

CLAIMS

~~What is claimed is:~~

1. A multispecific antigen-binding protein comprising at least two VL regions respectively paired with at least two VH regions to form at least two antigen-binding sites and at least two CH1 regions respectively paired with two CL regions,

wherein at least one CH1/CL pair comprises CH1/CL mutations to facilitate pairing selected from the group consisting of one or more of

- (1) a T192E (CH1) mutation and N137K and S114A (CL) mutations, and
- (2) L143Q and S188V (CH1) mutations, and V133T and S176V (CL) mutations, and
- (3) T192E, L143Q and S188V (CH1) mutations and N137K, S114A, V133T and S176V (CL) mutations,
- (4) a K221E (CH1) mutation and a E123K (CL) mutation,
- (5) T192E and K221E (CH1) mutations and N137K, S114A and E123K (CL) mutations,
- (6) a L143E, a L143D, a L143K, a L143R, or a L143H (CH1) mutation and a S176E, a S176D, a S176K, a S176R, or a S176H (CL) mutation,
- (7) a L124E, a L124D, a L124K, a L124R, or a L124H (CH1) mutation and a V133E, a V133D, a V133K, a V133R, or a V133H (CL) mutation,
- (8) a K228D (CH1) mutation and a D122K (CL) mutation, and
- (9) K221E and K228D (CH1) mutations and D122K and E123K (CL) mutations,

wherein when two CH1/CL pairs comprise mutations to facilitate pairing for two different VH/VL pairs, the two CH1/CL pairs do not comprise same mutations, and

wherein at least one VH/VL pair comprise opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and

wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region.

2. A multispecific antigen-binding protein comprising: of claim 1, wherein at least one CH1 region is operably linked to a heterodimerization domain.

- a) a first light chain (LC1)/heavy chain (HC1) pair comprising:
- (1) a first VL region (VL1) paired with first VH region (VH1) to form a first antigen-binding site;
 - (2) a first constant heavy chain region 1 (CH1-1) operatively linked to VH1 and a first constant light chain region (CL1) operatively linked to VL1; and
 - (3) a first heterodimerization domain (HD1); and
- b) a second light chain (LC2)/heavy chain (HC2) pair comprising:
- (4) a second VL region (VL2) paired with a second VH region (VH2) to form a second antigen-binding site;
 - (5) a second constant heavy chain region 1 (CH1-2) operatively linked to VH2 and a second constant light chain region (CL2) operatively linked to VL2; and
 - (6) a second heterodimerization domain (HD2);

wherein HD1 and HD2 heterodimerize,

wherein at least one or both of VL1 and VH1 pair and of VL2 and VH2 pair comprises opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region,

wherein at least one or both of CL1 and CH1-1 pair and of CL2 and CH1-2 pair comprises mutations to facilitate pairing, and

wherein when both of CL1 and CH1-1 pair and of CL2 and CH1-2 pair comprise mutations to facilitate pairing, the mutations in CH1-1 and CL1 to facilitate pairing are different from the mutations in CH1-2 and CL2 to facilitate pairing.

3. A multispecific antigen-binding protein of claims 1, wherein at least one CH1 region is operably linked to a heterodimerization domain.

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3. A multispecific antigen-binding protein comprising at least two polypeptide chains and forming at least two antigen-binding sites, wherein one polypeptide chain comprises a structure represented by the formula:

VL1-L1-VL2-L2-CL [I]

and one polypeptide chain comprises a structure represented by the formula:

VH2-L3-VH1-L4-CH1 [II]

wherein:

VL1 is a first immunoglobulin light chain variable domain;

VL2 is a second immunoglobulin light chain variable domain;

VH1 is a first immunoglobulin heavy chain variable domain;

VH2 is a second immunoglobulin heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin CH1 heavy chain constant domain; and

L1, L2, L3, and L4 are amino acid linkers,

wherein VH1 is paired with VL1, VH2 is paired with VL2, and CH1 is paired with CL,

wherein the polypeptide of formula I and the polypeptide of formula II form a cross-over light chain-heavy chain pair,

wherein one or more cysteine residues are engineered into the one or both of VH1/VL1 and VH2/VL2 pairs to form one or more disulfide bonds,

wherein at least one or both of VL1 and VH1 pair and of VL2 and VH2 pair comprises opposite charged mutations that facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H,

wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region, and

wherein the CH1 and CL domain pair comprise mutations that facilitate pairing.

54. A multispecific antigen-binding protein comprising four polypeptide chains that form four antigen-binding sites, wherein two polypeptide chains each comprises a structure represented by the formula:

VL1-L1-VL2-L2-CL [I]

and two polypeptide chains each comprises a structure represented by the formula:

VH2-L3-VH1-L4-CH1-Fc [II]

wherein:

VL1 is a first immunoglobulin light chain variable domain;

VL2 is a second immunoglobulin light chain variable domain;

VH1 is a first immunoglobulin heavy chain variable domain;

VH2 is a second immunoglobulin heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin CH1 heavy chain constant domain;

Fc comprises an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1, L2, L3, and L4 are amino acid linkers,

wherein VH1 is paired with VL1 to form a first antigen-binding site, VH2 is paired with VL2 to form a second antigen-binding site, and CH1 is paired with CL,

wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair,

wherein one or more cysteine residues are engineered into the one or both of VH1/VL1 and VH2/VL2 pairs to form one or more disulfide bonds,

wherein one or both of the VL1 and VH1 pair and the VL2 and VH2 pair comprise opposite charged mutations that facilitate pairing, said opposite charged mutation comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position

38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region, and

wherein one or both of the CH1 and CL domain pair comprises mutations that facilitate pairing, and

wherein when at least two CH1/CL pairs comprise mutations to facilitate pairing, then the mutation set in one CH1/CL pair is different from the mutation set in the other CH1/CL pair.

65. A multispecific antigen-binding protein comprising four polypeptide chains, that form three antigen-binding sites, wherein

a first polypeptide chain comprises a structure represented by the formula:

VL2-L1-VL1-L2-CL1 [I],

a second polypeptide chain comprises a structure represented by the formula:

VH1-L3-VH2-L4-CH1-1-hinge-CH2-CH3 [II],

a third polypeptide chain comprises a structure represented by the formula:

VH3-CH1-2-hinge-CH2-CH3 [III], and

a fourth polypeptide chain comprises a structure represented by the formula:

VL3-CL2 [IV],

wherein:

VL1 is a first immunoglobulin light chain variable domain;

VL2 is a second immunoglobulin light chain variable domain;

VL3 is a third immunoglobulin light chain variable domain;

VH1 is a first immunoglobulin heavy chain variable domain;

VH2 is a second immunoglobulin heavy chain variable domain;

VH3 is a third immunoglobulin heavy chain variable domain;

CL1 is a first immunoglobulin light chain constant domain;

CL2 is a second immunoglobulin light chain constant domain;

CH1-1 is a first immunoglobulin CH1 heavy chain constant domain;

CH1-2 is a second immunoglobulin CH1 heavy chain constant domain;

CH2 is an immunoglobulin CH2 heavy chain constant domain;

CH3 is an immunoglobulin CH3 heavy chain constant domain;

hinge is an immunoglobulin hinge region connecting the CH1 and CH2 domains;
and

L1, L2, L3, and L4 are amino acid linkers,

wherein the polypeptide of formula I and the polypeptide of formula II form a cross-over light chain-heavy chain pair, and

wherein one or more cysteine residues are engineered into the one or more of VH1/VL1, VH2/VL2, and VH3/VL3 pairs to form one or more disulfide bonds,

wherein one or both of the VL1 and VH1 pair and the VL2 and VH2 pair comprise opposite charged mutations that facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region,

wherein one or both of the CL1 and CH1-1 pair and the CL2 and CH1-2 pair comprise mutations that facilitate pairing, and

wherein when both of CL1 and CH1-1 pair and of CL2 and CH1-2 pair comprise mutations to facilitate pairing, the mutations in CH1-1 and CL1 are different than the mutations in CH1-2 and CL2.

76. A multispecific antigen-binding protein comprising

a) _____ a first light chain (LC1)/heavy chain (HC1) pair comprising:

(1) _____ a first VL region (VL1) paired with first VH region (VH1) to form a first antigen-binding site;

(2) _____ a first constant heavy chain region 1 (CH1-1) operatively linked to VH1 and a first constant light chain region (CL1) operatively linked to VL1, and

b) _____ a second light chain (LC2)/heavy chain (HC2) pair comprising:

(3) _____ a second VL region (VL2) paired with a second VH region (VH2) to form a second antigen-binding site; and

(4) _____ a second constant heavy chain region 1 (CH1-2) operatively linked to VH2 and a second constant light chain region (CL2) operatively linked to VL2,

wherein the C terminus of CH1-1 is operatively linked to the N terminus of VH2,

wherein one or more cysteine residues are engineered into the one or both of VH1/VL1 and VH2/VL2 pairs to form one or more disulfide bonds,

wherein one or both of the VL1 and VH1 pair and the VL2 and VH2 pair comprise opposite charged mutations that facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region,

wherein one or both of the CL1 and CH1-1 pair and the CL2 and CH1-2 pair comprise mutations that facilitate pairing, and

wherein when both of CL1 and CH1-1 pair and of CL2 and CH1-2 pair comprise mutations to facilitate pairing, the mutations in CH1-1 and CL1 are different than the mutations in CH1-2 and CL2.

~~87.~~ A multispecific antigen-binding protein ~~of any of claims 2 according to 7~~claim 6, wherein CH1 comprises a T192E mutation and CL comprises N137K and S114A mutations.

~~98.~~ A multispecific antigen-binding protein ~~of any of claims 2 according to 8~~claim 7, wherein CH1 comprises L143Q and S188V mutations and CL comprises V133T and S176V mutations.

~~109.~~ A multispecific antigen-binding protein ~~of any of claims 2 according to 9~~claim 8, wherein CH1 comprises T192E, L143Q and S188V mutations and CL comprises N137K, S114A, V133T and S176V mutations.

~~110.~~ A multispecific antigen-binding protein ~~of any of claims 2 according to 10~~claim 9, wherein CH1 comprises a K221E mutation and CL comprises a E123K mutation.

~~111.~~ A multispecific antigen-binding protein ~~of any of claims 2 according to 11~~claim 10, wherein CH1 comprises a K228D mutation and CL comprises a D122K mutation.

- ~~13~~12. A multispecific antigen-binding protein ~~of any of claims 2 according to 12~~claim 11, wherein CH1 comprises K221E and K228D mutations and CL comprises D122K and E123K mutations.
- ~~14~~13. A multispecific antigen-binding protein ~~of any of claims 2 according to 13~~claim 12, wherein CH1 comprises a L143E, a L143D, a L143K, a L143R, or a L143H mutation, and CL comprises a S176E, a S176D, a S176K, a S176R, or a S176H mutation
- ~~15~~14. A multispecific antigen-binding protein ~~of any of claims 2 according to 14~~claim 13, wherein CH1 comprises a L124E, a L124D, a L124K, a L124R, or a L124H mutation, and CL comprises a V133E, a V133D, a V133K, a V133R, or a V133H mutation.
- ~~16~~15. A multispecific antigen-binding protein ~~of any of claims 2 according to 15~~claim 14, wherein VH comprises a Q39E, a Q39D, a Q39K, a Q39R, or a Q39H mutation, and VL comprises a Q38E, a Q38D, a Q38K, a Q38R, or a Q38H mutation.
- ~~17~~16. A multispecific antigen-binding protein ~~of any of claims 2 according to 16~~claim 15, wherein at least one CH1/CL pair comprises CH1/CL mutations to facilitate pairing selected from the group consisting of one or more of:
- (1) a T192E (CH1) mutation and N137K and S114A (CL) mutations,
 - (2) L143Q and S188V (CH1) mutations, and V133T and S176V (CL) mutations,
 - (3) T192E, L143Q and S188V (CH1) mutations and N137K, S114A, V133T and S176V (CL) mutations,
 - (4) a K221E (CH1) mutation and a E123K (CL) mutation,
 - (5) T192E and K221E (CH1) mutations and N137K, S114A and E123K (CL) mutations,
 - (6) a L143E, a L143D, a L143K, a L143R, or a L143H (CH1) mutation and a S176E, a S176D, a S176K, a S176R, or a S176H (CL) mutation,
 - (7) a L124E, a L124D, a L124K, a L124R, or a L124H (CH1) mutation and a V133E, a V133D, a V133K, a V133R, or a V133H (CL) mutation,
 - (8) a K228D (CH1) mutation and a D122K (CL) mutation, and
 - (9) K221E and K228D (CH1) mutations and D122K and E123K (CL) mutations,
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wherein when at least two CH1/CL pairs comprise a mutation set to facilitate pairing for two different VH/VL pairs, then the at least two CH1/CL pairs do not comprise same mutations, and

wherein at least one VH/VL pair comprises opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region.

~~48~~17. An antigen-binding protein comprising:

an antigen-binding domain; and

a constant heavy chain CH1 region paired with a constant light chain CL region,

wherein the antigen-binding domain selectively binds to a target antigen, and wherein the CH1 region and CL region comprise one or both of:

a) a L143E, a L143D, a L143K, a L143R, or a L143H mutation in the CH1 region and a S176E, a S176D, a S176K, a S176R, or a S176H mutation in the CL region; and

b) a L124E, a L124D, a L124K, a L124R, or a L124H mutation in the CH1 region and a V133E, a V133D, a V133K, a V133R, or a V133H mutation in the CL region, wherein the mutated residue in the CH1 region has an opposite charge from the mutated residue in the CL region.

~~49~~18. The antigen-binding protein of claim ~~48~~17, further comprising CH1/CL mutations to facilitate pairing selected from the group consisting of one or more of:

(1) a T192E (CH1) mutation and N137K and S114A (CL) mutations,

(2) L143Q and S188V (CH1) mutations, and V133T and S176V (CL) mutations,

(3) T192E, L143Q and S188V (CH1) mutations and N137K, S114A, V133T and S176V (CL) mutations,

(4) a K221E (CH1) mutation and a E123K (CL) mutation,

(5) a K228D (CH1) mutation and a D122K (CL) mutation, and

(6) K221E and K228D (CH1) mutations and D122K and E123K (CL) mutations,

wherein when two CH1/CL pairs comprise mutations to facilitate pairing for two different VH/VL pairs, the two CH1/CL pairs do not comprise same mutations.

~~2019.~~ The antigen-binding protein of claim ~~18-17~~ or ~~1918~~, further comprising at least one VH/VL pair comprising opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region.

~~20.~~ An antigen-binding protein comprising:

~~an antigen-binding domain; and~~

~~a constant heavy chain CH1 region paired with a constant light chain CL region,~~

~~wherein the antigen-binding domain selectively binds to a target antigen, and wherein the CH1 region and CL region comprise one or both of:~~

~~a) a L143E, L143D, L143K, L143R, or L143H mutation in the CH1 region and a S176E, S176D, S176K, S176R, or S176H mutation in the CL region; and~~

~~b) a K221E and a K228D mutation in the CH1 region and a D122K and a E123K mutation in the CL region,~~

~~wherein the mutated residue in the CH1 region has an opposite charge from the mutated residue in the CL region.~~

~~21.~~ The antigen-binding protein of claim ~~20~~, further comprising at least one VH/VL pair comprising opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region.

~~21.~~ ~~An antigen-binding protein comprising:~~

~~an antigen-binding domain; and~~

~~a constant heavy chain CH1 region paired with a constant light chain CL region,~~

~~wherein the antigen-binding domain selectively binds to a target antigen, and wherein the CH1 region and CL region comprise one or both of:~~

a) a L143E, L143D, L143K, L143R, or L143H mutation in the CH1 region and a S176E, S176D, S176K, S176R, or S176H mutation in the CL region; and

b) a K221E and a K228D mutation in the CH1 region and a D122K and a E123K mutation in the CL region;

wherein the mutated residue in the CH1 region has an opposite charge from the mutated residue in the CL region.

~~22.~~ The antigen-binding protein of claim 21, further comprising at least one VH/VL pair comprising opposite charged mutations to facilitate pairing, said opposite charged mutations comprising (1) a mutated residue in the VH region at Kabat position 39 selected from E, D, K, R, or H, and (2) a mutated residue in the VL region at Kabat position 38 selected from E, D, K, R, or H, and wherein the mutated residue in the VH region has an opposite charge from the mutated residue in the VL region.

~~23~~²². A The multispecific antigen-binding protein or the antigen-binding protein ~~of~~according to any one of claims 1 to ~~22~~²¹, further comprising one or more cysteine residues engineered into one or several VH/VL pairs to form one or more disulfide bonds.

~~24.~~ A multispecific antigen-binding protein of claim 23, wherein one or both VH regions comprise one or both of 44C and 105C mutations, and one or both VL regions comprise one or both of 100C and 43C mutations.

~~25~~²³. A kit comprising one or more isolated nucleic acid molecules comprising one or more nucleotide sequences encoding the multispecific antigen-binding protein or the antigen-binding protein of any one of claims 1-~~24~~²².

~~26~~²⁴. A kit comprising one or more expression vectors comprising the one or more nucleic acid molecules of claim ~~25~~²³.

~~27~~²⁵. An isolated host cell comprising the one or more nucleic acid molecules of claim ~~25~~²³ or the one or more expression vectors of claim ~~24~~²².

~~26.~~

28. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the multispecific antigen-binding protein or the antigen-binding protein of any one of claims 1-~~24~~22.
- ~~29. A method of 27. Use of the multispecific antigen-binding protein or the antigen-binding protein of any one of claims 1-22 in an effective amount for treating a disorder in which antigen activity is detrimental; the method comprising administering to a subject in need thereof an effective amount of a multispecific antigen-binding protein of any one claims 1-24.~~
- ~~30~~28. The multispecific antigen-binding protein or the antigen-binding protein of any of claims 1 to ~~24~~16, 18-19, and 21-22, comprising three HCDRs for each VH region and three LCDRs for each VL region, and further comprising binding specificity to one or more target antigens or one or more target epitopes.