

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization

International Bureau

(43) International Publication Date

27 June 2019 (27.06.2019)



(10) International Publication Number

WO 2019/122052 A2

(51) International Patent Classification:

C07K 16/28 (2006.01)

(21) International Application Number:

PCT/EP20 18/086055

(22) International Filing Date:

20 December 2018 (20.12.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

17209205.8 21 December 2017 (21.12.2017) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

## Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ANTIBODIES BINDING TO HLA-A2/WT1

(57) Abstract: The present invention generally relates to antibodies that bind to HLA-A2/WT1, including bispecific antigen binding molecules e.g. for activating T cells. In addition, the present invention relates to polynucleotides encoding such antibodies, and vectors and host cells comprising such polynucleotides. The invention further relates to methods for producing the antibodies, and to methods of using them in the treatment of disease.

## Antibodies binding to HLA-A2/WT1

### Field of the Invention

The present invention generally relates to antibodies that bind to HLA-A2/WT1, including bispecific antigen binding molecules e.g. for activating T cells. In addition, the present invention relates to polynucleotides encoding such antibodies, and vectors and host cells comprising such 5 polynucleotides. The invention further relates to methods for producing the antibodies, and to methods of using them in the treatment of disease.

### Background

WT1 (Wilms tumor 1, Wilms tumor protein) is an oncogenic transcription factor involved in cell 10 proliferation, differentiation, as well as apoptosis and organ development, whose expression in normal adult tissue is rare (Hinrichs and Restifo, Nat Biotechnol (2013) 31, 999-1008). WT1 is, however, reported to be overexpressed in several types of haematological malignancies and a wide range of solid tumors (Van Driessche et al., Oncologist (2012) 17, 250-259). WT1 is a nuclear protein, localized intracellularly. Intracellular protein can be degraded in the proteasome, 15 processed and presented on the cell surface by major histocompatibility complex (MHC) I as T cell epitopes, and recognized by T cell receptors (TCR). As such, WT1-derived peptides such as WT1RMF (RMFPNAPYL) and WT1VLD (VLDFAPPGA) are presented in the context of HLA-A2 on the cell surface and can trigger T cell recognition.

Several approaches have been taken to exploit WT1 as target for cancer (immuno)therapy, 20 including the development of cancer vaccines based on WT1-derived peptide and adoptive T cell transfer or WT1-specific T cells. TCR-like antibodies against the HLA-A2/WT1<sub>RMF</sub> complex, including bispecific derivatives thereof, have also been generated (Dao et al., Sci Transl Med (2013) 5, 176ra33; WO2012/135854; Dao et al., Nat Biotechnol (2015) 33, 1079-1086; WO 2015/070061; WO 2017/060201).

25 Bispecific antibodies that bind to a surface antigen on target cells and an activating T cell antigen such as CD3 on T-cells (also called herein T cell bispecific antibodies or “TCBs”) hold great promise for the treatment of various cancers. The simultaneous binding of such an antibody to

both of its targets will force a temporary interaction between target cell and T cell, causing crosslinking of the T cell receptor and subsequent activation of any cytotoxic T cell and subsequent lysis of the target cell. Given their potency in target cell killing, the choice of target and the specificity of the targeting antibody is of utmost importance for T cell bispecific antibodies to avoid on- and off-target toxicities. Intracellular proteins such as WT1 represent attractive targets, but are only accessible to T cell receptor (TCR)-like antibodies that bind major histocompatibility complex (MHC) presenting peptide antigens derived from the intracellular protein on the cell surface. An inherent issue of TCR-like antibodies is potential cross-reactivity with MHC molecules per se, or MHC molecules presenting peptides other than the desired one, which could compromise organ or tissue selectivity.

### **Summary of the Invention**

The present invention provides novel antibodies, including bispecific antibodies, that bind HLA-A2/WT1 and have particularly favorable properties for therapeutic purposes.

The present inventors have developed novel antibodies with unexpected, improved properties, that bind to HLA-A2/WT1. In particular, the antibody binds HLA-A2/WT1 with good affinity and remarkable specificity. Furthermore, the inventors have developed bispecific antigen binding molecules that bind to HLA-A2/WT1 and an activating T cell antigen, incorporating the novel HLA-A2/WT1 antibody and combine good efficacy and producability with low toxicity and favorable pharmacokinetic properties.

In a first aspect the present invention provides an antibody that binds to HLA-A2/WT1, wherein the antibody comprises

(i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementary determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;

(ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

(iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;

(iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 5 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;

(v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;

10 (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 15 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 20 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70.

In one embodiment, the antibody comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino 25 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino 30 acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

(iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino

5 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

(v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

10 acid sequence of SEQ ID NO: 40;

(vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

15 (vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99%

20 or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino

25 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

In one embodiment, the antibody is an IgG, particularly an IgGi, antibody. In one embodiment, the antibody is a full-length antibody. In another embodiment, the antibody is an antibody fragment selected from the group of an Fv molecule, a scFv molecule, a Fab molecule, and a

30 F(ab')2 molecule. In one embodiment, the antibody is a multispecific antibody.

The invention also provides a bispecific antigen binding molecule, comprising

(a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1 and the first antigen binding moiety comprises

(i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO:

5 6;

(ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

10 (iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;

(iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;

15 (v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;

20 (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

25 (viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70; and

30 (b) a second antigen binding moiety which specifically binds to a second antigen.

In one embodiment, the first antigen binding moiety comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino

5 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

10 acid sequence of SEQ ID NO: 24;

(iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

15 (v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

(vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99%

20 or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino

25 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino

30 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

In one embodiment, the second antigen is CD3, particularly CD3 $\epsilon$ . In one embodiment, the second antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 115, a

5 HCDR 2 of SEQ ID NO: 116, and a HCDR 3 of SEQ ID NO: 117, and a VL comprising a LCDR 1 of SEQ ID NO: 118, a LCDR 2 of SEQ ID NO: 119 and a LCDR 3 of SEQ ID NO: 120.

In one embodiment, the VH of the second antigen binding moiety comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 121, and the VL of the second antigen binding moiety comprises an

10 amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 122. In one embodiment, the first and/or the second antigen

binding moiety is a Fab molecule. In one embodiment, the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1, particularly the variable domains VL and VH, of the Fab light chain and the Fab heavy chain are

15 replaced by each other. In one embodiment, the first antigen binding moiety is a Fab molecule wherein in the constant domain the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat) and the amino acid at

position 123 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat), and in the constant domain CH1 the amino acid at position 147 is

20 substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index). In one embodiment, the first and the second antigen binding moiety are fused to each other, optionally via a peptide

linker. In one embodiment, the first and the second antigen binding moiety are each a Fab

25 molecule and either (i) the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety, or (ii) the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-

terminus of the Fab heavy chain of the second antigen binding moiety. In one embodiment, the bispecific antigen binding molecule comprises a third antigen binding moiety. In one

30 embodiment, the third antigen moiety is identical to the first antigen binding moiety. In one embodiment, the bispecific antigen binding molecule comprises an Fc domain composed of a first and a second subunit. In one embodiment, the first, the second and, where present, the third antigen binding moiety are each a Fab molecule; and either (i) the second antigen binding moiety

is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety and the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first subunit of the Fc domain, or (ii) the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab

5 heavy chain of the second antigen binding moiety and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first subunit of the Fc domain; and the third antigen binding moiety, where present, is fused at the C-terminus of the Fab heavy chain to the N-terminus of the second subunit of the Fc domain. In one embodiment, the Fc domain is an IgG, particularly an IgGi, Fc domain. In one embodiment, the Fc domain is a  
10 human Fc domain. In one embodiment, an amino acid residue in the CH3 domain of the first subunit of the Fc domain is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which is positionable in a cavity within the CH3 domain of the second subunit, and an amino acid residue in the CH3 domain of the second subunit of the Fc domain is replaced with an amino acid  
15 residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit is positionable. In one embodiment, the Fc domain comprises one or more amino acid substitution that reduces binding to an Fc receptor and/or effector function.

According to another aspect of the invention there is provided one or more isolated  
20 polynucleotide(s) encoding an antibody or bispecific antigen binding molecule of the invention. The invention further provides one or more expression vector(s) comprising the isolated polynucleotide(s) of the invention, and a host cell comprising the isolated polynucleotide(s) or the expression vector(s) of the invention. In some embodiments the host cell is a eukaryotic cell, particularly a mammalian cell.

25 In another aspect is provided a method of producing an antibody that binds to HLA-A2/WT1, comprising the steps of a) culturing the host cell of the invention under conditions suitable for the expression of the antibody and b) recovering the antibody. The invention also encompasses an antibody that binds to HLA-A2/WT1 produced by the method of the invention.

The invention further provides a pharmaceutical composition comprising the antibody or  
30 bispecific antigen binding molecule of the invention and a pharmaceutically acceptable carrier.

Also encompassed by the invention are methods of using the antibody, bispecific antigen binding molecule and pharmaceutical composition of the invention. In one aspect the invention provides an antibody, bispecific antigen binding molecule or pharmaceutical composition according to the

invention for use as a medicament. In one aspect is provided an antibody, bispecific antigen binding molecule or pharmaceutical composition according to the invention for use in the treatment of a disease. In a specific embodiment the disease is cancer.

Also provided is the use of an antibody or bispecific antigen binding molecule according to the

5 invention in the manufacture of a medicament for the treatment of a disease; as well as a method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the antibody or bispecific antigen binding molecule according to the invention in a pharmaceutically acceptable form. In a specific embodiment the disease is cancer. In any of the above embodiments the individual preferably is  
10 a mammal, particularly a human.

### Brief Description of the Drawings

**Figure 1.** Exemplary configurations of the bispecific antigen binding molecules of the invention.

(A, D) Illustration of the “1+1 CrossMab” molecule. (B, E) Illustration of the “2+1 IgG Crossfab”  
15 molecule with alternative order of Crossfab and Fab components (“inverted”). (C, F) Illustration of the “2+1 IgG Crossfab” molecule. (G, K) Illustration of the “1+1 IgG Crossfab” molecule with alternative order of Crossfab and Fab components (“inverted”). (H, F) Illustration of the “1+1 IgG Crossfab” molecule. (I, M) Illustration of the “2+1 IgG Crossfab” molecule with two CrossFabs. (J, N) Illustration of the “2+1 IgG Crossfab” molecule with two CrossFabs and  
20 alternative order of Crossfab and Fab components (“inverted”). (O, S) Illustration of the “Fab-Crossfab” molecule. (P, T) Illustration of the “Crossfab-Fab” molecule. (Q, U) Illustration of the “(Fab)<sub>2</sub>-Crossfab” molecule. (R, V) Illustration of the “Crossfab-(Fab)<sub>2</sub>” molecule. (W, Y) Illustration of the “Fab-(Crossfab)<sub>2</sub>” molecule. (X, Z) Illustration of the “(Crossfab)<sub>2</sub>-Fab” molecule. Black dot: optional modification in the Fc domain promoting heterodimerization. ++, -  
25 -: amino acids of opposite charges optionally introduced in the CH1 and CF domains. Crossfab molecules are depicted as comprising an exchange of VH and VF regions, but may - in embodiments wherein no charge modifications are introduced in CH1 and CF domains - alternatively comprise an exchange of the CH1 and CF domains.

**Figure 2.** Illustration of the T-cell bispecific (TCB) antibody molecules prepared in the

30 Examples. All tested TCB antibody molecules were produced as “2+1 IgG CrossFab, inverted” with charge modifications (VH/VF exchange in CD3 binder, charge modifications in WT1 binders, EE = 147E, 213E; RK = 123R, 124K).

**Figure 3.** Binding of HLA-A2/WT1 IgG antibodies to peptide-pulsed T2 cells. (A) 11D06 IgG, (B) 33H09-IgG, (C) HB09-IgG, (D) 13B04-IgG, (E) 5E11-IgG, (F) 5C01-IgG, (G) HG06-IgG.

**Figure 4.** Activation of T cells by HLA-A2/WT1 x CD3 bispecific antibodies (TCBs) upon binding to peptide-pulsed T2 cells (NFAT reporter assay). (A) 11D06-TCB, (B) 33H09-TCB, (C) 11B09-TCB, (D) 13B04-TCB, (E) ESK1-TCB, (F) 5E11-TCB. (G) 5C01-TCB, (H) DP47GS-TCB.

**Figure 5.** Killing of peptide-pulsed T2 cells mediated by HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A) 11D06-TCB, (B) 33H09-TCB, (C) 13B04-TCB, (D) 11B09-TCB, (E) 33F05-TCB, (F) 5C01-TCB.

**Figure 6.** Killing of HLA-A2+WT1+ tumor cell lines mediated by HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A) Overview of cell lines. (B-E) Killing of cell lines by (B) 11D06-TCB, (C) 33H09-TCB, (D) 11B09-TCB, (E) 13B04-TCB. (F) Killing of SKM-1 cells by different TCBs. (G) Killing of BJAB cells by different TCBs.

**Figure 7.** Activation of T cells by HLA-A2/WT1 x CD3 bispecific antibodies (TCBs) upon binding to HLA-A2+WT1+ tumor cell lines. (A) SKM-1 cells, (B) BJAB cells.

**Figure 8.** No binding to off-target peptides by selected HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A-C) Binding to peptide-pulsed T2 cells by (A) 11D06-TCB, (B) 33H09-TCB, (C) ESK1-TCB. (D-E) Activation of T cells upon binding to peptide-pulsed T2 cells by (D) 11D06-TCB and (E) 33H09-TCB. (F) Overview of peptides.

**Figure 9.** No binding to additional off-target peptides by selected HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A-B) Activation of T cells upon binding to peptide-pulsed T2 cells by (A) 11D06-TCB and (B) 33H09-TCB. The 6 indicated off-target peptides were tested along with the RMF peptide. (C-G) Killing of peptide-pulsed T2 cells by (C, E) 11D06-TCB, (D, F) 33H09-TCB and (G) ESK1-TCB. The 19 indicated off-target peptides were tested along with the RMF and VLD peptides.

**Figure 10.** No killing of normal bone marrow-derived CD34+ stem cells mediated by selected HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A) 11D06-TCB, (B) 33H09-TCB.

**Figure 11.** Identification by alanine scan of binding residues in RMF peptide for selected HLA-A2/WT1 x CD3 bispecific antibodies (TCBs). (A) RMF native peptide, (B) RMF R1Y peptide, (C) RMF R1A peptide, (D) RMF M2A peptide, (E) RMF F3A peptide, (F) RMF P4A peptide, (G) RMF N5A peptide, (H) RMF A6G peptide, (I) RMF P7A peptide, (J) RMF Y8A peptide, (K) RMF F9A peptide. (F) Overview of peptides. (M) Fold change of EC<sub>50</sub> relative to EC<sub>50</sub> for the RMF native peptide. (N) Critical contact residues.

**Figure 12.** Pharmacokinetic profile of HLA-A2/WT1 x CD3 bispecific antibodies (11D06-TCB and 33H09-TCB) after single injection in NSG mice.

**Figure 13.** Efficacy study with HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) in SKM-1 xenograft in humanized mice. (A) Study design. (B) Treatment groups. (C) Tumor growth kinetics (mean) in all treatment groups. (D) Single tumor growth kinetics in the vehicle group. (E) Single tumor growth kinetics in the 11D06-TCB group. (F) Single tumor growth kinetics in the 33H09-TCB group. (G) Statistics. Calculations based on day 38 (vehicle as control group). Tumor growth inhibition (TGI): TGI > 100 → tumor regression, TGI = 100 → tumor stasis. Treatment to control ratio (TCR): TCR = 1 → no effect, TCR = 0 → complete regression.

**Figure 14.** Overview of crystal structures of HLA-A2/WT1 antibody - pMHC complexes. The antibodies (Fab fragments) are shown on top, with the heavy chain colored dark gray and the light chain colored light gray. Crystallized solvent atoms are not shown. (A) 1.98 Å resolution crystal structure of 5C01 Fab in complex with HFA-A02/VFD pMHC. Fab-pMHC contact area:  $\approx 476 \text{ Å}^2$ , peptide contribution:  $\approx 68 \text{ Å}^2$ . (B) 2.60 Å resolution crystal structure of 11D06 Fab in complex with HFA-A02/RMF pMHC. Fab-pMHC contact area:  $\approx 397 \text{ Å}^2$ , peptide contribution:  $\approx 107 \text{ Å}^2$ . (C) 3.05 Å resolution crystal structure of ESK1 Fab in complex with HFA-A02/RMF pMHC (published, PDB ID 4WUU). Fab-pMHC contact area:  $\approx 505 \text{ Å}^2$ , peptide contribution:  $\approx 60 \text{ Å}^2$ .

**Figure 15.** Close-up view on the 5C01 Fab - HFA-A2/WTT<sub>VLD</sub> pMHC binding interface. Essential chemical interactions between Fab and pMHC as identified by BIOVIA Discovery Studio 4.5 are highlighted. Solvent atoms are not shown.

**Figure 16.** Interface and interaction matrix of 5C01 Fab residues (rows) with HFA-A2/WT1<sub>LD</sub> pMHC residues (columns). N=near/neighboring, H=H-bond, Pi=Pi interactions, SB=salt bridge. Interface residues defined as residues that undergo a change in solvent-accessible surface area in absence/presence of the interaction partner.

**Figure 17.** Close-up view on the 11D06 Fab-HFA-A2/WT1<sub>RMF</sub> pMHC binding interface. Essential chemical interactions between Fab and pMHC as identified by BIOVIA Discovery Studio 4.5 are highlighted. Solvent atoms are not shown.

**Figure 18.** Interface and interaction matrix of 11D06 Fab residues (rows) with HFA-A2/WT1<sub>RMF</sub> pMHC residues (columns). N=near/neighboring, H=H-bond, Pi=Pi interactions, SB=salt bridge. Interface residues defined as residues that undergo a change in solvent-accessible surface area in absence/presence of the interaction partner.

**Figure 19.** Close-up view on the ESK1 Fab-HLA-A2/WT1<sub>RMF</sub> pMHC binding interface (PDB ID 4WUU). Essential chemical interactions between Fab and pMHC as identified by BIOVIA Discovery Studio 4.5 are highlighted. Solvent atoms are not shown.

**Figure 20.** Interface and interaction matrix of ESK1 Fab residues (rows) with HEA-A2/WT1<sub>RMF</sub> pMHC residues (columns). N=near/neighboring, H=H-bond, Pi=Pi interactions, SB=salt bridge. Interface residues defined as residues that undergo a change in solvent-accessible surface area in absence/presence of the interaction partner.

**Figure 21.** Killing of HFA-A2+/WT1+ SKM-1 cells mediated by HFA-A2/WT1 x CD3 bispecific antibodies with different CD3 binders.

10 **Figure 22.** Killing of RMF peptide-pulsed T2 cells mediated by HFA-A2/WT1 x CD3 bispecific antibodies (TCBs).

**Figure 23.** Assessment of binding to off-target peptides by HFA-A2/WT1 x CD3 bispecific antibodies (TCBs) Aali-TCB (A), Daniel-TCB (B), ESK1-TCB (C) and 11D06-TCB (D).

15 **Figure 24.** Pharmacokinetic profile of HFA-A2/WT1 x CD3 bispecific antibody 11D06-TCB (V9) after single injection in NSG mice.

**Figure 25.** Efficacy study with HFA-A2/WT1 x CD3 bispecific antibody 11D06-TCB (V9) in SKM-1 xenograft in humanized mice. (A) Tumor growth kinetics (mean) in all treatment groups. (B) Single tumor growth kinetics in the vehicle group. (C) Single tumor growth kinetics in the 11D06-TCB (V9) group. (D) Statistics. Calculations based on day 48 (vehicle as control group).

20 Tumor growth inhibition (TGI): TGI > 100 → tumor regression, TGI = 100 → tumor stasis. Treatment to control ratio (TCR): TCR = 1 → no effect, TCR = 0 → complete regression.

**Figure 26.** Activation of T cells by HFA-A2/WT1 x CD3 bispecific antibodies (TCBs) upon binding to CHO-K1 cells expressing a HEA-A02/WT1<sub>RMF</sub> pMHC complex (NFAT reporter assay). Solid line: 11D06-TCB (V9) ("2+1" format). Dashed line: analogous molecule (11D06 and V9 binders) in "1+1 CrossMab" format.

## Detailed Description of the Invention

### Definitions

Terms are used herein as generally used in the art, unless otherwise defined in the following.

30 As used herein, the term "antigen binding molecule" refers in its broadest sense to a molecule that specifically binds an antigenic determinant. Examples of antigen binding molecules are immunoglobulins and derivatives, e.g. fragments, thereof.

The term "bispecific" means that the antigen binding molecule is able to specifically bind to at least two distinct antigenic determinants. Typically, a bispecific antigen binding molecule comprises two antigen binding sites, each of which is specific for a different antigenic determinant. In certain embodiments the bispecific antigen binding molecule is capable of 5 simultaneously binding two antigenic determinants, particularly two antigenic determinants expressed on two distinct cells.

The term "valent" as used herein denotes the presence of a specified number of antigen binding sites in an antigen binding molecule. As such, the term "monovalent binding to an antigen" denotes the presence of one (and not more than one) antigen binding site specific for the antigen 10 in the antigen binding molecule.

An "antigen binding site" refers to the site, i.e. one or more amino acid residues, of an antigen binding molecule which provides interaction with the antigen. For example, the antigen binding site of an antibody comprises amino acid residues from the complementarity determining regions (CDRs). A native immunoglobulin molecule typically has two antigen binding sites, a Fab 15 molecule typically has a single antigen binding site.

As used herein, the term "antigen binding moiety" refers to a polypeptide molecule that specifically binds to an antigenic determinant. In one embodiment, an antigen binding moiety is able to direct the entity to which it is attached (e.g. a second antigen binding moiety) to a target site, for example to a specific type of tumor cell bearing the antigenic determinant. In another 20 embodiment an antigen binding moiety is able to activate signaling through its target antigen, for example a T cell receptor complex antigen. Antigen binding moieties include antibodies and fragments thereof as further defined herein. Particular antigen binding moieties include an antigen binding domain of an antibody, comprising an antibody heavy chain variable region and an antibody light chain variable region. In certain embodiments, the antigen binding moieties 25 may comprise antibody constant regions as further defined herein and known in the art. Useful heavy chain constant regions include any of the five isotypes:  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , or  $\mu$ . Useful light chain constant regions include any of the two isotypes:  $\kappa$  and  $\lambda$ .

As used herein, the term "antigenic determinant" or "antigen" refers to a site on a polypeptide macromolecule to which an antigen binding moiety binds, forming an antigen binding moiety- 30 antigen complex. Useful antigenic determinants can be found, for example, on the surfaces of tumor cells, on the surfaces of virus-infected cells, on the surfaces of other diseased cells, on the surface of immune cells, free in blood serum, and/or in the extracellular matrix (ECM).

The term "epitope" denotes the site on an antigen, either proteinaceous or non-proteinaceous, to which an antigen binding moiety binds. Epitopes can be formed both from contiguous amino acid stretches (linear epitope) or comprise non-contiguous amino acids (conformational epitope), e.g. coming in spatial proximity due to the folding of the antigen, i.e. by the tertiary folding of a

5 proteinaceous antigen. Linear epitopes are typically still bound by an antigen binding moiety after exposure of the proteinaceous antigen to denaturing agents, whereas conformational epitopes are typically destroyed upon treatment with denaturing agents. An epitope comprises at least 3, at least 4, at least 5, at least 6, at least 7, or 8-10 amino acids in a unique spatial conformation.

10 "CD3" refers to any native CD3 from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed CD3 as well as any form of CD3 that results from processing in the cell. The term also encompasses naturally occurring variants of CD3, e.g., splice variants or allelic variants. In one embodiment, 15 CD3 is human CD3, particularly the epsilon subunit of human CD3 (CD3e). The amino acid sequence of human CD3e is shown in UniProt ([www.uniprot.org](http://www.uniprot.org)) accession no. P07766 (version 189), or NCBI ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) RefSeq NP\_000724.1. See also SEQ ID NO: 107. The amino acid sequence of cynomolgus [Macaca fascicularis] CD3e is shown in NCBI GenBank no. BAB71849.1. See also SEQ ID NO: 108.

20 "WT1", also known as "Wilms tumor 1" or "Wilms tumor protein", refers to any native WT1 from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed WT1 as well as any form of WT1 that results from processing in the cell. The term also encompasses naturally occurring variants of WT1, e.g., 25 splice variants or allelic variants. In one embodiment, WT1 is human WT1, particularly the protein of SEQ ID NO: 106. Human WT1 is described in UniProt ([www.uniprot.org](http://www.uniprot.org)) accession no. P19544 (entry version 215), and an amino acid sequence of human WT1 is also shown in SEQ ID NO: 106.

By "VLD", "VLD peptide" or "WT1<sub>vix</sub>" is meant the WT1 derived peptide having the amino 30 acid sequence VLDFAPPGA (SEQ ID NO: 77; position 37-45 of the WT1 protein of SEQ ID NO: 106).

By "RMF", "RMF peptide" or "WT! <sub>RMF</sub>" is meant the WT1 derived peptide having the amino acid sequence RMFRNAPYL (SEQ ID NO: 78; position 126-134 of the WT1 protein of SEQ ID NO: 106).

"HLA-A2", "HLA-A\*02", "HLA-A02", or "HLA-A\*2" (used interchangeably) refers to a

5 human leukocyte antigen serotype in the HLA-A serotype group. The HLA-A2 protein (encoded by the respective HLA gene) constitutes the a chain of the respective class I MHC (major histocompatibility complex) protein, which further comprises a  $\beta$ 2 microglobulin subunit. A

specific HLA-A2 protein is HLA-A201 (also referred to as HLA-A0201, HLA-A02.01, or HLA-A\*02:01). In specific embodiments, the HLA-A2 protein described herein is HLA-A201

10 "HLA-A2/WT1" refers to a complex of a HLA-A2 molecule and a WT1 derived peptide (also referred to herein as a "WT1 peptide"), specifically the RMF or VLD peptide ("HLA-A2/WT1 <sub>RMF</sub>" and "HLA-A2/WT1 <sub>VLD</sub>", respectively). The antibody or bispecific antigen binding molecule of the present invention specifically binds to either the HLA-A2/WT1 <sub>RMF</sub> or the HLA-A2/WT1<sub>VLD</sub> complex.

15 By "specific binding" is meant that the binding is selective for the antigen and can be discriminated from unwanted or non-specific interactions. The ability of an antigen binding moiety to bind to a specific antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. surface plasmon resonance (SPR) technique (analyzed e.g. on a BIACore instrument) (Liljeblad

20 et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). Suitable assays for determining the specificity of the antibody and bispecific antigen binding molecule of the present invention are described herein, e.g. in Examples 4, 9 and

10 hereinbelow. In one embodiment, the extent of binding of an antigen binding moiety to an unrelated protein is less than about 10% of the binding of the antigen binding moiety to the 25 antigen as measured, e.g., by SPR. In certain embodiments, an antigen binding moiety that binds to the antigen, or an antigen binding molecule comprising that antigen binding moiety, has a dissociation constant ( $K_D$ ) of  $\leq 1 \mu\text{M}$ ,  $\leq 100 \text{ nM}$ ,  $\leq 10 \text{ nM}$ ,  $\leq 1 \text{ nM}$ ,  $\leq 0.1 \text{ nM}$ ,  $\leq 0.01 \text{ nM}$ , or  $\leq 0.001 \text{ nM}$  (e.g.  $10^{-8} \text{ M}$  or less, e.g. from  $10^{-8} \text{ M}$  to  $10^{-13} \text{ M}$ , e.g., from  $10^{-9} \text{ M}$  to  $10^{-13} \text{ M}$ ).

"Affinity" refers to the strength of the sum total of non-covalent interactions between a single

30 binding site of a molecule (e.g., a receptor) and its binding partner (e.g., a ligand). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., an antigen binding moiety and an antigen, or a receptor and its ligand). The affinity of a molecule X for its partner Y can

generally be represented by the dissociation constant (**KD**), which is the ratio of dissociation and association rate constants ( $k_{\text{off}}$  and  $k_{\text{on}}$ , respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by well established methods known in the art, including those described herein. A

5 particular method for measuring affinity is Surface Plasmon Resonance (SPR).

“Reduced binding”, for example reduced binding to an Fc receptor, refers to a decrease in affinity for the respective interaction, as measured for example by SPR. For clarity, the term includes also reduction of the affinity to zero (or below the detection limit of the analytic method), i.e. complete abolishment of the interaction. Conversely, “increased binding” refers to

10 an increase in binding affinity for the respective interaction.

An “activating T cell antigen” as used herein refers to an antigenic determinant expressed on the surface of a T lymphocyte, particularly a cytotoxic T lymphocyte, which is capable of inducing T cell activation upon interaction with an antigen binding molecule. Specifically, interaction of an antigen binding molecule with an activating T cell antigen may induce T cell activation by

15 triggering the signaling cascade of the T cell receptor complex. In a particular embodiment the activating T cell antigen is CD3, particularly the epsilon subunit of CD3 (see UniProt no. P07766 (version 189), NCBI RefSeq no. NP\_000724.1, SEQ ID NO: 107 for the human sequence; or UniProt no. Q95LI5 (version 49), NCBI GenBank no. BAB71849.1, SEQ ID NO: 108 for the cynomolgus [Macaca fascicularis] sequence).

20 “T cell activation” as used herein refers to one or more cellular response of a T lymphocyte, particularly a cytotoxic T lymphocyte, selected from: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, and expression of activation markers. Suitable assays to measure T cell activation are known in the art and described herein.

25 A “target cell antigen” as used herein refers to an antigenic determinant presented on the surface of a target cell, for example a cell in a tumor such as a cancer cell or a cell of the tumor stroma. In a particular embodiment, the target cell antigen is HLA-A2/WT1, particularly HLA-A2/WT1RMF or HLA-A2/WT1vLD, most particularly HLA-A2/WT1RMF.

30 As used herein, the terms “first”, “second” or “third” with respect to Fab molecules etc., are used for convenience of distinguishing when there is more than one of each type of moiety. Use of these terms is not intended to confer a specific order or orientation of the bispecific antigen binding molecule unless explicitly so stated.

By “fused” is meant that the components (e.g. a Fab molecule and an Fc domain subunit) are linked by peptide bonds, either directly or via one or more peptide linkers.

A “Fab molecule” refers to a protein consisting of the VH and CH1 domain of the heavy chain (the “Fab heavy chain”) and the VL and CL domain of the light chain (the “Fab light chain”) of an immunoglobulin.

By a “crossover” Fab molecule (also termed “Crossfab”) is meant a Fab molecule wherein the

5 variable domains or the constant domains of the Fab heavy and light chain are exchanged (i.e. replaced by each other), i.e. the crossover Fab molecule comprises a peptide chain composed of the light chain variable domain VL and the heavy chain constant domain 1 CH1 (VL-CH1, in N- to C-terminal direction), and a peptide chain composed of the heavy chain variable domain VH and the light chain constant domain CL (VH-CL, in N- to C-terminal direction). For clarity, in a  
10 crossover Fab molecule wherein the variable domains of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain constant domain 1 CH1 is referred to herein as the “heavy chain” of the (crossover) Fab molecule. Conversely, in a crossover Fab molecule wherein the constant domains of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain variable domain VH is  
15 referred to herein as the “heavy chain” of the (crossover) Fab molecule.

In contrast thereto, by a “conventional” Fab molecule is meant a Fab molecule in its natural format, i.e. comprising a heavy chain composed of the heavy chain variable and constant domains (VH-CH1, in N- to C-terminal direction), and a light chain composed of the light chain variable and constant domains (VL-CL, in N- to C-terminal direction).

20 The term “immunoglobulin molecule” refers to a protein having the structure of a naturally occurring antibody. For example, immunoglobulins of the IgG class are heterotetrameric glycoproteins of about 150,000 daltons, composed of two light chains and two heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable domain (VH), also called a variable heavy domain or a heavy chain variable region, followed by three constant  
25 domains (CH1, CH2, and CH3), also called a heavy chain constant region. Similarly, from N- to C-terminus, each light chain has a variable domain (VL), also called a variable light domain or a light chain variable region, followed by a constant light (CL) domain, also called a light chain constant region. The heavy chain of an immunoglobulin may be assigned to one of five types, called a (IgA),  $\delta$  (IgD),  $\epsilon$  (IgE),  $\gamma$  (IgG), or  $\mu$  (IgM), some of which may be further divided into  
30 subtypes, e.g.  $\gamma_1$  (IgG1),  $\gamma_2$  (IgG2),  $\gamma_3$  (IgG3),  $\gamma_4$  (IgG4),  $\alpha_1$  (IgA1) and  $\alpha_2$  (IgA2). The light chain of an immunoglobulin may be assigned to one of two types, called kappa ( $\kappa$ ) and lambda ( $\lambda$ ), based on the amino acid sequence of its constant domain. An immunoglobulin essentially consists of two Fab molecules and an Fc domain, linked via the immunoglobulin hinge region.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

5 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies comprised in the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal  
10 antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular  
15 method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

20 An "isolated" antibody is one which has been separated from a component of its natural environment, i.e. that is not in its natural milieu. No particular level of purification is required. For example, an isolated antibody can be removed from its native or natural environment. Recombinantly produced antibodies expressed in host cells are considered isolated for the purpose of the invention, as are native or recombinant antibodies which have been separated,  
25 fractionated, or partially or substantially purified by any suitable technique. As such, the antibodies and bispecific antigen binding molecules of the present invention are isolated. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC) methods. For  
30 review of methods for assessment of antibody purity, see, e.g., Flatman et al., *J. Chromatogr. B* 848:79-87 (2007).

The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>, diabodies, linear antibodies, single-chain antibody molecules (e.g. scFv), and single-domain antibodies. For a review of certain antibody fragments, see Hudson et al., *Nat Med* 9, 129-134 (2003). For a review of scFv fragments, see e.g. Pliickthun, in *The Pharmacology of Monoclonal Antibodies*,

10 vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and F(ab')<sub>2</sub> fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046. Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; 15 Hudson et al., *Nat Med* 9, 129-134 (2003); and Hollinger et al., *Proc Natl Acad Sci USA* 90, 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat Med* 9, 129-134 (2003). Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody 20 (Domantis, Inc., Waltham, MA; see e.g. U.S. Patent No. 6,248,516 B1). Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage), as described herein.

The term "antigen binding domain" refers to the part of an antibody that comprises the area 25 which specifically binds to and is complementary to part or all of an antigen. An antigen binding domain may be provided by, for example, one or more antibody variable domains (also called antibody variable regions). Particularly, an antigen binding domain comprises an antibody light chain variable domain (VF) and an antibody heavy chain variable domain (VH).

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or 30 light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VF, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See, e.g., Kindt et al., *Kuby Immunology*, 6<sup>th</sup> ed., W.H. Freeman

and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity. As used herein in connection with variable region sequences, "Kabat numbering" refers to the numbering system set forth by Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991).

5 As used herein, the amino acid positions of all constant regions and domains of the heavy and light chain are numbered according to the Kabat numbering system described in Kabat, et al., *Sequences of Proteins of Immunological Interest*, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991), referred to as "numbering according to Kabat" or "Kabat numbering" herein. Specifically the Kabat numbering system (see pages 647-660 of

10 Kabat, et al., *Sequences of Proteins of Immunological Interest*, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991)) is used for the light chain constant domain CL of kappa and lambda isotype and the Kabat EU index numbering system (see pages 661-723) is used for the heavy chain constant domains (CH1, Hinge, CH2 and CH3), which is herein further clarified by referring to "numbering according to Kabat EU index" in this case.

15 The term "hypervariable region" or "HVR", as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops") and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). Exemplary HVRs herein

20 include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991));

25 (c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3), 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. *J. Mol. Biol.* 262: 732-745 (1996)); and

30 (d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Rabat et al., *supra*.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3,

5 and FR4. Accordingly, the HVR and FR sequences generally appear in the following order in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in

10 which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. Such variable domains are referred to herein as "humanized variable region". A humanized antibody

15 optionally may comprise at least a portion of an antibody constant region derived from a human antibody. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity. A "humanized form" of an antibody, e.g. of a non-human antibody, refers to an antibody that has undergone

humanization. Other forms of "humanized antibodies" encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the

20 original antibody to generate the properties according to the invention, especially in regard to Clq binding and/or Fc receptor (FcR) binding.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition

25 of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. In certain embodiments, a human antibody is derived from a non-human transgenic mammal, for example a mouse, a rat, or a rabbit. In certain embodiments, a human antibody is derived from a hybridoma cell line. Antibodies or antibody fragments isolated

from human antibody libraries are also considered human antibodies or human antibody

30 fragments herein.

The "class" of an antibody or immunoglobulin refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1,

IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgAi, and IgA<sub>2</sub>. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively.

The term “Fc domain” or “Fc region” herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term

5 includes native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an IgG heavy chain might vary slightly, the human IgG heavy chain Fc region is usually defined to extend from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, antibodies produced by host cells may undergo post-translational cleavage of one or more, particularly one or two, amino acids from the C-terminus of the heavy chain.

10 Therefore an antibody produced by a host cell by expression of a specific nucleic acid molecule encoding a full-length heavy chain may include the full-length heavy chain, or it may include a cleaved variant of the full-length heavy chain (also referred to herein as a “cleaved variant heavy chain”). This may be the case where the final two C-terminal amino acids of the heavy chain are glycine (G446) and lysine (K447, numbering according to Kabat EU index). Therefore, the C-

15 terminal lysine (Lys447), or the C-terminal glycine (Gly446) and lysine (K447), of the Fc region may or may not be present. Amino acid sequences of heavy chains including Fc domains (or a subunit of an Fc domain as defined herein) are denoted herein without C-terminal glycine-lysine dipeptide if not indicated otherwise. In one embodiment of the invention, a heavy chain including a subunit of an Fc domain as specified herein, comprised in an antibody or bispecific

20 antigen binding molecule according to the invention, comprises an additional C-terminal glycine-lysine dipeptide (G446 and K447, numbering according to EU index of Kabat). In one embodiment of the invention, a heavy chain including a subunit of an Fc domain as specified herein, comprised in an antibody or bispecific antigen binding molecule according to the invention, comprises an additional C-terminal glycine residue (G446, numbering according to

25 EU index of Kabat). Compositions of the invention, such as the pharmaceutical compositions described herein, comprise a population of antibodies or bispecific antigen binding molecules of the invention. The population of antibodies or bispecific antigen binding molecules may comprise molecules having a full-length heavy chain and molecules having a cleaved variant heavy chain. The population of antibodies or bispecific antigen binding molecules may consist of

30 a mixture of molecules having a full-length heavy chain and molecules having a cleaved variant heavy chain, wherein at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the antibodies or bispecific antigen binding molecules have a cleaved variant heavy chain. In one embodiment of the invention a composition comprising a population of antibodies or bispecific

antigen binding molecules of the invention comprises an antibody or bispecific antigen binding molecule comprising a heavy chain including a subunit of an Fc domain as specified herein with an additional C-terminal glycine-lysine dipeptide (G446 and K447, numbering according to EU index of Kabat). In one embodiment of the invention a composition comprising a population of

5 antibodies or bispecific antigen binding molecules of the invention comprises an antibody or bispecific antigen binding molecule comprising a heavy chain including a subunit of an Fc domain as specified herein with an additional C-terminal glycine residue (G446, numbering according to EU index of Kabat). In one embodiment of the invention such a composition comprises a population of antibodies or bispecific antigen binding molecules comprised of  
10 molecules comprising a heavy chain including a subunit of an Fc domain as specified herein; molecules comprising a heavy chain including a subunit of a Fc domain as specified herein with an additional C-terminal glycine residue (G446, numbering according to EU index of Kabat); and molecules comprising a heavy chain including a subunit of an Fc domain as specified herein with an additional C-terminal glycine-lysine dipeptide (G446 and K447, numbering according to  
15 EU index of Kabat). Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991 (see also above). A “subunit” of an Fc domain as used herein refers to one of the two polypeptides forming the dimeric Fc  
20 domain, i.e. a polypeptide comprising C-terminal constant regions of an immunoglobulin heavy chain, capable of stable self-association. For example, a subunit of an IgG Fc domain comprises an IgG CH2 and an IgG CH3 constant domain.

A “modification promoting the association of the first and the second subunit of the Fc domain” is a manipulation of the peptide backbone or the post-translational modifications of an Fc  
25 domain subunit that reduces or prevents the association of a polypeptide comprising the Fc domain subunit with an identical polypeptide to form a homodimer. A modification promoting association as used herein particularly includes separate modifications made to each of the two Fc domain subunits desired to associate (i.e. the first and the second subunit of the Fc domain), wherein the modifications are complementary to each other so as to promote association of the  
30 two Fc domain subunits. For example, a modification promoting association may alter the structure or charge of one or both of the Fc domain subunits so as to make their association sterically or electrostatically favorable, respectively. Thus, (hetero)dimerization occurs between a polypeptide comprising the first Fc domain subunit and a polypeptide comprising the second

Fc domain subunit, which might be non-identical in the sense that further components fused to each of the subunits (e.g. antigen binding moieties) are not the same. In some embodiments the modification promoting association comprises an amino acid mutation in the Fc domain, specifically an amino acid substitution. In a particular embodiment, the modification promoting association comprises a separate amino acid mutation, specifically an amino acid substitution, in each of the two subunits of the Fc domain.

5 The term “effector functions” refers to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: Clq binding and complement dependent cytotoxicity (CDC), Fc receptor binding, 10 antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), cytokine secretion, immune complex-mediated antigen uptake by antigen presenting cells, down regulation of cell surface receptors (e.g. B cell receptor), and B cell activation.

15 As used herein, the terms “engineer, engineered, engineering”, are considered to include any manipulation of the peptide backbone or the post-translational modifications of a naturally occurring or recombinant polypeptide or fragment thereof. Engineering includes modifications of the amino acid sequence, of the glycosylation pattern, or of the side chain group of individual amino acids, as well as combinations of these approaches.

20 The term “amino acid mutation” as used herein is meant to encompass amino acid substitutions, deletions, insertions, and modifications. Any combination of substitution, deletion, insertion, and modification can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., reduced binding to an Fc receptor, or increased association with another peptide. Amino acid sequence deletions and insertions include amino- and/or carboxy-terminal deletions and insertions of amino acids. Particular amino acid mutations 25 are amino acid substitutions. For the purpose of altering e.g. the binding characteristics of an Fc region, non-conservative amino acid substitutions, i.e. replacing one amino acid with another amino acid having different structural and/or chemical properties, are particularly preferred. Amino acid substitutions include replacement by non-naturally occurring amino acids or by naturally occurring amino acid derivatives of the twenty standard amino acids (e.g. 4- 30 hydroxyproline, 3-methylhistidine, ornithine, homoserine, 5-hydroxylysine). Amino acid mutations can be generated using genetic or chemical methods well known in the art. Genetic methods may include site-directed mutagenesis, PCR, gene synthesis and the like. It is contemplated that methods of altering the side chain group of an amino acid by methods other

than genetic engineering, such as chemical modification, may also be useful. Various designations may be used herein to indicate the same amino acid mutation. For example, a substitution from proline at position 329 of the Fc domain to glycine can be indicated as 329G, G329, G329, P329G, or Pro329Gly.

5 "Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for  
10 purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, Clustal W, Megalign (DNASTAR) software or the FASTA program package. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the  
15 sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the ggsearch program of the FASTA package version 36.3.8c or later with a BLOSUM50 comparison matrix. The FASTA program package was authored by W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448; W. R. Pearson (1996) "Effective protein sequence comparison" Meth. Enzymol.  
20 266:227- 258; and Pearson et. al. (1997) Genomics 46:24-36, and is publicly available from [http://fasta.bioch.virginia.edu/fasta\\_www2/fasta\\_down.shtml](http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml). Alternatively, a public server accessible at [http://fasta.bioch.virginia.edu/fasta\\_www2/index.cgi](http://fasta.bioch.virginia.edu/fasta_www2/index.cgi) can be used to compare the sequences, using the ggsearch (global proteinprotein) program and default options (BLOSUM50; open: -10; ext: -2; Ktup = 2) to ensure a global, rather than local, alignment is performed.  
25 Percent amino acid identity is given in the output alignment header.

The term "polynucleotide" refers to an isolated nucleic acid molecule or construct, e.g. messenger RNA (mRNA), virally-derived RNA, or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (e.g. an amide bond, such as found in peptide nucleic acids (PNA). The term "nucleic acid molecule" refers to  
30 any one or more nucleic acid segments, e.g. DNA or RNA fragments, present in a polynucleotide.

By "isolated" nucleic acid molecule or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant

polynucleotide encoding a polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. An isolated polynucleotide includes a polynucleotide 5 molecule contained in cells that ordinarily contain the polynucleotide molecule, but the polynucleotide molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the present invention, as well as positive and negative strand forms, and double-stranded forms. Isolated polynucleotides or nucleic acids according to the present 10 invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

“Isolated polynucleotide (or nucleic acid) encoding [e.g. an antibody or bispecific antigen binding molecule of the invention]” refers to one or more polynucleotide molecules encoding 15 antibody heavy and light chains (or fragments thereof), including such polynucleotide molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term “expression cassette” refers to a polynucleotide generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a 20 particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. In certain embodiments, the expression cassette comprises polynucleotide sequences that encode 25 antibodies or bispecific antigen binding molecules of the invention or fragments thereof.

The term “vector” or “expression vector” refers to a DNA molecule that is used to introduce and direct the expression of a specific gene to which it is operably associated in a cell. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated 30 into the genome of a host cell into which it has been introduced. The expression vector of the present invention comprises an expression cassette. Expression vectors allow transcription of large amounts of stable mRNA. Once the expression vector is inside the cell, the ribonucleic acid molecule or protein that is encoded by the gene is produced by the cellular transcription and/or translation machinery. In one embodiment, the expression vector of the invention comprises an

expression cassette that comprises polynucleotide sequences that encode antibodies or bispecific antigen binding molecules of the invention or fragments thereof.

The terms "host cell", "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such

5 cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages.

Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein. A host cell is any type of

10 cellular system that can be used to generate the antibodies or bispecific antigen binding molecules of the present invention. Host cells include cultured cells, *e.g.* mammalian cultured cells, such as HEK cells, CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells,

P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal,

15 transgenic plant or cultured plant or animal tissue.

An "activating Fc receptor" is an Fc receptor that following engagement by an Fc domain of an antibody elicits signaling events that stimulate the receptor-bearing cell to perform effector functions. Human activating Fc receptors include FcyRIIIa (CD16a), FcyRI (CD64), FcyRIIa (CD32), and FcaRI (CD89).

20 Antibody-dependent cell-mediated cytotoxicity (ADCC) is an immune mechanism leading to the lysis of antibody-coated target cells by immune effector cells. The target cells are cells to which antibodies or derivatives thereof comprising an Fc region specifically bind, generally via the protein part that is N-terminal to the Fc region. As used herein, the term "reduced ADCC" is defined as either a reduction in the number of target cells that are lysed in a given time, at a

25 given concentration of antibody in the medium surrounding the target cells, by the mechanism of ADCC defined above, and/or an increase in the concentration of antibody in the medium surrounding the target cells, required to achieve the lysis of a given number of target cells in a given time, by the mechanism of ADCC. The reduction in ADCC is relative to the ADCC mediated by the same antibody produced by the same type of host cells, using the same standard

30 production, purification, formulation and storage methods (which are known to those skilled in the art), but that has not been engineered. For example the reduction in ADCC mediated by an antibody comprising in its Fc domain an amino acid substitution that reduces ADCC, is relative to the ADCC mediated by the same antibody without this amino acid substitution in the Fc

domain. Suitable assays to measure ADCC are well known in the art (see e.g. PCT publication no. WO 2006/082515 or PCT publication no. WO 2012/130831).

An "effective amount" of an agent refers to the amount that is necessary to result in a physiological change in the cell or tissue to which it is administered.

5 A "therapeutically effective amount" of an agent, e.g. a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. A therapeutically effective amount of an agent for example eliminates, decreases, delays, minimizes or prevents adverse effects of a disease.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, 10 domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g. humans and non-human primates such as monkeys), rabbits, and rodents (e.g. mice and rats). Particularly, the individual or subject is a human.

The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which 15 contains no additional components which are unacceptably toxic to a subject to which the composition would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

20 As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of a disease in the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or 25 indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies or bispecific antigen binding molecules of the invention are used to delay development of a disease or to slow the progression of a disease.

The term "package insert" is used to refer to instructions customarily included in commercial 30 packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

## **Detailed Description of the Embodiments**

The invention provides antibodies and bispecific antigen binding molecules that bind HLA-A2/WT1, particularly HLA-A2/WT1RMF or HLA-A2/WT1vLD, most particularly HLA-A2/WT1<sub>RMF</sub>, and have good affinity and specificity as required for therapeutic purposes. In addition, the molecules have also other favorable properties for therapeutic application, e.g. with respect to efficacy and/or safety as well as producibility.

### **HLA-A2/WT1 antibody**

The present inventors have developed novel antibodies that bind to HLA-A2/WT1 with particularly good affinity and specificity. For instance, as shown in the Examples, the inventors have developed antibodies that are remarkably selective for HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub>) over complexes of HLA-A2 with other, structurally similar peptides.

Thus, in certain aspects, the invention provides an antibody that binds to HLA-A2/WT1 and has any of the following features.

In one embodiment, the antibody has a monovalent affinity to HLA-A2/WT1 with a dissociation constant (KD) of lower than about 100 nM, lower than about 75 nM, or lower than about 50 nM.

In one embodiment, the antibody has a bivalent affinity (avidity) to HLA-A2/WT1 with an apparent KD of lower than about 1.5 nM, lower than about 1 nM, or lower than about 0.75 nM. In one embodiment, the bivalent affinity (avidity) of the antibody is at 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold higher than the monovalent affinity of the antibody in terms of (apparent) KD.

In one embodiment, the affinity is determined by Surface Plasmon Resonance (SPR) at 25°C. In one embodiment, the monovalent affinity is determined in a Fab molecule format of the antibody. In one embodiment, the bivalent affinity (avidity) is determined in an IgG molecule format of the antibody.

In a specific embodiment, the affinity of the antibody is determined as follows:

Experiments are performed at 25°C using PBST as running buffer (10 mM PBS, pH 7.4 and 0.005% (v/v) Tween 20). A ProteOn XPR36 biosensor equipped with GLC and GLM sensor chips and coupling reagents (10 mM sodium acetate pH 4.5, sulfo-N-hydroxysuccinimide [sulfo-NHS], 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride [EDC] and ethanolamine)

from BioRad Inc. (Hercules, CA) is used. Immobilizations are performed at 30 µl/min on a GLM chip. pAb (goat) anti human IgG, F(ab)2 specific antibody (Jackson ImmunoResearch) is coupled in vertical direction using a standard amine-coupling procedure: all six ligand channels are activated for 5 min with a mixture of EDC (200 mM) and sulfo-NHS (50 mM). Immediately

after the surfaces are activated, pAb (goat) anti human IgG, F(ab)2 specific antibody (50 pg/ml, 10 mM sodium acetate, pH 5) is injected across all six channels for 5 min. Finally, channels are blocked with a 5 min injection of 1 M ethanolamine-HCl (pH 8.5). The Fab variants are captured from *E.coli* supernatants by simultaneous injection along five of the separate horizontal channels

5 (30  $\mu$ l/min) for 5 min. Conditioned medium is injected along the sixth channel to provide an 'in-line' blank for double referencing purposes. One-shot kinetic measurements are performed by injection of a dilution series of HLA-WT1 (e.g. HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT!<sub>VLD</sub>) (100, 50, 25, 12.5, 6.25, 0 nM, 50 pl/min) for 2 min along the vertical channels. Dissociation is monitored for 3 min. Kinetic data is analyzed in ProteOn Manager v. 2.1. Processing of the  
10 reaction spot data involves applying an interspot-reference and a double-reference step using an inline buffer blank (Myszka, J Mol Recognit (1999) 12, 279-284). The processed data from replicate one-shot injections are fit to a simple 1:1 Langmuir binding model without mass transport (O'Shannessy et al., Anal Biochem (1993) 212, 457-468).

The antibody of the invention specifically binds to HLA-A2/WT1 (i.e. a complex of a HLA-A2  
15 molecule and a WT1-derived peptide). In some embodiments, the antibody of the invention specifically binds to HLA-A2/WT1<sub>RMF</sub> (i.e. a complex of a HLA-A2 molecule and the WT!<sub>RMF</sub> peptide). In a more specific embodiment, the antibody specifically binds to HLA-A201/WT1<sub>RMF</sub> (i.e. a complex of a HLA-A201 molecule and the WTTRMF peptide). Antibodies of the invention that bind to HLA-A2/WT1<sub>RMF</sub> include antibodies 11D06, 33H09 and 5E1 1 described herein. In  
20 other embodiments, the antibody of the invention specifically binds to HLA-A2/WT1<sub>v LD</sub> (i.e. a complex of a HLA-A2 molecule and the WT1<sub>v LD</sub> peptide). In a more specific embodiment, the antibody specifically binds to HLA-A201/WT1<sub>v LD</sub> (i.e. a complex of a HLA-A201 molecule and the WT1<sub>v LD</sub> peptide). Antibodies of the invention that bind to HLA-A2/WT1<sub>v LD</sub> include antibodies 11B09, 13B04 and 5C01 described herein.

25 In one embodiment, specific binding of the antibody is determined by flow cytometry using HLA-A2/peptide (e.g. WT!RMF or WT1<sub>v LD</sub> peptide)-expressing cells, particularly peptide-pulsed T2 cells.

In a specific embodiment, specific binding of the antibody is determined as follows:

T2 cells are prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life  
30 Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco, Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, Cat No. 15070-063) (complete medium). Cells are kept in a total volume of 10 ml in a tube, and incubated with 10  $\mu$ l of peptide (e.g. WT1 VLD peptide (SEQ ID NO: 77), or RMF peptide (SEQ ID NO: 78)) at  $10^{-2}$  M (final concentration of the

peptide: 10<sup>-5</sup>M) for 2 hours at 37°C with 5% CO<sub>2</sub>. After washing, cells are suspended in cold PBS and incubated with titrated concentration of antibody in IgG format (e.g. 10 µg/ml to 0.00064 pg/ml) for 1 hour at 4°C, followed by incubation with a secondary anti-human IgG-Fc phycoerythrin (PE)-conjugated antibody (Jackson Laboratories, Cat No. 109-116-098) for 30 min. Cells are acquired on FACS LSR II (BD), and data are presented as mean fluorescence intensity (MFI) of PE in Graphpad Prism.

In one embodiment, the antibody of the invention does not significantly bind to HLA-A2 alone (i.e. without a peptide) or HLA-A2 with a peptide other than a WT1 -derived peptide such as WT1RMF or WT1vLD.

10 In one embodiment, the antibody does not significantly bind to HLA-A2 in the absence of a WT1 -derived peptide, particularly WT1<sub>RMF</sub> or WT1v<sub>LD</sub>. In one embodiment, the antibody binds to HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>VLD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for binding to HLA-A2 in the absence of a WT1 -derived peptide  
15 (specifically WT1RMF or WT1vLD).

In one embodiment, the antibody binds to HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1v<sub>LD</sub>), but does not significantly bind to HLA-A2 with a peptide selected from the peptides in Table 5 (the peptides of SEQ ID NOS 79-105). In one embodiment, the antibody binds to HLA-A2/WT1 (specifically HLA-A2/WT1RMF or HLA-A2/WT1VLD), but does not  
20 significantly bind to HLA-A2 with any of the peptides in Table 5 (the peptides of SEQ ID NOS 79-105).

In one embodiment, the antibody binds to HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>VLD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for binding to HLA-A2 with a peptide selected from the peptides in Table 5 (the peptides of SEQ ID NOS 79-105). In one embodiment, the antibody binds to HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>VLD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for binding to HLA-A2 with any of the peptides in Table 5 (the peptides of SEQ ID NOS 79-105).

25 30 In one embodiment, the EC50 is determined flow cytometry using HLA-A2/peptide (e.g. WT1<sub>RMF</sub> or WT1v<sub>LD</sub> peptide)-expressing cells, particularly peptide-pulsed T2 cells.

In a specific embodiment, the EC50 is determined as follows:

T2 cells (ATCC, Cat. No. CRL-1992) are prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco, Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, Cat No. 15070-063) (complete medium). Cells are kept in a total volume of 10 ml in a tube, and incubated with 10  $\mu$ M of peptide (e.g. WT1 VLD peptide (SEQ ID NO: 77), or RMF peptide (SEQ ID NO: 78)) at  $10^{-2}$  M (final concentration of the peptide:  $10^5$  M) for 2 hours at 37°C with 5% CO<sub>2</sub>. After washing, cells are suspended in cold PBS and incubated with titrated concentration of antibody in IgG format (e.g. 10 pg/ml to 0.00064 pg/ml) for 1 hour at 4°C, followed by incubation with a secondary anti-human IgG-Fc phycoerythrin (PE)-conjugated antibody (Jackson Laboratories, Cat No. 109-116-098) for 30 min. Cells are acquired on FACS LSR II (BD), and data are presented as mean fluorescence intensity (MFI) of PE in Graphpad Prism. EC<sub>50</sub> values are calculated in Microsoft® Excel using the XLfit® add-on (ID Business Solutions, Guildford, UK).

In one aspect, the invention provides an antibody that competes for binding to HLA-A2/WT1, particularly HLA-A201/WT1<sub>RMF</sub>, with an antibody comprising a heavy chain variable region (VH) sequence of SEQ ID NO: 7, and a light chain variable region (VL) sequence of SEQ ID NO: 8.

Competition assays may be used to identify an antibody that competes with the antibody comprising the VH sequence of SEQ ID NO: 7 and the VL sequence of SEQ ID NO: 8 (the reference antibody) for binding to HLA-A2/WT1. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by the reference antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) “Epitope Mapping Protocols”, in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ) and are also described in the Examples herein. In an exemplary competition assay, immobilized HLA-A2/WT1 is incubated in a solution comprising a first labeled antibody that binds to HLA-A2/WT1 (e.g., the antibody comprising the VH sequence of SEQ ID NO: 7 and the VL sequence of SEQ ID NO: 8) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to HLA-A2/WT1. The second antibody may be present in a hybridoma supernatant. As a control, immobilized HLA-A2/WT1 is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to HLA-A2/WT1, excess unbound antibody is removed, and the amount of label associated with immobilized HLA-A2/WT1 is measured. If the amount of label associated with immobilized HLA-A2/WT1 is substantially reduced in the test sample relative to

the control sample, then that indicates that the second antibody is competing with the first antibody for binding to HLA-A2/WT1. See Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

In one aspect, the invention provides an antibody that binds to the same epitope of HLA-A2/WT1, particularly HLA-A2/WT1<sub>RMF</sub>, as an antibody comprising a heavy chain variable region (VH) sequence of SEQ ID NO: 7, and a light chain variable region (VL) sequence of SEQ ID NO: 8.

Screening for antibodies binding to a particular epitope (i.e., those binding to the same epitope) can be done using methods routine in the art such as, e.g., without limitation, alanine scanning, peptide blots (see Meth. Mol. Biol. 248 (2004) 443-463), peptide cleavage analysis, epitope excision, epitope extraction, chemical modification of antigens (see Prot. Sci. 9 (2000) 487-496), and cross-blocking (see “Antibodies”, Harlow and Lane (Cold Spring Harbor Press, Cold Spring Harb., NY).

Antigen Structure-based Antibody Profiling (ASAP), also known as Modification-Assisted Profiling (MAP), allows to bin a multitude of monoclonal antibodies specifically binding to HLA-A2/WT1 based on the binding profile of each of the antibodies from the multitude to chemically or enzymatically modified antigen surfaces (see, e.g., US 2004/0101920). The antibodies in each bin bind to the same epitope which may be a unique epitope either distinctly different from or partially overlapping with epitope represented by another bin.

Also competitive binding can be used to easily determine whether an antibody binds to the same epitope of HLA-A2/WT1 as, or competes for binding with, a reference anti HLA-A2/WT1 antibody. For example, an “antibody that binds to the same epitope” as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. Also for example, to determine if an antibody binds to the same epitope of HLA-A2/WT1 as a reference antibody, the reference antibody is allowed to bind to HLA-A2/WT1 under saturating conditions. After removal of the excess of the reference antibody, the ability of an anti HLA-A2/WT1 antibody in question to bind to HLA-A2/WT1 is assessed. If the antibody is able to bind to HLA-A2/WT1 after saturation binding of the reference antibody, it can be concluded that the antibody in question binds to a different epitope than the reference antibody. But, if the antibody in question is not able to bind to HLA-A2/WT1 after saturation binding of the reference antibody, then the antibody in question may bind to the same epitope as the epitope bound by the reference antibody. To confirm whether the

antibody in question binds to the same epitope or is just hampered from binding by steric reasons routine experimentation can be used (e.g., peptide mutation and binding analyses using ELISA, RIA, surface plasmon resonance, flow cytometry or any other quantitative or qualitative antibody-binding assay available in the art). This assay should be carried out in two set-ups, i.e.

5 with both of the antibodies being the saturating antibody. If, in both set-ups, only the first (saturating) antibody is capable of binding to HLA-A2/WT1, then it can be concluded that the anti-HLA-A2/WT1 antibody in question and the reference anti-HLA-A2/WT1 antibody compete for binding to HLA-A2/WT1.

In some embodiments two antibodies are deemed to bind to the same or an overlapping epitope 10 if a 1-, 5-, 10-, 20- or 100-fold excess of one antibody inhibits binding of the other by at least 50%, at least 75%, at least 90% or even 99% or more as measured in a competitive binding assay (see, e.g., Junghans et al., Cancer Res. 50 (1990) 1495-1502).

In some embodiments two antibodies are deemed to bind to the same epitope if essentially all 15 amino acid mutations in the antigen that reduce or eliminate binding of one antibody also reduce or eliminate binding of the other. Two antibodies are deemed to have “overlapping epitopes” if only a subset of the amino acid mutations that reduce or eliminate binding of one antibody reduce or eliminate binding of the other.

In one aspect, the invention provides an antibody that binds to HLA-A2/WT1, wherein the antibody binds to an epitope of HLA-A2/WT1, particularly HLA-A2/WT1<sub>RMF</sub>, according to any 20 of the following embodiments. The epitope may particularly be determined by crystal structure analysis.

In one embodiment, said epitope comprises at least three amino acid residues of the WT1 peptide, particularly the WTTRMF peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises at least four amino acid residues of the WT1 peptide, 25 particularly the WTTRMF peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises at least five amino acid residues of the WT1 peptide, particularly the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises at least six amino acid residues of the WT1 peptide, particularly the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

30 In one embodiment, said epitope comprises at least three amino acid residues of the WT1 peptide, wherein said at least three amino acid residues are selected from the amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises at least four amino acid residues of the WT1 peptide, wherein said at least four amino acid residues are selected from the amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

5 In one embodiment, said epitope comprises at least five amino acid residues of the WT1 peptide, wherein said at least five amino acid residues are selected from the amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises at least six amino acid residues of the WT1 peptide, 10 wherein said at least six amino acid residues are selected from the amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues R1, N5 and A6 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

15 In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, R65, K66 and Q155 of the HLA-A2 sequence shown in SEQ ID NO: 138.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid 20 residues E58, R65, K66 and Q155 of the HLA-A2 sequence shown in SEQ ID NO: 138, and amino acid residues corresponding to amino acid residues R1, N5 and A6 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, R65, K66, Q155 and D61 of the HLA-A2 sequence shown in SEQ ID NO: 138. In

25 one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, R65, K66, Q155 and A69 of the HLA-A2 sequence shown in SEQ ID NO: 138. In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, R65, K66, Q155 and A150 of the HLA-A2 sequence shown in SEQ ID NO: 138.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid 30 residues E58, R65, K66, Q155, and one or more of D61, A69 and A150 of the HLA-A2 sequence shown in SEQ ID NO: 138, and amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and/or Y8 of the WT!<sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, D61, G62, R65, K66, A69, A150, Q155, A158, T163, E166, W167 and R170 of the HLA-A2 sequence shown in SEQ ID NO: 138.

In one embodiment, said epitope comprises amino acid residues corresponding to amino acid residues E58, D61, G62, R65, K66, A69, A150, Q155, A158, T163, E166, W167 and R170 of the HLA-A2 sequence shown in SEQ ID NO: 138, and amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and/or Y8 of the WT! <sub>RMF</sub> peptide shown in SEQ ID NO: 78.

In one embodiment, said epitope does not comprise amino acid residues corresponding to amino acid residues G56, D106, W107, R108, E161, G162 and/or R169 of the HLA-A2 sequence shown in SEQ ID NO: 138.

In a further aspect the present invention provides an antibody that binds to HLA-A2/WT1, wherein the antibody comprises

- (i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;
- (ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;
- (iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;
- (iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;
- (v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;
- (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a

5 HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70.

10 In a particular embodiment, the antibody comprises a VH comprising a HCDR 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a VL comprising a LCDR 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6.

In another embodiment, the antibody comprises a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1

15 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14.

In a further embodiment, the antibody comprises a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22.

In still a further embodiment, the antibody comprises a VH comprising a HCDR 1 of SEQ ID

20 NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30.

In some embodiments, the antibody is a human antibody. In one embodiment, the VH is a human VH and/or the VL is a human VL. In one embodiment, the antibody comprises CDRs as in any of the above embodiments, and further comprises a human framework, e.g. a human

25 immunoglobulin framework.

In one embodiment, the antibody comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

30 acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino

5 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

(iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

10 acid sequence of SEQ ID NO: 32;

(v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

15 (vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99%

20 or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino

25 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

30 acid sequence of SEQ ID NO: 72.

In a particular embodiment, the antibody comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of

SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the antibody comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ

5 ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.

In a further embodiment, the antibody comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%,

10 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.

In still a further embodiment, the antibody comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32.

15 In one embodiment, the antibody comprises

(i) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the 20 amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

25 (iv) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

(v) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL sequence that is at least about 95%, 96%,

30 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

(vi) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the

5 amino acid sequence of SEQ ID NO: 63, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

10 In a particular embodiment, the antibody comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the antibody comprises a VH sequence that is at least about 95%, 96%,

15 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.

In a further embodiment, the antibody comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL

20 sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.

In still a further embodiment, the antibody comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

25 acid sequence of SEQ ID NO: 32.

In certain embodiments, a VH or VL sequence having at least 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative

to the reference sequence, but an antibody comprising that sequence retains the ability to bind to HLA-A2/WT1. In certain embodiments, a total of 1 to 10 amino acids have been substituted,

30 inserted and/or deleted in the VH (SEQ ID NO: 7, 15, 23, 31, 39, 47, 55, 63 or 71) and/or a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in the VL (SEQ ID NO: 8, 16, 24, 32, 40, 48, 56, 64 or 72). In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the antibody comprises the VH

sequence and/or the VL sequence indicated above, including post-translational modifications of that sequence.

In one embodiment, the antibody comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO: 7, and a VL comprising the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising the amino acid sequence of SEQ ID NO: 15, and a VL comprising the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising the amino acid sequence of SEQ ID NO: 23, and a VL comprising the amino acid sequence of SEQ ID NO: 24;

10 (iv) a VH comprising the amino acid sequence of SEQ ID NO: 31, and a VL comprising the amino acid sequence of SEQ ID NO: 32;

(v) a VH comprising the amino acid sequence of SEQ ID NO: 39, and a VL comprising the amino acid sequence of SEQ ID NO: 40;

15 (vi) a VH comprising the amino acid sequence of SEQ ID NO: 47, and a VL comprising the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising the amino acid sequence of SEQ ID NO: 55, and a VL comprising the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising the amino acid sequence of SEQ ID NO: 63, and a VL comprising the amino acid sequence of SEQ ID NO: 64; or

20 (ix) a VH comprising the amino acid sequence of SEQ ID NO: 71, and a VL comprising the amino acid sequence of SEQ ID NO: 72.

In one embodiment, the antibody comprises

(i) the VH sequence of SEQ ID NO: 7, and the VL sequence of SEQ ID NO: 8;

(ii) the VH sequence of SEQ ID NO: 15, and the VL sequence of SEQ ID NO: 16;

25 (iii) the VH sequence of SEQ ID NO: 23, and the VL sequence of SEQ ID NO: 24;

(iv) the VH sequence of SEQ ID NO: 31, and the VL sequence of SEQ ID NO: 32;

(v) the VH sequence of SEQ ID NO: 39, and the VL sequence of SEQ ID NO: 40;

(vi) the VH sequence of SEQ ID NO: 47, and the VL sequence of SEQ ID NO: 48;

(vii) the VH sequence of SEQ ID NO: 55, and the VL sequence of SEQ ID NO: 56;

30 (viii) the VH sequence of SEQ ID NO: 63, and the VL sequence of SEQ ID NO: 64; or

(ix) the VH sequence of SEQ ID NO: 71, and the VL sequence of SEQ ID NO: 72.

In a particular embodiment, the antibody comprises a VH comprising the amino acid sequence of SEQ ID NO: 7 and a VL comprising the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the antibody comprises a VH comprising the amino acid sequence of SEQ ID NO: 15 and a VL comprising the amino acid sequence of SEQ ID NO: 16.

In a further embodiment, the antibody comprises a VH comprising the amino acid sequence of SEQ ID NO: 23 and a VL comprising the amino acid sequence of SEQ ID NO: 24.

5 In still a further embodiment, the antibody comprises a VH comprising the amino acid sequence of SEQ ID NO: 31 and a VL comprising the amino acid sequence of SEQ ID NO: 32.

In a particular embodiment, the antibody comprises the VH sequence of SEQ ID NO: 7 and the VL sequence of SEQ ID NO: 8.

10 In another embodiment, the antibody comprises the VH sequence of SEQ ID NO: 15 and the VL sequence of SEQ ID NO: 16.

In a further embodiment, the antibody comprises the VH sequence of SEQ ID NO: 23 and the VL sequence of SEQ ID NO: 24.

In still a further embodiment, the antibody comprises the VH sequence of SEQ ID NO: 31 and the VL sequence of SEQ ID NO: 32.

15 In one embodiment, the antibody comprises a human constant region. In one embodiment, the antibody is an immunoglobulin molecule comprising a human constant region, particularly an IgG class immunoglobulin molecule comprising a human CH1, CH2, CH3 and/or CL domain. Exemplary sequences of human constant domains are given in SEQ ID NOs 112 and 113 (human kappa and lambda CL domains, respectively) and SEQ ID NO: 114 (human IgG1 heavy chain

20 constant domains CH1-CH2-CH3). In some embodiments, the antibody comprises a light chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 112 or SEQ ID NO: 113, particularly the amino acid sequence of SEQ ID NO: 112. In some embodiments, the antibody comprises a heavy chain constant region comprising an amino acid sequence that is at least about

25 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 114. Particularly, the heavy chain constant region may comprise amino acid mutations in the Fc domain as described herein.

In one embodiment, the antibody is a monoclonal antibody.

30 In one embodiment, the antibody is an IgG, particularly an IgG1, antibody. In one embodiment, the antibody is a full-length antibody.

In one embodiment, the antibody comprises an Fc domain, particularly an IgG Fc domain, more particularly an IgG1 Fc domain. In one embodiment the Fc domain is a human Fc domain. The Fc domain of the antibody may incorporate any of the features, singly or in combination,

described herein in relation to the Fc domain of the bispecific antigen binding molecule of the invention.

In another embodiment, the antibody is an antibody fragment selected from the group of an Fv molecule, a scFv molecule, a Fab molecule, and a F(ab')<sub>2</sub> molecule; particularly a Fab molecule.

5 In another embodiment, the antibody fragment is a diabody, a triabody or a tetrabody.

In a further aspect, the antibody according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in the sections below.

### Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent 10 to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc region, the oligosaccharide attached thereto may be altered.

Native antibodies produced by mammalian cells typically comprise a branched, biantennary 15 oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the 20 invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a non-fucosylated oligosaccharide, i.e. an oligosaccharide structure that lacks fucose attached (directly or indirectly) to an Fc region. Such non-fucosylated oligosaccharide (also referred to as “afucosylated” oligosaccharide) particularly is an N-linked oligosaccharide which lacks a fucose residue attached to the first 25 GlcNAc in the stem of the biantennary oligosaccharide structure. In one embodiment, antibody variants are provided having an increased proportion of non-fucosylated oligosaccharides in the Fc region as compared to a native or parent antibody. For example, the proportion of non-fucosylated oligosaccharides may be at least about 20%, at least about 40%, at least about 60%, at least about 80%, or even about 100% (i.e. no fucosylated oligosaccharides are present). The 30 percentage of non-fucosylated oligosaccharides is the (average) amount of oligosaccharides lacking fucose residues, relative to the sum of all oligosaccharides attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry,

as described in WO 2006/082515, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (EU numbering of Fc region residues); however, Asn297 may also be located about  $\pm$  3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such antibodies

5 having an increased proportion of non-fucosylated oligosaccharides in the Fc region may have improved Fc<sub>Y</sub>RIIIa receptor binding and/or improved effector function, in particular improved ADCC function. See, e.g., US 2003/0157108; US 2004/0093621.

Examples of cell lines capable of producing antibodies with reduced fucosylation include Fee 13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 10 (1986); US 2003/0157108; and WO 2004/056312, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8*, knockout CHO cells (see, e.g., Yamane-Ohnuki et al. *Biotech. Bioeng.* 87:614-622 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107), or cells with reduced or abolished activity of a GDP-fucose synthesis or transporter protein (see, e.g., US2004259150, US2005031613, 15 US2004132140, US2004 110282).

In a further embodiment, antibody variants are provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function as described above. Examples of such antibody variants are described, e.g., in Umana 20 et al., *Nat Biotechnol* 17, 176-180 (1999); Ferrara et al., *Biotechn Bioeng* 93, 851-861 (2006); WO 99/54342; WO 2004/065540, WO 2003/011878.

Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087; WO 1998/58964; and WO 1999/22764.

25 Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at 30 accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described

further herein. -Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541, 8,30,930, 7,855,275, 9,000,130, or W02016040856.

### Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain 5 additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, 10 poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The 15 polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative 20 will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *I'roc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited 25 to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

### Immunoconjugates

The invention also provides immunoconjugates comprising an anti-HLA-A2/WT1 antibody as described herein conjugated (chemically bonded) to one or more therapeutic agents such as 30 cytotoxic agents, chemotherapeutic agents, drugs, growth inhibitory agents, toxins (e.g., protein

toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more of the therapeutic agents mentioned above. The antibody

5 is typically connected to one or more of the therapeutic agents using linkers. An overview of ADC technology including examples of therapeutic agents and drugs and linkers is set forth in *Pharmacol Review* 68:3:19 (2016).

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to

10 diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, *sapaonaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the trichothecenes.

15 In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At<sup>211</sup>, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Bi<sup>212</sup>, P<sup>32</sup>, Pb<sup>212</sup> and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or

20 1123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP),  
25 succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HC1), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine  
30 compounds (such as 1,5-difluoro-2, 4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The

linker may be a “cleavable linker” facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., *Cancer Res.* 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

5 The immunoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from  
10 Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

#### Multispecific antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites, i.e., different epitopes on different antigens or different epitopes on  
15 the same antigen. In certain embodiments, the multispecific antibody has three or more binding specificities. In certain embodiments, one of the binding specificities is for HLA-A2/WT1 and the other (two or more) specificity is for any other antigen. In certain embodiments, bispecific antibodies may bind to two (or more) different epitopes of HLA-A2/WT1. Multispecific (e.g., bispecific) antibodies may also be used to localize cytotoxic agents or cells to cells which  
20 express HLA-A2/WT1. Multispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)) and “knob-in-hole” engineering (see, e.g., U.S. Patent No. 5,731,168, and Atwell et al., *J. Mol. Biol.* 270:26 (1997)). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (see, e.g., WO 2009/089004); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al., *J. Immunol.*, 148(5): 1547-1553 (1992) and WO 2011/034605); using the common light chain technology for circumventing the light chain mis-pairing problem (see, e.g., WO 98/50431); using “diabody” technology for making bispecific antibody fragments (see, e.g., Hollinger et al., *Proc. Natl. Acad.*

*Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (see, e.g. Gruber et al., */. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. *J. Immunol.* 147: 60 (1991).

Engineered antibodies with three or more antigen binding sites, including for example, “Octopus antibodies,” or DVD-Ig are also included herein (see, e.g. WO 2001/77342 and WO 2008/024715). Other examples of multispecific antibodies with three or more antigen binding sites can be found in WO 2010/115589, WO 2010/112193, WO 2010/136172, WO 2010/145792, and WO 2013/026831. The bispecific antibody or antigen binding fragment thereof also includes a “Dual Acting FAb” or “DAF” comprising an antigen binding site that binds to HLA-A2/WT1 as well as another different antigen, or two different epitopes of HLA-A2/WT1 (see, e.g., US 2008/0069820 and WO 2015/095539).

Multi-specific antibodies may also be provided in an asymmetric form with a domain crossover in one or more binding arms of the same antigen specificity, i.e. by exchanging the VH/VL domains (see e.g., WO 2009/080252 and WO 2015/150447), the CH1/CL domains (see e.g., WO 2009/080253) or the complete Fab arms (see e.g., WO 2009/080251, WO 2016/016299, also see Schaefer et al, *PNAS*, 108 (2011) 1187-1191, and Klein et al., *MAbs* 8 (2016) 1010-20). Asymmetrical Fab arms can also be engineered by introducing charged or non-charged amino acid mutations into domain interfaces to direct correct Fab pairing. See e.g., WO 2016/172485.

Various further molecular formats for multispecific antibodies are known in the art and are included herein (see e.g., Spiess et al., *Mol Immunol* 67 (2015) 95-106).

A particular type of multispecific antibodies, also included herein, are bispecific antibodies designed to simultaneously bind to a surface antigen on a target cell, e.g., a tumor cell, and to an activating, invariant component of the T cell receptor (TCR) complex, such as CD3, for retargeting of T cells to kill target cells. Hence, in certain embodiments, an antibody provided herein is a multispecific antibody, particularly a bispecific antibody, wherein one of the binding specificities is for HLA-A2/WT1 and the other is for CD3.

Examples of bispecific antibody formats that may be useful for this purpose include, but are not limited to, the so-called “BiTE” (bispecific T cell engager) molecules wherein two scFv molecules are fused by a flexible linker (see, e.g., WO2004/1 06381, WO2005/061547, WO2007/042261, and WO2008/1 19567, Nagorsen and Bauerle, *Exp Cell Res* 317, 1255-1260 (2011)); diabodies (Holliger et al., *Prot Eng* 9, 299-305 (1996)) and derivatives thereof, such as tandem diabodies (“TandAb”; Kipriyanov et al., *J Mol Biol* 293, 41-56 (1999)); “DART” (dual affinity retargeting) molecules which are based on the diabody format but feature a C-terminal

disulfide bridge for additional stabilization (Johnson et al., *J Mol Biol* 399, 436-449 (2010)), and so-called triomabs, which are whole hybrid mouse/rat IgG molecules (reviewed in Seimetz et al., *Cancer Treat Rev* 36, 458-467 (2010)). Particular T cell bispecific antibody formats included herein are described in WO 2013/026833, WO2013/026839, WO 2016/020309; Bacac et al.,

5 *Oncoimmunology* 5(8) (2016) e1203498.

### **Bispecific antigen binding molecules that bind to HLA-A2/WT1 and a second antigen**

The invention also provides a bispecific antigen binding molecule, i.e. an antigen binding molecule that comprises at least two antigen binding moieties capable of specific binding to two 10 distinct antigenic determinants (a first and a second antigen).

Based on the HLA-A2/WT1 antibodies they developed, the present inventors have developed bispecific antigen binding molecules that bind to HLA-A2/WT1 and a further antigen, particularly an activating T cell antigen such as CD3.

As shown in the Examples, these bispecific antigen binding molecules have a number of 15 remarkable properties, including good efficacy and low toxicity.

Thus, in certain aspects, the invention provides a bispecific antigen binding molecule, comprising (a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, and (b) a second antigen binding moiety which specifically binds to a second antigen, wherein the bispecific antigen binding molecule has any of the following 20 features.

The bispecific antigen binding molecule of the invention specifically induces T-cell mediated killing of cells expressing HLA-A2/WT1 (i.e. a complex of a HLA-A2 molecule and a WT1-derived peptide). In some embodiments, the bispecific antigen binding molecule of the invention specifically induces T-cell mediated killing of cells expressing HLA-A2/WT1<sub>RMF</sub> (i.e. a complex 25 of a HLA-A2 molecule and the WT1<sub>RMF</sub> peptide). In a more specific embodiment, the bispecific antigen binding molecule specifically induces T-cell mediated killing of cells expressing HLA-A2.01/WT1<sub>RMF</sub> (i.e. a complex of a HLA-A201 molecule and the WT1<sub>RMF</sub> peptide).

In one embodiment, induction of T-cell mediated killing by the bispecific antigen binding molecule is determined using HLA-A2/peptide (e.g. WT1<sub>RMF</sub> or WT1<sub>vLD</sub> peptide)-expressing 30 cells, particularly peptide-pulsed T2 cells, and measuring lactate dehydrogenase (LDH) release from said cells after incubation with the bispecific antigen binding molecule in the presence of T cells.

In a specific embodiment, induction of T-cell mediated killing by the bispecific antigen binding molecule is determined as follows:

T2 cells (ATCC, Cat. No. CRL-1992) are prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco,

5 Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, Cat No. 15070-063) (complete medium). Cells are kept in a total volume of 10 ml in a tube, and incubated with 10  $\mu$ l of peptide (e.g. WT1 VLD peptide (SEQ ID NO: 77), or RMF peptide (SEQ ID NO: 78)) at  $10^{-2}$  M (final concentration of the peptide:  $10^{-5}$  M) for 2 hours at 37°C with 5% CO<sub>2</sub>. Pan CD3<sup>+</sup> cells are purified from PBMCs isolated from buffy coat by Ficoll (GE Healthcare, Cat. No. 17-1440-03)

10 gradient centrifugation. Total CD3<sup>+</sup> T cells are purified by MACS (Miltenyi Biotec) using a Human Pan T cell Isolation Kit (Miltenyi Biotec, Cat. No. 130-096-535). The cytotoxicity assay is performed as follows: The peptide-pulsed cells (100  $\mu$ l) are seeded into a 96 well microtiter round bottom plate ( $3 \times 10^5$  cells/ml), co-cultured with 50  $\mu$ l of T cells ( $6 \times 10^6$  cells/ml), and with 50  $\mu$ l of titrated bispecific antigen binding molecule (e.g. at 40  $\mu$ g/ml to 0.00004 pg/ml) in 15 complete medium for 18 hours at 37°C with 5% CO<sub>2</sub>. Thereafter, 50  $\mu$ l of supernatant are transferred into a new white plate, and 25  $\mu$ l per well of CytoTox-Glo Luciferase Assay (Promega, Cat. No. G9291) are added for incubation at room temperature (RT) for 15 minutes. The luminescence signal (for measurement of LDH release as indicative of cell death) is read by EnVision (PerkinElmer). Data are presented as Relative Luminescence Unit (RLU).

20 The bispecific antigen binding molecule of the invention specifically activates T cells in the presence of cells expressing HLA-A2/WT1 (i.e. a complex of a HLA-A2 molecule and a WT1-derived peptide). In some embodiments, the bispecific antigen binding molecule of the invention specifically activates T cells in the presence of cells expressing HLA-A2/WTT<sub>RMF</sub> (i.e. a complex of a HLA-A2 molecule and the WT1<sub>RMF</sub> peptide). In a more specific embodiment, the 25 bispecific antigen binding molecule specifically activates T cells in the presence of cells expressing HLA-A201/WT1<sub>RMF</sub> (i.e. a complex of a HLA-A201 molecule and the WT1<sub>RMF</sub> peptide).

In one embodiment, activation of T cells by the bispecific antigen binding molecule is determined by measuring, particularly by flow cytometry, expression of CD25 and/or CD69 by T 30 cells after incubation with the bispecific antigen binding molecule in the presence of HLA-A2/peptide (e.g. WTTRMF or WTTVLD peptide)-expressing cells, particularly peptide-pulsed T2 cells.

In a specific embodiment, activation of T cells by the bispecific antigen binding molecule is determined as follows:

T2 cells (ATCC, Cat. No. CRL-1992) are prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco,

5 Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, Cat No. 15070-063) (complete medium). Cells are kept in a total volume of 10 ml in a tube, and incubated with 10  $\mu$ g of peptide (e.g. WT1 VLD peptide (SEQ ID NO: 77), or RMF peptide (SEQ ID NO: 78)) at  $10^{-2}$  M (final concentration of the peptide:  $10^5$  M) for 2 hours at 37°C with 5% CO<sub>2</sub>. Pan CD3<sup>+</sup> cells are purified from PBMCs isolated from buffy coat by Ficoll (GE Healthcare, Cat. No. 17-1440-03) 10 gradient centrifugation. Total CD3<sup>+</sup> T cells are purified by MACS (Miltenyi Biotec) using a Human Pan T cell Isolation Kit (Miltenyi Biotec, Cat. No. 130-096-535). The cytotoxicity assay is performed as follows: The peptide-pulsed cells (100  $\mu$ g) are seeded into a 96 well microtiter round bottom plate ( $3 \times 10^5$  cells/ml), co-cultured with 50  $\mu$ g of T cells ( $6 \times 10^6$  cells/ml), and with 50  $\mu$ g of titrated bispecific antigen binding molecule (e.g. at 40  $\mu$ g/ml to 0.00004 pg/ml) in 15 complete medium for 18 hours at 37°C with 5% CO<sub>2</sub>. Cells are harvested after 18 hours of co-incubation, and stained with antibodies against CD3 (Biolegend Cat. No. 300321), CD25 (Biolegend Cat. No. 302606) and CD69 (Biolegend Cat. No. 310914) to measure T cell activation by flow cytometry.

In another embodiment, activation of T cells by the bispecific antigen binding molecule is

20 determined using HLA-A2/peptide (e.g. WTTRMF or WTTVLD peptide)-expressing cells, particularly peptide-pulsed T2 cells, and a reporter T cell line, particularly a Jurkat T cell line that expresses a luciferase reporter driven by an NFAT (nuclear factor of activated T cells) response element.

In a specific embodiment, activation of T cells by the bispecific antigen binding molecule is

25 determined as follows:

T2 cells (ATCC, Cat. No. CRL-1992) are prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco,

Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, Cat No. 15070-063) (complete medium). Cells are kept in a total volume of 10 ml in a tube, and incubated with 10  $\mu$ g of peptide (e.g. WT1 VLD peptide (SEQ ID NO: 77), or RMF peptide (SEQ ID NO: 78)) at  $10^{-2}$  M (final concentration of the peptide:  $10^5$  M) for 2 hours at 37°C with 5% CO<sub>2</sub>. After washing, 90  $\mu$ g of the peptide-pulsed cells in a cell suspension of  $2.2 \times 10^5$  cells/ml are seeded into a 96 well microtiter round bottom plate (20,000 cells/well, TPP, Cat. No. 92097), co-cultured with 50  $\mu$ g of

Jurkat cells that express luciferase under the promoter of NFAT (Jurkat-NFAT; Promega, Cat. No. CS176501) (cell suspension of 2x10<sup>6</sup> cells/ml), and with 10 µl of titrated bispecific antigen binding molecule (e.g. at 100 pg/ml to 0.0064 µg/ml in PBS) for 16 hours at 37°C with 5% CO<sub>2</sub>. Thereafter, 50 µl of supernatant are removed, and replaced with 100 p1 per well of Bright-Glo

5 Luciferase Assay (Promega, Cat. No. E2620) for incubation at room temperature (RT). Five minutes later, 180 p1 of supernatant are transferred into a new white plate to measure luminescence signal by EnVision (PerkinElmer). Data are presented as Relative Luminescence Unit (RLU).

In one embodiment, the bispecific antigen binding molecule of the invention does not 10 significantly induce T cell mediated killing of, or activate T cells in the presence of, cells expressing HLA-A2 alone (i.e. without a peptide) or HLA-A2 with a peptide other than a WT1-derived peptide such as WT1<sub>RMF</sub> or WT1<sub>LD</sub>.

In one embodiment, the bispecific antigen binding does not significantly induce T cell mediated killing of, or activate T cells in the presence of, cells expressing HLA-A2 in the absence of a 15 WT1-derived peptide, particularly WT1<sub>RMF</sub> or WT1<sub>LD</sub>. In one embodiment, the bispecific antigen binding molecule induces T cell mediated killing of, and/or activates T cells in the presence of, cells expressing HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>LD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for induction of T cell mediated 20 killing of, or activation of T cells in the presence of, cells expressing HLA-A2 in the absence of a WT1-derived peptide (specifically WT1<sub>RMF</sub> or WT1<sub>LD</sub>).

In one embodiment, the bispecific antigen binding molecule induces T cell mediated killing of, and/or activates T cells in the presence of, cells expressing HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>LD</sub>), but does not significantly induce T cell mediated killing of, 25 or activate T cells in the presence of, cells expressing to HLA-A2 with a peptide selected from the peptides in Table 5 (the peptides of SEQ ID NOS 79-105). In one embodiment, the bispecific antigen binding molecule induces T cell mediated killing of, and/or activates T cells in the presence of, cells expressing to HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>LD</sub>), but does not significantly induce T cell mediated killing of, or activate T cells in 30 the presence of, cells expressing to HLA-A2 with any of the peptides in Table 5 (the peptides of SEQ ID NOS 79-105).

In one embodiment, the bispecific antigen binding molecule induces T cell mediated killing of, and/or activates T cells in the presence of, cells expressing HLA-A2/WT1 (specifically HLA-

A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>LD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for induction of T cell mediated killing of, and/or activation of T cells in the presence of, cells expressing to HLA-A2 with a peptide selected from the peptides in Table 5 (the peptides of SEQ ID NOS 79-105). In

5 one embodiment, the bispecific antigen binding molecule induces T cell mediated killing of, and/or activates T cells in the presence of, cells expressing HLA-A2/WT1 (specifically HLA-A2/WT1<sub>RMF</sub> or HLA-A2/WT1<sub>LD</sub>) with an EC50 that is at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75 or at least 100 times lower than the EC50 for induction of T cell mediated killing of, and/or activation of T cells in the presence of, cells expressing to HLA-

10 A2 with any of the peptides in Table 5 (the peptides of SEQ ID NOS 79-105).

In one embodiment, induction of T cell mediated killing and/or activation of T cells is determined as described above, and the EC50 is calculated in Microsoft® Excel using the XLfit® add-on (ID Business Solutions, Guildford, UK).

15 According to particular embodiments of the invention, the antigen binding moieties comprised in the bispecific antigen binding molecule are Fab molecules (i.e. antigen binding domains composed of a heavy and a light chain, each comprising a variable and a constant domain). In one embodiment, the first and/or the second antigen binding moiety is a Fab molecule. In one embodiment, said Fab molecule is human. In a particular embodiment, said Fab molecule is 20 humanized. In yet another embodiment, said Fab molecule comprises human heavy and light chain constant domains.

Preferably, at least one of the antigen binding moieties is a crossover Fab molecule. Such modification reduces mispairing of heavy and light chains from different Fab molecules, thereby improving the yield and purity of the bispecific antigen binding molecule of the invention in 25 recombinant production. In a particular crossover Fab molecule useful for the bispecific antigen binding molecule of the invention, the variable domains of the Fab light chain and the Fab heavy chain (VF and VH, respectively) are exchanged. Even with this domain exchange, however, the preparation of the bispecific antigen binding molecule may comprise certain side products due to a so-called Bence Jones-type interaction between mispaired heavy and light chains (see Schaefer 30 et al, PNAS, 108 (2011) 11187-11191). To further reduce mispairing of heavy and light chains from different Fab molecules and thus increase the purity and yield of the desired bispecific antigen binding molecule, charged amino acids with opposite charges may be introduced at specific amino acid positions in the CH1 and CF domains of either the Fab molecule(s) binding

to the first antigen (HLA-A2/WT1), or the Fab molecule binding to the second antigen (e.g. an activating T cell antigen such as CD3), as further described herein. Charge modifications are made either in the conventional Fab molecule(s) comprised in the bispecific antigen binding molecule (such as shown e.g. in Figures 1 A-C, G-J), or in the VH/VL crossover Fab molecule(s)

5 comprised in the bispecific antigen binding molecule (such as shown e.g. in Figure 1 D-F, K-N) (but not in both). In particular embodiments, the charge modifications are made in the conventional Fab molecule(s) comprised in the bispecific antigen binding molecule (which in particular embodiments bind(s) to the first antigen, i.e. HLA-A2/WT1).

In a particular embodiment according to the invention, the bispecific antigen binding molecule is  
10 capable of simultaneous binding to the first antigen (i.e. HLA-A2/WT1), and the second antigen (e.g. an activating T cell antigen, particularly CD3). In one embodiment, the bispecific antigen binding molecule is capable of crosslinking a T cell and a target cell by simultaneous binding HLA-A2/WT1 and an activating T cell antigen. In an even more particular embodiment, such simultaneous binding results in lysis of the target cell, particularly a HLA-A2/WT1 expressing  
15 tumor cell. In one embodiment, such simultaneous binding results in activation of the T cell. In other embodiments, such simultaneous binding results in a cellular response of a T lymphocyte, particularly a cytotoxic T lymphocyte, selected from the group of: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, and expression of activation markers. In one embodiment, binding of the bispecific antigen binding molecule to the  
20 activating T cell antigen, particularly CD3, without simultaneous binding to HLA-A2/WT1 does not result in T cell activation.

In one embodiment, the bispecific antigen binding molecule is capable of re-directing cytotoxic activity of a T cell to a target cell. In a particular embodiment, said re-direction is independent of MHC-mediated peptide antigen presentation by the target cell and and/or specificity of the T cell.  
25 Particularly, a T cell according to any of the embodiments of the invention is a cytotoxic T cell. In some embodiments the T cell is a CD4<sup>+</sup> or a CD8<sup>+</sup> T cell, particularly a CD8<sup>+</sup> T cell.

#### First antigen binding moiety

The bispecific antigen binding molecule of the invention comprises at least one antigen binding moiety, particularly a Fab molecule, that binds to HLA-A2/WT1 (first antigen). In certain  
30 embodiments, the bispecific antigen binding molecule comprises two antigen binding moieties, particularly Fab molecules, which bind to HLA-A2/WT1. In a particular such embodiment, each of these antigen binding moieties binds to the same antigenic determinant. In an even more

particular embodiment, all of these antigen binding moieties are identical, i.e. they comprise the same amino acid sequences including the same amino acid substitutions in the CH1 and CL domain as described herein (if any). In one embodiment, the bispecific antigen binding molecule comprises not more than two antigen binding moieties, particularly Fab molecules, which bind to

5 HLA-A2/WT1.

In particular embodiments, the antigen binding moiety(ies) which bind to HLA-A2/WT1 is/are a conventional Fab molecule. In such embodiments, the antigen binding moiety(ies) that binds to a second antigen is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CH1 and CL of the Fab heavy and light  
10 chains are exchanged / replaced by each other.

In alternative embodiments, the antigen binding moiety(ies) which bind to HLA-A2/WT1 is/are a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CH1 and CL of the Fab heavy and light chains are exchanged / replaced by each other. In such embodiments, the antigen binding moiety(ies) that  
15 binds a second antigen is a conventional Fab molecule.

The HLA-A2/WT1 binding moiety is able to direct the bispecific antigen binding molecule to a target site, for example to a specific type of tumor cell that expresses HLA-A2/WT1.

The first antigen binding moiety of the bispecific antigen binding molecule may incorporate any of the features, singly or in combination, described herein in relation to the antibody that binds  
20 HLA-A2/WT1, unless scientifically clearly unreasonable or impossible.

Thus, in one aspect, the invention provides a bispecific antigen binding molecule, comprising (a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1 and the first antigen binding moiety comprises

25 (i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO:  
6;  
30 (ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

(iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;

(iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 5 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;

(v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;

10 (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of 15 SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 20 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70, and

(b) a second antigen binding moiety that binds to a second antigen.

In a particular embodiment, the first antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and 25 a VL comprising a LCDR 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6.

In another embodiment, the first antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ 30 ID NO: 14.

In a further embodiment, the first antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL

comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22.

In still a further embodiment, the first antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30.

In some embodiments, the first antigen binding moiety is (derived from) a human antibody. In one embodiment, the VH is a human VH and/or the VL is a human VL. In one embodiment, the first antigen binding moiety comprises CDRs as in any of the above embodiments, and further comprises a human framework, e.g. a human immunoglobulin framework.

In one embodiment, the first antigen binding moiety comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

(iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

(v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

(vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino

5 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino

10 acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

15 In a particular embodiment, the first antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8.

20 In another embodiment, the first antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.

25 In a further embodiment, the first antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.

30 In still a further embodiment, the first antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at

least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32. In one embodiment, the first antigen binding moiety comprises

(i) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%,

5 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the 10 amino acid sequence of SEQ ID NO: 23, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

(iv) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

15 (v) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

(vi) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL sequence that is at least about 95%, 96%,

20 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the 25 amino acid sequence of SEQ ID NO: 63, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

30 In a particular embodiment, the first antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the first antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.

5 In a further embodiment, the first antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.

In still a further embodiment, the first antigen binding moiety comprises a VH sequence that is at 10 least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32.

In one embodiment, the first antigen binding moiety comprises  
(i) a VH comprising the amino acid sequence of SEQ ID NO: 7, and a VL comprising the amino 15 acid sequence of SEQ ID NO: 8;  
(ii) a VH comprising the amino acid sequence of SEQ ID NO: 15, and a VL comprising the amino acid sequence of SEQ ID NO: 16;  
(iii) a VH comprising the amino acid sequence of SEQ ID NO: 23, and a VL comprising the amino acid sequence of SEQ ID NO: 24;  
20 (iv) a VH comprising the amino acid sequence of SEQ ID NO: 31, and a VL comprising the amino acid sequence of SEQ ID NO: 32;  
(v) a VH comprising the amino acid sequence of SEQ ID NO: 39, and a VL comprising the amino acid sequence of SEQ ID NO: 40;  
(vi) a VH comprising the amino acid sequence of SEQ ID NO: 47, and a VL comprising the 25 amino acid sequence of SEQ ID NO: 48;  
(vii) a VH comprising the amino acid sequence of SEQ ID NO: 55, and a VL comprising the amino acid sequence of SEQ ID NO: 56;  
(viii) a VH comprising the amino acid sequence of SEQ ID NO: 63, and a VL comprising the amino acid sequence of SEQ ID NO: 64; or  
30 (ix) a VH comprising the amino acid sequence of SEQ ID NO: 71, and a VL comprising the amino acid sequence of SEQ ID NO: 72.

In one embodiment, the first antigen binding moiety comprises

(i) the VH sequence of SEQ ID NO: 7, and the VL sequence of SEQ ID NO: 8;

- (ii) the VH sequence of SEQ ID NO: 15, and the VL sequence of SEQ ID NO: 16;
- (iii) the VH sequence of SEQ ID NO: 23, and the VL sequence of SEQ ID NO: 24;
- (iv) the VH sequence of SEQ ID NO: 31, and the VL sequence of SEQ ID NO: 32;
- (v) the VH sequence of SEQ ID NO: 39, and the VL sequence of SEQ ID NO: 40;
- 5 (vi) the VH sequence of SEQ ID NO: 47, and the VL sequence of SEQ ID NO: 48;
- (vii) the VH sequence of SEQ ID NO: 55, and the VL sequence of SEQ ID NO: 56;
- (viii) the VH sequence of SEQ ID NO: 63, and the VL sequence of SEQ ID NO: 64; or
- (ix) the VH sequence of SEQ ID NO: 71, and the VL sequence of SEQ ID NO: 72.

In a particular embodiment, the first antigen binding moiety comprises a VH comprising the 10 amino acid sequence of SEQ ID NO: 7 and a VL comprising the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the first antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 15 and a VL comprising the amino acid sequence of SEQ ID NO: 16.

15 In a further embodiment, the first antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 23 and a VL comprising the amino acid sequence of SEQ ID NO: 24.

In still a further embodiment, the first antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 31 and a VL comprising the amino acid sequence of SEQ 20 ID NO: 32.

In a particular embodiment, the first antigen binding moiety comprises the VH sequence of SEQ ID NO: 7 and the VL sequence of SEQ ID NO: 8.

In another embodiment, the first antigen binding moiety comprises the VH sequence of SEQ ID NO: 15 and the VL sequence of SEQ ID NO: 16.

25 In a further embodiment, the first antigen binding moiety comprises the VH sequence of SEQ ID NO: 23 and the VL sequence of SEQ ID NO: 24.

In still a further embodiment, the first antigen binding moiety comprises the VH sequence of SEQ ID NO: 31 and the VL sequence of SEQ ID NO: 32.

In one embodiment, the first antigen binding moiety comprises a human constant region. In one 30 embodiment, the first antigen binding moiety is a Fab molecule comprising a human constant region, particularly a human CH1 and/or CL domain. Exemplary sequences of human constant domains are given in SEQ ID NOs 112 and 113 (human kappa and lambda CL domains, respectively) and SEQ ID NO: 114 (human IgGi heavy chain constant domains CH1-CH2-CH3).

In some embodiments, the first antigen binding moiety comprises a light chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 112 or SEQ ID NO: 113, particularly the amino acid sequence of SEQ ID NO: 112. Particularly, the light chain constant region may 5 comprise amino acid mutations as described herein under “charge modifications” and/or may comprise deletion or substitutions of one or more (particularly two) N-terminal amino acids if in a crossover Fab molecule. In some embodiments, the first antigen binding moiety comprises a heavy chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the CH1 domain sequence comprised in the amino acid 10 sequence of SEQ ID NO: 114. Particularly, the heavy chain constant region (specifically CH1 domain) may comprise amino acid mutations as described herein under “charge modifications”.

#### Second antigen binding moiety

The bispecific antigen binding molecule of the invention comprises at least one antigen binding moiety, particularly a Fab molecule, that binds to a second antigen (different from HLA-15 A2/WT1).

In particular embodiments, the antigen binding moiety that binds the second antigen is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CH1 and CL of the Fab heavy and light chains are exchanged / replaced by each other. In such embodiments, the antigen binding moiety(ies) that 20 binds to the first antigen (i.e. HLA-A2/WT1) is preferably a conventional Fab molecule. In embodiments where there is more than one antigen binding moiety, particularly Fab molecule, that binds to HLA-A2/WT1 comprised in the bispecific antigen binding molecule, the antigen binding moiety that binds to the second antigen preferably is a crossover Fab molecule and the antigen binding moieties that bind to HLA-A2/WT1 are conventional Fab molecules.

25 In alternative embodiments, the antigen binding moiety that binds to the second antigen is a conventional Fab molecule. In such embodiments, the antigen binding moiety(ies) that binds to the first antigen (i.e. HLA-A2/WT1) is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CH1 and CL of the Fab heavy and light chains are exchanged / replaced by each other. In embodiments where there 30 is more than one antigen binding moiety, particularly Fab molecule, that binds to a second antigen comprised in the bispecific antigen binding molecule, the antigen binding moiety that

binds to HLA-A2/WT1 preferably is a crossover Fab molecule and the antigen binding moieties that bind to the second antigen are conventional Fab molecules.

In some embodiments, the second antigen is an activating T cell antigen (also referred to herein as an “activating T cell antigen binding moiety, or activating T cell antigen binding Fab molecule”). In a particular embodiment, the bispecific antigen binding molecule comprises not more than one antigen binding moiety capable of specific binding to an activating T cell antigen. In one embodiment the bispecific antigen binding molecule provides monovalent binding to the activating T cell antigen.

In particular embodiments, the second antigen is CD3, particularly human CD3 (SEQ ID NO: 107) or cynomolgus CD3 (SEQ ID NO: 108), most particularly human CD3. In one embodiment the second antigen binding moiety is cross-reactive for (i.e. specifically binds to) human and cynomolgus CD3. In some embodiments, the second antigen is the epsilon subunit of CD3 (CD3 epsilon).

In one embodiment, the second antigen binding moiety comprises a HCDR 1 of SEQ ID NO: 115, a HCDR 2 of SEQ ID NO: 116, a HCDR 3 of SEQ ID NO: 117, a LCDR 1 of SEQ ID NO: 118, a LCDR 2 of SEQ ID NO: 119 and a LCDR 3 of SEQ ID NO: 120.

In one embodiment, the second antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 115, a HCDR 2 of SEQ ID NO: 116, and a HCDR 3 of SEQ ID NO: 117, and a VL comprising a LCDR 1 of SEQ ID NO: 118, a LCDR 2 of SEQ ID NO: 119 and a LCDR 3 of SEQ ID NO: 120.

In some embodiments, the second antigen binding moiety is (derived from) a humanized antibody. In one embodiment, the VH is a humanized VH and/or the VL is a humanized VL. In one embodiment, the second antigen binding moiety comprises CDRs as in any of the above embodiments, and further comprises an acceptor human framework, e.g. a human immunoglobulin framework or a human consensus framework.

In one embodiment, the second antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 121. In one embodiment, the second antigen binding moiety comprises a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 122.

In one embodiment, the second antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:

121, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 122.

In one embodiment, the VH of the second antigen binding moiety comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid

5 sequence of SEQ ID NO: 121, and the VL of the second antigen binding moiety comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 122.

In one embodiment, the second antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 121, and a VL comprising the amino acid sequence of SEQ ID

10 NO: 122.

In one embodiment, the second antigen binding moiety comprises the VH sequence of SEQ ID NO: 121, and the VL sequence of SEQ ID NO: 122.

In a particular embodiment, the second antigen binding moiety comprises a HCDR 1 of SEQ ID NO: 130, a HCDR 2 of SEQ ID NO: 131, a HCDR 3 of SEQ ID NO: 132, a LCDR 1 of SEQ ID

15 NO: 133, a LCDR 2 of SEQ ID NO: 134 and a LCDR 3 of SEQ ID NO: 135.

In another particular embodiment, the second antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 130, a HCDR 2 of SEQ ID NO: 131, and a HCDR 3 of SEQ ID NO: 132, and a VL comprising a LCDR 1 of SEQ ID NO: 133, a LCDR 2 of SEQ ID NO: 134 and a LCDR 3 of SEQ ID NO: 135.

20 In some embodiments, the second antigen binding moiety is (derived from) a humanized antibody. In one embodiment, the VH is a humanized VH and/or the VL is a humanized VL. In one embodiment, the second antigen binding moiety comprises CDRs as in any of the above embodiments, and further comprises an acceptor human framework, e.g. a human immunoglobulin framework or a human consensus framework.

25 In one embodiment, the second antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 136. In one embodiment, the second antigen binding moiety comprises a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 137.

30 In a particular embodiment, the second antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 136, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 137.

In another particular embodiment, the VH of the second antigen binding moiety comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 136, and the VL of the second antigen binding moiety comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100%

5 identical to the amino acid sequence of SEQ ID NO: 137.

In one embodiment, the second antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 136, and a VL comprising the amino acid sequence of SEQ ID NO: 137.

In one embodiment, the second antigen binding moiety comprises the VH sequence of SEQ ID 10 NO: 136, and the VL sequence of SEQ ID NO: 137.

In one embodiment, the second antigen binding moiety comprises a human constant region. In one embodiment, the second antigen binding moiety is a Fab molecule comprising a human constant region, particularly a human CH1 and/or CL domain. Exemplary sequences of human constant domains are given in SEQ ID NOs 112 and 113 (human kappa and lambda CL domains, 15 respectively) and SEQ ID NO: 114 (human IgGi heavy chain constant domains CH1-CH2-CH3).

In some embodiments, the second antigen binding moiety comprises a light chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 112 or SEQ ID NO: 113, particularly the amino acid sequence of SEQ ID NO: 112. Particularly, the light chain constant region may 20 comprise amino acid mutations as described herein under “charge modifications” and/or may comprise deletion or substitutions of one or more (particularly two) N-terminal amino acids if in a crossover Fab molecule.. In some embodiments, the second antigen binding moiety comprises a heavy chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the CH1 domain sequence comprised in the amino 25 acid sequence of SEQ ID NO: 114. Particularly, the heavy chain constant region (specifically CH1 domain) may comprise amino acid mutations as described herein under “charge modifications”.

In some embodiments, the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1, particularly the variable domains VL 30 and VH, of the Fab light chain and the Fab heavy chain are replaced by each other (i.e. according to such embodiment, the second antigen binding moiety is a crossover Fab molecule wherein the variable or constant domains of the Fab light chain and the Fab heavy chain are exchanged). In

one such embodiment, the first (and the third, if any) antigen binding moiety is a conventional Fab molecule.

In one embodiment, not more than one antigen binding moiety that binds to the second antigen (e.g. an activating T cell antigen such as CD3) is present in the bispecific antigen binding

5 molecule (i.e. the bispecific antigen binding molecule provides monovalent binding to the second antigen).

### Charge modifications

The bispecific antigen binding molecules of the invention may comprise amino acid substitutions in Fab molecules comprised therein which are particularly efficient in reducing mispairing of

10 light chains with non-matching heavy chains (Bence-Jones-type side products), which can occur in the production of Fab-based bi-/multispecific antigen binding molecules with a VH/VL exchange in one (or more, in case of molecules comprising more than two antigen-binding Fab

15 molecules) of their binding arms (see also PCT publication no. WO 2015/150447, particularly the examples therein, incorporated herein by reference in its entirety). The ratio of a desired bispecific antigen binding molecule compared to undesired side products, in particular Bence

Jones-type side products occurring in bispecific antigen binding molecules with a VH/VL domain exchange in one of their binding arms, can be improved by the introduction of charged amino acids with opposite charges at specific amino acid positions in the CH1 and CL domains (sometimes referred to herein as “charge modifications”).

20 Accordingly, in some embodiments wherein the first and the second antigen binding moiety of the bispecific antigen binding molecule are both Fab molecules, and in one of the antigen binding moieties (particularly the second antigen binding moiety) the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other,

i) in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is

25 substituted by a positively charged amino acid (numbering according to Kabat), and wherein in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 or the amino acid at position 213 is substituted by a negatively charged amino acid (numbering according to Kabat EU index); or

ii) in the constant domain CL of the second antigen binding moiety the amino acid at position

30 124 is substituted by a positively charged amino acid (numbering according to Kabat), and wherein in the constant domain CH1 of the second antigen binding moiety the amino acid at

position 147 or the amino acid at position 213 is substituted by a negatively charged amino acid (numbering according to Kabat EU index).

The bispecific antigen binding molecule does not comprise both modifications mentioned under i) and ii). The constant domains CL and CH1 of the antigen binding moiety having the VH/VL exchange are not replaced by each other (i.e. remain unexchanged).

In a more specific embodiment,

i) in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index); or

ii) in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In one such embodiment, in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In a further embodiment, in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In a particular embodiment, in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) and the amino acid at position 123 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and

in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

5 In a more particular embodiment, in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by lysine (K) (numbering according to Kabat), and in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Kabat EU index) and the amino acid at  
10 position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index).

In an even more particular embodiment, in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by arginine (R) (numbering according to Kabat), and in the constant domain CH1 of the first antigen binding moiety the amino acid at  
15 position 147 is substituted by glutamic acid (E) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index).

In particular embodiments, if amino acid substitutions according to the above embodiments are made in the constant domain CL and the constant domain CH1 of the first antigen binding  
20 moiety, the constant domain CL of the first antigen binding moiety is of kappa isotype.

Alternatively, the amino acid substitutions according to the above embodiments may be made in the constant domain CL and the constant domain CH1 of the second antigen binding moiety instead of in the constant domain CL and the constant domain CH1 of the first antigen binding moiety. In particular such embodiments, the constant domain CL of the second antigen binding  
25 moiety is of kappa isotype.

Accordingly, in one embodiment, in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 or the amino acid at position 213 is

substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In a further embodiment, in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine 5 (H) (numbering according to Kabat), and in the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In still another embodiment, in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine

10 (H) (numbering according to Kabat) and the amino acid at position 123 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and in the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted independently by glutamic 15 acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

In one embodiment, in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by lysine (K) (numbering according to Kabat), and in the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 is

20 substituted by glutamic acid (E) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index).

In another embodiment, in the constant domain CL of the second antigen binding moiety the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by arginine (R) (numbering according to Kabat), and in

25 the constant domain CH1 of the second antigen binding moiety the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index).

In a particular embodiment, the bispecific antigen binding molecule of the invention comprises

30 (a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, specifically HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of

SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6, and

(b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen

5 binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

wherein in the constant domain CL of the first antigen binding moiety the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat) (in a particular embodiment independently by lysine (K) or arginine (R))

10 and the amino acid at position 123 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat) (in a particular embodiment independently by lysine (R) or arginine (R)), and in the constant domain CH1 of the first antigen binding moiety

the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted

15 independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index).

In another particular embodiment, the bispecific antigen binding molecule of the invention comprises

(a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-

20 A2/WT1, specifically HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14, and

(b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

wherein in the constant domain CL of the first antigen binding moiety the amino acid at position

30 124 is substituted independently by lysine (R), arginine (R) or histidine (H) (numbering according to Rabat) (in a particular embodiment independently by lysine (R) or arginine (R)) and the amino acid at position 123 is substituted independently by lysine (R), arginine (R) or histidine (H) (numbering according to Rabat) (in a particular embodiment independently by

lysine (K) or arginine (R)), and in the constant domain CH1 of the first antigen binding moiety the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Kabat EU index).

#### Bispecific antigen binding molecule formats

The components of the bispecific antigen binding molecule according to the present invention can be fused to each other in a variety of configurations. Exemplary configurations are depicted in **Figure 1**.

10 In particular embodiments, the antigen binding moieties comprised in the bispecific antigen binding molecule are Fab molecules. In such embodiments, the first, second, third etc. antigen binding moiety may be referred to herein as first, second, third etc. Fab molecule, respectively. In one embodiment, the first and the second antigen binding moiety of the bispecific antigen binding molecule are fused to each other, optionally via a peptide linker. In particular 15 embodiments, the first and the second antigen binding moiety are each a Fab molecule. In one such embodiment, the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety. In another such embodiment, the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety. In 20 embodiments wherein either (i) the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety or (ii) the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety, additionally the Fab light chain of the first antigen binding moiety and the Fab light chain of the second antigen binding 25 moiety may be fused to each other, optionally via a peptide linker.

A bispecific antigen binding molecule with a single antigen binding moiety (such as a Fab molecule) capable of specific binding to a target cell antigen such as HFA-A2/WT1 (for example as shown in **Figure 1A, D, G, H, K, L**) is useful, particularly in cases where internalization of the target cell antigen is to be expected following binding of a high affinity antigen binding 30 moiety. In such cases, the presence of more than one antigen binding moiety specific for the target cell antigen may enhance internalization of the target cell antigen, thereby reducing its availability.

In other cases, however, it will be advantageous to have a bispecific antigen binding molecule comprising two or more antigen binding moieties (such as Fab molecules) specific for a target cell antigen (see examples shown in **Figure 1B, 1C, 1E, 1F, 1I, 1J, 1M or 1N**), for example to optimize targeting to the target site or to allow crosslinking of target cell antigens.

5 Accordingly, in particular embodiments, the bispecific antigen binding molecule according to the present invention comprises a third antigen binding moiety.

In one embodiment, the third antigen binding moiety binds to the first antigen, i.e. HLA-A2/WT1. In one embodiment, the third antigen binding moiety is a Fab molecule.

10 In particular embodiments, the third antigen moiety is identical to the first antigen binding moiety.

The third antigen binding moiety of the bispecific antigen binding molecule may incorporate any of the features, singly or in combination, described herein in relation to the first antigen binding moiety and/or the antibody that binds HLA-A2/WT1, unless scientifically clearly unreasonable or impossible.

15 In one embodiment, the third antigen binding moiety comprises

(i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO:

20 **6;**

(ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

(iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a

25 HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;

(iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;

30 (v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;

(vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a

5 HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

10 (ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70.

In a particular embodiment, the third antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and

15 a VL comprising a LCDR 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6.

In another embodiment, the third antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ

20 ID NO: 14.

In a further embodiment, the third antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22.

25 In still a further embodiment, the third antigen binding moiety comprises a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30.

In some embodiments, the third antigen binding moiety is (derived from) a human antibody. In

30 one embodiment, the VH is a human VH and/or the VL is a human VL. In one embodiment, the third antigen binding moiety comprises CDRs as in any of the above embodiments, and further comprises a human framework, e.g. a human immunoglobulin framework.

In one embodiment, the third antigen binding moiety comprises (i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:

5 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

10 (iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

15 (iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

20 (v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

25 (vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

30 (viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

- 5 In a particular embodiment, the third antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8.
- 10 In another embodiment, the third antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.
- 15 In a further embodiment, the third antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.
- 20 In still a further embodiment, the third antigen binding moiety comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32.
- 25 In one embodiment, the third antigen binding moiety comprises
  - (i) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;
  - (ii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

5 (iv) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

(v) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

10 (vi) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL sequence that is at least about 95%, 96%,

15 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

In a particular embodiment, the third antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to

25 the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the third antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16.

30 In a further embodiment, the third antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24.

In still a further embodiment, the third antigen binding moiety comprises a VH sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32.

- 5 In one embodiment, the third antigen binding moiety comprises
  - (i) a VH comprising the amino acid sequence of SEQ ID NO: 7, and a VL comprising the amino acid sequence of SEQ ID NO: 8;
  - (ii) a VH comprising the amino acid sequence of SEQ ID NO: 15, and a VL comprising the amino acid sequence of SEQ ID NO: 16;
- 10 (iii) a VH comprising the amino acid sequence of SEQ ID NO: 23, and a VL comprising the amino acid sequence of SEQ ID NO: 24;
- (iv) a VH comprising the amino acid sequence of SEQ ID NO: 31, and a VL comprising the amino acid sequence of SEQ ID NO: 32;
- 15 (v) a VH comprising the amino acid sequence of SEQ ID NO: 39, and a VL comprising the amino acid sequence of SEQ ID NO: 40;
- (vi) a VH comprising the amino acid sequence of SEQ ID NO: 47, and a VL comprising the amino acid sequence of SEQ ID NO: 48;
- (vii) a VH comprising the amino acid sequence of SEQ ID NO: 55, and a VL comprising the amino acid sequence of SEQ ID NO: 56;
- 20 (viii) a VH comprising the amino acid sequence of SEQ ID NO: 63, and a VL comprising the amino acid sequence of SEQ ID NO: 64; or
- (ix) a VH comprising the amino acid sequence of SEQ ID NO: 71, and a VL comprising the amino acid sequence of SEQ ID NO: 72.

In one embodiment, the third antigen binding moiety comprises

- 25 (i) the VH sequence of SEQ ID NO: 7, and the VL sequence of SEQ ID NO: 8;
- (ii) the VH sequence of SEQ ID NO: 15, and the VL sequence of SEQ ID NO: 16;
- (iii) the VH sequence of SEQ ID NO: 23, and the VL sequence of SEQ ID NO: 24;
- (iv) the VH sequence of SEQ ID NO: 31, and the VL sequence of SEQ ID NO: 32;
- (v) the VH sequence of SEQ ID NO: 39, and the VL sequence of SEQ ID NO: 40;
- 30 (vi) the VH sequence of SEQ ID NO: 47, and the VL sequence of SEQ ID NO: 48;
- (vii) the VH sequence of SEQ ID NO: 55, and the VL sequence of SEQ ID NO: 56;
- (viii) the VH sequence of SEQ ID NO: 63, and the VL sequence of SEQ ID NO: 64; or
- (ix) the VH sequence of SEQ ID NO: 71, and the VL sequence of SEQ ID NO: 72.

In a particular embodiment, the third antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 7 and a VL comprising the amino acid sequence of SEQ ID NO: 8.

In another embodiment, the third antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 15 and a VL comprising the amino acid sequence of SEQ ID NO: 16.

In a further embodiment, the third antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 23 and a VL comprising the amino acid sequence of SEQ ID NO: 24.

10 In still a further embodiment, the third antigen binding moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 31 and a VL comprising the amino acid sequence of SEQ ID NO: 32.

In a particular embodiment, the third antigen binding moiety comprises the VH sequence of SEQ ID NO: 7 and the VL sequence of SEQ ID NO: 8.

15 In another embodiment, the third antigen binding moiety comprises the VH sequence of SEQ ID NO: 15 and the VL sequence of SEQ ID NO: 16.

In a further embodiment, the third antigen binding moiety comprises the VH sequence of SEQ ID NO: 23 and the VL sequence of SEQ ID NO: 24.

20 In still a further embodiment, the third antigen binding moiety comprises the VH sequence of SEQ ID NO: 31 and the VL sequence of SEQ ID NO: 32.

In one embodiment, the third antigen binding moiety comprises a human constant region. In one embodiment, the third antigen binding moiety is a Fab molecule comprising a human constant region, particularly a human CH1 and/or CL domain. Exemplary sequences of human constant domains are given in SEQ ID NOs 112 and 113 (human kappa and lambda CL domains, 25 respectively) and SEQ ID NO: 114 (human IgGi heavy chain constant domains CH1-CH2-CH3).

30 In some embodiments, the third antigen binding moiety comprises a light chain constant region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 112 or SEQ ID NO: 113, particularly the amino acid sequence of SEQ ID NO: 112. Particularly, the light chain constant region may comprise amino acid mutations as described herein under “charge modifications” and/or may comprise deletion or substitutions of one or more (particularly two) N-terminal amino acids if in a crossover Fab molecule. In some embodiments, the third antigen binding moiety comprises a heavy chain constant region comprising an amino acid sequence that is at least about 95%, 96%,

97%, 98%, 99% or 100% identical to the CH1 domain sequence comprised in the amino acid sequence of SEQ ID NO: 114. Particularly, the heavy chain constant region (specifically CH1 domain) may comprise amino acid mutations as described herein under “charge modifications”.

In particular embodiments, the third and the first antigen binding moiety are each a Fab molecule

5 and the third antigen binding moiety is identical to the first antigen binding moiety. Thus, in these embodiments the first and the third antigen binding moiety comprise the same heavy and light chain amino acid sequences and have the same arrangement of domains (i.e. conventional or crossover)). Furthermore, in these embodiments, the third antigen binding moiety comprises the same amino acid substitutions, if any, as the first antigen binding moiety. For example, the  
10 amino acid substitutions described herein as “charge modifications” will be made in the constant domain CF and the constant domain CH1 of each of the first antigen binding moiety and the third antigen binding moiety. Alternatively, said amino acid substitutions may be made in the constant domain CF and the constant domain CH1 of the second antigen binding moiety (which in particular embodiments is also a Fab molecule), but not in the constant domain CF and the  
15 constant domain CH1 of the first antigen binding moiety and the third antigen binding moiety.

Like the first antigen binding moiety, the third antigen binding moiety particularly is a conventional Fab molecule. Embodiments wherein the first and the third antigen binding moieties are crossover Fab molecules (and the second antigen binding moiety is a conventional Fab molecule) are, however, also contemplated. Thus, in particular embodiments, the first and

20 the third antigen binding moieties are each a conventional Fab molecule, and the second antigen binding moiety is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VF or the constant domains CF and CH1 of the Fab heavy and light chains are exchanged / replaced by each other. In other embodiments, the first and the third antigen binding moieties are each a crossover Fab molecule and the second antigen binding  
25 moiety is a conventional Fab molecule.

If a third antigen binding moiety is present, in a particular embodiment the first and the third antigen moiety bind to HFA-A2/WT1, and the second antigen binding moiety binds to a second antigen, particularly an activating T cell antigen, more particularly CD3, most particularly CD3 epsilon.

30 In particular embodiments, the bispecific antigen binding molecule comprises an Fc domain composed of a first and a second subunit. The first and the second subunit of the Fc domain are capable of stable association.

The bispecific antigen binding molecule according to the invention can have different configurations, i.e. the first, second (and optionally third) antigen binding moiety may be fused to each other and to the Fc domain in different ways. The components may be fused to each other directly or, preferably, via one or more suitable peptide linkers. Where fusion of a Fab 5 molecule is to the N-terminus of a subunit of the Fc domain, it is typically via an immunoglobulin hinge region.

In some embodiments, the first and the second antigen binding moiety are each a Fab molecule and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain. In such embodiments, the first 10 antigen binding moiety may be fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety or to the N-terminus of the other one of the subunits of the Fc domain. In particular such embodiments, said first antigen binding moiety is a conventional Fab molecule, and the second antigen binding moiety is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or 15 the constant domains CL and CH1 of the Fab heavy and light chains are exchanged / replaced by each other. In other such embodiments, said first Fab molecule is a crossover Fab molecule and the second Fab molecule is a conventional Fab molecule.

In one embodiment, the first and the second antigen binding moiety are each a Fab molecule, the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain, and the first antigen binding moiety 20 is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety. In a specific embodiment, the bispecific antigen binding molecule essentially consists of the first and the second Fab molecule, the Fc domain composed of a first and a second subunit, and optionally one or more peptide linkers, wherein the first Fab 25 molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second Fab molecule, and the second Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain. Such a configuration is schematically depicted in **Figures 1G and IK** (with the second antigen binding domain in these examples being a VH/VL crossover Fab molecule). Optionally, the Fab light 30 chain of the first Fab molecule and the Fab light chain of the second Fab molecule may additionally be fused to each other.

In another embodiment, the first and the second antigen binding moiety are each a Fab molecule and the first and the second antigen binding moiety are each fused at the C-terminus of the Fab

heavy chain to the N-terminus of one of the subunits of the Fc domain. In a specific embodiment, the bispecific antigen binding molecule essentially consists of the first and the second Fab molecule, the Fc domain composed of a first and a second subunit, and optionally one or more peptide linkers, wherein the first and the second Fab molecule are each fused at the C-terminus  
5 of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain. Such a configuration is schematically depicted in Figures 1A and 1D (in these examples with the second antigen binding domain being a VH/VL crossover Fab molecule and the first antigen binding moiety being a conventional Fab molecule). The first and the second Fab molecule may be fused to the Fc domain directly or through a peptide linker. In a particular embodiment the first and the  
10 second Fab molecule are each fused to the Fc domain through an immunoglobulin hinge region. In a specific embodiment, the immunoglobulin hinge region is a human IgG1 hinge region, particularly where the Fc domain is an IgG1 Fc domain.

In some embodiments, the first and the second antigen binding moiety are each a Fab molecule and the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain. In such embodiments, the second antigen binding moiety may be fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety or (as described above) to the N-terminus of the other one of the subunits of the Fc domain. In particular such embodiments, said first antigen binding moiety is a conventional Fab molecule, and the second antigen binding  
15 moiety is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CL and CH1 of the Fab heavy and light chains are exchanged / replaced by each other. In other such embodiments, said first Fab molecule is a crossover Fab molecule and the second Fab molecule is a conventional Fab molecule.  
20

In one embodiment, the first and the second antigen binding moiety are each a Fab molecule, the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain, and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety. In a specific embodiment, the bispecific antigen binding molecule essentially consists of the first and the second Fab molecule, the Fc domain composed of a first  
25 and a second subunit, and optionally one or more peptide linkers, wherein the second Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first Fab molecule, and the first Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or the second subunit of the Fc domain. Such a  
30

configuration is schematically depicted in **Figures 1H** and **1L** (in these examples with the second antigen binding domain being a VH/VL crossover Fab molecule and the first antigen binding moiety being a conventional Fab molecule). Optionally, the Fab light chain of the first Fab molecule and the Fab light chain of the second Fab molecule may additionally be fused to each other.

5 In some embodiments, a third antigen binding moiety, particularly a third Fab molecule, is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first or second subunit of the Fc domain. In particular such embodiments, said first and third Fab molecules are each a conventional Fab molecule, and the second Fab molecule is a crossover Fab molecule as

10 described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CL and CH1 of the Fab heavy and light chains are exchanged / replaced by each other. In other such embodiments, said first and third Fab molecules are each a crossover Fab molecule and the second Fab molecule is a conventional Fab molecule.

In a particular such embodiment, the second and the third antigen binding moiety are each fused

15 at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain, and the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second Fab molecule. In a specific embodiment, the bispecific antigen binding molecule essentially consists of the first, the second and the third Fab molecule, the Fc domain composed of a first and a second subunit, and optionally one or

20 more peptide linkers, wherein the first Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second Fab molecule, and the second Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first subunit of the Fc domain, and wherein the third Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the second subunit of the Fc domain. Such a configuration is

25 schematically depicted in **Figure 1B** and **1E** (in these examples with the second antigen binding moiety being a VH/VL crossover Fab molecule, and the first and the third antigen binding moiety being a conventional Fab molecule), and **Figure 1J** and **1N** (in these examples with the second antigen binding moiety being a conventional Fab molecule, and the first and the third antigen binding moiety being a VH/VL crossover Fab molecule). The second and the third Fab

30 molecule may be fused to the Fc domain directly or through a peptide linker. In a particular embodiment the second and the third Fab molecule are each fused to the Fc domain through an immunoglobulin hinge region. In a specific embodiment, the immunoglobulin hinge region is a human IgGi hinge region, particularly where the Fc domain is an IgGi Fc domain. Optionally,

the Fab light chain of the first Fab molecule and the Fab light chain of the second Fab molecule may additionally be fused to each other.

In another such embodiment, the first and the third antigen binding moiety are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain,

5 and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety. In a specific embodiment, the bispecific antigen binding molecule essentially consists of the first, the second and the third Fab molecule, the Fc domain composed of a first and a second subunit, and optionally one or more peptide linkers, wherein the second Fab molecule is fused at the C-terminus of the Fab 10 heavy chain to the N-terminus of the Fab heavy chain of the first Fab molecule, and the first Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first subunit of the Fc domain, and wherein the third Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the second subunit of the Fc domain. Such a configuration is schematically depicted in **Figure 1C** and **1F** (in these examples with the second antigen binding 15 moiety being a VH/VL crossover Fab molecule, and the first and the third antigen binding moiety being a conventional Fab molecule) and in **Figure II** and **1M** (in these examples with the second antigen binding moiety being a conventional Fab molecule, and the first and the third antigen binding moiety being a VH/VL crossover Fab molecule). The first and the third Fab molecule may be fused to the Fc domain directly or through a peptide linker. In a particular 20 embodiment the first and the third Fab molecule are each fused to the Fc domain through an immunoglobulin hinge region. In a specific embodiment, the immunoglobulin hinge region is a human IgGi hinge region, particularly where the Fc domain is an IgGi Fc domain. Optionally, the Fab light chain of the first Fab molecule and the Fab light chain of the second Fab molecule may additionally be fused to each other.

25 In configurations of the bispecific antigen binding molecule wherein a Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of each of the subunits of the Fc domain through an immunoglobulin hinge regions, the two Fab molecules, the hinge regions and the Fc domain essentially form an immunoglobulin molecule. In a particular embodiment the immunoglobulin molecule is an IgG class immunoglobulin. In an even more particular 30 embodiment the immunoglobulin is an IgGi subclass immunoglobulin. In another embodiment the immunoglobulin is an IgG<sub>4</sub> subclass immunoglobulin. In a further particular embodiment the immunoglobulin is a human immunoglobulin. In other embodiments the immunoglobulin is a

chimeric immunoglobulin or a humanized immunoglobulin. In one embodiment, the immunoglobulin comprises a human constant region, particularly a human Fc region.

In some of the bispecific antigen binding molecule of the invention, the Fab light chain of the first Fab molecule and the Fab light chain of the second Fab molecule are fused to each other,

5 optionally via a peptide linker. Depending on the configuration of the first and the second Fab molecule, the Fab light chain of the first Fab molecule may be fused at its C-terminus to the N-terminus of the Fab light chain of the second Fab molecule, or the Fab light chain of the second Fab molecule may be fused at its C-terminus to the N-terminus of the Fab light chain of the first Fab molecule. Fusion of the Fab light chains of the first and the second Fab molecule further  
10 reduces mispairing of unmatched Fab heavy and light chains, and also reduces the number of plasmids needed for expression of some of the bispecific antigen binding molecules of the invention.

The antigen binding moieties may be fused to the Fc domain or to each other directly or through a peptide linker, comprising one or more amino acids, typically about 2-20 amino acids. Peptide

15 linkers are known in the art and are described herein. Suitable, non-immunogenic peptide linkers include, for example,  $(G_4S)_n$ ,  $(SG_4)_n$ ,  $(G_4S)_n$  or  $G_4(SG_4)_n$  peptide linkers. "n" is generally an integer from 1 to 10, typically from 2 to 4. In one embodiment said peptide linker has a length of at least 5 amino acids, in one embodiment a length of 5 to 100, in a further embodiment of 10 to 50 amino acids. In one embodiment said peptide linker is  $(GxS)_n$  or  $(GxS)_nG_m$  with G=glycine,  
20 S=serine, and (x=3, n= 3, 4, 5 or 6, and m=0, 1, 2 or 3) or (x=4, n=2, 3, 4 or 5 and m= 0, 1, 2 or 3), in one embodiment x=4 and n=2 or 3, in a further embodiment x=4 and n=2. In one embodiment said peptide linker is  $(G_4S)_2$ . A particularly suitable peptide linker for fusing the Fab light chains of the first and the second Fab molecule to each other is  $(G_4S)_2$ . An exemplary peptide linker suitable for connecting the Fab heavy chains of the first and the second Fab  
25 fragments comprises the sequence (D)-(G<sub>4</sub>S)<sub>2</sub> (SEQ ID NOs 110 and 111). Another suitable such linker comprises the sequence (G<sub>4</sub>S)<sub>4</sub>. Additionally, linkers may comprise (a portion of) an immunoglobulin hinge region. Particularly where a Fab molecule is fused to the N-terminus of an Fc domain subunit, it may be fused via an immunoglobulin hinge region or a portion thereof, with or without an additional peptide linker.

30 In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the

heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit ( $VL_{(2)}-CHI_{(2)}-CH2-CH3(-CH4)$ ), and a polypeptide wherein the Fab heavy chain of the first Fab molecule shares a carboxy-terminal peptide bond with an Fc domain subunit ( $VH_{(i)}-CHI_{(i)}-CH2-CH3(-CH4)$ ). In some embodiments

5 the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $VH_{(2)}-CL_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ). In certain embodiments the polypeptides are covalently linked, e.g., by a disulfide bond.

10 In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in

15 turn shares a carboxy-terminal peptide bond with an Fc domain subunit ( $VH_{(2)}-CL_{(2)}-CH2-CH3(-CH4)$ ), and a polypeptide wherein the Fab heavy chain of the first Fab molecule shares a carboxy-terminal peptide bond with an Fc domain subunit ( $VH_{(i)}-CHI_{(i)}-CH2-CH3(-CH4)$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-

20 terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule ( $VL_{(2)}-CHI_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ). In certain embodiments the polypeptides are covalently linked, e.g., by a disulfide bond.

In some embodiments, the bispecific antigen binding molecule comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit ( $VL_{(2)}-CHI_{(2)}-VH_{(i)}-CHI_{(i)}-CH2-CH3(-CH4)$ ). In other

25 embodiments, the bispecific antigen binding molecule comprises a polypeptide wherein the Fab heavy chain of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain variable region of the second Fab molecule which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the

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second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit ( $\text{VH}_{(i)}-\text{CHI}_{(i)}-\text{VL}_{(2)}-\text{CHI}_{(2)}-\text{CH2-CH3(-CH4)}$ ).

In some of these embodiments the bispecific antigen binding molecule further comprises a

5 crossover Fab light chain polypeptide of the second Fab molecule, wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $\text{VH}_{(2)}-\text{CL}_{(2)}$ ), and the Fab light chain polypeptide of the first Fab molecule ( $\text{VL}_{(i)}-\text{CL}_{(i)}$ ). In others of these embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable 10 region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule which in turn shares a carboxy-terminal peptide bond with the Fab light chain polypeptide of the first Fab molecule ( $\text{VH}_{(2)}-\text{CL}_{(2)}-\text{VL}_{(i)}-\text{CL}_{(i)}$ ), or a polypeptide wherein the Fab light chain polypeptide of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the second Fab 15 molecule which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $\text{VL}_{(i)}-\text{CL}_{(i)}-\text{VH}_{(2)}-\text{CL}_{(2)}$ ), as appropriate.

The bispecific antigen binding molecule according to these embodiments may further comprise

(i) an Fc domain subunit polypeptide ( $\text{CH2-CH3(-CH4)}$ ), or (ii) a polypeptide wherein the Fab 20 heavy chain of a third Fab molecule shares a carboxy-terminal peptide bond with an Fc domain subunit ( $\text{VH}_{(3)}-\text{CHI}_{(3)}-\text{CH2-CH3(-CH4)}$ ) and the Fab light chain polypeptide of a third Fab molecule ( $\text{VL}_{(3)}-\text{CL}_{(3)}$ ). In certain embodiments the polypeptides are covalently linked, e.g., by a disulfide bond.

In some embodiments, the bispecific antigen binding molecule comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal 25 peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second

Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit ( $\text{VH}_{(2)}-\text{CL}_{(2)}-\text{VH}_{(i)}-\text{CHI}_{(i)}-\text{CH2-CH3(-CH4)}$ ). In other 30 embodiments, the bispecific antigen binding molecule comprises a polypeptide wherein the Fab heavy chain of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the second Fab molecule which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second

Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit (VH<sub>(i)</sub>-CH1<sub>(i)</sub>-VH<sub>(2)</sub>-CL<sub>(2)</sub>-CH2-CH3(-CH4)).

In some of these embodiments the bispecific antigen binding molecule further comprises a

5 crossover Fab light chain polypeptide of the second Fab molecule, wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (VL<sub>(2)</sub>-CH1<sub>(2)</sub>), and the Fab light chain polypeptide of the first Fab molecule (VL<sub>(i)</sub>-CL<sub>(i)</sub>). In others of these embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable  
10 region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule which in turn shares a carboxy-terminal peptide bond with the Fab light chain polypeptide of the first Fab molecule (VL<sub>(2)</sub>-CH1<sub>(2)</sub>-VL<sub>(i)</sub>-CL<sub>(i)</sub>), or a polypeptide wherein the Fab light chain polypeptide of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the second Fab  
15 molecule which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (VL<sub>(i)</sub>-CL<sub>(i)</sub>-VH<sub>(2)</sub>-CL<sub>(2)</sub>), as appropriate.

The bispecific antigen binding molecule according to these embodiments may further comprise

(i) an Fc domain subunit polypeptide (CH2-CH3(-CH4)), or (ii) a polypeptide wherein the Fab heavy chain of a third Fab molecule shares a carboxy-terminal peptide bond with an Fc domain  
20 subunit (VH<sub>(3)</sub>-CH1<sub>(3)</sub>-CH2-CH3(-CH4)) and the Fab light chain polypeptide of a third Fab molecule (VL<sub>(3)</sub>-CL<sub>(3)</sub>). In certain embodiments the polypeptides are covalently linked, e.g., by a disulfide bond.

In certain embodiments, the bispecific antigen binding molecule does not comprise an Fc domain.

In particular such embodiments, said first and, if present third Fab molecules are each a  
25 conventional Fab molecule, and the second Fab molecule is a crossover Fab molecule as described herein, i.e. a Fab molecule wherein the variable domains VH and VL or the constant domains CL and CH1 of the Fab heavy and light chains are exchanged / replaced by each other.

In other such embodiments, said first and, if present third Fab molecules are each a crossover Fab molecule and the second Fab molecule is a conventional Fab molecule.

30 In one such embodiment, the bispecific antigen binding molecule essentially consists of the first and the second antigen binding moiety, and optionally one or more peptide linkers, wherein the first and the second antigen binding moiety are both Fab molecules and the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy

chain of the second antigen binding moiety. Such a configuration is schematically depicted in **Figures 1O and 1S** (in these examples with the second antigen binding domain being a VH/VL crossover Fab molecule and the first antigen binding moiety being a conventional Fab molecule).

In another such embodiment, the bispecific antigen binding molecule essentially consists of the

5 first and the second antigen binding moiety, and optionally one or more peptide linkers, wherein the first and the second antigen binding moiety are both Fab molecules and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety. Such a configuration is schematically depicted in **Figures 1P and 1T** (in these examples with the second antigen binding domain being a VH/VL

10 crossover Fab molecule and the first antigen binding moiety being a conventional Fab molecule).

In some embodiments, the first Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second Fab molecule, and the bispecific antigen binding molecule further comprises a third antigen binding moiety, particularly a third Fab molecule, wherein said third Fab molecule is fused at the C-terminus of the Fab heavy chain to

15 the N-terminus of the Fab heavy chain of the first Fab molecule. In certain such embodiments, the bispecific antigen binding molecule essentially consists of the first, the second and the third Fab molecule, and optionally one or more peptide linkers, wherein the first Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second Fab molecule, and the third Fab molecule is fused at the C-terminus of the Fab heavy chain to

20 the N-terminus of the Fab heavy chain of the first Fab molecule. Such a configuration is schematically depicted in **Figures 1Q and 1U** (in these examples with the second antigen binding domain being a VH/VL crossover Fab molecule and the first and the antigen binding moiety each being a conventional Fab molecule), or **Figures 1X and 1Z** (in these examples with the second antigen binding domain being a conventional Fab molecule and the first and the third

25 antigen binding moiety each being a VH/VL crossover Fab molecule).

In some embodiments, the second Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first Fab molecule, and the bispecific antigen binding molecule further comprises a third antigen binding moiety, particularly a third Fab molecule, wherein said third Fab molecule is fused at the N-terminus of the Fab heavy chain

30 to the C-terminus of the Fab heavy chain of the first Fab molecule. In certain such embodiments, the bispecific antigen binding molecule essentially consists of the first, the second and the third Fab molecule, and optionally one or more peptide linkers, wherein the second Fab molecule is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the

first Fab molecule, and the third Fab molecule is fused at the N-terminus of the Fab heavy chain to the C-terminus of the Fab heavy chain of the first Fab molecule. Such a configuration is schematically depicted in **Figures 1R** and **IV** (in these examples with the second antigen binding domain being a VH/VL crossover Fab molecule and the first and the antigen binding moiety each being a conventional Fab molecule), or **Figures 1W** and **1Y** (in these examples with the second antigen binding domain being a conventional Fab molecule and the first and the third antigen binding moiety each being a VH/VL crossover Fab molecule).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain of the first Fab molecule shares a carboxy-

10 terminal peptide bond with the Fab light chain variable region of the second Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region) ( $VH_{(i)}-CHI_{(i)}-VL_{(2)}-CHI_{(2)}$ ). In some embodiments the bispecific antigen binding molecule further  
15 comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $VH_{(2)}-CL_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention

20 comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule ( $VL_{(2)}-CHI_{(2)}-VH_{(i)}-CHI_{(i)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $VH_{(2)}-CL_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(1)}$ ).

25 30 In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain,

wherein the heavy chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule ( $VH_{(2)}-CL_{(2)}-VH_{(i)}-CHI_{(i)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of the second Fab

5 molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule ( $VL_{(2)}-CHI_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule ( $VL_{(2)}-CHI_{(2)}-VH_{(i)}-CHI_{(i)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a

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15 polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $VH_{(2)}-CL_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(1)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab light chain variable region of the second Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover

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25 Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region) ( $VH_{(3)}-CHI_{(3)}-VH_{(i)}-CHI_{(i)}-VL_{(2)}-CHI_{(2)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule ( $VH_{(2)}-CL_{(2)}$ ) and the Fab light chain polypeptide of

30 the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ). In some embodiments the bispecific antigen binding molecule further comprises the Fab light chain polypeptide of a third Fab molecule ( $VL_{(3)}-CL_{(3)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain of a third Fab molecule shares a carboxy-

terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the second Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab

5 heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region) (VH<sub>(3)</sub>-CHI<sub>(3)</sub>-VH<sub>(i)</sub>-CHI<sub>(i)</sub>-VH<sub>(2)</sub>-CL<sub>(2)</sub>). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule (VL<sub>(2)</sub>-CHI<sub>(2)</sub>) and the Fab light chain polypeptide of the first

10 Fab molecule (VL<sub>(i)</sub>-CL<sub>(i)</sub>). In some embodiments the bispecific antigen binding molecule further comprises the Fab light chain polypeptide of a third Fab molecule (VL<sub>(3)</sub>-CL<sub>(3)</sub>).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab light chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second

15 Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of a third Fab molecule (VL<sub>p</sub>-CHI<sub>(2)</sub>-VH<sub>(i)</sub>-CHI<sub>(i)</sub>-VH<sub>(3)</sub>-CHI<sub>(3)</sub>). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (VH<sub>(2)</sub>-CL<sub>(2)</sub>) and the Fab light chain polypeptide of the first

20 Fab molecule (VL<sub>(i)</sub>-CL<sub>(i)</sub>). In some embodiments the bispecific antigen binding molecule further comprises the Fab light chain polypeptide of a third Fab molecule (VL<sub>(3)</sub>-CL<sub>(3)</sub>).

25 In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain variable region of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the second Fab molecule (i.e. the second Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in

30 turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of a third Fab molecule (VH<sub>(2)</sub>-CL<sub>(2)</sub>-VH<sub>(i)</sub>-CHI<sub>(i)</sub>-VH<sub>(3)</sub>-CHI<sub>(3)</sub>). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of

the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the second Fab molecule ( $VL_{(2)}-CHI_{(2)}$ ) and the Fab light chain polypeptide of the first Fab molecule ( $VL_{(i)}-CL_{(i)}$ ). In some embodiments the bispecific antigen binding molecule further comprises the Fab light chain polypeptide of a third Fab molecule ( $VL_{(3)}-CL_{(3)}$ ).

5 In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain variable region of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the first Fab molecule (i.e. the first Fab molecule comprises a crossover Fab heavy chain,

10 wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab light chain variable region of a third Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of a third Fab molecule (i.e. the third Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region)

15 ( $VH_{(2)}-CHI_{(2)}-VL_{(i)}-CHI_{(i)}-VL_{(3)}-CHI_{(3)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the first Fab molecule ( $VH_{(i)}-CL_{(i)}$ ) and the Fab light chain polypeptide of the second Fab molecule ( $VL_{(2)}-CL_{(2)}$ ). In some embodiments the bispecific antigen binding molecule

20 further comprises a polypeptide wherein the Fab heavy chain variable region of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of a third Fab molecule ( $VH_{(3)}-CL_{(3)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain of the second Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the first Fab molecule (i.e. the first Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of a third Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of a third Fab molecule (i.e. the third Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain constant region is replaced by a light chain constant region) ( $VH_{(2)}-CHI_{(2)}-VH_{(i)}-CL_{(i)}-VH_{(3)}-CL_{(3)}$ ). In some embodiments the bispecific antigen

binding molecule further comprises a polypeptide wherein the Fab light chain variable region of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the first Fab molecule ( $VL_{(i)}-CH_{(i)}$ ) and the Fab light chain polypeptide of the second Fab molecule ( $VL_{(2)}-CL_{(2)}$ ). In some embodiments the bispecific antigen binding molecule

5 further comprises a polypeptide wherein the Fab light chain variable region of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of a third Fab molecule ( $VL_{(3)}-CH_{(3)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab light chain variable region of a third Fab molecule

10 shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of a third Fab molecule (i.e. the third Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab light chain variable region of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain constant region

15 of the first Fab molecule (i.e. the first Fab molecule comprises a crossover Fab heavy chain, wherein the heavy chain variable region is replaced by a light chain variable region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the second Fab molecule ( $VL(3)-CH(3)-VL(i)-CH(i)-VH(2)-CH(2)$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region

20 of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of the first Fab molecule ( $VH_{(i)}-CL_{(i)}$ ) and the Fab light chain polypeptide of the second Fab molecule ( $VL_{(2)}-CL_{(2)}$ ). In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab heavy chain variable region of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of

25 a third Fab molecule ( $VH_{(3)}-CL_{(3)}$ ).

In certain embodiments the bispecific antigen binding molecule according to the invention comprises a polypeptide wherein the Fab heavy chain variable region of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab light chain constant region of a third Fab molecule (i.e. the third Fab molecule comprises a crossover Fab heavy chain, wherein the heavy

30 chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain variable region of the first Fab molecule, which in turn shares a carboxy-terminal peptide bond with the Fab light chain constant region of the first Fab molecule (i.e. the first Fab molecule comprises a crossover Fab heavy

chain, wherein the heavy chain constant region is replaced by a light chain constant region), which in turn shares a carboxy-terminal peptide bond with the Fab heavy chain of the second Fab molecule  $(VH_{(3)}-CL_{(3)}-VH_{(i)}-CL_{(i)}-VH_{(2)}-CH1_{(2)})$ . In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of the first Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of the first Fab molecule  $(VL_{(i)}-CH1_{(i)})$  and the Fab light chain polypeptide of the second Fab molecule  $(VL_{(2)}-CL_{(2)})$ . In some embodiments the bispecific antigen binding molecule further comprises a polypeptide wherein the Fab light chain variable region of a third Fab molecule shares a carboxy-terminal peptide bond with the Fab heavy chain constant region of a third Fab molecule  $(VL_{(3)}-CH1_{(3)})$ .

In one embodiment, the invention provides a bispecific antigen binding molecule comprising

- a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;
- b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;
- c) an Fc domain composed of a first and a second subunit;

wherein

- (i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c), or
- (ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In a particular embodiment, the invention provides a bispecific antigen binding molecule comprising

a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule

5 comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;

10 b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;

15 c) a third antigen binding moiety that binds to the first antigen and is identical to the first antigen binding moiety; and

d) an Fc domain composed of a first and a second subunit;

wherein

(i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to

20 the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d), or

(ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain

25 to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d).

In another embodiment, the invention provides a bispecific antigen binding molecule comprising

30 a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of

SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;

c) an Fc domain composed of a first and a second subunit;

10 wherein

(i) the first antigen binding moiety under a) and the second antigen binding moiety under b) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In one embodiment, the invention provides a bispecific antigen binding molecule comprising

15 a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;

c) an Fc domain composed of a first and a second subunit;

wherein

(i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c), or

(ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

5 In a particular embodiment, the invention provides a bispecific antigen binding molecule comprising

10 a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

15 b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;

20 c) a third antigen binding moiety that binds to the first antigen and is identical to the first antigen binding moiety; and

d) an Fc domain composed of a first and a second subunit;

wherein

25 (i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d), or

(ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d).

In another embodiment, the invention provides a bispecific antigen binding molecule comprising a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary

5 determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is

10 an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1 of the Fab light chain and the Fab heavy chain are replaced by each other;

15 c) an Fc domain composed of a first and a second subunit;

wherein

(i) the first antigen binding moiety under a) and the second antigen binding moiety under b) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In all of the different configurations of the bispecific antigen binding molecule according to the

20 invention, the amino acid substitutions described herein, if present, may either be in the CH1 and CL domains of the first and (if present) the third antigen binding moiety/Fab molecule, or in the CH1 and CL domains of the second antigen binding moiety/Fab molecule. Preferably, they are in the CH1 and CL domains of the first and (if present) the third antigen binding moiety/Fab molecule. In accordance with the concept of the invention, if amino acid substitutions as

25 described herein are made in the first (and, if present, the third) antigen binding moiety/Fab molecule, no such amino acid substitutions are made in the second antigen binding moiety/Fab molecule. Conversely, if amino acid substitutions as described herein are made in the second antigen binding moiety/Fab molecule, no such amino acid substitutions are made in the first (and, if present, the third) antigen binding moiety/Fab molecule. Amino acid substitutions are

30 particularly made in bispecific antigen binding molecules comprising a Fab molecule wherein the variable domains VL and VH1 of the Fab light chain and the Fab heavy chain are replaced by each other.

In particular embodiments of the bispecific antigen binding molecule according to the invention, particularly wherein amino acid substitutions as described herein are made in the first (and, if present, the third) antigen binding moiety/Fab molecule, the constant domain CL of the first (and, if present, the third) Fab molecule is of kappa isotype. In other embodiments of the bispecific antigen binding molecule according to the invention, particularly wherein amino acid substitutions as described herein are made in the second antigen binding moiety/Fab molecule, the constant domain CL of the second antigen binding moiety/Fab molecule is of kappa isotype. In some embodiments, the constant domain CL of the first (and, if present, the third) antigen binding moiety/Fab molecule and the constant domain CL of the second antigen binding moiety/Fab molecule are of kappa isotype.

In one embodiment, the invention provides a bispecific antigen binding molecule comprising

- a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementary determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;
- b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;
- c) an Fc domain composed of a first and a second subunit;

wherein in the constant domain CL of the first antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first antigen binding moiety under a) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Rabat EU index); and

wherein

- (i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the

second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c), or

(ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the

5 first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In a particular embodiment, the invention provides a bispecific antigen binding molecule comprising

a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-

10 A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of

15 SEQ ID NO: 6;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

20 c) a third antigen binding moiety that binds to the first antigen and is identical to the first antigen binding moiety; and

d) an Fc domain composed of a first and a second subunit;

wherein in the constant domain CL of the first antigen binding moiety under a) and the third

antigen binding moiety under c) the amino acid at position 124 is substituted by lysine (K)

25 (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most particularly by arginine (R)), and wherein

in the constant domain CH1 of the first antigen binding moiety under a) and the third antigen

binding moiety under c) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by

30 glutamic acid (E) (numbering according to Rabat EU index); and

wherein

(i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the

second antigen binding moiety under b) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d), or

(ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain

5 to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d).

In another embodiment, the invention provides a bispecific antigen binding molecule comprising

10 a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity

15 determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab

20 light chain and the Fab heavy chain are replaced by each other;

c) an Fc domain composed of a first and a second subunit;

wherein in the constant domain CL of the first antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most

25 particularly by arginine (R)), and wherein in the constant domain CH1 of the first antigen binding moiety under a) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Rabat EU index); and

wherein the first antigen binding moiety under a) and the second antigen binding moiety under b)

30 are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In one embodiment, the invention provides a bispecific antigen binding molecule comprising

a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

5 b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

10 c) an Fc domain composed of a first and a second subunit;  
wherein in the constant domain CL of the first antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first antigen binding moiety under a) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Rabat EU index); and

15 20 wherein

(i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c), or

25 (ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In a particular embodiment, the invention provides a bispecific antigen binding molecule  
30 comprising

a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule comprising a heavy chain variable region (VH) comprising a heavy chain complementary

determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

5 b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

10 c) a third antigen binding moiety that binds to the first antigen and is identical to the first antigen binding moiety; and

15 d) an Fc domain composed of a first and a second subunit;

wherein in the constant domain CL of the first antigen binding moiety under a) and the third antigen binding moiety under c) the amino acid at position 124 is substituted by lysine (K) (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first antigen binding moiety under a) and the third antigen binding moiety under c) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Rabat EU index); and

20 wherein

(i) the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d), or

25 (ii) the second antigen binding moiety under b) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety under a), and the first antigen binding moiety under a) and the third antigen binding moiety under c) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under d).

In another embodiment, the invention provides a bispecific antigen binding molecule comprising a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1 RMF, and the first antigen binding moiety is a Fab molecule

comprising a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO:

5 13 and a LCDR 3 of SEQ ID NO: 14;

b) a second antigen binding moiety that binds to a second antigen, wherein the second antigen is an activating T cell antigen, particularly CD3, more particularly CD3 epsilon, and the second antigen binding moiety is a Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other;

10 c) an Fc domain composed of a first and a second subunit;

wherein in the constant domain CL of the first antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Rabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Rabat) (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first antigen

15 binding moiety under a) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Rabat EU index); and

wherein the first antigen binding moiety under a) and the second antigen binding moiety under b) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits

20 of the Fc domain under c).

According to any of the above embodiments, components of the bispecific antigen binding molecule (e.g. Fab molecules, Fc domain) may be fused directly or through various linkers, particularly peptide linkers comprising one or more amino acids, typically about 2-20 amino acids, that are described herein or are known in the art. Suitable, non-immunogenic peptide

25 linkers include, for example,  $(G_4S)_n$ ,  $(SG_4)_n$ ,  $(G_4S)_n$  or  $G_4(SG_4)_n$  peptide linkers, wherein n is generally an integer from 1 to 10, typically from 2 to 4.

In a particular aspect, the invention provides a bispecific antigen binding molecule comprising

a) a first and a third antigen binding moiety that binds to a first antigen; wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1<sub>RMF</sub>, and wherein the first and the second antigen

30 binding moiety are each a (conventional) Fab molecule comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8;

b) a second antigen binding moiety that binds to a second antigen; wherein the second antigen is CD3 and wherein the second antigen binding moiety is Fab molecule wherein the variable domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other, comprising (i) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:

5 121 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 122, or (ii) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 136 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 137 (particularly a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 136 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 137);

10 c) an Fc domain composed of a first and a second subunit;

wherein

in the constant domain CL of the first and the third antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Kabat)

15 (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first and the third antigen binding moiety under a) the amino acid at position 147 is substituted by glutamic acid (E) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index);

and wherein further

20 the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the second antigen binding moiety under b) and the third antigen binding moiety under a) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

25 In a further aspect, the invention provides a bispecific antigen binding molecule comprising

a) a first and a third antigen binding moiety that binds to a first antigen; wherein the first antigen is HLA-A2/WT1, particularly HLA-A2/WT1<sub>RMF</sub>, and wherein the first and the second antigen binding moiety are each a (conventional) Fab molecule comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 15 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16;

30 b) a second antigen binding moiety that binds to a second antigen; wherein the second antigen is CD3 and wherein the second antigen binding moiety is Fab molecule wherein the variable

domains VL and VH of the Fab light chain and the Fab heavy chain are replaced by each other, comprising (i) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 121 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 122, or (ii) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 136

5 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 137 (particularly a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 136 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 137);

c) an Fc domain composed of a first and a second subunit;

wherein

10 in the constant domain CL of the first and the third antigen binding moiety under a) the amino acid at position 124 is substituted by lysine (K) (numbering according to Kabat) and the amino acid at position 123 is substituted by lysine (K) or arginine (R) (numbering according to Kabat) (most particularly by arginine (R)), and wherein in the constant domain CH1 of the first and the third antigen binding moiety under a) the amino acid at position 147 is substituted by glutamic

15 acid (E) (numbering according to Kabat EU index) and the amino acid at position 213 is substituted by glutamic acid (E) (numbering according to Kabat EU index);

and wherein further

the first antigen binding moiety under a) is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety under b), and the

20 second antigen binding moiety under b) and the third antigen binding moiety under a) are each fused at the C-terminus of the Fab heavy chain to the N-terminus of one of the subunits of the Fc domain under c).

In one embodiment according to these aspects of the invention, in the first subunit of the Fc

domain the threonine residue at position 366 is replaced with a tryptophan residue (T366W), and

25 in the second subunit of the Fc domain the tyrosine residue at position 407 is replaced with a valine residue (Y407V) and optionally the threonine residue at position 366 is replaced with a serine residue (T366S) and the leucine residue at position 368 is replaced with an alanine residue (L368A) (numberings according to Kabat EU index).

In a further embodiment according to these aspects of the invention, in the first subunit of the Fc

30 domain additionally the serine residue at position 354 is replaced with a cysteine residue (S354C) or the glutamic acid residue at position 356 is replaced with a cysteine residue (E356C) (particularly the serine residue at position 354 is replaced with a cysteine residue), and in the

second subunit of the Fc domain additionally the tyrosine residue at position 349 is replaced by a cysteine residue (Y349C) (numberings according to Kabat EU index).

In still a further embodiment according to these aspects of the invention, in each of the first and

the second subunit of the Fc domain the leucine residue at position 234 is replaced with an

5 alanine residue (L234A), the leucine residue at position 235 is replaced with an alanine residue (L235A) and the proline residue at position 329 is replaced by a glycine residue (P329G) (numbering according to Kabat EU index).

In still a further embodiment according to these aspects of the invention, the Fc domain is a human IgGi Fc domain.

10 In particular specific embodiment, the bispecific antigen binding molecule comprises a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 123, a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 125, a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99%

15 identical to the sequence of SEQ ID NO: 139, and a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO:

140. In a further particular specific embodiment, the bispecific antigen binding molecule comprises a polypeptide comprising the amino acid sequence of SEQ ID NO: 123, a polypeptide comprising the amino acid sequence of SEQ ID NO: 125, a polypeptide comprising the amino

20 acid sequence of SEQ ID NO: 139 and a polypeptide comprising the amino acid sequence of SEQ ID NO: 140.

In another specific embodiment, the bispecific antigen binding molecule comprises a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 123, a polypeptide comprising an amino acid sequence that is at least

25 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 124, a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 125, and a polypeptide comprising an amino acid sequence that is at

least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 129. In a further particular specific embodiment, the bispecific antigen binding molecule comprises a polypeptide

30 comprising the amino acid sequence of SEQ ID NO: 123, a polypeptide comprising the amino acid sequence of SEQ ID NO: 124, a polypeptide comprising the amino acid sequence of SEQ ID NO: 125 and a polypeptide comprising the amino acid sequence of SEQ ID NO: 129.

In another specific embodiment, the bispecific antigen binding molecule comprises a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 126, a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 127, a polypeptide 5 comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 128, and a polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 129. In a further specific embodiment, the bispecific antigen binding molecule comprises a polypeptide comprising the amino acid sequence of SEQ ID NO: 126, a polypeptide comprising the amino acid sequence of SEQ ID NO: 127, a polypeptide comprising the amino acid sequence of SEQ 10 ID NO: 128 and a polypeptide comprising the amino acid sequence of SEQ ID NO: 129.

### **Fc domain**

In particular embodiments, the bispecific antigen binding molecule of the invention comprises an 15 Fc domain composed of a first and a second subunit. It is understood, that the features of the Fc domain described herein in relation to the bispecific antigen binding molecule can equally apply to an Fc domain comprised in an antibody of the invention.

The Fc domain of the bispecific antigen binding molecule consists of a pair of polypeptide chains comprising heavy chain domains of an immunoglobulin molecule. For example, the Fc 20 domain of an immunoglobulin G (IgG) molecule is a dimer, each subunit of which comprises the CH2 and CH3 IgG heavy chain constant domains. The two subunits of the Fc domain are capable of stable association with each other. In one embodiment, the bispecific antigen binding molecule of the invention comprises not more than one Fc domain.

In one embodiment, the Fc domain of the bispecific antigen binding molecule is an IgG Fc 25 domain. In a particular embodiment, the Fc domain is an IgGi Fc domain. In another embodiment the Fc domain is an IgG<sub>4</sub> Fc domain. In a more specific embodiment, the Fc domain is an IgG<sub>4</sub> Fc domain comprising an amino acid substitution at position S228 (Kabat EU index numbering), particularly the amino acid substitution S228P. This amino acid substitution reduces *in vivo* Fab arm exchange of IgG<sub>4</sub> antibodies (see Stubenrauch et al., Drug Metabolism and 30 Disposition 38, 84-91 (2010)). In a further particular embodiment, the Fc domain is a human Fc domain. In an even more particular embodiment, the Fc domain is a human IgGi Fc domain. An exemplary sequence of a human IgGi Fc region is given in SEQ ID NO: 109.

### Fc domain modifications promoting heterodimerization

Bispecific antigen binding molecules according to the invention comprise different antigen binding moieties, which may be fused to one or the other of the two subunits of the Fc domain, thus the two subunits of the Fc domain are typically comprised in two non-identical polypeptide chains. Recombinant co-expression of these polypeptides and subsequent dimerization leads to 5 several possible combinations of the two polypeptides. To improve the yield and purity of bispecific antigen binding molecules in recombinant production, it will thus be advantageous to introduce in the Fc domain of the bispecific antigen binding molecule a modification promoting the association of the desired polypeptides.

Accordingly, in particular embodiments, the Fc domain of the bispecific antigen binding 10 molecule according to the invention comprises a modification promoting the association of the first and the second subunit of the Fc domain. The site of most extensive protein-protein

interaction between the two subunits of a human IgG Fc domain is in the CH3 domain of the Fc domain. Thus, in one embodiment said modification is in the CH3 domain of the Fc domain.

There exist several approaches for modifications in the CH3 domain of the Fc domain in order to 15 enforce heterodimerization, which are well described e.g. in WO 96/27011, WO 98/050431, EP 1870459, WO 2007/110205, WO 2007/147901, WO 2009/089004, WO 2010/129304,

WO 2011/90754, WO 2011/143545, WO 2012058768, WO 2013157954, WO 2013096291.

Typically, in all such approaches the CH3 domain of the first subunit of the Fc domain and the CH3 domain of the second subunit of the Fc domain are both engineered in a complementary

20 manner so that each CH3 domain (or the heavy chain comprising it) can no longer homodimerize with itself but is forced to heterodimerize with the complementarily engineered other CH3 domain (so that the first and second CH3 domain heterodimerize and no homodimers between the two first or the two second CH3 domains are formed). These different approaches for improved heavy chain heterodimerization are contemplated as different alternatives in combination with

25 the heavy-light chain modifications (e.g. VH and VL exchange/replacement in one binding arm and the introduction of substitutions of charged amino acids with opposite charges in the CH1/CL interface) in the bispecific antigen binding molecule which reduce heavy/light chain mispairing and Bence Jones-type side products.

In a specific embodiment said modification promoting the association of the first and the second 30 subunit of the Fc domain is a so-called “knob-into-hole” modification, comprising a “knob” modification in one of the two subunits of the Fc domain and a “hole” modification in the other one of the two subunits of the Fc domain.

The knob-into-hole technology is described e.g. in US 5,731,168; US 7,695,936; Ridgway et al., Prot Eng 9, 617-621 (1996) and Carter, J Immunol Meth 248, 7-15 (2001). Generally, the method involves introducing a protuberance (“knob”) at the interface of a first polypeptide and a corresponding cavity (“hole”) in the interface of a second polypeptide, such that the 5 protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones 10 (e.g. alanine or threonine).

Accordingly, in a particular embodiment, in the CH3 domain of the first subunit of the Fc domain of the bispecific antigen binding molecule an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which is positionable in a cavity within the CH3 domain of 15 the second subunit, and in the CH3 domain of the second subunit of the Fc domain an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit is positionable.

Preferably said amino acid residue having a larger side chain volume is selected from the group 20 consisting of arginine (R), phenylalanine (F), tyrosine (Y), and tryptophan (W).

Preferably said amino acid residue having a smaller side chain volume is selected from the group consisting of alanine (A), serine (S), threonine (T), and valine (V).

The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis.

25 In a specific embodiment, in (the CH3 domain of) the first subunit of the Fc domain (the “knobs” subunit) the threonine residue at position 366 is replaced with a tryptophan residue (T366W), and in (the CH3 domain of) the second subunit of the Fc domain (the “hole” subunit) the tyrosine residue at position 407 is replaced with a valine residue (Y407V). In one embodiment, in the second subunit of the Fc domain additionally the threonine residue at position 366 is replaced 30 with a serine residue (T366S) and the leucine residue at position 368 is replaced with an alanine residue (F368A) (numberings according to Rabat EU index).

In yet a further embodiment, in the first subunit of the Fc domain additionally the serine residue at position 354 is replaced with a cysteine residue (S354C) or the glutamic acid residue at

position 356 is replaced with a cysteine residue (E356C) (particularly the serine residue at position 354 is replaced with a cysteine residue), and in the second subunit of the Fc domain additionally the tyrosine residue at position 349 is replaced by a cysteine residue (Y349C) (numberings according to Kabat EU index). Introduction of these two cysteine residues results in

5 formation of a disulfide bridge between the two subunits of the Fc domain, further stabilizing the dimer (Carter, J Immunol Methods 248, 7-15 (2001)).

In a particular embodiment, the first subunit of the Fc domain comprises the amino acid substitutions S354C and T366W, and the second subunit of the Fc domain comprises the amino acid substitutions Y349C, T366S, F368A and Y407V (numbering according to Kabat EU

10 index).

In a particular embodiment the antigen binding moiety that binds to the second antigen (e.g. an activating T cell antigen) is fused (optionally via the first antigen binding moiety, which binds to HFA-A2/WT1, and/or a peptide linker) to the first subunit of the Fc domain (comprising the “knob” modification). Without wishing to be bound by theory, fusion of the antigen binding

15 moiety that binds a second antigen, such as an activating T cell antigen, to the knob-containing subunit of the Fc domain will (further) minimize the generation of antigen binding molecules comprising two antigen binding moieties that bind to an activating T cell antigen (steric clash of two knob-containing polypeptides).

Other techniques of CH3-modification for enforcing the heterodimerization are contemplated as

20 alternatives according to the invention and are described e.g. in WO 96/27011, WO 98/050431, EP 1870459, WO 2007/110205, WO 2007/147901, WO 2009/089004, WO 2010/129304, WO 2011/90754, WO 2011/143545, WO 2012/058768, WO 2013/157954, WO 2013/096291.

In one embodiment, the heterodimerization approach described in EP 1870459, is used alternatively. This approach is based on the introduction of charged amino acids with opposite

25 charges at specific amino acid positions in the CH3/CH3 domain interface between the two subunits of the Fc domain. One preferred embodiment for the bispecific antigen binding molecule of the invention are amino acid mutations R409D; K370E in one of the two CH3 domains (of the Fc domain) and amino acid mutations D399K; E357K in the other one of the CH3 domains of the Fc domain (numbering according to Kabat EU index).

30 In another embodiment, the bispecific antigen binding molecule of the invention comprises amino acid mutation T366W in the CH3 domain of the first subunit of the Fc domain and amino acid mutations T366S, F368A, Y407V in the CH3 domain of the second subunit of the Fc domain, and additionally amino acid mutations R409D; K370E in the CH3 domain of the first

subunit of the Fc domain and amino acid mutations D399K; E357K in the CH3 domain of the second subunit of the Fc domain (numberings according to Kabat EU index).

In another embodiment, the bispecific antigen binding molecule of the invention comprises amino acid mutations S354C, T366W in the CH3 domain of the first subunit of the Fc domain

5 and amino acid mutations Y349C, T366S, L368A, Y407V in the CH3 domain of the second subunit of the Fc domain, or said bispecific antigen binding molecule comprises amino acid mutations Y349C, T366W in the CH3 domain of the first subunit of the Fc domain and amino acid mutations S354C, T366S, F368A, Y407V in the CH3 domains of the second subunit of the Fc domain and additionally amino acid mutations R409D; K370E in the CH3 domain of the first

10 subunit of the Fc domain and amino acid mutations D399K; E357K in the CH3 domain of the second subunit of the Fc domain (all numberings according to Kabat EU index).

In one embodiment, the heterodimerization approach described in WO 2013/157953 is used alternatively. In one embodiment, a first CH3 domain comprises amino acid mutation T366K and a second CH3 domain comprises amino acid mutation F351D (numberings according to

15 Kabat EU index). In a further embodiment, the first CH3 domain comprises further amino acid mutation F351K. In a further embodiment, the second CH3 domain comprises further an amino acid mutation selected from Y349E, Y349D and F368E (preferably F368E) (numberings according to Kabat EU index).

In one embodiment, the heterodimerization approach described in WO 2012/058768 is used

20 alternatively. In one embodiment a first CH3 domain comprises amino acid mutations F351Y, Y407A and a second CH3 domain comprises amino acid mutations T366A, K409F. In a further embodiment the second CH3 domain comprises a further amino acid mutation at position T411, D399, S400, F405, N390, or K392, e.g. selected from a) T411N, T411R, T411Q, T411K, T411D, T411E or T411W, b) D399R, D399W, D399Y or D399K, c) S400E, S400D, S400R, or 25 S400K, d) F405I, F405M, F405T, F405S, F405V or F405W, e) N390R, N390K or N390D, f) K392V, K392M, K392R, K392F, K392F or K392E (numberings according to Kabat EU index).

In a further embodiment a first CH3 domain comprises amino acid mutations L351Y, Y407A and a second CH3 domain comprises amino acid mutations T366V, K409F. In a further embodiment, a first CH3 domain comprises amino acid mutation Y407A and a second CH3

30 domain comprises amino acid mutations T366A, K409F. In a further embodiment, the second CH3 domain further comprises amino acid mutations K392E, T411E, D399R and S400R (numberings according to Kabat EU index).

In one embodiment, the heterodimerization approach described in WO 2011/143545 is used alternatively, e.g. with the amino acid modification at a position selected from the group consisting of 368 and 409 (numbering according to Kabat EU index).

In one embodiment, the heterodimerization approach described in WO 2011/090762, which also

5 uses the knobs-into-holes technology described above, is used alternatively. In one embodiment a first CH3 domain comprises amino acid mutation T366W and a second CH3 domain comprises amino acid mutation Y407A. In one embodiment, a first CH3 domain comprises amino acid mutation T366Y and a second CH3 domain comprises amino acid mutation Y407T (numberings according to Kabat EU index).

10 In one embodiment, the bispecific antigen binding molecule or its Fc domain is of IgG<sub>2</sub> subclass and the heterodimerization approach described in WO 2010/129304 is used alternatively.

In an alternative embodiment, a modification promoting association of the first and the second subunit of the Fc domain comprises a modification mediating electrostatic steering effects, e.g. as described in PCT publication WO 2009/089004. Generally, this method involves replacement

15 of one or more amino acid residues at the interface of the two Fc domain subunits by charged amino acid residues so that homodimer formation becomes electrostatically unfavorable but heterodimerization electrostatically favorable. In one such embodiment, a first CH3 domain comprises amino acid substitution of K392 or N392 with a negatively charged amino acid (e.g. glutamic acid (E), or aspartic acid (D), preferably K392D or N392D) and a second CH3 domain

20 comprises amino acid substitution of D399, E356, D356, or E357 with a positively charged amino acid (e.g. lysine (K) or arginine (R), preferably D399K, E356K, D356K, or E357K, and more preferably D399K and E356K). In a further embodiment, the first CH3 domain further comprises amino acid substitution of K409 or R409 with a negatively charged amino acid (e.g. glutamic acid (E), or aspartic acid (D), preferably K409D or R409D). In a further embodiment

25 the first CH3 domain further or alternatively comprises amino acid substitution of K439 and/or K370 with a negatively charged amino acid (e.g. glutamic acid (E), or aspartic acid (D)) (all numberings according to Kabat EU index).

In yet a further embodiment, the heterodimerization approach described in WO 2007/147901 is used alternatively. In one embodiment, a first CH3 domain comprises amino acid mutations

30 K253E, D282K, and K322D and a second CH3 domain comprises amino acid mutations D239K, E240K, and K292D (numberings according to Kabat EU index).

In still another embodiment, the heterodimerization approach described in WO 2007/110205 can be used alternatively.

In one embodiment, the first subunit of the Fc domain comprises amino acid substitutions K392D and K409D, and the second subunit of the Fc domain comprises amino acid substitutions D356K and D399K (numbering according to Kabat EU index).

Fc domain modifications reducing Fc receptor binding and/or effector function

5 The Fc domain confers to the bispecific antigen binding molecule (or the antibody) favorable pharmacokinetic properties, including a long serum half-life which contributes to good accumulation in the target tissue and a favorable tissue-blood distribution ratio. At the same time it may, however, lead to undesirable targeting of the bispecific antigen binding molecule (or the antibody) to cells expressing Fc receptors rather than to the preferred antigen-bearing cells.

10 Moreover, the co-activation of Fc receptor signaling pathways may lead to cytokine release which, in combination with the T cell activating properties (e.g. in embodiments of the bispecific antigen binding molecule wherein the second antigen binding moiety binds to an activating T cell antigen) and the long half-life of the bispecific antigen binding molecule, results in excessive activation of cytokine receptors and severe side effects upon systemic administration. Activation

15 of (Fc receptor-bearing) immune cells other than T cells may even reduce efficacy of the bispecific antigen binding molecule (particularly a bispecific antigen binding molecule wherein the second antigen binding moiety binds to an activating T cell antigen) due to the potential destruction of T cells e.g. by NK cells.

Accordingly, in particular embodiments, the Fc domain of the bispecific antigen binding molecule according to the invention exhibits reduced binding affinity to an Fc receptor and/or reduced effector function, as compared to a native IgGi Fc domain. In one such embodiment the Fc domain (or the bispecific antigen binding molecule comprising said Fc domain) exhibits less than 50%, preferably less than 20%, more preferably less than 10% and most preferably less than 5% of the binding affinity to an Fc receptor, as compared to a native IgGi Fc domain (or a bispecific antigen binding molecule comprising a native IgGi Fc domain), and/or less than 50%, preferably less than 20%, more preferably less than 10% and most preferably less than 5% of the effector function, as compared to a native IgGi Fc domain (or a bispecific antigen binding molecule comprising a native IgGi Fc domain). In one embodiment, the Fc domain (or the bispecific antigen binding molecule comprising said Fc domain) does not substantially bind to an Fc receptor and/or induce effector function. In a particular embodiment the Fc receptor is an Fey receptor. In one embodiment the Fc receptor is a human Fc receptor. In one embodiment the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc

receptor is an activating human Fey receptor, more specifically human FcyRIIIa, FcyRI or FcyRIIa, most specifically human FcyRIIIa. In one embodiment the effector function is one or more selected from the group of CDC, ADCC, ADCP, and cytokine secretion. In a particular embodiment, the effector function is ADCC. In one embodiment, the Fc domain domain exhibits 5 substantially similar binding affinity to neonatal Fc receptor (FcRn), as compared to a native IgGi Fc domain domain. Substantially similar binding to FcRn is achieved when the Fc domain (or the bispecific antigen binding molecule comprising said Fc domain) exhibits greater than about 70%, particularly greater than about 80%, more particularly greater than about 90% of the binding affinity of a native IgGi Fc domain (or the bispecific antigen binding molecule 10 comprising a native IgGi Fc domain) to FcRn.

In certain embodiments the Fc domain is engineered to have reduced binding affinity to an Fc receptor and/or reduced effector function, as compared to a non-engineered Fc domain. In particular embodiments, the Fc domain of the bispecific antigen binding molecule comprises one or more amino acid mutation that reduces the binding affinity of the Fc domain to an Fc receptor 15 and/or effector function. Typically, the same one or more amino acid mutation is present in each of the two subunits of the Fc domain. In one embodiment, the amino acid mutation reduces the binding affinity of the Fc domain to an Fc receptor. In one embodiment, the amino acid mutation reduces the binding affinity of the Fc domain to an Fc receptor by at least 2-fold, at least 5-fold, or at least 10-fold. In embodiments where there is more than one amino acid mutation that 20 reduces the binding affinity of the Fc domain to the Fc receptor, the combination of these amino acid mutations may reduce the binding affinity of the Fc domain to an Fc receptor by at least 10-fold, at least 20-fold, or even at least 50-fold. In one embodiment the bispecific antigen binding molecule comprising an engineered Fc domain exhibits less than 20%, particularly less than 10%, more particularly less than 5% of the binding affinity to an Fc receptor as compared to a 25 bispecific antigen binding molecule comprising a non-engineered Fc domain. In a particular embodiment, the Fc receptor is an Fey receptor. In some embodiments, the Fc receptor is a human Fc receptor. In some embodiments, the Fc receptor is an activating Fc receptor. In a specific embodiment, the Fc receptor is an activating human Fey receptor, more specifically human FcyRIIIa, FcyRI or FcyRIIa, most specifically human FcyRIIIa. Preferably, binding to 30 each of these receptors is reduced. In some embodiments, binding affinity to a complement component, specifically binding affinity to Clq, is also reduced. In one embodiment, binding affinity to neonatal Fc receptor (FcRn) is not reduced. Substantially similar binding to FcRn, i.e. preservation of the binding affinity of the Fc domain to said receptor, is achieved when the Fc

domain (or the bispecific antigen binding molecule comprising said Fc domain) exhibits greater than about 70% of the binding affinity of a non-engineered form of the Fc domain (or the bispecific antigen binding molecule comprising said non-engineered form of the Fc domain) to FcRn. The Fc domain, or bispecific antigen binding molecules of the invention comprising said

5 Fc domain, may exhibit greater than about 80% and even greater than about 90% of such affinity. In certain embodiments, the Fc domain of the bispecific antigen binding molecule is engineered to have reduced effector function, as compared to a non-engineered Fc domain. The reduced effector function can include, but is not limited to, one or more of the following: reduced complement dependent cytotoxicity (CDC), reduced antibody-dependent cell-mediated  
10 cytotoxicity (ADCC), reduced antibody-dependent cellular phagocytosis (ADCP), reduced cytokine secretion, reduced immune complex-mediated antigen uptake by antigen-presenting cells, reduced binding to NK cells, reduced binding to macrophages, reduced binding to monocytes, reduced binding to polymorphonuclear cells, reduced direct signaling inducing apoptosis, reduced crosslinking of target-bound antibodies, reduced dendritic cell maturation, or  
15 reduced T cell priming. In one embodiment, the reduced effector function is one or more selected from the group of reduced CDC, reduced ADCC, reduced ADCP, and reduced cytokine secretion. In a particular embodiment, the reduced effector function is reduced ADCC. In one embodiment the reduced ADCC is less than 20% of the ADCC induced by a non-engineered Fc domain (or a bispecific antigen binding molecule comprising a non-engineered Fc domain).  
20 In one embodiment, the amino acid mutation that reduces the binding affinity of the Fc domain to an Fc receptor and/or effector function is an amino acid substitution. In one embodiment, the Fc domain comprises an amino acid substitution at a position selected from the group of E233, L234, L235, N297, P331 and P329 (numberings according to Kabat EU index). In a more specific embodiment, the Fc domain comprises an amino acid substitution at a position selected  
25 from the group of L234, L235 and P329 (numberings according to Kabat EU index). In some embodiments, the Fc domain comprises the amino acid substitutions L234A and L235A (numberings according to Kabat EU index). In one such embodiment, the Fc domain is an IgGi Fc domain, particularly a human IgGi Fc domain. In one embodiment, the Fc domain comprises an amino acid substitution at position P329. In a more specific embodiment, the amino acid substitution is P329A or P329G, particularly P329G (numberings according to Kabat EU index).  
30 In one embodiment, the Fc domain comprises an amino acid substitution at position P329 and a further amino acid substitution at a position selected from E233, F234, F235, N297 and P331 (numberings according to Kabat EU index). In a more specific embodiment, the further amino

acid substitution is E233P, L234A, L235A, L235E, N297A, N297D or P331S. In particular embodiments, the Fc domain comprises amino acid substitutions at positions P329, L234 and L235 (numberings according to Kabat EU index). In more particular embodiments, the Fc domain comprises the amino acid mutations L234A, L235A and P329G (“P329G LALA”, 5 “PGLALA” or “LALAPG”). Specifically, in particular embodiments, each subunit of the Fc domain comprises the amino acid substitutions L234A, L235A and P329G (Kabat EU index numbering), i.e. in each of the first and the second subunit of the Fc domain the leucine residue at position 234 is replaced with an alanine residue (L234A), the leucine residue at position 235 is replaced with an alanine residue (L235A) and the proline residue at position 329 is replaced by a 10 glycine residue (P329G) (numbering according to Kabat EU index).

. In one such embodiment, the Fc domain is an IgGi Fc domain, particularly a human IgGi Fc domain. The “P329G LALA” combination of amino acid substitutions almost completely abolishes Fey receptor (as well as complement) binding of a human IgGi Fc domain, as described in PCT publication no. WO 2012/130831, which is incorporated herein by reference in 15 its entirety. WO 2012/130831 also describes methods of preparing such mutant Fc domains and methods for determining its properties such as Fc receptor binding or effector functions.

IgG<sub>4</sub> antibodies exhibit reduced binding affinity to Fc receptors and reduced effector functions as compared to IgGi antibodies. Hence, in some embodiments, the Fc domain of the bispecific antigen binding molecules of the invention is an IgG<sub>4</sub> Fc domain, particularly a human IgG<sub>4</sub> Fc 20 domain. In one embodiment, the IgG<sub>4</sub> Fc domain comprises amino acid substitutions at position S228, specifically the amino acid substitution S228P (numberings according to Kabat EU index). To further reduce its binding affinity to an Fc receptor and/or its effector function, in one embodiment, the IgG<sub>4</sub> Fc domain comprises an amino acid substitution at position F235, specifically the amino acid substitution F235E (numberings according to Kabat EU index). In 25 another embodiment, the IgG<sub>4</sub> Fc domain comprises an amino acid substitution at position P329, specifically the amino acid substitution P329G (numberings according to Kabat EU index). In a particular embodiment, the IgG<sub>4</sub> Fc domain comprises amino acid substitutions at positions S228, F235 and P329, specifically amino acid substitutions S228P, F235E and P329G (numberings according to Kabat EU index). Such IgG<sub>4</sub> Fc domain mutants and their Fey receptor binding 30 properties are described in PCT publication no. WO 2012/130831, incorporated herein by reference in its entirety.

In a particular embodiment, the Fc domain exhibiting reduced binding affinity to an Fc receptor and/or reduced effector function, as compared to a native IgGi Fc domain, is a human IgGi Fc

domain comprising the amino acid substitutions L234A, L235A and optionally P329G, or a human IgG<sub>4</sub> Fc domain comprising the amino acid substitutions S228P, L235E and optionally P329G (numberings according to Kabat EU index).

In certain embodiments, N-glycosylation of the Fc domain has been eliminated. In one such embodiment, the Fc domain comprises an amino acid mutation at position N297, particularly an amino acid substitution replacing asparagine by alanine (N297A) or aspartic acid (N297D) (numberings according to Kabat EU index).

In addition to the Fc domains described hereinabove and in PCT publication no. WO 2012/130831, Fc domains with reduced Fc receptor binding and/or effector function also include

10 those with substitution of one or more of Fc domain residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056) (numberings according to Kabat EU index). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called “DANA” Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

15 Mutant Fc domains can be prepared by amino acid deletion, substitution, insertion or modification using genetic or chemical methods well known in the art. Genetic methods may include site-specific mutagenesis of the encoding DNA sequence, PCR, gene synthesis, and the like. The correct nucleotide changes can be verified for example by sequencing.

Binding to Fc receptors can be easily determined e.g. by EFISA, or by Surface Plasmon 20 Resonance (SPR) using standard instrumentation such as a BIACore instrument (GE Healthcare), and Fc receptors such as may be obtained by recombinant expression. Alternatively, binding affinity of Fc domains or bispecific antigen binding molecules comprising an Fc domain for Fc receptors may be evaluated using cell lines known to express particular Fc receptors, such as human NK cells expressing Fcyllla receptor.

25 Effector function of an Fc domain, or a bispecific antigen binding molecule comprising an Fc domain, can be measured by methods known in the art. Examples of *in vitro* assays to assess ADCC activity of a molecule of interest are described in U.S. Patent No. 5,500,362; Hellstrom et al. Proc Natl Acad Sci USA 83, 7059-7063 (1986) and Hellstrom et al., Proc Natl Acad Sci USA 82, 1499-1502 (1985); U.S. Patent No. 5,821,337; Bruggemann et al., J Exp Med 166, 1351-30 1361 (1987). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTITM non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA); and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI)). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and

Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g. in a animal model such as that disclosed in Clynes et al., Proc Natl Acad Sci USA 95, 652-656 (1998).

In some embodiments, binding of the Fc domain to a complement component, specifically to 5 Clq, is reduced. Accordingly, in some embodiments wherein the Fc domain is engineered to have reduced effector function, said reduced effector function includes reduced CDC. Clq binding assays may be carried out to determine whether the Fc domain, or the bispecific antigen binding molecule comprising the Fc domain, is able to bind Clq and hence has CDC activity.

See e.g., Clq and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess 10 complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J Immunol Methods 202, 163 (1996); Cragg et al., Blood 101, 1045-1052 (2003); and Cragg and Glennie, Blood 103, 2738-2743 (2004)).

FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., *Int'l. Immunol.* 18(12): 1759-1769 (2006); WO 15 2013/120929).

## Polynucleotides

The invention further provides isolated polynucleotides encoding an antibody or bispecific antigen binding molecule as described herein or a fragment thereof. In some embodiments, said 20 fragment is an antigen binding fragment.

The polynucleotides encoding antibodies or bispecific antigen binding molecules of the invention may be expressed as a single polynucleotide that encodes the entire antibody or bispecific antigen binding molecule or as multiple (e.g., two or more) polynucleotides that are co-expressed. Polypeptides encoded by polynucleotides that are co-expressed may associate 25 through, e.g., disulfide bonds or other means to form a functional antibody or bispecific antigen binding molecule. For example, the light chain portion of an antibody or bispecific antigen binding molecule may be encoded by a separate polynucleotide from the portion of the antibody or bispecific antigen binding molecule comprising the heavy chain of the antibody or bispecific antigen binding molecule. When co-expressed, the heavy chain polypeptides will associate with 30 the light chain polypeptides to form the antibody or bispecific antigen binding molecule. In another example, the portion of the antibody or bispecific antigen binding molecule comprising one of the two Fc domain subunits and optionally (part of) one or more Fab molecules could be encoded by a separate polynucleotide from the portion of the antibody or bispecific antigen

binding molecule comprising the other of the two Fc domain subunits and optionally (part of) a Fab molecule. When co-expressed, the Fc domain subunits will associate to form the Fc domain.

In some embodiments, the isolated polynucleotide encodes the entire antibody or bispecific antigen binding molecule according to the invention as described herein. In other embodiments, the isolated polynucleotide encodes a polypeptide comprised in the antibody or bispecific antigen binding molecule according to the invention as described herein.

In certain embodiments the polynucleotide or nucleic acid is DNA. In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA). RNA of the present invention may be single stranded or double stranded.

### **Recombinant Methods**

Antibodies or bispecific antigen binding molecules of the invention may be obtained, for example, by solid-state peptide synthesis (e.g. Merrifield solid phase synthesis) or recombinant production. For recombinant production one or more polynucleotide encoding the antibody or bispecific antigen binding molecule (fragment), e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such polynucleotide may be readily isolated and sequenced using conventional procedures. In one embodiment a vector, preferably an expression vector, comprising one or more of the polynucleotides of the invention is provided. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of an antibody or bispecific antigen binding molecule (fragment) along with appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, N.Y. (1989); and Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, N.Y (1989). The expression vector can be part of a plasmid, virus, or may be a nucleic acid fragment. The expression vector includes an expression cassette into which the polynucleotide encoding the antibody or bispecific antigen binding molecule (fragment) (i.e. the coding region) is cloned in operable association with a promoter and/or other transcription or translation control elements. As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a

coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' untranslated regions, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g. on a single vector, or in separate polynucleotide constructs, e.g. on separate 5 (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, e.g. a vector of the present invention may encode one or more polypeptides, which are post- or co-translationally separated into the final proteins via proteolytic cleavage. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions, either fused or unfused to a polynucleotide encoding the 10 antibody or bispecific antigen binding molecule (fragment) of the invention, or variant or derivative thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain. An operable association is when a coding region for a gene product, e.g. a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the 15 influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with 20 the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription 25 termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein. A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions, which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (e.g. the immediate 30 early promoter, in conjunction with intron-A), simian virus 40 (e.g. the early promoter), and retroviruses (such as, e.g. Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit  $\beta$ -globin, as well as other sequences capable of controlling gene expression in eukaryotic

cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as inducible promoters (e.g. promoters inducible tetracyclins). Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination

5 codons, and elements derived from viral systems (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence). The expression cassette may also include other features such as an origin of replication, and/or chromosome integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal repeats (ITRs).

10 Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention. For example, if secretion of the antibody or bispecific antigen binding molecule is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding an antibody or bispecific antigen

15 binding molecule of the invention or a fragment thereof. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus

20 of the polypeptide, which is cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, e.g. an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse  $\beta$ -glucuronidase.

25 DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the antibody or bispecific antigen binding molecule may be included within or at the ends of the antibody or bispecific antigen binding molecule (fragment) 30 encoding polynucleotide.

In a further embodiment, a host cell comprising one or more polynucleotides of the invention is provided. In certain embodiments a host cell comprising one or more vectors of the invention is

provided. The polynucleotides and vectors may incorporate any of the features, singly or in combination, described herein in relation to polynucleotides and vectors, respectively. In one such embodiment a host cell comprises (e.g. has been transformed or transfected with) one or more vector comprising one or more polynucleotide that encodes (part of) an antibody or

5 bispecific antigen binding molecule of the invention. As used herein, the term "host cell" refers to any kind of cellular system which can be engineered to generate the antibody or bispecific antigen binding molecule of the invention or fragments thereof. Host cells suitable for replicating and for supporting expression of antibodies or bispecific antigen binding molecules are well known in the art. Such cells may be transfected or transduced as appropriate with the particular  
10 expression vector and large quantities of vector containing cells can be grown for seeding large scale fermenters to obtain sufficient quantities of the antibody or bispecific antigen binding molecule for clinical applications. Suitable host cells include prokaryotic microorganisms, such as *E. coli*, or various eukaryotic cells, such as Chinese hamster ovary cells (CHO), insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation  
15 is not needed. After expression, the polypeptide may be isolated from the bacterial cell paste in a soluble fraction and can be further purified. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized", resulting in the production of a polypeptide with a partially or fully human glycosylation pattern.

20 See Gerngross, *Nat Biotech* 22, 1409-1414 (2004), and Li et al., *Nat Biotech* 24, 210-215 (2006). Suitable host cells for the expression of (glycosylated) polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant  
25 cell cultures can also be utilized as hosts. See e.g. US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7);  
30 human embryonic kidney line (293 or 293T cells as described, e.g., in Graham et al., *J Gen Virol* 36, 59 (1977)), baby hamster kidney cells (BHK), mouse sertoli cells (TM4 cells as described, e.g., in Mather, *Biol Reprod* 23, 243-251 (1980)), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HELA), canine kidney cells

(MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumor cells (MMT 060562), TRI cells (as described, e.g., in Mather et al., Annals N.Y. Acad Sci 383, 44-68 (1982)), MRC 5 cells, and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including dhfr<sup>-</sup> CHO cells (Urlaub et 5 al., Proc Natl Acad Sci USA 77, 4216 (1980)); and myeloma cell lines such as YO, NS0, P3X63 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki and Wu, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003). Host cells include cultured cells, e.g., mammalian cultured 10 cells, yeast cells, insect cells, bacterial cells and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is a eukaryotic cell, preferably a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., Y0, NS0, Sp20 cell).

Standard technologies are known in the art to express foreign genes in these systems. Cells 15 expressing a polypeptide comprising either the heavy or the light chain of an antigen binding domain such as an antibody, may be engineered so as to also express the other of the antibody chains such that the expressed product is an antibody that has both a heavy and a light chain.

In one embodiment, a method of producing an antibody or bispecific antigen binding molecule according to the invention is provided, wherein the method comprises culturing a host cell 20 comprising a polynucleotide encoding the antibody or bispecific antigen binding molecule, as provided herein, under conditions suitable for expression of the antibody or bispecific antigen binding molecule, and optionally recovering the antibody or bispecific antigen binding molecule from the host cell (or host cell culture medium).

The components of the bispecific antigen binding molecule (or the antibody) of the invention 25 may be genetically fused to each other. The bispecific antigen binding molecule can be designed such that its components are fused directly to each other or indirectly through a linker sequence. The composition and length of the linker may be determined in accordance with methods well known in the art and may be tested for efficacy. Examples of linker sequences between different components of bispecific antigen binding molecules are provided herein. Additional sequences 30 may also be included to incorporate a cleavage site to separate the individual components of the fusion if desired, for example an endopeptidase recognition sequence.

The antibody or bispecific antigen binding molecule of the invention generally comprise at least an antibody variable region capable of binding an antigenic determinant. Variable regions can

form part of and be derived from naturally or non-naturally occurring antibodies and fragments thereof. Methods to produce polyclonal antibodies and monoclonal antibodies are well known in the art (see e.g. Harlow and Lane, "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory, 1988). Non-naturally occurring antibodies can be constructed using solid phase-peptide synthesis, can be produced recombinantly (e.g. as described in U.S. patent No. 4,186,567) or can be obtained, for example, by screening combinatorial libraries comprising variable heavy chains and variable light chains (see e.g. U.S. Patent. No. 5,969,108 to McCafferty).

Any animal species of antibody, antibody fragment, antigen binding domain or variable region may be used in the antibody or bispecific antigen binding molecule of the invention. Non-

10 limiting antibodies, antibody fragments, antigen binding domains or variable regions useful in the present invention can be of murine, primate, or human origin. If the antibody or bispecific antigen binding molecule is intended for human use, a chimeric form of antibody may be used

wherein the constant regions of the antibody are from a human. A humanized or fully human form of the antibody can also be prepared in accordance with methods well known in the art (see

15 e. g. U.S. Patent No. 5,565,332 to Winter). Humanization may be achieved by various methods including, but not limited to (a) grafting the non-human (e.g., donor antibody) CDRs onto human (e.g. recipient antibody) framework and constant regions with or without retention of critical framework residues (e.g. those that are important for retaining good antigen binding affinity or antibody functions), (b) grafting only the non-human specificity-determining regions (SDRs or

20 a-CDRs; the residues critical for the antibody-antigen interaction) onto human framework and constant regions, or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332:323-329 (1988);

25 Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri et al., *Methods* 36:25-34 (2005) (describing specificity determining region (SDR) grafting); Padlan, *Mol. Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*,

30 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling). Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular

subgroup of light or heavy chain variable regions (see, e.g., Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr Opin Pharmacol* 5, 368-74 (2001) and Lonberg, *Curr Opin Immunol* 20, 450-459 (2008). Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyxue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein,

*Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3): 185-91 (2005).

Human antibodies may also be generated by isolation from human antibody libraries, as described herein.

5 Antibodies useful in the invention may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. Methods for screening combinatorial libraries are reviewed, e.g., in Lerner et al. in *Nature Reviews* 16:498-508 (2016). For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are  
10 reviewed, e.g., in Frenzel et al. in *mAbs* 8:1177-1194 (2016); Bazan et al. in *Human Vaccines and Immunotherapeutics* 8:1817-1828 (2012) and Zhao et al. in *Critical Reviews in Biotechnology* 36:276-289 (2016) as well as in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and in Marks and Bradbury in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ,  
15 2003).

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al. in *Annual Review of Immunology* 12: 433-455 (1994). Phage typically display antibody fragments, either as single-  
20 chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al. in *EMBO Journal* 12: 725-734 (1993). Finally, naive libraries can  
25 also be made synthetically by cloning unarranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter in *Journal of Molecular Biology* 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent Nos. 5,750,373; 7,985,840; 7,785,903 and 8,679,490 as  
30 well as US Patent Publication Nos. 2005/0079574, 2007/0117126, 2007/0237764 and 2007/0292936. Further examples of methods known in the art for screening combinatorial libraries for antibodies with a desired activity or activities include ribosome and mRNA display, as well as methods for antibody display and selection on bacteria, mammalian cells, insect cells

or yeast cells. Methods for yeast surface display are reviewed, e.g., in Scholler et al. in *Methods in Molecular Biology* 503:135-56 (2012) and in Cherf et al. in *Methods in Molecular Biology* 1319:155-175 (2015) as well as in the Zhao et al. in *Methods in Molecular Biology* 889:73-84 (2012). Methods for ribosome display are described, e.g., in He et al. in *Nucleic Acids Research*

5 25:5132-5134 (1997) and in Hanes et al. in *PNAS* 94:4937-4942 (1997).

Antibodies or bispecific antigen binding molecules prepared as described herein may be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography, size exclusion chromatography, and the like. The actual conditions used to purify a particular protein will depend, in part, on 10 factors such as net charge, hydrophobicity, hydrophilicity etc., and will be apparent to those having skill in the art. For affinity chromatography purification, an antibody, ligand, receptor or antigen can be used to which the antibody or bispecific antigen binding molecule binds. For example, for affinity chromatography purification of antibodies or bispecific antigen binding molecules of the invention, a matrix with protein A or protein G may be used. Sequential Protein 15 A or G affinity chromatography and size exclusion chromatography can be used to isolate an antibody or bispecific antigen binding molecule essentially as described in the Examples. The purity of the antibody or bispecific antigen binding molecule can be determined by any of a variety of well known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like.

20

### **Compositions, Formulations, and Routes of Administration**

In a further aspect, the invention provides pharmaceutical compositions comprising any of the antibodies or bispecific antigen binding molecules provided herein, e.g., for use in any of the below therapeutic methods. In one embodiment, a pharmaceutical composition comprises any of 25 the antibodies or bispecific antigen binding molecules provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises any of the antibodies or bispecific antigen binding molecules provided herein and at least one additional therapeutic agent, e.g., as described below.

Further provided is a method of producing an antibody or bispecific antigen binding molecule of 30 the invention in a form suitable for administration in vivo, the method comprising (a) obtaining an antibody or bispecific antigen binding molecule according to the invention, and (b) formulating the antibody or bispecific antigen binding molecule with at least one

pharmaceutically acceptable carrier, whereby a preparation of antibody or bispecific antigen binding molecule is formulated for administration in vivo.

Pharmaceutical compositions of the present invention comprise a therapeutically effective amount of antibody or bispecific antigen binding molecule dissolved or dispersed in a

5 pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that are generally non-toxic to recipients at the dosages and concentrations employed, i.e. do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that contains an antibody or  
10 bispecific antigen binding molecule and optionally an additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required  
15 by FDA Office of Biological Standards or corresponding authorities in other countries. Preferred compositions are lyophilized formulations or aqueous solutions. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, buffers, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g. antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, antioxidants, proteins, drugs,  
20 drug stabilizers, polymers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the  
25 therapeutic or pharmaceutical compositions is contemplated.

An antibody or bispecific antigen binding moelcule of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration.

Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or  
30 subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

Parenteral compositions include those designed for administration by injection, e.g. subcutaneous, intradermal, intralesional, intravenous, intraarterial intramuscular, intrathecal or intraperitoneal injection. For injection, the antibodies or bispecific antigen binding molecules of the invention may be formulated in aqueous solutions, preferably in physiologically compatible

5 buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the antibodies or bispecific antigen binding molecules may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. Sterile injectable solutions are prepared by incorporating the antibodies or bispecific antigen binding molecules of  
10 the invention in the required amount in the appropriate solvent with various of the other ingredients enumerated below, as required. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the  
15 preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The composition must be  
20 stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Suitable pharmaceutically acceptable carriers include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine;  
25 preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone;  
30 amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or

non-ionic surfactants such as polyethylene glycol (PEG). Aqueous injection suspensions may contain compounds which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, dextran, or the like. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for

5 the preparation of highly concentrated solutions. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl cleats or triglycerides, or liposomes.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation

10 techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (18th Ed. Mack Printing Company, 1990). Sustained-release

15 preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g. films, or microcapsules. In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum

20 monostearate, gelatin or combinations thereof.

In addition to the compositions described previously, the antibodies or bispecific antigen binding molecules may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the antibodies or bispecific antigen binding

25 molecules may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions comprising the antibodies or bispecific antigen binding molecules of the invention may be manufactured by means of conventional mixing, dissolving, 30 emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the proteins into

preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The antibodies or bispecific antigen binding molecules may be formulated into a composition in a free acid or base, neutral or salt form. Pharmaceutically acceptable salts are salts that

5 substantially retain the biological activity of the free acid or base. These include the acid addition salts, e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, 10 ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

### **Therapeutic Methods and Compositions**

15 Any of the antibodies or bispecific antigen binding molecules provided herein may be used in therapeutic methods. Antibodies or bispecific antigen binding molecules of the invention may be used as immunotherapeutic agents, for example in the treatment of cancers.

For use in therapeutic methods, antibodies or bispecific antigen binding molecules of the invention would be formulated, dosed, and administered in a fashion consistent with good 20 medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

In one aspect, antibodies or bispecific antigen binding molecules of the invention for use as a 25 medicament are provided. In further aspects, antibodies or bispecific antigen binding molecules of the invention for use in treating a disease are provided. In certain embodiments, antibodies or bispecific antigen binding molecules of the invention for use in a method of treatment are provided. In one embodiment, the invention provides an antibody or bispecific antigen binding molecule as described herein for use in the treatment of a disease in an individual in need 30 thereof. In certain embodiments, the invention provides an antibody or bispecific antigen binding molecule for use in a method of treating an individual having a disease comprising administering to the individual a therapeutically effective amount of the antibody or bispecific antigen binding molecule. In certain embodiments the disease to be treated is a proliferative disorder. In a

particular embodiment the disease is cancer. In certain embodiments the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-cancer agent if the disease to be treated is cancer. In further embodiments, the invention provides an antibody or bispecific antigen binding molecule

5 as described herein for use in inducing lysis of a target cell, particularly a tumor cell. In certain embodiments, the invention provides an antibody or bispecific antigen binding molecule for use in a method of inducing lysis of a target cell, particularly a tumor cell, in an individual comprising administering to the individual an effective amount of the antibody or bispecific antigen binding molecule to induce lysis of a target cell. An “individual” according to any of the  
10 above embodiments is a mammal, preferably a human.

In a further aspect, the invention provides for the use of an antibody or bispecific antigen binding molecule of the invention in the manufacture or preparation of a medicament. In one embodiment the medicament is for the treatment of a disease in an individual in need thereof. In a further embodiment, the medicament is for use in a method of treating a disease comprising  
15 administering to an individual having the disease a therapeutically effective amount of the medicament. In certain embodiments the disease to be treated is a proliferative disorder. In a particular embodiment the disease is cancer. In one embodiment, the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-cancer agent if the disease to be treated is cancer. In a further  
20 embodiment, the medicament is for inducing lysis of a target cell, particularly a tumor cell. In still a further embodiment, the medicament is for use in a method of inducing lysis of a target cell, particularly a tumor cell, in an individual comprising administering to the individual an effective amount of the medicament to induce lysis of a target cell. An “individual” according to any of the above embodiments may be a mammal, preferably a human.

25 In a further aspect, the invention provides a method for treating a disease. In one embodiment, the method comprises administering to an individual having such disease a therapeutically effective amount of an antibody or bispecific antigen binding molecule of the invention. In one embodiment a composition is administered to said individual, comprising the antibody or bispecific antigen binding molecule of the invention in a pharmaceutically acceptable form. In  
30 certain embodiments the disease to be treated is a proliferative disorder. In a particular embodiment the disease is cancer. In certain embodiments the method further comprises administering to the individual a therapeutically effective amount of at least one additional

therapeutic agent, e.g., an anti-cancer agent if the disease to be treated is cancer. An "individual" according to any of the above embodiments may be a mammal, preferably a human.

In a further aspect, the invention provides a method for inducing lysis of a target cell, particularly a tumor cell. In one embodiment the method comprises contacting a target cell with

5 an antibody or bispecific antigen binding molecule of the invention in the presence of a T cell, particularly a cytotoxic T cell. In a further aspect, a method for inducing lysis of a target cell, particularly a tumor cell, in an individual is provided. In one such embodiment, the method comprises administering to the individual an effective amount of an antibody or bispecific antigen binding molecule to induce lysis of a target cell. In one embodiment, an "individual" is a

10 human.

In certain embodiments the disease to be treated is a proliferative disorder, particularly cancer.

Non-limiting examples of cancers include haematological cancer such as leukemia, bladder cancer, brain cancer, head and neck cancer, pancreatic cancer, biliary cancer, thyroid cancer, lung cancer, breast cancer, ovarian cancer, uterine cancer, cervical cancer, endometrial cancer,

15 esophageal cancer, colon cancer, colorectal cancer, rectal cancer, gastric cancer, prostate cancer, skin cancer, squamous cell carcinoma, sarcoma, bone cancer, and kidney cancer. Other cell proliferation disorders that may be treated using an antibody or bispecific antigen binding molecule of the present invention include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal,

20 parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. Also included are pre-cancerous conditions or lesions and cancer metastases.

In certain embodiments the cancer is chosen from the group consisting of haematological cancer (such as leukemia), kidney cancer, bladder cancer, skin cancer, lung cancer, colorectal cancer,

25 breast cancer, brain cancer, head and neck cancer and prostate cancer. In one embodiment, the cancer is a haematological cancer, particularly leukemia, most particularly acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML). A skilled artisan readily recognizes that in many cases the antibody or bispecific antigen binding molecule may not provide a cure but may only provide partial benefit. In some embodiments, a physiological change having some

30 benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of antibody or bispecific antigen binding molecule that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount". The subject, patient, or individual in need of treatment is typically a mammal, more specifically a human.

In some embodiments, an effective amount of an antibody or bispecific antigen binding molecule of the invention is administered to a cell. In other embodiments, a therapeutically effective amount of an antibody or bispecific antigen binding molecule of the invention is administered to an individual for the treatment of disease.

5 For the prevention or treatment of disease, the appropriate dosage of an antibody or bispecific antigen binding molecule of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the route of administration, the body weight of the patient, the type of antibody or bispecific antigen binding molecule, the severity and course of the disease, whether the antibody or bispecific antigen  
10 binding molecule is administered for preventive or therapeutic purposes, previous or concurrent therapeutic interventions, the patient's clinical history and response to the antibody or bispecific antigen binding molecule, and the discretion of the attending physician. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject. Various dosing  
15 schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

The antibody or bispecific antigen binding molecule is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 pg/kg to 15 mg/kg (e.g. 0.1 mg/kg - 10 mg/kg) of antibody or bispecific antigen binding  
20 molecule can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 pg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease  
25 symptoms occurs. One exemplary dosage of the antibody or bispecific antigen binding molecule would be in the range from about 0.005 mg/kg to about 10 mg/kg. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg body weight, about 5 microgram/kg body weight, about 10 microgram/kg body weight, about 50 microgram/kg body weight, about 100 microgram/kg body weight, about 200 microgram/kg body weight, about 350  
30 microgram/kg body weight, about 500 microgram/kg body weight, about 1 milligram/kg body weight, about 5 milligram/kg body weight, about 10 milligram/kg body weight, about 50 milligram/kg body weight, about 100 milligram/kg body weight, about 200 milligram/kg body weight, about 350 milligram/kg body weight, about 500 milligram/kg body weight, to about

1000 mg/kg body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg body weight to about 100 mg/kg body weight, about 5 microgram/kg body weight to about 500 milligram/kg body weight, etc., can be administered, based on the numbers described above.

5 Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody or bispecific antigen binding molecule). An initial higher loading dose, followed by one or more lower doses may be  
10 administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The antibodies or bispecific antigen binding molecules of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the antibodies or bispecific antigen binding molecules of the invention, or  
15 pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays, such as cell culture assays. A dose can then be formulated in animal models to  
20 achieve a circulating concentration range that includes the IC50 as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data, e.g., animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

25 Dosage amount and interval may be adjusted individually to provide plasma levels of the antibodies or bispecific antigen binding molecules which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 50 mg/kg/day, typically from about 0.5 to 1 mg/kg/day. Therapeutically effective plasma levels may be achieved by administering multiple doses each day. Levels in plasma may be measured, for  
30 example, by HPLC.

In cases of local administration or selective uptake, the effective local concentration of the antibodies or bispecific antigen binding molecules may not be related to plasma concentration.

One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

A therapeutically effective dose of the antibodies or bispecific antigen binding molecules described herein will generally provide therapeutic benefit without causing substantial toxicity.

5     Toxicity and therapeutic efficacy of an antibody or bispecific antigen binding molecule can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used to determine the LD50 (the dose lethal to 50% of a population) and the ED50 (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which can be expressed as  
10    the ratio LD50/ED50. Antibodies or bispecific antigen binding molecules that exhibit large therapeutic indices are preferred. In one embodiment, the antibody or bispecific antigen binding molecule according to the present invention exhibits a high therapeutic index. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages suitable for use in humans. The dosage lies preferably within a range of circulating  
15    concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon a variety of factors, e.g., the dosage form employed, the route of administration utilized, the condition of the subject, and the like. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see, e.g., Fingl et al., 1975, in: *The Pharmacological Basis of Therapeutics*, Ch. 1, p.  
20    1, incorporated herein by reference in its entirety).

The attending physician for patients treated with antibodies or bispecific antigen binding molecules of the invention would know how and when to terminate, interrupt, or adjust administration due to toxicity, organ dysfunction, and the like. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not  
25    adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated, with the route of administration, and the like. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency will also vary according to the age, body weight, and response of the individual patient.

30

### **Other Agents and Treatments**

The antibodies and bispecific antigen binding molecules of the invention may be administered in combination with one or more other agents in therapy. For instance, an antibody or bispecific

antigen binding molecule of the invention may be co-administered with at least one additional therapeutic agent. The term "therapeutic agent" encompasses any agent administered to treat a symptom or disease in an individual in need of such treatment. Such additional therapeutic agent may comprise any active ingredients suitable for the particular indication being treated,

5 preferably those with complementary activities that do not adversely affect each other. In certain embodiments, an additional therapeutic agent is an immunomodulatory agent, a cytostatic agent, an inhibitor of cell adhesion, a cytotoxic agent, an activator of cell apoptosis, or an agent that increases the sensitivity of cells to apoptotic inducers. In a particular embodiment, the additional therapeutic agent is an anti-cancer agent, for example a microtubule disruptor, an antimetabolite, 10 a topoisomerase inhibitor, a DNA intercalator, an alkylating agent, a hormonal therapy, a kinase inhibitor, a receptor antagonist, an activator of tumor cell apoptosis, or an antiangiogenic agent. Such other agents are suitably present in combination in amounts that are effective for the purpose intended. The effective amount of such other agents depends on the amount of antibody or bispecific antigen binding molecule used, the type of disorder or treatment, and other factors 15 discussed above. The antibodies or bispecific antigen binding molecules are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

Such combination therapies noted above encompass combined administration (where two or 20 more therapeutic agents are included in the same or separate compositions), and separate administration, in which case, administration of the antibody or bispecific antigen binding molecule of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antibodies or bispecific antigen binding molecules of the invention may also be used in combination with radiation therapy.

25

### **Articles of Manufacture**

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the 30 container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for

example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody or bispecific antigen binding molecule of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture

5 may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody or bispecific antigen binding molecule of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be

10 used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

15

### **Methods and Compositions for Diagnostics and Detection**

In certain embodiments, any of the anti-HLA-A2/WT1 antibodies provided herein is useful for detecting the presence of HLA-A2/WT1 in a biological sample. The term “detecting” as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological

20 sample comprises a cell or tissue, such as prostate tissue.

In one embodiment, an anti-HLA-A2/WT1 antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of HLA-A2/WT1 in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with an anti-HLA-A2/WT1 antibody as described herein under conditions

25 permissive for binding of the anti-HLA-A2/WT1 antibody to HLA-A2/WT1, and detecting whether a complex is formed between the anti-HLA-A2/WT1 antibody and HLA-A2/WT1. Such method may be an *in vitro* or *in vivo* method. In one embodiment, an anti-HLA-A2/WT1 antibody is used to select subjects eligible for therapy with an anti-HLA-A2/WT1 antibody, e.g. where HLA-A2/WT1 is a biomarker for selection of patients.

30 Exemplary disorders that may be diagnosed using an antibody of the invention include cancer, particularly prostate cancer.

In certain embodiments, labeled anti-HLA-A2/WT1 antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric,

electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^{3}\text{H}$ , and  $^{131}\text{I}$ , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine 5 and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that 10 employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

## Examples

The following are examples of methods and compositions of the invention. It is understood that 15 various other embodiments may be practiced, given the general description provided above.

### **Example 1. Generation of Fab binders to HLA-A2/WT1**

#### Selection and screening of anti-HLA-A2/WT1 Fabs

Anti-HLA-A2/WT1 Fabs were selected by phage display from synthetic Fab libraries based on 20 entirely human frameworks with sequence diversity in CDR3 of VF (3 different lengths) and VH domains (6 different lengths).

Selection rounds (biopanning) were performed in solution according to the following protocol: 1. pre-clearing of  $10^{12}$  phagemid particles per library pool on neutravidin coated 96well plates coated with 500 nM of an unrelated biotinylated HFA-A2/WT1<sub>LD</sub> complex, 2. incubation of the 25 non-HFA-A2/WT1<sub>LD</sub>-binding phagemid particles with 100 nM biotinylated HFA-A2/WT1<sub>RMF</sub> complex for 0.5 h in a total volume of 800  $\mu\text{l}$ , 3. capture of biotinylated HFA-A2/WT1<sub>RMF</sub> and specifically binding phage by adding 80  $\mu\text{l}$  of streptavidin-coated magnetic particles for 20 min on a shaker, 4. washing of respective magnetic particles 5-10x with 1 ml PBS/Tween 20 and 5-10x with 1 ml PBS using a magnetic particle separator, 5. elution of phage particles by addition 30 of 1 ml 100 mM triethylamine (TEA) for 5-10 min and neutralization by addition of an 1/2

volume of 1 M Tris/HCl pH 7.4, 6. re-infection of log-phase *E. coli* TG1 cells with the eluted phage particles, infection with helperphage VCSM13, incubation on a shaker at 30°C over night and subsequent PEG/NaCl precipitation of phagemid particles to be used in the next selection round.

5 Selections were carried out over 3 to 4 rounds using constant antigen concentrations of 100 nM.

In addition to selection campaigns with constant antigen concentrations, further selection campaigns were carried out with decreasing antigen concentrations of 100 nM, 50 nM, 10 nM and 5 nM in order to select for antibodies with lower affinities.

HLA-A2/WT1 binding assays: sandwich ELISA for characterisation of Fabs obtained by phage

10 display

Individual clones were bacterially expressed as 1 ml cultures in 96-well format and supernatants were subjected to a screening by ELISA. Specific binders were defined as having signals higher than 5 x background for HLA-A2/WT1<sub>RMF</sub> and signals lower than 3 x background for HLA-

A2/WT1<sub>v</sub><sub>LD</sub>. More precisely, neutravidin 96 well strip plates (Thermo Fisher) were coated with

15 10 nM of HLA-A2/WTW or 50 nM HLA-A2/WT 1<sub>v</sub><sub>LD</sub> at 37°C for 30 min, followed by blocking of the plate with 2% (w/v) milk-phosphate-buffered saline (MPBS) (200 µl/well) for 1 h at room temperature. The plate was washed 3 times with PBS, then Fab containing bacterial supernatants were added and the plate was incubated at room temperature for 1h. After another 3 washing steps with PBS, anti-FLAG-HRP secondary antibody (Sigma, Cat. No. A8592)

20 ((1:4000) was added and the plate was incubated on a shaker for 1 h at room temperature. The plate was washed 3 times with PBS and developed by adding 100 µl/well BM Blue POD (Roche). The enzymatic reaction was stopped by adding 50 µl/well 1 M H<sub>2</sub>S<sub>4</sub>. The OD was read at 450 nm (reference at 650 nm) for a final read-out of OD<sub>450-650</sub>. ELISA-positive clones were subjected to the kinetic screening experiment described below.

25 HLA-A2/WT1 binding assays: surface plasmon resonance for kinetic characterisation of Fabs obtained by phage display

Specific binders were identified by surface plasmon resonance-screening of Fab-containing bacterial culture supernatants using a ProteOn XPR36 biosensor (BioRad). In brief, after infection of log-phase *E. coli* TG1 cells with the eluted phage particles, single colony forming

units (cfu) were plated and picked for inoculation of 1 ml expression cultures in 96-deep well plates.

All experiments were performed at 25°C using PBST as running buffer (10 mM PBS, pH 7.4 and 0.005% (v/v) Tween 20). A ProteOn XPR36 biosensor equipped with GLC and GLM sensor 5 chips and coupling reagents (10 mM sodium acetate pH 4.5, sulfo-N-hydroxysuccinimide [sulfo-NHS], 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride [EDC] and ethanolamine) from BioRad Inc. (Hercules, CA) were used.

Immobilizations were performed at 30  $\mu$ l/min on a GLM chip. pAb (goat) anti human IgG, F(ab)2 specific antibody (Jackson ImmunoResearch) was coupled in vertical direction using a 10 standard amine-coupling procedure: all six ligand channels were activated for 5 min with a mixture of EDC (200 mM) and sulfo-NHS (50 mM). Immediately after the surfaces were activated, pAb (goat) anti human IgG, F(ab)2 specific antibody (50 pg/ml, 10 mM sodium acetate, pH 5) was injected across all six channels for 5 min. Finally, channels were blocked with a 5 min injection of 1 M ethanolamine-HCl (pH 8.5). Final immobilization levels were similar on 15 all channels, ranging from 11000 to 11500 RU. The Fab variants were captured from *E.coli* supernatants by simultaneous injection along five of the separate horizontal channels (30  $\mu$ l/min) for 5 min and resulted in levels ranging from 200 to 900 RU, depending on the concentration of Fab in supernatant. Conditioned medium was injected along the sixth channel to provide an 'in-line' blank for double referencing purposes. One-shot kinetic measurements were performed by 20 injection of a dilution series of HLA-A2/WTTRMF or HLA-A2/WTTVLD (100, 50, 25, 12.5, 6.25, 0 nM, 50  $\mu$ l/min) for 2 min along the vertical channels. Dissociation was monitored for 3 min. Kinetic data were analyzed in ProteOn Manager v. 2.1. Processing of the reaction spot data involved applying an interspot-reference and a double-reference step using an inline buffer blank 25 (Myszka, J Mol Recognit (1999) 12, 279-284). The processed data from replicate one-shot injections were fit to a simple 1:1 Langmuir binding model without mass transport (O'Shannessy et al., Anal Biochem (1993) 212, 457-468).

For measurements of IgG from supernatants of HEK productions in 6-well format, the IgG variants were captured from HEK293 supernatants by simultaneous injection along five of the separate horizontal channels (30  $\mu$ l/min) for 5 min and resulted in levels ranging from 200 to 400 30 RU. Conditioned medium was injected along the sixth channel to provide an 'in-line' blank for double referencing purposes. One-shot kinetic measurements were performed by injection of a

dilution series of HLA-A2/WTW or HLA-A2/WT 1vLD (100, 50, 25, 12.5, 6.25, 0 nM, 50 pL/min) for 3 min along the vertical channels. Dissociation was monitored for 5 min. Kinetic data were analyzed as described above.

Based on binding profile and measured specificity to bind to the antigen, binders were shortlisted 5 and measured in cell binding assays.

Sequences of all selected binders (11D06, 33H09, 13B04, 11B09, 33F05, 5E11, 13E08, 5C01, 11G06) are provided in the sequence listing included herein and summarized in Table 1 below.

**Table 1.** Amino acid sequences of selected HLA-A2/WT1 binders.

Binder	SEQ ID NO							
	HCDR1	HCDR2	HCDR3	VH	LCDR1	LCDR2	LCDR3	VL
11D06	1	2	3	7	4	5	6	8
33H09	9	10	11	15	12	13	14	16
13B04	17	18	19	23	20	21	22	24
11B09	25	26	27	31	28	29	30	32
33F05	33	34	35	39	36	37	38	40
5E11	41	42	43	47	44	45	46	48
13E08	49	50	51	55	52	53	54	56
5C01	57	58	59	63	60	61	62	64
11G06	65	66	67	71	68	69	70	72

10 **Example 2. Preparation of HLA-A2/WT1 IgG and HLA-A2/WT1 x CD3 bispecific antibodies**

Cloning

The cDNAs encoding the proteins were cloned into a vector system (Evitria) using conventional (non-PCR based) cloning techniques. The vector plasmids were gene synthesized. Plasmid DNA 15 was prepared under low-endotoxin conditions based on anion exchange chromatography. DNA concentration was determined by measuring the absorption at a wavelength of 260 nm.

Correctness of the sequences was verified with Sanger sequencing (with up to two sequencing reactions per plasmid depending on the size of the cDNA).

### Production

IgG antibodies and bispecific antibodies were generated by transient transfection of HEK293

5 EBNA cells (cultivated in suspension serum free in Excell culture medium). Cells were centrifuged and medium replaced by pre-warmed CD CHO medium. Expression vectors were mixed in CD CHO medium, polyethyleneimine (PEI) was added, the solution vortexed and incubated for 10 minutes at room temperature. Afterwards, cells were mixed with the DNA/PEI solution, transferred to shake flask and incubated for 3 hours at 37°C in an incubator with a 5%  
10  $CO_2$  atmosphere. After the incubation, Excell medium with supplements was added. One day after transfection supplements were added. Cell supernatants were harvested after 7 days and purified by standard methods.

Alternatively, suspension-adapted CHO K1 cells (originally from ATCC and adapted to serum-free growth in suspension culture) were used for production. The seed was grown in eviGrow

15 medium, a chemically defined, animal-component free, serum-free medium. Cells were transfected with eviFect transfection reagent, and cells were grown after transfection in eviMake2, an animal-component free, serum-free medium. Supernatant was harvested by centrifugation and subsequent filtration (0.2 pm filter).

### Purification

20 Proteins were purified from filtered cell culture supernatants referring to standard protocols. In brief, Fc containing proteins were purified from cell culture supernatants by affinity chromatography using Protein A (HiTrap ProteinA HP column, GE Healthcare). Elution was achieved at pH 3.0 followed by immediate neutralization of the sample. The protein was concentrated and aggregated protein was separated from monomeric protein by size exclusion  
25 chromatography (HiLoad Superdex 200 column, GE Healthcare) in 20 mM histidine, 140 mM sodium chloride, pH 6.0.

### Analytics

The concentration of purified proteins was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence

according to Pace et al. (Protein Science, 1995, 4, 2411-1423). Purity and molecular weight of the proteins were analyzed by CE-SDS in the presence and absence of a reducing agent using a LabChipGXII system (Caliper Lifescience). Determination of the aggregate content was performed by HPLC chromatography using analytical size-exclusion column (TSKgel G3000 SW XL, Tosoh) equilibrated in a 25 mM K<sub>2</sub>HP0<sub>4</sub>, 125 mM NaCl, 200mM L-arginine monohydrochloride, pH 6.7 running buffer at 25°C.

CD3 bispecific antibodies are also referred to herein a “T cell bispecific antibodies” or “TCBs”. A schematic illustration of the bispecific antibodies prepared in this example is given in Figure 2.

Exemplary sequences of TCBs are given in SEQ ID NOS 123, 124, 125 and 129 (11D06 TCB) and SEQ ID NOS 126, 127, 128 and 129 (33H09-TCB). Other TCBs were constructed in an analogous manner, using the VH and VL sequences of the corresponding HLA-A2/WT1 binders.

As controls, a HLA-A2/WT1 x CD3 bispecific antibody (TCB) based on a binder similar to antibody ESK1 (Dao et al., Sci Transl Med (2013) 5, l76ra33; WO2012/135854) - referred to herein as “ESK1-TCB” (see SEQ ID NOS 73 and 74 for the variable region sequences) - as well as an untargeted TCB (see SEQ ID NOS 75 and 76 for the variable region sequences) were prepared.

All molecules were produced and purified following the same method. The final quality was very good for all molecules with almost 100% monomer content and 100% purity on CE-SDS.

20 **Example 3. Biochemical analysis of affinity and avidity of HLA-A2/WT1 x CD3 bispecific antibodies**

For determination of affinity of HLA-A2/WT1 x CD3 bispecific antibodies to HLA-A2/WT1<sub>RMF</sub>, surface plasmon resonance (SPR) experiments were performed at 25°C on a Biacore T200 with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% Surfactant P20 (GE Healthcare)). Anti-human Fc specific antibody (GE Healthcare, Cat. No. BR-1008-39) was directly immobilized by amine coupling on a CM5 chip (GE Healthcare). The bispecific constructs were captured for 30 s at 5 nM. A three-fold dilution series of the HLA-A2/WT1<sub>RMF</sub> complex in HBS-EP (1.03 to 250 nM) was passed over the ligand at 30 µl/min for 120 sec to record the association phase. The dissociation phase was monitored for 120 s and triggered by

switching from the sample solution to HBS-EP. The chip surface was regenerated after every cycle using an injection of 3M MgCl<sub>2</sub> at 10  $\mu$ l/min for 30 sec. Bulk refractive index differences were corrected for by subtracting the response obtained on the reference flow cell which contains the anti-human Fc antibody, but without bispecific construct captured on it. The affinity constants were derived from the kinetic rate constants by fitting to a 1:1 Langmuir binding using the BIAeval software (GE Healthcare).

For determination of avidity and specificity of the HLA-A2/WT1 x CD3 bispecific antibodies to HLA-A2/WT1 RMF, SPR experiments were again performed at 25°C on a Biacore T200 with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% Surfactant P20 (GE Healthcare)). Anti-His antibody (Penta His, Qiagen Cat. No. 34660) was directly immobilized by amine coupling on a CM5 chip (GE Healthcare). The HLA-A2/WT1RMF or HLA-A2/WT1v<sub>LD</sub> was captured for 30 sec and 10  $\mu$ l/min at 5 or 10 nM (for the bispecific antibody or IgG measurement, respectively). A 3-fold dilution series of the bispecific constructs in HBS-EP (1.23 to 100 nM) were passed over the ligand at 30  $\mu$ l/ $\mu$ min for 120 sec to record the association phase. The dissociation phase was monitored for 240 sec and triggered by switching from the sample solution to HBS-EP. The chip surface was regenerated after every cycle using an injection of 10 mM glycine pH 2 for 60 sec. Bulk refractive index differences were corrected for by subtracting the response obtained on the reference flow cell (which contains the anti-His antibody, but without HLA-A2/WT1<sub>RMF</sub> captured on it). Even though it is a 2:1 interaction (analyte is bivalent) the affinity constants were derived from the kinetic rate constants by fitting to a 1:1 Langmuir binding using the BIAeval software (GE Healthcare). This results in an apparent KD representing the avidity of the interaction.

The results of these experiments are summarized in Table 2 below. Both tested HLA-A2/WT1 antibodies bind to HLA-A2/WT1<sub>RMF</sub> with double-digit nanomolar (monovalent) affinity / three-digit picomolar (bivalent) affinity (avidity), and keep the same affinity/avidity when converted from IgG to bispecific format. While the affinity of the two tested antibodies differs by about a factor two, avidity of both molecules is in the same range.

No binding of the tested HLA-A2/WT1 antibodies to HLA-A2/WT1v<sub>LD</sub> was detected (data not shown).

**Table 2.** Summary of affinity and avidity data as determined by SPR for selected HLA-A2/WT1 IgG and HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to HLA-A2/WT! RMF .

Analyte	ligand	Affinity			Avidity		
		ka (1/Ms)	kd (1/s)	KD	ka (1/Ms) average stdev	kd (1/s) average stdev	Apparent KD (pM) average stdev
33H09-hulgG1	RMF	1.67 10 <sup>6</sup>	1.16 10 <sup>-1</sup>	70 nM	<b>4.74 10<sup>6</sup></b> 6.5 10 <sup>4</sup>	<b>3.57 10<sup>-3</sup></b> 2.35 10 <sup>-4</sup>	<b>750</b> 50
		1.8 10 <sup>6</sup>	1.24 10 <sup>-1</sup>	69 nM			
33H09-TCB	RMF	2.67 10 <sup>6</sup>	1.85 10 <sup>-1</sup>	70 nM	<b>3.3 10<sup>7</sup></b> 5.85 10 <sup>6</sup>	<b>1.81 10<sup>-2</sup></b> 6.45 10 <sup>-3</sup>	<b>540</b> 110
		2.32 10 <sup>6</sup>	1.61 10 <sup>-1</sup>	69 nM			
11D06-hulgG1	RMF	1.08 10 <sup>6</sup>	3.78 10 <sup>-2</sup>	35 nM	<b>2.97 10<sup>6</sup></b> 1.01 10 <sup>5</sup>	<b>1.81 10<sup>-3</sup></b> 1.28 10 <sup>-4</sup>	<b>610</b> 20
		1.04 10 <sup>6</sup>	3.57 10 <sup>-2</sup>	34 nM			
11D06-TCB	RMF	1.07 10 <sup>6</sup>	3.85 10 <sup>-2</sup>	36 nM	<b>7.05 10<sup>6</sup></b> 1.87 10 <sup>6</sup>	<b>4.98 10<sup>-3</sup></b> 1.39 10 <sup>-3</sup>	<b>710</b> 80
		1.12 10 <sup>6</sup>	3.82 10 <sup>-2</sup>	34 nM			

5 For determination of affinity of HLA-A2/WT1 x CD3 bispecific antibodies to CD3, surface plasmon resonance (SPR) experiments were performed at 25°C on a Biacore T200 with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% Surfactant P20 (GE Healthcare)). Anti-human Fab specific antibody (GE Healthcare, Cat. No. 28-9583-25) was directly immobilized by amine coupling on a CM5 chip (GE Healthcare). The bispecific constructs were captured for 40 s at 5 nM. A three-fold dilution series of the CD3 $\epsilon$  $\delta$ -Fc fusion molecule in HBS-EP (12.35 to 3000 nM) was passed over the bispecific antibodies at 30  $\mu$ l/min for 240 sec to record the association phase. The dissociation phase was monitored for 240 s and triggered by switching from the sample solution to HBS-EP. The chip surface was regenerated after every cycle using a double injection of 10 mM glycine-HCl pH 2.1 at 30  $\mu$ l/min for 60 sec.

10 Bulk refractive index differences were corrected for by subtracting the response obtained on the reference flow cell (which contains the anti-human Fab antibody, but without bispecific construct captured on it). The affinity constants were derived from the kinetic rate constants by fitting to a 1:1 Langmuir binding using the BIAeval software (GE Healthcare).

15

The results are summarized in Table 3. Since the CD3 binder is identical in both tested bispecific molecules, as expected their affinity to CD3 is essentially the same.

**Table 3.** Summary of affinity data as determined by SPR for selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to CD3.

Analyte	ligand	Affinity		
		ka (1/Ms)	kd (1/s)	KD
33H09-TCB	huCD3-Fc	2.74 10 <sup>4</sup>	2.93 10 <sup>-3</sup>	110 nM
		2.66 10 <sup>4</sup>	2.77 10 <sup>-3</sup>	100 nM
11D06-TCB	huCD3-Fc	2.59 10 <sup>4</sup>	3.01 10 <sup>-3</sup>	120 nM
		2.53 10 <sup>4</sup>	2.82 10 <sup>-3</sup>	110 nM

5 **Example 4. Selection of IgG binders for specificity for HLA-A2/WT1 peptide RMF or VLD.**

We first measured the binding specificity on peptide-pulsed T2 cells by the IgG binders generated from phage display by flow cytometry. Briefly, T2 cells were prepared as a cell suspension at 10<sup>6</sup> cells/ml in IMDM medium (Gibco by Life Technologies, Cat No. 31980-048), supplemented with 10% FBS (Gibco, Cat No. 16140-071) +1% Penicillin-Streptomycin (Gibco, 10 Cat No. 15070-063) (complete medium). Cells were kept in a total volume of 10 ml in a tube, and incubated with 10 µM of peptide (WT1 p37-45 VLD peptide (SEQ ID NO: 77), or p126-134 RMF peptide (SEQ ID NO: 78)) at 10<sup>-2</sup> M (final concentration of the peptide: 10<sup>-5</sup> M) for 2 hours at 37°C with 5% CO<sub>2</sub>. After washing, cells were suspended in cold PBS and incubated with titrated concentration of IgG binders (10 pg/ml to 0.00064 pg/ml) for 1 hour at 4°C, followed by 15 incubation with a secondary anti-human IgG-Fc phycoerythrin (PE)-conjugated antibody (Jackson Laboratories, Cat No. 109-116-098) for 30 min. Cells were acquired on FACS LSR II (BD), and data are presented as mean fluorescence intensity (MFI) of PE in Graphpad Prism.

As shown in Figure 3, 11D06-IgG, 33H09-IgG and 5E1 l-IgG all bind to RMF-peptide pulsed T2 cells, but not to unpulsed or VLD peptide-pulsed T2 cells. In contrast, HB09-IgG, 13B04-IgG 20 and 5C01-IgG (but not HG06-IgG) all bind specifically to VLD-peptide, but not to RMF-peptide. Based on these data binders for conversion to CD3 bispecific antibodies (TCBs) were selected.

**Example 5. Activation of T cells in a NFAT-Jurkat reporter assay upon binding of HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to peptide-pulsed T2 cells**

To check the specificity of the HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”), a reporter cell line, Jurkat cells that express luciferase under the promoter of NFAT (Jurkat-NFAT;

5 Promega Cat. No. CS176501), was used to measure activation of T cells when TCBs bind to peptide-pulsed T2 cells (ATCC, Cat. No. CRL-1992). Briefly, T2 cells were prepared as a cell suspension at  $10^6$  cells/ml in IMDM medium (Gibco by Life Technologies, Cat. No. 31980-048), supplemented with 10% FBS (Gibco, Cat. No. 16140-071) + 1% Penicillin-Streptomycin (Gibco, Cat. No. 15070-063) (complete medium). Cells were kept in a total volume of 10 ml in a tube, 10 and incubated with 10  $\mu$ l of peptide (WT1 p37-45 VLD peptide, or pI26-I34 RMF peptide) at  $10^{-2}$ M (final concentration of the peptide:  $10^{-5}$ M) for 2 hours at 37°C with 5%  $\text{CO}_2$ . After washing, 90  $\mu$ l of the peptide-pulsed cells in a cell suspension of  $2.2 \times 10^5$  cells/ml were seeded into a 96 well microtiter round bottom plate (20,000 cells/well, TPP, Cat. No. 92097), co-cultured with 50  $\mu$ l of Jurkat-NFAT (cell suspension of  $2 \times 10^6$  cells/ml), and with 10  $\mu$ l of titrated TCB (at 100 pg/ml to 0.0064 pg/ml in PBS) for 16 hours at 37°C with 5%  $\text{CO}_2$ . Thereafter, 50  $\mu$ l of supernatant were removed, and replaced with 100  $\mu$ l per well of Bright-Glo Luciferase Assay (Promega, Cat. No. E2620) for incubation at room temperature (RT). Five minutes later, 180  $\mu$ l of supernatant were transferred into a new white plate to measure luminescence signal by EnVision (PerkinElmer). Data are presented as Relative Luminescence Unit (RLU).

20 As shown in Figure 4, the TCBs based on the 11D06 and the 33H09 binder (11D06-TCB and 33H09-TCB, respectively) specifically recognize the HLA-A2/WT1<sub>RMF</sub> complex and activate NFAT on reporter Jurkat cells only in the presence of T2 cells pulsed with the RMF peptide. In contrast thereto, the control TCB based on the ESK1-like binder (ESK1-TCB) did not show specificity for RMF peptide. Also the TCB based on the 5E11 binder (5E11-TCB) showed 25 activation of NFAT reporter T cells in the presence of both RMF and VLD peptide-pulsed T2 cells, thus this TCB was eliminated for the next round of screening. There were also some VLD peptide-specific TCBs identified such as the ones based on the 11B09 and the 13B04 binder (11B09-TCB and 13B04-TCB, respectively). As a negative control, untargeted TCB (DP47GS-TCB) was used in the assay, which did not activate NFAT on reporter Jurkat cells in the 30 presence of RMF- or VLD-peptide pulsed T2 cells. 5C01-TCB showed recognition of VLD peptide, but cross-reacted with RMF peptide at higher concentrations.

**Example 6. T cell cytotoxicity mediated by HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) upon binding to peptide-pulsed T2 cells**

Next, we measured the cytotoxicity of the TCBs. The target cells were peptide-pulsed T2 cells as 5 described in Example 5. The effector cells were pan CD3<sup>+</sup> cells purified from PBMCs isolated from huffy coat by Ficoll (GE Healthcare, Cat. No. 17-1440-03) gradient centrifugation. Total CD3<sup>+</sup> T cells were purified by MACS (Miltenyi Biotec) using a Human Pan T cell Isolation Kit (Miltenyi Biotec, Cat. No. 130-096-535). The cytotoxicity assay was performed as follows: The peptide-pulsed cells (100  $\mu$ l) were seeded into a 96 well microtiter round bottom plate (3x10<sup>5</sup> 10 cells/ml), co-cultured with 50  $\mu$ l of T cells (6x10<sup>6</sup> cells/ml), and with 50  $\mu$ l of titrated TCB (at 40  $\mu$ g/ml to 0.00004  $\mu$ g/ml) in complete medium for 18 hours at 37°C with 5% CO<sub>2</sub>. Thereafter, 50  $\mu$ l of supernatant were transferred into a new white plate, and 25  $\mu$ l per well of CytoTox-Glo 15 Luciferase Assay (Promega, Cat. No. G9291) were added for incubation at room temperature (RT) for 15 minutes. The luminescence signal (for measurement of LDH release as indicative of cell death) was read by EnVision (PerkinElmer). Data are presented as Relative Luminescence Unit (RLU).

Figure 5 shows the TCB-mediated specific T cell killing of RMF- or VLD-expressing target cells. We found that both 11D06-TCB and 33H09-TCB showed specific killing on RMF peptide-pulsed T2 cells. 33F05-TCB did not mediate specific killing of RMF or VLD peptide-pulsed 20 cells. In addition, 13B04-TCB and 11B09-TCB mediated potent killing on VLD peptide-pulsed T2 cells. 5C01-TCB showed killing of VLD-pulsed T2 cells, but also of RMF-pulsed cells at higher concentration, consistent with the observations in the NFAT reporter assay (Figure 4).

**Example 7. T cell cytotoxicity mediated by HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) upon binding to WT1<sup>+</sup> cell lines**

To confirm the specific killing by the TCBs, we performed the cytotoxicity assay on WT1<sup>+</sup> tumor cell lines. The HLA-A2<sup>+</sup>WT1<sup>+</sup> cell lines were SKM-1 cells (DZMZ No. ACC 547), and CHO cells transfected with HLA-A2/WT1<sub>RMF</sub> complex (CHO-WT1) (in-house). The negative controls were HLA-A2<sup>+</sup>WT1 cells: BJAB (DZMZ No. ACC 757), ARH-77 (DZMZ No. ACC

512), and CHO cells transfected with HLA-A2/MAGE-A4 complex (CHO-MAGEA4) (in-house), as well as a HLA-A2<sup>+</sup>WT1<sup>+</sup> cell line: K562 (ATCC No. CLL-243) (Figure 6A). The cytotoxicity assay was performed as described in Example 6. Both 11D06-TCB and 33H09-TCB showed potent killing on SKM-1 and CHO-WT1 cells, but not on any HLA-A2<sup>+</sup>WT1<sup>-</sup> cells and  
5 HLA-A2<sup>+</sup>WT1<sup>+</sup> cells, indicating that these two TCBs have specificity for WT1 peptide RMF (Figure 6 B, C). In contrast thereto, none of the VLD peptide- specific TCBs showed killing on HLA-A2<sup>+</sup>WT1<sup>+</sup> cell lines, and if they showed low potency killing at high concentration (10 pg/ml) on the WT1<sup>+</sup> cell lines, the same degree of killing was seen on WTT cell lines as well (Figure 6 D, E). Based on these functional data, we selected 11D06-TCB and 33H09-TCB for  
10 further evaluation.

We also compared our selected TCBs with ESK1-TCB for their killing activity of the tumor target cells. Interestingly, though ESK1-TCB achieved similar potency in mediating killing of SKM-1 cells as compared to 11D06-TCB and 33H09-TCB, it also induced killing on HFA-A2<sup>+</sup>WT-1<sup>-</sup> BJAB cells, indicating that ESK1-TCB binding is not restricted to HEA-A2/WT1<sub>RMF</sub>  
15 complex (Figure 6F, G)

We tested the killing of multiple tumor cell lines that are HFA-A2+WT1+. Based on the EC<sub>50</sub> value for the killing, cell lines were categorized into those that were killed with an EC<sub>50</sub> of < 1 pM (marked in Table 4 with “++”), those that were killed with an EC<sub>50</sub> of > 1  $\mu$ M and < 5 pM (marked in Table 4 with “+”), and those that were essentially not killed (marked in Table 4 with  
20 “no”).

All together, the selected TCBs 11D06-TCB and 33H09-TCB mediated T cell cytotoxicity on 6 out of 13 HLA-A2<sup>+</sup>WT1<sup>+</sup> cell lines. The lack of killing on the other cell lines was likely due to the low expression of WT1 presented on the cell surface by MHC I. Table 4 shows the results for 11D06-TCB. The results for 33H09-TCB were similar (with EC<sub>50</sub> values being slightly higher  
25 than for 11D06-TCB).

**Table 4.** Killing of WT1+ cell lines by selected TCBs.

Cell line name	HLA-A2	WT1	Disease of Tissue Origin	Killing by 11D06-TCB (RMF-specific)	EC <sub>50</sub> (uM) in vitro killing by 11D06-TCB	K-Ras mutation
SKM-1	+	+	Acute myeloid leukemia	++	0.09--0.35	yes
T98G	+	+	Glioblastoma	++	0.8--1.7	no
MDA-MB-231	+	+	Breast adenocarcinoma	++	0.5--2.8	yes
SW620	+	+	colorectal adenocarcinoma	+	4.8	yes
SW480	+	+	colorectal adenocarcinoma	+	2.9	yes
SET-2	+	+	Essential thrombocythemia	(+)* if treated with Decitabine	(0.005)	no
CTV-1	+	+	Leukemia, acute myeloid	no		no
BV173	+	+	acute leukemia, chronic myeloid	no		no
A-375	+	+	Melanoma	no		no
LN-18	+	+	Glioblastoma	no		no
U-266	+	+	myeloma	no		no
OVCAR3	+	+	ovarian carcinoma	no		no
Nalm6	+	+	B cell precursor leukemia	no		no

**Example 8. T cell activation mediated by HLA-A2/WT1 x CD3 bispecific antibodies****5 (“TCBs”) upon binding to WT1+ cell lines**

A prerequisite of TCB-mediated cytotoxicity on WT1<sup>+</sup> target cells is that T cells are activated to acquire effector function. We measured the activation status of T cells by flow cytometry in the co-culture of T cells and HLA-A2<sup>+</sup>WT1<sup>-</sup> cells SKM-1 or HLA-A2<sup>+</sup>WT1<sup>-</sup> cells BJAB in the presence of the two selected TCBs, 33H09-TCB and 11D06-TCB, during the in vitro killing assay as described in Example 7. Cells were harvested after 18 hours of co-incubation, and stained with antibodies against CD3 (Biolegend Cat. No. 300321), CD25 (Biolegend Cat. No. 302606) and CD69 (Biolegend Cat. No. 310914) to measure T cell activation by flow cytometry.

As shown in Figure 7, both TCBs induced up-regulation of CD69 and CD25 on CD3<sup>+</sup> T cells upon binding to SKM-1 cells, but not BJAB cells, indicating that the specific recognition by TCBs of HLA-A2/WT1<sup>RMF</sup> complex presented by SKM-1 cells triggers CD3-mediated activation of T cells, eventually leading to the specific lysis of target cells.

**Example 9. No binding of selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to reported off-target peptides**

A major potential risk in developing T cell receptor (TCR)-like antibodies is the cross-reactivity of the antibody with peptide homologues presented from proteins expressed by normal tissue. As

5 it was reported by Ataie et al. (J Mol Biol. (2016) 428:194-205) that ESK1 antibody binds to sequence-similar peptides derived from protein MED13L and PIGQ, we tested our TCBs for their cross-reactivity to these two peptides. The sequences of these peptides are shown in Figure 8F, and SEQ ID NOs 79 and 80. In a flow cytometry-based binding assay, as described in Example 4, we found that both 11D06-TCB and 33H09-TCB do not bind to PIGQ peptide. The 10 binding to MED13L peptide by both TCBs is nearly 100 fold less as compared to the native WT1 RMF peptide (Figure 8 A, B). Although ESK1-TCB binds to RMF peptide in a low affinity, it binds to PIGQ peptide in a similar manner and even more strongly to MED13F (Figure 8C), consistent with the reported binding activity of the ESK1 IgG (Ataie et al., J Mol Biol. (2016) 428:194-205).

15 We also tested cross-reactivity using the NFAT-reporter assay as described in Example 5. In line with the binding data, the strength of NFAT activation on Jurkat cells upon binding to MED13F and PIGQ is at least 100 fold weaker than the activation upon binding to WT1 RMF peptide by 11D06-TCB and 33H09-TCB (Figure 8 D, E).

20 **Example 10. No binding of selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to unidentified off-target peptides**

Cross-reactivity against homologous peptides (sharing key recognition residues) derived from normal tissue may cause serious and not readily predictable toxicities by TCR-like antibody or TCR-based T cell therapy (see e.g. Linette et al, Blood (2013) 122:863-71; reporting fatal

25 toxicity of MAGE A3-TCR-therapy due to off-target reactivity against a protein expressed by cardiac tissue). Specificity of these agents is therefore critical. To thoroughly define specificity of our antibodies, based on the crystal structure data which confirms the positions in the RMF peptide involved in binding of 11D06 (see Example 15), we extended our search for potential off-target peptides with similar amino acid sequence as WT1 RMF peptide (SEQ ID NO: 78) by 30 masking RxxPNxxYx in the peptidome databases (Swissprot and TrEMBL). We found

additional 25 peptides derived from different proteins which all have high predicted affinity to HLA-A2 (Table 5). We then tested the cross-reactivity of our selected TCBs on the peptide-pulsed T2 cells using the NFAT-reporter assay (peptide 1-6) or T cell cytotoxicity assay (peptide 7-25), as described above.

5 For the first 6 peptides tested, there was no cross-reactivity to any of the 6 peptides by 11D06-TCB. 33H09-TCB showed some recognition to ARHGEF11, but the degree of NFAT-activation was >100 fold lower as compared to native RMF peptide (Figure 9A, B). Similarly, the cross-reactivity to the other 19 off-target peptides was at least 100 fold lower than to the native RMF peptide, as measured by direct T cell killing of peptide-pulsed T2 cells (Figure 9 C, D). This  
10 result was confirmed in a second experiment, wherein also ESK1-TCB was tested (Figure 9 E-G).

Taken together, we confirmed that 11D06 and 33H09 (but not the ESK1-like binder) show specificity to the WT1 RMF peptide, and not to the potential off-target peptides detected in the peptidome.

**Table 5.** Additional, newly identified off-target peptides.

Peptide	Peptide sequence	Gene name	SEQ ID NO
1	RLFPNLPEL	ARHGEF11	81
2	RMFPNKYSL	PRDM16	82
3	AMDPNAAYV	SERPINA6	83
4	RMGPNIYEL	NIPSNAP1	84
5	NMPPNFPYI	TAF3	85
6	YTIPNHPYL	U4	86
7	RLFPNAKFL	TPST1	87
8	RMVPRAVYL	IGFBP5	88
9	KMVPSPYIYL	RALGPS1	89
10	RIFPSYSYL	ZNF382	90
11	RLFPNSKFL	TPST2	91
12	KMTPCIPYL	RALGPS2	92
13	SMFPSLKYL	TBCE	93
14	RLLPSAPTL	KIFC2	94
15	RLRPHVPYL	SLC16A8	95
16	RMNPNSPSI	ERH	96
17	RVFNNRWYL	ZBTB47	97
18	RLQLNNPYL	MIOS	98
19	RMFFNGRYI	ATP6V0A2	99
20	RLSPNRPPL	PKD1	100

21	ETFPNSWYL	NAAFADF1	101
22	GLKPNAYL	ROBO1	102
23	RQFPNASLI	RECQF	103
24	YIFPNCPFF	SERPINA2	104
25	RFRINFPYF	FAM220A	105

**Example 11. No cytotoxicity on CD34<sup>+</sup> stem cells mediated by selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”)**

It has been reported that CD34<sup>+</sup> hematopoietic stem cells are WT1 positive (Ramani and Cowell,

5 J. Path (1996) 179:162-8), therefore it can potentially be harmful if the TCBs would bind them. To study whether CD34<sup>+</sup> cells endogenously present the RMF peptide, we obtained HFA-A2<sup>+</sup> bone marrow derived CD34<sup>+</sup> cells from 3 donors from Fonza, and tested our selected TCBs in a killing assay as described above in Example 7. Whereas 11D06-TCB and 33H09-TCB mediate potent killing on SKM-1 cells, they had no killing activity on the CD34<sup>+</sup> stem cells, indicating 10 that these stem cells may not present the RMF peptide in the context of HFA-A2 (Figure 10).

**Example 12. Definition of binding residues of selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) by alanine scan assay**

It is clear that our TCBs have different binding to RMF peptide and functional activity as

15 compared to ESK1-TCB. To characterize the potential binding motifs of the RMF peptide by the TCBs, we set up an alanine scan assay using peptide arrays derived from native sequences by individually replacing each amino acid with an alanine, and measured the NFAT-reporter signal by T2 cells pulsed with these peptides (Figure 11 A-F). Data are presented as RFU across increasing concentrations of TCB. We plotted the fold change of EC<sub>50</sub> relative to the EC<sub>50</sub> for the 20 native peptide, and considered a fold change of >10 as significant, meaning the respective amino acid residue of the RMF peptide might be critical for recognition by the 11D06-TCB and 33H09-TCB (Figure 11M). We concluded that both 11D06-TCB and 33H09-TCB have critical contact with residues R1, F3, N5 and A6 (Figure 11N), while ESK1 was shown to have close contact to RMF peptide R1, P4 and N5 (Ataie et al., J Mol Biol. (2016) 428:194-205).

**Example 13. Pharmacokinetic profile of HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) after single injection in NSG mice**

A single dose of 0.5 mg/kg of 33H09-TCB or 11D06-TCB was injected into NSG mice. All mice were injected i.v. with 200  $\mu$ l of the appropriate solution. To obtain the proper amount of 5 compounds per 200  $\mu$ l, the stock solution was diluted with histidine buffer. Three mice per time point were bled at 10 min, 1 hr, 3 hrs, 6 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 6 days, 8 days, 10 days and 12 days. The injected compound was analyzed in serum samples by ELISA. Biotinylated 10 anti-huCD3-CDR antibody (Roche Diagnostics, Penzberg, Germany), test sample, digoxigenin-labelled anti-huFc antibody and anti-digoxigenin detection antibody (peroxidase (POD)) were added stepwise to a 96-well streptavidin-coated microtiter plate and incubated after every step for 1 h at room temperature. The plate was washed three times after each step to remove 15 unbound substances. Finally, the peroxidase-bound complex is visualized by adding ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution to form a colored reaction product. The reaction product intensity, which was photometrically determined at 405 nm (with reference wavelength at 490 nm), is proportional to the analyte concentration in the serum. The result (Figure 12) showed a stable PK-behaviour for both clones tested which suggested a once 15 weekly schedule for subsequent efficacy studies.

**Example 14. Efficacy study with HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) in 20 SKM-1 xenograft in humanized mice**

The first efficacy study of HLA-A2/WT1 x CD3 bispecific antibodies was aimed at comparing two different WT1 binders (11D06 and 33H09) in terms of efficacy in a human acute myeloid leukemia (AML) xenograft (SKM-1; HLA-A2+, WT-1+) in fully humanized NSG mice.

SKM-1 cells (human AML) were originally obtained from ATCC and deposited in the Roche 25 Glycart internal cell bank. The cells were cultured in RPMI + 10% FCS + 1% glutamine in a water-saturated atmosphere at 5% CO<sub>2</sub>. In vitro passage 15 was used for subcutaneous injection at a viability of 98% with Matrigel (1:1 ratio).

Female NSG mice, age 4-5 weeks at start of the experiment (Jackson Laboratory) were maintained under specific-pathogen-free condition with daily cycles of 12 h light / 12 h darkness 30 according to committed guidelines (GV-Solas; Felasa; TierschG). The experimental study protocol was reviewed and approved by local government. After arrival, animals were

maintained for one week to get accustomed to the new environment and for observation. Continuous health monitoring was carried out on a regular basis.

Female NSG mice were injected i.p. with 15 mg/kg of busulfan followed one day later by an i.v. injection of  $1 \times 10^5$  human hematopoietic stem cells isolated from cord blood. At week 14-16 after

5 stem cell injection mice were bled sublingual and blood was analyzed by flow cytometry for successful humanization. Efficiently engrafted mice were randomized according to their human T cell frequencies into the different treatment groups. At that time, mice were injected with SKM-1 tumor cells s.c. as illustrated in Figure 13A and treated once weekly with the compounds or histidine buffer (vehicle) when tumor size reached approximately  $150 \text{ mm}^3$  (day 10). All mice  
10 were injected i.v. with  $200 \mu\text{l}$  of the appropriate solution. To obtain the proper amount of compounds per  $200 \mu\text{l}$ , the stock solutions were diluted with histidine buffer when necessary. Tumor growth was measured three times weekly using a caliper and tumor volume was calculated as follows:

15  $T_v: (W^2/2) \times L$  *(W: Width, L: Length)*

Tumor growth Inhibition (TGI) as well as Tumor to control ratio (TCR) were calculated as follows:

20  $TGI: \frac{100 - \text{Av}(T_{\text{treatment}}^{[\text{day x}]} - T_{\text{treatment}}^{[\text{baseline}]})}{\text{Av}(T_{\text{Vehicle}}^{[\text{day x}]} - T_{\text{Vehicle}}^{[\text{baseline}]})} \times 100$

$$TCR: \frac{\text{Av}(T_{\text{treatment}}^{[\text{day x}]})}{\text{Av}(T_{\text{Vehicle}}^{[\text{day x}]})}$$

25

Figure 13 shows the tumor growth kinetics (mean) in all treatment groups (Figure 13C) as well as the single tumor growth kinetics in each group (Figure 13 D-F). As shown, both TCBs exhibit tumor growth inhibition, with clone 11D06 showing the strongest tumor growth inhibition with a TGI of 101.3 (Figure 13G).

30

**Example 15. Crystal structure of HLA-A2/WT1 antibody / pMHC complexes**

Fab fragments were prepared by incubation of antibodies for 72 hours at 25°C in 50 mM Tris pH 8.0, 150 mM NaCl with 1.05 U plasmin (Roche, Cat. No. 602361) per mg. Cleaved Fc was separated from Fab fragments using a 4.5 mL CaptureSelect CH1- affinity column (BAC BV,

5 Cat. No. 191.3120) equilibrated with 50 mM Tris, 100 mM glycine, 150 mM NaCl, pH 8.0. Fab fragments were eluted from the column with 50 mM Tris, 100 mM glycine, 150 mM NaCl, pH 2.0 and neutralized with 0.5M sodium phosphate pH8.0 before loading on a size exclusion column S75 (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.4. Quality control was performed by doing analytical size exclusion (column Tosoh, TSK-Gel 10 G3000SWXL, on an Agilent HPLC 1200 system) and CE- SDS (Caliper LabChip GXII, Perkin Elmer) under non - reducing and reduced conditions. Purified Fab fragments were frozen in liquid nitrogen and stored at -80 °C.

*Crystallization, data collection and structure determination of the 5C01 antibody / pMHC complex*

15 *Crystallization.* The antibody / pMHC complex (Fab 5C01 HLA-A02/WT1<sub>VLD</sub> pMHC) was prepared by mixing a 1.2-fold molar excess of HLA-A2/WT1 Fab fragment based on the 5C01 binder (Fab 5C01) with HFA-A2/WTTVLD peptide complex (HFA-A02/WTTVLD pMHC). After 1 hour incubation at 4°C the mixture was concentrated to 10 mg/ml. Initial crystallization trials were performed in sitting drop vapor diffusion setups at 21°C. Crystals appeared within 1 day 20 out of 0.2 M sodium-tartrate, 20% polyethyleneglycol (PEG) 3350 with 10% 2-methyl-2,4-pentanediol (MPD) added to the crystallization droplet to improve crystal quality. Crystals were harvested directly from the screening plate without any further optimization step.

25 *Data collection and structure determination.* For data collection crystals were flash frozen at 100K in precipitant solution containing 15% glycerol. Diffraction data were collected at a wavelength of 1.0000 Å using a PIFATUS 6M detector at the beamline X10SA of the Swiss Light Source (Villigen, Switzerland). Data were processed with XDS (Kabsch, W. *Acta Cryst. D66*, 133-144 (2010)) and scaled with SADABS (BRUKER). The crystals of the complex belong to space group P2i2i2 with cell axes of a= 158.94 Å, b= 49.12 Å, c= 128.63 Å and diffract to a resolution of 1.98 Å. The structure was determined by molecular replacement with 30 PHASER (McCoy, A.J, Grosse-Kunstleve, R.W., Adams, P.D., Storoni, F.C., and Read, R.J. *J. Appl. Cryst.* 40, 658-674 (2007)) using the coordinates of the crystal structure with Protein Data

Bank (PDB) entry 4N05 and an in house Fab structure as search models. Difference electron density was used to place peptide and to change amino acids according to the sequence differences by real space refinement. Structures were refined with programs from the CCP4 suite (Collaborative Computational Project, Number 4 *Acta Cryst.* D50, 760-763 (1994)) and 5 BUSTER (Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Roversi, P., Sharff, A., Smart, O.S., Vonrhein, C., Womack, T.O. (2011). Buster version 2.9.5 Cambridge, United Kingdom : Global Phasing Ltd). Manual rebuilding was done with COOT (Emsley, P., Lohkamp, B., Scott, W.G. and Cowtan, K. *Acta Cryst* D66, 486-501 (2010)).

Data collection and refinement statistics are summarized in Table 6.

10 **Table 6.** Data collection and refinement statistics for Fab 5C01 HLA-A02/WT! <sub>VLD</sub> pMHC.

5C01-HLA-A02/ WT!VLD pMHC	
<b>Data collection</b>	
Space group	P2i2i2
Cell dimensions	
$a, b, c$ (Å)	158.94, 49.12, 128.63
(°)	90, 90, 90
Resolution (Å)	1.98
$R_{\text{sym}}$ Of $R_{\text{merge}}$	0.11
$11\sigma l$	12.01 (0.53)
Completeness (%)	99.9 (99.9)
Redundancy	6.61 (6.76)
<b>Refinement</b>	
Resolution (Å)	48.9 - 1.98
No. reflections	71103
$R_{\text{work}} / R_{\text{free}}$	19.12/24.01
No. atoms	
Protein	6374
Water	528
fi-factors	
Protein	66.32
Water	59.27
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.07

\*Values in parentheses are for highest-resolution shell.

*Structure of Fab 5C01 in complex with HLA-A02/WT1 VLD*

In order to characterize the interaction details of the VLD peptide with Fab 5C01, the binding epitope and paratope of 5C01 with HLA-A02, we determined the crystal structure of the complex of 5C01 with HLA-A02/WT<sub>1V</sub><sub>LD</sub> pMHC at a resolution of 1.98 Å (Figure 14A). The 5 structure reveals Fab 5C01 to bind to pMHC by main contributions of the CDR1 and CDR3 of the light chain and by all CDRs of the heavy chain. From the VLD peptide (SEQ ID NO: 77), the side chains of residues Val1, Phe4 and Pro7 are in direct contact to the Fab. All other side chains of the peptide point towards the HLA-A02 molecule. Contribution of the peptide to the contact surface area is ~68 Å<sup>2</sup> whereas a total Fab-pMHC contact area of ~476 Å<sup>2</sup> is observed. A close-up of the Fab 5C01-pMHC interface is shown in Figure 15.

10 Analysis of the binding interface with the program PISA (E. Krissinel and K. Henrick (2007), J. Mol. Biol. 372, 774-797) reveals an interaction pattern of Fab 5C01 with the HLA-A02 via 8 hydrogen bonds, Pi-Pi interactions and van der Waals contacts. Side chain hydrogen bonds are formed between residues of heavy chain CDR3 (Trp97) and CDR2 (Ser52, Ser53) with Glu63 15 and Glu66 of HLA-A02. Further hydrogen bonds are established by light chain backbone atoms of Tyr91 and Ile93 with Glu155. The complex is in addition stabilized via formation of Pi-Pi interactions of light chain residues Trp32 and Trp94 with HLA-A02 side chains of Glu155 and His151. The N-terminal valine of the VLD peptide entertains hydrogen bonds through the backbone nitrogen to Tyr171 of HLA-A02. Its side chain is oriented towards a pocket formed by 20 Glu63 and Trp167 of HLA-A02 and Trp97 of the heavy chain of Fab 5C01. In addition Phe4 of the peptide makes edge to face interactions with Tyr167 of the heavy chain. A schematic Fab 5C01-pMHC interaction matrix summarizing the contacts is shown in Figure 16.

*Crystallization, data collection and structure determination of the 11D06 antibody/pMHC complex*

25 *Crystallization.* The antibody / pMHC complex (Fab 11D06 HLA-A02/WTT<sub>1RMF</sub> pMHC) was prepared by mixing a 1:1 molar amount of HLA-A2/WT1 Fab fragment based on the 11D06 binder (Fab 11D06) with HLA-A2/WT1RMF peptide complex (HLA-A02/WT1RMF pMHC). After 4 hours of incubation at 21°C the mixture was concentrated to 20 mg/ml. Initial crystallization trials were performed in sitting drop vapor diffusion setups at 21°C. Crystals appeared within 4 30 days out of 0.1 M Tris pH 8.0, 20% PEG 4000. Crystals were harvested directly from the screening plate without any further optimization step.

*Data collection and structure determination.* Data was collected, processed and scaled as described above. The crystals of the complex belong to space group P2i with cell axes of  $a=54.11\text{ \AA}$ ,  $b=67.00\text{ \AA}$ ,  $c=139.36\text{ \AA}$  with  $\beta=90.57^\circ$  and diffract to a resolution of  $2.64\text{ \AA}$ . The structure was determined by molecular replacement with PHASER using the coordinates of an in house Fab and MHC complex structure as search model. Difference electron density was used to place peptide and to change amino acids according to the sequence differences by real space refinement. Structure refinement and manual rebuilding were done as described above.

Data collection and refinement statistics are summarized in Table 7.

**Table 7.** Data collection and refinement statistics for 11D06 HLA-A02/WT<sup>1</sup> <sub>RMF</sub> pMHC.

11D06 HLA-A02/ WT <sub>RMF</sub> pMHC	
<b>Data collection</b>	
Space group	P2i
Cell dimensions	
$a, b, c\text{ (\AA)}$	54.11, 67.00, 139.36
( $^\circ$ )	90, 90.57, 90
Resolution ( $\text{\AA}$ )	2.64
$R_{\text{sym}}$ $\text{O}\Gamma$ //merge	0.10
$I/\sigma I$	13.10 (0.82)
Completeness (%)	99.9 (99.8)
Redundancy	3.79 (3.85)
<b>Refinement</b>	
Resolution ( $\text{\AA}$ )	48.3 - 2.64
No. reflections	29606
//work / Rfree	17.10/23.00
No. atoms	
Protein	6395
Water	250
//-factors	
Protein	67.52
Water	57.46
R.m.s. deviations	
Bond lengths ( $\text{\AA}$ )	0.010
Bond angles ( $^\circ$ )	1.20

10 \*Values in parentheses are for highest-resolution shell.

*Structure of Fab 11D06 in complex with HLA-A02/RMF pMHC*

We determined the crystal structure of the complex of Fab 11D06 with HFA-A02/RMF pMHC at a resolution of 2.64 Å (Figure 14B). The structure shows Fab 11D06 binds to pMHC by contributions of all CDRs. RMF peptide (SEQ ID NO: 78) side chains of residues Arg1, Met2,

5 Pro4, Asn5, Ala6 and Tyr8 are in direct contact to light and heavy chain of the Fab. The remaining side chains of the peptide point towards the HFA-A02 molecule. Contribution of the peptide to the contact surface area is -107 Å<sup>2</sup>. The total Fab-pMHC contact area corresponds to -397 Å<sup>2</sup>. A close-up of the Fab HD06-pMHC interface is shown in Figure 17.

Analysis of the binding interface with the program PISA reveals an interaction pattern of Fab 10 11D06 with HFA-A02 via 4 hydrogen bonds, numerous Pi-Pi interactions and van der Waals contacts. Hydrogen bonds are observed between residues of heavy chain CDR1 (Ser30, Ser31) and CDR3 (GlylOOA) with Glu58 and Arg65 of HFA-A02. Further hydrogen bonds are established by light chain residue Asp50 with HFA-A02 Arg65. Beside others, Trp1OO of the heavy chain provides van der Waals and Pi-Pi contacts to Arg65 and Fys66 of the HFA-A02 and 15 to Pro4 of the RMF peptide. The N-terminal arginine of the RMF peptide points with its side chain into a polar pocket formed by Ser31, Glu97 and the backbone carbonyl of Ile96 of the heavy chain of 11D06. Additional polar contacts to 11D06 and HFA-A02 are entertained by the RMF peptide residue Asn5 which is part of a hydrogen bonding network to Trp32 of light chain CDR1, Glu92 of light chain CDR3 together with Glu155 of the HFA-A02. A schematic Fab 20 HD06-pMHC interaction matrix summarizing the contacts is shown in Figure 18.

*3D structure of ESK1 retrieved from the public database:*

For comparison, the publicly available crystal structure of antibody (Fab) ESK1 binding to HLA-A02/RMF pMHC (PDB ID 4WUU;

25 <http://www.rcsb.org/pdb/explore/explore.do?structureId=4WUU>) was analyzed analogously with regard to its Fab-pMHC contacts. Figure 14C shows the Fab ESK1 with HLA-A02/RMF pMHC crystal structure from the same angle (aligned on the HLA-A02 part) as the crystal structures of Fab 5C01 with HLA-A02/VLD pMHC and Fab 11D06 with HFA-A02/RMF pMHC (Figures 14 A and B). A close-up of the Fab ESK1-pMHC interface is shown in Figure 19, and a schematic 30 Fab ESK1-pMHC interaction matrix summarizing the contacts is shown in Figure 20.

The structural comparison reveals that 5C01 and particularly 11D06 cover and bind to a larger fraction of the respective WT1 peptide than ESK1, which forms specific contacts exclusively with the N-terminal Arg of the RMF peptide while the remainder of the binding interface is provided by HLA-A02. Based on these observations one can conclude that 5C01 and 11D06 5 should be less likely to tolerate off-target peptides than ESK1 as they create significantly more steric hindrance for peptides with non-VLD or non-RMF-like sidechains on the exposed positions.

All graphical representations of the crystal structures were created with BIOVIA Discovery Studio 4.5, Dassault Systemes BIOVIA.

10

#### *Epitope and paratope residues (5 Å radius)*

A summary of the epitope and paratope for the binders 5C01, 11D06 and ESK1 is shown below. Epitope and paratope (definition based on 5 Å neighborhood radius) residues are highlighted in bold italic script. CDR residues are highlighted with grey background.

15 **HLA-A02**

**5C01** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAAASQRMEPRAPWIEQEGPEYW

**11D06** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAAASQRMEPRAPWIEQEGPEYW

**ESK1** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAAASQRMEPRAPWIEQEGPEYW

1-60

20 **5C01** DGET/GTVKAHSQTHRVDLGLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDG

**11D06** **DGETRKVKAI** SQTHRVDLGLRG YYNQSEAGSHTVQRMYGCDVGSDWRFLRG YHQ YAYDG

**ESK1** DGETRKVKAHSQTHR VDLGLRG YYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQ YAYDG

61-120

**5C01** KDYIALKEDLRSWTAADMAAQTTKHKWEAAtfEAEigLRAFLEGrCVEWLRRYLENGKETLQ

25 **11D06** KDY1A1,KEDI.RSWTAADMAAQTTKHKWEAAGVAEGLRAY|EGTCVEWLrj?YIENGKETI.Q

**ESK1** KDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEigL/MYLEGrCVEWLRRYLENGKETLQ

121-180

**5C01** RTDAPKTHMTHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT

**11D06** RTDAPKTHMTHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT

30 **ESK1** RTDAPKTHMTHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT

181-240

**5C01** FQKWA AVVVPSGQEQRYTCH VQHEGLPKPLTLRWE

**11D06** FQKWA AVVVPSGQEQRYTCHVQHEGLPKPLTLRWE

**ESK1** FQKWAAWPS GQEQR YTCHVQHEGLPKPLTLR WE

241-275

### Peptide

5 **5C01** VLDFAPPGA

**11D06** RMFPNAPYL

**ESK1** RMFPNAPYL

1-9

### 10 Fab Heavy Chain

**5C01** EVQLLESGGGLV QPGGSLRLS CAAS (i/TISSLANISWVRQAPGKGFFWVSA ISGS GGS TYY

**11D06** QVQLVQSGAEVKKPGSSVKVSCk. (S) (G)ISSLSSYAIISWVRQ APGQGLEWM (G)I/I/F(G)TANY

**ESK1** QMQLVQSGAEVKEPGESLRISCKGSi (Y)YSLFLM WISWVRQMPGKGLEWM (G)RY/DPGYSYSTY

1-60

15 **5C01** A(L)SYK (I)RFTISRDN SKNTLYLQMNSLR AEDTA VYYCAK (G)S H(Y) SY(A)FL(Y) VGQGTLVTVS

**11D06** A(Q)I FQGRVTITADKSTSTAYMEFSSLRSEDTAVYYCARS I (L)H (H)GGFDYWGQGTTVTVS

**ESK1** SPSFQG (I)IITISAMrSTSTAYLQWNSLKASDTAMYYCARYm (S)GYYDW (F)PWGQGTLVTVS

61-120

**5C01** SASTKGPS VFPLAPS SKSTSGGT AALGCL VKD YFPEP VTV SWNSGALT SGVHTFP AVLQS

20 **11D06** SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS

**ESK1** SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS

121-180

**5C01** SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

**11D06** SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

25 **ESK1** SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS

181-224

### Fab Light Chain

**5C01** DIQMTQSPSTLSASVGDRVTIT (RAS) (S) SIS SWL AWYQQKPGKAPKLLI YDASSI LES

30 **11D06** DIQMTQSPSTLSASVGDRVTITI (RAS) Q M.S SWL AWYQQKPGKAPKLLI YDASSI LES

**ESK1** QAVVTQP PSASGTPGQRVTIS \* (S)I SSSMG 'SN /VNWYQQVPGTAPKLLn (S)VNQRPS

1-60

**5C01** GVPSRFGSGSGTEFTLTISLQPDDFATY^ (C)QQYSI WFPYIFG (^G)TKVEIKRT VAAPS

**11D06** GVPSRFGSGSGTEFTLTISLQPDDFAT^ (Y)CQQYI: DYIIFG (^G)TKVEIKRT VAAPS

35 **ESK1** GVPDRFSGS/SrSGrSASLAISGLQSEDEADY YCAA (W)AS7..VGWA (F)GGG TK1/TVL GQPKANP

61-120

**5C01** VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS

**11D06** VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS

**ESK1** TVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYA

121-180

**5C01** LSSTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC  
**11D06** LSSTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC  
**ESK1** ASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

5 181-220

*Residues involved in chemical interactions*

A summary of the residues involved in chemical interaction for the binders 5C01, 11D06 and ESK1 is shown below. Residues involved in specific chemical interactions (compare Figures 16, 10 18 and 20) are highlighted in bold italic script. CDR residues are highlighted with grey background.

**HLA-A02**

**5C01** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAASQRMEPRAPWIEQEGPEYW  
**11D06** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAASQRMEPRAPWIEQEGPEYW  
15 **ESK1** GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAASQRMEPRAPWIEQEGPEYW  
1-60  
**5C01** DGETRKVKAHSQTHRVDLGLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDG  
**11D06** DGETRKVKAHSQTHRVDLGLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDG  
**ESK1** DGETRKVKAHSQTHRVDLGLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDG  
20 61-120  
**5C01** KDYIALKEDLRSWTAADMAAQTTKHKWEAA*HVAEQLR4*YLEGTCVEWLRRYLENGKE TLQ  
**11D06** KDYIALKEDLRSWTAADMAAQTTKHKWEAA*HVAEQLRA*YLEGTCVEWLRRYLENGKETLQ  
**ESK1** KDYIALKEDLRSWTAADMAAQTTKHKWEAA*HVAEigLRA*YLEGTCVEWLRRYLENGKETLQ  
121-180  
25 **5C01** RTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT  
**11D06** RTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT  
**ESK1** RTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGT  
181-240  
**5C01** FQKWAAVVPSGQEQRYTCH VQHEGLPKPLTLRWE  
30 **11D06** FQKWAAVVPSGQEQRYTCHVQHEGLPKPLTLRWE  
**ESK1** FQKWAAVWPS GQEQRYTCHVQHEGLPKPLTLRWE  
241-275

**Peptide**

35 **5C01** VLDEAPPGA  
**11D06** RMFPNAPYL  
**ESK1** RMFPNAPYL

**Fab Heavy Chain**5C01 EVQLLESGGGLV QPGGLRLS CAASG~~F~~~~F~~SS~~I~~AMS~~W~~VRQAPGKGLEW VS~~A~~~~I~~~~S~~~~S~~~~C~~STYY5 11D06 QVQLVQSGAEVKPGSSVKVSCKAS( ~~S~~~~C~~~~I~~~~I~~~~S~~~~S~~Y~~A~~IS~~W~~VRQAPGQGLEWIVK~~I~~~~I~~~~P~~~~I~~~~F~~~~G~~ITANYESK1 QMQLVQSGAEVKEPGESLRISCKGSi ~~I~~~~Y~~~~S~~~~I~~~~T~~~~M~~~~W~~IS~~W~~VRQMPGKGLEWM( ~~G~~~~R~~~~V~~~~D~~~~G~~~~I~~SYSTY

1-60

5C01 ~~A~~~~L~~~~S~~~~Y~~~~K~~~~C~~IRFTISRDN SKNTLYLQMNSLR AEDTAVYYCAK~~G~~~~s~~~~W~~~~I~~~~Y~~~~S~~~~T~~~~A~~~~N~~~~D~~~~Y~~~~W~~~~G~~~~Q~~~~G~~~~T~~~~L~~~~V~~~~T~~~~V~~~~S~~11D06 ~~A~~~~Q~~~~K~~~~F~~~~Q~~~~G~~~~R~~~~V~~~~T~~~~I~~~~T~~ ADKSTST AYMELS SLRSEDT AVYYCAk ~~S~~~~I~~~~E~~~~I~~~~Y~~~~H~~~~I~~~~F~~~~D~~~~Y~~~~W~~~~G~~~~Q~~~~G~~~~T~~~~T~~~~V~~~~T~~~~V~~~~S~~10 ESK1 ~~S~~~~P~~~~S~~~~F~~~~Q~~~~G~~~~I~~~~H~~~~V~~~~T~~~~I~~~~S~~~~A~~~~D~~~~K~~~~S~~~~T~~~~S~~~~A~~~~Y~~~~L~~~~Q~~~~W~~~~N~~~~S~~~~L~~~~K~~~~A~~~~S~~~~D~~~~T~~~~A~~~~M~~~~Y~~~~Y~~~~C~~~~A~~ ~~Y~~~~Q~~~~Y~~~~S~~~~C~~~~I~~~~Y~~~~I~~~~D~~~~W~~~~F~~~~L~~~~P~~~~W~~~~G~~~~Q~~~~G~~~~T~~~~L~~~~V~~~~T~~~~V~~~~S~~

61-120

5C01 SASTKGPSVFPLAPS SKSTSGGT AALGCL VKD YFPEP VTV SWNSGALT SGVHTFP AVLQS

11D06 SASTKGPSVFPLAPSSKSTSGGTAA~~L~~~~G~~~~C~~~~L~~~~V~~~~K~~~~D~~~~Y~~~~F~~~~P~~~~E~~~~P~~~~V~~~~T~~~~V~~~~W~~~~N~~~~S~~~~G~~~~A~~~~L~~~~T~~~~S~~~~G~~~~V~~~~H~~~~T~~~~F~~~~P~~~~A~~~~V~~~~L~~~~Q~~~~S~~ESK1 SASTKGPSVFPLAPSSKSTSGGTAA~~L~~~~G~~~~C~~~~L~~~~V~~~~K~~~~D~~~~Y~~~~F~~~~P~~~~E~~~~P~~~~V~~~~T~~~~V~~~~W~~~~N~~~~S~~~~G~~~~A~~~~L~~~~T~~~~S~~~~G~~~~V~~~~H~~~~T~~~~F~~~~P~~~~A~~~~V~~~~L~~~~Q~~~~S~~

15 121-180

5C01 SGLYSLSSVVTV~~P~~~~S~~~~S~~~~L~~~~G~~~~T~~~~Q~~~~T~~~~Y~~~~I~~~~C~~~~N~~~~V~~~~N~~~~H~~~~K~~~~P~~~~S~~~~N~~~~T~~~~K~~~~V~~~~D~~~~K~~~~V~~~~E~~~~P~~~~K~~~~S~~11D06 SGLYSLSSVVTV~~P~~~~S~~~~S~~~~L~~~~G~~~~T~~~~Q~~~~T~~~~Y~~~~I~~~~C~~~~N~~~~V~~~~N~~~~H~~~~K~~~~P~~~~S~~~~N~~~~T~~~~K~~~~V~~~~D~~~~K~~~~V~~~~E~~~~P~~~~K~~~~S~~ESK1 SGLYSLSSVVTV~~P~~~~S~~~~S~~~~L~~~~G~~~~T~~~~Q~~~~T~~~~Y~~~~I~~~~C~~~~N~~~~V~~~~N~~~~H~~~~K~~~~P~~~~S~~~~N~~~~T~~~~K~~~~V~~~~D~~~~K~~~~R~~~~V~~~~E~~~~P~~~~K~~~~S~~

18 1-224

20

**Fab Light Chain**5C01 DIQMTQSPSTLSASVGDRVTITi ~~C~~~~R~~~~A~~~~S~~ Q~~s~~~~I~~~~S~~ ~~S~~~~W~~~~L~~~~A~~~~W~~~~Y~~~~U~~~~Q~~~~K~~~~P~~~~G~~~~K~~~~A~~~~P~~~~K~~~~L~~~~I~~~~Y~~~~I~~~~Y~~~~A~~~~S~~~~S~~~~L~~~~E~~~~S~~11D06 DIQMTQSPSTLSASVGDRVTIT( ~~C~~~~R~~~~A~~~~S~~ Q~~S~~~~I~~~~S~~ ~~S~~~~W~~~~L~~~~A~~~~W~~~~Y~~~~Q~~~~Q~~~~K~~~~P~~~~G~~~~K~~~~A~~~~P~~~~K~~~~L~~~~I~~~~Y~~~~I~~~~Y~~~~A~~~~S~~~~S~~~~L~~~~E~~~~S~~ESK1 QAVVTQP PSASGTPGQRVTISI ~~C~~~~S~~~~C~~ ~~S~~~~S~~~~M~~~~G~~~~.S~~~~V~~ ~~T~~~~V~~~~N~~~~W~~~~Y~~~~Q~~~~Q~~~~V~~~~P~~~~G~~~~T~~~~A~~~~P~~~~K~~~~L~~~~I~~~~Y~~~~.S~~~~N~~~~N~~~~Q~~~~R~~~~P~~~~S~~

25 1-60

5C01 GVP~~S~~RGSGSGTEFTLT~~I~~~~S~~~~L~~~~Q~~~~P~~~~D~~~~F~~~~A~~~~T~~~~Y~~ ~~C~~~~Q~~~~Q~~~~I~~~~S~~~~I~~~~W~~~~F~~~~P~~~~Y~~~~I~~~~F~~~~G~~~~Q~~~~G~~~~I~~~~K~~~~V~~~~E~~~~I~~~~K~~~~R~~~~T~~~~V~~~~A~~~~P~~~~S~~11D06 GVP~~S~~RGSGSGTEFTLT~~I~~~~G~~~~S~~~~L~~~~Q~~~~P~~~~D~~~~F~~~~A~~~~T~~~~Y~~ ~~Y~~~~C~~~~Q~~~~O~~~~Y~~~~E~~ ~~L~~~~Y~~~~T~~~~I~~~~F~~~~C~~~~K~~~~G~~~~I~~~~K~~~~V~~~~E~~~~I~~~~K~~~~R~~~~T~~~~V~~~~A~~~~P~~~~S~~ESK1 GVP~~D~~RGSGSKSGTSASLAISGLQSEDEADY ~~Y~~~~C~~~~A~~~~A~~~~W~~~~I~~~~D~~~~S~~~~L~~~~N~~~~G~~~~W~~~~W~~~~I~~~~Y~~~~J~~~~G~~~~G~~~~T~~~~K~~~~L~~~~T~~~~V~~~~L~~~~G~~~~Q~~~~P~~~~K~~~~A~~~~N~~~~P~~

61-120

30 5C01 VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS

11D06 VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS

ESK1 TVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYA

121-180

5C01 LSSTLTL~~S~~KADYE~~K~~~~H~~~~K~~~~V~~~~Y~~~~A~~~~C~~~~E~~~~V~~~~T~~~~H~~~~Q~~~~G~~~~L~~~~S~~~~P~~~~V~~~~T~~~~K~~~~S~~~~F~~~~N~~~~R~~~~G~~~~E~~~~C~~35 11D06 LSSTLTL~~S~~KADYE~~K~~~~H~~~~K~~~~V~~~~Y~~~~A~~~~C~~~~E~~~~V~~~~T~~~~H~~~~Q~~~~G~~~~L~~~~S~~~~P~~~~V~~~~T~~~~K~~~~S~~~~F~~~~N~~~~R~~~~G~~~~E~~~~C~~ESK1 ASSYLSLTPEQWKSHRSYSCQVT~~H~~~~E~~~~G~~~~S~~~~T~~~~V~~~~E~~~~K~~~~T~~~~V~~~~A~~~~P~~~~T~~~~E~~~~C~~~~S~~

18 1-220

**Example 16. Comparison of HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) with different CD3 binders**

We compared the potency of killing activities of 11D06-TCB with different CD3 binders, using SKM-1 cells in an assay as described above (Example 7). CH2527 is the CD3 binder of the

5 TCBs used in the previous experiments (see SEQ ID NOs 121 and 122 for the VH and VL sequences). V9 is a CD3 binder described Rodrigues et al., Int J Cancer Suppl (1992) 7, 45-50, and WO 1992/22653 (see SEQ ID NOs 136 and 137 for the VH and VL sequences), and 40G5C is described in WO 2015/095392 (see SEQ ID NOs 184 and 185 (“hu40G5c”) of WO 2015/095392 for the VH and VL sequences).

10 The affinity of CD3 binders CH2527, 40G5C and V9 are shown in Table 8.

As shown in Figure 21, we observed that 11D06-TCB (CH2527) showed the same potency of T cell-mediated killing on HLA-A2<sup>+</sup>WT1<sup>+</sup> SKM-1 cell lines as 11D06-TCB (V9), whereas 11D06-TCB (40G5C) showed strongly reduced killing potency.

**Table 8.** Affinities of CD3 binders compared in this experiment.

CD3 clone	Cross-reactivity	Affinity (Kd) [nM]
CH2527	Hu-Cyno	85-130
40G5C	Hu-Cyno	390-460
V9	Hu only	35-50

15

**Example 17. T cell cytotoxicity mediated by HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) upon binding to RMF peptide-pulsed T2 cells**

The killing activity of 11D06-TCB (V9) (see SEQ ID NOs 123, 125, 139, 140 for full sequences) was compared to analogous TCBs with different HLA/WT1 binders (“Aali” and “Daniel”, see

20 WO 2017/060201, SEQ ID NOs 5 (VH) and 6 (VL) and SEQ ID NOs 35 (VH) and 36 (VL), respectively). The ESK1-TCB and the untargeted DP47-TCB were also included as controls. The experiment was performed as described in Example 6. The RMF peptide-pulsed cells (100 µ,

2x10<sup>5</sup> cells/ml) were co-cultured with 50 µg of T cells (2x10<sup>6</sup> cells/ml), and with serial dilutions of TCB (50 µg) for 24 hours at 37°C with 5% CO<sub>2</sub>.

Figure 22 shows the TCB-mediated specific T cell killing of RMF-expressing target cells. We found that 11D06-TCB and ESK1-TCB mediated potent killing on RMF peptide-pulsed T2 cells, whereas the TCBs based on the HLA/WT1 binders “Aali” and “Daniel” (Aali-TCB and Daniel-TCB) showed weak killing of RMF-pulsed cells only at the highest concentrations.

#### **Example 18. No binding of selected HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) to off-target peptides**

10 We also compared the cross-reactivity of 11D06-TCB (V9), Aali-TCB, Daniel-TCB and ESK1-TCB with the off-target peptides described in Examples 9 and 10.

For this experiment, Jurkat NFAT reporter cells expressing an anti-PGLALA chimeric antigen receptor (CAR) were used (CAR J assay, see PCT application claiming priority from European patent application no. EP17209201.7, incorporated herein by reference in its entirety). The anti-15 PGLALA CAR recognizes the P329G L234A L235A (“PGLALA”, EU numbering) mutations in the Fc region of the TCBs. Peptide-pulsed T2 cells as described above were used as target cells. The principle of the assay is to co-culture the Jurkat-NFAT engineered effector cells with target cells. Only upon simultaneous binding of the TCBs to the CAR (via the PGLALA mutation) and the target antigen, the NFAT promoter is activated and leads to increasing luciferase expression 20 in the Jurkat effector cells. Upon addition of an adequate substrate, active Firefly Luciferase leads to emission of luminescence, which can be measured as a signal of CAR-mediated activation.

Briefly, target cells were harvested and viability determined. 20 000 target cells/well were plated in a round-bottom, 96-well-plate (Greiner bio-one, #650180) in 100 µg medium. Non-pulsed T2 25 cells were used as negative control. 50 µl/well of diluted TCB were added to the target cells. Subsequently, Jurkat-NFAT reporter cells were harvested and viability assessed, resuspended in cell culture medium and added to tumor cells at 100 000 cells/well (50 µl/well) to obtain a final effector-to-target (E:T) ratio of 5:1 and a final volume of 200 µg per well. Cells were incubated for 20 h at 37°C in a humidified incubator. At the end of the incubation time, the plates were

adapted to room temperature (about 15 min). 125  $\mu$ l of media/well was removed from the top and 25  $\mu$ l/well of One-Glo Luciferase (Promega #E6120) was added, and mixed. 100  $\mu$ l/well of the mixture was then transferred to 96 well flat bottom plate (Greiner bio-one, #655098) and the plate was incubated for 15 min in the dark before luminescence was detected using Perkin Elmer.

5 As shown in Figure 23, Aali-TCB (A), Daniel-TCB (B) and ESK1-TCB (C) resulted in weaker activation of Jurkat NFAT reporter cell line (corresponding to weaker killing shown in Figure 22) and moreover, a smaller window between recognition of RMF peptide and other off-target peptides comparing to 11D06-TCB (V9) (D).

10 **Example 19. Pharmacokinetic profile of HLA-A2/WT1 x CD3 bispecific antibody (11D06-TCB (V9)) after single injection in NSG mice**

A single dose of 1 mg/kg of 11D06-TCB (V9) was injected into humanized and tumor-bearing NSG mice. Mice were injected i.v. with 200  $\mu$ l of TCB, diluted with histidine buffer. Three mice per time point were bled at 10 min, 6 h, 24 h, 72 h and 7 days. The injected compound was 15 analyzed in serum samples by ELISA as described in Example 13.

The result (Figure 24) showed a stable PK-behaviour for the tested TCB, which suggested a once weekly schedule for subsequent efficacy studies.

20 **Example 20. Efficacy study with HLA-A2/WT1 x CD3 bispecific antibody (11D06-TCB (V9)) in SKM-1 xenograft in humanized mice**

This efficacy study was aimed to evaluate the efficacy of 11D06-TCB (V9) in a human AML xenograft (SKM-1) in fully humanized NSG mice. The experiment was performed as described in Example 14.

Figure 25 shows the tumor growth kinetics (mean, Figure 25A) as well as the single tumor 25 growth kinetics in each group (Figure 25 B, C). As shown, the TCB exhibits tumor growth inhibition a TGI of 78 at study day 48 (Figure 25D).

**Example 21. Comparison of HLA-A2/WT1 x CD3 bispecific antibodies (“TCBs”) with different molecular formats**

We compared the activity of 11D06-TCB (V9) with an analogous molecule in a “1+1 CrossMab” format (as depicted in Figure 1A). Activity was monitored using a cell-based functional assay

5 which detects TCB-mediated activation of a reporter cell line (Jurkat NFAT) in the presence of target cells in a dose-dependent manner. The assay was performed essentially as described in Example 5 above, using as target cells CHO-K1 cells expressing a HLA-A02/WT1<sub>RMF</sub> pMHC complex. Reporter cell activation occurs upon simultaneous binding of the TCB to the HLA-A02/WT1<sub>RMF</sub> pMHC complex on target cells and to the CD3 $\epsilon$  unit of T cell receptor (TCR) on 10 reporter cells, which leads to hyper-clustering of CD3 and thereby to TCR activation. Subsequent initiation of corresponding intracellular signaling pathways results in activation of transcription factor NFAT which induces expression of a NFAT-driven luciferase reporter gene. Activity of luciferase reporter is measured upon addition of substrate by luminescence read-out.

The result of this experiment is shown in Figure 26. Relative light units (RLU) reflecting target- 15 dependent reporter cell activation are plotted against antibody concentration. As can be seen in Figure 26, the molecule in the “1+1” format showed a lower activity than the “2+1” format in this assay.

## Amino Acid Sequences

	Sequence	SEQ ID NO
11D06 HCDR1	SYAIS	1
11D06 HCDR2	GIPIFGTANYAQKFQG	2
11D06 HCDR3	SIELWWGGFDY	3
11D06 LCDR1	RASQSISSWLA	4
11D06 LCDR2	DASSLES	5
11D06 LCDR3	QQYEDYTT	6
11D06 VH	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARSIELWWGGFDYWQGTTVTVSS	7
11D06 VL	DIQMTQSPSTLSASVGDRVITCRASQSISSWLA YQQKPG KAPKLLIYDASSLES GVPSRFS SGSGTEFTLT IGSLQPDDFA TYYCQQYEDYTTFGQGTKVEIK	8
33H09 HCDR1	SYAIS	9
33H09 HCDR2	GIPIFGTANYAQKFQG	10
33H09 HCDR3	GSYDLFSLDY	11
33H09 LCDR1	RASQSISSWLA	12
33H09 LCDR2	DASSLES	13
33H09 LCDR3	QQYYDGIT	14
33H09 VH	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARGSYDLFSLDYWQGTTVTVSS	15
33H09 VL	DIQMTQSPSTLSASVGDRVITCRASQSISSWLA YQQKPG KAPKLLIYDASSLES GVPSRFS SGSGTEFTLT ISSLQPDDFA TYYCQQYYDGITFGQGTKVEIK	16
13B04 HCDR1	SYYWS	17
13B04 HCDR2	YIYYSGSTNYNPSLKS	18
13B04 HCDR3	VSYNGLDY	19
13B04 LCDR1	RASQSISSWLA	20
13B04	DASSLES	21

LCDR2		
13B04 LCDR3	QQYNMWNPVT	22
13B04 VH	EVQLLESGPGLVKPSETLSLTCTVSGGSISYYWSWIRQPPG KGLEWIGIYYSGSTNYNPSLKSRSVTISVDTSKNQFSLKLSS VTAADTAVYYCARVSYNGLDYWGQGTLTVSS	23
13B04 VL	DIQMTQSPSTLSASVGDRVITCRASQSISSWLA YQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSSLQPD DDFA TYYCQQY NMWN PVT FGQG TKVEIK	24
11B09 HCDR1	SYAIS	25
11B09 HCDR2	GIPIFGTANYAQKFQG	26
11B09 HCDR3	VPGRWYGAMDY	27
11B09 LCDR1	RASQSISSWLA	28
11B09 LCDR2	DASSLES	29
11B09 LCDR3	QQEDDYPLT	30
11B09 VH	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPFGTANYAQKFQGRV TITADKSTSTAYM ELSSLRSE DTAVYYCARV PGRWYGAMDY WGQGTT TVSS	31
11B09 VL	DIQMTQSPSTLSASVGDRVITCRASQSISSWLA YQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSSLQPD DDFA TYYCQQ E DDYPLT FGQG TKVEIK	32
33F05 HCDR1	SYYWS	33
33F05 HCDR2	YIYYSGSTNYNPSLKS	34
33F05 HCDR3	SYYEAFDY	35
33F05 LCDR1	QGDSLRSYYAS	36
33F05 LCDR2	GKNNRPS	37
33F05 LCDR3	NSPDMNGNAV	38
33F05 VH	QVQLQESGPGLVKPSETLSLTCTVSGGSINSYYWSWIRQPP GKGL E WIGIYYSGSTNYNPSLKSRS VTISVDT SKNQFSLKLS SVTAADTA VYYCARS YY EA FDY WGQG TL TVSS	39
33F05 VL	SSELTQDPAVSVALGQT VRITCQGD SLRSYYAS WYQQKPG QAPVLVIY GKNNRPS GIPDR FSG SSSG NTAS LT ITGA QA EDE ADYYC NSP DMNGNA VFGGG TKL TVL	40
5E11 HCDR1	SYAIS	41
5E11 HCDR2	GIPIFGTANYAQKFQG	42
5E11 HCDR3	SSYDLYSF DY	43
5E11 LCDR1	RASQSISSWLA	44
5E11 LCDR2	DASSLES	45
5E11 LCDR3	QQYSF PP MIT	46
5E11 VH	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA	47

	PGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARSSYDLYSF DYWGQGTTVTVSS	
5E11 VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSLQPDDFA TYYCQQYSFPPMITFGQG TKVEIK	48
13E08 HCDR1	SYAMS	49
13E08 HCDR2	AISGGSTYYADSVKG	50
13E08 HCDR3	TYPYTGSFDY	51
13E08 LCDR1	RASQSISSWLA	52
13E08 LCDR2	DASSLES	53
13E08 LCDR3	QQNYNYPPT	54
13E08 VH	EVQLLESGGGLVQPGGSLRLSCAAS GFTFSSYAM SWVRQA PGKGLEWVSAISGGSTYYADSV KGRFTISRDNSKNTLYL QMNSLRAEDTAVYYCA KTY TGSFDYWGQG TLVTVSS	55
13E08 VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSLQPDDFA TYYCQQNYNYPPTFGQG TKVEIK	56
5C01 HCDR1	SYAMS	57
5C01 HCDR2	AISGGSTYYADSVKG	58
5C01 HCDR3	G SWVSYAFDY	59
5C01 LCDR1	RASQSISSWLA	60
5C01 LCDR2	DASSLES	61
5C01 LCDR3	QQYSIWFPYT	62
5C01 VH	EVQLLESGGGLVQPGGSLRLSCAAS GFTFSSYAM SWVRQA PGKGLEWVSAISGGSTYYADSV KGRFTISRDNSKNTLYL QMNSLRAEDTAVYYCA KG GSWVSYAFDYWGQG TLVTVSS	63
5C01 VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSLQPDDFA TYYCQQYSIWFPYT FGQG TKVEIK	64
11G06 HCDR1	SYAIS	65
11G06 HCDR2	GIIPIFGTANYAQKFQG	66
11G06 HCDR3	TGPYYGAFDY	67
11G06 LCDR1	RASQSISSWLA	68
11G06 LCDR2	DASSLES	69
11G06 LCDR3	QQGFRGYT	70
11G06 VH	QVQLVQSGAEVKKPGSSVKVSCKASGG TSSY AISWVRQA PGQGLEWMGGIPIFGTANYAQKFQGR VTITADKSTSTAYM ELSSLRSEDTAVYYCART GPYYGAFDYWGQG TTVTVSS	71
11G06 VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPG KAPKLLIYDASSLES GVPSRFSGSGSGTEFTLT ISSLQPDDFA	72

	TYYCQQGFRGYTFGQGTKVEIK	
ESK1 VH	QMQLVQSGAEVKEPGESLRISCKSGYSFTNFWISWVRQM PGKGLEWMGRVDPGYSYSTYSPSPFQGHVTISADKSTSTAY LQWNSLKASDTAMYCARVQYSGYYDWFDPWGQGTLVT VSS	73
ESK1 VL	QAWTQPPSASCTPGQRTTISCSSNIGSNTVNWYQQVPG TAPKLLIYSNNQRPSGVPDFSGSKSGTSASLAISGLQSEDE ADYYCAAWDDSLNGWVFGGGTKLTVL	74
Untargeted VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA PGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYL QMNSLRAEDTAVYYCAKGSGFDYWGGQGTLTVSS	75
Untargeted VL	EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKP GQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDF AVYYCQQYGSPLTFGQGTLTVSS	76
VLD peptide	VLDFAAPPAGA	77
RMF peptide	RMFPNAPYL	78
MED13L	RMFPPTPSL	79
PIGQ	RMFPGEVAL	80
ARHGEF11	RLFPNLPEL	81
PRDM16	RMFPNKYSL	82
SERPINA6	AMDPNAAYV	83
NIPSNAP1	RMGPNIYEL	84
TAF3	NMPPNFYI	85
U4	YTIPNHPYL	86
TPST1	RLFPNAKFL	87
IGFBP5	RMVPRAVYL	88
RALGPS1	KMVPsipYL	89
ZNF382	RIFPSYSYL	90
TPST2	RLFPNSKFL	91
RALGPS2	KMTPCIPYL	92
TBCE	SMFPSLKYL	93
KIFC2	RLLPSAPTL	94
SLC16A8	RLRPHVPYL	95
ERH	RMNPNSPSI	96
ZBTB47	RVFNNRWYL	97
MIOS	RLQLNNPYL	98
ATP6V0A2	RMFFNGRYI	99
PKD1	RLSPNRPPL	100
NAALADL1	ETFPNSWYL	101
ROBO1	GLKPNAIYL	102
RECQL	RQFPNASLI	103
SERPINA2	YIFPNCPFL	104
FAM220A	RLRINFYPL	105
Human WT1	MGSDVRDLNALLPAVPSLGGGGCALPVGAAQWAPVLD FAPPGASAYGSLGGPAPPAPPAPPAPPHSFIKQEPEWGGA EPHEEQCLSAFTVHFSGQFTGTAGACRYGPFGPPPSQASS GQARMFPNAPYLPSCLESQPAIRNQGYSTVTFDGTPSYGHT PSHHAACQFPNHSFKHEDPMGQQGSLGEQQYSVPPPVGCH TPTDSCTGSQALLRTPYSSDNLYQMTSQLECMTNQMNL	106

	GATLKGVAAGSSSVKWTGQSNHSTGYESDNHTTPILCG AQYRIHTHGVFRGIQDVRRVPGVAPTLVRSASETSEKPFM CAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFKDCERR FSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTR THTGKTSEKPFSCRWPSCQKKFARSDELVRHHNMQRNM TKLQLAL	
Human CD3	MQSGTHWRVLGLCLLSVGVGQDGNEEMGGITQTPYKVS ISGTTVILTCPQYPGSEILWQHNDKNIGGDEDDKNIGSDEDH LSLKEFSELEQSGYYVCYPRGSKPEDANFYLYLRARVCENC MEMDVMSVATIVIVDICITGGLLLVYYWSKNRKAACP TRGAGAGGRQRGQNKERPPPVPNPDYEPIRGQRDLYSGL NQRRI	107
Cynomolgus CD3	MQSGTRWRVLGLCLLSIGVGQDGNEEMGSITQTPYQVSI SGTTVILTCSQHLGSEAQWQHNGKNEDSGDRLFLPEFSE MEQSGYYVCYPRGSNPEDASHHLYLKARVCENCMEMDV MAVATIVIVDICITLGLLLVYYWSKNRKAACP AGGRQRGQNKERPPPVPNPDYEPIRGQRDLYSGLNQRRI	108
hIgG1 Fc region	DKTHTCPCPAPELLGGPSVFLFPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI KGQPREPVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWE SNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSP	109
linker	GGGGSGGGGS	110
linker	DGGGGSGGGGS	111
Human kappa CL domain	RTVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPREAKVQW KVDNALQSGNSQESVTEQDSKDSTYSLSSTTLSKADYEK HKVYACEVTHQGLSSPVTKSFNRGEC	112
Human lambda CL domain	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYP GAVTVAW KADSSPVKAGVETTPSKQSNN YAASSYLSLTPEQWKSH RSYSCQVT THEGSTVEKTVAPTECS	113
Human IgG1 heavy chain constant region (CH1- CH2-CH3)	ASTKGPSVFLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSW NSGALTSGVHTFP AVLQSSGLYSLSSV VTPSSSLGTQTYIC NVNHKPSNTK VDKKVEPKSCDK THTCPCPAPEL GGPSVF LFPPKPKDTLM ISRTPEV TCVVVDV SHEDPEV KFNWY VDG VEVHNA KTPREE QYNSTYR VVS VLT VLHQDW LNGKEY K CKV SNK ALP API EKTI KGQ PRE PVY TLPP SRDEL TKN QV SLT CLV KG F YPSD IA VE WE SNG QPEN NYK TPP VLD SD GS FFLY SKLT VDK SRW QQGN V F SCS VMHE ALHN HYT QK SL SP	114
CD3 HCDR1	TYAMN	115
CD3 HCDR2	RIRSKYNNYATYYADSVKG	116
CD3 HCDR3	HGNFGNSYVSWFAY	117
CD3 LCDR1	GSSTGAVTTSNYAN	118
CD3 LCDR2	GTNKRAP	119
CD3 LCDR3	ALWYSNLWV	120
CD3 VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQA PGKGLEWVSRIRSKYNNYATYYADSVKG RFTISRDDSKNT LYLQMNSLRAEDTA VYYCVRHGNFGNSYV SWFAYWGQG	121

	TLTVVSS	
CD3 VL	QAVVTQEPLTVSPGGTVLTCGSSTGAVTSNYANWVQE KPGQAFRGLIGCTNKRAPGTPARFSGSLLGGKAALTLSGAQ PEDEAEYYCALWYSNLWVFGGGTKLTVL	122
WT1 11D06 VH-CH1(EE)- Fc(hole, PGLALA)	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARSIELWWGGFDYWQGQTTVTVSSA STKGPSVFLAPSSKSTSGGTAAALGCLVEDYFPEPVTVSWN SGALTSGVHTFPALQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDEKVEPKSCDKHTCPCPAPEAAGGPSVFL FPPPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALGAPIEKTISKAKGQPREPVQVCTLPPSRDELTKNQ VSLSCAVKGFPYPSDIAVEWESNGQPENNYKTPPVLDSDGS FFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SP	123
WT1 11D06 VH-CH1(EE)- CD3 VL-CH1- Fc(knob, PGLALA)	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARSIELWWGGFDYWQGQTTVTVSSA STKGPSVFLAPSSKSTSGGTAAALGCLVEDYFPEPVTVSWN SGALTSGVHTFPALQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDEKVEPKSCDGCGGGSGGGGSQAVTQEPL TVSPGGTVLTCGSSTGAVTSNYANWVQEKGQAFRGLI GGTNKRAPGTPARFSGSLLGGKAALTLSGAQPEDEAEYYC ALWYSNLWVFGGGTKLTVLSSASTKGPSVFLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPALQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKVEPK SCDKHTCPCPAPEAAGGPSVFLPPKPKDLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKA KGQPREPVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAV EWESNGQPENNYKTPPVLDSDGSFFYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSP	124
WT1 11D06 VL-CL(RK)	DIQMTQSPSTLSAVGDRVTITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESGVPSRFSGSGSGTEFTLTIGSLQPDDFA TYYCQQYEDYTFQGQGTKVEIKRTVAAPSVFIFPPSDRKLK SGTASVVCLNNFYPREAKVQWKVDNALQSGNSQESVTE QDSKDSTYSLSTTLSKADYEKHKVYACEVTHQGLSSPVT KSFNRGEC	125
WT1 33H09 VH-CH1(EE)- Fc(hole, PGLALA)	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARGSYDLFSLDYWGQGQTTVTVSSAS TKGPSVFLAPSSKSTSGGTAAALGCLVEDYFPEPVTVSWNS GALTSGVHTFPALQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDEKVEPKSCDKHTCPCPAPEAAGGPSVFLF PPPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALGAPIEKTISKAKGQPREPVQVCTLPPSRDELTKNQV SLSACAVKGFPYPSDIAVEWESNGQPENNYKTPPVLDSDGSF FLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP	126

	P	
WT1 33H09 VH-CH1(EE)- CD3 VL-CH1- Fc(knob, PGLALA)	QVQLVQSGAEVKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPIFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARGSYDLFSLDYWGQGTTVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNS GALTSGVHTFPALQSSGLYSLSSVTVPSSLGTQTYICNV NHKPSNTKVDEKVEPKSCDGGGGSGGGGSQAVVTQEPSLT VSPGGTVTLCGSSTGAVTTSNYANWVQEKGQAFRGLIG GTNKRAPGTPARFSGSLLGGKAALTSGAQPEDEAEYYCA LWYSNLWVFGGGTKLTVLSSASTKGPSVFPLAPSSKSTSGG TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPALQSSG LYSLSSVTVPSSLGTQTYICNVNHKPSNTKVDDKKVEPKS CDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTIKAK GQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSP	127
WT1 33H09 VL-CL(RK)	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESQVPSRFSGSGSGTEFTLTISSSLQPDFA TYYCQQYYDGITFGQGTKVEIKRTVAAPSVFIFPPSDRKLKS GTASVVCLNNFYPREAKVQWKVDNALQSGNSQESVTEQ DSKDSTYSLSSTLTLKADYEKHKVYACEVTHQGLSSPVTK SFNRGEC	128
CD3 VH-CL	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQA PGKGLEWVSRIRSKYNNYATYYADSVKGRFTISRDDSKNT LYLQMNSLRAEDTAVYYCVRHGNFGNSYVWFAYWGQG TLTVVSSASVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPR EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL KADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	129
CD3 HCDR1 (V9)	GYTMN	130
CD3 HCDR2 (V9)	LINPYKGVSTYNQKFKD	131
CD3 HCDR3 (V9)	SGYYGDSDWYFDV	132
CD3 LCDR1 (V9)	RASQDIRNYLN	133
CD3 LCDR2 (V9)	YTSRLES	134
CD3 LCDR3 (V9)	QQGNTLPWT	135
CD3 VH (V9)	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQ APGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTA YLQMNSLRAEDTAVYYCARSQYGDSDWYFDVWGQGTL VTVSS	136
CD3 VL (V9)	DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPG KAPKLLIYYTSRLESQVPSRFSGSGSGTDYTLTISSLQPEDFA TYYCQQGNTLPWTFGQGTKVEIK	137

HLA-A2	GHSMRYYFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSAA SQRMEPRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGT LRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAY DGKDYIALKEDLSWTAADMAAQTTKHKWEAAHVAEQL RAYLEGTCVEWLRRYLENGKETLQRTDAPKTHMTHAVS DHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRP AGDGTFFQKWAAVVPSGQEQRYTCHVQHEGLPKPLTLRW E	138
WT1 11D06 VH-CH1(EE)- CD3 (V9) VL- CH1-Fc(knob, PGLALA)	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQA PGQGLEWMGGIIPFGTANYAQKFQGRVTITADKSTSTAYM ELSSLRSEDTAVYYCARSIELWWGGFDYWQGQTTVTVSSA STKGPSVFLAPSSKSTSGGTAAALGCLVEDYFPEPVTVSWN SGALTSGVHTFPALQSSGLYSLSSVVTVPSSLGTQTYICN VNHKPSNTKVDEKVEPKSCDGGGGSGGGGSDIQMTQSPSS LSASVGDRVITCRASQDIRNYLNWYQQKPGKAPKLLIYYT SRLESGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNT LPWTFGQGTKVEIKSSASTKGPSVFLAPSSKSTSGGTAAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPALQSSGLYSLSS VVTVPSSLGTQTYICNVNHHKPSNTKVDDKVEPKSCDKTH TCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQP PQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNG QPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSP	139
CD3 (V9) VH-CL	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQ APGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTA YLQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTL VTVSSASVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	140

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

### Claims

1. An antibody that binds to HLA-A2/WT1, wherein the antibody binds an epitope of HLA-A2/WT1, particularly HLA-A2/WT1<sub>RMF</sub>, comprising at least three amino acid residues of the 5 WT1 peptide in HLA-A2/WT1, particularly the WT1<sub>RMF</sub> peptide.
2. The antibody of claim 1, wherein said at least three amino acid residues are selected from the amino acid residues corresponding to amino acid residues R1, M2, P4, N5, A6 and Y8 of the WT1RMF peptide.
3. An antibody that binds to HLA-A2/WT1, wherein the antibody comprises 10 (i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementarity determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO: 6;
- 15 (ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;
- 20 (iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;
- 25 (iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;
- 30 (v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;
- (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;
- 35 (vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR

5 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70.

4. The antibody of claim 3, wherein the antibody comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

20 (iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

(v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

(vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

(vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino

5 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

(ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino

10 acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

5. The antibody of any one of claims 1 to 4, wherein the antibody is an IgG, particularly an IgGi, antibody.

6. The antibody of any one of claims 1 to 5, wherein the antibody is a full-length antibody.

7. The antibody of any one of claims 1 to 5, wherein the antibody is an antibody fragment

15 selected from the group of an Fv molecule, a scFv molecule, a Fab molecule, and a F(ab')2 molecule.

8. The antibody of any one of claims 1 to 7, wherein the antibody is a multispecific antibody.

9. A bispecific antigen binding molecule, comprising

20 (a) a first antigen binding moiety that binds to a first antigen, wherein the first antigen is HLA-A2/WT1 and the first antigen binding moiety comprises

(i) a heavy chain variable region (VH) comprising a heavy chain complementary determining region (HCDR) 1 of SEQ ID NO: 1, a HCDR 2 of SEQ ID NO: 2, and a HCDR 3 of SEQ ID NO: 3, and a light chain variable region (VL) comprising a light chain complementary determining region (LCDR) 1 of SEQ ID NO: 4, a LCDR 2 of SEQ ID NO: 5 and a LCDR 3 of SEQ ID NO:

25 6;

(ii) a VH comprising a HCDR 1 of SEQ ID NO: 9, a HCDR 2 of SEQ ID NO: 10, and a HCDR 3 of SEQ ID NO: 11, and a VL comprising a LCDR 1 of SEQ ID NO: 12, a LCDR 2 of SEQ ID NO: 13 and a LCDR 3 of SEQ ID NO: 14;

(iii) a VH comprising a HCDR 1 of SEQ ID NO: 17, a HCDR 2 of SEQ ID NO: 18, and a HCDR 3 of SEQ ID NO: 19, and a VL comprising a LCDR 1 of SEQ ID NO: 20, a LCDR 2 of SEQ ID NO: 21 and a LCDR 3 of SEQ ID NO: 22;

(iv) a VH comprising a HCDR 1 of SEQ ID NO: 25, a HCDR 2 of SEQ ID NO: 26, and a HCDR 5 3 of SEQ ID NO: 27, and a VL comprising a LCDR 1 of SEQ ID NO: 28, a LCDR 2 of SEQ ID NO: 29 and a LCDR 3 of SEQ ID NO: 30;

(v) a VH comprising a HCDR 1 of SEQ ID NO: 33, a HCDR 2 of SEQ ID NO: 34, and a HCDR 3 of SEQ ID NO: 35, and a VL comprising a LCDR 1 of SEQ ID NO: 36, a LCDR 2 of SEQ ID NO: 37 and a LCDR 3 of SEQ ID NO: 38;

10 (vi) a VH comprising a HCDR 1 of SEQ ID NO: 41, a HCDR 2 of SEQ ID NO: 42, and a HCDR 3 of SEQ ID NO: 43, and a VL comprising a LCDR 1 of SEQ ID NO: 44, a LCDR 2 of SEQ ID NO: 45 and a LCDR 3 of SEQ ID NO: 46;

(vii) a VH comprising a HCDR 1 of SEQ ID NO: 49, a HCDR 2 of SEQ ID NO: 50, and a HCDR 3 of SEQ ID NO: 51, and a VL comprising a LCDR 1 of SEQ ID NO: 52, a LCDR 2 of 15 SEQ ID NO: 53 and a LCDR 3 of SEQ ID NO: 54;

(viii) a VH comprising a HCDR 1 of SEQ ID NO: 57, a HCDR 2 of SEQ ID NO: 58, and a HCDR 3 of SEQ ID NO: 59, and a VL comprising a LCDR 1 of SEQ ID NO: 60, a LCDR 2 of SEQ ID NO: 61 and a LCDR 3 of SEQ ID NO: 62; or

(ix) a VH comprising a HCDR 1 of SEQ ID NO: 65, a HCDR 2 of SEQ ID NO: 66, and a HCDR 20 3 of SEQ ID NO: 67, and a VL comprising a LCDR 1 of SEQ ID NO: 68, a LCDR 2 of SEQ ID NO: 69 and a LCDR 3 of SEQ ID NO: 70, and

(b) a second antigen binding moiety which specifically binds to a second antigen.

10. The bispecific antigen binding molecule of claim 9, wherein the first antigen binding moiety comprises

25 (i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8;

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 30 100% identical to the amino acid sequence of SEQ ID NO: 15, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 16;

(iii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 23, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 24;

5 (iv) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 31, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 32;

10 (v) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 39, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 40;

15 (vi) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 47, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 48;

20 (vii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 55, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 56;

(viii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 63, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 64; or

25 (ix) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 71, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 72.

30 11. The bispecific antigen binding molecule of claim 9 or 10, wherein the second antigen is CD3, particularly CD3 $\epsilon$ .

12. The bispecific antigen binding molecule of claim 11, wherein the second antigen binding moiety comprises

(i) a VH comprising a HCDR 1 of SEQ ID NO: 115, a HCDR 2 of SEQ ID NO: 116, and a HCDR 3 of SEQ ID NO: 117, and a VL comprising a LCDR 1 of SEQ ID NO: 118, a LCDR 2 of SEQ ID NO: 119 and a LCDR 3 of SEQ ID NO: 120; or

(ii) a VH comprising a HCDR 1 of SEQ ID NO: 130, a HCDR 2 of SEQ ID NO: 131, and a

5 HCDR 3 of SEQ ID NO: 132, and a VL comprising a LCDR 1 of SEQ ID NO: 133, a LCDR 2 of SEQ ID NO: 134 and a LCDR 3 of SEQ ID NO: 135.

13. The bispecific antigen binding molecule of claim 12, wherein the second antigen binding moiety comprises

(i) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or

10 100% identical to the amino acid sequence of SEQ ID NO: 21, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 22; or

(ii) a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or

15 100% identical to the amino acid sequence of SEQ ID NO: 136, and a VL comprising an amino

acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 137.

14. The bispecific antigen binding molecule of any one of claims 9 to 13, wherein the first and/or the second antigen binding moiety is a Fab molecule.

15. The bispecific antigen binding molecule of any one of claims 9 to 14, wherein the second

20 antigen binding moiety is a Fab molecule wherein the variable domains VL and VH or the constant domains CL and CH1, particularly the variable domains VL and VH, of the Fab light chain and the Fab heavy chain are replaced by each other.

16. The bispecific antigen binding molecule of any one of claims 9 to 15, wherein the first antigen binding moiety is a Fab molecule wherein in the constant domain the amino acid at

25 position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat) and the amino acid at position 123 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Rabat), and in the constant domain CH1 the amino acid at position 147 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index) and the amino acid at position 213 is substituted independently by glutamic acid (E), or aspartic acid (D) (numbering according to Rabat EU index).

17. The bispecific antigen binding molecule of any one of claims 9 to 16, wherein the first and the second antigen binding moiety are fused to each other, optionally via a peptide linker.

18. The bispecific antigen binding molecule of any one of claims 9 to 17, wherein the first and the second antigen binding moiety are each a Fab molecule and wherein either (i) the second

5 antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety, or (ii) the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety.

19. The bispecific antigen binding molecule of any one of claims 9 to 18, comprising a third  
10 antigen binding moiety.

20. The bispecific antigen binding molecule of claim 19, wherein the third antigen moiety is identical to the first antigen binding moiety.

21. The bispecific antigen binding molecule of any one of claims 9 to 20, comprising an Fc domain composed of a first and a second subunit.

15 22. The bispecific antigen binding molecule of claim 21, wherein the first, the second and, where present, the third antigen binding moiety are each a Fab molecule; and wherein either (i) the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the first antigen binding moiety and the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus 20 of the first subunit of the Fc domain, or (ii) the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the Fab heavy chain of the second antigen binding moiety and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the first subunit of the Fc domain; and wherein the third antigen binding moiety, where present, is fused at the C-terminus of the 25 Fab heavy chain to the N-terminus of the second subunit of the Fc domain.

23. The bispecific antigen binding molecule of claim 21 or 22, wherein the Fc domain is an IgG, particularly an IgGi, Fc domain.

24. The bispecific antigen binding molecule of any one of claims 21 to 23, wherein the Fc domain is a human Fc domain.

25. The bispecific antigen binding molecule of any one of claims 21 to 24, wherein an amino acid residue in the CH3 domain of the first subunit of the Fc domain is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which is positionable in a cavity within the CH3 domain of the second subunit, and an amino acid residue in the CH3 domain of the second subunit of the Fc domain is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit is positionable.

5 26. The bispecific antigen binding molecule of any one of claims 21 to 25, wherein the Fc domain comprises one or more amino acid substitution that reduces binding to an Fc receptor and/or effector function.

10 27. One or more isolated polynucleotide encoding the antibody or bispecific antigen binding molecule of any one of claims 1 to 26.

15 28. One or more vector, particularly expression vector, comprising the polynucleotide(s) of claim 27.

29. A host cell comprising the polynucleotide(s) of claim 27 or the vector(s) of claim 28.

30. A method of producing an antibody that binds to HLA-A2/WT1, comprising the steps of a) culturing the host cell of claim 29 under conditions suitable for the expression of the antibody and b) optionally recovering the antibody.

20 31. An antibody that binds to HLA-A2/WT1, produced by the method of claim 30.

32. A pharmaceutical composition comprising the antibody or bispecific antigen binding molecule of any one of claims 1 to 26 or 31 and a pharmaceutically acceptable carrier.

33. The antibody or bispecific antigen binding molecule of any one of claims 1 to 26 or 31 or the pharmaceutical composition of claim 30 for use as a medicament.

25 34. The antibody or bispecific antigen binding molecule of any one of claims 1 to 26 or 31 or the pharmaceutical composition of claim 30 for use in the treatment of a disease.

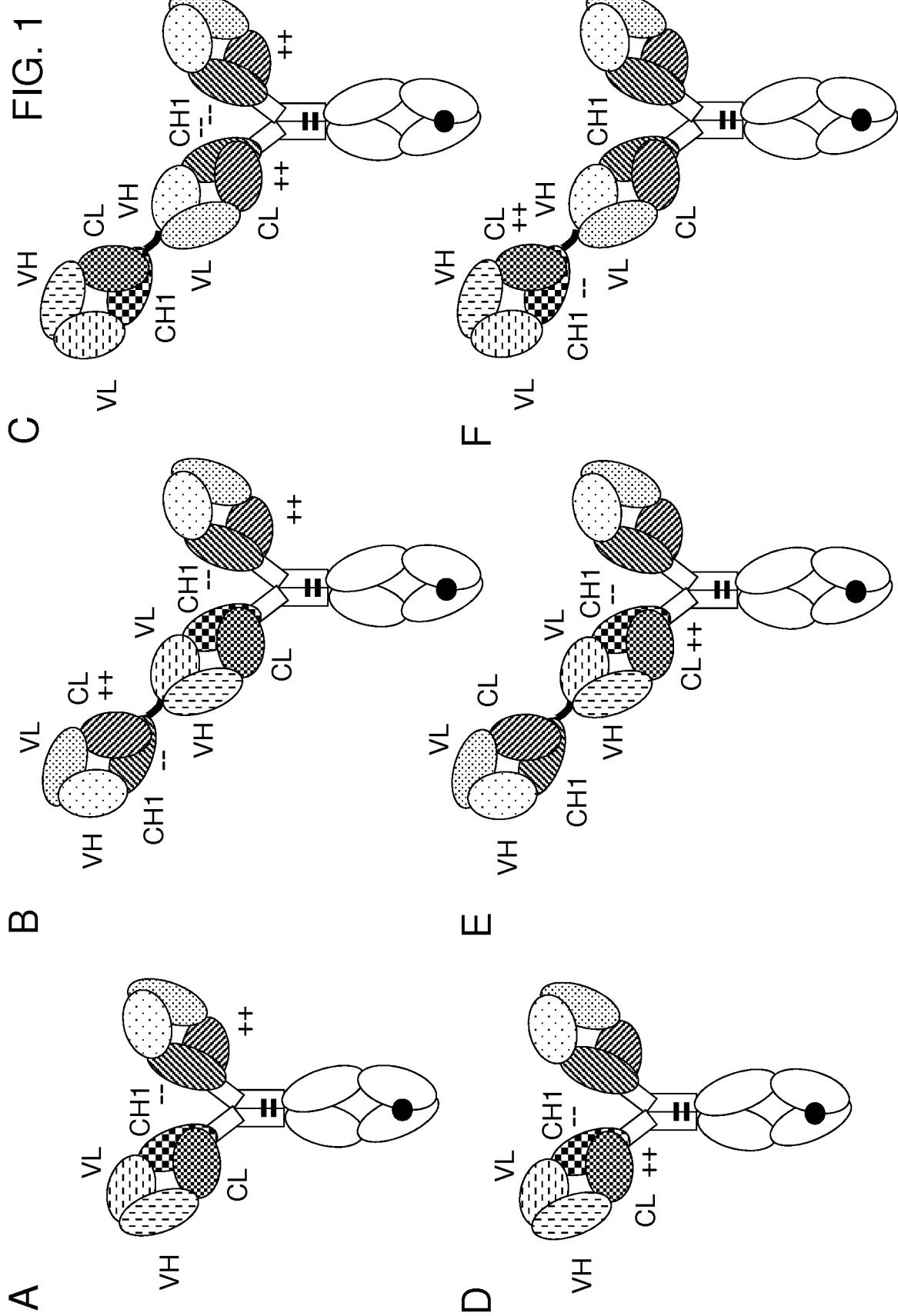
35. The antibody, bispecific antigen binding molecule or pharmaceutical composition of claim 34, wherein the disease is cancer.

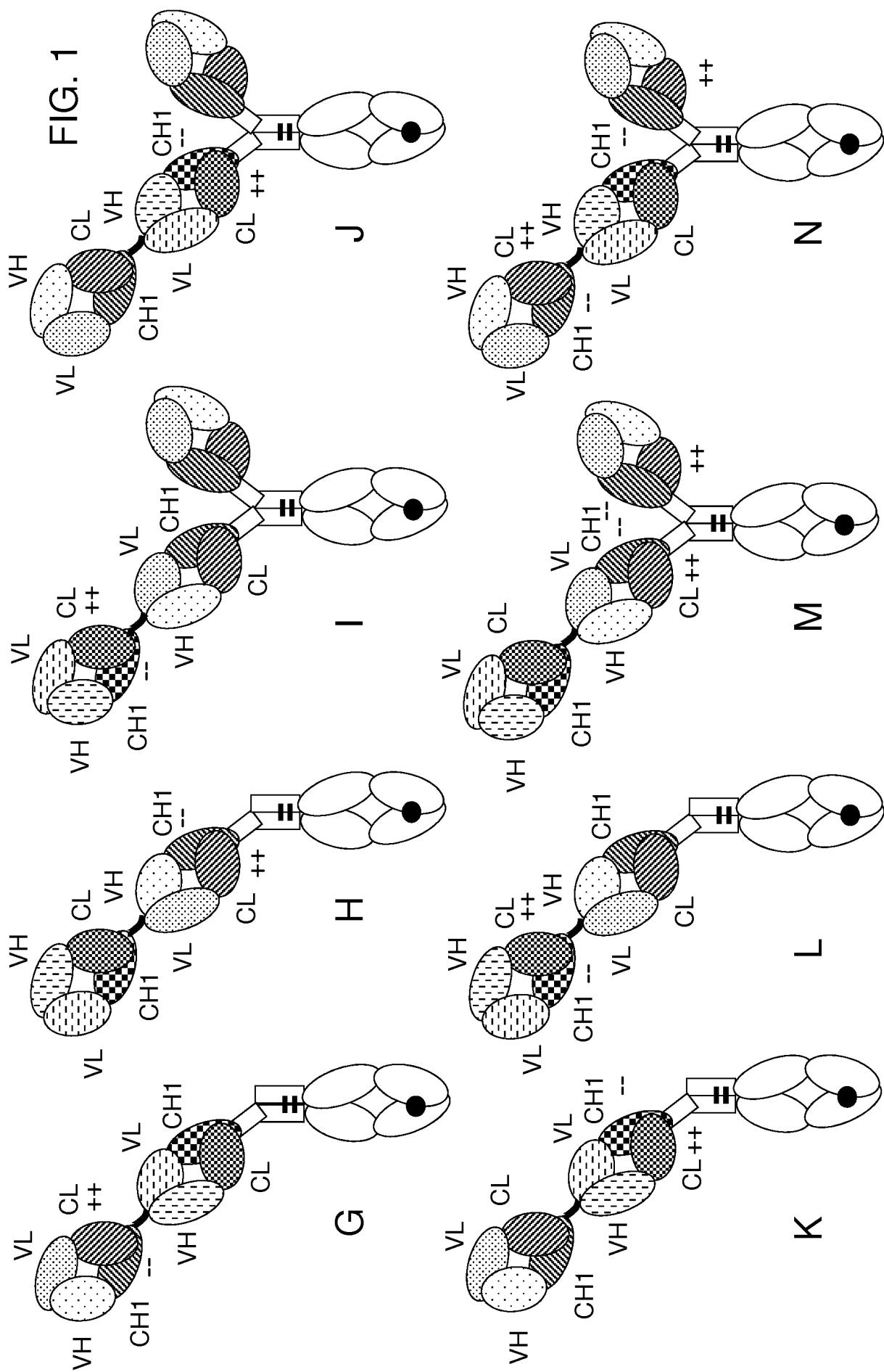
36. Use of the antibody or bispecific antigen binding molecule of any one of claims 1 to 26 or 31 in the manufacture of a medicament for the treatment of a disease.

37. A method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the antibody or 5 bispecific antigen binding molecule of any one of claims 1 to 26 or 31 in a pharmaceutically acceptable form.

38. The use of claim 36 or the method of claim 37, wherein said disease is cancer.

39. The invention as described hereinbefore.





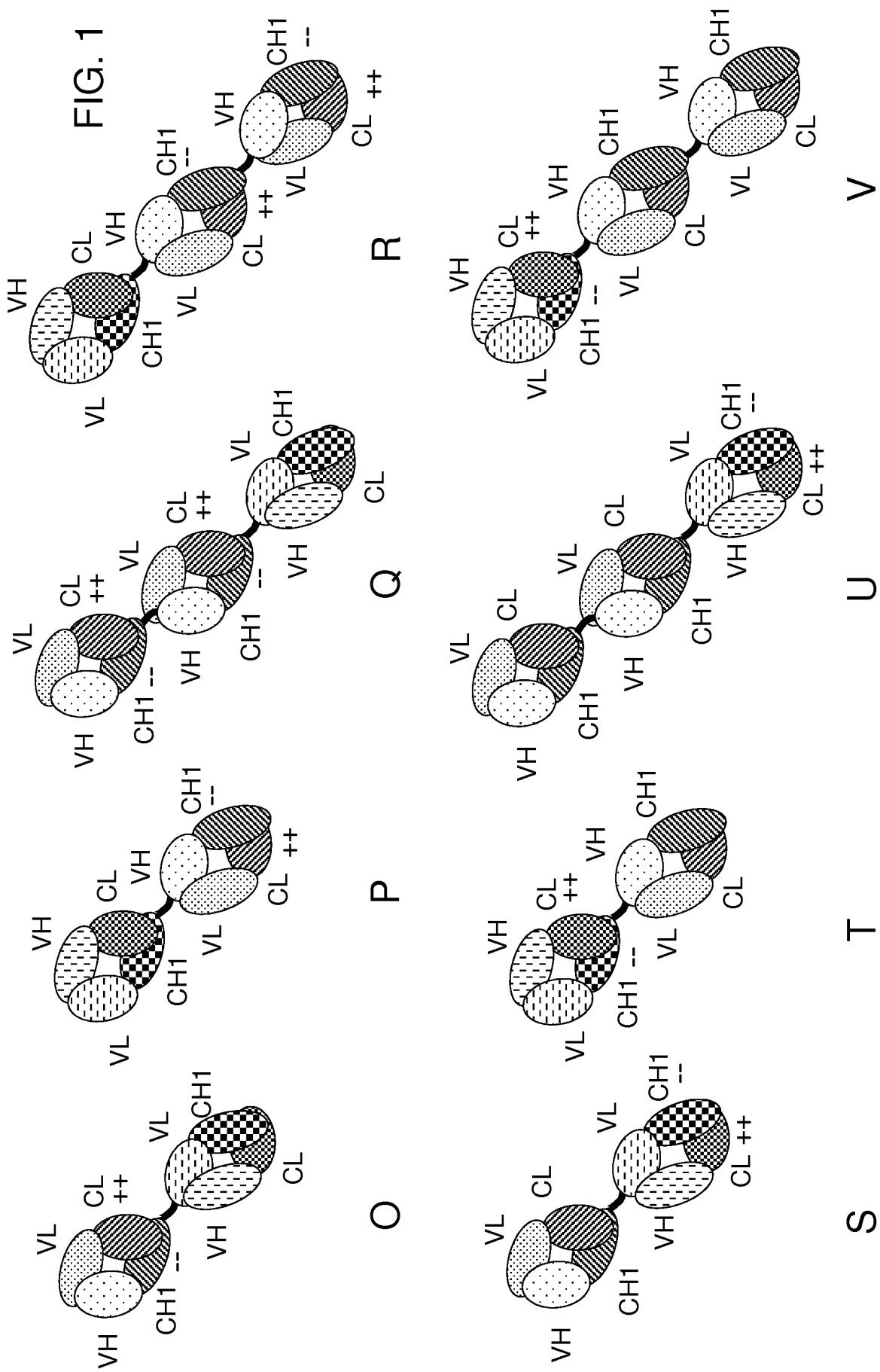
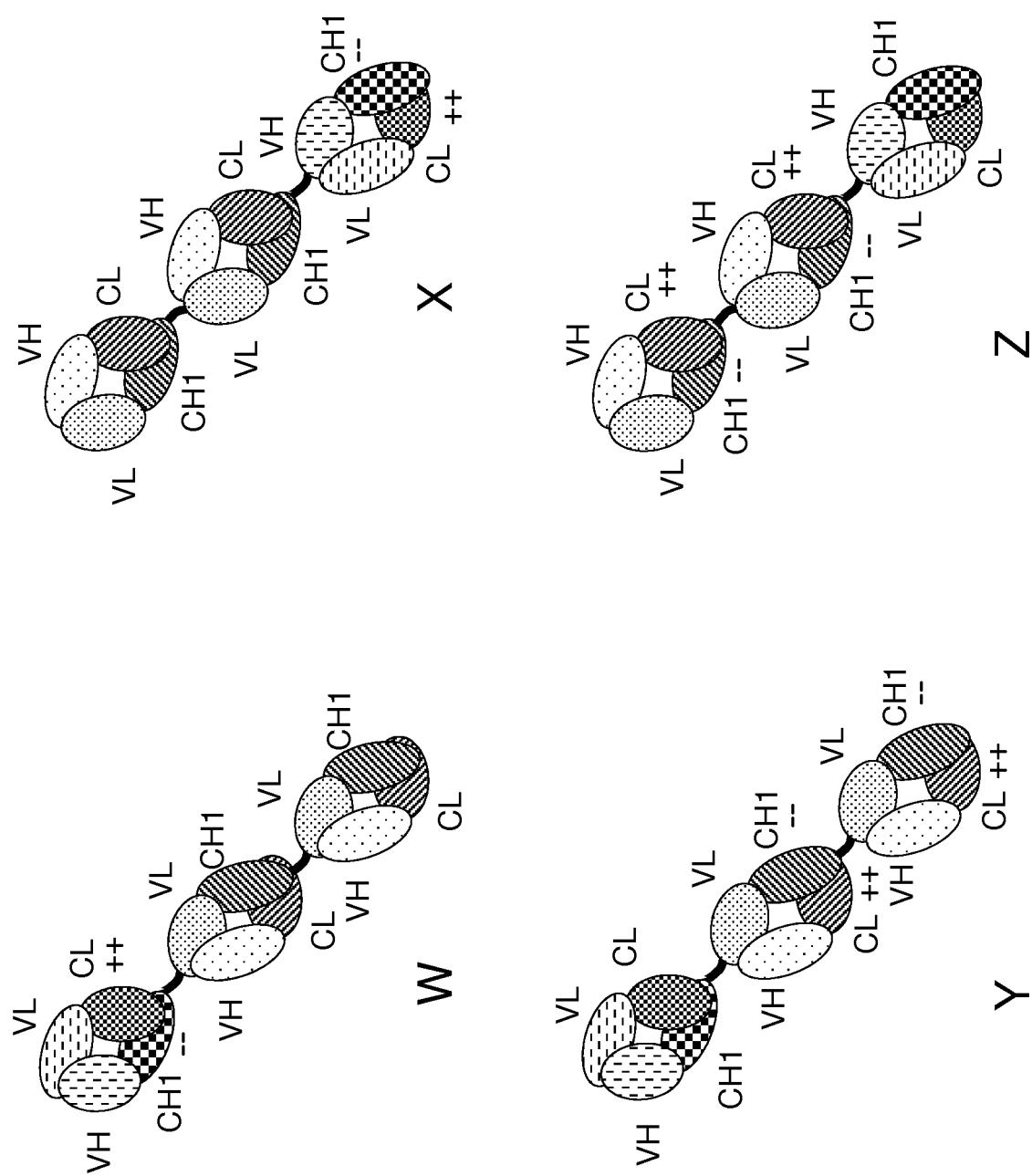


FIG. 1



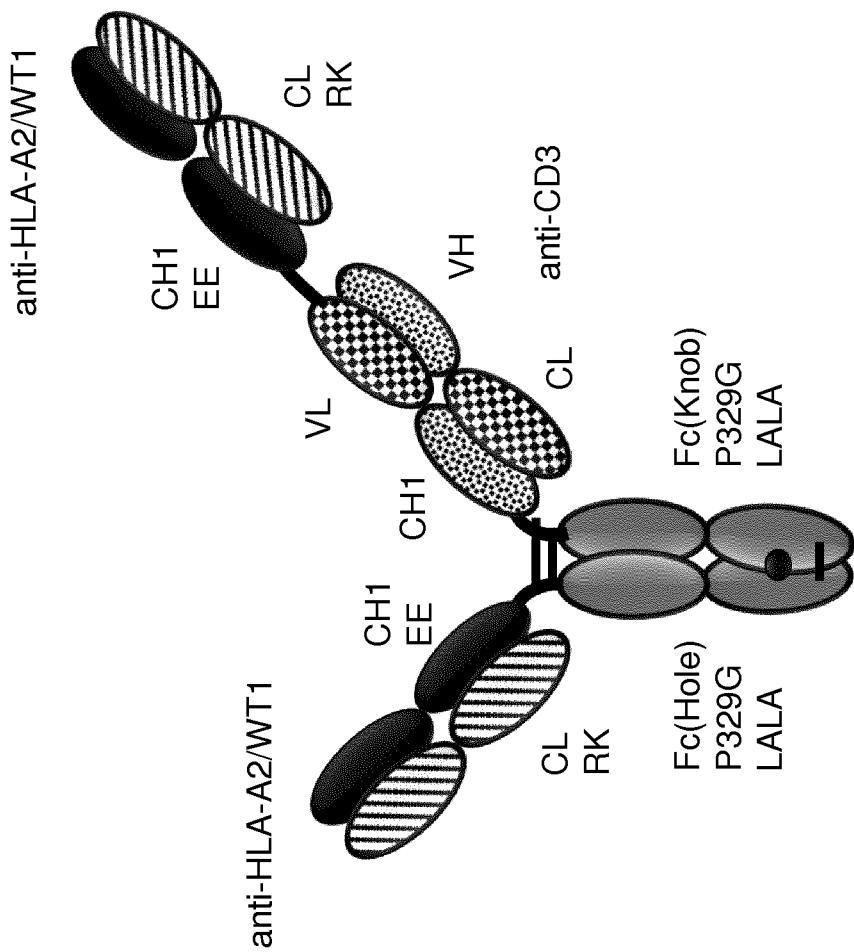


FIG. 2

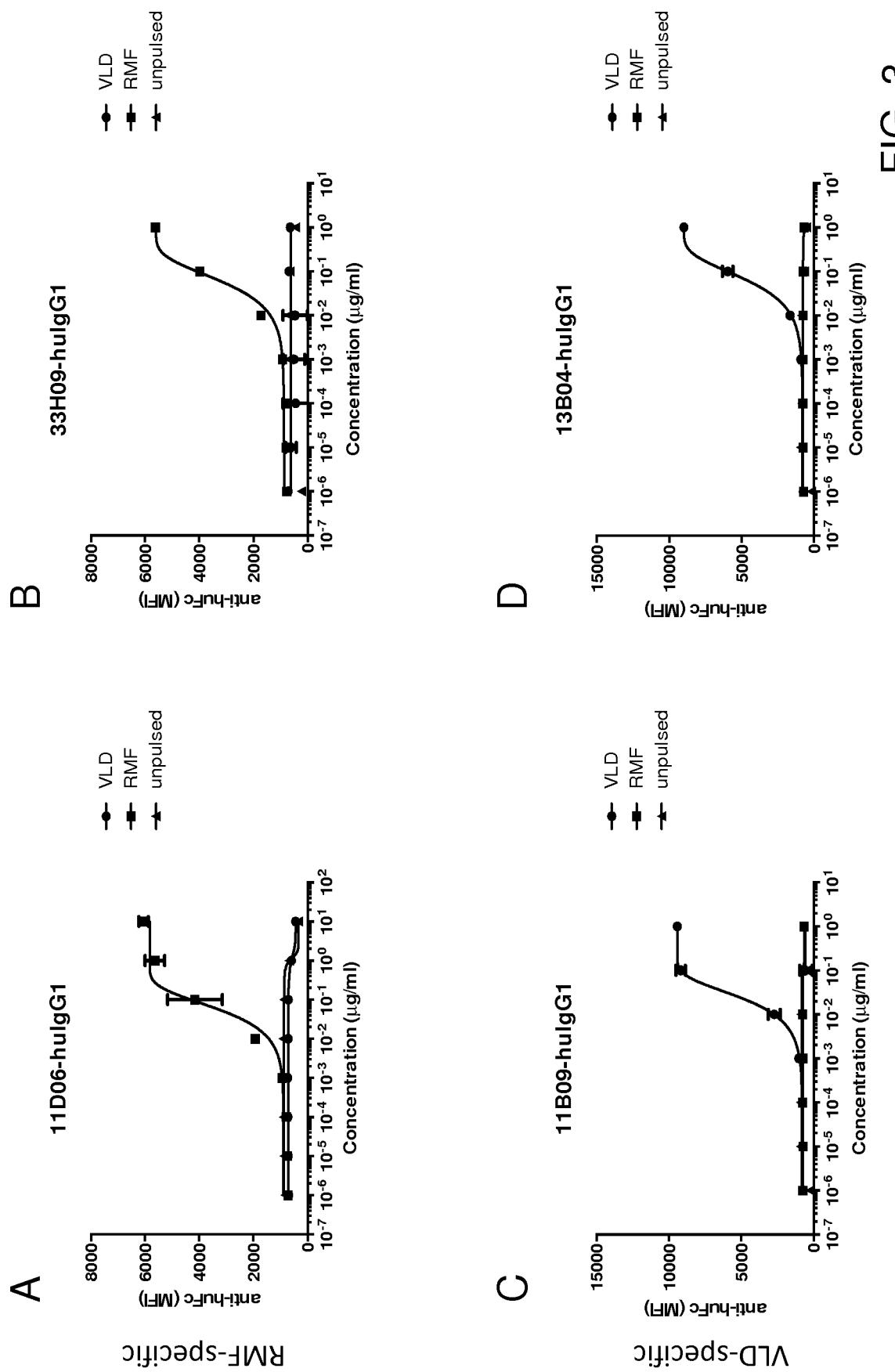
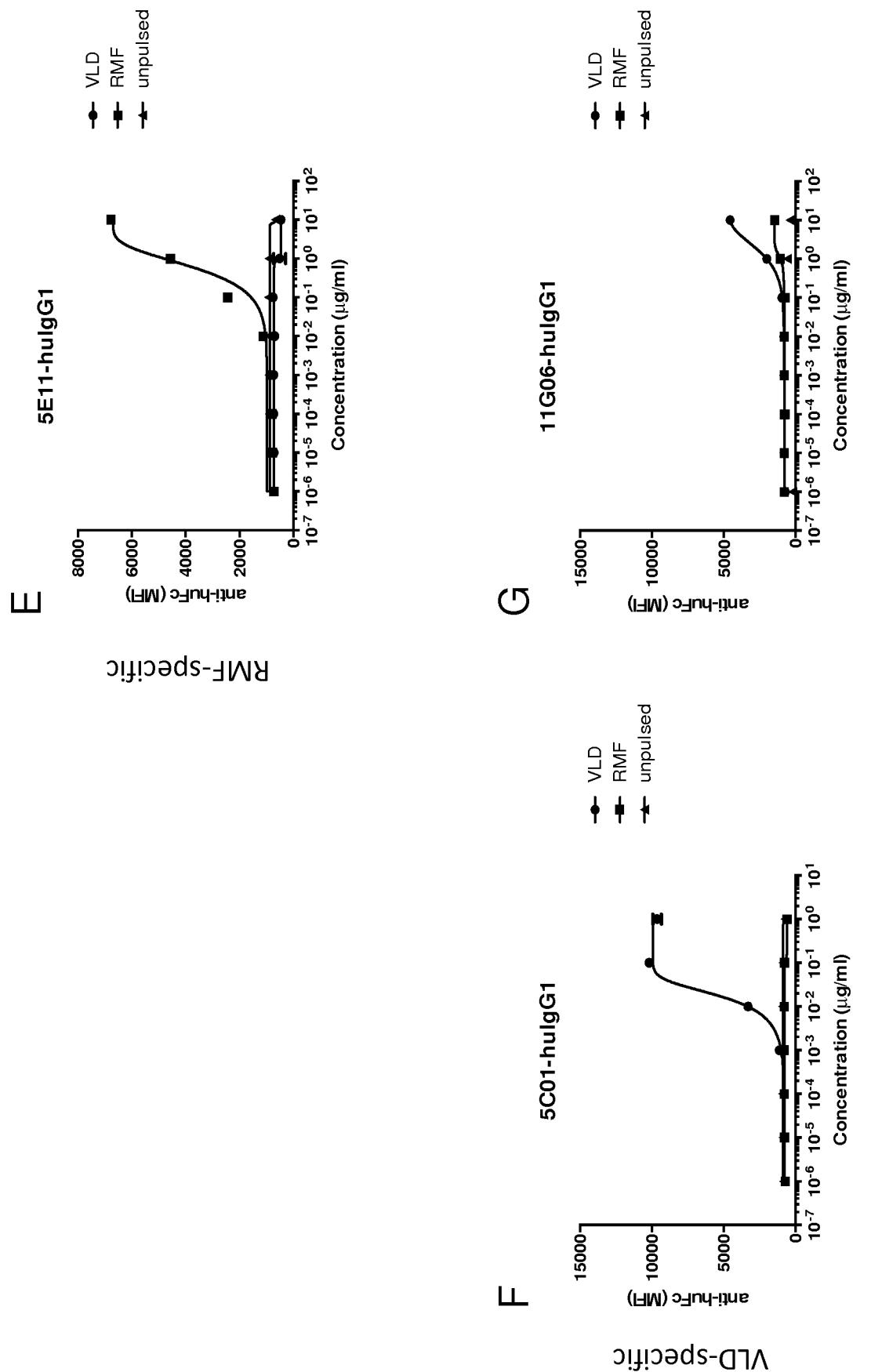


FIG. 3



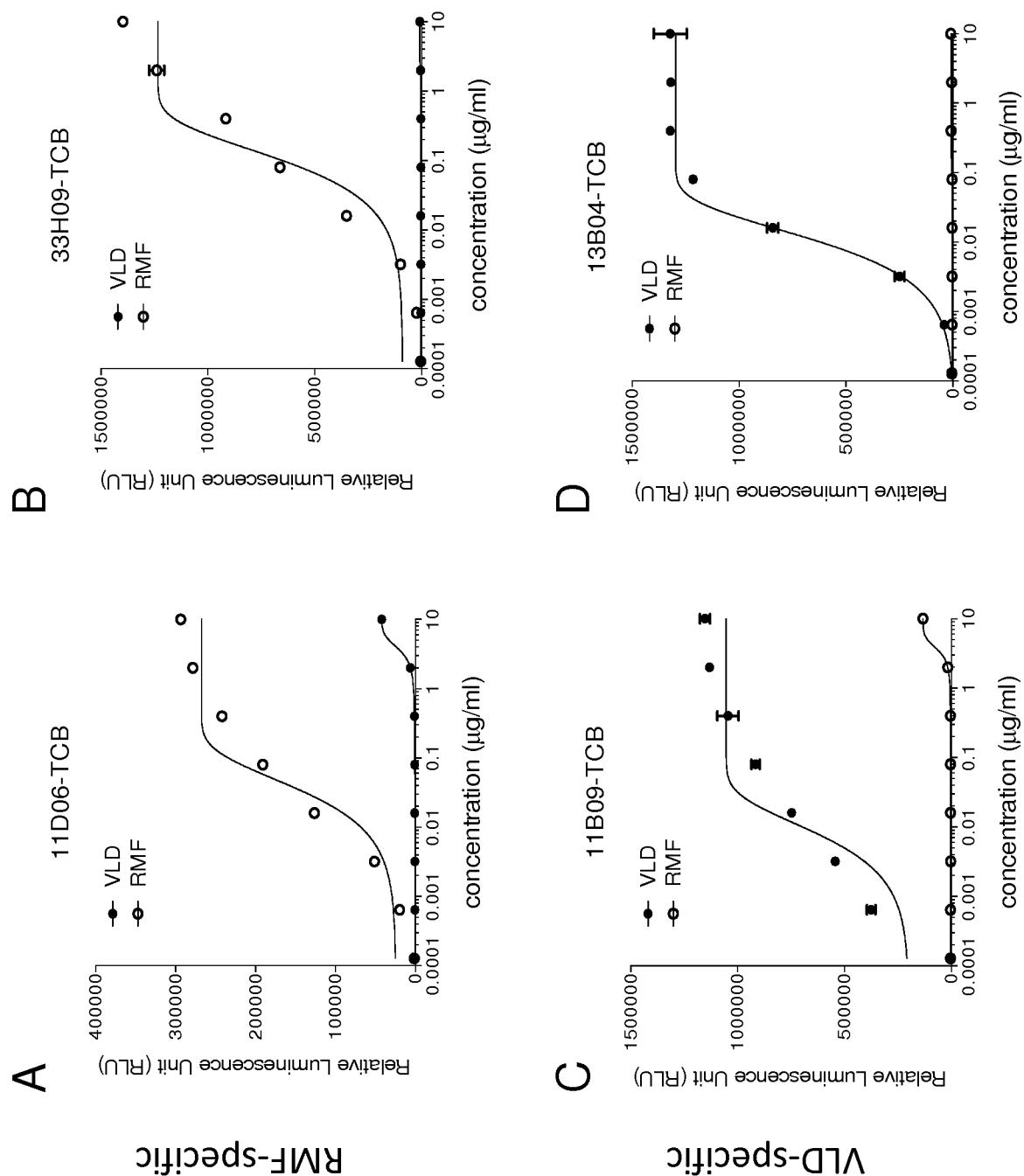


FIG. 4

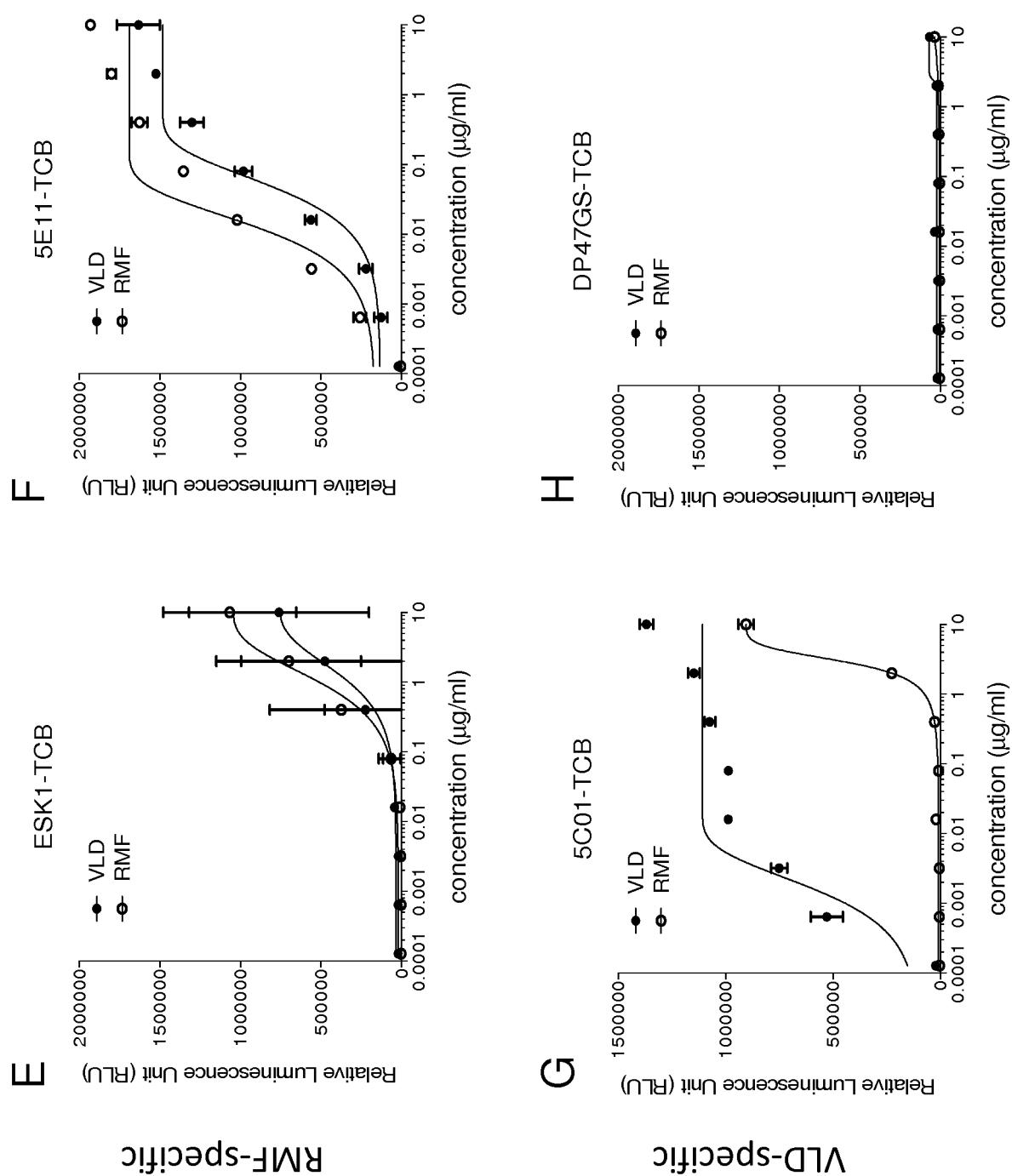


FIG. 5

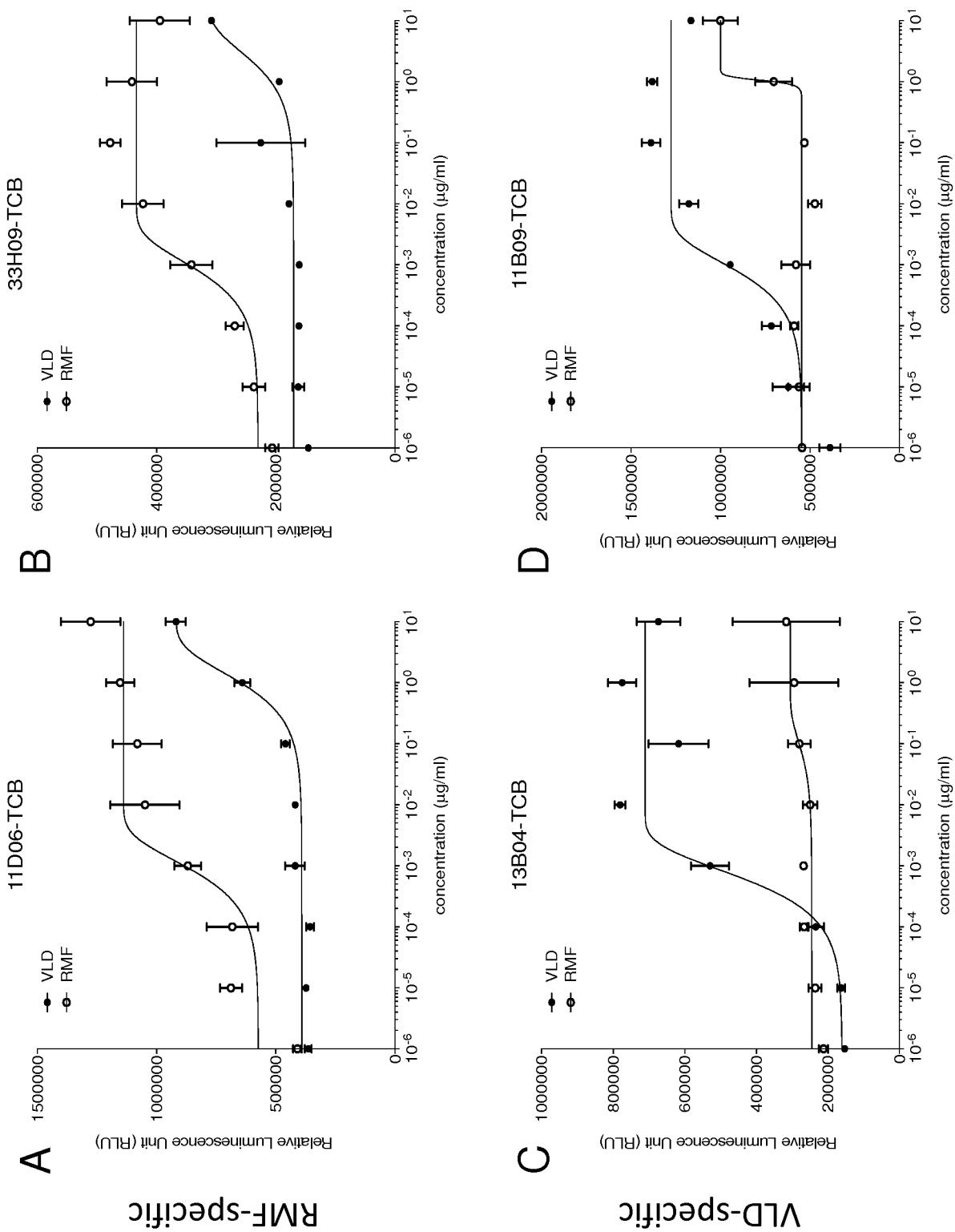


FIG. 5

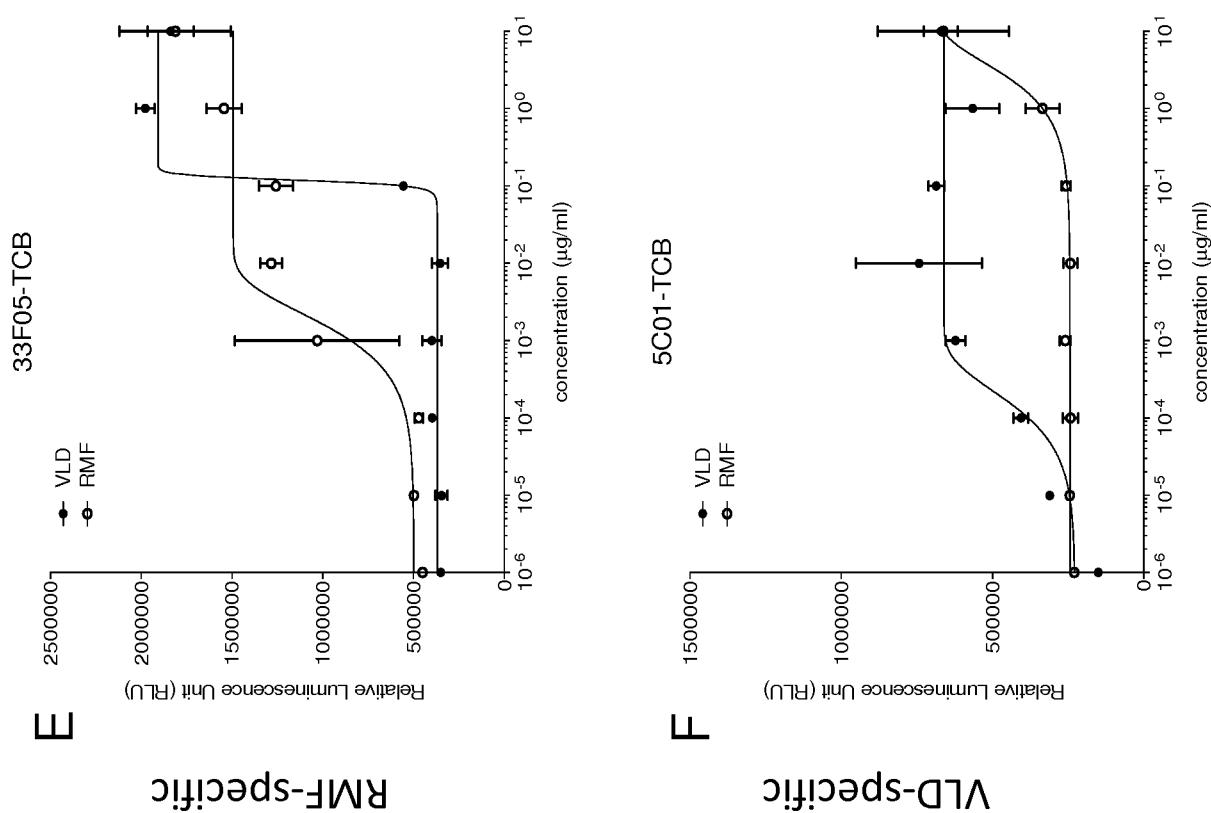
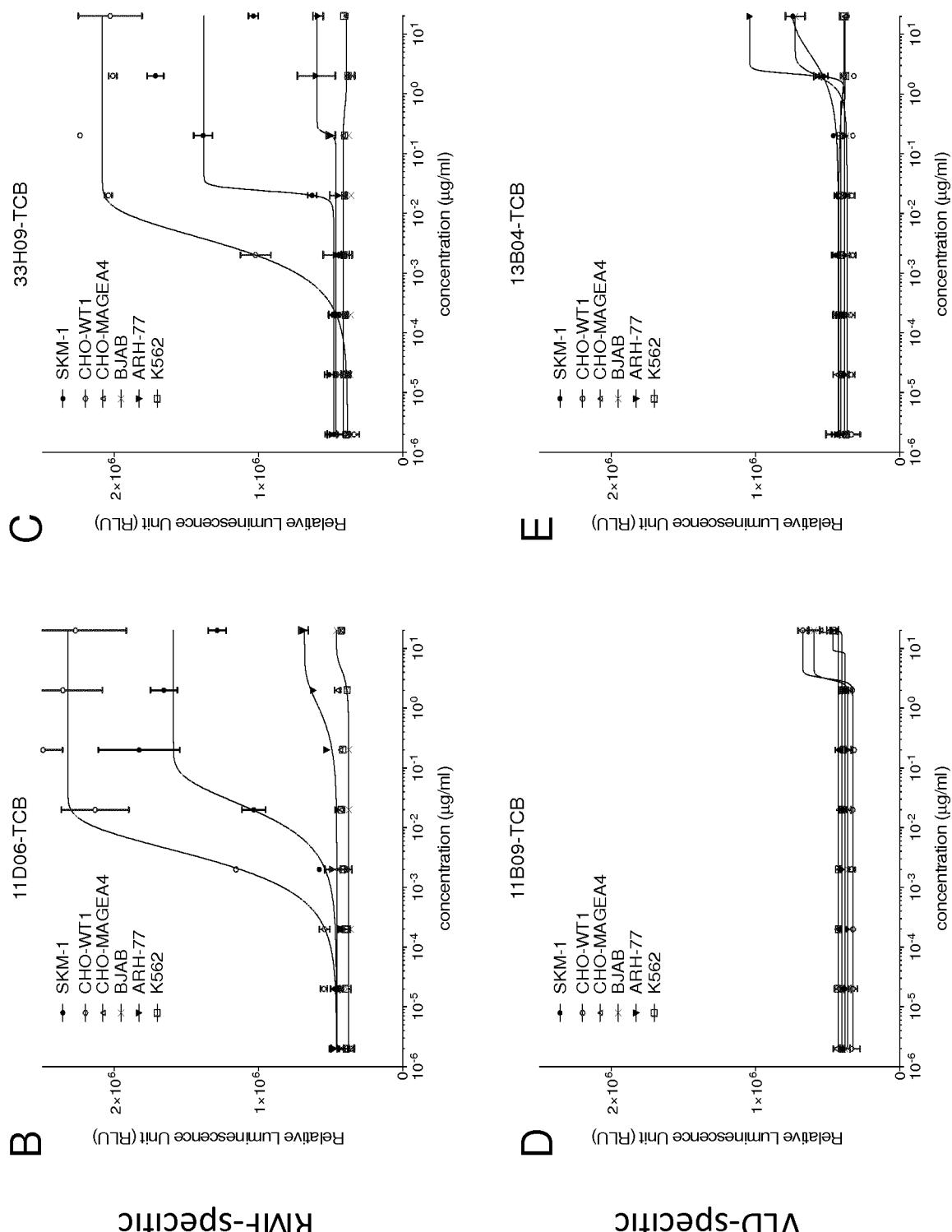


FIG. 6

**A**

Cell line	HLA-A2	WT1 (mRNA)
SKM-1	+	+
CHO-WT1	+	+
CHO- MAGE-A4	+	-
BJAB	+	-
ARH-77	+	-
K562	-	+

FIG. 6



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BJAB

G

33H09-TCB  
11D06-TCB  
ESK1-TCB

Relative Luminescence Unit (RLU)

10<sup>-6</sup> 10<sup>-5</sup> 10<sup>-4</sup> 10<sup>-3</sup> 10<sup>-2</sup> 10<sup>-1</sup> 10<sup>0</sup> 10<sup>1</sup>  
concentration (μg/ml)

SKM-1

F

33H09-TCB  
11D06-TCB  
ESK1-TCB

Relative Luminescence Unit (RLU)

10<sup>-6</sup> 10<sup>-5</sup> 10<sup>-4</sup> 10<sup>-3</sup> 10<sup>-2</sup> 10<sup>-1</sup> 10<sup>0</sup> 10<sup>1</sup>  
concentration (μg/ml)

FIG. 6

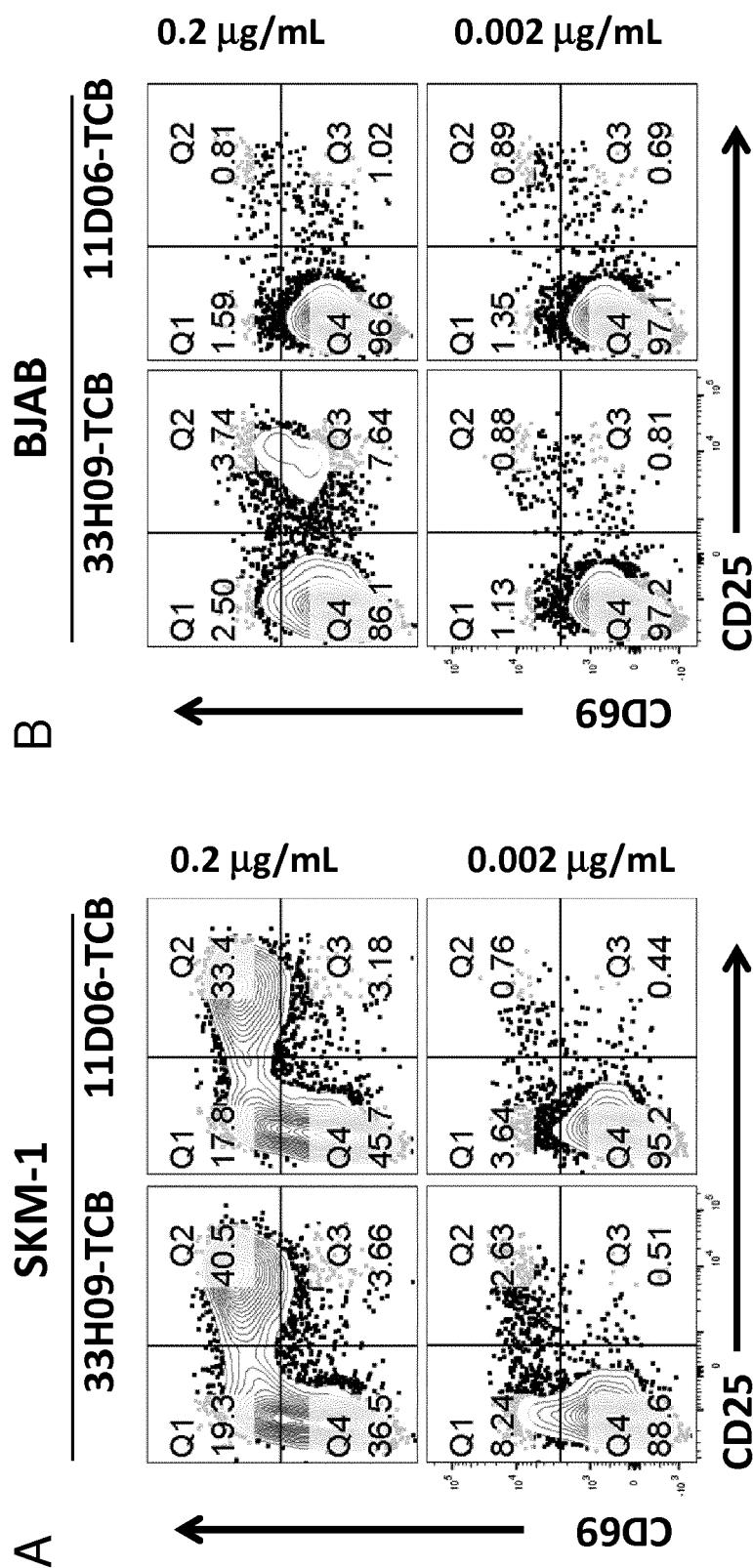
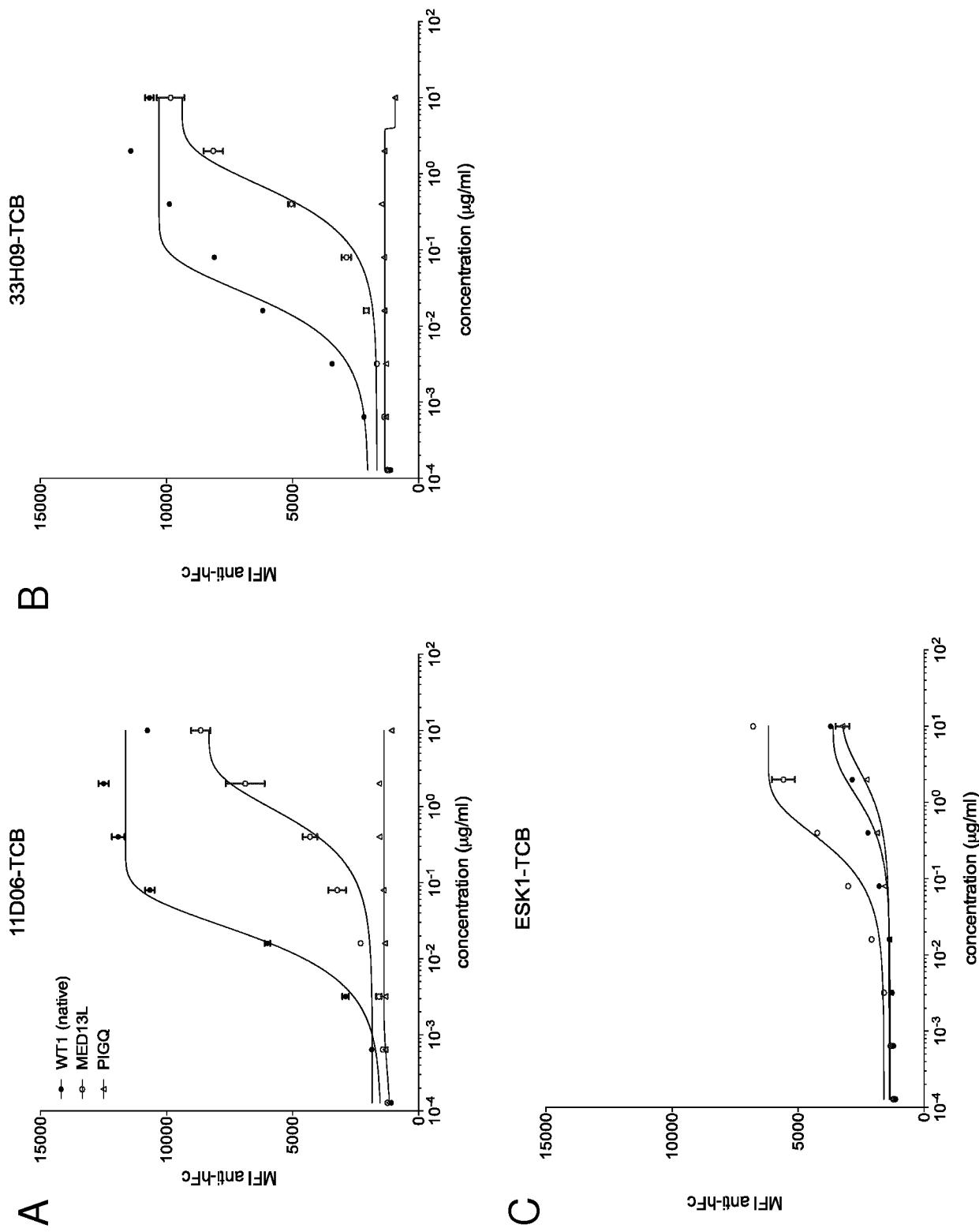


FIG. 7

FIG. 8



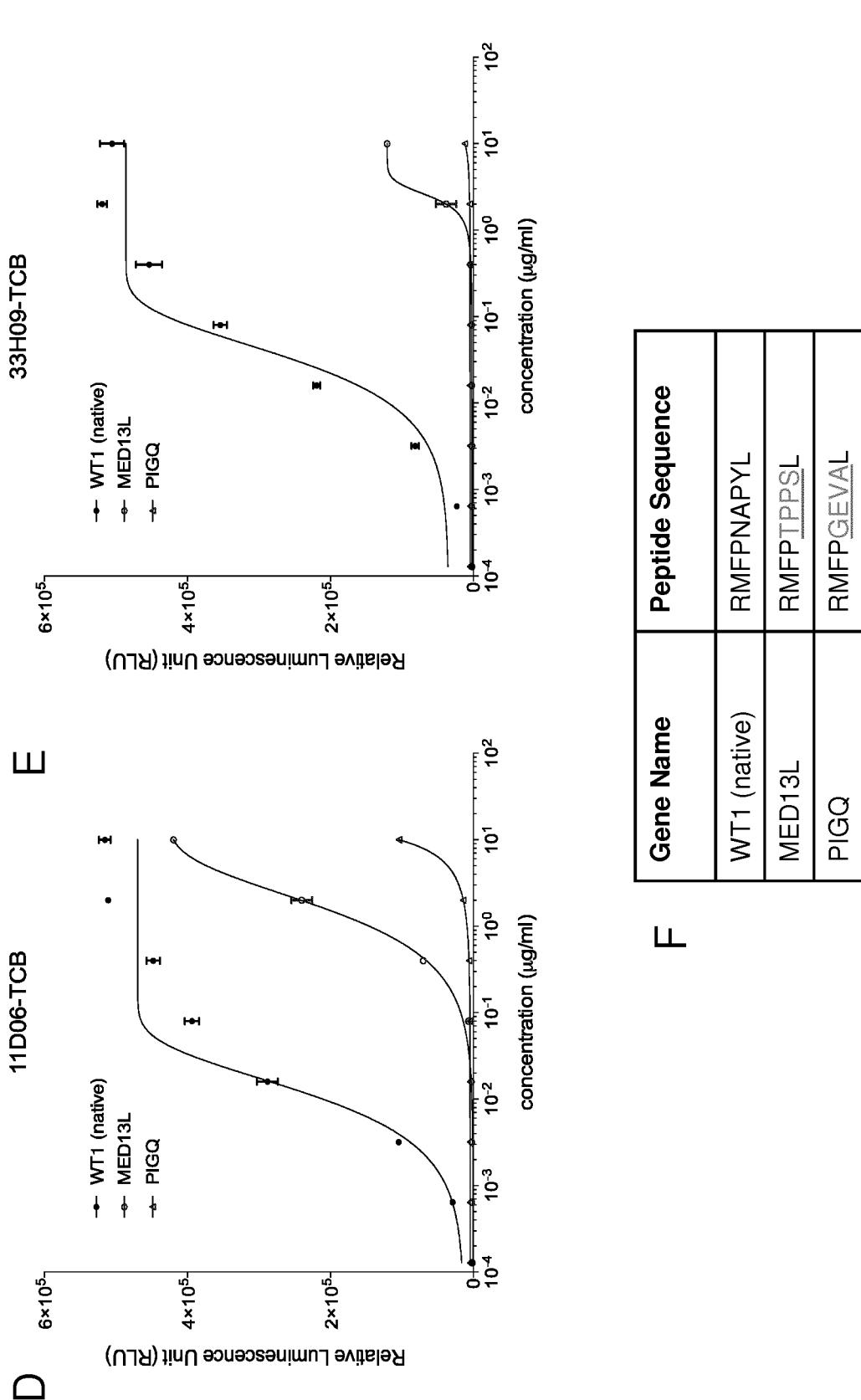


FIG. 8

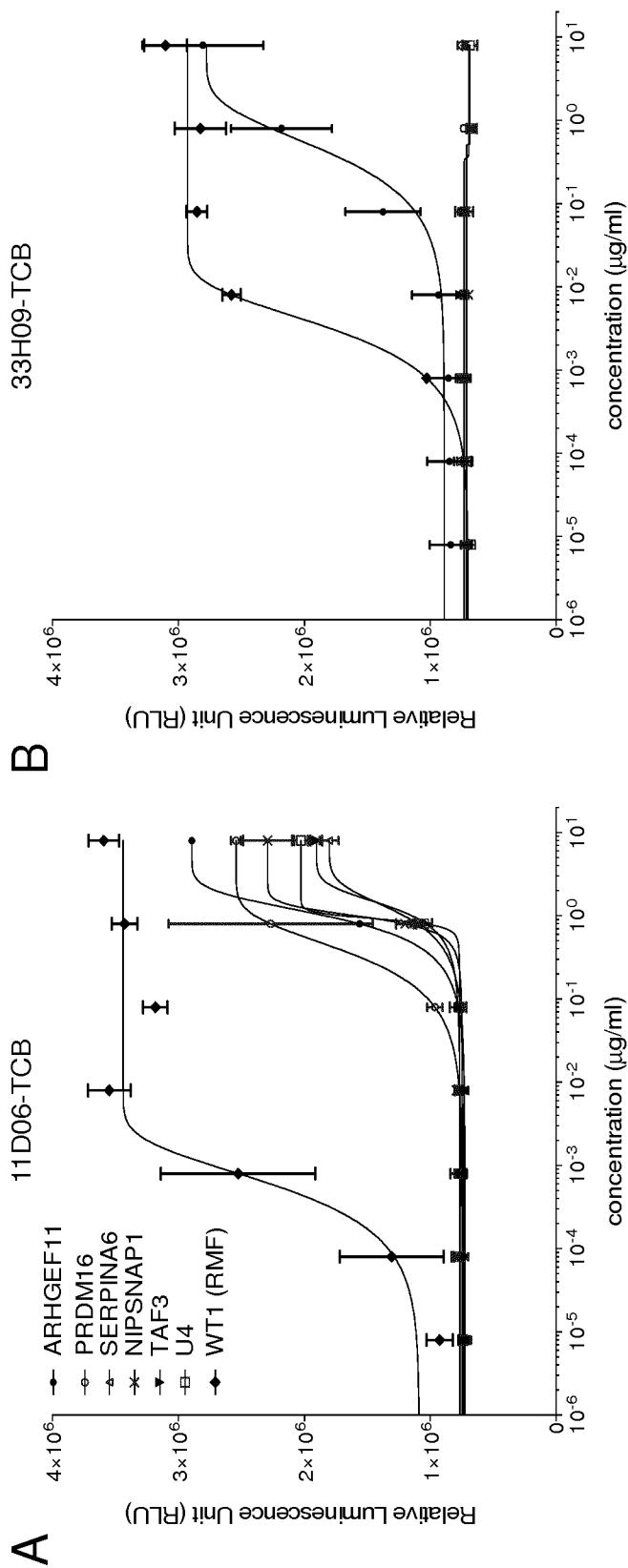


FIG. 9

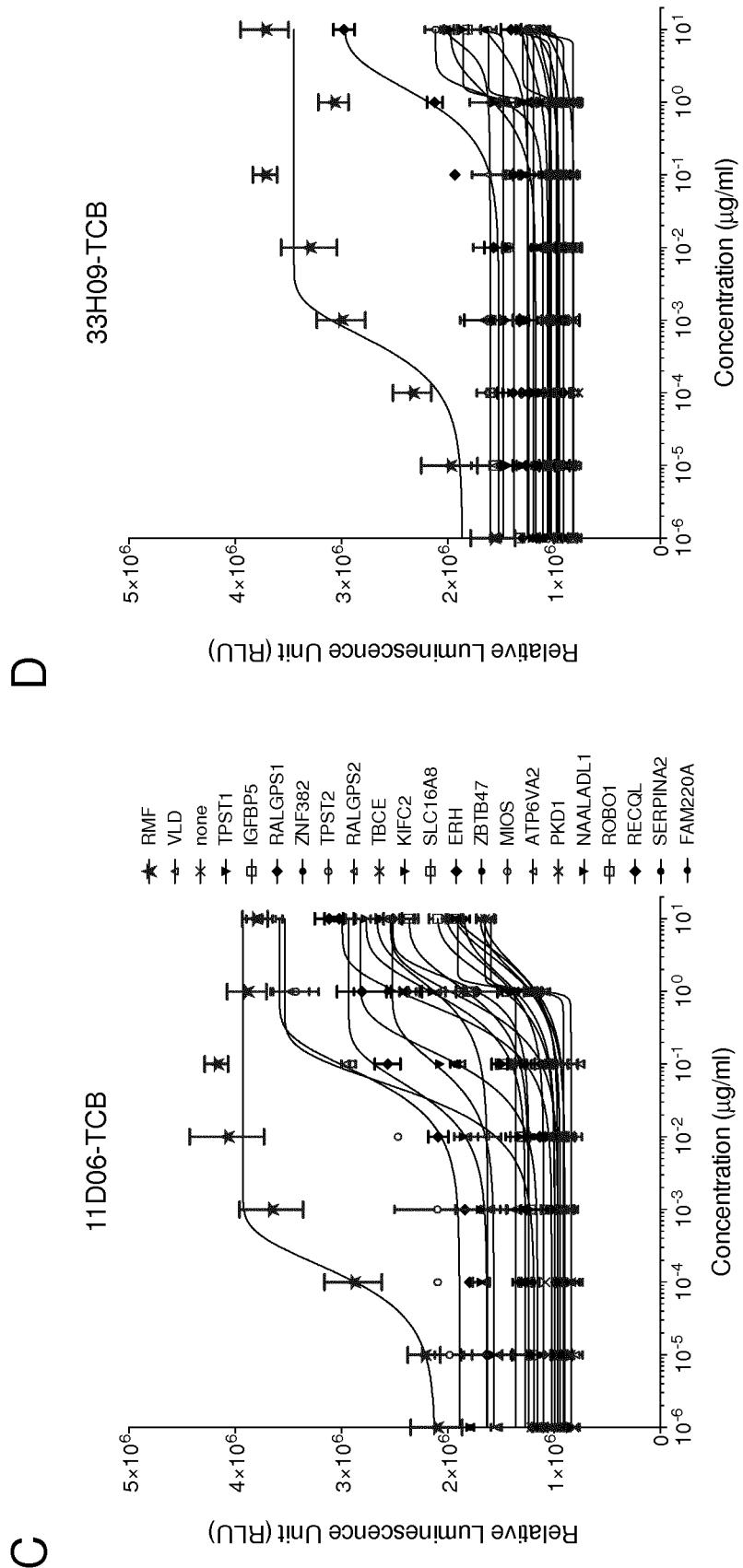
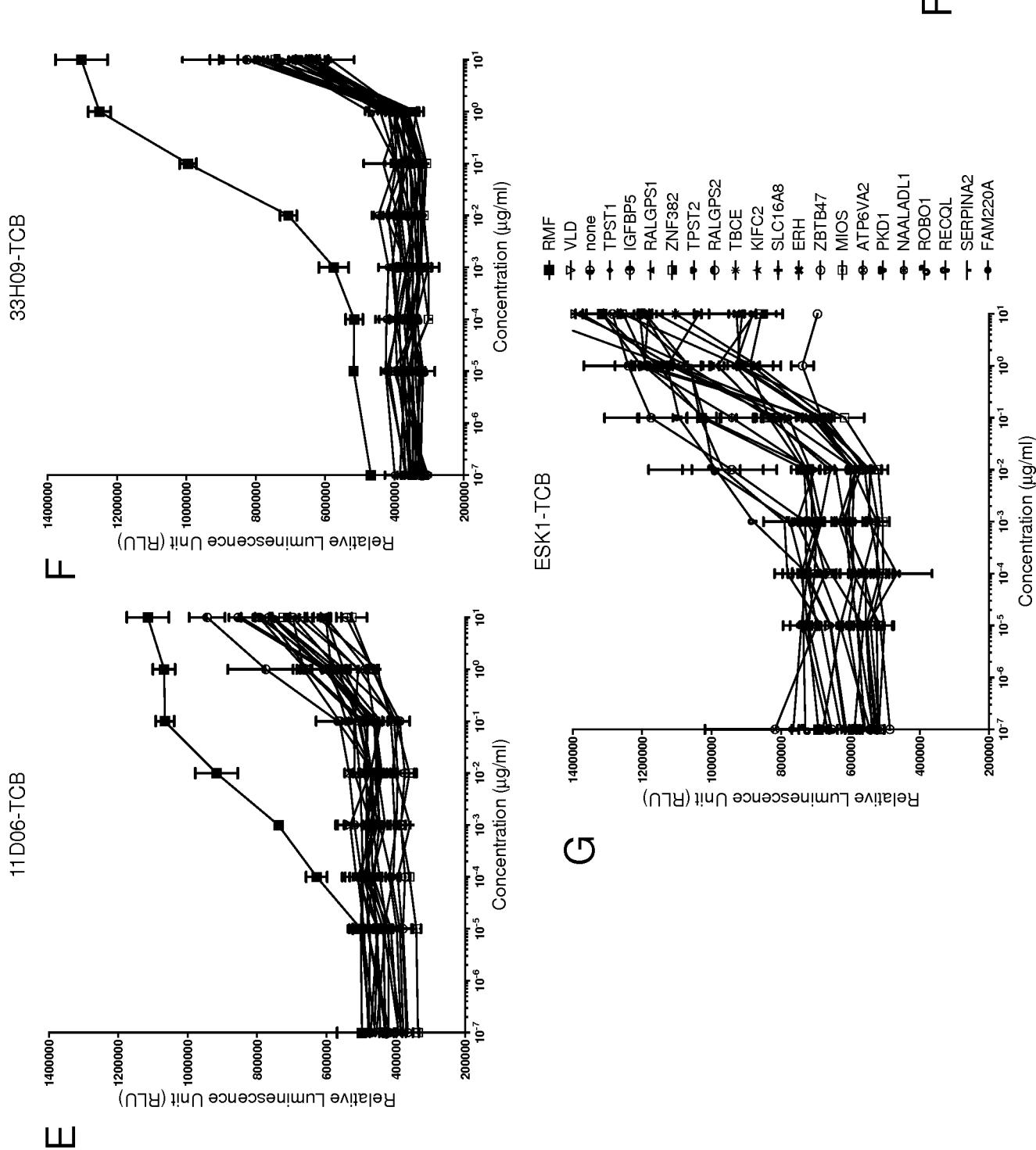


FIG. 9



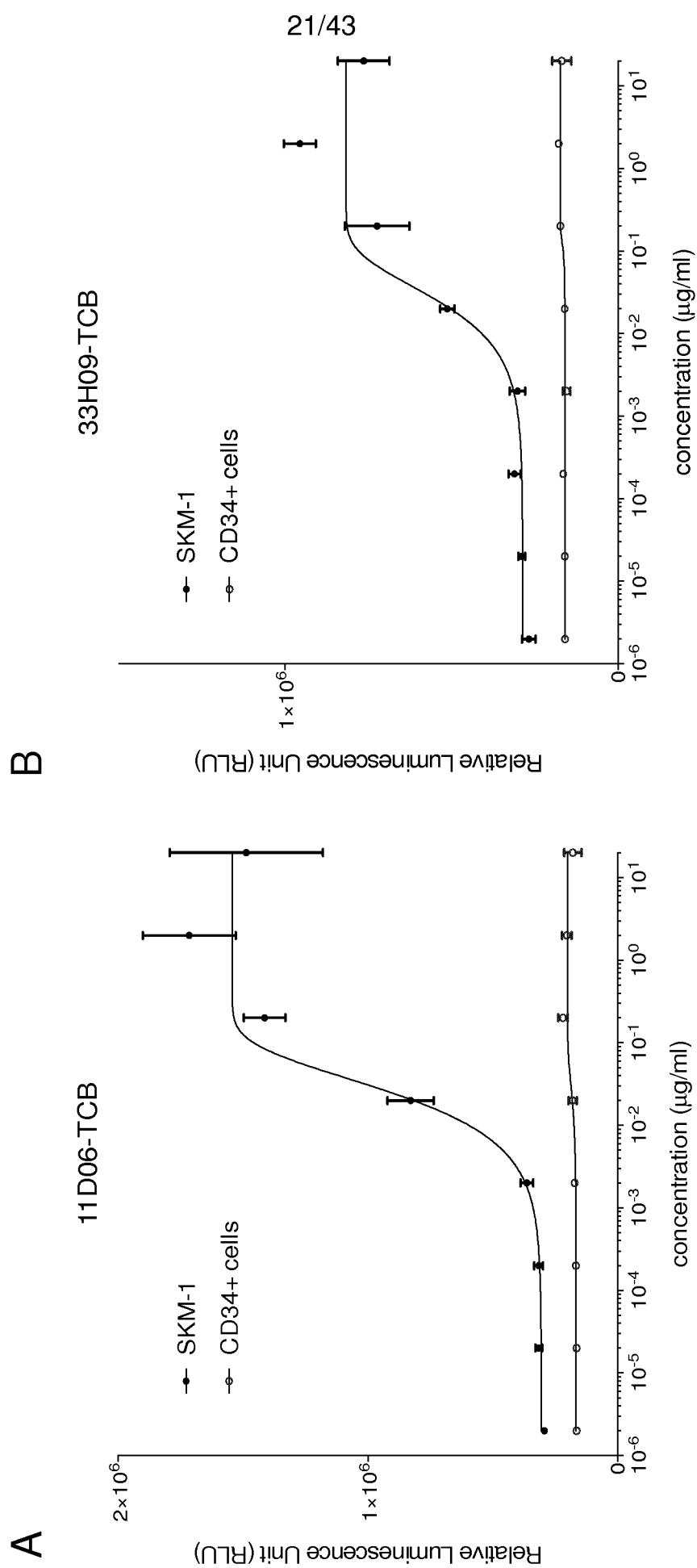


FIG. 10

FIG. 11

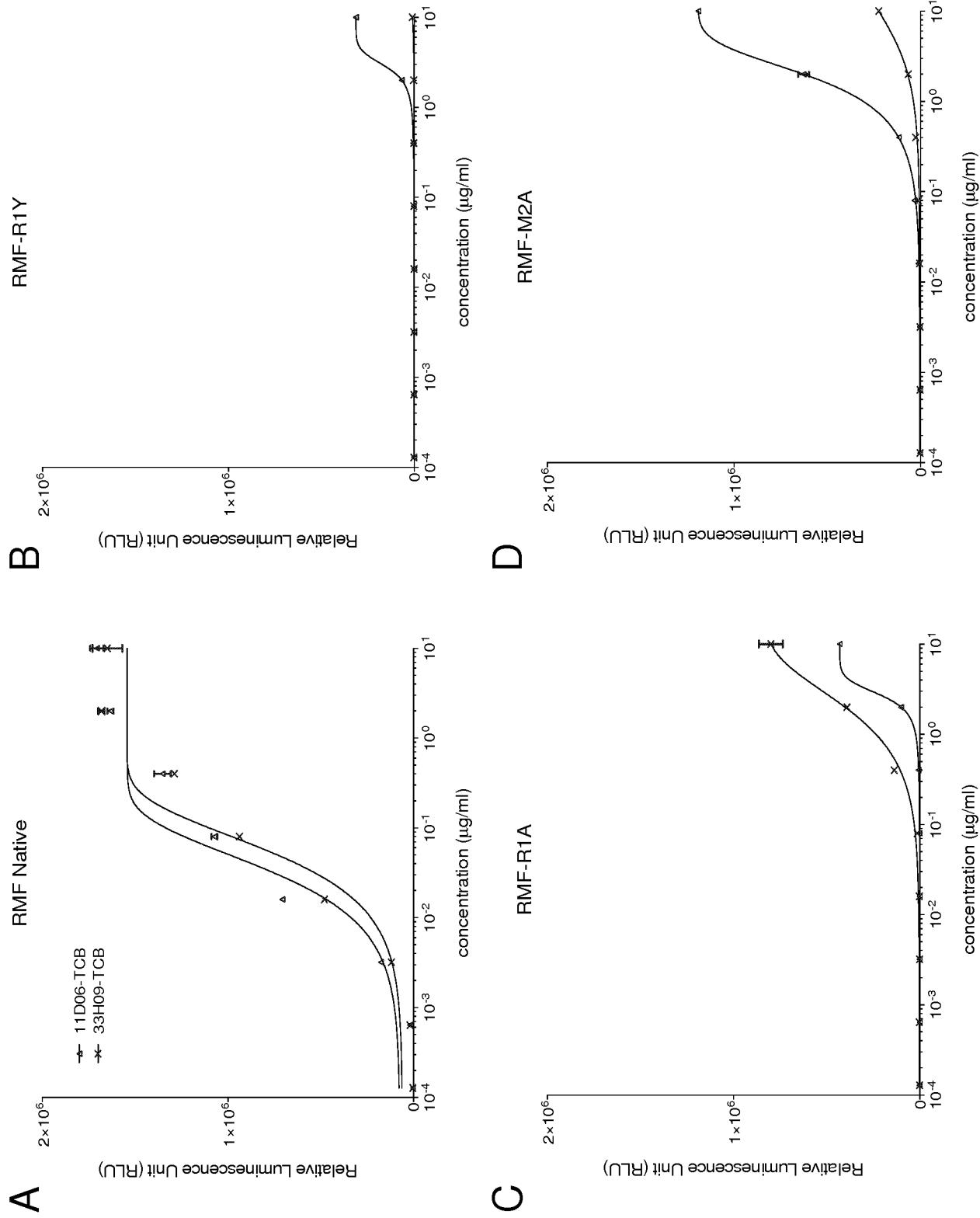
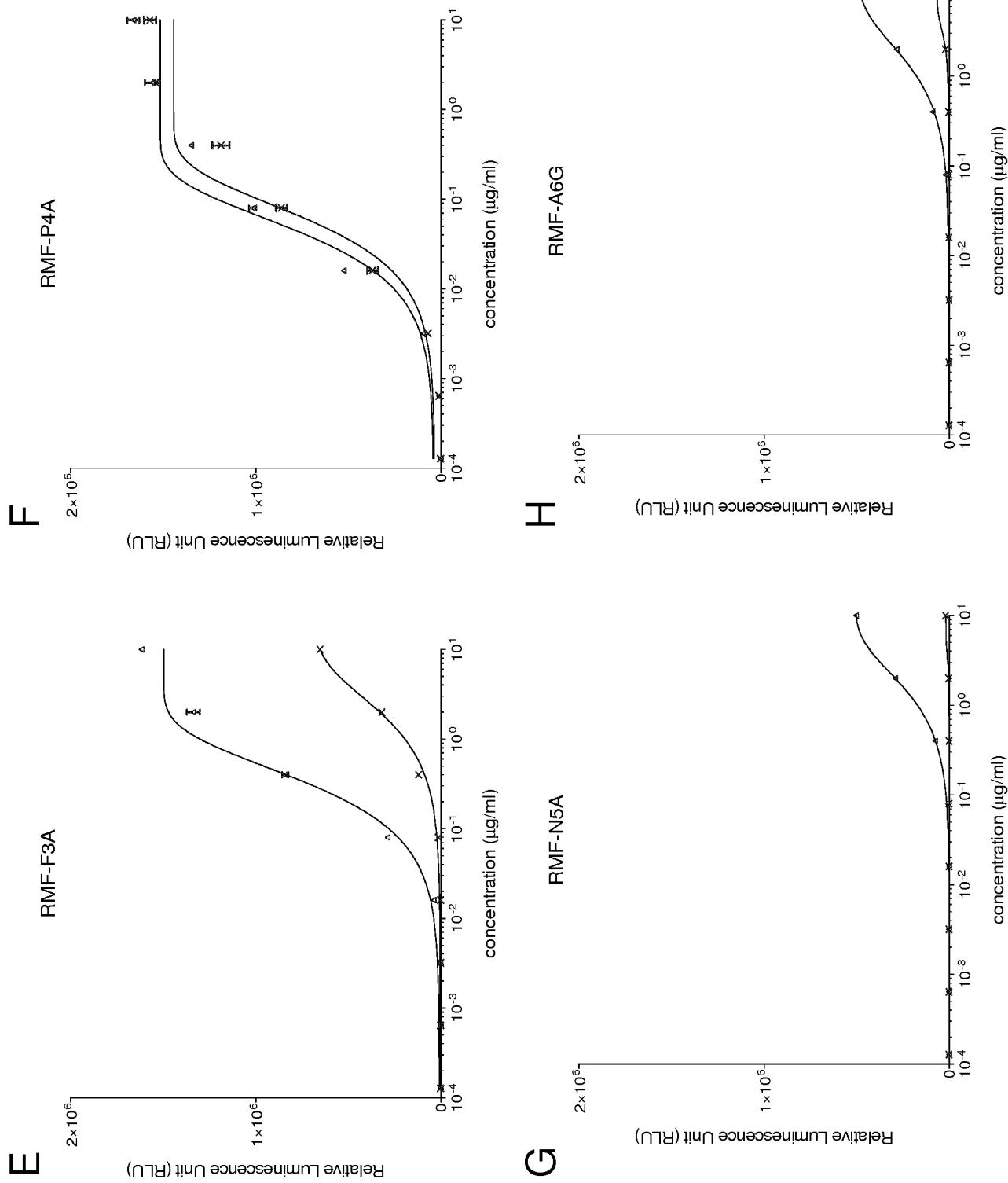


FIG. 11



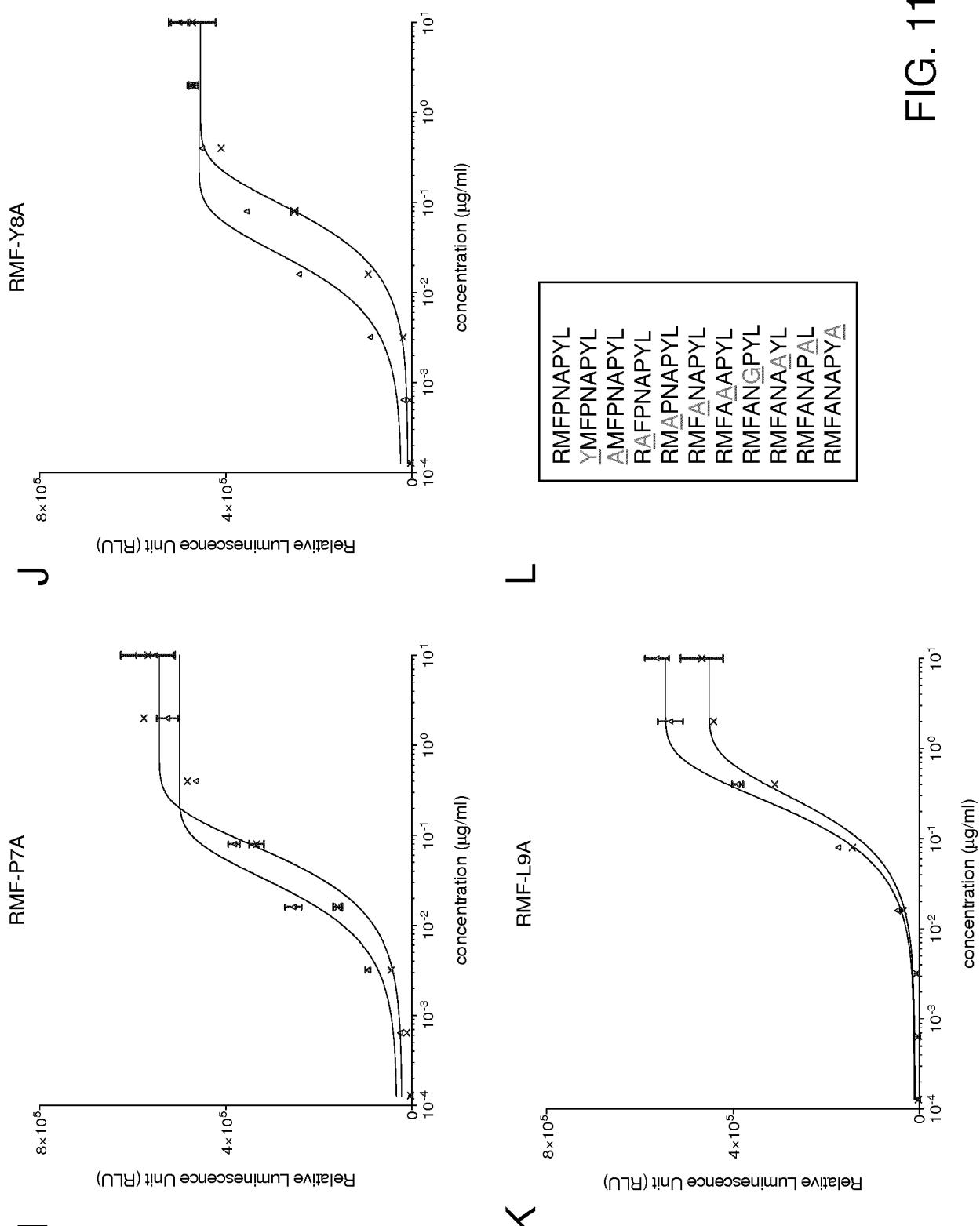


FIG. 11

M	EC <sub>50</sub> fold change	N		
		11D06- TCB	33H09- TCB	11D06-TCB RMFPNAPYL
R1Y	69.41	48.87	33H09-TCB RMFPNAPYL	33H09-TCB RMFPNAPYL
R1A	67.46	33.34		
M2A	49.07	49.14		
F3A	9.86	31.65		
P4A	1.20	1.38		
N5A	93.26	110.18		
A6G	90.84	101.61		
P7A	1.55	2.01		
Y8A	1.33	2.68		
L9A	10.88	7.38		

FIG. 11

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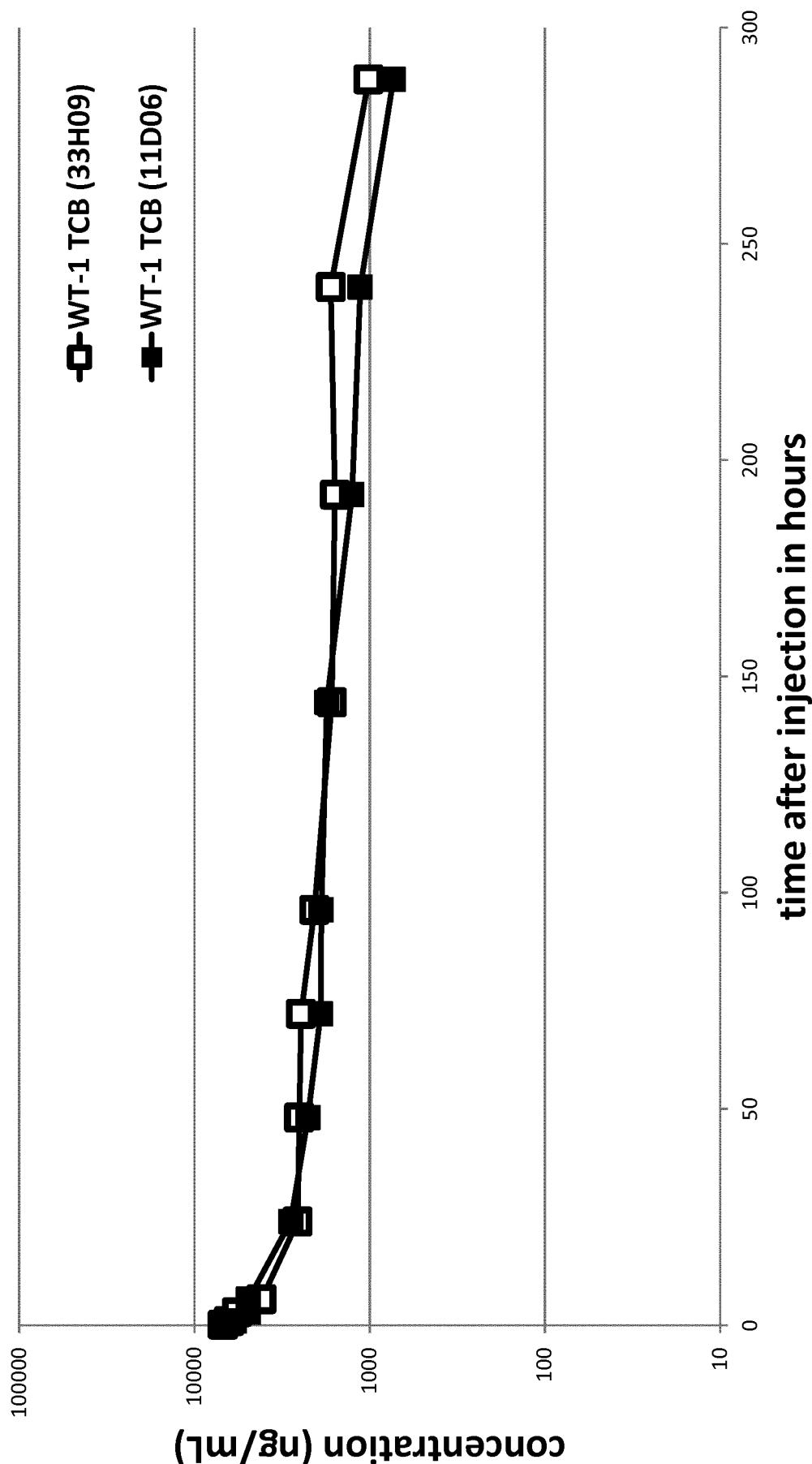


FIG. 12

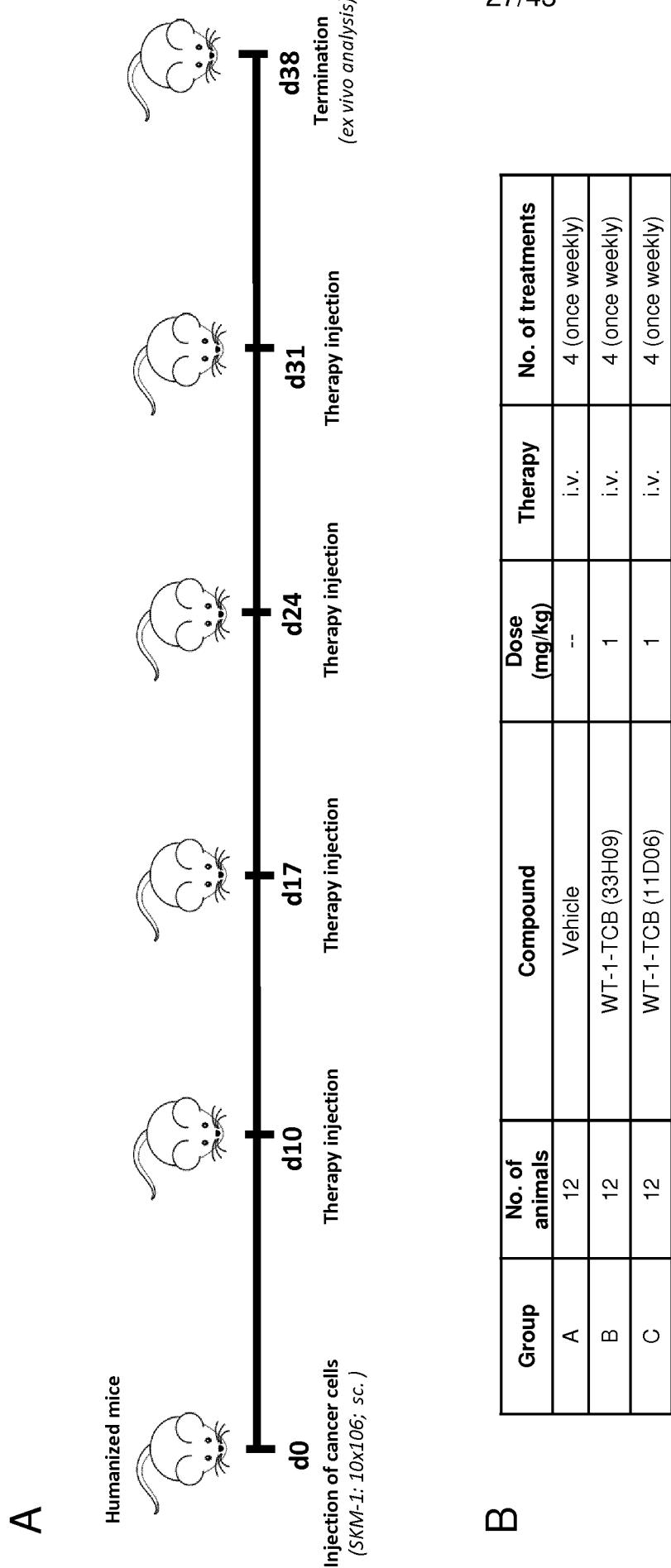
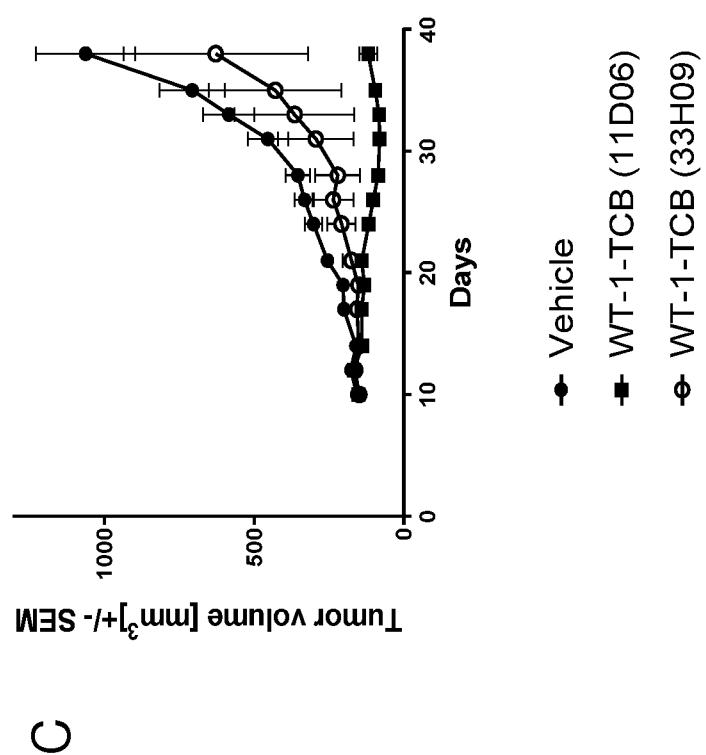


FIG. 13

FIG. 13



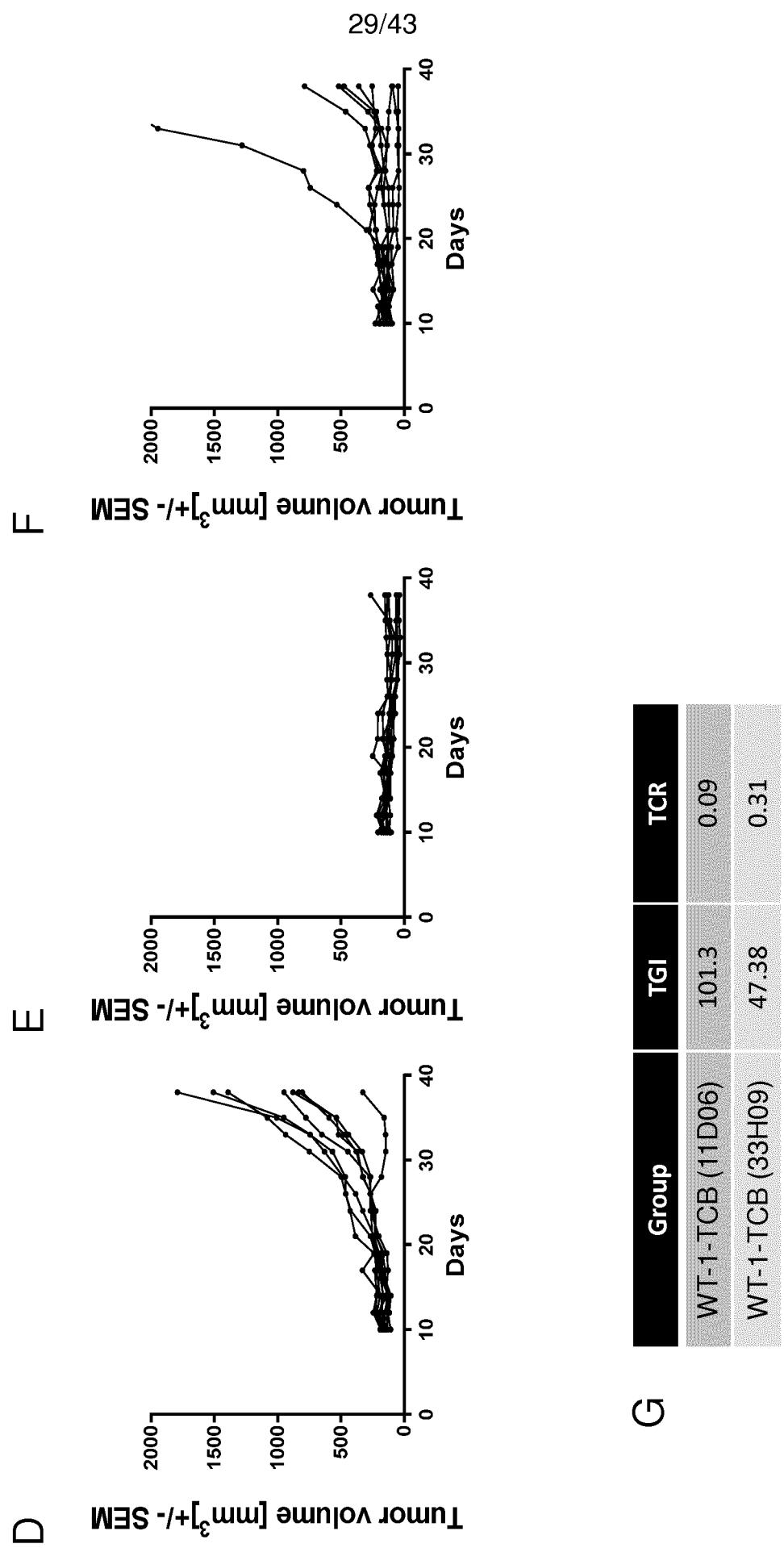


FIG. 13

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FIG. 14

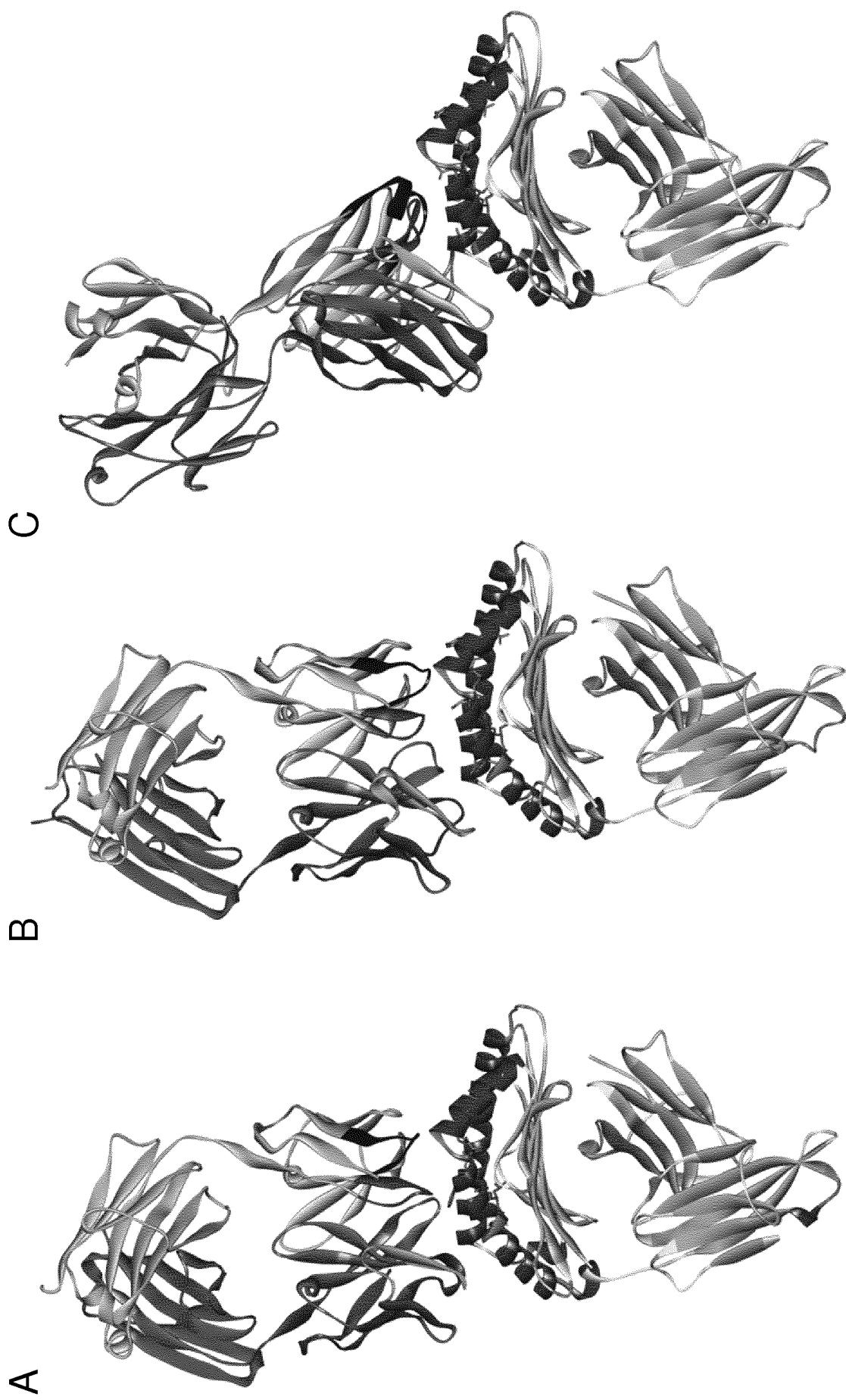
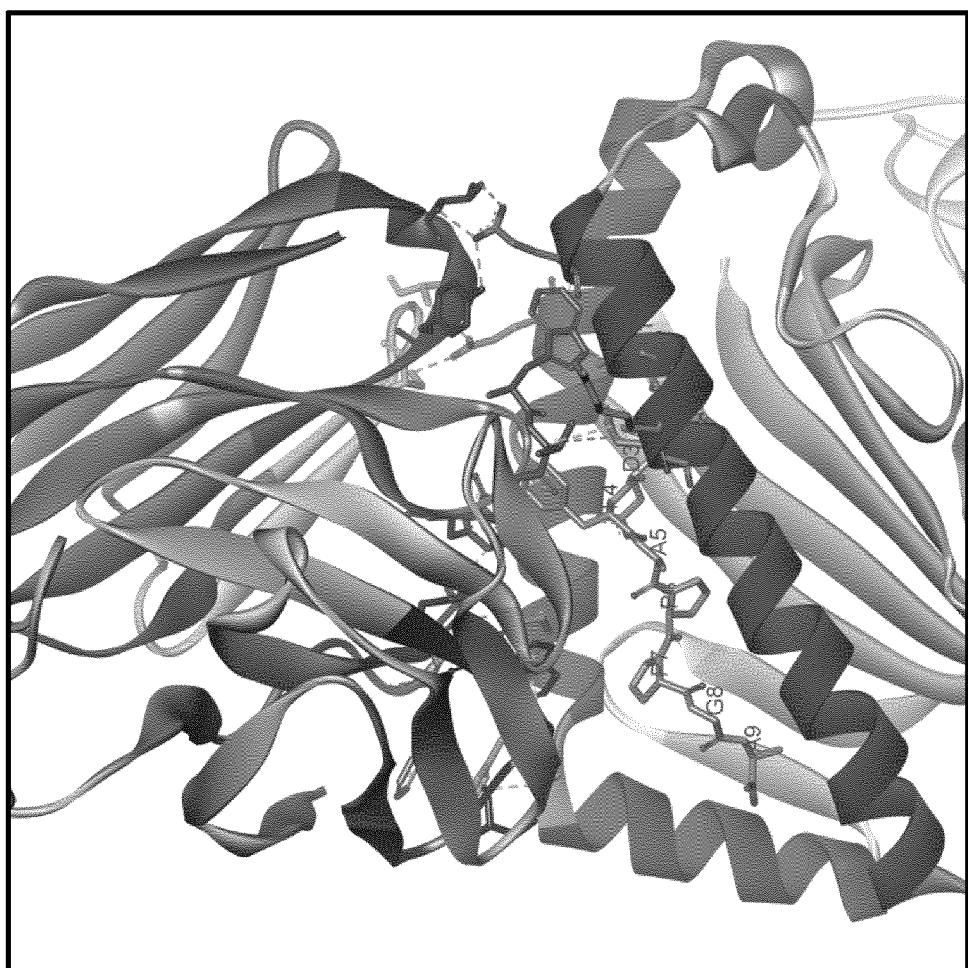


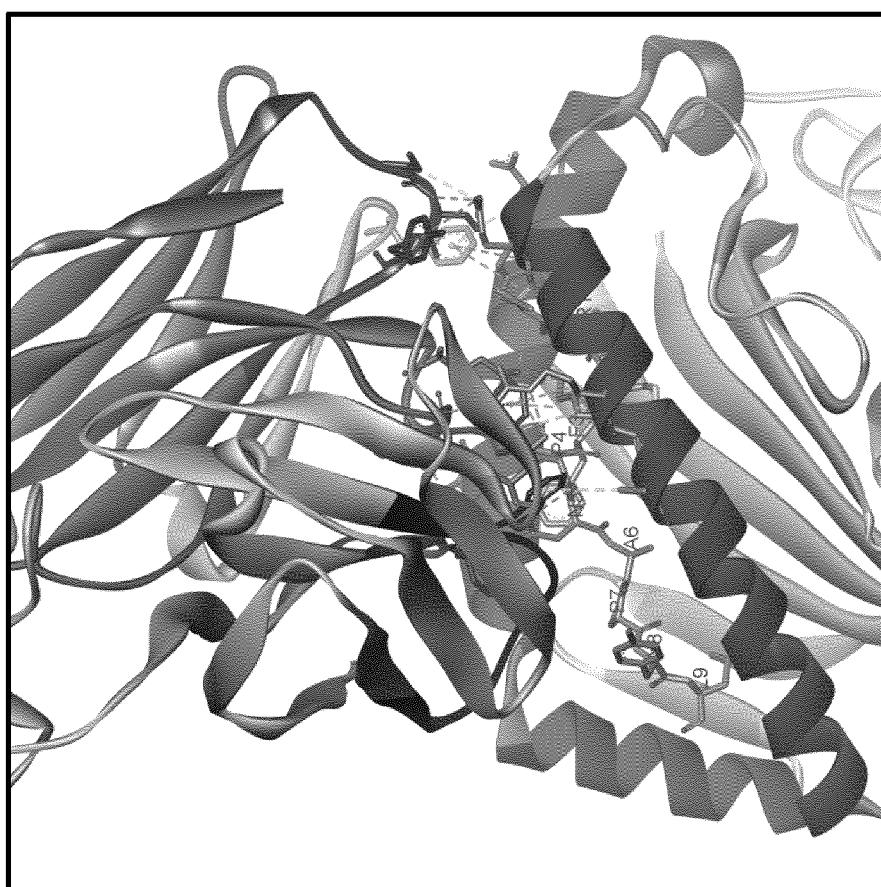
FIG. 15



Kabat↓	E58	Y59	G62	E63	R65	K66	A149	A150	H151	V152	E154	Q155	A158	Y159	G162	E166	W167	R170	V1	F4	P7
CDR-H1	F27	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H1	T28	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H1	S31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H1	Y32	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	S52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	G52A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	S53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	G54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	G55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	S56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2	Y58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3	W97	N	N	H	-	N	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N
CDR-H3	V98	-	N	-	N	N	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-
CDR-H3	S99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P1	-	-
CDR-H3	Y100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P1	-	-
CDR-L1	Q27	-	-	-	-	N	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1	S28	-	-	-	-	N	H	N	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1	S30	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	N	-	-
CDR-L1	W32	-	-	-	-	-	-	P1	N	N	P1	-	-	-	-	-	-	-	N	-	-
CDR-L2	S56	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3	Y91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3	S92	-	-	-	-	-	-	N	N	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3	I93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3	W94	-	-	-	-	-	-	-	-	P1	-	N	N	-	-	-	-	-	-	-	-

FIG. 16

FIG. 17



Kabat↓	E58	D61	G62	R65	K66	A69	A150	Q155	A158	T163	E166	W167	R170	R1	M2	P4	N5	A6	Y8
CDR-H1 S30	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H1 S31	H	-	-	-	-	-	-	-	-	N	-	N	-	-	-	-	-	-	-
CDR-H1 Y32	Pi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2 I52	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-H2 I53	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-H2 F54	-	-	-	-	-	-	-	-	-	N	Pi	Pi	-	-	-	-	-	-	-
CDR-H2 T56	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-H3 I96	N	N	N	N	-	-	-	-	-	N	-	-	SB	N	N	-	-	-	-
CDR-H3 E97	-	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-H3 W99	-	-	-	-	-	-	-	-	-	Pi	N	-	-	-	-	-	-	-	-
CDR-H3 W100	-	-	N	Pi	Pi	-	-	-	-	-	-	-	Pi	-	-	-	-	-	-
CDR-H3 G100A	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1 Q27	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1 S28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N
CDR-L1 L30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N
CDR-L1 W32	-	-	N	N	Pi	-	-	-	-	-	-	-	-	-	-	-	H	H	-
CDR-L2 Y49	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L2 D50	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3 Y91	-	-	N	-	-	-	-	-	-	N	-	-	-	-	-	-	-	H	-
CDR-L3 E92	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-L3 D93	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
CDR-L3 Y94	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-

FIG. 18

FIG. 19



Kabat ↓	E55	G56	E58	Y59	G62	E63	R65	K66	D106	W107	R108	Q155	A158	Y159	E161	G162	T163	E166	W167	R169	R170	R1	P4
CDR-H1 W33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2 R50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2 D52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2 Y54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H2 Y56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 Y97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 S98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 G99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 Y100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 Y100A	H	N	H	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-H3 W100C	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1 G29	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L1 S30	-	-	-	H	-	N	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-
CDR-L1 N31	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-
CDR-L1 T32	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L2 S50	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LC-FW3 G68	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3 W91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pi	-
CDR-L3 D93	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N
CDR-L3 S94	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-
CDR-L3 N95A	-	-	-	-	-	-	-	-	-	-	H	N	-	-	-	-	-	-	-	-	-	-	-

FIG. 20

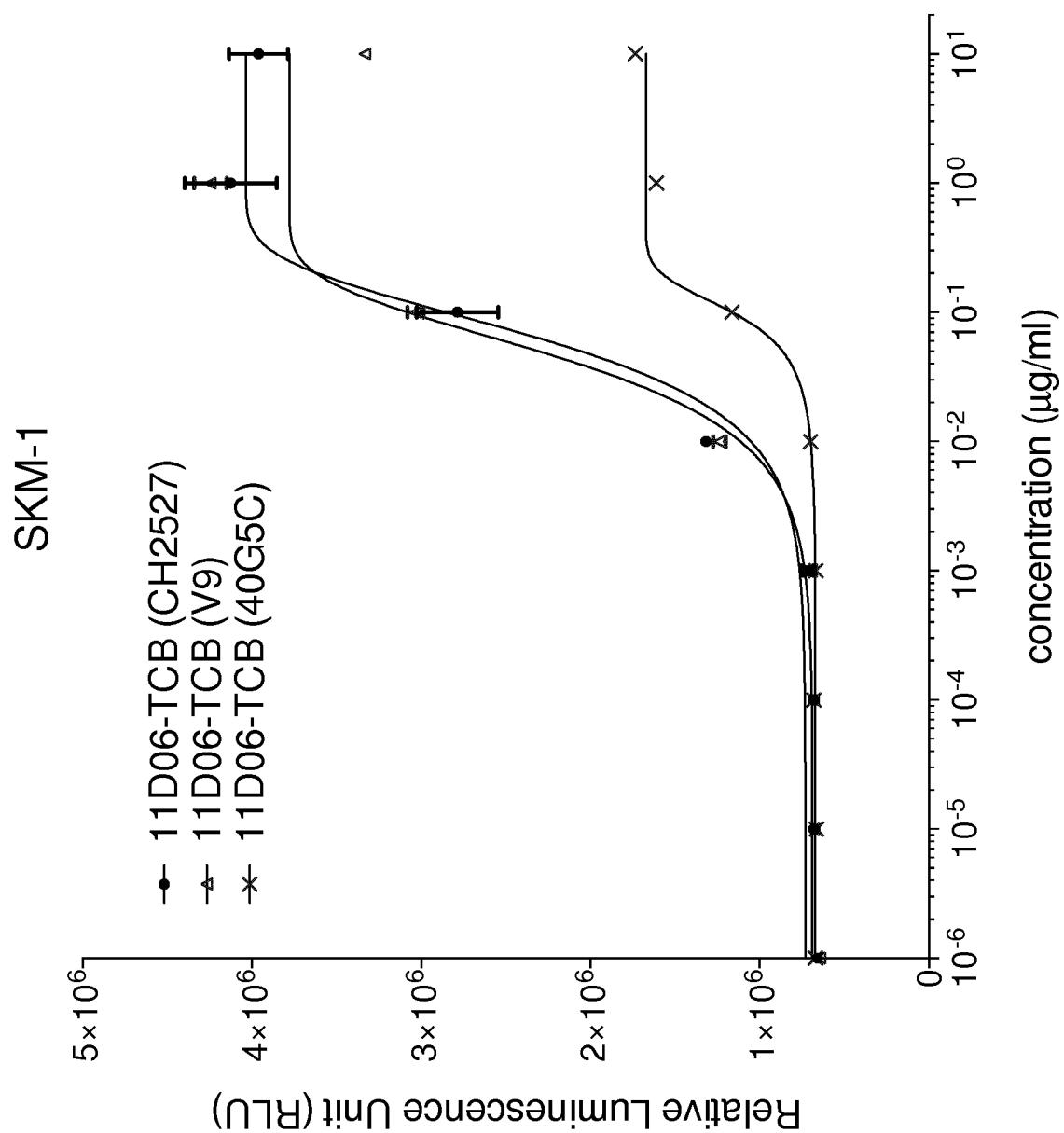


FIG. 21

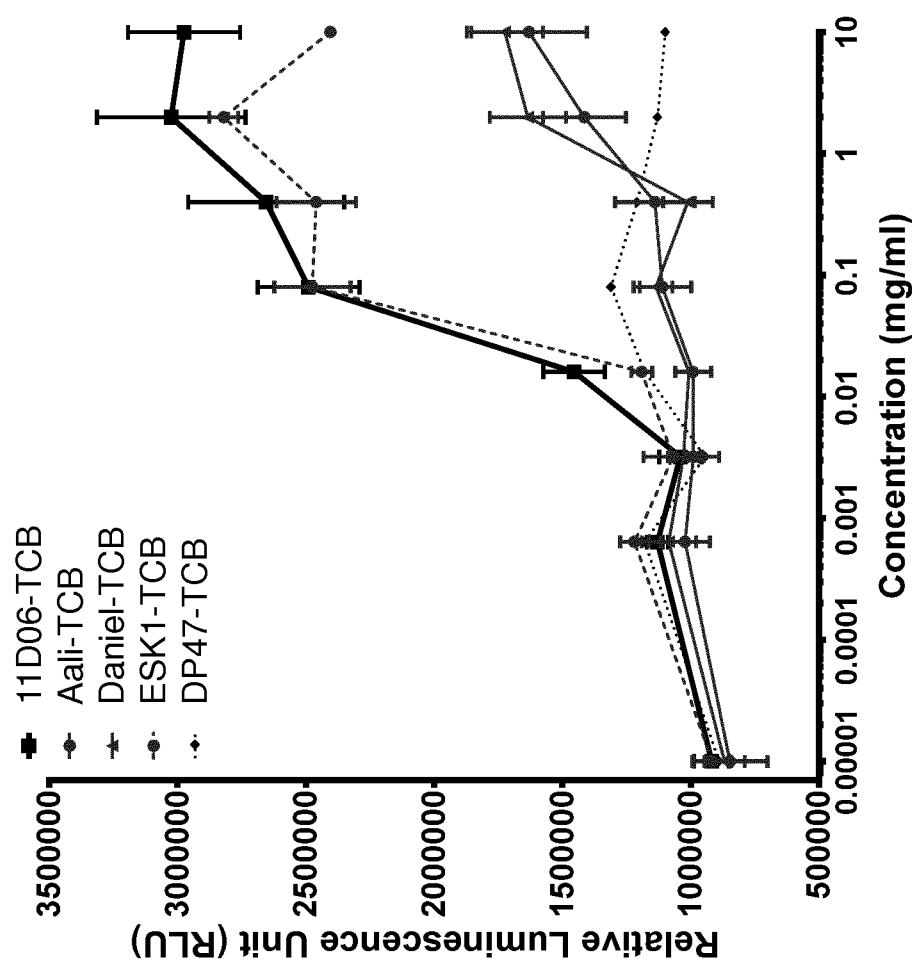


FIG. 22

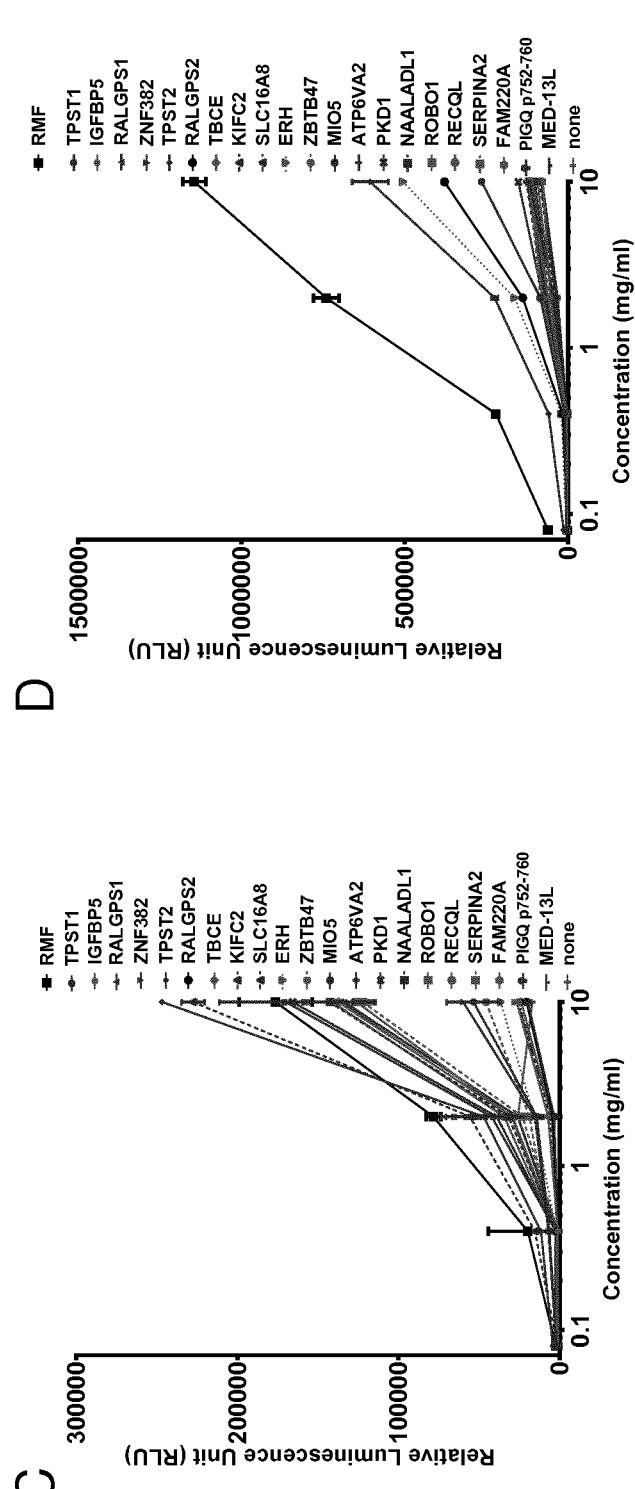
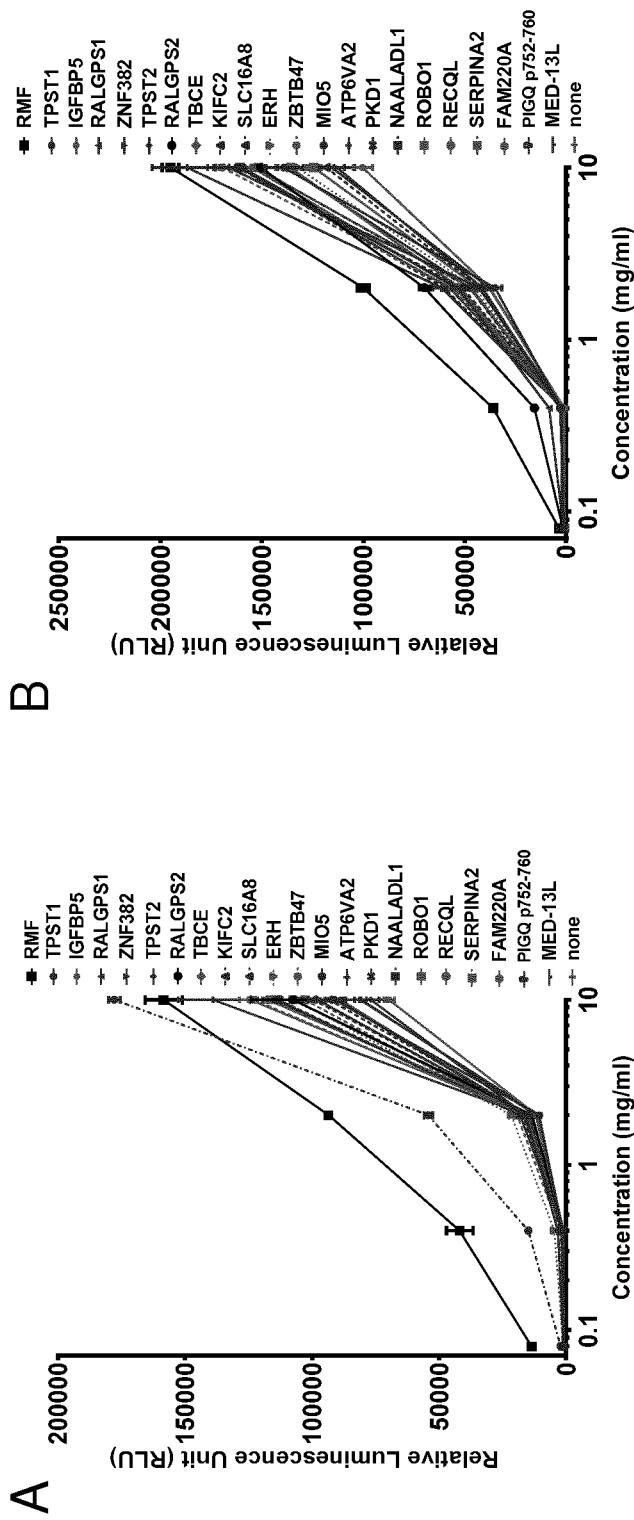


FIG. 23

FIG. 24

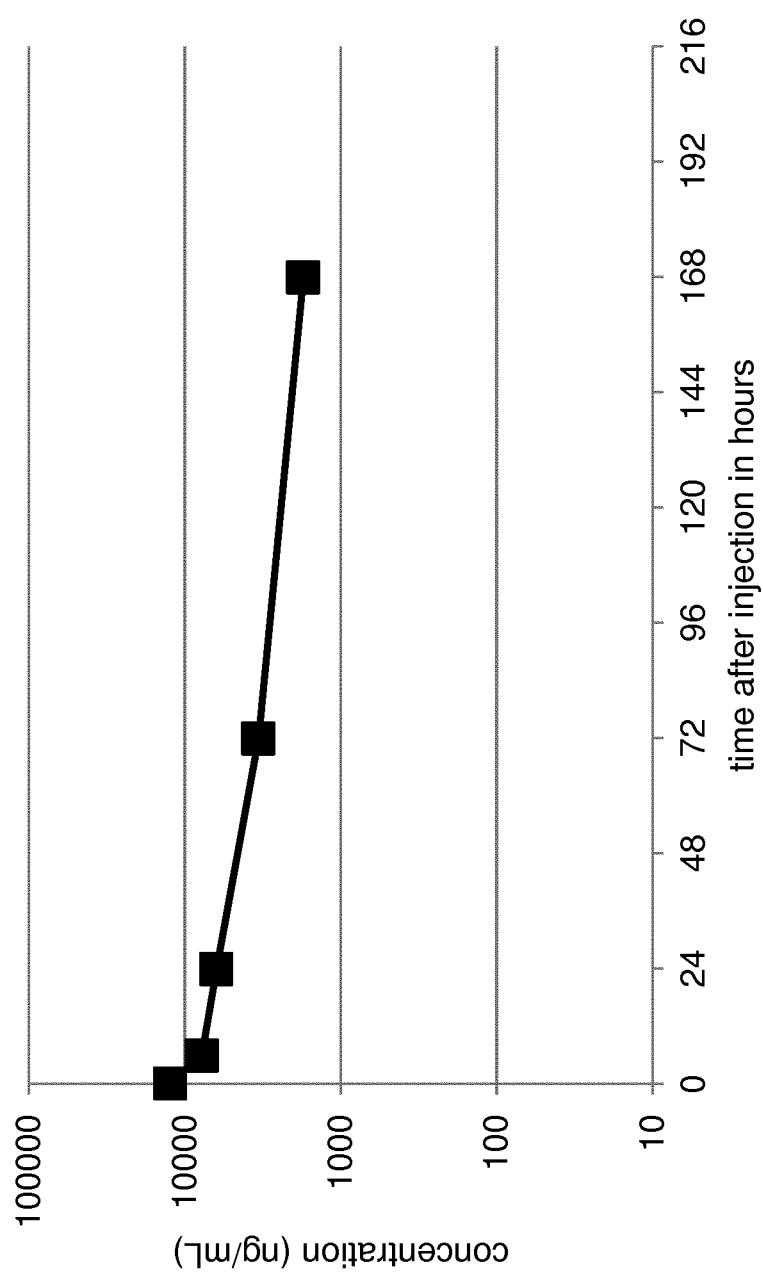
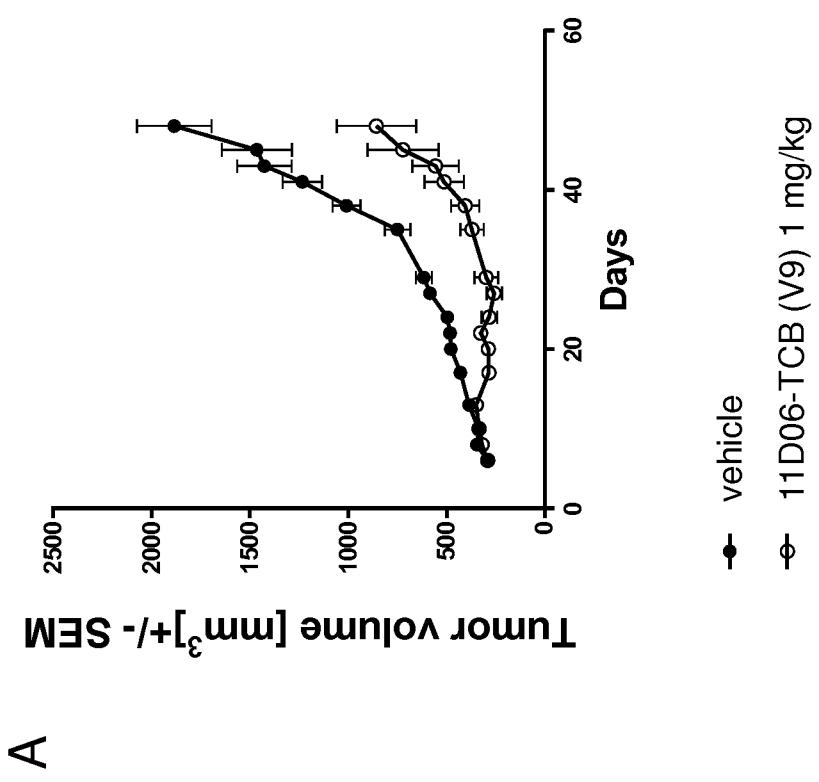


FIG. 25



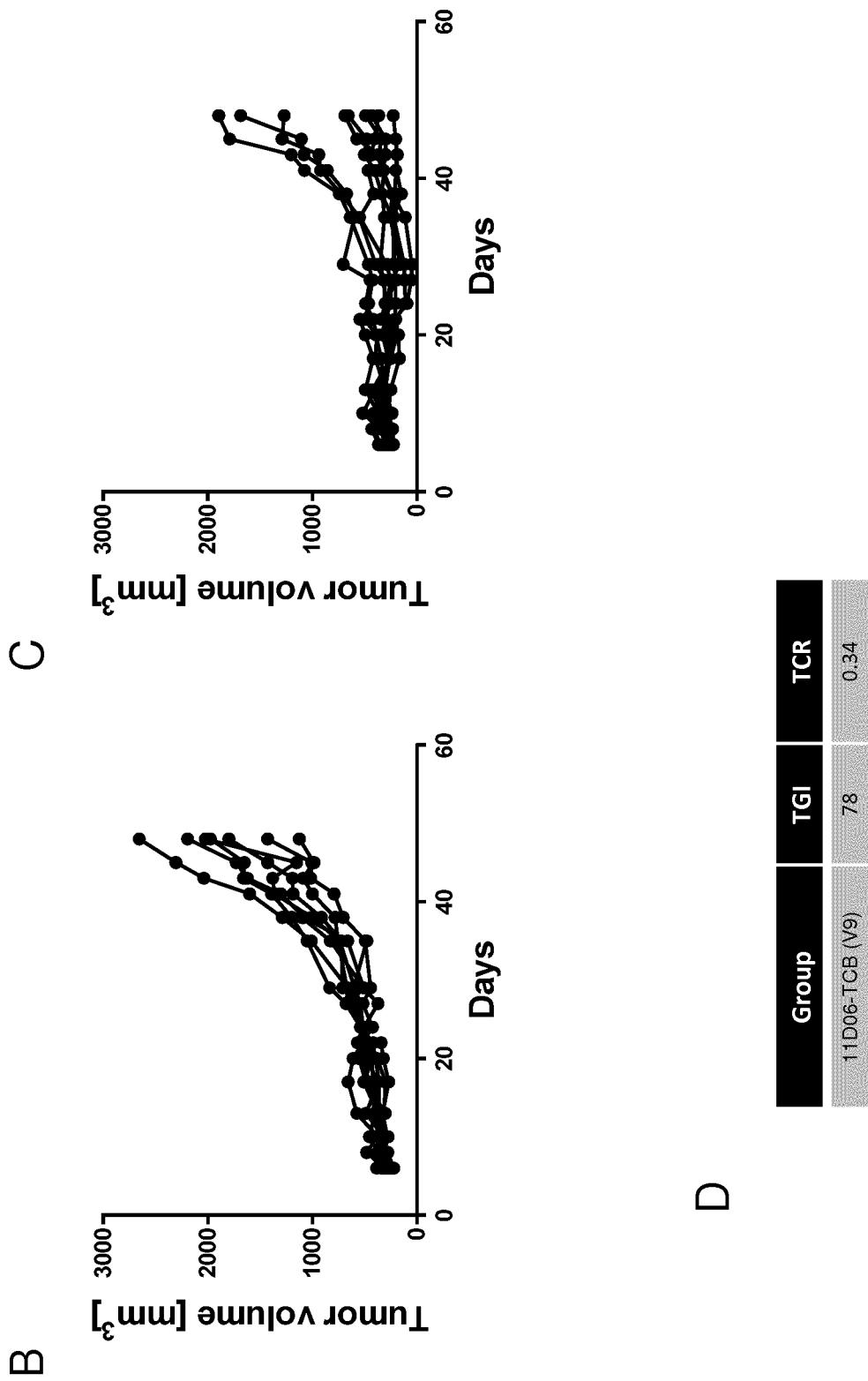


FIG. 25

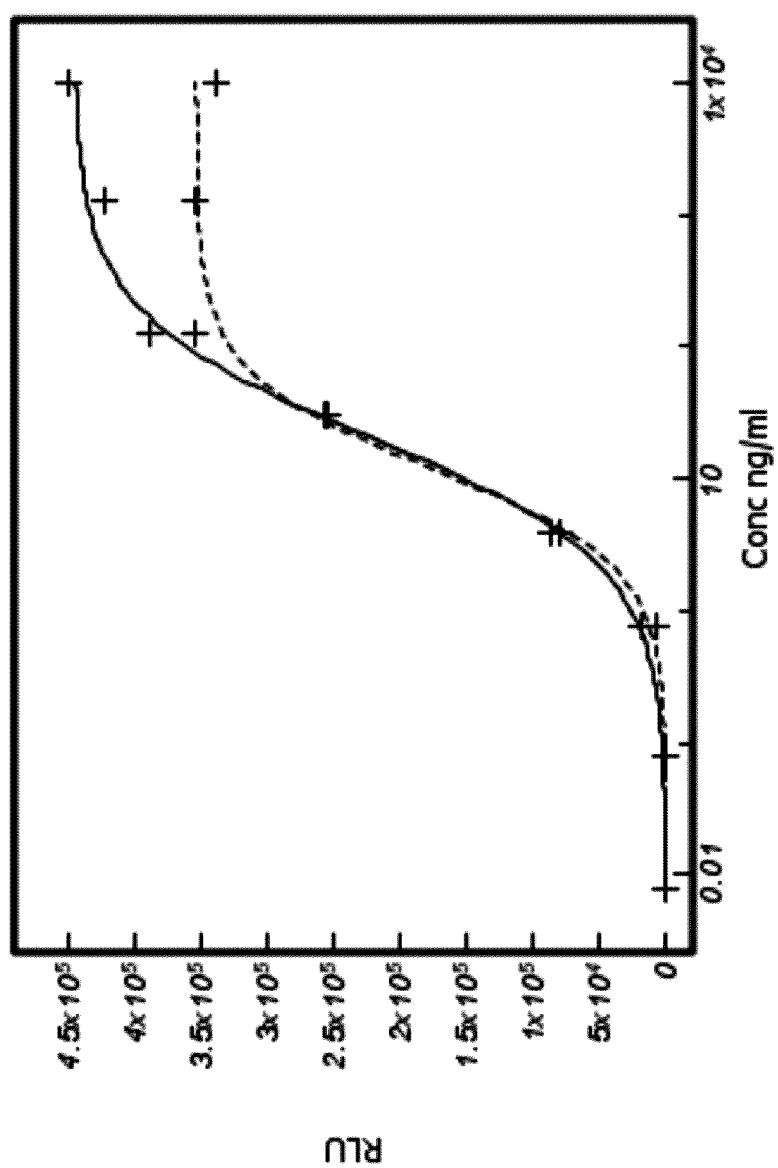


FIG. 26