprising at least one CD25 ISVD or the pharmaceutical composition thereof.

[00366] The multispecific CD25 binding proteins described herein can exploit the CD25 shuttling mechanism discovered herein and the methods enable the efficient and selective degradation of various target proteins, including pathogenic proteins, proteins secreted by tumors, autoantibodies, inflammatory proteins, interleukins, and signaling molecules. The disclosed methods can be used for the development of targeted therapies with precise control over the degradation of specific molecules for the treatment of diseases such as autoimmune disorders, cancer, and inflammatory conditions.

[00367] By facilitating the degradation of target proteins, the multispecific binding proteins of the disclosure are useful, *inter alia*, for the treatment, prevention and/or amelioration of any disease or disorder associated with or mediated by target protein expression, signaling, or activity, or treatable by CD25-mediated degradation of the target protein within the lysosome. For example, the present disclosure

provides methods for treating autoimmune disease, cancer (tumor growth inhibition), chronic viral infections, and other disease by administering the multispecific binding proteins described herein to a patient in need of such treatment.

[00368] The multispecific binding proteins of the present disclosure are useful for the treatment, prevention, and/or amelioration of disease or disorder or condition such as autoimmune disease, a viral infection, or cancer and/or for ameliorating at least one symptom associated with such disease, disorder or condition. In the context of the methods of treatment described herein, the multispecific binding proteins can be administered as a monotherapy (i.e., as the only therapeutic agent) or in combination with one or more additional therapeutic agents (examples of which are described elsewhere herein).

[00369] In certain aspects, a multispecific binding protein described herein is useful for treating subjects suffering from a disease selected from a group consisting of: cancer, autoimmune disease, inflammatory disorder, infectious disease, and neurodegenerative disorder.

Additional Terms

Native Fc

[00370] The term “native Fc” as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain a hinge region. The original immunoglobulin source of the native Fc can be of human origin and can be any of the immunoglobulins, such as IgG1 or IgG2. Native Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, and IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, and IgA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term “native Fc” as used herein is generic to the monomeric, dimeric, and multimeric forms.

Kabat and EU numbering

[00371] Amino acid positions in a heavy chain constant region, including amino acid positions in the CH1, hinge, CH2, CH3, and CL domains, can be numbered according to the Kabat index numbering system (see Kabat et al., in "Sequences of Proteins of Immunological Interest", U.S. Dept. Health and Human Services, 5th edition, 1991). Alternatively, antibody amino acid positions can be numbered according to the EU index numbering system (see Kabat et al.).

Native residue

[00372] As used herein, the term "native residue" refers to an amino acid residue that occurs naturally at a particular amino acid position of a polypeptide and which has not been modified, introduced, or altered by the hand of man. As used herein, the term "altered protein," "altered polypeptide," "modified protein" or "modified polypeptide" shall refer to polypeptides and/or proteins comprising at least one amino acid substitution, deletion and/or addition relative to the native (i.e., wild-type) amino acid sequence, and/or a mutation that results in altered glycosylation (e.g., hyperglycosylation, hypoglycosylation and/or aglycosylation) at one or more amino acid positions relative to the native (i.e., wild-type) amino acid sequence.

Specifically binds

[00373] A polypeptide (e.g., an immunoglobulin, an antibody, an ISVD, a VHH, or generally an antigen binding molecule or an antigen fragment thereof) that can "bind to" or "specifically bind to" that "has affinity for" and/or that "has specificity for" a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "against" or "directed against" said epitope, antigen or protein or is a "binding" molecule with respect to such epitope, antigen or protein, or is said to be "anti"-epitope, "anti"-antigen or "anti"-protein (e.g., "anti"- CD25 and/or "anti"- a target protein e.g., TNF α).

[00374] A polypeptide that "specifically binds" e.g., can bind to an antigen with a dissociation constant (KD) of at most about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, or less, and/or to bind to an antigen with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen. Specific binding of an antibody can be to a target antigen through the CDR sequences. An antibody can also specifically bind to FcRs, such as FcRn or Fc γ RIIIa through the Fc region.

[00375] The terms “specificity”, “binding specifically” or “specific binding” refer to the number of different target molecules, such as antigens, from the same organism to which a particular binding unit, such as an ISVD, can bind with sufficiently high affinity (e.g., an ISVD that specifically binds to CD25 or to a target protein. “Specificity”, “binding specifically” or “specific binding” are used interchangeably herein with “selectivity”, “binding selectively” or “selective binding”.

[00376] Binding units, such as ISVDs, specifically bind to their designated targets. The specificity/selectivity of a binding unit can be determined based on affinity. The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given by the KD, or dissociation constant, which has units of mol/liter (or M). The affinity can also be expressed as an association constant, KA, which equals 1/KD and has units of (mol/liter)⁻¹ (or M⁻¹).

[00377] In some aspects, a monospecific or a multispecific binding protein of the disclosure comprises at least two CD25 ISVDs that specifically bind the same or different CD25 protein(s). In some aspects, the at least two CD25 ISVDs specifically bind the same CD25 protein. In some aspects, the at least two CD25 ISVDs bind to the same or different contacts on the same CD25 protein.

[00378] In some aspects, a multispecific binding protein of the disclosure comprises at least two ISVDs that specifically bind the same or different target proteins(s). In some aspects, the at least two ISVDs bind to the same or different contacts on the same target protein (e.g., at least two ISVDs bind to the same contacts on a TNF α).

Affinity

[00379] Affinity is a measure for the binding strength between a binding moiety (e.g., a first or a second binding moiety as described herein) and a binding site on the target molecule (e.g., CD25 or a target protein). The lower the value of the KD, the stronger the binding strength between a target molecule and a targeting moiety. Typically, binding units used in the present technology, such as ISVDs, will bind to their targets with a dissociation constant (KD) of 10⁻⁵ to 10⁻¹² moles/liter or less, 10⁻⁷ to 10⁻¹² moles/liter or less, or 10⁻⁸ to 10⁻¹² moles/liter (i.e., with an association constant (KA) of 10⁵ to 10¹² liter/moles or more, 10⁷ to 10¹² liter/moles or more, or 10⁸ to 10¹² liter/moles). Any KD value greater than 10⁻⁴ mol/liter (or any KA value lower than 10⁴ liters/mol) is generally considered to indicate non-specific binding.

The K_D for biological interactions, such as the binding of immunoglobulin sequences to an antigen, which are considered specific are typically in the range of 10^{-5} moles/liter (10000 nM or 10 μ M) to 10^{-12} moles/liter (0.001 nM or 1 pM) or less.

[00380] Accordingly, specific and/or selective binding may mean that using the same measurement method, e.g., SPR- a binding unit (or polypeptide comprising the same) binds to CD25 with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and binds to related CD25 members with a K_D value greater than 10^{-4} moles/liter.

[00381] Specific binding to a certain target from a certain species does not exclude that the binding unit can also specifically bind to the analogous target from a different species. For example, specific binding to human CD25 does not exclude that the binding unit or a polypeptide comprising the same can also specifically bind to CD25 from cynomolgus monkeys ("cyno").

[00382] In some aspects, the monospecific or multispecific binding protein of the disclosure comprises least one CD25 ISVD that specifically binds human CD25. In some aspects, the multispecific binding protein of the disclosure comprises at least one target protein ISVD that specifically binds human target protein (e.g., human TNF α).

[00383] Specific binding of a binding unit (or a binding subunit) to its designated target can be determined in any suitable manner, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned further herein.

Dissociation constant

[00384] The dissociation constant (K_D) of a binding protein can be determined, for example, by surface plasmon resonance. Generally, surface plasmon resonance analysis measures real-time binding interactions between ligand (a target antigen on a biosensor matrix) and analyte (a binding protein in solution) by surface plasmon resonance (SPR) using the Biacore system (Cytiva Life Sciences, Marlborough, MA) or Carterra LSA platform (Carterra, Salt Lake City, UT). Surface plasmon analysis can also be performed by immobilizing the analyte (binding protein on a biosensor matrix) and presenting the ligand (target antigen). The term " K_D " as used herein

refers to the dissociation constant of the interaction between a particular binding protein and a target antigen.

Valency

[00385] As used herein the term “valency” refers to the number of potential target binding sites in either in a monospecific or multispecific binding protein. Each target binding site specifically binds one target molecule or specific site on a target molecule. When a polypeptide comprises more than one target binding site, each target binding site can specifically bind the same or different molecules (*e.g.*, can bind to more than one target protein, or different epitopes on the same target protein). A subject multispecific binding protein has at least one binding site (*e.g.*, 1, 2, 3, or more) specific for CD25. A subject a multispecific binding protein has at least one binding site (*e.g.*, 1, 2, 3, 4, or more) for a target protein.

[00386] A “multivalent” binding site refers to a binding site that has two or more binding sites that bind the same antigen or target protein. For example, a multivalent CD25 ISVD refers to two or more ISVD(s) that each specifically bind the same or different contacts of the a CD25. For example, a multivalent target protein ISVD that each specifically bind the same or different contacts of a target protein.

Specificity

[00387] The term “specificity” refers to the ability to specifically bind (*e.g.*, immunoreact with) a given target protein or antigen (*e.g.*, CD25). A subject multispecific binding protein contains two or more binding sites (*e.g.*, 2, 3, 4, 5, or more) which specifically bind the same or different binding sites. In certain aspects, a subject multispecific binding protein is specific for two different (*e.g.*, non-overlapping) target binding sites.

[00388] In certain aspects, the monospecific and/or multispecific binding proteins of the disclosure employ CD25 binding moieties which bind to human CD25 but not to CD25 from other species. Alternatively, the multispecific binding protein employ a CD25 binding moiety which binds to human CD25 and to CD25 from one or more non-human species (*e.g.*, cynomolgus CD25). For example, the monospecific and/or multispecific binding protein can bind to human CD25 and can bind or not bind, as the case can be, to one or more of mouse, rat, guinea pig, hamster, gerbil, pig, cat, dog, rabbit, goat, sheep, cow, horse, camel, cynomolgus (cyno), marmoset, rhesus or chimpanzee CD25. In certain aspects, the monospecific

and/or multispecific binding protein can bind to human CD25, but do not bind to rat and mouse CD25. In other aspects, the monospecific and/or multispecific binding proteins of the disclosure bind to human CD25, and to rat and mouse CD25, with similar binding affinities. In other aspects, the monospecific and/or multispecific binding proteins of the disclosure bind to human CD25 and to cyno CD25, with similar binding affinities but not to CD25 from other species.

Competition, Binding, Blocking

[00389] The following generally describes a suitable FACS assay for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent blocks or is capable of blocking. It will be appreciated that the assay can be used with any of the immunoglobulin single variable domains and polypeptides described herein. The FACS instrument (e.g., FACS Canto; Becton Dickinson) is operated in line with the manufacturer's recommendations.

[00390] To evaluate the "blocking" or "competition" between two binding agents (such as an ISVD and a natural ligand or another binding agent) for binding CD25, a FACS competition experiment can be performed using cells (such as e.g. Flp-In™-293 cells) overexpressing human or cyno CD25 and the parental cells as background cell line. Different detection reagents can be used including e.g. monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, cat# F1804), monoclonal anti-C-myc antibody (Sigma-Aldrich, cat# WH0004609M2), monoclonal ANTI-HIS TAG antibody (Sigma-Aldrich, cat# SAB1305538), each labeled differently. A wide range of fluorophores can be used as labels in flow cytometry (such as e.g. PE (R-Phycoerythrin), 7-aminoactinomycin D (7-AAD), Acridine Orange, various forms of Alexa Fluor, Allophycocyanin (APC), AmCyan, Aminocoumarin, APC Cy5, APC Cy7, APC-H7, APC/Alexa Fluor 750, AsRed2, Azami-Green, Azurite, B ODIPY FL C5-ceramide, BCECF-AM, Bis-oxonol DiBAC2(3), BODIPY-FL, Calcein, Calcein AM, Caroxy-H2DCFDA, Cascade Blue, Cascade Yellow, Cell Tracker Green, Cerulean, CFSE, Chromomycin A3, CM-H2DCFDA, Cy2, Cy3, Cy3.5, Cy3B, Cy5, Cy5.5, Cy7, CyPet, DAF-FM DAF-FM diacetate, DAPI, DCFH (2'7'Dichlorodihydrofluorescein), DHR, Dihydrocalcein AM, Dihydrorhodamine, Dihydrothidium, DiLC1(5), DiOC6(3), DiOC7(3), dKeima-Red, DRAQ5, Dronpa-Green, various forms of DsRed dTomato, various forms of DyLight, E.coli BioParticles AF488, E2-Crimson, E2-Orange,

EBFP2, ECFP, various forms of eFluor, EGFP, EGFP*, Emerald, eqFP650, eqFP670, ER-Tracker Blue-White DPX, Ethidium Bromide, Express2, EYFP, Fc OxyBurst Green, Fc OxyBurst Green 123, FITC, Fluo-3, Fluo-4, Fluorescein, Fura-2, Fura-Red, GFPuv, H2DCFDA, HcRed1, Hoechst Blue (33258), Hoechst Red (33342), Hydroxycoumarin, HyPer, Indo-1, Indo-1 Blue (Low Ca²⁺), Indo-1 Violet (High Ca²⁺), iRFP, J-Red, JC-1, JC-9, Katushka (TurboFP635), Katushka2 Kusabira-Orange, LDS 751, Lissamine Rhodamine B, various forms of Live/Dead, Lucifer yellow, Lucifer Yellow CH, Lyso Tracker Blue, Lyso Tracker Green, Lyso Tracker Red, mAmertrine, Marina Blue, mBanana, mCFP, mCherry, mCitrine, Methoxycoumarin, mHoneyDew, Midoriishi-Cyan, Mithramycin, Mito Tracker Deep Red, Mito Tracker Green, Mito Tracker Orange, Mito Tracker Red, MitoFluor Green, mKate (TagFP635), mKate2, mKeima, mKeima-Red, mKO, mKOk, mNeptune, Monochlorobimane, mOrange, mOrange2, mRaspberry, mPlum, mRFP1, mStrawberry, mTangerine, mTurquoise, mTFP1, mTFP1 (Teal), NBD, OxyBurst Green H2DCFDA, OxyBurst Green H2HFF BSA, Pacific Blue, PE (R-Phycoerythrin), PE Cy5, PE Cy5.5, PE Cy7, PE Texas Red, PE-Cy5 conjugates, PE-Cy7 conjugates, PerCP (Peridinin chlorophyll protein), PerCP Cy5.5, PhiYFP, PhiYFP-m, Propidium Iodide (PI), various forms of Qdot, Red 613, RFP Tomato, Rhod-2, S65A, S65C, S65L, S65T, Singlet Oxygen Sensor Green, Sirius, SNARF, Superfolder GFP, SYTOX Blue, SYTOX Green, SYTOX Orange, T-Sapphire, TagBFP, TagCFP, TagGFP, TagRFP, TagRFP657, TagYFP, tdTomato, Texas Red, Thiazole Orange, TMRE, TMRM, Topaz, TOTO-1, TO-PRO-1, TRITC, TRITC TruRed, TurboFP602, TurboFP635, TurboGFP, TurboRFP, TurboYFP, Venus, Vybrant CycleDye Violet, Wild Type GFP, X-Rhodamin, Y66F, Y66H, Y66W, YOYO-1, YPet, ZsGreen1, ZsYellow1, Zymosan A BioParticles AF488 (see more at: <http://www.thefcn.org/flow-fluorochromes>). Fluorophores, or simply “fluors”, are typically attached to the antibody (e.g. the immunoglobulin single variable domain) that recognizes CD25 or to the antibody that is used as detection reagent. Various conjugated antibodies are available, such as (without being limiting) for example antibodies conjugated to Alexa Fluor®, DyLight®, Rhodamine, PE, FITC, and Cy3. Each fluorophore has a characteristic peak excitation and emission wavelength. The combination of labels which can be used will depend on the wavelength of the lamp(s) or laser(s) used to excite the fluorophore and on the detectors available.

[00391] To evaluate the competition between two test binding agents (termed A and B) for binding to CD25, a dilution series of cold (without any label) binding agent A is added to (e.g. 200 000) cells together with the labeled binding agent B*. The concentration of binding agent B* in the test mix should be high enough to readily saturate the binding sites on CD25 expressed on the cells. The concentration of binding agent B* that saturates the binding sites for that binding agent on CD25 expressed on the cells can be determined with a titration series of binding agent B* on the CD25 positive cells and determination of the EC50 value for binding. In order to work at saturating concentration, binding agent B* can be used at 100x the EC50 concentration.

[00392] After incubation of the cells with the mixture of binding agent A and binding agent B* and cells wash, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded. A separate solution of binding agent B* is also prepared. Binding agent B* in this solution should be in the same buffer and at the same concentration as in the test mix (with binding agent A and B*). This separate solution is also added to the cells. After incubation and cells wash, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded. A reduction of fluorescence for the cells incubated with the mixture of binding agent A and B* compared to the fluorescence for the cells incubated with the separate solution of binding agent B* indicates that binding agent A blocks binding by binding agent B* to CD25 positive cells

[00393] A cross-blocking immunoglobulin, antibody, ISVD, polypeptide or other binding agent is one which will bind to e.g., a CD25 or a target protein described herein in the above competition FACS such that during the assay and in the presence of the second binding agent the recorded fluorescence is between 80% and 0.1% (e.g. 80% to 4%) of the maximum fluorescence (measured for the separate labelled immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent), specifically between 75% and 0.1% (e.g. 75% to 4%) of the maximum fluorescence, and more specifically between 70% and 0.1% (e.g. 70% to 4%) of maximum fluorescence (as just defined above).

[00394] The competition between two test binding agents (termed A* and B*) for binding to e.g., a CD25 or a target protein described herein can also be evaluated

by adding both binding agents, each labeled with a different fluorophore, to the CD25 positive cells. After incubation and cells wash, read out can be performed on a FACS. A gate is set for each fluorophore and the total amount of channel fluorescence is recorded. Reduction and/or absence of fluorescence of one of the fluorophores indicates blocking by the binding agents for binding to CD25 expressed on the cells.

[00395] Other methods for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent directed against a target blocks, is capable of blocking, competitively binds or is competitive as defined herein are described e.g. in Xiao-Chi Jia et al. (Journal of Immunological Methods 288: 91–98, 2004), Miller et al. (Journal of Immunological Methods 365: 118–125, 2011).

About or approximately

[00396] The term “about” or “approximately” means within about 20%, such as within about 10%, within about 5%, or within about 1% or less of a given value or range.

[00397] As used herein, “administer” or “administration” refers to the act of injecting or otherwise physically delivering a substance as it exists outside the body (e.g., a monospecific and/or multispecific binding protein provided herein) into a patient, such as by, but not limited to, pulmonary (e.g., inhalation), mucosal (e.g., intranasal), intradermal, intravenous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art. When a disease, or a symptom thereof, is being managed or treated, administration of the substance typically occurs after the onset of the disease or symptoms thereof. When a disease, or symptom thereof, is being prevented, administration of the substance typically occurs before the onset of the disease or symptoms thereof and can be continued chronically to defer or reduce the appearance or magnitude of disease-associated symptoms.

Composition

[00398] As used herein, the term “composition” is intended to encompass a product containing the specified ingredients (e.g., a monospecific or a multispecific binding protein composition provided herein) in, optionally, the specified amounts, as

well as any product which results, directly or indirectly, from combination of the specified ingredients in, optionally, the specified amounts.

Effective amount

[00399] “Effective amount” means the amount of active pharmaceutical agent (e.g., a monospecific or a multispecific binding protein of the present disclosure) sufficient to effectuate a desired physiological outcome in an individual in need of the agent. The effective amount can vary among individuals depending on the health and physical condition of the individual to be treated, the taxonomic group of the individuals to be treated, the formulation of the composition, assessment of the individual's medical condition, and other relevant factors.

Subject or patient

[00400] As used herein, the terms “subject” and “patient” are used interchangeably. As used herein, a subject can be a mammal, such as a non-primate (e.g., cows, pigs, horses, cats, dogs, rats, etc.) or a primate (e.g., monkey and human). In certain aspects, the term “subject,” as used herein, refers to a vertebrate, such as a mammal. Mammals include, without limitation, humans, non-human primates, wild animals, feral animals, farm animals, sport animals, and pets.

Therapy and Pharmaceutical Compositions

[00401] As used herein, the term “therapy” refers to any protocol, method and/or agent that can be used in the prevention, management, treatment and/or amelioration of a disease or a symptom related thereto. In some aspects, the term “therapy” refers to any protocol, method and/or agent that can be used in the modulation or depletion of a target protein from the circulation of a subject. In some aspects, the terms “therapies” and “therapy” refer to a biological therapy, supportive therapy, and/or other therapies useful in the prevention, management, treatment and/or amelioration of a disease or a symptom related thereto, known to one of skill in the art such as medical personnel. In other aspects, the terms “therapies” and “therapy” refer to a biological therapy, supportive therapy, and/or other therapies useful in the modulation of an immune response to an inflammatory or autoimmune diseases in a subject or a symptom related thereto known to one of skill in the art such as medical personnel.

[00402] As used herein, the terms “treat,” “treatment” and “treating” refer to the reduction or amelioration of the progression, severity, and/or duration of a disease or a symptom related thereto, resulting from the administration of one or more therapies (including, but not limited to, the administration of one or more prophylactic or therapeutic agents, such as the administration of a monospecific or multispecific binding protein provided herein). The term “treating,” as used herein, can also refer to altering the disease course of the subject being treated. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptom(s), diminishment of direct or indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

[00403] The route of administration of a monospecific or multispecific binding protein of the current disclosure can be oral, parenteral, by inhalation, or topical, or other suitable method. The term parenteral as used herein includes intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal administration. While all these forms of administration are clearly contemplated as being within the scope of the current disclosure, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection can comprise a buffer (e.g., acetate, phosphate or citrate buffer), a surfactant (e.g., polysorbate), optionally a stabilizer agent (e.g., human albumin), etc. However, in other methods compatible with the teachings herein, the monospecific or multispecific binding protein can be delivered directly to the site of the adverse tissue thereby increasing the exposure of the diseased tissue to the therapeutic agent.

[00404] In certain aspects, a pharmaceutical composition comprises either a monospecific or a multispecific binding protein described herein and a pharmaceutically acceptable carrier or diluent. In certain aspects, a method of depleting a CD25 from the cell surface and/or a target protein comprises administering to a subject an effective amount of a monospecific and/or a multispecific binding protein of the disclosure or the pharmaceutical composition comprising said binding protein.

[00405] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil,

and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the compositions and methods of the current disclosure, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1 M, e.g., 0.05M phosphate buffer, or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives can also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will typically be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

[00406] In many cases, isotonic agents will be included, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00407] In any case, sterile injectable solutions can be prepared by incorporating an active compound (e.g., a monospecific or a multispecific binding protein by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, exemplary methods of preparation include vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Such articles of manufacture will typically have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to autoimmune or neoplastic disorders.

[00408] Effective doses of a monospecific or a multispecific binding protein composition of the present disclosure for the treatment of the conditions described above vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or another animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic mammals can also be treated. Treatment dosages can be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

[00409] Monospecific or multispecific binding proteins of the current disclosure can be administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of a target protein in the patient. Alternatively, multispecific binding protein can be administered as a sustained release formulation, in which case less frequent administration is required. For multispecific binding protein, dosage and frequency vary depending on the half-life of the multispecific binding protein in the patient.

[00410] The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, compositions containing the present monospecific binding protein, multispecific binding protein, or a cocktail thereof are administered to a patient not already in the disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactic effective dose." In this use, the precise amounts again depend upon the patient's state of health and general immunity, but generally range from about 0.1 to about 25 mg per dose, especially about 0.5 to about 2.5 mg per dose. A relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage (e.g., from about 1 to 400 mg/kg of a multispecific binding protein per dose) at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, or until the patient shows partial or complete amelioration of disease symptoms. Thereafter, the patient can be administered a prophylactic regime.

[00411] A pharmaceutical composition in accordance with the present disclosure can comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, nontoxic buffers, preservatives and the like. For the purposes of the instant application, a pharmaceutically effective amount of a monospecific or multispecific binding protein disclosed herein, shall be held to mean an amount sufficient to achieve effective binding to a target protein and to achieve a benefit, e.g., to ameliorate symptoms of a disease or disorder.

Determining Multispecific Binding Protein Binding and Specificity

[00412] The binding affinity of a first cell surface binding moiety comprising a CD25 antibody or an antigen binding fragment thereof that binds to CD25, or a second binding moiety that binds to the target protein can each be assessed using, e.g., surface plasmon resonance, ELISA, or other suitable method (see Shields *et al.* (2001) J. Biol. Chem., 276:6591-6604.)

[00413] In some aspects, the binding constant K_D of a monospecific or a multispecific binding protein for CD25 can be above that of the wild-type control by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold or higher. The wild-type control can be a CD25 antibody or an antigen binding fragment thereof. The binding constant K_D of a monospecific or a multispecific binding protein for CD25

or a target protein can be substantially the same (*i.e.*, $\pm 50\%$) as the wild-type control or above it.

[00414] In some aspects, the binding constant K_D of a monospecific or a multispecific binding protein of the disclosure for a target protein can be substantially the same (*i.e.*, $\pm 50\%$) as the wild-type control or below it.

[00415] In some aspects, the binding constant K_D of a monospecific or a multispecific binding protein for a target protein can be above that of the wild-type control by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold or higher. The wild-type control can be an antibody or an antigen binding fragment thereof that binds to the target protein. The binding constant K_D of a monospecific or a multispecific binding protein for the target protein can be substantially the same (*i.e.*, $\pm 50\%$) as the wild-type control or above it.

[00416] The binding specificity of a monospecific or a multispecific binding protein to CD25 or a target protein can be determined by, *e.g.*, flow cytometry, western blotting, or another suitable method. In some aspects, a monospecific or a multispecific binding protein is directed against or specifically binds a CD25. In some aspects, a multispecific binding protein is directed against or specifically binds a target protein. A monospecific or a multispecific binding protein can be either specific to a human CD25 or can cross-react with corresponding targets from other species. A monospecific or a multispecific binding protein can be either specific to a human target protein or can cross-react with corresponding targets from other species.

[00417] In some aspects, the binding constant K_D of a monospecific or a multispecific binding protein comprising a CD25 antibody or an antigen binding fragment thereof can be above that of the wild-type control by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold or higher. The wild-type control can either be a CD25 antibody or an antigen binding fragment or an antibody or an antigen binding fragment that binds the target protein. The binding constant K_D of a monospecific or a multispecific binding protein for a CD25 or a target protein can be substantially the same (*i.e.*, $\pm 50\%$) as the wild-type control or above it.

[00418] The binding specificity of a monospecific or a multispecific binding protein to a CD25 or to a target protein can be determined by, *e.g.*, flow cytometry, western blotting, or another suitable method. In some aspects, a monospecific or a multispecific binding protein specifically binds to a CD25 and/or a target protein.

[00419] In some aspects, certain pharmacokinetic parameters of a monospecific or a multispecific binding protein of the disclosure are same or better than those of wild-type control. For example, in some aspects, elimination half-life ($t_{1/2}$) and/or the area under the concentration curve (AUC) can be substantially the same (*i.e.*, $\pm 50\%$) as the wild-type control or above it. Pharmacokinetic parameters can be measured in humans or using an appropriate animal model (See, *e.g.*, Shargel *et al.* (1995) *Applied Biopharmaceutics and Pharmacokinetics*, 4th ed., McGraw-Hill/Appleton.)

Polynucleotides

[00420] A “nucleic acid molecule” (used interchangeably with “nucleic acid” or “polynucleotide”) is a chain of nucleotide monomers linked to each other via a phosphate backbone to form a nucleotide sequence. A nucleic acid may be used to transform/transfect a host cell or host organism, *e.g.*, for expression and/or production of a polypeptide. Suitable hosts or host cells for production purposes will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. A host or host cell comprising a nucleic acid encoding the polypeptide of the present technology is also encompassed by the present technology.

[00421] A nucleic acid may be for example DNA, RNA, or a hybrid thereof, and may also comprise (*e.g.*, chemically) modified nucleotides, like PNA. It can be single- or double-stranded. In one aspect, it is in the form of double-stranded DNA. For example, the nucleotide sequences of the present technology may be genomic DNA, cDNA.

[00422] The nucleic acids of the present technology can be prepared or obtained in a manner known per se, and/or can be isolated from a suitable natural source. Nucleotide sequences encoding naturally occurring (poly)peptides can for example be subjected to site-directed mutagenesis, so as to provide a nucleic acid molecule encoding polypeptide with sequence variation. Also, as will be clear to the skilled person, to prepare a nucleic acid, also several nucleotide sequences, such as at least one nucleic acid with a nucleotide sequence encoding a targeting moiety and for example, a nucleic acid with a nucleotide sequence encoding one or more linkers can be linked together in a suitable manner (*e.g.*, a nucleic acid encoding an amino acid linker sequence).

[00423] Techniques for generating nucleic acids will be clear to the skilled person and may for instance include, but are not limited to, automated DNA synthesis; site-directed mutagenesis; combining two or more naturally occurring and/or synthetic sequences (or two or more parts thereof), introduction of mutations that lead to the expression of a truncated expression product; introduction of one or more restriction sites (e.g. to create cassettes and/or regions that may easily be digested and/or ligated using suitable restriction enzymes), and/or the introduction of mutations by means of a PCR reaction using one or more “mismatched” primers.

[00424] Methods of making a monospecific or multispecific binding protein comprising expressing these polynucleotides are also provided. Polynucleotides encoding a multispecific binding protein or variants thereof disclosed herein are typically inserted in an expression vector for introduction into host cells that can be used to produce the desired quantity of the claimed monospecific or multispecific binding protein. Accordingly, in certain aspects, the disclosure provides expression vectors comprising polynucleotides disclosed herein and host cells comprising these vectors and polynucleotides.

[00425] In one aspect, polynucleotides (i.e., nucleic acid molecules) encode either a monospecific or a multispecific binding protein described herein or variants thereof are provided. A polynucleotide variant as used herein is about 50, 75, 80, 85, 90, 93, 95, 98, 99% or more identical to a polynucleotide that encodes a multispecific binding protein described herein. Also provided is a nucleic acid molecule encoding the ISVD and/or polypeptide of the present technology.

[00426] In some aspects, nucleic acid molecules encode an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein.

[00427] In some aspects, a nucleic acid molecule encodes an amino acid sequence for at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell. In some aspects, a nucleic acid molecule encodes an amino acid sequence encoding a CD25 ISVD comprises at least 80%, 85%, 90%, or 95% identity to an amino acid sequence set forth in SEQ ID NO: 72.

Expression vector and host cell

[00428] Also provided is a vector comprising the nucleic acid molecule encoding the ISVD and/or polypeptide of the present technology. A vector as used herein is a vehicle suitable for carrying genetic material into a cell. A vector includes naked nucleic acids, such as plasmids or mRNAs, or nucleic acids embedded into a bigger structure, such as liposomes or viral vectors.

[00429] In some aspects, vectors comprise at least one nucleic acid that is optionally linked to one or more regulatory elements, such as for example one or more suitable promoter(s), enhancer(s), terminator(s), etc.). In one aspect, the vector is an expression vector, i.e. a vector suitable for expressing an encoded polypeptide or construct under suitable conditions, e.g. when the vector is introduced into a (e.g. human) cell. DNA-based vectors include the presence of elements for transcription (e.g. a promoter and a polyA signal) and translation (e.g. Kozak sequence).

[00430] In one aspect, in the vector, said at least one nucleic acid and said regulatory elements are "operably linked" to each other, by which is generally meant that they are in a functional relationship with each other. For instance, a promoter is considered "operably linked" to a coding sequence if said promoter is able to initiate or otherwise control/regulate the transcription and/or the expression of a coding sequence (in which said coding sequence should be understood as being "under the control of" said promoter). Generally, when two nucleotide sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

[00431] In one aspect, any regulatory elements of the vector are such that they are capable of providing their intended biological function in the intended host cell or host organism. For instance, a promoter, enhancer or terminator should be "operable" in the intended host cell or host organism, by which is meant that for example said promoter should be capable of initiating or otherwise controlling/regulating the transcription and/or the expression of a nucleotide sequence - e.g. a coding sequence - to which it is operably linked.

[00432] The term "vector" or "expression vector" is used herein for the purposes of the specification and claims, to mean vectors used in accordance with the present disclosure as a vehicle for introducing into and expressing the

polynucleotide sequence encoding a monospecific or a multispecific binding protein polypeptide in a cell. As known to those skilled in the art, such vectors can easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant disclosure will comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired gene and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

[00433] *In vitro* production allows scale-up to give large amounts of the desired polypeptides. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, e.g., in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g., in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography.

[00434] One or more genes encoding a monospecific or a multispecific binding protein can also be expressed non-mammalian cells such as bacteria or yeast or plant cells. In this regard it will be appreciated that various unicellular non-mammalian microorganisms such as bacteria can also be transformed; i.e. those capable of being grown in cultures or fermentation. Bacteria, which are susceptible to transformation, include members of the enterobacteriaceae, such as strains of *Escherichia coli* or *Salmonella*; *Bacillaceae*, such as *Bacillus subtilis*; *Pneumococcus*; *Streptococcus*, and *Haemophilus influenzae*. It will further be appreciated that, when expressed in bacteria, the polypeptides can become part of inclusion bodies. The polypeptides must be isolated, purified and then assembled into functional molecules.

[00435] In addition to prokaryotes, eukaryotic cells can also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used although a number of other strains are commonly available.

[00436] In some aspects, (non-human) host cells or (non-human) host organisms can express the ISVD and/or polypeptide of the present disclosure, or comprise the nucleic acid encoding the ISVD and/or polypeptide of the present disclosure, and/or the vector comprising the nucleic acid molecule encoding the ISVD and/or polypeptide of the present disclosure.

[00437] Suitable host cells or host organisms are e.g., any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. Specific examples include HEK293 cells, CHO cells, *Escherichia coli* or *Pichia pastoris*. In one aspect, the cell host is *Pichia pastoris*.

[00438] In some aspects, a vector comprises nucleic acid molecules encoding an amino acid sequence for a monospecific binding protein comprising at least one CD25 ISVD that specifically binds CD25 on the surface of a CD25 positive cell.

[00439] In some aspects, a vector comprises nucleic acid molecules encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein.

[00440] In some aspects, at least two vectors comprise nucleic acid molecules encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein.

[00441] In some aspects, two vectors comprise a multispecific binding protein of the disclosure. In some aspects, the first vector comprises a nucleic acid molecule encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell. In some aspects, the second vector comprises a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein. In some aspects, two vectors comprise a multispecific binding protein of the disclosure.

[00442] In some aspects, a cell comprises a vector comprising a nucleic acid molecule encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein. In some aspects, a cell comprises two vectors comprising nucleic acid molecules encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein. In some aspects, a cell comprises at least two vectors comprising

nucleic acid molecules encoding an amino acid sequence for a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell; and b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein.

Methods of producing a binding protein comprising an ISVD or polypeptide

[00443] The present disclosure provides a method for producing the monospecific or multispecific binding protein as described herein comprising a ISVD and/or polypeptide. The method can comprise transforming and/or transfecting a host cell or host organism with a nucleic acid encoding the ISVD and/or polypeptide, expressing the ISVD and/or polypeptide in the host cell or host organism, and optionally followed by one or more isolation and/or purification steps. As used herein the binding protein comprises the ISVD and/or the polypeptide, (which is encoded by the nucleic acid molecule(s)) that either selectively binds to a CD25 and/or to a target protein or antigen of interest (e.g., a TNF α). The monospecific binding proteins of the disclosure bind to CD25 whereas the multispecific binding proteins of the disclosure bind to CD25 and a target protein.

[00444] In an aspect, the disclosure provides a method of producing a monospecific or multispecific binding protein described herein comprising: a) expressing in a host cell or host organism or in another suitable expression system, a nucleic acid sequence encoding a ISVD and/or polypeptide; optionally followed by: b) isolating and/or purifying the ISVD and/or polypeptide.

[00445] In another aspect, the disclosure provides a method of producing a monospecific or multispecific binding protein described herein comprising: a) cultivating and/or maintaining a (non-human) host or host cell that is capable of expressing the ISVD and/or polypeptide and/or that comprises a nucleic acid or a genetic construct encoding the ISVD and/or polypeptide, under suitable circumstances that are such that said (non-human) host or host cell is expresses the ISVD and/or polypeptide; optionally followed by: b) isolating and/or purifying the ISVD and/or polypeptide produced.

[00446] The ISVD, the polypeptide, or a genetic construct encoding the ISVD or the polypeptide comprising the nucleic acid molecule or vector as described herein,

or the composition comprising the ISVD, polypeptide, construct, nucleic acid molecule or vector of the present disclosure are useful as a medicament.

[00447] The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicant reserves the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

[00448] While the present disclosure has been described with reference to the specific aspects thereof, it should be understood by those skilled in the art that various changes can be made and equivalents can be substituted without departing from the true spirit and scope of the application. It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods described herein can be made using suitable equivalents without departing from the scope of the aspects disclosed herein. In addition, many modifications can be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present disclosure. All such modifications are intended to be within the scope of the claims appended hereto. Having now described certain aspects in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting.

EXAMPLES

[00449] The present disclosure is further illustrated by the following examples which should not be construed as further limiting. The contents of the Sequence Listing, figures and all references, patents, and published patent applications cited throughout this application are expressly incorporated herein by reference.

Example 1. CD25 positive multispecific binding protein design and construction

Introduction

[00450] Two different multispecific binding protein constructs were designed each containing a first binding moiety targeting CD25 and a second binding moiety targeting TNF α . The first binding moiety targeting CD25 was an anti-CD25 half antibody derived from one of two different commercially available IgG1 monoclonal antibody specific for human CD25. The second binding moiety targeting TNF α was an anti-TNF α half antibody derived from a commercially available IgG1 monoclonal antibody specific for human tumor necrosis factor. The CH3 domain of the Fc region of each half-antibody was engineered to have either a knob (protuberance) or a hole cavity to promote heterodimerization. **Figure 1A** is a schematic drawing of steps in CD25-mediated internalization and endocytic trafficking routes for CD25, a multispecific binding protein (e.g., TNF α /CD25 bispecific antibody), and a target protein (e.g., a TNF α).

Material and Methods

[00451] The halfbodies (also referred to as half-antibodies) CD25 Ab1, CD25 Ab2 and TNF α Ab were expressed in mammalian expression systems (HEK293 and CHO cell lines). The supernatants were purified *via* Protein A resin such as Mabselect Sure and buffer exchanged to PBS pH 7.4. Concentration of each halfbody was measured at A280. Knob and Hole halfbodies were then mixed at exact 1:1 molar ratio in 0.5 M L-Arg buffer pH 8.5. The mixture was incubated at 37 °C for 1 hr. Reducing agents such as Glutathione at 200-fold molar excess were added to the mixture and incubated for additional 3 hrs. The reduced mixture was slowly re-oxidized by removing the reducing agents using techniques such as centrifugal filter with 50 kDa MWCO. The re-oxidized solutions were polished *via* prep-grade SEC using a TSKgel SW2000 column on an GE Healthcare AKTA Pure HPLC system. Final product was quality controlled *via* aSEC to have at least about or greater than 99% purity. Sequence identity was confirmed by mass spectroscopy (MS) and Labchip.

Results

[00452] *Pre-polishing:* The half antibodies (containing either knob or hole mutations) were cloned into an expression plasmid and the resulting purified homodimers are displayed in **Table 2** below.

Table 2. Expression and Purification of antibody halves

Name	A280	Mass ext.	MW (Da)	Concentration (mg/ml)
TNF α Ab	7.96	1.54	72,696.67	5.1688
CD25 Ab1	3.00	1.4	71,814	2.14
CD25 Ab2	2.60	1.42	71,741.7	1.83

[00453] A cell-free reaction adding the complementary knob and hole containing halves were performed in two identical replicates (*i.e.*, two lots) and the protein concentration after heterodimerization is shown in **Table 3** below.

Table 3. Bispecific binding protein yield after heterodimerization reaction

Name	A280	Mass ext.	MW (Da)	Concentration (mg/ml)	Yield (mg)
TNF α /CD25 Ab1 (Lot 1)	2.12	1.47	144,493.0	1.44	1.5
TNF α /CD25 Ab1 (Lot 2)	1.18	1.47	144,493.0	0.8	1
TNF α /CD25 Ab2 (Lot 1)	1.67	1.48	144,398	1.12	1.5
TNF α /CD25 Ab2 (Lot 2)	1.45	1.48	144,398	0.97	1

[00454] To assess aggregation and oligomeric state of the bispecific binding protein size exclusion chromatography (SEC) was performed prior to the polishing step. The chromatograms as shown in **Figures 2 and 3** display the elution profile obtained prior to polishing step for the TNF α /CD25 bispecific Ab1 and the TNF α /CD25 bispecific Ab2 , respectively.

[00455] The expected and observed mass prior to the polishing step for the bispecific binding proteins was also analyzed by mass spectrometry. The expected mass for the TNF α /CD25 bispecific Ab1 is 144,482 Daltons (Da) and the observed mass as shown in **Figure 4** was 144,479 Da. The expected mass for the TNF α /CD25 bispecific Ab2 is 144,409 Da and the observed mass as shown in **Figure 5** was 144,411 Da.

[00456] The supernatants collected pre-polishing for both constructs were also analyzed by using LabChip as shown in **Figure 6** (lane 2: TNF α /CD25 bispecific Ab1 ; lane 3: TNF α /CD25 bispecific Ab 2. Heterodimers were visible as a band above the ladder band of 119 kDa.

[00457] *Post-polishing:* After the initial purification and characterization of the two constructs, two separate pools were created and compared. QC results from aSEC and MS showed the two pools had near identical characteristics. The two pools were nonetheless registered into two separate lots. A polishing step using a subsequent SEC step using an ÄKTA system was performed with the two lots to further remove high and low molecular weight species to achieve final constructs with high purity.

[00458] After polishing, the elution profiles of the both constructs and each respective lot were obtained and are displayed in the SEC-HPLC chromatograms shown in **Figures 7A and 7B and Figures 8A and 8B** for the TNF α /CD25 bispecific Ab1 and for the TNF α /CD25 bispecific Ab2, respectively.

[00459] The supernatants collected post-polishing for both constructs and each respective lot were also analyzed by using LabChip as shown in **Figure 9** (lane 1 and 2: TNF α /CD25 bispecific Ab1; lane 3 and 4: TNF α /CD25 bispecific Ab2 .

[00460] The results of mass spectrometry analysis for each construct and each lot post-polishing are reported in **Figures 10-13**. **Figure 10** is the mass spectrometry results for the TNF α /CD25 bispecific Ab1, lot 1. The observed mass was 144,481 Da. **Figure 11** is the results for lot 2 with and observed mass was 144,480 Da. **Figure 12** is the mass spectrometry results for the anti- TNF α /CD25 bispecific Ab2, lot 1. The observed mass for bispecific binding protein are the same at 144,409 Da. **Figure 13** shows the results for lot 2, also with an observed mass at 144,409 Da.

[00461] The summary analytics for the post-polished constructs are reported in **Table 4 and 5**. **Table 4** reports the size and purity of each construct as and the respective lots as highlighted in gray. **Table 5** reports the total concentration and volume obtain post-polishing for each construct.

Table 4: Bispecific binding protein construct size and purity post-polishing

Name	Name	Size [kDa]	Purity (%)
TNF α /CD25 bispecific Ab1	A7	95.13	0.89
	A7	152.67	2.7
	A7	172.96	96.41

TNF α /CD25 bispecific Ab2	B7	96.8	1.13
	B7	150.81	2.18
	B7	170.81	96.69
	C7	33.59	0.49
	C7	96.13	1.3
	C7	153.05	2.9
	C7	172.59	95.3
	D7	0.47	
	D7	91.31	0.49
	D7	95.91	0.85
	D7	149.83	2.5
	D7	169.64	96.16

Table 5. Bispecific binding protein constructs concentration and volume post-polishing

Name	Concentration (mg/ml)	Volume (mL)
TNF α /CD25 bispecific Ab1	1.12	2.18
TNF α /CD25 bispecific Ab2	1.05	2.35
Anti-TNP_hyb_1B7.11Hz_hlgG1_dK	11	0.1

Conclusion

[00462] Each construct was purified and expressed at a high purity for downstream functional analysis.

Example 2: Bispecific binding proteins bind to CD25 positive cells

Introduction

[00463] In this Example the TNF α /CD25 bispecific antibodies purified in Example 1 were analyzed for the ability to specifically bind CD25 at the cell surface of a CD25 positive cell. Initial experiments were performed using either wild-type Human Embryonic Kidney Cells (HEK-WT) or HEK cells that were transfected with the human IL-2 complex (IL-2 alpha, beta, and gamma chains) (HEK-IL2) (see **Figure 15**).

Methods

[00464] HEK WT or HEK-IL2 cells were stained with the indicated antibody followed by APC labeled anti-human IgG antibody. Then flowcytometry was conducted.

Results

[00465] As shown in **Figure 15**, after incubating the exemplary TNF α /CD25 bispecific antibodies with either HEK-WT or a HEK- IL2 the amount of CD25 cell surface expression was analyzed by flow cytometric analysis. Two different TNF α /CD25 bispecific antibodies bound to the HEK-IL2 cells that are CD25 positive but not to HEK-WT cells examined as a function of CD25 surface expression demonstrated by the mean fluorescence intensity (MFI) shift. The binding of TNF α /CD25 bispecific antibodies was specific because when HEK-WT or HEK-IL2 incubation was performed with IgG1 controls (anti-TNF IgG1 or anti-TNP IgG1) no shift in CD25 cell surface expression was observed.

Conclusion

[00466] The multispecific binding proteins designed herein can bind to CD25 on CD25 positive cells, *e.g.*, HEK-IL2 cells (**Figure 15**).

Example 3: Internalization of TNF α by CD25 expressing cells treated with TNF α /CD25 bispecific antibodies**Introduction**

[00467] Examples 3 and 4 tested the hypothesis that an exemplar CD25 targeting multispecific binding proteins of the disclosure (TNF α /CD25 bispecific Ab1 and TNF α /CD25 bispecific Ab2) can be utilized to shuttle target proteins to CD25 expressing cells for internalization and ultimately for degradation of the target proteins *via* the lysosomal pathway. Accordingly, two different TNF α /CD25 bispecific binding proteins as described in Examples 1 and 2 were analyzed for the ability to bind TNF α and subsequently be internalized by CD25 as part of the IL2 complex. A schematic of the binding and internalization steps of fluorescently labeled TNF α (TNF-biotin-alexaflour647) *via* binding a TNF α /CD25 bispecific antibody used to track the trafficking steps of binding and subsequent internalization is shown in **Figure 14**.

[00468] As shown in **Figure 14**, the TNF α /CD25 bispecific antibody binds to TNF α and to CD25 that is part of the human IL-2 complex on CD25 positive cells. Subsequently, the TNF α /CD25 bispecific antibody plus the TNF α (*i.e.*, the target protein) bound to CD25 is internalized.

Methods

[00469] A competition assay was conducted using commercially available anti-CD25 antibody (clone M-A251) and either (1) TNF α /CD25 bispecific Ab1 or (2) TNF α /CD25 bispecific Ab2. HEK-WT and HEK-IL2 cells were incubated with excessive antibody indicated in the middle column in the table and washed. Then the cells were stained with PE-conjugated anti-CD25 antibody (clone M-A251).

[00470] *TNF α internalization assay by flow cytometry:* HEK-WT cells and HEK-IL2 cells that stably expressed surface human IL-2 were plated at a concentration of 7.5×10^4 cells/well in 48 well plates. Recombinant biotinylated TNF α at a final concentration of 50 nM, streptavidin-alexa Fluor 647 at a final concentration of 100 nM, and anti-TNF α antibody, TNF α /CD25 bispecific Ab1, or TNF α /CD25 bispecific Ab2 at a final concentration of 5 or 50 nM were added sequentially to the 48-well plate. After the cells were cultured at 37 °C for 4 hours, they were washed twice with cold phosphate buffered saline (PBS), then flow cytometry was conducted to assess alexa Fluor 647 fluorescence. The same method using U-bottom 96 well plates at 2×10^5 cells /well was used for TNF α /CD25 bispecific antibody at a final concentration of 5 or 50 nM with 24-hour peripheral blood mononuclear cells (PBMC). Flowcytometry data were analyzed with FlowJo.

Results

[00471] As shown in **Figure 16**, HEK-WT or HEK-IL2 cells were incubated with biotinylated TNF α , streptavidin-alexafluor647 plus either of the two TNF α /CD25 bispecific antibodies or controls at a concentration of 5 nM or 50 nM. Subsequently, HEK-WT and HEK-IL2 cells were analyzed for intracellular expression of CD25. As shown in the first four plots in the right panel only the two TNF α /CD25 bispecific antibodies at the 5 and 50 nM concentration show a change in CD25 expression determined by the MFI shift.

[00472] The results of **Figure 16** were further corroborated by using the same experimental set-up to probe for TNF α but in human donor peripheral blood mononuclear cells (PBMC). As shown in **Figure 20**, TNF α was internalized after incubation with two different TNF α /CD25 bispecific antibodies and activated PBMCs from two different human donors ("donor 1" and "donor 2"; compare upper right quadrant of flow cytometric plots of PMBCs incubated either with TNF α /CD25 bispecific antibody or with controls). CD3⁺CD4⁺ T cells gated from the total PMBCs, as shown in **Figure 21**, show the same internalization pattern as the total PMBCs.

[00473] Flow cytometric plots, in **Figures 20** and **21** show the amount of CD25 using anti-CD25 Ab (clone M-A251) (x-axis) and TNF α complex (y-axis) staining in PMBCs at 4 and 24 hour after incubation with: (1) either TNF α /CD25 bispecific Ab1 or (2) TNF α /CD25 bispecific Ab2 plus TNF α or with an antibody control plus TNF α . The results shown in **Figure 22** confirmed that neither bispecific antibodies tested competed with the anti-CD25 antibody (clone M-A251).

Conclusion

[00474] The CD25 targeting multispecific binding proteins designed herein bind to a target protein, TNF α , and to human IL-2 complex containing CD25. The IL-2 complex containing CD25 plus the TNF α /CD25 bispecific antibody bound to the TNF α are then internalized by CD25 positive cells (see **Figure 16, 20, and 21**).

Example 4: TNF α is degraded in the lysosome after CD25 targeting multispecific binding protein plus TNF α cargo is internalized by CD25 positive cells

Introduction

[00475] This Example tests degradation of a target protein in the lysosome after the internalization of a complex comprising an exemplary CD25 targeting multispecific binding protein bound to TNF α and CD25, by CD25 positive cells.

Methods

[00476] *TNF α degradation assay by western blotting:* HEK-WT or HEK-IL2 cells were plated at a concentration of 2×10^4 cells /well in flat-bottom 96 well plates. Recombinant TNF α at a final concentration of 50 nM, and anti-TNF α antibody or anti- TNF α /CD25 bispecific antibody at a final concentration of 25 nM were added sequentially to the 96-well plate. After the cells were cultured at 37 °C for 2 hours,

they were washed twice with media and lysate was produced using radioimmunoprecipitation assay (RIPA) buffer. A few cells remained in culture for an additional 2, 8 or 22 hours in the presence of DMSO or 100 nM Bafilomycin A (a potent lysosome inhibitor). At each time point, the cells were washed twice with cold PBS then RIPA buffer to produce a cell lysate. Western blotting was employed to detect TNF α or β -actin with the prepared lysate.

Results

[00477] Cellular internalization and degradation of TNF α by two different TNF α /CD25 bispecific antibodies described herein were tested by incubating CD25 positive (HEK-IL2) cells with TNF α plus the TNF α /CD25 bispecific Ab1 or TNF α /CD25 bispecific Ab2 for 2, 8, or 22 hours in either the cell or outside of the cell as measured by probing for TNF α in the cell lysates (**Figure 17 and 18**) or in the cell supernatants (**Figure 19**), respectively.

[00478] At the earliest time point, the 2-hr post incubation period, higher concentration of TNF α was detected in HEK-IL2 cell lysates in the samples incubated with one of the two different TNF α /CD25 bispecific antibodies compared to samples incubated with the control antibodies, demonstrating that TNF α was internalized in the CD25 positive cell. *Compare* western blot gel lanes 7-10 with lanes 11-12 in both **Figure 17** and **Figure 18** probed with an anti-TNF α antibody. As early as the 8-hr post incubation period, degradation of TNF α was detected in the HEK-IL2 cell lysates incubated with either of the two different TNF α /CD25 bispecific antibodies. See western blot gel lanes 15 and 16 in **Figure 17** and **Figure 18**. When a potent lysosome inhibitor (bafilomycin) was applied the samples TNF α was not degraded at either the 8-hr or the 22-hr time point demonstrating that target proteins are degraded *via* the lysosomal degradation pathway.

[00479] Additionally, the intracellular degradation of TNF α as shown by the western blot of CD25 positive cell lysate samples that were incubated with a TNF α /CD25 bispecific antibody was also commensurate with a depletion of the TNF α from the supernatant of HEK-IL2 cells. By the 48 and 72-hour time point post-incubation the supernatant of HEK-IL2 cells had a decreased in the level of TNF α in the samples that were incubated with a TNF α /CD25 bispecific antibody but not the control samples as shown in **Figure 19**.

Conclusion

[00480] Target proteins, e.g., TNF α , are internalized by a CD25 positive cell when bound to a CD25 targeting multispecific binding protein described herein (see **Figures 15-18**). The target protein then traffics to the lysosome for subsequent degradation (see **Figures 17 and 18**). The degradation of the target protein is commensurate with a depletion of the target protein in the surrounding supernatant (see **Figure 19**).

Example 5: CD25 targeting multispecific binding protein is internalized and traffics to the lysosome**Introduction**

[00481] Example 5 analyzes the internalization and trafficking steps of the anti-TNF α /CD25 bispecific antibodies (Ab1 and Ab2) designed in Example 1 and 2 *via* cellular microscopy further corroborating the mechanism of action described in Examples 3 and 4 and depicted in **Figure 1A**. Experiments were performed utilizing either HEK-WT or HEK-IL2 cells described in Example 2.

Methods***CD25 internalization with an anti-TNF α /CD25 bispecific antibody analysis by confocal microscope***

[00482] HEK-WT or HEK-IL2 cells that stably expressed human CD25 were incubated with the following bispecific antibody complex: 25 nM anti-TNF α /CD25 bispecific antibody, 50 nM biotinylated TNF α , and 100 nM streptavidin conjugated to AlexaFluor 488 at 37°C for a 1-, 2-, or 4-hour time course in cell culture media in the dark. After the incubation, the media were removed, and the cells were washed with PBS.

[00483] Cells were then fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer, at pH 7.4) at room temperature for 30 minutes and cytospun onto glass slides. Samples were then permeabilized with 0.5% Triton X-100 in PBS, washed three times with PBS, and stained and incubated with primary rabbit anti-Early Endosome Antigen 1 (EEA1, endosome marker) and mouse anti-LAMP-1 (lysosome marker) antibodies. After incubation with primary antibodies, samples were washed three times with PBS and stained and incubated with hoeschst (nucleus marker) as well as with fluorescent

goat anti-rabbit and goat anti-mouse secondary antibodies. Following staining samples were washed and mounted using AquaPoly/Mount. To protect against photobleaching during the fixation and staining process, all incubations were carried out in the dark as described previously (Piepenhagen, Microsc Res Tech. 2010).

[00484] All micrographs were acquired using a Zeiss LSM880 confocal microscope (Carl Zeiss, White Plains, NY) equipped with a 40X Plan-Apo water immersion objective. Alexa Fluor 488 was excited using the 488-nm line of an argon laser and detected using a 515–565-nm band pass filter. Other fluorescent labels were excited using different nm laser lines and were detected using appropriate band-pass filters. High resolution images were captured using the Zeiss Airyscan detector array, and channels were acquired sequentially to minimize spectral overlap. One or two fields were randomly picked per sample and optical stacks were recorded. All images were acquired using identical parameters. Images were analyzed in Cellprofiler (Cimini lab, Broad Institute) using maximum intensity projections of the optical stacks. Data were plotted and statistics were calculated using RStudio (version 2022.12.0, Posit Software).

Live cell imaging with pH-dependent dye

[00485] HEK-WT or HEK-IL2 cells that stably express surface human CD25 were plated at a concentration of 200,000 cells/well in 35 mm² glass-bottom microwell dishes that had been coated with poly-d-lysine at 1mg/mL. Cells were allowed to rest overnight to attach to dishes. Recombinant biotinylated TNF α at a final concentration of 50nM, streptavidin-pHrodo red at a final concentration of 100nM, and anti-TNF α /CD25 bispecific antibody at a final concentration of 25nM were added to each dish immediately before imaging. Hoescht (nuclear dye) was added to media at a final dilution of 1:5000 was added to media of some samples at a final concentration of 1X from a 1000X stock solution. Dishes were immediately taken for live cell imaging in a stage top incubator system set to 37°C with 5% CO₂ and added humidity.

[00486] All micrographs were acquired using a Zeiss LSM780 confocal microscope (Carl Zeiss, White Plains, NY) equipped with a 20X Plan-Apo air immersion objective. Five fields of view were randomly chosen from each dish and optical stacks were acquired every 3 minutes for up to two hours. pHrodo red dye was excited using the 561nm laser line and was detected using a bandpass filter.

LysoView633 was excited using the 633nm laser line and Hoescht was excited using the 405nm laser line. They were detected using appropriate bandpass filters. Images were acquired using identical parameters. Live imaging data was analyzed in Cellprofiler (Cimini lab, Broad Institute) using maximum intensity projections of the optical stacks. Data were plotted and statistics were calculated using RStudio (version 2022.12.0, Posit Software).

Results

[00487] To assess *via* confocal microscopy the cellular internalization and lysosomal trafficking steps of the target protein, HEK-WT or HEK-IL2 cells were incubated with a bispecific antibody complex which comprised biotinylated human TNF α , streptavidin conjugated to AlexaFluor488 (streptavidin-AF488), and an anti-TNF α /CD25 bispecific antibody (either Ab1 or Ab2) as depicted in **Figure 23**.

[00488] After incubation of the bispecific antibody complex (the complex either with Ab1 or Ab2) with cells for 1-, 2-, or 4-hours samples were fixed and co-stained with endosome and lysosome markers using rabbit anti-EEA1 and mouse anti-LAMP1 antibodies, fluorescently labeled goat anti-rabbit and goat anti-mouse secondary antibodies, as well as a nuclear marker (Hoechst) as shown in representative maximum intensity projection images (**Figures 24-27**). As shown in **Figure 28 and 29** there was internalization of the bispecific antibody complex as early as 1-hour post incubation that overlapped with LAMP1 and/or EEA1 markers (see also **Figure 31 and 32, middle and right columns**) which continued to accumulate at the 2- and 4- hour post incubation time point (**Figure 33**). Most of the bispecific antibody complex colocalized with lysosomal marker, LAMP1, at the 4-hour incubation timepoint (**Figure 33**).

[00489] Unlike pH-insensitive fluorophores or pH-sensitive fluorophores that are brightly fluorescent at neutral pH, the fluorogenic nature of pHrodo red dye provides a ratiometric sensor for measuring the pH change in internal vesicular compartments like lysosomes and endosomes. Accordingly, the bispecific antibody complex described in **Figure 23** was modified by either conjugation to streptavidin-AF488 (**Figure 34 top**) or to pHrodo-red to the bispecific antibody (**Figure 34 bottom**) to confirm by a second method that the bispecific antibody complex was indeed taken up by HEK-IL2 cells and shuttled into low pH internal compartments (e.g., lysosomes and

endosomes). Maximum intensity projection images captured over a 100-minute live time course of HEK-IL2 cells incubated with the CD25 bispecific antibody complex conjugated either to streptavidin-AF488 and pHrodo-red are shown in **Figure 35** (Ab2) and **36** (Ab1) and **37** (Ab2) and **38** (Ab1), respectively.

[00490] **Figure 39** quantifies the AlexaFluor 488-labeled streptavidin (top) and pHrodo red-labeled streptavidin (bottom) MFI intensity in live HEK+IL2 cells imaged over time with gray shadow denotes 95% confidence interval for bispecific antibody complex containing wither Ab2 or Ab1. Although Ab1 peak was later than the peak for Ab2, both Ab1 and Ab2 multispecific antibody complexes obtained a pHrodo-label signal which further supports that the complex is in low pH compartments like lysosomes and late endosomes.

Example 6: Generation of a novel CD25 ISVD

Introduction

[00491] This Example generated and tested the design and binding properties of a novel CD25 binding protein, i.e., a CD25 ISVD.

Methods

[00492] *Immunization.* After approval of the Ethical Committee of the Ablynx Camelid Facility (LA1400575), 2 llamas and 2 alpacas were immunized with plasmid DNA encoding human CD25. Boost immunizations were performed using plasmid DNA encoding cynomolgus CD25 for all 4 animals. Serum titers were determined by ELISA using recombinant human and cynomolgus CD25 proteins.

[00493] *Library generation.* Pre-boost and post-boost libraries were constructed in pAX629. cDNA was prepared from 90 µg total RNA extracted from the PBL samples of the camelids using SuperScript III First-Strand Synthesis System and the included random hexamer primers (Invitrogen, Cat. No. 18080051). Nucleotide sequences encoding VHH proteins were amplified from the cDNA by PCR using forward primers ABL051 (GGCTGAGCTGGGTGGTCCTGG; SEQ ID NO: 107) and ABL052 (GGCTGAGTTTGGTGGTCCTGG; SEQ ID NO: 108) and reverse ABL003 (GGTACGTGCTGTTGAAGTGTTC; SEQ ID NO: 109). The 700 base pair (bp) amplicons amplified from the IgG2 and IgG3 encoding cDNAs were isolated from agarose gel and subsequently used as templates in a nested PCR reaction using forward primers Fw_Nb_MfeI (GAGGTGCAATTGGTGGAGTCTGGGGG; SEQ ID

NO: 110) and reverse primer Rev_Lib_Eco91I (TGAGGAGACGGTGACCAGGGT; SEQ ID NO: 111).

[00494] The PCR products were subsequently digested with restriction enzymes, MfeI and Eco91I, and ligated via the corresponding restriction sites into phagemid vector pAX629, followed by transformation of *E. coli* strain TG1 (Lucigen, Cat. No. 60502) with the ligation product via electroporation. pAX629 is an expression vector derived from pUC119, which contains the LacZ promoter, a *E. coli* phage pIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multi-cloning site, and the gene3 leader sequence. In frame with the VHH protein coding sequence, the vector also encodes a C-terminal 3xFLAG-tag and a His6-tag. After superinfection of the *E. coli* TG1 library clones with helper phage, either VCSM13 (Stratagene) or Hyperphage (PROGEN). pAX629 allows for production of phage particles displaying the individual VHH proteins as a fusion protein with the pIII protein. Rescue with VCSM13 helper phage resulted in most phage particles displaying none, or one, VHH protein copy. Rescue with Hyperphage resulted in phage particles where all pIII proteins (3 to 5 copies) display a VHH protein.

[00495] *Selection.* In-solution selection campaigns were performed with the help of the KingFisher purification instrument. The eluted phage was amplified in *E. coli* ER2738 cells and rescued with helper phage (Hyperphage or VCSM13) and used for a subsequent selection round. In each round of selection, a phage titration was performed. For the screening of individual colonies certain outputs were plated out on agar plates with carbenicillin, which were then picked with the help of a K8 Colony Picker and grown in 96-deep-well plates. With the help of the TECAN Fluent 80 µl of this overnight culture of each clone was mixed with 40 µl of 60% glycerol to create a PMP (Primary Master Plate). The PMP was stored at -80°C. In this case the standard 96-well induction and peri extract preparation protocol was followed.

[00496] Preferred clones were selected based on: binding to human CD25, cross reactivity for binding to cynoCD25, and germline and family amino acid sequence diversity.

[00497] *Screening strategy.* VHH protein-containing periplasmic extracts for screening purposes, were generated by inoculating 5 µL of the TG1 glycerol stocks stored in plasma membrane permeabilizers (PMPs) in 96 deep-well plates (ABgene Cat. No. AB-0932) in 1 mL/well 2xTY media supplemented with 100 µg/mL carbenicillin (Thermo Fisher Scientific, Cat. No. BP2648-5) and 0.1% glucose. VHH

protein expression was induced at OD₆₀₀ ~0.5 with 1 mM IPTG (Thermo Fisher Scientific, Cat. No. R0392) for 4 hours, whereafter the cells were pelleted and frozen overnight at -20°C. The frozen cell pellets were resuspended in D-PBS (Gibco, Cat. No. 14190094) in 1/10th of the original culture volume and incubated at 4°C for 1 hour under gentle shaking conditions. Then, the extracted cells were pelleted and the supernatants, containing the proteins secreted into the periplasmic space, were stored at -20°C until use.

[00498] For the screening experiments a multiplex binding assay was performed which relied on the use of wild-type HEK-Blue™ cells at 20000 cells/well, HEK-Blue™ IL-2 cells at 20000 cells/well, His-tagged recombinant human, cyno, and mouse extracellular domain of CD25, all at 1000 beads/well. The generation of the plates for screening was done following a specific layout in which a negative control was included, along with cell binding VHH compounds (CBN1 and CBN2). During screening, the ratio of each sample over the in-plate negative control (IRR00156 and IRR00283) was calculated per plate using Spotfire (Tibco). All VHH proteins with a ratio > 3 were classified as active binders to the specified target.

[00499] *Expression of VHH proteins in E. coli.* Sequence analysis of VHH proteins from phage display selection outputs was done according to commonly known procedures, e.g., Pardon et al. (2014), A general protocol for the generation of Nanobodies for structural biology, Nat Protoc, vol. 9: 674.

[00500] VHH protein encoding DNA fragments, obtained by PCR with specific combinations of forward FR1 and reverse FR4 primers each carrying a unique restriction site, were digested with the appropriate restriction enzymes, and ligated into the matching cloning cassettes of VHH protein expression vectors. The ligation mixtures were then used to transform electrocompetent or chemically competent *E. coli* TG1 cells (Lucigen, Cat. No. 60502), which were grown under the appropriate antibiotic selection pressure. Resistant clones were verified by Sanger sequencing of plasmid DNA (LGC Genomics).

[00501] For expression and later purification of VHH proteins by transformed *E. coli* TG1 cells, cells were grown for 2 hours at 37°C followed by 29 hours at 30°C (250 rpm) in a baffled shaker flask containing "5052" auto-induction medium. Cells were pelleted by centrifugation (20 minutes, 4500 rpm, 4°C), the supernatant was discarded, and pellets were frozen over night at -20°C. The frozen cell pellets were then dissolved in DPBS (Gibco, Cat. No. 14190-094) at 1/12.5th of the original culture

volume and incubated at 4°C for 1 hour while gently rotating, to disrupt the outer membrane of the cells. The cells were pelleted again (20 minutes, 8500 rpm, 4°C) and the supernatant, containing the VHH proteins, was collected and filtered to immediately proceed with purification.

[00502] *Generic purification of VHH proteins.* His6-tagged VHH proteins were purified by immobilized metal affinity chromatography (IMAC) on Ni Sepharose® Excel (Cytiva, Cat. No. 17-3712-05) resin with imidazole elution followed by a desalting step (PD MidiTrap columns with Sephadex G25 resin, Cytiva, Cat. No. 28-9180-08) and if necessary, preparative size exclusion chromatography (SEC) (Superdex 75 Increase 10/300 GL column, Cytiva, Cat. No. 29-1487-21) in D-PBS. To this end, robotic stations or ÄKTA purification systems were used.

[00503] *Flow cytometry-based target binding.* Binding of purified VHH proteins to human and cyno targets. CD25, expressed on the cell surface, was examined by flow cytometry. HEK293-MZA cells expressing human or cyno CD25 were thawed, washed with assay buffer (PBS, 2% FBS, 0.05% NaN₃), and seeded in 384-well Bio-One V-bottom plates (Greiner, Cat. No. 781280), with a total of 4.104 cells seeded per well. After washing, serial dilutions of VHH proteins (starting at 1 µM, 3-fold dilution, 11 points, diluted in assay buffer), were added and incubated for 30 minutes at 4 °C. Plates were then washed 3 times and cells were incubated with mouse anti-FLAG (Sigma-Aldrich, Cat. No. F1804) (1000-fold diluted in assay buffer) for 30 minutes at 4 °C. Subsequently, plates were washed 3 times and cells were incubated with goat anti-mouse Fc APC (Jackson ImmunoResearch, Cat. No. 115-135-164) (100-fold diluted in assay buffer) for 30 minutes at 4 °C. Plates were finally washed 3 times and cells were resuspended in DAPI solution (BD Biosciences, Cat. No. 564907, 5000-fold diluted in assay buffer). Cell suspensions were analyzed with iQue Screener PLUS 3 (Intellicyt). In each washing step, plates were centrifuged at 300xg for 2 minutes at 4 °C, supernatants discarded, and buffer dispensed by microplate washer (BioTek). Other reagents were added either manually or with ViaFlo (Integra).

[00504] For characterization of VHH compound target binding, EC₅₀ of binding curves are estimated by dose response modelling. Curves were fit using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.).

[00505] *Flow cytometry-based ligand competition assay.* The ability of VHH proteins to inhibit the interaction between human IL-2 and human or cyno CD25

expressed on cell surfaces was assessed with flow cytometry. HEK293-MZA cells expressing human or cyno CD25 were thawed, washed with assay buffer (PBS, 2% FBS, 0.05% NaN₃) and seeded in 384-well Bio-One V-bottom plates (Greiner, Cat. No. 781280), with a total of 104 cells seeded per well. After washing, VHH proteins (serial dilution with final concentrations starting from 1 μ M, 3-fold dilution, 11 points, in assay buffer) were premixed with biotinylated IL-2 (Acrobiosystems, IL2-H82E4) at final concentration of 30 nM ($\approx$ EC₃₀) and added to the cells for 90 minutes at 4°C on a shaker at 600 rpm.

[00506] Plates were washed 3 times and cells were incubated in streptavidin-phycoerythrin, 1000-fold diluted in assay buffer (BD - Pharmingen, Cat. No. 554061) for 30 minutes at 4°C. Subsequently, plates were washed 3 times and cells were resuspended in DAPI solution (BD Biosciences, Cat. No. 564907, 5000-fold diluted in assay buffer). Cell suspensions were analyzed with iQue Screener PLUS 3 (Intellicyt). In each washing step, plates were centrifuged at 300xg for 2 minutes at 4°C, supernatants were discarded, and buffer was dispensed by microplate washer (BioTek). Other reagents were added either manually or with ViaFlo (Integra). As for VHH protein characterization the IC₅₀ was estimated by dose response modelling. Curves were fit using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.).

[00507] *Affinity determination.* Affinity of VHH compounds binding to human and cyno CD25 was determined by surface plasmon resonance (SPR) on Biacore. Human and cyno CD25 were directly immobilized on different channels of a CM5 chip. Activation was done by an 480s injection of 200 mM EDC/50 mM sulfo-NHS, and deactivation by a 480s injection of 1 M ethanolamine. Flow rate during activation and deactivation was 10 μ L/min. Human and cyno CD25 were immobilized for 350s at 2.0 μ g/mL at a flow rate of 10 μ L/min in 10 mM acetate pH 5.0. VHH compounds were flowed over as analytes at different concentrations ranging from 0.78 to 500 nM to determine affinities. Data analysis was carried out within the Biacore Insight Evaluation Software.

Results

[00508] *Immunization and generation of CD25 ISVD libraries.* The serum titers confirmed that the immune response for the pre-boost and post-boost immunizations were of suitable quality for all the libraries against human CD25 (ECD, His tag) and

cyno CD25 (ECD, His tag), but low to moderate for murine CD25 (ECD, His tag). Regarding the post-boost libraries there was an increase in the immune response for the libraries of 1 alpaca and the 2 llamas.

[00509] The VHH building block for the CD25 ISVD, A0448013B05, was derived from the first immunization library of Alpaca 102 after selection on recombinant Fc fused cyno CD25 followed by secondary selection on biotinylated human CD25 using hyperphage.

[00510] *CD25 ISVD Sequence Selection.* After selection and screening all possible hits were sequenced and a panel of 25 monovalent building blocks were produced for further characterization. From this panel the CD25 ISVD, A0448013B05, was selected as possible candidate for binding cell surface CD25 internalization. This ISVD is VHH2 germline VHH compound. Sequence of the CD25 ISVD, A0448013B05, is provided in Table 6 below.

Table 6: Sequence information of IL2Ra VHH compound including ID, family, germline and parental amino acid sequence.

Sample ID	Family	Germline	Sequence
A0448013B05 SEQ ID NO: 72 CD25 ISVD	26	VHH2	EVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAM GWYRQAPGKQRELVASITGGGSTNYADSVKGRF TISRDNAKNTVYLQMNSLKPEDTATYYCNIYRNVI PGRLSWGQGTLLTVSS

[00511] *CD25 Affinity determination via SPR.* SPR was used to define the affinity of individual CD25 VHH compounds to the human target protein CD25. Affinity of the CD25 ISVD, A0448013B05, was in the range of 17 nM. In this setup species cross reactivity for binding to cyno CD25 was explored in parallel and cyno species cross reactivity was observed within predefined threshold of fold difference as compared to affinity to the human protein below 10-fold, more specifically a 2-fold difference is observed as reported in Table 7.

Table 7: SPR based affinity determination of A0448013B05 to human and cyno IL2Ra

Sample ID	Affinity human CD25			Affinity cyno CD25			Fold-Difference KD Cyno/ Human
	ka (1/Ms)	Kd (1/s)	KD (M)	Ka (1/Ms)	Kd (1/s)	KD (M)	
A0448013B05	2.00E+05	3.40E-03	1.70E-08	1.20E+05	4.10E-03	3.50E-08	2.06

[00512] *Binding of CD25 ISVD to cell surface human and cyno CD25 by flow cytometry.* Flow cytometry binding of the selected CD25 ISVD was explored using flow cytometry on cells overexpressing human or cyno CD25 at the cell surface. Dose dependent binding of both the CD25 ISVD (A0448013B05) was observed for both human and cyno CD25 expressed on HEK293-MZA cells. In contrast, CD25 ISVD did not bind and was not detected on parental HEK293 cells. Under the applied conditions, comparable binding EC₅₀ was observed for binding to both human and cyno CD25 as shown in **Figure 41** and **Table 8** shown below.

Table 8: EC₅₀ values of CD25 ISVD (A0448013B05) to human and cyno CD25 stably expressed on HEK293-MZA cells

Target	Bottom	Top	EC ₅₀ (M)	95% LCI EC ₅₀ (M)	95% UCI EC ₅₀ (M)
Human IL2R α	62232	1775781	3.60E-09	2.41E-09	5.97E-09
Cyno IL2R α	42122	2051835	4.74E-09	4.37E-09	5.18E-09

[00513] *Ligand binding competition of IL-2 versus CD25 ISVD to cell surface CD25 measured by flow cytometry.* Competition of the selected CD25 ISVD (A0448013B05) versus biotinylated human IL-2 for cell surface ligand binding to human and cyno CD25 was measured using flow cytometry on HEK293-MZA cells expressing human or cyno CD25. Fixed concentration of biotinylated human IL-2 at 30 nM was added to the cells in combination with different concentrations of the CD25 ISVD (A0448013B05). Binding of biotinylated human IL-2 to the cells was assessed using PE-labelled streptavidin. These data demonstrate that the CD25 ISVD (A0448013B05) competed with IL-2 for binding to CD25. Under the applied conditions, more than 10-fold difference in IC₅₀ is observed for competition of human IL-2 binding to cyno CD25 as compared to human CD25 as shown by comparing left and right graphs of **Figure 42**. See *also* **Table 9** below which tabulates graphical data presented in **Figure 42**. Albeit accurate determination of fold difference in IC₅₀ was hampered by incomplete curve for competition with human IL-2 binding on cyno CD25.

Table 9: IC₅₀ values of CD25 ISVD (A0448013B05) in competition with human IL-2 for binding to human and cyno CD25 stably expressed on HEK293-MZA cells

Target	Bottom	Top	IC ₅₀ (M)	95% LCI IC ₅₀ (M)	95% UCI IC ₅₀ (M)
human CD25	4456	33300	7.13E-09	2.64E-09	2.48E-08
cyno CD25	8102	61400	8.41E-08*	4.35E-08	4.49E-07

LCI: lower confidence interval, UCI: upper confidence interval

Conclusion

[00514] The novel CD25 ISVD generated and tested in this example binds to both human and cyno cell surface expressed CD25 and is a competitive inhibitor for CD25 binding. Further, it is envisaged that this novel monospecific CD25 ISVD can also be incorporated into a CD25 multispecific binding protein as described herein and this was further interrogated in Example 7 as described below.

Example 7: Internalization and lysosomal targeting of TNF α by CD25 expressing cells treated with a TNF α /CD25 bispecific ISVD

Introduction

[00515] This example investigates whether the CD25 ISVD designed in Example 6 can be operatively linked to a second binding moiety that specifically binds to the target protein, e.g., TNF α , to form a CD25 multispecific protein which has the same potential to internalize and degrade a target protein utilizing the CD25 lysosomal shuttle mechanism described herein and schematized in **Figure 1A**. Further, this example tests whether incorporation of a binding moiety that utilizes a multivalent ISVD (i.e., at least two ISVDs) which bind to the same target protein (i.e., at least two TNF ISVDs that both specifically bind to TNF) as well as the incorporation of a serum albumin ISVD into the design of the multispecific binding proteins described herein can be engineered into CD25 multispecific binding protein designs.

Methods

[00516] Applicable methods for generation of a ISVD that specifically target a protein (e.g., TNF α) can be found in Example 6. The linkage utilized to operatively link the CD25 ISVD, the multivalent TNF ISVD, and the serum albumin ISVD are described throughout the specification for example the section entitled, "*Linker*

Region.” Methods for western blot analysis of internalization and degradation of the target protein can be found in Example 4. The multispecific binding protein format of the binding protein is shown in **Table 13** below.

Table 13: Description of a multispecific binding protein of the disclosure

Name	Description	Polypeptide Linkage Order (N' to C' terminal domain)
Nb59 (SEQ ID NO: 119)	a multispecific binding protein comprising: a) a CD25 ISVD (e.g., SEQ ID NO: 72); b) a target protein ISVD (using two TNF ISVDs); and c) a serum albumin (Alb23002) binding ISVD (e.g., SEQ ID NO:88)	CD25-TNF-TNF-Alb
Nb60 (SEQ ID NO: 120)	a multispecific binding protein control protein comprising: a) a CD25 ISVD (e.g., SEQ ID NO: 72); b) an irrelevant (IRR) ISVD (using two IRR ISVD); and c) a serum albumin (Alb23002) binding ISVD (e.g., SEQ ID NO:88)	CD25-IRR-IRR-Alb

[00517] The Nb59 multispecific binding protein of the disclosure described in **Table 13** is encoded by amino acid sequence set forth in SEQ ID NO: 119. The amino acid sequence set forth in SEQ ID NO: 119 is:
DVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAMGWYRQAPGKQRELVASITGG
GSTNYADSVKGRFTISRDNKNTVYLQMNSLKPEDTATYYCNIYRNVIPGRLSWGQ
GTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESG
GGVQPGGSLRLSCAASGFTFSDYWMYWRQAPGKGLEWVSEINTNGLITKYPD
SVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCARSPSGFNRGQGTTLVTVSSGG
GGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVQPGGSL
RLSCAASGFTFSDYWMYWRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISR
DNKNTLYLQMNSLRPEDTALYYCARSPSGFNRGQGTTLVTVSSGGGGSGGGSEVQ
LVESGGGVQPGGSLRLSCAASGFTFRSFGMSWRQAPGKGPEWSSISGSGSD
TLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTTLTVS
SA.

[00518] The Nb60 multispecific binding protein of the disclosure described in **Table 13** is encoded by amino acid sequence set forth in SEQ ID NO: 120. The amino acid sequence set forth in SEQ ID NO: 120 is:

DVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAMGWYRQAPGKQRELVASITGG
 GSTNYADSVKGRFTISRDNANKNTVYQLQMNSLKPEDTATYYCNIYRNVIPGRLSWGQ
 GTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESG
 GGWVQPGGSLRLSCAASGLTFSTNPMYWYRQAPGKQRELVASISSRGITNYADSV
 KGRFTISRDNANKNTVYQLQMNSLRPEDTALYYCRLASLSSGTVYWGGQTLVTVSSG
 GGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGG
 SLRLSCAASGLTFSTNPMYWYRQAPGKQRELVASISSRGITNYADSVKGRFTISR
 DANKNTVYQLQMNSLRPEDTALYYCRLASLSSGTVYWGGQTLVTVSSGGGGSGGG
 EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWSSISGS
 GSDTLYADSVKGRFTISRDNANKNTLYQLQMNSLRPEDTALYYCTIGGSLRSSQGT
 LVTVSSA.

Results

[00519] Cellular internalization and degradation of TNF α by the TNF α /CD25 bispecific ISVD was tested by incubating CD25 positive (HEK-IL2) cells with TNF α plus the TNF α /CD25 bispecific ISVD for 8 or 22 hours in the cell or outside of the cell as measured by probing for TNF α in the cell lysates (**Figure 43**) or in the cell supernatants (**Figure 44**), respectively.

[00520] At the earliest time point, the 2-hr post incubation period, higher concentration of TNF α was detected in HEK-IL2 cell lysates in the samples incubated with the TNF α /CD25 bispecific ISVD when compared to samples incubated with the control ISVD (Nb60), demonstrating that TNF α was internalized via the CD25 ISVD binding moiety into the CD25 positive cell (**Figure 43**). As early as the 8-hr post incubation period, degradation of TNF α was detected in the HEK-IL2 cell lysates incubated with the TNF α /CD25 bispecific ISVD. When a potent lysosome inhibitor (bafilomycin) was applied the samples TNF α was not degraded at either the 8-hr or the 22-hr time point demonstrating that target proteins are degraded via the lysosomal degradation pathway (**Figure 43**).

[00521] Additionally, the intracellular degradation of TNF α as shown by the western blot of CD25 positive cell lysate samples that were incubated with a TNF α /CD25 bispecific ISVD was also commensurate with a depletion of the TNF α from the supernatant of HEK-IL2 cells. By the 48 and 72-hour time point post-incubation the supernatant of HEK-IL2 cells had a decreased in the level of TNF α in

the samples that were incubated with a TNF α /CD25 bispecific antibody but not the control samples as shown in **Figure 44**.

Conclusion

[00522] Target proteins, e.g., TNF α , are internalized by a CD25 positive cell when bound to a CD25 targeting multispecific binding protein regardless of the binding format. Further, this example demonstrates the successful incorporation of a binding moiety that utilizes a multivalent ISVD (i.e., at least two ISVDs) which bind to the target protein (i.e., at least two TNF ISVDs) as well as the incorporation and utilization of a serum albumin ISVD (Alb23002) into the design of the multispecific binding proteins described herein.

SEQUENCE TABLE

Table 14 - Amino acid sequences

SEQ ID NO / Description	Amino Acid sequence
SEQ ID NO: 1	GGGG
SEQ ID NO: 2	GGGGG
SEQ ID NO: 3	GGGGGG
SEQ ID NO: 4	GGGGGGG
SEQ ID NO: 5	GGGGGGGG
SEQ ID NO: 6	GGGGS
SEQ ID NO: 7	GGGSGGGGS
SEQ ID NO: 8	GGGGGS
SEQ ID NO: 9	KERE
SEQ ID NO: 10	KQRE
SEQ ID NO: 11	GLEW
SEQ ID NO: 12	KEREL
SEQ ID NO: 13	KEREF
SEQ ID NO: 14	KQREL
SEQ ID NO: 15	KQREF
SEQ ID NO: 16	KEREG
SEQ ID NO: 17	KQREW
SEQ ID NO: 18	KQREG
SEQ ID NO: 19	TERE
SEQ ID NO: 20	TEREL
SEQ ID NO: 21	TQRE
SEQ ID NO: 22	TQREL
SEQ ID NO: 23	KECE
SEQ ID NO: 24	KECEL
SEQ ID NO: 25	KECER
SEQ ID NO: 26	KQCE
SEQ ID NO: 27	KQCEL
SEQ ID NO: 28	RERE
SEQ ID NO: 29	REREG

SEQ ID NO / Description	Amino Acid sequence
SEQ ID NO: 30	RQRE
SEQ ID NO: 31	RQREL
SEQ ID NO: 32	RQREF
SEQ ID NO: 33	RQREW
SEQ ID NO: 34	QERE
SEQ ID NO: 35	QEREG
SEQ ID NO: 36	QQRE
SEQ ID NO: 37	QQREW
SEQ ID NO: 38	QQREL
SEQ ID NO: 39	QQREF
SEQ ID NO: 40	KGRE
SEQ ID NO: 41	KGREG
SEQ ID NO: 42	KDRE
SEQ ID NO: 43	KDREV
SEQ ID NO: 44	DECKL
SEQ ID NO: 45	NVCEL
SEQ ID NO: 46	GVEW
SEQ ID NO: 47	EPEW
SEQ ID NO: 48	GLER
SEQ ID NO: 49	DQEW
SEQ ID NO: 50	DLEW
SEQ ID NO: 51	GIEW
SEQ ID NO: 52	ELEW
SEQ ID NO: 53	GPEW
SEQ ID NO: 54	EWLP
SEQ ID NO: 55	GP ER
SEQ ID NO: 56	(GGGGS)N
SEQ ID NO: 57	GGGGS GGGGS GGGGS
SEQ ID NO: 58	GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 59	AAA
SEQ ID NO: 60	SGGS GGS
SEQ ID NO: 61	GGGGS GGS
SEQ ID NO: 62	GGGGS GGGGS
SEQ ID NO: 63	GGGGS GGGGS GGGGS GGS
SEQ ID NO: 64	GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 65	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 66	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 67	GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS
SEQ ID NO: 68	EPKSCDKHTCPPCP
SEQ ID NO: 69	GGGSGGGSEPKSCDKHTCPPCP
SEQ ID NO: 70	EPKTPKPQAAA
SEQ ID NO: 71	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPR CPEPKSCDTPPPCPRCP
SEQ ID NO: 72 CD25 ISVD	EVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAMGWYRQAPGKQ RELVASITGGGSTNYADSVKGRFTISRDNKNTVYLQMNSLKPEDT ATYYCNIYRNVIPGRLSWGQGLTVTVSS
SEQ ID NO: 73 Alb8	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDNKNTTLYLQMNSLRPED TAVYYCTIGGSLSRSSQGTLTVTVSS

SEQ ID NO / Description	Amino Acid sequence
SEQ ID NO: 74 Alb23	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKG PEWVSSISGSGSDTLYADSVKGRFTISRDN SKNTLYLQMNSLRPED TAVYYCTIGGSLSRSSQGT LVTVSS
SEQ ID NO: 75 Alb129	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TATYYCTIGGSLSRSSQGT LVTVSSA
SEQ ID NO: 76 Alb132	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKG PEWVSSISGSGSDTLYADSVKGRFTISRDN SKNTLYLQMNSLRPED TATYYCTIGGSLSRSSQGT LVTVSSA
SEQ ID NO: 77 Alb11	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSRSSQGT LVTVSS
SEQ ID NO: 78 Alb11 (S112K)-A	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSRSSQGT LVKVSSA
SEQ ID NO: 79 Alb82	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSS
SEQ ID NO: 80 Alb82-A	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSA
SEQ ID NO: 81 Alb82-AA	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSAA
SEQ ID NO: 82 Alb82-AAA	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSAAA
SEQ ID NO: 83 Alb82-G	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSG
SEQ ID NO: 84 Alb82-GG	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSGG
SEQ ID NO: 85 Alb82-GGG	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSGGG
SEQ ID NO: 86 Alb223	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKG PEWVSSISGSGSDTLYADSVKGRFTISRDN SKNTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSSA
SEQ ID NO: 87 AlbX00001	EVQLVESGGGVVQPGGSLRLSCAASGLTFSSYAMGWFRQAPGKE RERVVSI SRGGGYTTYADSVKGRFTISRDN SENTVYLQMNSLRPED TALYYCAAARYWATGSEYEFDYWGQGT LVTVSS
SEQ ID NO: 88 Alb23002	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKG PEWVSSISGSGSDTLYADSVKGRFTISRDN SKNTLYLQMNSLRPED TALYYCTIGGSLSRSSQGT LVTVSS
SEQ ID NO: 89 ISVD C-terminus	VTVSS(X)n
SEQ ID NO: 90	VKVSS

SEQ ID NO / Description	Amino Acid sequence
ISVD C-terminus	
SEQ ID NO: 91 ISVD C-terminus	VQVSS
SEQ ID NO: 92 ISVD C-terminus	VTVKS
SEQ ID NO: 93 ISVD C-terminus	VTVQS
SEQ ID NO: 94 ISVD C-terminus	VKVKS
SEQ ID NO: 95 ISVD C-terminus	VKVQS
SEQ ID NO: 96 ISVD C-terminus	VQVKS
SEQ ID NO: 97 ISVD C-terminus	VQVQS
SEQ ID NO: 98 ISVD C-terminus	VTVSSA
SEQ ID NO: 99 ISVD C-terminus	VKVSSA
SEQ ID NO: 100 C-terminus	VQVSSA
SEQ ID NO: 101 C-terminus	VTVKSA
SEQ ID NO: 102 C-terminus	VTVQSA
SEQ ID NO: 103 C-terminus	VKVKSA
SEQ ID NO: 104 C-terminus	VKVQSA
SEQ ID NO: 105 C-terminus	VQVKSA
SEQ ID NO: 106 C-terminus	VQVQSA
SEQ ID NO: 107 forward primer ABL051	GGCTGAGCTGGGTGGTCCTGG
SEQ ID NO: 108 forward primer ABL052	GGCTGAGTTTGGTGGTCCTGG
SEQ ID NO: 109 reverse primer ABL003	GGTACGTGCTGTTGAACTGTTCC
SEQ ID NO: 110 forward primer Fw_Nb_Mfel	GAGGTGCAATTGGTGGAGTCTGGGGG
SEQ ID NO: 111 reverse primer Rev_Lib_Eco91I	TGAGGAGACGGTGACCAGGGT).
SEQ ID NO: 112 FR1	EVQLVESGGGLVQAGGSLRLTCAAS
SEQ ID NO: 113 CDR1	RNIFSSNAMG

SEQ ID NO / Description	Amino Acid sequence
SEQ ID NO: 114 FR2	WYRQAPGKQRELVA
SEQ ID NO: 115 CDR2	SITGGGSTN
SEQ ID NO: 116 FR3	YADSVKGRFTISRDNKNTVYLQMNSLKPEDTATYYCNI
SEQ ID NO: 117 CDR3	YRNVIPGRLS
SEQ ID NO: 118 FR4	WGQGTTLTVSS
SEQ ID NO: 119 Nb59 multispecific binding protein amino acid sequence	DVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAMGWYRQAPGKQ RELVASITGGGSTNYADSVKGRFTISRDNKNTVYLQMNSLKPEDT ATYYCNIYRNVIPGRLSWGQGTTLTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAA SGFTFSDYWMYWRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTI SRDNKNTLYLQMNSLRPEDTALYYCARSPSGFNRGQGTTLTVSS GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVE SGGGVVQPGGSLRLSCAASGFTFSDYWMYWRQAPGKGLEWVS EINTNGLITKYPDSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYC ARSPSGFNRGQGTTLTVSSGGGGSGGGGSEVQLVESGGGVVQPG GSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTL YADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSS QGTTLTVSSA
SEQ ID NO: 120 Nb60 multispecific binding protein amino acid sequence	DVQLVESGGGLVQAGGSLRLTCAASRNIFSSNAMGWYRQAPGKQ RELVASITGGGSTNYADSVKGRFTISRDNKNTVYLQMNSLKPEDT ATYYCNIYRNVIPGRLSWGQGTTLTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAA SGLTFSTNPMYWRQAPGKQRELVASISSRGITNYADSVKGRFTIS RDNSKNTVYLQMNSLRPEDTALYYCRLASLSSGTVYWQGTTLTV SSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQL VESGGGVVQPGGSLRLSCAASGLTFSTNPMYWRQAPGKQRELVA SISRGITNYADSVKGRFTISRDNKNTVYLQMNSLRPEDTALYYC RLASLSSGTVYWQGTTLTVSSGGGGSGGGGSEVQLVESGGGVVQ PGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSD TLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTTLTVSSA

CLAIMS

1. A method for degrading a target protein, comprising:
contacting a CD25 positive cell with a multispecific binding protein, wherein the multispecific binding protein comprises:
 - a) a first cell surface binding moiety that specifically binds to CD25 on the surface of the CD25 positive cell, wherein the first cell surface binding moiety comprises at least one immunoglobulin domain or an antigen binding fragment thereof; and
 - b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to the target protein,
wherein specific binding of the multispecific binding protein to the CD25 facilitates the internalization of the target protein into the CD25 positive cell.
2. The method of claim 1, wherein the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein.
3. The method of claim 1 or 2, wherein the CD25 and/or the multispecific binding protein is recycled back to the surface of the cell independent of the target protein.
4. The method of any one of claims 1-3, wherein the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell.
5. The method of any one of claims 1-4, wherein the CD25 positive cell is a neoplastic cell.
6. The method of any one of claims 1-5, wherein the CD25 positive cell is a T-cell or a NK cell.
7. The method of any one of claims 1-6, wherein the multispecific binding protein exhibits increased degradation of the target protein compared to a reference binding polypeptide.

8. The method of claim 7, wherein the reference binding polypeptide does not comprise the first cell surface binding moiety that specifically binds to CD25 but is otherwise identical to the multispecific binding protein.
9. The method of claim 7 or 8, wherein the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide.
10. The method of any one of claims 7-9, wherein the multispecific binding protein degrades the target protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide.
11. The method of any one of claims 1-10, wherein the first cell surface binding moiety binds an extracellular domain of CD25.
12. The method of any one of claims 1-11, wherein the first cell surface binding moiety does not inhibit the binding of IL-2 to CD25.
13. The method of any one of claims 1-12, wherein the first cell surface binding moiety does not inhibit the signaling of IL-2 *via* CD25.
14. The method of any one of claims 1-13, wherein the first cell surface binding moiety comprises at least one CD25 specific variable domain.
15. The method of claim 14, wherein the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide.
16. The method of claim 15, wherein the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide *via* an amino acid linker.
17. The method of 16, wherein the amino acid linker is at least 90% identical to an amino acid linker sequence encoded by an amino acid linker sequence set forth in Table 10.

18. The method of any one of claims 15-17, wherein the first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.
19. The method of any one of claims 1-18, wherein the first cell surface binding moiety comprises a fusion protein comprising a CD25 specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25:Fc fusion polypeptide or a variant thereof.
20. The method of any one of claims 1-19, wherein the second binding moiety that specifically binds to the target protein comprises at least one target specific variable domain.
21. The method of claim 20, wherein the target specific variable domain is operatively linked to a second Fc domain polypeptide.
22. The method of claim 21, wherein the target specific variable domain is operatively linked to a second Fc domain polypeptide *via* an amino acid linker.
23. The method of claim 21 or 22, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker encoded by an amino acid linker sequence set forth in Table 10.
24. The method of any one of claims 19-23, wherein the second Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.
25. The method of any one of claims 1-24, wherein the second binding moiety comprises a fusion protein comprising a target specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-target:Fc fusion polypeptide or a variant thereof.
26. The method of any one of claims 1-25, wherein the second binding moiety that specifically binds to the target protein comprises an antibody or an antigen binding fragment thereof.

27. The method of claim 26, wherein the first and second IgG Fc domain polypeptides dimerize to form the multispecific binding protein.
28. The method of claim 27, wherein the first and second IgG Fc domain polypeptides dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof.
29. The method of claim 27 or 28, wherein the first IgG Fc domain polypeptide comprises a knob substitution, and the second IgG Fc domain polypeptide comprises a hole substitution or wherein the first IgG Fc domain polypeptide comprises a hole substitution, and the second IgG Fc domain polypeptide comprises a knob substitution.
30. The method of any one of claims 28-29, wherein the knob substitution is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W).
31. The method of any one of claims 28-30, wherein the hole substitution is selected from the group consisting of alanine (A), asparagine (N), aspartic acid (D), glycine (G), serine (S), threonine (T), and valine (V).
32. The method of any one of claims 1-31, wherein the first cell surface binding moiety and the second binding moieties of the multispecific binding moiety each independently comprises a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®.
33. The method of claim 32, wherein the scFV is a linear scFV or a tandem scFV.
34. The method of claim 32 or 33, wherein the multispecific binding protein further comprises a Fc domain or a variant thereof.

35. The method of any one of claims 32-34, wherein the ISVD is a VHH, a VH, or a VNAR.

36. The method of claim 35, wherein the ISVD is a humanized VHH, a camelized VH, a camelized human VH, a domain antibody, a single domain antibody, or a dAb.

37. The method of anyone of claims 32-36, wherein the second binding moiety comprises at least one ISVD.

38. The method of claim 37, wherein the second binding moiety comprises at least two ISVDs.

39. The method of claim 38, wherein the second binding moiety comprises at least two ISVDs that bind the same target protein.

40. The method of claim 39, wherein the at least two ISVDs bind to the same or different epitopes on the same target protein.

41. The method of any one of claims 1-40, wherein the target protein is a membrane-associated target protein, a soluble target protein, or both.

42. The method of claim 41, wherein the target protein is expressed on the surface of the same or a different a CD25 positive cell.

43. The method of claims 1-42, wherein the target protein is expressed on the surface of a neoplastic cell and/or an immune cell.

44. The method of any one of claims 1-43, wherein the target protein is expressed on a T-cell.

45. The method of claim 44, wherein the T-cell is an activated T cell or a regulatory T (Treg) cell.

46. The method of claims 1-45, wherein the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein.
47. The method of any one of claims 1-46, wherein the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein.
48. The method of any one of claims 1-47, wherein the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.
49. The method of any one of claims 1-48, wherein the multispecific binding protein exhibits pH-dependent binding to CD25 on the CD25 positive cell.
50. The method of any one of claims 1-49, wherein the multispecific binding protein exhibits pH-dependent binding to the target protein.
51. The method of any one of claims 1-50, wherein the multispecific binding protein exhibits reduced binding at acidic pH.
52. The method of any one of claims 1-51, wherein the first cell surface binding moiety of the multispecific binding protein binds to CD25 on the CD25 positive cell with an affinity from about 100 pM to about 1 μ M.
53. The method of any one of claims 1-52, wherein the second binding moiety of the multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M.

54. The method of any one of claims 1-53, wherein the multispecific binding protein comprises one or more mutations or glycan modifications to modulate Fc mediated effector function.
55. The method of any one of claims 1-54, wherein the multispecific binding protein comprises one or more mutations to modulate serum half-life.
56. A multispecific binding protein comprising:
- a) a first cell surface binding moiety that specifically binds to CD25 on the surface of a CD25 positive cell, wherein the first cell surface binding moiety comprises an immunoglobulin domain; and
 - b) a second binding moiety that is operatively linked to the first cell surface binding moiety and that specifically binds to a target protein,
- wherein specific binding of the multispecific binding protein to the CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.
57. The multispecific binding protein of claim 56, wherein the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein.
58. The multispecific binding protein of claim 56 or 57, wherein the CD25 and/or the multispecific binding protein is recycled back to the surface of the cell independent of the target protein.
59. The multispecific binding protein of any one of claims 56-58, wherein the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell.
60. The multispecific binding protein of any one of claims 56-59, wherein the CD25 positive cell is a neoplastic cell.
61. The multispecific binding protein of any one of claims 56-60, wherein the CD25 positive cell is a T-cell or a NK cell.

62. The multispecific binding protein of any one of claims 56-61, wherein the multispecific binding protein exhibits increased degradation of the target protein compared to a reference binding polypeptide.
63. The multispecific binding protein of claim 62, wherein the reference binding polypeptide does not comprise the first cell surface binding moiety that specifically binds to CD25 but is otherwise identical to the multispecific binding protein.
64. The multispecific binding protein of claim 62 or 63, wherein the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide.
65. The multispecific binding protein of any one of claims 62-64, wherein the multispecific binding protein degrades the target protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide.
66. The multispecific binding protein of any one of claims 56-65, wherein the first cell surface binding moiety binds an extracellular domain of CD25.
67. The multispecific binding protein of any one of claims 56-66, wherein the first cell surface binding moiety does not inhibit the binding of IL-2 to CD25.
68. The multispecific binding protein of any one of claims 56-67, wherein the first cell surface binding moiety does not inhibit the signaling of IL-2 *via* CD25.
69. The multispecific binding protein of any one of claims 56-68, wherein the first cell surface binding moiety comprises at least one CD25 specific variable domain.
70. The multispecific binding protein of claim 69, wherein the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide.

71. The multispecific binding protein of claim 70, wherein the CD25 specific variable domain is operatively linked to a first Fc domain polypeptide *via* an amino acid linker.
72. The multispecific binding protein of 71, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker encoded by an amino acid linker sequence set forth in Table 10.
73. The multispecific binding protein of claim 70-72, wherein the first Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.
74. The multispecific binding protein of any one of claims 56-73, wherein the first cell surface binding moiety comprises a fusion protein comprising a CD25 specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-CD25:Fc fusion polypeptide or a variant thereof.
75. The multispecific binding protein of any one of claims 56-74, wherein the second binding moiety that specifically binds to the target protein comprises at least one target specific variable domain.
76. The multispecific binding protein of claim 74 or 75, wherein the target specific variable domain is operatively linked to a second Fc domain polypeptide.
77. The multispecific binding protein of claim 76, wherein the target specific variable domain is operatively linked to a second Fc domain polypeptide *via* an amino acid linker.
78. The multispecific binding protein of 77, wherein the amino acid linker is at least 90% identical to an amino acid linker sequence encoded by an amino acid linker sequence set forth in Table 10.
79. The multispecific binding protein of any one of claims 76-78, wherein the second Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide.

80. The multispecific binding protein of any one of claims 56-79, wherein the second binding moiety comprises a fusion protein comprising a target specific variable domain and a fragment crystallizable (Fc) domain or a variant thereof, to form an anti-target:Fc fusion polypeptide or a variant thereof.
81. The multispecific binding protein of any one of claims 56-80, wherein the second binding moiety that specifically binds to the target protein comprises an antibody or an antigen binding fragment thereof.
82. The multispecific binding protein of claim 81, wherein the first and second IgG Fc domain polypeptides dimerize to form the multispecific binding protein.
83. The multispecific binding protein of claim 82, wherein the first and second IgG Fc domain polypeptides dimerize by knobs-into-holes interactions, Fab arm exchange (FAE), electrostatic steering interactions, hydrophobic interactions, or any combination thereof.
84. The multispecific binding protein of claim 82 or 83, wherein the first IgG Fc domain polypeptide comprises a knob substitution, and the second IgG Fc domain polypeptide comprises a hole substitution or wherein the first IgG Fc domain polypeptide comprises a hole substitution, and the second IgG Fc domain polypeptide comprises a knob substitution.
85. The multispecific binding protein of any one of claims 83-84, wherein the knob substitution is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W).
86. The multispecific binding protein of any one of claims 83-85, wherein the hole substitution is selected from the group consisting of alanine (A), asparagine (N), aspartic acid (D), glycine (G), serine (S), threonine (T), and valine (V).
87. The multispecific binding protein of any one of claims 56-86, wherein the first cell surface binding moiety and the second binding moieties of the multispecific binding moiety each independently comprises a VH, a Fab, a Fab', a F(ab')₂, a

variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®.

88. The multispecific binding protein of claim 87, wherein the scFV is a linear scFV or a tandem scFV.

89. The multispecific binding protein of claim 87 or 88, wherein the ISVD is a VHH, humanized VHH, a camelized VH, a single domain antibody, a domain antibody, a dAb, or a VNAR.

90. The multispecific binding protein of claim 89, wherein the second binding moiety comprises at least one ISVD.

91. The multispecific binding protein of claim 90, wherein the second binding moiety comprises at least two ISVDs.

92. The multispecific binding protein of claim 91, wherein the second binding moiety comprises at least two ISVDs that bind the same target protein.

93. The multispecific binding protein of claim 91, wherein the at least two ISVDs bind to the same or different epitope on the same target protein.

94. The multispecific binding protein of any one of claims 87-93, wherein the multispecific binding protein further comprises a Fc domain or a variant thereof.

95. The multispecific binding protein of any one of claims 56-94, wherein the target protein is a membrane-associated target protein, a soluble target protein, or both.

96. The multispecific binding protein of claim 95, wherein the target protein is expressed on the surface of the same or a different a CD25 positive cell.

97. The multispecific binding protein of any one of claims 56-96, wherein the target protein is expressed on the surface of a neoplastic cell and/or an immune cell.

98. The multispecific binding protein of any one of claims 56-97, wherein the target protein is expressed on a T-cell.

99. The multispecific binding protein of claim 98, wherein the T-cell is an activated T cell or a regulatory T (Treg) cell.

100. The multispecific binding protein of any one of claims 56-99, wherein the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein.

101. The multispecific binding protein of any one of claims 56-100, wherein the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein.

102. The multispecific binding protein of any one of claims 56-101, wherein the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.

103. The multispecific binding protein any one of claims 56-102, wherein the multispecific binding protein exhibits pH-dependent binding to CD25 on the CD25 positive cell.

104. The multispecific binding protein any one of claims 56-103, wherein the multispecific binding protein exhibits pH-dependent binding to the target protein.

105. The multispecific binding protein any one of claims 56-104, wherein the multispecific binding protein exhibits reduced binding at acidic pH.
106. The multispecific binding protein any one of claims 56-105, wherein the first cell surface binding moiety of the multispecific binding protein binds to CD25 on the CD25 positive cell with an affinity from about 100 pM to about 1 μ M.
107. The multispecific binding protein any one of claims 56-106, wherein the second binding moiety of the multispecific binding protein binds to the target protein with an affinity from about 100 pM to about 1 μ M.
108. The multispecific binding protein any one of claims 56-107, wherein the multispecific binding protein comprises one or more mutations or glycan modifications to modulate Fc mediated effector function.
109. The multispecific binding protein any one of claims 56-108, wherein the multispecific binding protein comprises one or more mutations to modulate serum half-life.
110. A pharmaceutical composition comprising the multispecific binding protein of any one of the preceding claims and a pharmaceutically acceptable carrier or diluent.
111. A nucleic acid molecule encoding the multispecific binding protein of any one of claims 1-110.
112. A vector comprising the nucleic acid molecule of claim 111.
113. A cell comprising the nucleic acid molecule of claim 111 or the vector of claim 112.
114. A method of depleting a target protein comprising administering to a subject an effective amount of the multispecific binding protein of any one of claims 56-109 or the pharmaceutical composition of claim 110.

115. The method of claim 114, wherein the multispecific binding protein is internalized by the CD25 positive cell.
116. The method of claim 115, wherein an amount of multispecific binding protein internalized by the CD25 positive cell is greater than an amount of a reference binding polypeptide that does not comprise the first cell surface binding moiety.
117. The method of any one of claims 114-116, wherein the target protein is selectively depleted from a target tissue or circulation of the subject.
118. The method of claim 117, wherein administering the multispecific binding protein results in at least about 10%, 20, 30%, 40%, 50%, 75%, or 90% depletion of the target protein from the target tissue or circulation of the subject.
119. The method of treating a disease comprising administering an effective amount of a multispecific binding protein of any one of claims 56-109 or the pharmaceutical composition of claim 110 to a subject.
120. The method of claim 119, wherein the disease is selected from a group consisting of: cancer, autoimmune disease, inflammatory disorder, infectious disease, and neurodegenerative disorder.
121. A binding protein comprising at least one ISVD that specifically binds to a CD25 (CD25 ISVD) on the surface of a CD25 positive cell.
122. The binding protein of claim 121, wherein the CD25 ISVD binds an extracellular domain of a CD25 protein.
123. The binding protein of claim 121 or 122, wherein the cell comprises human or cynomolgus CD25 protein (hCD25 or cynoCD25, respectively).
124. The binding protein of any one of claims 121-123, wherein the binding protein is cross-reactive to hCD25 or cynoCD25 but not to CD25 from other species.

125. The binding protein of any one of claims 121-124, wherein the binding protein specifically binds to hCD25 and cynoCD25.

126. The binding protein of any one of claims 121-125, wherein the binding protein is an antagonist of CD25 activity.

127. The binding protein of claim 126, wherein the binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25.

128. The binding protein of claim 127, wherein the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay.

129. The binding protein of any one of claims 121-128, wherein the CD25 ISVD binding to CD25 on the cell surface does not result in CD25 degradation.

130. The binding protein of any one of claims 121-129, wherein the CD25 positive cell is an immune cell, a white blood cell, or a hematopoietic cell.

131. The method of any one of claims 121-130, wherein the CD25 positive cell is a neoplastic cell.

132. The method of any one of claims 121-131, wherein the CD25 positive cell is a T-cell or a NK cell.

133. The binding protein of any one of claims 121-132, wherein the binding protein comprises at least two CD25 ISVDs.

134. The binding protein of claim 133, wherein the at least two CD25 ISVDs bind the same CD25 protein.

135. The binding protein of claim 134, wherein the at least two CD25 ISVDs bind to the same or different epitopes on the same CD25 protein.

136. The binding protein of any one of claims 133-135, wherein the at least two CD25 ISVDs are operatively linked via an amino acid linker sequence.

137. The binding protein of any one of claims 121-136, wherein the binding protein specifically binds to hCD25 with:

(a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 2×10^{-8} , 1.7×10^{-8} , 1.5×10^{-8} , 1×10^{-8} , 5×10^{-9} , 1×10^{-9} ;

(b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 3.5×10^{-3} , 3.4×10^{-3} , 1×10^{-3} , 5×10^{-4} , 10^{-4} ; or

(c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.5×10^5 , 2.0×10^5 , 5×10^5 , 10^6 ,

as measured by surface plasmon resonance (SPR).

138. The binding protein of any one of claims 121-137, wherein the binding protein specifically binds to cynoCD25 with:

(a) a K_D (M) of between 5×10^{-8} and 10^{-9} , between 2×10^{-8} and 10^{-9} , such as of about 5×10^{-8} , 3.5×10^{-8} , 10^{-8} , 5×10^{-9} , 10^{-9} ;

(b) a k_d (1/s) of between 10^{-2} and 10^{-4} , between 5×10^{-3} and 10^{-3} , such as of about 5×10^{-3} , 4×10^{-3} , 10^{-3} , 5×10^{-4} , 10^{-4} ; or

(c) a k_a (1/Ms) of between 10^5 and 10^6 , between 10^5 and 5×10^5 , such as of about 1.0×10^5 , 1.2×10^5 , 5×10^5 , 1×10^6 ,

as measured by surface plasmon resonance (SPR).

139. The binding protein of any one of claims 121-138, wherein the binding protein has an EC_{50} value for binding to human or cyno CD25 on HEK293-MZA cells of less than $10^{-8}M$, such as less than $5 \cdot 10^{-9}M$, such as between $5 \cdot 10^{-9}M$ and $2 \times 10^{-9}M$, as measured in a FACS binding assay.

140. The binding protein of any one of claims 121-139, wherein the binding protein has an IC_{50} value in competition with IL-2 for binding to

- human CD25 on HEK293-MZA cells of less than $10^{-8}M$, such as between $10^{-8}M$ and $10^{-9}M$.

- cyno CD25 on HEK293-MZA cells of less than $10^{-7}M$, such as between $10^{-7}M$ and $10^{-8}M$,

as measured in a FACS competition assay.

141. The binding protein of any one of claims 121-140, wherein the CD25 ISVD, consists essentially of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 (AbM numbering) has an amino acid sequence selected from:
 - a) the amino acid sequence of SEQ ID NO: 113;
 - b) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 113; or
 - c) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 113;

and

- CDR2 (AbM numbering) has an amino acid sequence selected from:
 - d) the amino acid sequence of SEQ ID NO: 115;
 - e) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 115; or
 - f) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 115;

and

- CDR3 (AbM numbering) has an amino acid sequence selected from:
 - g) the amino acid sequence of SEQ ID NO: 117;
 - h) amino acid sequences that have at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 117; or
 - i) amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 117.

142. The binding protein of claims 141, in which:

- CDR1 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 113;
- CDR2 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 115;

and

- CDR3 (AbM numbering) has an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 117.

143. The binding protein of any one of claims 121-142, wherein the CD25 ISVD comprises at least 80%, 85%, 90%, or 95% identical to an amino acid sequence set forth in SEQ ID NO: 72.

144. The binding protein of any one of claims 141-143, wherein the CD25 ISVD consists essentially of SEQ ID NO: 72.

145. The binding protein of any one of claims 121-144, where the CD25 ISVD consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb.

146. The binding protein of claim 145, wherein the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation.

147. The binding protein of any one of claims 121-146, wherein the VHH sequence is a humanized VHH sequence.

148. The binding protein of claim 121-147, wherein the binding protein is operatively linked to a Fc domain polypeptide or variant thereof.

149. The binding protein of claim 148, wherein the binding protein is operatively linked to a Fc domain polypeptide or variant thereof *via* an amino acid linker sequence.

150. The binding protein claim 149, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

151. The binding protein of claim 148-150, wherein the Fc domain polypeptide is an immunoglobulin G (IgG) Fc domain polypeptide or variant thereof.

152. The binding protein of any one of claims 121-151, wherein the binding protein comprises a fusion protein comprising a CD25 ISVD and a fragment crystallizable

(Fc) domain or a variant thereof, to form an anti-CD25 ISVD:Fc fusion polypeptide or a variant thereof.

153. The binding protein of claim 121-152, wherein the binding protein is operatively linked to a serum albumin ISVD.

154. The binding protein of claim 153, wherein the serum albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88.

155. The binding protein of claim 154, wherein the serum albumin ISVD consists essentially of SEQ ID NO: 88.

156. The binding protein of any one of claims 153-155, wherein the CD25 ISVD is operatively linked to the serum albumin ISVD via an amino acid linker sequence.

157. The binding protein claim 156, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

158. The binding protein of any one of claims 121-157, further comprising one or more other groups, residues, moieties or binding units, optionally linked *via* one or more amino acid linker sequence(s).

159. The binding protein of claim 158, wherein one or more other groups, residues, moieties or binding units are ISVDs.

160. The binding protein of claim 159, wherein one or more other groups, residues, moieties or binding units are chosen from the group consisting of VHHs, humanized VHHs, camelized VHs, domain antibodies, single domain antibodies and dAbs.

161. The binding protein of any one of claims 121-160, wherein the binding protein comprises one or more mutations or glycan modifications to modulate Fc mediated effector function.

162. The binding protein of any one of claims 121-161, wherein the binding protein comprises one or more mutations to modulate serum half-life.

163. A pharmaceutical composition comprising the binding protein of any one of claims 121-162 and a pharmaceutically acceptable carrier or diluent.

164. A nucleic acid molecule encoding the multispecific binding protein of any one of claims 121-162.

165. A vector comprising the nucleic acid molecule of claim 164.

166. A cell comprising the nucleic acid molecule of claim 164 or the vector of claim 165.

167. A method of treating a neoplastic disorder in a subject comprising administering to a subject an effective amount of the binding protein of any one of claims 121-162 or the pharmaceutical composition of claim 163.

168. A method of treating an autoimmune disorder comprising administering to a subject an effective amount of the binding protein of any one of claims 121-162 or the pharmaceutical composition of claim 163.

169. A method of depleting human antigen-specific CD25⁺ regulatory T cells in a subject comprising administering to a subject an effective amount of the binding protein of any one of claims 121-162 or the pharmaceutical composition of claim 163.

170. The binding protein of any one of claims 121-162, wherein the binding protein is a multispecific binding protein.

171. The binding protein of claim 170, wherein the multispecific binding protein comprises at least one CD25 ISVD and a second binding moiety that specifically binds to a target protein.

172. The binding protein of claim 171, wherein the binding of the multispecific binding protein to the cell surface CD25 facilitates internalization of the target protein bound to the multispecific binding protein into the CD25 positive cell.

173. The binding protein of claim 172, wherein the target protein bound to the multispecific binding protein traffics to lysosomes for degradation of the target protein.

174. The binding protein of any one of claims 170-173, wherein the target protein is an immune checkpoint protein, a cancer antigen, and/or an immunomodulatory protein.

175. The binding protein of any one of claims 170-175, wherein the target protein is selected from the group consisting of: an antibody, an autoantibody, an inflammatory protein, an interleukin, a cytokine, an interferon, a tumor necrosis factor (TNF), a growth factor, a hormone, a neurotransmitter, a lipid mediator, an activating factor, an extracellular matrix (ECM) protein, a Wnt protein, a member of the Transforming Growth Factor-beta (TGF- β) Family, a Notch ligand, a Fc receptor-like protein 3 (FcRL3), and an immune checkpoint protein.

176. The binding protein of any one of claims 170-175, wherein the target protein is associated with a disease selected from the group consisting of: a cancer, an autoimmune disease, an inflammatory disorder, an infectious disease, and a neurodegenerative disorder.

177. The binding protein of any one of claims 170-176, wherein the second binding moiety that specifically binds to the target protein comprises at least one antigen binding fragment.

178. The binding protein of any one of claims 170-177, wherein the target antigen binding fragment comprises at least one of a VH, a Fab, a Fab', a F(ab')₂, a variable fragment (Fv), a disulfide-stabilized Fv (dsFv), a single-chain Fv (scFv), a diabody, a

triabody, an immunoglobulin single variable domain (ISVD), or an AFFIBODY®, a linear scFV, or a tandem scFV.

179. The binding protein of claim 178, wherein the target antigen binding fragment comprises at least one ISVD that specifically binds the target protein.

180. The binding protein of claim 179, wherein the target antigen binding fragment comprises at least two ISVDs that specifically bind the target protein.

181. The binding protein of any one of claims 178-180, wherein the target protein ISVD(s) consists essentially of a VHH, a humanized VHH, a camelized VH, a domain antibody, a single domain antibody, or a dAb.

182. The binding protein of claim 181, wherein the VHH sequence is a humanized VHH sequence or a VHH sequence that has been obtained by affinity maturation.

183. The binding protein of any one of claims 177-182, wherein the VHH sequence is a humanized VHH sequence.

184. The binding protein of any one of claims 180-183, wherein at least two target protein ISVDs are operatively linked via an amino acid linker sequence.

185. The binding protein of claim 184, wherein the at least two target protein ISVDs bind the same target protein.

186. The binding protein of claim 185, wherein the at least two target protein ISVDs bind to the same or different epitopes on the same target protein.

187. The binding protein of claim 170-186, wherein the binding protein is operatively linked to a serum albumin ISVD.

188. The binding protein of claim 187, wherein the serum albumin ISVD is an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 73-88.

189. The binding protein of claim 188, wherein the serum albumin ISVD consists essentially of SEQ ID NO: 88.

190. The binding protein of any one of claims 170-189, wherein the second binding moiety that specifically binds to the target protein is operatively linked to the serum albumin ISVD via an amino acid linker sequence.

191. The binding protein claim 190, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker sequence amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

192. The binding protein of any one of claims 170-191, wherein the binding protein is encoded by an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 119.

193. A multispecific binding protein comprising:

- a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD;
- b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD; and
- c) a third binding moiety that specifically binds to serum albumin ISVD.

194. A multispecific binding protein comprising:

- a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a second binding moiety;
- b) the second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a third binding moiety; and
- c) the third binding moiety that specifically binds to serum albumin ISVD.

195. A multispecific binding protein comprising:

a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a second binding moiety;

b) the second binding moiety that specifically binds to a target protein comprising at least two target protein ISVDs wherein one of the at least two target protein ISVDs are linked to the N- and/or C-terminal end of a third binding moiety; and

c) the third binding moiety that specifically binds to serum albumin ISVD.

196. The multispecific binding protein of claim 194 or 195, wherein the multispecific binding protein comprises at least two target protein ISVDs and the at least two target protein ISVDs are at least two TNF ISVDs that both specifically bind to TNF.

197. The multispecific binding protein of claim 196, wherein the at least two TNF ISVDs bind the same or different TNF epitopes.

198. The multispecific binding protein of any one of claims 193-197, wherein the serum albumin ISVD comprises SEQ ID NO: 88.

199. The multispecific binding protein of any one of claims 192-198, wherein the multispecific binding protein comprises an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence set forth in SEQ ID NO: 119.

200. The multispecific binding protein of any one of claims 170-199, wherein the multispecific binding protein exhibits increased degradation of the target protein by at least 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 percent compared to the reference binding polypeptide.

201. The multispecific binding protein of any one of claims 170-200, wherein the multispecific binding protein degrades the target protein at least 2, 3, 4, 5, 10, 24, 48, or 72 hours faster compared to the reference binding polypeptide.

202. The binding protein of any one of claims 170-201, wherein the CD25 ISVD(s) and the target protein ISVD(s) are operatively linked to a first and second Fc domain polypeptide, respectively.

203. The binding protein of claim 202, wherein the CD25 ISVD(s) and the target protein ISVD(s) are operatively linked to the first and second Fc domain polypeptide *via* an amino acid linker sequence.

204. The binding protein of claim 203, wherein the amino acid linker sequence is at least 90% identical to an amino acid linker sequence encoded by an amino acid sequence set forth in Table 10.

205. The binding protein of any one of claims 202-204, wherein the first and second Fc domain polypeptides each comprise a first and a second IgG domain that dimerize to form the multispecific binding protein.

206. A multispecific binding protein comprising:

a) a first cell surface binding moiety that specifically binds to CD25 comprising at least one CD25 ISVD wherein the at least one CD25 ISVD is linked to the N- and/or C- terminal end of a first IgG; and

b) a second binding moiety that specifically binds to a target protein comprising at least one target protein ISVD wherein the at least one target protein ISVD is linked to the N- and/or C-terminal end of a second IgG; and wherein each the first and second IgG domains heterodimerize to form a Fc domain or variant thereof.

207. A pharmaceutical composition comprising the binding protein of any one of claims 170-206 and a pharmaceutically acceptable carrier or diluent.

208. A nucleic acid molecule encoding the multispecific binding protein of any one of claims 170-206.

209. A vector comprising the nucleic acid molecule of claim 208.

210. A cell comprising the nucleic acid molecule of claim 208 or the vector of claim 209.

211. A method of treating a disease comprising administering an effective amount of the multispecific binding protein of any one of claims 170-206 or the pharmaceutical composition of claim 207 to a subject.

212. The method of claim 211, wherein the disease is selected from a group consisting of: neoplastic disorder, cancer, autoimmune disease, inflammatory disorder, infectious disease, and neurodegenerative disorder.

213. The method of any one of claims 1-11, wherein the binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25.

214. The method of claim 214, wherein the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay.

215. The multispecific binding protein of any one of claims 56-67, wherein the binding protein is a competitive inhibitor of soluble human IL-2 binding to CD25.

216. The multispecific binding protein of claim 215, wherein the binding protein blocks binding of human IL-2 to hCD25 on the cell surface by at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% as determined by a competitive binding assay.

217. The method of claim 9 or 10, wherein the multispecific binding protein exhibits increased degradation of the target protein compared to the reference binding polypeptide as assessed by confocal microscopy.

218. The multispecific binding protein of claim 64 or 65, wherein the multispecific binding protein exhibits increased degradation of the target protein compared to the reference binding polypeptide as assessed by confocal microscopy.