

CLAIMS

1. A heterodimer comprising an Fc receptor (FcR)-binding member and one or more recognition moieties covalently linked to the FcR-binding member, wherein said FcR-binding member comprises a first polypeptide and a second polypeptide joined to each other by one or more disulfide bonds, the first polypeptide and the second polypeptide each comprising at least a portion of an immunoglobulin heavy chain constant region; and wherein said first polypeptide and second polypeptide comprise at least five amino acid substitutions at the following positions according to the Kabat EU index:

T366 and D399 in the first polypeptide and L351, Y407 and K409 in the second polypeptide;

whereby the first and second polypeptides have higher dimerization affinity for each other than self, wherein the five amino acid substitutions occur in the two CH3 domains of the immunoglobulin heavy chain constant region of both the first polypeptide and the second polypeptide, and comprise any of the following members of Group V a)-h):

Group V:

a) T366L and D399R in the first polypeptide and L351E, Y407L and K409V in the second polypeptide;

b) T366L and D399C in the first polypeptide and L351G, Y407L and K409C in the second polypeptide;

c) T366L and D399C in the first polypeptide and L351Y, Y407A and K409P in the second polypeptide;

d) T366P and D399N in the first polypeptide and L351V, Y407P and K409S in the second polypeptide;

e) T366W and D399G in the first polypeptide and L351D, Y407P and K409S in the second polypeptide;

f) T366P and D399I in the first polypeptide and L351P, Y407F and K409F in the second polypeptide;

g) T366V and D399T in the first polypeptide and L351K, Y407T and K409Q in the second polypeptide; and

h) T366L and D399A in the first polypeptide and L351W, Y407H and K409R in the second polypeptide,

wherein the FcR-binding member comprises an Fc domain, and the Fc domain is derived from an IgG Fc domain which comprises one selected from the group consisting of an IgG1 Fc domain, an IgG2 Fc domain, an IgG3 Fc domain, and an IgG4 Fc domain.

2. The heterodimer of claim 1, wherein the one or more recognition moieties comprise at least one of fragment antigen-binding (Fab) fragments, single chain variable (scFv) fragments, extracellular domains of membrane receptors, peptide ligands of cell membrane receptors, cytokines, coagulation factors, affinity tags, and combinations thereof.

3. The heterodimer of claim 2, wherein the one or more recognition moieties comprise two recognition moieties, and wherein the two recognition moieties each comprise Fab fragments.

4. The heterodimer of claim 3, wherein the two Fab fragments are non-identical.

5. The heterodimer of claim 4, wherein the heterodimer comprises an immunoglobulin-like molecule.

6. The heterodimer of any of claims 4-5, wherein the heterodimer comprises a bispecific antibody.

7. The heterodimer of claim 2, wherein the one or more recognition moieties comprise two recognition moieties, and wherein the two recognition moieties comprise a Fab fragment and a scFv fragment.

8. The heterodimer of any of claims 1-7, wherein less than 50% by weight of only one of the first and second polypeptides and the cognate recognition moiety of said first or second polypeptide exist in a homodimeric form, when present in an aqueous solution under physiological conditions containing 1 mM dithiothreitol, based on the weight of all polypeptidyl forms in the solution, provided that said solution is essentially free of polypeptides other than said first or second polypeptide and the cognate recognition moiety thereof.

9. The heterodimer of any of claims 1-8, wherein the at least five amino acid substitutions comprise T366L and D399R in the first polypeptide and L351E, Y407L and K409V in the second polypeptide.

10. The heterodimer of claim 4, wherein the heterodimer comprises a first heavy chain and a second heavy chain, and a first light chain and a second light chain, wherein the first heavy chain and the second heavy chain are non-identical, and wherein the first light chain and the second light chain are non-identical.

11. The heterodimer of claim 7, wherein the Fab fragment and the scFv fragment are selected from a pair of recognition moieties selected from Group VII a)-d):
Group VII:

- a) Fab specifically binding to HER2 and scFv specifically binding to CD3;
 - b) Fab specifically binding to Trop2 and scFv specifically binding to CD3;
 - c) Fab specifically binding to CD20 and scFv specifically binding to CD3;
- and
- d) Fab specifically binding to PD-L1 and scFv specifically binding to CD3.

12. The heterodimer of claim 4, wherein the two non-identical Fab fragments comprise a first Fab fragment that specifically binds to HER2 and a second Fab fragment that specifically binds to CD20.

13. The heterodimer of any one of claims 1-12, wherein at least one of the first polypeptide and the second polypeptide comprises amino acid sequences set forth in: SEQ ID NOs: 16, 18, 20, 22, 24, 26, 28 or 30, and the other polypeptide comprises the amino acid sequence set forth in: SEQ ID NOs: 17, 19, 21, 23, 25, 27, 29 or 31.

14. The heterodimer of any one of claims 1-13, wherein the FcR-binding member is capable of binding to an Fc receptor selected from the group consisting of FcγRI, FcγRIIA, FcγRIIB1, FcγRIIB2, FcγRIIIA, FcγRIIIB, FcεRI, FcεRII, FcαRI, Fcα/μR, FcRn, and combinations thereof.

15. The heterodimer of claim 14, wherein the FcR-binding member is capable of binding to FcRn.

16. The heterodimer of claim 14, wherein binding to the Fc receptor triggers ADCC.

17. A method of producing the heterodimer of any one of claims 1-16 comprising

- 1) expressing one or more nucleic acids encoding the first polypeptide in a first host cell and one or more nucleic acids encoding the second polypeptide in a second host cell, the first host cell and the second host cell being separated from one another;
- 2) reducing the first polypeptide and the second polypeptides while separated;
- 3) combining the first polypeptide and the second polypeptide to form a resultant mixture;
- 4) oxidizing the resultant mixture; and
- 5) recovering the formed heterodimer.

18. A method of producing a heterodimer comprising a bivalent heterologous immunoglobulin having a first heavy chain, a second heavy chain, a first light chain and a second light chain, wherein the first heavy chain and the second heavy chain are non-identical, wherein the first light chain and the second light chain are nonidentical,

wherein a portion of the first heavy chain and the second heavy chain form an Fc region of the bivalent heterologous immunoglobulin, comprising:

1) expressing one or more nucleic acids encoding the first heavy chain and the first light chain in a first host cell and one or more nucleic acids encoding the second heavy chain and the second light chain in a second host cell, the first host cell and the second host cell being separated from one another;

2) reducing the first heavy chain and first light chain together and the second heavy chain and second light chain together;

3) combining the first heavy chain, first light chain, second heavy chain, and second light chain to form a resultant mixture;

4) oxidizing the resultant mixture; and

5) recovering the formed heterodimer,

wherein the Fc region comprises at least five amino acid substitutions, the at least five amino acid substitutions comprise any one of the following members from Group X a)-h):

Group X:

a) T366L and D399R in the first heavy chain and L351E, Y407L and K409V in the second heavy chain;

b) T366L and D399C in the first heavy chain and L351G, Y407L and K409C in the second heavy chain;

c) T366L and D399C in the first heavy chain and L351Y, Y407A and K409P in the second heavy chain;

d) T366P and D399N in the first heavy chain and L351V, Y407P and K409S in the second heavy chain;

e) T366W and D399G in the first polypeptide and L351D, Y407P and K409S in the second heavy chain;

f) T366P and D399I in the first polypeptide and L351P, Y407F and K409F in the second heavy chain

g) T366V and D399T in the first heavy chain and L351K, Y407T and K409Q in the second heavy chain; and

h) T366L and D399A in the first heavy chain and L351W, Y407H and K409R in the second heavy chain.

19. The method of any of claims 17-18, wherein the one or more nucleic acids are contained within a vector or system of vectors.

20. The method of claim 19, wherein the vector or system of vectors comprises plasmid vectors pX0GC modified from pCDNA vectors.

21. The method of claim 20, wherein the vector or system of vectors are introduced into a cell or cells.

22. The method of claim 21, wherein the cell or cells comprises one of HEK293, HEK293T, HEK293F or CHO, CHO-S, CHO-dhfr-, CHO/DG44 and Expi CHO cells.

23. The method of any of claims 17 and 18, wherein the reducing step comprises:

- i) adding a reductant, wherein the reductant comprises one of 2-aminoethanethiol, dithiothreitol (DTT), three (2 - carboxyethyl) phosphine, derivatives thereof, and combinations thereof;
- ii) reducing for at least about 3 hours at about 4 °C; and
- iii) removing the reductant.

24. The method of claim 23 wherein the reductant comprises DTT at a concentration of about 0.1mM or higher.

25. The method of any of claims 23-24 wherein the reductant is removed *via* a desalting column.

26. The method of any of claims 17, 18, and 23-25 wherein the oxidizing step comprises oxidizing by air for at least about 5 hours.

27. The method of any of claims 17, 18, and 23-25 wherein the oxidizing step comprises:

- i) addition of an oxidant comprising L-dehydroascorbic acid, its derivatives, or combinations thereof
- ii) oxidizing for at least about 5 hours at about 4 °C; and
- iii) removing the oxidant.

28. The method of claim 27 wherein the oxidant comprises L-dehydroascorbic acid at a concentration of about 0.5mM or higher.

29. The method of any of claims 17, 18 and 23-26, further comprising a separation step.

30. A nucleic acid encoding at least one of the first polypeptide, the second polypeptide and a fragment thereof of any one of claims 1-16.

31. The nucleic acid of claim 30, wherein the nucleic acid comprises one selected from SEQ ID NOs: 32, 34, 36, 38, 40, 42, 44, 46, 47, 50, 52, 54, 56, 58, 60, 62 and 64-67.

32. A vector or system of vectors comprising the nucleic acid of any of claims 30-31.

33. The vector or system of vectors of claim 32, wherein the vector or system of vectors comprise plasmid vectors pX0GC modified from pCDNA vectors.

34. A cell comprising the vector or system of vectors of any of claims 32-33.

35. The cell of claim 34, wherein the cell comprises one of HEK293, HEK293T, HEK293F or CHO, CHO-S, CHO-dhfr-, CHO/DG44 and ExpiCHO cells.

36. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and one of:

- i) the heterodimer of any one of claims 1-16;
- ii) the nucleic acid of any one of claims 30-31;
- iii) the vector or system of vectors of any one of claims 32-33;
- iv) the cell of any one of claims 34-35; and
- v) combinations of i) to iv).

37. Use of the pharmaceutical composition of claim 36 in the preparation of a medicament for treating or preventing a disease or disorder in a subject,

wherein the disease or disorder comprises one or more of autoimmune diseases, immune response against grafts, allergic reactions, infections, neurodegenerative diseases, neoplastic diseases and cellular proliferative disorders, arthritis, rheumatoid arthritis, psoriasis, multiple sclerosis (MS), ulcerative colitis, Crohn's disease, systemic lupus erythematosus (SLE), glomerulonephritis, dilated cardiomyopathy-like diseases, Sjogren's syndrome, allergic contact dermatitis, polymyositis, scleroderma, periarteritis nodosa, rheumatic fever, vitiligo, insulin-dependent diabetes mellitus, Behcet's syndrome, chronic thyroiditis, Parkinson's disease, Huntington's disease, Machado-Joseph disease, amyotrophic lateral sclerosis (ALS), Creutzfeldt - Jakob disease, leukemia, lymphoma, myeloma, brain tumors, head and neck squamous cell carcinoma, non-small cell lung cancer (NSCLC), nasopharyngeal cancer, esophageal cancer, stomach cancer, pancreatic cancer, gallbladder cancer, liver cancer, colorectal cancer, breast cancer, ovarian cancer, cervical cancer, endometrial cancer, uterine sarcoma, prostate cancer, bladder cancer, renal cell carcinoma, melanoma, and combinations thereof.