

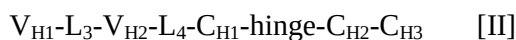
CLAIMS

What is claimed is:

1. A binding protein comprising four polypeptide chains that form three antigen binding sites that specifically bind one or more target proteins, wherein a first polypeptide chain of the binding protein comprises a structure represented by the formula:



and a second polypeptide chain of the binding protein comprises a structure represented by the formula:



and a third polypeptide chain of the binding protein comprises a structure represented by the formula:



and a fourth polypeptide chain of the binding protein comprises a structure represented by the formula:



wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{L3} is a third immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

V_{H3} is a third immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain;

C_{H2} is an immunoglobulin C_{H2} heavy chain constant domain;

C_{H3} is an immunoglobulin C_{H3} heavy chain constant domain;

hinge is an immunoglobulin hinge region connecting the C_{H1} and C_{H2} domains; and

L_1 , L_2 , L_3 and L_4 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; wherein V_{H1} and V_{L1} form a binding pair and a first antigen binding site, V_{H2} and V_{L2} form a binding pair and a second antigen binding site; and V_{H3} and V_{L3} form a binding pair and a third antigen binding site.

2. The binding protein of claim 1, wherein (a) L_1 , L_2 , L_3 and L_4 each independently are zero amino acids in length or comprise a sequence selected from the group consisting of GGGGSGGGGS (SEQ ID NO:104), GGGGSGGGSGGGGS (SEQ ID NO:105), S, RT, TKGPS (SEQ ID NO:106), GQPKAAP (SEQ ID NO: 175), and GSGSSGSGG (SEQ ID NO:148); or (b) L_1 , L_2 , L_3 and L_4 each independently comprise a sequence selected from the group consisting of GGGGSGGGGS (SEQ ID NO:104), GGGGSGGGSGGGGS (SEQ ID NO:105), S, RT, TKGPS (SEQ ID NO:106), GQPKAAP (SEQ ID NO: 175), and GSGSSGSGG (SEQ ID NO:148).

3. The binding protein of claim 2, wherein

(a) L_1 comprises the sequence GQPKAAP (SEQ ID NO: 175), L_2 comprises the sequence TKGPS (SEQ ID NO:106), L_3 comprises the sequence S, and L_4 comprises the sequence RT;

(b) L_1 comprises the sequence GGGGSGGGGS (SEQ ID NO:104), L_2 comprises the sequence GGGGSGGGGS (SEQ ID NO:104), L_3 is 0 amino acids in length, and L_4 is 0 amino acids in length; or

(c) L_1 comprises the sequence GSGSSGSGG (SEQ ID NO:148), L_2 comprises the sequence GSGSSGSGG (SEQ ID NO:148), L_3 is 0 amino acids in length, and L_4 is 0 amino acids in length.

4. The binding protein of claim 1, wherein L_1 , L_2 , L_3 , and/or L_4 comprise the sequence Asp-Lys-Thr-His-Thr (SEQ ID NO: 525).

5. The binding protein of claim 1, wherein the binding protein is trispecific and capable of specifically binding three different antigen targets.

6. The binding protein of any one of claims 1-5, wherein the binding protein specifically binds three target proteins that correspond to two target proteins on T cells and to one tumor target protein.
7. The binding protein of claim 6, wherein one of said target proteins on T cells is CD3.
8. The binding protein of claim 6 or claim 7, wherein one of said target proteins on T cells is CD28.
9. The binding protein of any one of claims 6-8, wherein said tumor target protein is CD38.
10. The binding protein of any one of claims 1-5, wherein the binding protein specifically binds three target proteins that correspond to two target proteins on T cells and to one target protein selected from the group consisting of A2AR, APRIL, ATPDase, BAFF, BAFFR, BCMA, BlyS, BTK, BTLA, B7DC, B7H1, B7H4, B7H5, B7H6, B7H7, B7RP1, B7-4, C3, C5, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL15, CCL17, CCL19, CCL20, CCL21, CCL24, CCL25, CCL26, CCR3, CCR4, CD3, CD19, CD20, CD23, CD24, CD27, CD28, CD38, CD39, CD40, CD70, CD80, CD86, CD122, CD137, CD137L, CD152, CD154, CD160, CD272, CD273, CD274, CD275, CD276, CD278, CD279, CDH1, chitinase, CLEC9, CLEC9L, CRTH2, CSF-1, CSF-2, CSF-3, CX3CL1, CXCL12, CXCL13, CXCR3, DNGR-1, ectonucleoside triphosphate diphosphohydrolase 1, EGFR, ENTPD1, FCER1A, FCER1, FLAP, FOLH1, Gi24, GITR, GITRL, GM-CSF, Her2, HHLA2, HMGB1, HVEM, ICOSLG, IDO, IFN α , IgE, IGF1R, IL2Rbeta, IL1, IL1A, IL1B, IL1F10, IL2, IL4, IL4Ra, IL5, IL5R, IL6, IL7, IL7Ra, IL8, IL9, IL9R, IL10, rhIL10, IL12, IL13, IL13Ra1, IL13Ra2, IL15, IL17, IL17Rb, IL18, IL22, IL23, IL25, IL27, IL33, IL35, ITGB4, ITK, KIR, LAG3, LAMP1, leptin, LPFS2, MHC class II, NCR3LG1, NKG2D, NTPDase-1, OX40, OX40L, PD-1H, platelet receptor, PROM1, S152, SISP1, SLC, SPG64, ST2, STEAP2, Syk kinase, TACI, TDO, T14, TIGIT, TIM3, TLR, TLR2, TLR4, TLR5, TLR9, TMEF1, TNFa, TNFRSF7, Tp55, TREM1, TSLP, TSLPR, TWEAK, VEGF, VISTA, Vstm3, WUCAM, and XCR1.
11. The binding protein of claim 1, wherein the binding protein is capable of inhibiting the function of one or more target proteins.

12. The binding protein of claim 11, wherein the one or more target proteins are selected from the group consisting of A2AR, APRIL, ATPDase, BAFF, BAFFR, BCMA, BlyS, BTK, BTLA, B7DC, B7H1, B7H4, B7H5, B7H6, B7H7, B7RP1, B7-4, C3, C5, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL15, CCL17, CCL19, CCL20, CCL21, CCL24, CCL25, CCL26, CCR3, CCR4, CD3, CD19, CD20, CD23, CD24, CD27, CD28, CD38, CD39, CD40, CD70, CD80, CD86, CD122, CD137, CD137L, CD152, CD154, CD160, CD272, CD273, CD274, CD275, CD276, CD278, CD279, CDH1, chitinase, CLEC9, CLEC91, CRTH2, CSF-1, CSF-2, CSF-3, CX3CL1, CXCL12, CXCL13, CXCR3, DNGR-1, ectonucleoside triphosphate diphosphohydrolase 1, EGFR, ENTPD1, FCER1A, FCER1, FLAP, FOLH1, Gi24, GITR, GITRL, GM-CSF, Her2, HHLA2, HMGB1, HVEM, ICOSLG, IDO, IFN α , IgE, IGF1R, IL2Rbeta, IL1, IL1A, IL1B, IL1F10, IL2, IL4, IL4Ra, IL5, IL5R, IL6, IL7, IL7Ra, IL8, IL9, IL9R, IL10, rhIL10, IL12, IL13, IL13Ra1, IL13Ra2, IL15, IL17, IL17Rb, IL18, IL22, IL23, IL25, IL27, IL33, IL35, ITGB4, ITK, KIR, LAG3, LAMP1, leptin, LPFS2, MHC class II, NCR3LG1, NKG2D, NTPDase-1, OX40, OX40L, PD-1H, platelet receptor, PROM1, S152, SISP1, SLC, SPG64, ST2, STEAP2, Syk kinase, TACI, TDO, T14, TIGIT, TIM3, TLR, TLR2, TLR4, TLR5, TLR9, TMEF1, TNFa, TNFRSF7, Tp55, TREM1, TSLP, TSLPR, TWEAK, VEGF, VISTA, Vstm3, WUCAM, and XCR1.

13. The binding protein of claim 1 or claim 6, wherein V_{H1} comprises a CDR-H1 comprising the amino acid sequence of SEQ ID NO:28, a CDR-H2 comprising the amino acid sequence of SEQ ID NO:29, and a CDR-H3 comprising the amino acid sequence of SEQ ID NO:30; V_{L1} comprises a CDR-L1 comprising the amino acid sequence of SEQ ID NO:46, a CDR-L2 comprising the amino acid sequence of SEQ ID NO:47, and a CDR-L3 comprising the amino acid sequence of SEQ ID NO:48; and V_{H2} comprises a CDR-H1 comprising the amino acid sequence of SEQ ID NO:34, a CDR-H2 comprising the amino acid sequence of SEQ ID NO:35, and a CDR-H3 comprising the amino acid sequence of SEQ ID NO:36; V_{L2} comprises a CDR-L1 comprising the amino acid sequence of SEQ ID NO:52, a CDR-L2 comprising the amino acid sequence of SEQ ID NO:53, and a CDR-L3 comprising the amino acid sequence of SEQ ID NO:54.

14. The binding protein of claim 1, wherein the C_{H3} domain of the second polypeptide chain comprises amino acid substitutions at positions corresponding to positions 354 and 366 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are S354C and T366W; and wherein the C_{H3} domain of the third polypeptide chain comprises amino acid substitutions at positions corresponding to positions 349, 366, 368, and 407 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are Y349C, T366S, L368A, and Y407V.
15. The binding protein of claim 1, wherein the C_{H3} domain of the second polypeptide chain comprises amino acid substitutions at positions corresponding to positions 349, 366, 368, and 407 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are Y349C, T366S, L368A, and Y407V; and wherein the C_{H3} domain of the third polypeptide chain comprises amino acid substitutions at positions corresponding to positions 354 and 366 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are S354C and T366W.
16. The binding protein of any one of claims claim 1, wherein the C_{H3} domains of the second and the third polypeptide chains both comprise amino acid substitutions at positions corresponding to positions 428 and 434 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are M428L and N434S.
17. The binding protein of any one of claims 1 and 14-16, wherein the C_{H3} domains of the second and the third polypeptide chains are human IgG1 or IgG4 C_{H3} domains, and wherein only one of the C_{H3} domains comprises amino acid substitutions at positions corresponding to positions 435 and 436 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are H435R and Y436F.
18. The binding protein of any one of claims 1 and 14-17, wherein the C_{H3} domains of the second and the third polypeptide chains are human IgG4 C_{H3} domains, and wherein the C_{H3} domains each comprise amino acid substitutions at positions corresponding to positions 228 and 409 of human IgG4 according to EU Index, wherein the amino acid substitutions are S228P and R409K.

19. The binding protein of any one of claims 1 and 14-18, wherein the C_{H3} domains of the second and the third polypeptide chains are human IgG4 C_{H3} domains, and wherein the C_{H3} domains each comprise amino acid substitutions at positions corresponding to positions 234 and 235 of human IgG4 according to EU Index, wherein the amino acid substitutions are F234A and L235A.

20. The binding protein of any one of claims 1 and 14-18, wherein the C_{H3} domains of the second and the third polypeptide chains are human IgG1 C_{H3} domains, and wherein the C_{H3} domains each comprise amino acid substitutions at positions corresponding to positions 234 and 235 of human IgG4 according to EU Index, wherein the amino acid substitutions are L234A and L235A.

21. The binding protein of claim 1, wherein V_{H1} and V_{L1} form a first antigen binding site that specifically binds human CD3, wherein V_{H2} and V_{L2} form a second antigen binding site that specifically binds human CD28, and wherein V_{H3} and V_{L3} form a third antigen binding site that specifically binds a human tumor target protein.

22. The binding protein of claim 1, wherein V_{H1} and V_{L1} form a first antigen binding site that specifically binds human CD28, wherein V_{H2} and V_{L2} form a second antigen binding site that specifically binds human CD3, and wherein V_{H3} and V_{L3} form a third antigen binding site that specifically binds a human tumor target protein.

23. The binding protein of claim 21 or claim 22, wherein the third antigen binding site specifically binds a human tumor target protein selected from the group consisting of CD19, CD20, CD38, Her2, and LAMP1.

24. The binding protein of any one of claims 21-23, wherein the antigen binding site that specifically binds CD3 comprises:

(a) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 152 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 153; or

(b) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 154 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 155.

25. The binding protein of any one of claims 21-24, wherein the antigen binding site that specifically binds CD28 comprises:

(a) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 160 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 161; or

(b) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 162 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 163.

26. The binding protein of any one of claims 21-25, wherein the antigen binding site that specifically binds a tumor target protein comprises:

(a) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 156 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 157;

(b) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 158 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 159;

(c) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 164 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 165;

(d) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 150 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 151; or

(e) a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 166 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 167.

27. The binding protein of any one of claims 1-26, wherein:

(a) the C_L domain of the first polypeptide chain is a human kappa C_L domain, and the C_L domain of the fourth polypeptide chain is a human lambda C_L domain; or

(b) the C_L domain of the first polypeptide chain is a human lambda C_L domain, and the C_L domain of the fourth polypeptide chain is a human kappa C_L domain.

28. The binding protein of claim 1, wherein the first polypeptide chain comprises a lambda C_L domain; wherein the C_{H3} domain of the second polypeptide chain comprises amino acid substitutions at positions corresponding to positions 354 and 366 of human IgG1 according to EU Index, wherein the amino acid substitutions are S354C and T366W; wherein the C_{H3} domain of the third polypeptide chain comprises amino acid substitutions at positions corresponding to positions 349, 366, 368, 407, 435, and 436 of human IgG1 according to EU Index, wherein the amino acid substitutions are Y349C, T366S, L368A, Y407V, H435R, and Y436F; and wherein the fourth polypeptide chain comprises a kappa C_L domain.

29. A binding protein comprising a first polypeptide chain, a second polypeptide chain, a third polypeptide chain and a fourth polypeptide chain wherein:

(a) the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 4; the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 3; the third polypeptide chain comprises the amino acid sequence of SEQ ID NO: 1; and the fourth polypeptide chain comprises the amino acid sequence of SEQ ID NO: 2;

(b) the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 10; the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 9; the third polypeptide chain comprises the amino acid sequence of SEQ ID NO: 1; and the fourth polypeptide chain comprises the amino acid sequence of SEQ ID NO: 2;

(c) the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 4; the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 3; the third

third polypeptide chain comprises the amino acid sequence of SEQ ID NO: 85; and the fourth polypeptide chain comprises the amino acid sequence of SEQ ID NO: 86;

(t) the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 4; the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 3; the third polypeptide chain comprises the amino acid sequence of SEQ ID NO: 114; and the fourth polypeptide chain comprises the amino acid sequence of SEQ ID NO: 115; or

(u) the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 10; the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 9; the third polypeptide chain comprises the amino acid sequence of SEQ ID NO: 114; and the fourth polypeptide chain comprises the amino acid sequence of SEQ ID NO: 115.

30. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the binding protein of any one of claims 1-29.

31. An expression vector comprising the nucleic acid molecule of claim 30.

32. An isolated host cell comprising the nucleic acid molecule of claim 30.

33. An isolated host cell comprising the expression vector of claim 31.

34. The isolated host cell of claim 32 or claim 33, wherein the host cell is a mammalian cell or an insect cell.

35. A pharmaceutical composition comprising the binding protein of any one of claims 1-29 and a pharmaceutically acceptable carrier.

36. The binding protein of any one of claims 1-29 or the composition of claim 35 for use in preventing and/or treating cancer in a patient.

37. The binding protein for use or the composition for use of claim 36, wherein the binding protein comprises one antigen binding site that specifically binds a T-cell surface protein and another antigen binding site that specifically binds a tumor target protein.

38. The binding protein for use or the composition for use of claim 37, wherein the binding protein comprises a first antigen binding site that specifically binds CD3, a second antigen binding site that specifically binds CD28, and a third antigen binding site that specifically binds

a tumor target protein selected from the group consisting of CD19, CD20, CD38, Her2, and LAMP1.

39. The binding protein for use or the composition for use of any one of claims 36-38, wherein the binding protein is co-administered with a chemotherapeutic agent.

40. The binding protein for use or the composition for use of any one of claims 36-39, wherein the patient is a human.

41. The binding protein for use or the composition for use of claim 36, wherein the binding protein is capable of inhibiting the function of one or more target proteins selected from the group consisting of A2AR, APRIL, ATPDase, BAFF, BAFFR, BCMA, BlyS, BTK, BTLA, B7DC, B7H1, B7H4, B7H5, B7H6, B7H7, B7RP1, B7-4, C3, C5, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL15, CCL17, CCL19, CCL20, CCL21, CCL24, CCL25, CCL26, CCR3, CCR4, CD3, CD19, CD20, CD23, CD24, CD27, CD28, CD38, CD39, CD40, CD70, CD80, CD86, CD122, CD137, CD137L, CD152, CD154, CD160, CD272, CD273, CD274, CD275, CD276, CD278, CD279, CDH1, chitinase, CLEC9, CLEC91, CRTH2, CSF-1, CSF-2, CSF-3, CX3CL1, CXCL12, CXCL13, CXCR3, DNGR-1, ectonucleoside triphosphate diphosphohydrolase 1, EGFR, ENTPD1, FCER1A, FCER1, FLAP, FOLH1, Gi24, GITR, GITRL, GM-CSF, Her2, HHLA2, HMGB1, HVEM, ICOSLG, IDO, IFN α , IgE, IGF1R, IL2Rbeta, IL1, IL1A, IL1B, IL1F10, IL2, IL4, IL4Ra, IL5, IL5R, IL6, IL7, IL7Ra, IL8, IL9, IL9R, IL10, rhIL10, IL12, IL13, IL13Ra1, IL13Ra2, IL15, IL17, IL17Rb, IL18, IL22, IL23, IL25, IL27, IL33, IL35, ITGB4, ITK, KIR, LAG3, LAMP1, leptin, LPFS2, MHC class II, NCR3LG1, NKG2D, NTPDase-1, OX40, OX40L, PD-1H, platelet receptor, PROM1, S152, SISP1, SLC, SPG64, ST2, STEAP2, Syk kinase, TACI, TDO, T14, TIGIT, TIM3, TLR, TLR2, TLR4, TLR5, TLR9, TMEF1, TNFa, TNFRSF7, Tp55, TREM1, TSLP, TSLPR, TWEAK, VEGF, VISTA, Vstm3, WUCAM, and XCR1.

42. A method of purifying a binding protein produced by a host cell, comprising:

(a) producing the binding protein of any one of claims 1 and 14-26 in a host cell, wherein only one of the C_{H3} domain of the second polypeptide chain and the C_{H3} domain of the third polypeptide chain comprises amino acid substitutions at positions corresponding to positions 435 and 436 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are H435R and Y436F;

(b) contacting the binding protein produced in (a) with Protein A; and
(c) eluting the binding protein from Protein A under conditions suitable for isolating the binding protein away from binding proteins comprising either 0 or 2 C_{H3} domains comprising the amino acid substitutions are H435R and Y436F.

43. The method of claim 42, wherein the C_L domain of the first polypeptide chain is a human kappa C_L domain, and the C_L domain of the fourth polypeptide chain is a human lambda C_L domain; or the C_L domain of the first polypeptide chain is a human lambda C_L domain, and the C_L domain of the fourth polypeptide chain is a human kappa C_L domain, and the method further comprises:

(d) contacting the binding protein eluted in (c) with a kappa light chain affinity medium; and

(e) eluting the binding protein from the kappa light chain affinity medium under conditions suitable for isolating the binding protein away from binding proteins comprising only lambda C_L domains.

44. The method of claim 43, further comprising, after (e):

(f) contacting the binding protein eluted in (e) with a lambda light chain affinity medium; and

(g) eluting the binding protein from the lambda light chain affinity medium under conditions suitable for isolating the binding protein away from binding proteins comprising only kappa C_L domains.

45. The method of claim 42, wherein the C_L domain of the first polypeptide chain is a human kappa C_L domain, and the C_L domain of the fourth polypeptide chain is a human lambda C_L domain; or the C_L domain of the first polypeptide chain is a human lambda C_L domain, and the C_L domain of the fourth polypeptide chain is a human kappa C_L domain, and the method further comprises:

(d) contacting the binding protein eluted in (c) with a lambda light chain affinity medium; and

(e) eluting the binding protein from the lambda light chain affinity medium under conditions suitable for isolating the binding protein away from binding proteins comprising only kappa C_L domains.

46. The method of claim 45, further comprising, after (e):

(f) contacting the binding protein eluted in (e) with a kappa light chain affinity medium; and

(g) eluting the binding protein from the kappa light chain affinity medium under conditions suitable for isolating the binding protein away from binding proteins comprising only lambda C_L domains.

47. The method of any one of claims 42-46, wherein the first polypeptide chain comprises a lambda C_L domain; wherein the C_{H3} domain of the second polypeptide chain comprises amino acid substitutions at positions corresponding to positions 354 and 366 of human IgG1 according to EU Index, wherein the amino acid substitutions are S354C and T366W; wherein the C_{H3} domain of the third polypeptide chain comprises amino acid substitutions at positions corresponding to positions 349, 366, 368, 407, 435, and 436 of human IgG1 according to EU Index, wherein the amino acid substitutions are Y349C, T366S, L368A, Y407V, H435R, and Y436F; and wherein the fourth polypeptide chain comprises a kappa C_L domain.

48. The method of any one of claims 42-47, wherein the binding protein is detected in one or more of (c) and (e) using hydrophobic interaction chromatography (HIC).

49. The method of any one of claims 42-48, wherein the C_{H3} domains of the second and the third polypeptide chains are human IgG1 or IgG4 C_{H3} domains.