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(71) Applicant (for all designated States except US): **ROCHE GLYCART AG** [CH/CH]; Wagistrasse 18, CH-8952 Schlieren (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **AUER, Johannes** [DE/DE]; Angerstrasse 7, 82445 Schwaigen (DE). **BRUENKER, Peter** [DE/CH]; Burgwiesenstrasse 3c, CH-8335 Hittnau (CH). **FAUTI, Tanja** [DE/CH]; Ruetihofstrasse 46, CH-8049 Zuerich (CH). **JAEGER, Christiane** [DE/CH]; Guggenbuehlstrasse 12, CH-8304 Wallisellen (CH). **KLEIN, Christian** [DE/CH]; Chruetzacherweg 41, CH-8906 Bonstetten (CH). **SCHAEFER, Wolfgang** [DE/DE]; Tannhaeuserring 190, 68199 Mannheim (DE). **SUSTMANN, Claudio** [DE/DE]; Penzberger Straße 3,

81373 München (DE). **UMANA, Pablo** [CR/CH]; Felsenrainstrasse 28, CH-8832 Wollerau (CH).

(74) Agent: **BRODBECK, Michel**; Grenzacherstrasse 124, CH-4070 Basel (CH).

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(54) Title: BISPECIFIC ANTIGEN BINDING MOLECULES

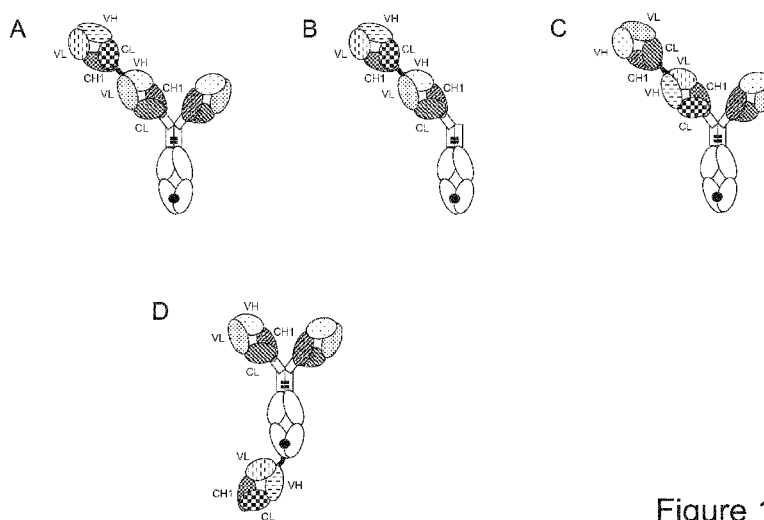


Figure 1

(57) Abstract: The present invention generally relates to novel bispecific antigen binding molecules. In addition, the present invention relates to polynucleotides encoding such bispecific antigen binding molecules, and vectors and host cells comprising such polynucleotides. The invention further relates to methods for producing the bispecific antigen binding molecules of the invention, and to methods of using these bispecific antigen binding molecules in the treatment of disease.

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BISPECIFIC ANTIGEN BINDING MOLECULES

Field of the Invention

The present invention generally relates to bispecific antigen binding molecules. In addition, the present invention relates to polynucleotides encoding such bispecific antigen binding molecules, and vectors and host cells comprising such polynucleotides. The invention further relates to
5 methods for producing the bispecific antigen binding molecules of the invention, and to methods of using these bispecific antigen binding molecules in the treatment of disease.

Background

Bi- or multispecific antibodies capable of binding two or more antigens are known in the art. Such multispecific binding proteins can be generated by hybridoma cell fusion, chemical
10 conjugation or recombinant DNA techniques.

Bispecific antibodies are of great interest for therapeutic applications, as they allow the simultaneous binding and inactivation of two or more target antigens, thereby obviating the need for combination therapies. Another promising application of bispecific antibodies is as engagers of immune effector cells e.g. for cellular cancer immunotherapy. For this purpose, bispecific
15 antibodies are designed which bind to a surface antigen on target cells and, for example, to an activating component of the T cell receptor (TCR) complex. The simultaneous binding of such an antibody to both of its targets will force a temporary interaction between target cell and T cell, causing activation of any cytotoxic T lymphocyte (CTL) and subsequent lysis of the target cell. Hence, the immune response is re-directed to the target cells, independently of peptide antigen
20 presentation by the target cell or the specificity of the T cell as required for normal MHC-restricted activation of CTLs. In this context it is important that CTLs are only activated when a target cell is presenting the bispecific antibody to them, i.e. when the immunological synapse is mimicked, and not simply upon binding of the antibody to the T cell antigen.

A variety of recombinant multispecific antibody formats have been developed in the recent past,
25 including, for example, tetravalent IgG-single-chain variable fragment (scFv) fusions (see e.g. Coloma & Morrison, Nat Biotechnol 15, 159-163 (1997)), tetravalent IgG-like dual-variable

domain antibodies (Wu et al., Nat Biotechnol 25, 1290-1297 (2007)), or bivalent rat/mouse hybrid bispecific IgGs (see e.g. Lindhofer et al., J Immunol 155, 219-225 (1995)).

Also several bispecific formats wherein the antibody core structure (IgA, IgD, IgE, IgG or IgM) is no longer retained have been made. Examples include diabodies (see e.g. Holliger et al., Proc Natl Acad Sci USA 90, 6444-6448 (1995)), tandem scFv molecules (see e.g. Bargou et al., Science 321, 974-977 (2008)), and various derivatives thereof.

The multitude of formats that are being developed shows the great potential attributed to bispecific antibodies. The task of generating bispecific antibodies suitable for a particular purpose is, however, by no means trivial and subject to a number of considerations. For example, the valency and geometry of the antibody needs to be appropriately chosen, depending on the characteristics of the target antigens and the intended effect. As for all therapeutic antibodies, efficacy and toxicity have to be balanced, which requires i.a. minimization of immunogenicity and optimization of pharmacokinetic properties of the antibody. Also, the desirability of Fc-mediated effects has to be considered. Furthermore, the production of bispecific antibody constructs at a clinically sufficient quantity and purity poses a major challenge, as the homodimerization of antibody heavy chains and/or the mispairing of antibody heavy and light chains of different specificities upon co-expression decreases the yield of the correctly assembled construct and results in a number of non-functional side products from which the desired bispecific antibody may be difficult to separate.

Given the increasing number of possible applications of bispecific antibodies, and the difficulties and disadvantages associated with currently available bispecific antibodies, there remains a need for novel, improved formats of such molecules.

Summary of the Invention

In a first aspect, the invention provides a bispecific antigen binding molecule, comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- a) the bispecific antigen binding molecule provides monovalent binding to the first and/or the second antigen,
- b) the first Fab fragment, the second Fab fragment and the first Fc domain subunit are fused to each other, and

c) in the first and/or the second Fab fragment one of the following replacements is made: (i) the variable domains VL and VH are replaced by each other, (ii) the constant domains CL and CH1 are replaced by each other, or (iii) both the variable and constant domains VL-CL and VH-CH1 are replaced by each other,

5 provided that not the same replacement is made in the first and the second Fab fragment.

In particular embodiments, the first Fab fragment is fused at its C-terminus to the N-terminus of the second Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit. In a more specific embodiment, the first Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the second Fab fragment, which is in
10 turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit.

In other embodiments, the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit. In a more specific embodiment, the second Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the first Fab fragment, which
15 is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit. In still other embodiments, the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fc domain subunit, which is in turn fused at its C-terminus to the N-terminus of the first Fab fragment. In a more specific embodiment, the second Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit,
20 which is in turn fused at its C-terminus to the N-terminus of the heavy chain of the first Fab fragment.

In embodiments wherein either the first Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the second Fab fragment which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit, or the second
25 Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the first Fab fragment which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit, additionally the Fab light chain of the first Fab fragment and the Fab light chain of the second Fab fragment may be fused to each other, optionally via a peptide linker.

30 In one embodiment, the replacement is made in the first Fab fragment. In some embodiments, the replacement is a replacement of the variable domains VL and VH by each other. In other embodiments the replacement is a replacement of the constant domains CL and CH1 by each other.

In one embodiment the bispecific antigen binding molecule essentially consists of the first Fab fragment, the second Fab fragment, the Fc domain, and optionally one or more peptide linkers.

In particular embodiments, the bispecific antigen binding molecule comprises a third Fab fragment which specifically binds to the first or the second antigen. In one embodiment, the third Fab fragment is fused to the second Fc domain subunit. In a more specific embodiment, the third Fab fragment is fused at its C-terminus to the N-terminus of the second Fc domain subunit. In an even more specific embodiment, the third Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the second Fc domain subunit. In one embodiment, the third Fab fragment specifically binds to the second antigen. In some embodiments the second Fab fragment, the third Fab fragment and the Fc domain are part of an immunoglobulin molecule. In a specific such embodiment, the immunoglobulin molecule is an IgG class immunoglobulin molecule, more specifically an IgG1 or IgG4 subclass immunoglobulin molecule. In one embodiment, the immunoglobulin molecule is a human immunoglobulin molecule. In one embodiment, the bispecific antigen binding molecule essentially consists of a first Fab fragment which specifically binds to the first antigen, an immunoglobulin molecule which specifically binds to the second antigen, and optionally one or more peptide linkers.

In one embodiment, the same replacement is made in Fab fragments that specifically bind to the same antigen. In a further embodiment, a replacement is made only in the first Fab fragment. In one embodiment, the bispecific antigen binding molecule provides monovalent binding to the first antigen. In one embodiment, the bispecific antigen binding molecule does not comprise a single-chain Fab fragment.

In certain embodiments, the Fc domain comprises a modification promoting the association of the first and second Fc domain subunit. In a specific such 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 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 is an IgG Fc domain, specifically an IgG1 or IgG4 Fc domain. In one embodiment the Fc domain is human. In certain embodiments, the Fc domain is engineered to have altered binding affinity to an Fc receptor and/or altered effector function, as compared to a non-engineered Fc domain.

According to another aspect of the invention there is provided an isolated polynucleotide encoding a bispecific antigen binding molecule of the invention or a fragment thereof. The invention also encompasses polypeptides encoded by the polynucleotides of the invention. The invention further provides an expression vector comprising the isolated polynucleotide of the invention, and a host cell comprising the isolated polynucleotide or the expression vector of the invention. In some embodiments the host cell is a eukaryotic cell, particularly a mammalian cell. In another aspect is provided a method of producing the bispecific antigen binding molecule of the invention, comprising the steps of a) culturing the host cell of the invention under conditions suitable for the expression of the bispecific antigen binding molecule and b) recovering the bispecific antigen binding molecule. The invention also encompasses a bispecific antigen binding molecule produced by the method of the invention.

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

Also encompassed by the invention are methods of using the bispecific antigen binding molecule and pharmaceutical composition of the invention. In one aspect the invention provides a bispecific antigen binding molecule or a pharmaceutical composition of the invention for use as a medicament. In one aspect is provided a bispecific antigen binding molecule or a pharmaceutical composition according to the invention for use in the treatment of a disease in an individual in need thereof. In a specific embodiment the disease is cancer.

Also provided is the use of a bispecific antigen binding molecule of the invention for the manufacture of a medicament for the treatment of a disease in an individual in need thereof; 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 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 a mammal, particularly a human.

Brief Description of the Drawings

FIGURE 1. Illustration of exemplary formats of the bispecific antigen binding molecules of the invention. (A) “2+1” format, with Crossfab fragment of different specificity fused to N-terminus of a Fab fragment comprised in an antibody (“2+1 IgG Crossfab (N-terminal)”). (B) “1+1” format, with Crossfab fragment of different specificity fused to N-terminus of a Fab fragment

comprised in an antibody lacking the second Fab fragment (“1+1 IgG Crossfab (N-terminal)”).
(C) “2+1” format as in (A), wherein the order of the Crossfab fragment, and the Fab fragment to
which the Crossfab fragment is fused, is inverted (“2+1” IgG Crossfab (N-terminal), inverted”).
(D) “2+1” format, with Crossfab fragment of different specificity fused to C-terminus of an Fc
5 domain subunit comprised in an antibody (“2+1 IgG Crossfab (C-terminal)”). Black dot: optional
modification in the Fc domain promoting heterodimerization.

FIGURE 2. (A, B) SDS PAGE (4-12% Tris-Acetate (A) or 4-12% Bis/Tris (B), NuPage
Invitrogen, Coomassie-stained) of “1+1 IgG Crossfab (N-terminal), Fc(hole) P329G LALA /
Fc(knob) wt” (anti-MCSP/anti-huCD3) (see SEQ ID NOs 1, 2, 3 and 4), non reduced (A) and
10 reduced (B). (C) Analytical size exclusion chromatography (Superdex 200 10/300 GL GE
Healthcare; 2 mM MOPS pH 7.3, 150 mM NaCl, 0.02% (w/v) NaCl; 50 µg sample injected) of
“1+1 IgG Crossfab (N-terminal), Fc(hole) P329G LALA / Fc(knob) wt” (anti-MCSP/anti-
huCD3).

FIGURE 3. (A, B) SDS PAGE (4-12% Bis/Tris, NuPage Invitrogen, Coomassie-stained) of “2+1
15 IgG Crossfab (N-terminal)” (anti-MCSP/anti-huCD3) (see SEQ ID NOs 1, 3, 4 and 5), non
reduced (A) and reduced (B). (C) Analytical size exclusion chromatography (Superdex 200
10/300 GL GE Healthcare; 2 mM MOPS pH 7.3, 150 mM NaCl, 0.02% (w/v) NaCl; 50 µg
sample injected) of “2+1 IgG Crossfab (N-terminal)” (anti-MCSP/anti-huCD3).

FIGURE 4. (A, B) SDS PAGE (4-12% Bis/Tris, NuPage Invitrogen, Coomassie-stained) of “2+1
20 IgG Crossfab (N-terminal), inverted” (anti-CEA/anti-huCD3) (see SEQ ID NOs 3, 8, 9 and 10),
non reduced (A) and reduced (B). (C) Analytical size exclusion chromatography (Superdex 200
10/300 GL GE Healthcare; 2 mM MOPS pH 7.3, 150 mM NaCl, 0.02% (w/v) NaCl; 50 µg
sample injected) of “2+1 IgG Crossfab (N-terminal), inverted” (anti-CEA/anti-huCD3).

FIGURE 5. (A, B) Capillary electrophoresis (CE)-SDS gel analysis of “2+1 IgG Crossfab (C-
25 terminal)” (anti-c-Met/anti-Her3) (see SEQ ID NOs 11, 12, 13, 14), non reduced (A) and
reduced (B).

FIGURE 6. Simultaneous binding of bispecific constructs to the D3 domain of human MCSP
and human CD3γ(G₄S)₅CD3ε–AcTev–Fc(knob)–Avi/Fc(hole). (A) Biacore assay setup; (B)
measurement of “2+1 IgG Crossfab (N-terminal)”.

30 FIGURE 7. Levels of different cytokines measured in the supernatant of whole blood after
treatment with 1 nM of different CD3-MCSP bispecific constructs (“2+1 IgG Crossfab (N-
terminal)”, “(scFv)₂”) or corresponding control IgGs in the presence (A, B) or absence (C, D) of
Colo-38 tumor cells for 24 hours.

FIGURE 8. Surface expression level of the late activation marker CD25 on cynomolgus CD8⁺ T cells from two different animals (cyno Nestor, cyno Nobu) after 43 hours incubation with the indicated concentrations of the “2+1 IgG Crossfab (N-terminal)” bispecific construct (targeting cynomolgus CD3 and human MCSP), in the presence or absence of human MCSP-expressing
5 MV-3 tumor target cells (E:T ratio = 3:1). As controls, the reference IgGs (anti-cynomolgus CD3 IgG, anti-human MCSP IgG) or the unphysiologic stimulus PHA-M were used.

FIGURE 9. Killing (as measured by LDH release) of MDA-MB-435 tumor cells upon co-culture with human pan T cells (E:T ratio = 5:1) and activation for 20 hours by different concentrations of the “2+1 IgG Crossfab (N-terminal)” and “(scFv)₂” bispecific molecules and corresponding
10 IgGs.

FIGURE 10. Killing (as measured by LDH release) of MDA-MB-435 tumor cells upon co-culture with human pan T cells (E:T ratio = 5:1), and activation for 21 hours by different concentrations of the bispecific constructs and corresponding IgGs. The CD3-MCSP bispecific “2+1 IgG Crossfab (N-terminal)” and “1+1 IgG Crossfab (N-terminal)” constructs, the “(scFv)₂”
15 molecule and corresponding IgGs were compared.

FIGURE 11. Killing (as measured by LDH release) of huMCSP-positive MV-3 melanoma cells upon co-culture with human PBMCs (E:T ratio = 10:1), treated with different CD3-MCSP bispecific constructs (“2+1 IgG Crossfab (N-terminal)” and “(scFv)₂”) for ~26 hours.

FIGURE 12. Exemplary configurations of bispecific antigen binding molecules of the invention
20 having a linked light chain. (A) Illustration of the “2+1 IgG Crossfab (N terminal), linked light chain” molecule. (B) Illustration of the “1+1 IgG Crossfab (N-terminal), linked light chain” molecule. (C) Illustration of the “2+1 IgG Crossfab (N-terminal), inverted, linked light chain” molecule. (D) Illustration of the “1+1 IgG Crossfab (N-terminal), inverted, linked light chain” molecule.

25 FIGURE 13. CE-SDS analyses. Electropherogram shown as SDS PAGE of “2+1 IgG Crossfab (N-terminal), linked light chain” (lane 1: reduced, lane 2: non-reduced).

FIGURE 14. Analytical size exclusion chromatography of “2+1 IgG Crossfab (N-terminal), linked light chain” (final product). 20 µg sample “2+1 IgG Crossfab (N-terminal), linked light chain” were injected.

30 FIGURE 15. Killing (as measured by LDH release) of MCSP-positive MV-3 tumor cells upon co-culture by human PBMCs (E:T ratio = 10:1), treated with different CD3-MCSP bispecific constructs for ~ 44 hours. Human PBMCs were isolated from fresh blood of healthy volunteers.

FIGURE 16. Killing (as measured by LDH release) of MCSP-positive Colo-38 tumor cells upon co-culture by human PBMCs (E:T ratio = 10:1), treated with different CD3-MCSP bispecific constructs for ~22 hours. Human PBMCs were isolated from fresh blood of healthy volunteers.

5 FIGURE 17. Killing (as measured by LDH release) of MCSP-positive Colo-38 tumor cells upon co-culture by human PBMCs (E:T ratio = 10:1), treated with different CD3-MCSP bispecific constructs for ~22 hours. Human PBMCs were isolated from fresh blood of healthy volunteers.

FIGURE 18. Killing (as measured by LDH release) of MCSP-positive WM266-4 cells upon co-culture by human PBMCs (E:T ratio = 10:1), treated with different CD3-MCSP bispecific constructs for ~22 hours. Human PBMCs were isolated from fresh blood of healthy volunteers.

10 FIGURE 19. Surface expression level of the early activation marker CD69 (A) and the late activation marker CD25 (B) on human CD8⁺ T cells after 22 hours incubation with 10 nM, 80 pM or 3 pM of different CD3-MCSP bispecific constructs in the presence or absence of human MCSP-expressing Colo-38 tumor target cells (E:T ratio = 10:1).

FIGURE 20. CE-SDS analyses. (A) Electropherogram shown as SDS-PAGE of 1+1 IgG Crossfab (N-terminal); VL/VH exchange (LC007/V9): a) non-reduced, b) reduced. (B) 15 Electropherogram shown as SDS-PAGE of 1+1 CrossMab; CL/CH1 exchange (LC007/V9): a) reduced, b) non-reduced. (C) Electropherogram shown as SDS-PAGE of 2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (LC007/V9): a) reduced, b) non-reduced. (D) Electropherogram shown as SDS-PAGE of 2+1 IgG Crossfab (N-terminal); VL/VH exchange 20 (M4-3 ML2/V9): a) reduced, b) non-reduced. (E) Electropherogram shown as SDS-PAGE of 2+1 IgG Crossfab (N-terminal); CL/CH1 exchange (M4-3 ML2/V9): a) reduced, b) non-reduced. (F) Electropherogram shown as SDS-PAGE of 2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (CH1A1A/V9): a) reduced, b) non-reduced.

FIGURE 21. Surface expression level of the early activation marker CD69 (A) or the late 25 activation marker CD25 (B) on human CD4⁺ or CD8⁺ T cells after 24 hours incubation with the indicated concentrations of the CD3/MCSP “1+1 CrossMab”, “1+1 IgG Crossfab (N-terminal)” and “2+1 IgG Crossfab (N-terminal)” constructs. The assay was performed in the presence or absence of MV-3 target cells, as indicated.

FIGURE 22. Killing (as measured by LDH release) of MKN-45 (A) or LS-174T (B) tumor cells 30 upon co-culture with human PBMCs (E:T ratio = 10:1) and activation for 28 hours by different concentrations of the “2+1 IgG Crossfab (N-terminal), inverted (VL/VH)” versus the “2+1 IgG Crossfab (N-terminal), inverted (CL/CH1)” construct.

FIGURE 23. Killing (as measured by LDH release) of WM266-4 tumor cells upon co-culture with human PBMCs (E:T ratio = 10:1) and activation for 26 hours by different concentrations of the “2+1 IgG Crossfab (N-terminal) (VL/VH)” versus the “2+1 IgG Crossfab (N-terminal) (CL/CH1)” construct.

5 FIGURE 24. Killing (as measured by LDH release) of MV-3 tumor cells upon co-culture with human PBMCs (E:T ratio = 10:1) and activation for 27 hours by different concentrations of the “2+1 IgG Crossfab (N-terminal) (VH/VL)” versus the “2+1 IgG Crossfab (N-terminal) (CL/CH1)” constructs.

FIGURE 25. Killing (as measured by LDH release) of human MCSP-positive WM266-4 (A) or
10 MV-3 (B) tumor cells upon co-culture with human PBMCs (E:T ratio = 10:1) and activation for 21 hours by different concentrations of the “2+1 IgG Crossfab (N-terminal)”, the “1+1 CrossMab”, and the “1+1 IgG Crossfab (N-terminal)”, as indicated.

FIGURE 26. Binding of bispecific constructs to human CD3, expressed by Jurkat cells (A), or to human CEA, expressed by LS-174T cells (B) as determined by FACS. As a control, the
15 equivalent maximum concentration of the reference IgGs and the background staining due to the labeled 2ndary antibody (goat anti-human FITC-conjugated AffiniPure F(ab')₂ Fragment, Fcγ Fragment-specific, Jackson Immuno Research Lab # 109-096-098) were assessed as well.

FIGURE 27. Binding of bispecific constructs to human CD3, expressed by Jurkat cells, or to human MCSP, expressed by WM266-4 tumor cells (B) as determined by FACS.

20

Detailed Description of the Invention

Definitions

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

As used herein, the term "antigen binding molecule" refers in its broadest sense to a molecule
25 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 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
30 embodiments the bispecific antigen binding molecule is capable of simultaneously binding two antigenic determinants, particularly two antigenic determinants expressed on two distinct cells.

As used herein, the term "antigenic determinant" is synonymous with "antigen" and "epitope," and refers to a site (e.g. a contiguous stretch of amino acids or a conformational configuration made up of different regions of non-contiguous amino acids) on a polypeptide macromolecule to which an antigen binding moiety binds, forming an antigen binding moiety-antigen complex.

5 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 proteins referred to as antigens herein (e.g. MCSP, FAP, CEA, EGFR, CD33, CD3, c-Met, Her3) can be any native form the proteins from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g. mice and rats), unless otherwise indicated. In a particular embodiment the antigen is a human protein. Where reference is made to a specific protein herein, the term encompasses the "full-length", unprocessed protein as well as any form of the protein that results from processing in the cell. The term also encompasses naturally occurring variants of the protein, e.g. splice variants or allelic variants. Exemplary human proteins useful as antigens
10 include, but are not limited to: Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), also known as Chondroitin Sulfate Proteoglycan 4 (UniProt no. Q6UVK1, NCBI Accession no. NP_001888); Fibroblast Activation Protein (FAP), also known as Seprase (Uni Prot nos. Q12884, Q86Z29, Q99998, NCBI Accession no. NP_004451); Carcinoembryonic antigen (CEA), also known as Carcinoembryonic antigen-related cell adhesion molecule 5 (UniProt no. P06731, NCBI Accession no. NP_004354); CD33, also known as gp67 or Siglec-3 (UniProt no. P20138, NCBI Accession nos. NP_001076087, NP_001171079); Epidermal Growth Factor Receptor (EGFR), also known as ErbB-1 or Her1 (UniProt no. P0053, NCBI Accession nos. NP_958439, NP_958440), CD3, particularly the epsilon subunit of CD3 (UniProt no. P07766, NCBI Accession no. NP_000724); c-Met, also known as Hepatocyte Growth Factor Receptor (UniProt
15 no. P08581, NCBI Accession nos. NP_000236, NP_001120972) and Her3, also known as ErbB-3 (UniProt no. P21860, NCBI Accession nos. NP_001973, NP_001005915). In certain embodiments the bispecific antigen binding molecule of the invention binds to an epitope of an first antigen or a second antigen that is conserved among the first antigen or second antigen from different species.

30 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 antibody 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 on a BIAcore instrument) (Liljeblad et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). In one embodiment, the extent of binding of an antibody to an unrelated protein is less than about 10% of the binding of the antibody to the antigen as measured, e.g., by SPR. In certain embodiments, an antibody or a fragment thereof that binds to the antigen 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} M or less, e.g. from 10^{-8} M to 10^{-13} M , e.g., from 10^{-9} M to 10^{-13} M).

“Affinity” refers to the strength of the sum total of non-covalent interactions between a single 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 (K_D), which is the ratio of dissociation and association rate constants (k_{off} and k_{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 particular method for measuring affinity is Surface Plasmon Resonance (SPR).

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 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 fragment 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. 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 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: α , δ , ϵ , γ , or μ . Useful light chain constant regions include any of the two isotypes: κ and λ .

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

As used herein, the term "single-chain" refers to a molecule comprising amino acid monomers linearly linked by peptide bonds. By a single-chain Fab fragment is meant a Fab molecule wherein the Fab light chain and the Fab heavy chain are connected by a peptide linker to form a
10 single peptide chain.

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 region (VH), also
15 called a variable heavy domain or a heavy chain variable domain, followed by a hinge region (HR) and three constant domains (CH1, CH2, and CH3), also called a heavy chain constant region. In case of an IgE class immunoglobulin the heavy chain additionally has a CH4 domain. Hence, an immunoglobulin heavy chain is a polypeptide consisting in N-terminal to C-terminal direction of the following domains: VH-CH1-HR-CH2-CH3-(CH4). Similarly, from N- to C-
20 terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain, also called a light chain constant region. Hence, an immunoglobulin light chain is a polypeptide consisting in N-terminal to C-terminal direction of the following domains: VL-CL. The heavy chain of an immunoglobulin may be assigned to one of five types, called α (IgA), δ (IgD), ϵ (IgE), γ (IgG),
25 or μ (IgM), some of which may be further divided into subtypes, e.g. γ_1 (IgG₁), γ_2 (IgG₂), γ_3 (IgG₃), γ_4 (IgG₄), α_1 (IgA₁) and α_2 (IgA₂). The light chain of an immunoglobulin may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain. An immunoglobulin essentially consists of two Fab fragments and an Fc domain, linked via the immunoglobulin hinge region.

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

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')₂, 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. Plückthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg 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')₂ 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; 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 (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.

A "Fab fragment" 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. A Fab fragment being fused to another protein is, in its unmodified form, fused at its heavy chain C- or N-terminus. Consequently, where the variable domains VH and VL are replaced by each other, the Fab fragment is fused at the C-terminus of the CH1 domain or the N-terminus of the VL domain. Similarly, where the constant domains CH1 and CL are replaced by each other, the Fab fragment is fused at the C-terminus of the CL domain or the N-terminus of the VH domain, and where the complete Fab heavy chain (VH-CH1) and Fab light chain (VL-CL) are replaced by each other, the Fab fragment is fused at its light chain C- or N-terminus.

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

The term "antigen binding domain" refers to the part of an antibody that comprises the area 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 region (VL) and an antibody heavy chain variable region (VH).

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, 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*, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity.

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 and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the complementarity determining regions (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. Hypervariable regions (HVRs) are also referred to as "complementarity determining regions" (CDRs), and these terms are used herein interchangeably in reference to portions of the variable region that form the antigen binding regions. This particular region has been described by Kabat et al., U.S. Dept. of Health and Human Services, *Sequences of Proteins of Immunological Interest* (1983) and by Chothia et al., *J Mol Biol* 196:901-917 (1987), where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

TABLE 1. CDR Definitions¹

CDR	Kabat	Chothia	AbM ²
V _H CDR1	31-35	26-32	26-35
V _H CDR2	50-65	52-58	50-58
V _H CDR3	95-102	95-102	95-102
V _L CDR1	24-34	26-32	24-34
V _L CDR2	50-56	50-52	50-56
V _L CDR3	89-97	91-96	89-97

¹ Numbering of all CDR definitions in Table 1 is according to the numbering conventions set forth by Kabat et al. (see below).

5 ² "AbM" with a lowercase "b" as used in Table 1 refers to the CDRs as defined by Oxford Molecular's "AbM" antibody modeling software.

Kabat et al. also defined a numbering system for variable region sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable region sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless otherwise specified, references to the numbering of specific amino acid residue positions in an antibody variable region are according to the Kabat numbering system.

The polypeptide sequences of the sequence listing are not numbered according to the Kabat numbering system. However, it is well within the ordinary skill of one in the art to convert the numbering of the sequences of the Sequence Listing to Kabat numbering.

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

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, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , 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 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, the C-terminal lysine (Lys447) of the Fc region may or may not be present. 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. A “subunit” of an Fc domain as used herein refers to one of the two polypeptides forming the dimeric Fc 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 region.

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 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 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. Fab fragments) 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.

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: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding, 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.

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
5 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.

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
10 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 are amino acid substitutions. For the purpose of altering e.g. the binding characteristics of an Fc
15 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-hydroxyproline, 3-methylhistidine, ornithine, homoserine, 5-hydroxylysine). Amino acid
20 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
25 substitution from proline at position 329 of the Fc domain to glycine can be indicated as 329G, G329, G₃₂₉, P329G, or Pro329Gly.

As used herein, term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain of two or more amino acids, and does not refer to a specific length of the product.
30 Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression

modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis. A polypeptide of the invention may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded.

By an "isolated" polypeptide or a variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

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 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 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.

The term "vector" or "expression vector" is synonymous with "expression construct" and 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 target cell. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated 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 target 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 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 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 cellular system that can be used to generate the bispecific antigen binding molecules of the present invention. Host cells include cultured cells, *e.g.* mammalian cultured cells, such as 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, 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 Fc γ RIIIa (CD16a), Fc γ RI (CD64), Fc γ RIIa (CD32), and Fc α RI (CD89).

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 (or increased) ADCC" is defined as either a reduction (increase) in the number of target cells that are lysed in a

given time, at a given concentration of antibody in the medium surrounding the target cells, by the mechanism of ADCC defined above, and/or an increase (reduction) 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 (increase) in ADCC is relative to the ADCC mediated by the same antibody produced by the same type of host cells, using the same standard 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 patent application no. PCT/EP2012/055393).

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.

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, 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 contains no additional components which are unacceptably toxic to a subject to which the formulation 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.

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 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, bispecific antigen binding molecules of the invention are used
5 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 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.

10 Detailed Description of the Embodiments

The invention provides a bispecific antigen binding molecule, comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- 15 a) the bispecific antigen binding molecule provides monovalent binding to the first and/or the second antigen,
- b) the first Fab fragment, the second Fab fragment and the first Fc domain subunit are fused to each other, and
- c) in the first and/or the second Fab fragment one of the following replacements is made: (i)
20 the variable domains VL and VH are replaced by each other, (ii) the constant domains CL and CH1 are replaced by each other, or (iii) both the variable and constant domains VL-CL and VH-CH1 are replaced by each other,
provided that not the same replacement is made in the first and the second Fab fragment.

25 Bispecific antigen binding molecule formats

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

In particular embodiments, the first Fab fragment is fused at its C-terminus to the N-terminus of the second Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc
30 domain subunit (see examples in Figure 1A and 1B). In one such embodiment, the second Fab fragment is fused to the first Fc domain subunit via an immunoglobulin hinge region. In a further such embodiment, the first Fab fragment is fused to the second Fab fragment via a peptide linker.

In one embodiment, the first Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the second Fab fragment, which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit.

In other embodiments, the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit (see example in Figure 1C). In one such embodiment, the first Fab fragment is fused to the first Fc domain subunit via an immunoglobulin hinge region. In a further such embodiment, the second Fab fragment is fused to the first Fab fragment via a peptide linker. In one embodiment, the second Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the first Fab fragment, which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit.

In some embodiments wherein either the first Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the second Fab fragment which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit, or the second Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the first Fab fragment which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit, additionally the Fab light chain of the first Fab fragment and the Fab light chain of the second Fab fragment are fused to each other, optionally via a peptide linker (see examples in Figure 12).

According to these embodiments, two Fab fragments of different specificity are fused to each other, one of which is in turn fused to an Fc domain subunit. This configuration allows for a geometry (e.g. distance, angle between the Fab fragments) different from the classical bispecific immunoglobulin format with the two Fab fragments of the immunoglobulin molecule having different specificities. For example, the inventors found that this configuration is more suitable than the classical bispecific immunoglobulin format for mimicking an immunological synapse between a T cell and a target cell, as required if the bispecific antigen binding molecule is to be used for T cell engagement and re-direction (data not shown).

In other embodiments, the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fc domain subunit, which is in turn fused at its C-terminus to the N-terminus of the first Fab fragment (see example in Figure 1D). In one such embodiment, the second Fab fragment is fused to the first Fc domain subunit via an immunoglobulin hinge region. In a further such embodiment, the first Fab fragment is fused to the first Fc domain subunit via a peptide linker. In one embodiment, the second Fab fragment is fused at the C-terminus of its heavy chain to the N-

terminus of the first Fc domain subunit, which is in turn fused at its C-terminus to the N-terminus of the heavy chain of the first Fab fragment. According to these embodiments, two Fab fragments of different specificity are fused to the two termini of an Fc domain subunit. Again, this configuration allows for a distinct geometry which might be advantageous for particular applications. In one embodiment the bispecific antigen binding molecule essentially consists of the first Fab fragment, the second Fab fragment, the Fc domain, and optionally one or more peptide linkers.

The bispecific antigen binding molecule according to the invention provides monovalent binding to at least one of the two antigens it binds to. Monovalent binding is important, for example in cases where internalization of the target antigen is to be expected following binding of a high affinity antigen binding molecule. In such cases, the presence of more than one antigen binding moiety specific for the target antigen may enhance internalization of the antigen, thereby reducing its availability. Furthermore, monovalent binding is essential where crosslinking of target antigen is not desired. For example in bispecific antigen binding molecules for T cell engagement and re-direction, bivalent binding to an activating T cell antigen such as CD3 could lead to activation of the T cell even in the absence of target cells.

In other cases, however, bivalent binding might be desirable, for example to increase binding affinity, optimize targeting to the target site or allow crosslinking of a target antigen.

Accordingly, in particular embodiments, the bispecific antigen binding molecule comprises a third Fab fragment which specifically binds to the first or the second antigen. In one embodiment, the third Fab fragment is fused to the second Fc domain subunit. In a more specific embodiment, the third Fab fragment is fused at its C-terminus to the N-terminus of the second Fc domain subunit. In an even more specific embodiment, the third Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the second Fc domain subunit. In one embodiment, the third Fab fragment is fused to the second Fc domain subunit via an immunoglobulin hinge region. In one embodiment, the third Fab fragment specifically binds to the second antigen.

In some embodiments the second Fab fragment, the third Fab fragment and the Fc domain are part of an immunoglobulin molecule. In embodiments where the third Fab fragment specifically binds to the second antigen, the immunoglobulin molecule is an immunoglobulin molecule which specifically binds to the second antigen. In a specific such embodiment, the immunoglobulin molecule is an IgG class immunoglobulin molecule, more specifically an IgG1 or IgG4 subclass immunoglobulin molecule. In one specific embodiment the immunoglobulin molecule is an IgG4 molecule comprising an amino acid substitution at position S228 (Kabat

numbering), particularly the amino acid substitution S228P. This amino acid substitution reduces in vivo Fab arm exchange of IgG₄ antibodies (see Stubenrauch et al., Drug Metabolism and Disposition 38, 84-91 (2010)). In one embodiment, the immunoglobulin molecule is a human immunoglobulin molecule. In one embodiment, the bispecific antigen binding molecule
5 essentially consists of a first Fab fragment which specifically binds to the first antigen, an immunoglobulin molecule which specifically binds to the second antigen, and optionally one or more peptide linkers.

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

According to any of the above embodiments, components of the bispecific antigen binding molecule (e.g. Fab fragments, Fc domain subunit) may be linked directly or through various linkers, particularly peptide linkers comprising one or more amino acids, typically about 2-20
20 amino acids, that are described herein or are known in the art. Suitable, non-immunogenic peptide linker include, for example, (G₄S)_n, (SG₄)_n, (G₄S)_n or G₄(SG₄)_n peptide linkers, wherein n is generally a number between 1 and 10, typically between 2 and 4. A particularly suitable peptide linker for fusing the light chains of the first and the second Fab fragment to each other is (G₄S)₂. Additionally, peptide linkers may comprise (a portion of) an immunoglobulin hinge
25 region. An exemplary such linker is EPKSC(D)-(G₄S)₂ (SEQ ID NOs 72 and 73). Particularly where a Fab fragment is linked to the N-terminus of an Fc domain subunit, it may be linked via an immunoglobulin hinge region or a portion thereof, with or without an additional peptide linker.

In certain embodiments the bispecific antigen binding molecule comprises a polypeptide wherein
30 a VL region shares a carboxy-terminal peptide bond with a CH1 region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with an immunoglobulin heavy chain (VH-CH1-HR-CH2-CH3-(CH4)). In some of these embodiments, the bispecific antigen binding molecule further comprises an antibody light

chain (VL-CL) and/or a polypeptide wherein a VH region shares a carboxy terminal peptide bond with a CL region. In some of these embodiments, the bispecific antigen binding molecule further comprises a polypeptide wherein a VH region shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a Fab light chain (VL-CL).

In other embodiments the bispecific antigen binding molecule comprises a polypeptide wherein a Fab heavy chain (VH-CH1) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VL region, which in turn shares a carboxy-terminal peptide bond with a CH1 region, which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit including an immunoglobulin hinge region (HR-CH2-CH3-(CH4)).

In some of these embodiments, the bispecific antigen binding molecule further comprises an antibody light chain (VL-CL) and/or a polypeptide wherein a VH region shares a carboxy terminal peptide bond with a CL region. In some of these embodiments, the bispecific antigen binding molecule further comprises a polypeptide wherein a Fab light chain (VL-CL) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VH region, which in turn shares a carboxy-terminal peptide bond with a CL region.

In certain embodiments the bispecific antigen binding molecule comprises a polypeptide wherein a VH region shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with an immunoglobulin heavy chain (VH-CH1-HR-CH2-CH3-(CH4)). In some of these embodiments, the bispecific antigen binding molecule further comprises an antibody light chain (VL-CL) and/or a polypeptide wherein a VL region shares a carboxy terminal peptide bond with a CH1 region. In some of these embodiments, the bispecific antigen binding molecule further comprises a polypeptide wherein a VL region shares a carboxy-terminal peptide bond with a CH1 region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a Fab light chain (VL-CL).

In other embodiments the bispecific antigen binding molecule comprises a polypeptide wherein a Fab heavy chain (VH-CH1) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VH region, which in turn shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond with an Fc domain subunit including an immunoglobulin hinge region (HR-CH2-CH3-(CH4)). In some of these embodiments, the bispecific antigen binding molecule further comprises an

antibody light chain (VL-CL) and/or a polypeptide wherein a VL region shares a carboxy terminal peptide bond with a CH1 region. In some of these embodiments, the bispecific antigen binding molecule further comprises a polypeptide wherein a Fab light chain (VL-CL) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VL region, which in turn shares a carboxy-terminal peptide bond with a CH1 region.

In still other embodiments, the bispecific antigen binding molecule comprises a polypeptide wherein an immunoglobulin heavy chain ((VH-CH1-HR-CH2-CH3-(CH4)) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VH region, which in turn shares a carboxy-terminal peptide bond with a CL region.

In one embodiment, the bispecific antigen binding molecule further comprises an immunoglobulin heavy chain ((VH-CH1-HR-CH2-CH3-(CH4)). In another embodiment, the bispecific antigen binding molecule further comprises an Fc domain subunit, optionally including an antibody hinge region ((HR)-CH2-CH3-(CH4)). In some of these embodiments, the bispecific antigen binding molecule further comprises an antibody light chain (VL-CL) and/or a polypeptide wherein a VL region shares a carboxy terminal peptide bond with a CH1 region.

Fab fragments

The antigen binding molecule of the invention is bispecific, i.e. it comprises at least two antigen binding moieties capable of specific binding to two distinct antigenic determinants. In a particular embodiment, the bispecific antigen binding molecule is capable of simultaneous binding to two distinct antigenic determinants. According to the invention, the antigen binding moieties are Fab fragments (i.e. antigen binding domains composed of a heavy and a light chain, each comprising a variable and a constant region). In one embodiment said Fab fragments are human. In another embodiment said Fab fragments are humanized. In yet another embodiment said Fab fragments comprise human heavy and light chain constant regions.

According to the invention, at least one of the Fab fragments is a "Crossfab" fragment, wherein the variable and/or constant domains of the Fab heavy and light chain are exchanged. Such modifications prevent mispairing of heavy and light chains from different Fab fragments, thereby improving the yield and purity of the bispecific antigen binding molecule of the invention in recombinant production. In other words, the problem of heavy and light chain mispairing in bispecific antibody production is overcome by the exchange of heavy and light chain variable and/or constant domains within one or more Fab fragments of the bispecific antigen binding

molecule, so that Fab fragments of different specificity do not have identical domain arrangement and consequently do not “interchange” light chains.

Possible replacements include the following: (i) the variable domains of the Fab heavy and light chain (VH and VL) are replaced by each other; (ii) the constant domains of the Fab heavy and light chain (CH1 and CL) are replaced by each other; or (iii) the Fab heavy and light chain (VH-CH1 and VL-CL) are replaced by each other.

To achieve the desired result, i.e. prevention of mispairing of heavy and light chains of different specificity, not the same replacement must be made in Fab fragments of different specificity. For example, in a Fab fragment which specifically binds to a first antigen, the heavy and light chain variable domains may be exchanged, while in a Fab fragment which specifically binds to a second antigen, the heavy and light chain constant region may be exchanged. As another example, in a Fab fragment which specifically binds to a first antigen, no replacement may be made, while in a Fab fragment which specifically binds to a second antigen, the heavy and light chain variable domains may be exchanged.

In a particular embodiment, the same replacement is made in Fab fragments of the same specificity (i.e. in Fab fragments which specifically bind to the same antigen). A replacement need not be made in all Fab fragments comprised in the bispecific antigen binding molecule. For example in embodiments wherein there are three Fab fragments, it is sufficient to make a replacement only in the Fab fragment having a different specificity from the other two Fab fragments. Specifically, in embodiments wherein the bispecific antigen binding molecule comprises a third Fab fragment which binds to the first antigen, a replacement is made only in the second Fab fragment. Similarly, in embodiments wherein the bispecific antigen binding molecule comprises a third Fab fragment which binds to the second antigen, a replacement is made only in the first Fab fragment.

In particular embodiments, a replacement is made in the first Fab fragment. In one such embodiment, no further replacement is made. In some embodiments, the replacement is a replacement of the variable domains VL and VH by each other. In other embodiments the replacement is a replacement of the constant domains CL and CH1 by each other. In still other embodiments, the replacement is a replacement of both the variable and constant domains VL-CL and VH-CH1 by each other.

In a particular embodiment, the bispecific antigen binding molecule provides monovalent binding to the first antigen. In one embodiment, the bispecific antigen binding molecule does not comprise a single chain Fab fragment.

In a particular aspect, the present invention provides a bispecific antigen binding molecule comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- 5 a) the bispecific antigen binding molecule provides monovalent binding to the first antigen,
- b) the first Fab fragment is fused at its C-terminus to the N-terminus of the second Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit,
- c) in the first Fab fragment the constant domains CL and CH1 are replaced by each other, and
- 10 d) the bispecific antigen binding molecule optionally comprises a third Fab fragment which specifically binds to the second antigen and is fused at its C-terminus to the N-terminus of the second Fc domain subunit.

In another aspect, the present invention provides a bispecific antigen binding molecule comprising a first Fab fragment which specifically binds to a first antigen, a second Fab
15 fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- a) the bispecific antigen binding molecule provides monovalent binding to the first antigen,
- b) the first Fab fragment is fused at its C-terminus to the N-terminus of the second Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain
20 subunit,
- c) in the first Fab fragment the variable domains VL and VH are replaced by each other, and
- d) the bispecific antigen binding molecule optionally comprises a third Fab fragment which specifically binds to the second antigen and is fused at its C-terminus to the N-terminus of the second Fc domain subunit.

25 In a further particular aspect, the invention provides a bispecific antigen binding molecule, comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- a) the bispecific antigen binding molecule provides monovalent binding to the first antigen,
- 30 b) the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit,
- c) in the first Fab fragment the constant domains CL and CH1 are replaced by each other, and

d) the bispecific antigen binding molecule optionally comprises a third Fab fragment which specifically binds to the second antigen and is fused at its C-terminus to the N-terminus of the second Fc domain subunit.

In a further aspect, the invention provides a bispecific antigen binding molecule, comprising a
5 first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- a) the bispecific antigen binding molecule provides monovalent binding to the first antigen,
- b) the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fab
10 fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit,
- c) in the first Fab fragment the variable domains VL and VH are replaced by each other, and
- d) the bispecific antigen binding molecule optionally comprises a third Fab fragment which specifically binds to the second antigen and is fused at its C-terminus to the N-terminus of
15 the second Fc domain subunit.

In yet a further aspect, the invention provides a bispecific antigen binding molecule, comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen, and an Fc domain composed of a first and a second subunit capable of stable association; wherein

- a) the bispecific antigen binding molecule provides monovalent binding to the first antigen,
- b) the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fc domain
20 subunit, which is in turn fused at its C-terminus to the N-terminus of the first Fab fragment,
- c) in the first Fab fragment the constant domains CL and CH1 are replaced by each other, and
- d) the bispecific antigen binding molecule optionally comprises a third Fab fragment which
25 specifically binds to the second antigen and is fused at its C-terminus to the N-terminus of the second Fc domain subunit.

Fc domain

The Fc domain of the bispecific antigen binding molecule consists of a pair of polypeptide
30 chains comprising heavy chain domains of an antibody molecule. For example, the Fc 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. The bispecific antigen binding molecule of the invention comprises not more than one Fc domain.

In one embodiment according to the invention the Fc domain of the bispecific antigen binding molecule is an IgG Fc domain. In a particular embodiment the Fc domain is an IgG₁ Fc domain.

5 In another embodiment the Fc domain is an IgG₄ Fc domain. In a more specific embodiment, the Fc domain is an IgG₄ Fc domain comprising an amino acid substitution at position S228 (Kabat numbering), particularly the amino acid substitution S228P. This amino acid substitution reduces in vivo Fab arm exchange of IgG₄ antibodies (see Stubenrauch et al., Drug Metabolism and Disposition 38, 84-91 (2010)). In a further particular embodiment the Fc domain is human. An
10 exemplary sequence of a human IgG₁ Fc region is given in SEQ ID NO: 71.

Fc domain modifications promoting heterodimerization

Bispecific antigen binding molecules according to the invention comprise different Fab
15 fragments, 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 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
20 domain of the bispecific antigen binding molecule a modification promoting the association of the desired polypeptides.

Accordingly, in particular embodiments, the Fc domain comprises a modification promoting the association of the first and the second Fc domain subunit. A modification may be present in the first Fc domain subunit and/or the second Fc domain subunit.

25 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.

In a specific embodiment said modification 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”
30 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 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).

5 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 (e.g. alanine or threonine).

Accordingly, in a particular embodiment, in the CH3 domain of the first Fc domain subunit of the bispecific antigen binding molecule an amino acid residue is replaced with an amino acid
10 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 in the CH3 domain of the second Fc domain subunit 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
15 CH3 domain of the first subunit is positionable.

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.

In a specific embodiment, in the CH3 domain of the first subunit of the Fc domain the threonine residue at position 366 is replaced with a tryptophan residue (T366W), and in the CH3 domain of
20 the second subunit of the Fc domain 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 with a serine residue (T366S) and the leucine residue at position 368 is replaced with an alanine residue (L368A).

In yet a further embodiment, in the first subunit of the Fc domain additionally the serine residue
25 at position 354 is replaced with a cysteine residue (S354C), and in the second subunit of the Fc domain additionally the tyrosine residue at position 349 is replaced by a cysteine residue (Y349C). Introduction of these two cysteine residues results in 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)).

30 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 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.

Fc domain modifications altering Fc receptor binding and/or effector function

In certain embodiments, the Fc domain is engineered to have altered binding affinity to an Fc
5 receptor and/or altered effector function, as compared to a non-engineered Fc domain.

Binding to Fc receptors can be easily determined e.g. by ELISA, or by Surface Plasmon
Resonance (SPR) using standard instrumentation such as a BIAcore instrument (GE Healthcare),
and Fc receptors such as may be obtained by recombinant expression. A suitable such binding
assay is described herein. Alternatively, binding affinity of Fc domains or bispecific antigen
10 binding molecules comprising an Fc domain for Fc receptors may be evaluated using cell lines
known to express particular Fc receptors, such as NK cells expressing FcγIIIa receptor.

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. Suitable *in vitro* assays to assess ADCC
activity of a molecule of interest are described in PCT publication no. WO 2006/082515 or PCT
15 patent application no. PCT/EP2012/055393, incorporated herein by reference in their entirety.
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).

20 In some embodiments binding of the Fc domain to a complement component, specifically to C1q,
is altered. Accordingly, in some embodiments wherein the Fc domain is engineered to have
altered effector function, said altered effector function includes altered CDC. C1q binding assays
may be carried out to determine whether the bispecific antigen binding molecule is able to bind
C1q and hence has CDC activity. See e.g., C1q and C3c binding ELISA in WO 2006/029879
25 and WO 2005/100402. To assess 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)).

a) Decreased Fc receptor binding and/or effector function

30 The Fc domain confers to the bispecific antigen binding molecule 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 to cells expressing Fc receptors

rather than to the preferred antigen-bearing cells. Moreover, the activation of Fc receptor signaling pathways may lead to cytokine release and severe side effects upon systemic administration.

Accordingly, in particular embodiments, the Fc domain of the bispecific antigen binding molecule 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 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 non-engineered Fc domain (or a bispecific antigen binding molecule comprising a non-engineered 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 non-engineered Fc domain domain (or a bispecific antigen binding molecule comprising a non-engineered Fc domain). In one embodiment, the Fc domain 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 Fc γ 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 Fc γ receptor, more specifically human Fc γ RIIIa, Fc γ RI or Fc γ RIIa, most specifically human Fc γ RIIIa. 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 substantially similar binding affinity to neonatal Fc receptor (FcRn), as compared to a non-engineered Fc 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 non-engineered Fc domain (or the bispecific antigen binding molecule comprising a non-engineered Fc domain) to FcRn.

In certain 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 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 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 a particular embodiment the Fc receptor is an Fc γ receptor. In some embodiments the Fc receptor is a human Fc receptor. In some 5 embodiments the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc receptor is an activating human Fc γ receptor, more specifically human Fc γ RIIIa, Fc γ RI or Fc γ RIIa, most specifically human Fc γ RIIIa. Preferably, binding to each of these receptors is reduced. In some embodiments binding affinity to a complement component, specifically 10 binding affinity to C1q, is also reduced. In one embodiment binding affinity to neonatal Fc receptor (FcRn) is not reduced.

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 15 complement dependent cytotoxicity (CDC), reduced antibody-dependent cell-mediated 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 20 apoptosis, reduced crosslinking of target-bound antibodies, reduced dendritic cell maturation, or 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 25 domain (or a bispecific antigen binding molecule comprising a non-engineered Fc domain).

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. In a more specific embodiment the Fc domain comprises an 30 amino acid substitution at a position selected from the group of L234, L235 and P329. In some embodiments the Fc domain comprises the amino acid substitutions L234A and L235A. In one such embodiment, the Fc domain is an IgG₁ Fc domain, particularly a human IgG₁ 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. 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, L234, L235, N297 and P331. In a more specific embodiment the further amino acid substitution is E233P, L234A, L235A, L235E,
5 N297A, N297D or P331S. In particular embodiments the Fc domain comprises amino acid substitutions at positions P329, L234 and L235. In more particular embodiments the Fc domain comprises the amino acid mutations L234A, L235A and P329G ("P329G LALA"). In one such embodiment, the Fc domain is an IgG₁ Fc domain, particularly a human IgG₁ Fc domain. The "P329G LALA" combination of amino acid substitutions almost completely abolishes Fcγ
10 receptor binding of a human IgG₁ Fc domain, as described in PCT patent application no. PCT/EP2012/055393, incorporated herein by reference in its entirety. PCT/EP2012/055393 also describes methods of preparing such mutant Fc domains and methods for determining its properties such as Fc receptor binding or effector functions.

IgG₄ antibodies exhibit reduced binding affinity to Fc receptors and reduced effector functions as
15 compared to IgG₁ antibodies. Hence, in some embodiments the Fc domain of the bispecific antigen binding molecules of the invention is an IgG₄ Fc domain, particularly a human IgG₄ Fc domain. In one embodiment the IgG₄ Fc domain comprises amino acid substitutions at position S228, specifically the amino acid substitution S228P. To further reduce its binding affinity to an Fc receptor and/or its effector function, in one embodiment the IgG₄ Fc domain comprises an
20 amino acid substitution at position L235, specifically the amino acid substitution L235E. In another embodiment, the IgG₄ Fc domain comprises an amino acid substitution at position P329, specifically the amino acid substitution P329G. In a particular embodiment, the IgG₄ Fc domain comprises amino acid substitutions at positions S228, L235 and P329, specifically amino acid substitutions S228P, L235E and P329G. Such IgG₄ Fc domain mutants and their Fcγ receptor
25 binding properties are described in PCT patent application no. PCT/EP2012/055393, 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 IgG₁ Fc domain, is a human IgG₁ Fc domain comprising the amino acid substitutions L234A, L235A and optionally P329G, or a
30 human IgG₄ Fc domain comprising the amino acid substitutions S228P, L235E and optionally P329G.

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).

In addition to the Fc domains described hereinabove and in PCT patent application no. 5 PCT/EP2012/055393, Fc domains with reduced Fc receptor binding and/or effector function also include 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). 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. 10 7,332,581).

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.

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b) Increased Fc receptor binding and/or effector function

Conversely, there may be situations where it is desirable to maintain or even enhance Fc receptor binding and/or effector functions of the bispecific antigen binding molecules, for example when the bispecific antigen binding molecule is targeted to a highly specific tumor antigen. Hence, in 20 certain embodiments the Fc domain of the bispecific antigen binding molecules of the invention is engineered to have increased binding affinity to an Fc receptor. Increased binding affinity may be an increase in the binding affinity of the Fc domain to the Fc receptor by at least 2-fold, at least 5-fold, or at least 10-fold. In one embodiment the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc receptor is an Fc γ receptor, particularly a human Fc γ receptor. 25 In one embodiment the Fc receptor is selected from the group of Fc γ RIIIa, Fc γ RI and Fc γ RIIa. In a particular embodiment the Fc receptor is Fc γ RIIIa.

In one such embodiment the Fc domain is engineered to have an altered oligosaccharide structure compared to a non-engineered Fc domain. In a particular such embodiment the Fc domain comprises an increased proportion of non-fucosylated oligosaccharides, compared to a 30 non-engineered Fc domain. In a more specific embodiment, at least about 50%, more particularly at least about 70%, of the N-linked oligosaccharides in the Fc domain of the bispecific antigen binding molecule are non-fucosylated. The non-fucosylated oligosaccharides may be of the hybrid or complex type. In another specific embodiment the Fc domain comprises an increased

proportion of bisected oligosaccharides, compared to a non-engineered Fc domain. In a more specific embodiment, at least about 35%, particularly at least about 50%, more particularly at least about 70%, of the N-linked oligosaccharides in the Fc domain of the bispecific antigen binding molecule are bisected. The bisected oligosaccharides may be of the hybrid or complex type. In yet another specific embodiment the Fc domain comprises an increased proportion of bisected, non-fucosylated oligosaccharides, compared to a non-engineered Fc domain. In a more specific embodiment, at least about 15%, more particularly at least about 25%, at least about 35% or at least about 50%, of the N-linked oligosaccharides in the Fc domain of the bispecific antigen binding molecule are bisected, non-fucosylated. The bisected, non-fucosylated oligosaccharides may be of the hybrid or complex type.

The oligosaccharide structures in the bispecific antigen binding molecule Fc domain can be analysed by methods well known in the art, e.g. by MALDI TOF mass spectrometry as described in Umana et al., *Nat Biotechnol* 17, 176-180 (1999) or Ferrara et al., *Biotechn Bioeng* 93, 851-861 (2006). The percentage of non-fucosylated oligosaccharides is the amount of oligosaccharides lacking fucose residues, relative to all oligosaccharides attached to Asn 297 (e.g. complex, hybrid and high mannose structures) and identified in an N-glycosidase F treated sample by MALDI TOF MS. Asn 297 refers to the asparagine residue located at about position 297 in the Fc domain (EU numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in immunoglobulins. The percentage of bisected, or bisected non-fucosylated, oligosaccharides is determined analogously.

Modification of the glycosylation in the Fc domain of the bispecific antigen binding molecule may result from production of the bispecific antigen binding molecule in a host cell that has been manipulated to express altered levels of one or more polypeptides having glycosyltransferase activity.

In one embodiment the Fc domain of the bispecific antigen binding molecule is engineered to have an altered oligosaccharide structure, as compared to a non-engineered Fc domain, by producing the bispecific antigen binding molecule in a host cell having altered activity of one or more glycosyltransferase. Glycosyltransferases include for example $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII), $\beta(1,4)$ -galactosyltransferase (GalT), $\beta(1,2)$ -N-acetylglucosaminyltransferase I (GnTI), $\beta(1,2)$ -N-acetylglucosaminyltransferase II (GnTII) and $\alpha(1,6)$ -fucosyltransferase. In a specific embodiment the Fc domain of the bispecific antigen binding molecule is engineered to comprise an increased proportion of non-fucosylated

oligosaccharides, as compared to a non-engineered Fc domain, by producing the bispecific antigen binding molecule in a host cell having increased $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII) activity. In an even more specific embodiment the host cell additionally has increased α -mannosidase II (ManII) activity. The glycoengineering methodology that can be used for glycoengineering bispecific antigen binding molecules of the present invention has been described in greater detail in Umana et al., Nat Biotechnol 17, 176-180 (1999); Ferrara et al., Biotechn Bioeng 93, 851-861 (2006); WO 99/54342 (U.S. Pat. No. 6,602,684; EP 1071700); WO 2004/065540 (U.S. Pat. Appl. Publ. No. 2004/0241817; EP 1587921), WO 03/011878 (U.S. Pat. Appl. Publ. No. 2003/0175884), the content of each of which is expressly incorporated herein by reference in its entirety.

Generally, any type of cultured cell line, including the cell lines discussed herein, can be used to generate cell lines for the production of bispecific antigen binding molecules with altered glycosylation pattern. Particular cell lines include CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, and other mammalian cells. In certain embodiments, the host cells have been manipulated to express increased levels of one or more polypeptides having $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII) activity. In certain embodiments the host cells have been further manipulated to express increased levels of one or more polypeptides having α -mannosidase II (ManII) activity. In a specific embodiment, the polypeptide having GnTIII activity is a fusion polypeptide comprising the catalytic domain of GnTIII and the Golgi localization domain of a heterologous Golgi resident polypeptide. Particularly, said Golgi localization domain is the Golgi localization domain of mannosidase II. Methods for generating such fusion polypeptides and using them to produce antibodies with increased effector functions are disclosed in Ferrara et al., Biotechn Bioeng 93, 851-861 (2006) and WO 2004/065540, the entire contents of which are expressly incorporated herein by reference.

The host cells which contain a coding sequence of a bispecific antigen binding molecule of the invention and/or a coding sequence of a polypeptide having glycosyltransferase activity, and which express the biologically active gene products, may be identified e.g. by DNA-DNA or DNA-RNA hybridization, the presence or absence of "marker" gene functions, assessing the level of transcription as measured by the expression of the respective mRNA transcripts in the host cell, or detection of the gene product as measured by immunoassay or by its biological activity - methods which are well known in the art. GnTIII or Man II activity can be detected e.g. by employing a lectin which binds to biosynthesis products of GnTIII or ManII, respectively. An

example for such a lectin is the E₄-PHA lectin which binds preferentially to oligosaccharides containing bisecting GlcNAc. Biosynthesis products (i.e. specific oligosaccharide structures) of polypeptides having GnTIII or ManII activity can also be detected by mass spectrometric analysis of oligosaccharides released from glycoproteins produced by cells expressing said
5 polypeptides. Alternatively, a functional assay which measures the increased effector function and/or increased Fc receptor binding, mediated by bispecific antigen binding molecules produced by the cells engineered with the polypeptide having GnTIII or ManII activity may be used.

In another embodiment the Fc domain is engineered to comprise an increased proportion of non-
10 fucosylated oligosaccharides, as compared to a non-engineered Fc domain, by producing the bispecific antigen binding molecule in a host cell having decreased $\alpha(1,6)$ -fucosyltransferase activity. A host cell having decreased $\alpha(1,6)$ -fucosyltransferase activity may be a cell in which the $\alpha(1,6)$ -fucosyltransferase gene has been disrupted or otherwise deactivated, e.g. knocked out (see Yamane-Ohnuki et al., *Biotech Bioeng* 87, 614 (2004); Kanda et al., *Biotechnol Bioeng*
15 94(4), 680-688 (2006); Niwa et al., *J Immunol Methods* 306, 151-160 (2006)).

Other examples of cell lines capable of producing defucosylated bispecific antigen binding molecules include Lec13 CHO cells deficient in protein fucosylation (Ripka et al., *Arch Biochem Biophys* 249, 533-545 (1986); US Pat. Appl. No. US 2003/0157108; and WO 2004/056312, especially at Example 11). The bispecific antigen binding molecules of the present
20 invention can alternatively be glycoengineered to have reduced fucose residues in the Fc domain according to the techniques disclosed in EP 1 176 195 A1, WO 03/084570, WO 03/085119 and U.S. Pat. Appl. Pub. Nos. 2003/0115614, 2004/093621, 2004/110282, 2004/110704, 2004/132140, US Pat. No. 6,946,292 (Kyowa), e.g. by reducing or abolishing the activity of a GDP-fucose transporter protein in the host cells used for bispecific antigen binding molecule
25 production.

Glycoengineered bispecific antigen binding molecules of the invention may also be produced in expression systems that produce modified glycoproteins, such as those taught in WO 2003/056914 (GlycoFi, Inc.) or in WO 2004/057002 and WO 2004/024927 (Greenovation).

In one embodiment the Fc domain of the bispecific antigen binding molecule is engineered to
30 have increased effector function, compared to a non-engineered Fc domain. The increased effector function can include, but is not limited to, one or more of the following: increased complement dependent cytotoxicity (CDC), increased antibody-dependent cell-mediated cytotoxicity (ADCC), increased antibody-dependent cellular phagocytosis (ADCP), increased

cytokine secretion, increased immune complex-mediated antigen uptake by antigen-presenting cells, increased binding to NK cells, increased binding to macrophages, increased binding to monocytes, increased binding to polymorphonuclear cells, increased direct signaling inducing apoptosis, increased crosslinking of target-bound antibodies, increased dendritic cell maturation,
5 or increased T cell priming.

In one embodiment the increased effector function is one or more selected from the group of increased CDC, increased ADCC, increased ADCP, and increased cytokine secretion. In a particular embodiment the increased effector function is increased ADCC. In one embodiment
10 ADCC induced by an engineered Fc domain (or a bispecific antigen binding molecule comprising an engineered Fc domain) is a least 2-fold increased as compared to ADCC induced by a non-engineered Fc domain (or a bispecific antigen binding molecule comprising a non-engineered Fc domain).

Antigens

15 The bispecific antigen binding molecules of the invention may bind to a variety of antigens. In certain embodiments the first and/or second antigen is an antigen associated with a pathological condition, such as an antigen presented on a tumor cell, on a virus-infected cell, or at a site of inflammation. Suitable antigens include cell surface antigens (for example, but not limited to, cell surface receptors), antigens free in blood serum, and/or antigens in the extracellular matrix.

20 In particular embodiments the antigen is a human antigen.

Non-limiting examples of antigens include tumor antigens such as MAGE, MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-C017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific
25 Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3,
30 MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, Her3, p21ras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, β -catenin and γ -catenin, p120ctn, gp100 Pmel117, PRAME,

NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, Smad family of tumor antigens, Imp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2; ECM antigens such as syndecan, heparanase, integrins, osteopontin, link, cadherins, laminin, laminin type EGF, lectin, fibronectin and its alternatively spliced domains (e.g. the Extra Domain B), notch, various forms of tenascin (e.g. tenascin C) and its alternatively spliced domains (e.g. the A1 or A2 domain of tenascin-C), and matrixin; Fibroblast Activation Protein (FAP), Epidermal Growth Factor Receptor (EGFR), CD2 (T-cell surface antigen), CD3 (heteromultimer associated with the TCR), CD19, CD22 (B-cell receptor), CD23 (low affinity IgE receptor), CD25 (IL-2 receptor α chain), CD30 (cytokine receptor), CD33 (myeloid cell surface antigen), CD40 (tumor necrosis factor receptor), IL-6R (IL6 receptor), CD20, Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Insulin-like growth factor-1 receptor (IGF-1R), and PDGF β R (β platelet-derived growth factor receptor). In a specific embodiment the first and second antigen are selected from the group of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Her3, c-Met, Carcinoembryonic Antigen (CEA), CD33 and CD3.

In a particular embodiment the first antigen is CD3, particularly human or cynomolgus CD3, most particularly human CD3. In some embodiments, the first antigen is the epsilon subunit of CD3. In one embodiment, the first Fab fragment can compete with monoclonal antibody H2C (described in PCT publication no. WO2008/119567) for binding an epitope of CD3. In a particular embodiment, the first Fab fragment can compete with monoclonal antibody SP34 (described in Pessano et al., EMBO J 4, 337-340 (1985)) for binding an epitope of CD3. In another embodiment, the first Fab fragment can compete with monoclonal antibody V9 (described in Rodrigues et al., Int J Cancer Suppl 7, 45-50 (1992) and US patent no. 6,054,297) for binding an epitope of CD3. In yet another embodiment, the first Fab fragment can compete with monoclonal antibody FN18 (described in Nooij et al., Eur J Immunol 19, 981-984 (1986)) for binding an epitope of CD3. In one embodiment, the first Fab fragment is specific for CD3 and comprises the heavy chain CDR1 of SEQ ID NO: 77, the heavy chain CDR2 of SEQ ID NO: 78, the heavy chain CDR3 of SEQ ID NO: 79, the light chain CDR1 of SEQ ID NO: 81, the light chain CDR2 of SEQ ID NO: 82, and the light chain CDR3 of SEQ ID NO: 83. In a further embodiment, the Fab fragment that is specific for CD3 comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to

SEQ ID NO: 80 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 84, or variants thereof that retain functionality. In another embodiment, the first Fab fragment is specific for CD3 and comprises the heavy chain CDR1 of SEQ ID NO: 104, the heavy chain CDR2 of SEQ ID NO: 105, the heavy chain CDR3 of SEQ ID NO: 106, the light chain CDR1 of SEQ ID NO: 108, the light chain CDR2 of SEQ ID NO: 109, and the light chain CDR3 of SEQ ID NO: 110. In a further embodiment, the Fab fragment that is specific for CD3 comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 107 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 111, or variants thereof that retain functionality.

In a particular embodiment according to the invention, the bispecific antigen binding molecule is capable of simultaneous binding to a target cell antigen, particularly a tumor cell antigen, and CD3. In one embodiment, the bispecific antigen binding molecule is capable of crosslinking a T cell and a target cell by simultaneous binding to a target cell antigen and CD3. In an even more particular embodiment, such simultaneous binding results in lysis of the target cell, particularly a 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 CD3 without simultaneous binding to the target cell antigen 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. 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⁺ or a CD8⁺ T cell, particularly a CD8⁺ T cell.

In one embodiment, the first antigen is c-Met, particularly human c-Met. In one embodiment, the first Fab fragment can compete with monoclonal antibody 5D5 (described e.g. in US patent no. 7,476,724, which is incorporated herein by reference in its entirety) for binding an epitope of c-Met. In one embodiment, the first Fab fragment is specific for c-Met and comprises the heavy chain CDR1 of SEQ ID NO: 63, the heavy chain CDR2 of SEQ ID NO: 64, the heavy chain CDR3 of SEQ ID NO: 65, the light chain CDR1 of SEQ ID NO: 67, the light chain CDR2 of

SEQ ID NO: 68, and the light chain CDR3 of SEQ ID NO: 69. In a further embodiment, the Fab fragment that is specific for c-Met comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 66 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%,
5 97%, 98%, 99% or 100% identical to SEQ ID NO: 70, or variants thereof that retain functionality.

In particular embodiments the second antigen is a tumor-associated antigen, specifically an antigen presented on a tumor cell or a cell of the tumor stroma. In one such embodiment the second antigen is selected from the group of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Her3, CD33, and Carcinoembryonic Antigen (CEA).
10

In one embodiment the second antigen is Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP). In another embodiment second and optionally the third Fab fragment can compete with monoclonal antibody LC007 (see SEQ ID NOs 18 and 22) for binding to an epitope of MCSP. In one embodiment, the Fab fragment that is specific for MCSP comprises the
15 heavy chain CDR1 of SEQ ID NO: 15, the heavy chain CDR2 of SEQ ID NO: 16, the heavy chain CDR3 of SEQ ID NO: 17, the light chain CDR1 of SEQ ID NO: 19, the light chain CDR2 of SEQ ID NO: 20, and the light chain CDR3 of SEQ ID NO: 21. In a further embodiment, the Fab fragment that is specific for MCSP comprises a heavy chain variable region sequence that is
20 at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 18 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 22, or variants thereof that retain functionality. In another embodiment second and optionally the third Fab fragment can compete with monoclonal antibody M4-3 ML2 (see SEQ ID NOs 99 and 103) for binding to an epitope of
25 MCSP. In one embodiment, the Fab fragment that is specific for MCSP comprises the heavy chain CDR1 of SEQ ID NO: 96, the heavy chain CDR2 of SEQ ID NO: 97, the heavy chain CDR3 of SEQ ID NO: 98, the light chain CDR1 of SEQ ID NO: 100, the light chain CDR2 of SEQ ID NO: 101, and the light chain CDR3 of SEQ ID NO: 102. In a further embodiment, the Fab fragment that is specific for MCSP comprises a heavy chain variable region sequence that is
30 at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 99 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 103, or variants thereof that retain functionality.

In yet another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 1, the polypeptide sequence of SEQ ID NO: 2, the polypeptide sequence of SEQ ID NO: 3 and the polypeptide sequence of SEQ ID NO: 4, or variants thereof that retain functionality. In a further embodiment the bispecific antigen binding molecule
5 comprises the polypeptide sequence of SEQ ID NO: 1, the polypeptide sequence of SEQ ID NO: 3, the polypeptide sequence of SEQ ID NO: 4 and the polypeptide sequence of SEQ ID NO: 5, or variants thereof that retain functionality. In yet another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 4, the polypeptide sequence of SEQ ID NO: 5, the polypeptide sequence of SEQ ID NO: 6 and the polypeptide
10 sequence of SEQ ID NO: 7, or variants thereof that retain functionality. In still another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 4, the polypeptide sequence of SEQ ID NO: 5, the polypeptide sequence of SEQ ID NO: 1 and the polypeptide sequence of SEQ ID NO: 85, or variants thereof that retain functionality. In a further embodiment the bispecific antigen binding molecule comprises the polypeptide
15 sequence of SEQ ID NO: 1, the polypeptide sequence of SEQ ID NO: 3, the polypeptide sequence of SEQ ID NO: 4 and the polypeptide sequence of SEQ ID NO: 86, or variants thereof that retain functionality. In still a further embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 4, the polypeptide sequence of SEQ ID NO: 87, the polypeptide sequence of SEQ ID NO: 89 and the polypeptide sequence of SEQ ID NO:
20 90, or variants thereof that retain functionality. In a further embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 3, the polypeptide sequence of SEQ ID NO: 91, the polypeptide sequence of SEQ ID NO: 92 and the polypeptide sequence of SEQ ID NO: 93, or variants thereof that retain functionality. In still another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ
25 ID NO: 87, the polypeptide sequence of SEQ ID NO: 91, the polypeptide sequence of SEQ ID NO: 93 and the polypeptide sequence of SEQ ID NO: 94, or variants thereof that retain functionality.

In one embodiment the second antigen is Carcinoembryonic Antigen (CEA). In another embodiment the second and optionally the third Fab fragment can compete with monoclonal
30 antibody CH1A1A for binding to an epitope of CEA. See PCT publication WO 2011/023787, incorporated herein by reference in its entirety. In one embodiment, the Fab fragment that is specific for CEA comprises the heavy chain CDR1 of SEQ ID NO: 39, the heavy chain CDR2 of SEQ ID NO: 40, the heavy chain CDR3 of SEQ ID NO: 41, the light chain CDR1 of SEQ ID

NO: 43, the light chain CDR2 of SEQ ID NO: 44, and the light chain CDR3 of SEQ ID NO: 45. In a further embodiment, the Fab fragment that is specific for CEA comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 42 and a light chain variable region sequence that is at least
5 about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 46, or variants thereof that retain functionality.

In yet another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 3, the polypeptide sequence of SEQ ID NO: 8, the polypeptide sequence of SEQ ID NO: 9 and the polypeptide sequence of SEQ ID NO: 10, or variants thereof
10 that retain functionality. In still another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 9, the polypeptide sequence of SEQ ID NO: 10, the polypeptide sequence of SEQ ID NO: 87 and the polypeptide sequence of SEQ ID NO: 95, or variants thereof that retain functionality.

In one embodiment the second antigen is Her3. In another embodiment the second and optionally
15 the third Fab fragment can compete with monoclonal antibody Mab 205.10 for binding to an epitope of Her3. See PCT publication no. WO 2011/076683, incorporated herein by reference in its entirety. In one embodiment, the Fab fragment that is specific for Her3 comprises the heavy chain CDR1 of SEQ ID NO: 55, the heavy chain CDR2 of SEQ ID NO: 56, the heavy chain CDR3 of SEQ ID NO: 57, the light chain CDR1 of SEQ ID NO: 59, the light chain CDR2 of
20 SEQ ID NO: 60, and the light chain CDR3 of SEQ ID NO: 61. In a further embodiment, the Fab fragment that is specific for Her3 comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 58 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%,
25 97%, 98%, 99% or 100% identical to SEQ ID NO: 62, or variants thereof that retain functionality.

In yet another embodiment the bispecific antigen binding molecule comprises the polypeptide sequence of SEQ ID NO: 11, the polypeptide sequence of SEQ ID NO: 12, the polypeptide sequence of SEQ ID NO: 13 and the polypeptide sequence of SEQ ID NO: 14, or variants thereof that retain functionality.

30 In one embodiment the second antigen is epidermal growth factor receptor (EGFR). In another embodiment the second and optionally the third Fab fragment can compete with monoclonal antibody GA201 for binding to an epitope of EGFR. See PCT publication WO 2006/082515, incorporated herein by reference in its entirety. In one embodiment, the Fab fragment that is

specific for EGFR comprises the heavy chain CDR1 of SEQ ID NO: 23, the heavy chain CDR2 of SEQ ID NO: 24, the heavy chain CDR3 of SEQ ID NO: 25, the light chain CDR1 of SEQ ID NO: 27, the light chain CDR2 of SEQ ID NO: 28, and the light chain CDR3 of SEQ ID NO: 29. In a further embodiment, the Fab fragment that is specific for EGFR comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 26 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 30, or variants thereof that retain functionality.

In one embodiment the second antigen is fibroblast activation protein (FAP). In another embodiment the second and optionally the third Fab fragment can compete with monoclonal antibody 3F2 for binding to an epitope of FAP. See PCT publication WO 2012/020006, incorporated herein by reference in its entirety. In one embodiment, the Fab fragment that is specific for FAP comprises the heavy chain CDR1 of SEQ ID NO: 31, the heavy chain CDR2 of SEQ ID NO: 32, the heavy chain CDR3 of SEQ ID NO: 33, the light chain CDR1 of SEQ ID NO: 35, the light chain CDR2 of SEQ ID NO: 36, and the light chain CDR3 of SEQ ID NO: 37. In a further embodiment, the Fab fragment that is specific for FAP comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 34 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 38, or variants thereof that retain functionality.

In one embodiment the second antigen is CD33. In one embodiment, the Fab fragment that is specific for CD33 comprises the heavy chain CDR1 of SEQ ID NO: 47, the heavy chain CDR2 of SEQ ID NO: 48, the heavy chain CDR3 of SEQ ID NO: 49, the light chain CDR1 of SEQ ID NO: 51, the light chain CDR2 of SEQ ID NO: 52, and the light chain CDR3 of SEQ ID NO: 53. In a further embodiment, the Fab fragment that is specific for CD33 comprises a heavy chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 50 and a light chain variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 54, or variants thereof that retain functionality.

30

Polynucleotides

The invention further provides isolated polynucleotides encoding a bispecific antigen binding molecule as described herein or a fragment thereof. The polynucleotides encoding bispecific

antigen binding molecules of the invention may be expressed as a single polynucleotide that encodes the entire 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 through, e.g., disulfide bonds or other means to form a functional

5 bispecific antigen binding molecule. For example, the light chain portion of a Fab fragment may be encoded by a separate polynucleotide from the portion of the bispecific antigen binding molecule comprising the heavy chain portion of the Fab fragment, an Fc domain subunit and optionally (part of) another Fab fragment. When co-expressed, the heavy chain polypeptides will associate with the light chain polypeptides to form the Fab fragment. In another example, the

10 portion of the bispecific antigen binding molecule comprising one of the two Fc domain subunits and optionally (part of) one or more Fab fragments could be encoded by a separate polynucleotide from the portion of the bispecific antigen binding molecule comprising the other of the two Fc domain subunits and optionally (part of) a Fab fragment. When co-expressed, the Fc domain subunits will associate to form the Fc domain.

15 In one embodiment, an isolated polynucleotide of the invention encodes the first Fc domain subunit, the heavy chain of the second Fab fragment and the heavy chain of the first Fab fragment. In a more specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a VL region shares a carboxy-terminal peptide bond with a CH1 region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-

20 terminal peptide bond with an immunoglobulin heavy chain (VH-CH1-HR-CH2-CH3-(CH4)). In another specific embodiment the isolated polynucleotide encodes a polypeptide wherein a Fab heavy chain (VH-CH1) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VL region, which in turn shares a carboxy-terminal peptide bond with a CH1 region, which in turn shares a carboxy-terminal peptide bond

25 with an Fc domain subunit including an immunoglobulin hinge region (HR-CH2-CH3-(CH4)). In yet another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a VH region shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with an immunoglobulin heavy chain (VH-CH1-HR-CH2-CH3-(CH4)). In still

30 another specific embodiment the isolated polynucleotide encodes a polypeptide wherein a Fab heavy chain (VH-CH1) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a VH region, which in turn shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond

with an Fc domain subunit including an immunoglobulin hinge region (HR-CH2-CH3-(CH4)). In yet another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein an immunoglobulin heavy chain ((VH-CH1-HR-CH2-CH3-(CH4)) shares a carboxy-terminal peptide bond with a peptide linker, which in turn shares a carboxy-terminal peptide bond with a
5 VH region, which in turn shares a carboxy-terminal peptide bond with a CL region.

In further embodiments, an isolated polynucleotide of the invention encodes the second Fc domain subunit and optionally the heavy chain of a third Fab fragment. In a specific embodiment, the isolated polynucleotide encodes an immunoglobulin heavy chain ((VH-CH1-HR-CH2-CH3-(CH4)). In another specific embodiment, the isolated polynucleotide encodes an
10 Fc domain subunit, optionally including an antibody hinge region ((HR)-CH2-CH3-(CH4)).

In still further embodiments, an isolated polynucleotide of the invention encodes one or more light chain comprised in the bispecific antigen binding molecule. In a specific embodiment, the isolated polynucleotide encodes an immunoglobulin light chain (VL-CL). In another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a VL region shares a
15 carboxy-terminal peptide bond with a CH1 region. In yet another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a VH region shares a carboxy-terminal peptide bond with a CL region. In still another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a VH region shares a carboxy-terminal peptide bond with a CL region, which in turn shares a carboxy-terminal peptide bond with a Fab light chain (VL-CL). In
20 yet another specific embodiment, the isolated polynucleotide encodes a polypeptide wherein a Fab light chain (VL-CL) shares a carboxy-terminal peptide bond with a VH region, which in turn shares a carboxy-terminal peptide bond with a CL region.

In another embodiment, the present invention is directed to an isolated polynucleotide encoding a bispecific antigen binding molecule of the invention or a fragment thereof, wherein the
25 polynucleotide comprises a sequence that encodes a variable region sequence as shown in SEQ ID NOs 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70, 80, 84, 99, 103, 107 and 111. In another embodiment, the present invention is directed to an isolated polynucleotide encoding a bispecific antigen binding molecule or fragment thereof, wherein the polynucleotide comprises a sequence that encodes a polypeptide sequence as shown in SEQ ID NOs 1, 2, 3, 4, 5, 6, 7, 8, 9,
30 10, 11, 12, 13, 14, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 and 95. In another embodiment, the invention is directed to an isolated polynucleotide encoding a bispecific antigen binding molecule of the invention or a fragment thereof, wherein the polynucleotide comprises a sequence that encodes a variable region sequence that is at least about 80%, 85%, 90%, 95%,

96%, 97%, 98%, or 99% identical to an amino acid sequence in SEQ ID NOs 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70, 80, 84, 99, 103, 107 or 111. In another embodiment, the invention is directed to an isolated polynucleotide encoding a bispecific antigen binding molecule or fragment thereof, wherein the polynucleotide comprises a sequence that encodes a polypeptide sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence in SEQ ID NOs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95. The invention encompasses an isolated polynucleotide encoding a bispecific antigen binding molecule of the invention or a fragment thereof, wherein the polynucleotide comprises a sequence that encodes the variable region sequence of SEQ ID NOs 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70, 80, 84, 99, 103, 107 or 111 with conservative amino acid substitutions. The invention also encompasses an isolated polynucleotide encoding a bispecific antigen binding molecule or fragment thereof, wherein the polynucleotide comprises a sequence that encodes the polypeptide sequence of SEQ ID NOs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95 with conservative amino acid substitutions.

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.

20 **Recombinant Methods**

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 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 a 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 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 (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 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 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 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 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 early promoter, in conjunction with
5 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 $\hat{\alpha}$ -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
10 (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 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
15 chromosome integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal repeats (ITRs).

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
20 of the bispecific antigen binding molecule is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding a bispecific antigen 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
25 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 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
30 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 β -glucuronidase. Exemplary amino acid sequences of secretory signal peptides are given in SEQ ID NOs 74-76.

DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the bispecific antigen binding molecule may be included within
5 or at the ends of the bispecific antigen binding molecule (fragment) 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

10 such embodiment a host cell comprises (e.g. has been transformed or transfected with) a vector comprising a polynucleotide that encodes (part of) a 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 bispecific antigen binding molecules of the invention or fragments thereof. Host cells suitable for replicating and for supporting expression of bispecific antigen

15 binding molecules are well known in the art. Such cells may be transfected or transduced as appropriate with the particular expression vector and large quantities of vector containing cells can be grown for seeding large scale fermenters to obtain sufficient quantities of the 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

20 (CHO), insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation 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

25 glycosylation pathways have been "humanized", resulting in the production of a polypeptide with a partially or fully human glycosylation pattern. 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

30 strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant 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 PLANTIBODIESTM 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); 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⁻ CHO cells (Urlaub et 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 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., YO, NS0, Sp20 cell).

Standard technologies are known in the art to express foreign genes in these systems. Cells 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 a bispecific antigen binding molecule according to the invention is provided, wherein the method comprises culturing a host cell comprising a polynucleotide encoding the bispecific antigen binding molecule, as provided herein, under conditions suitable for expression of the bispecific antigen binding molecule, and recovering the bispecific antigen binding molecule from the host cell (or host cell culture medium).

The components of the bispecific antigen binding molecule are genetically fused to each other. 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 found in the sequences provided herein. Additional sequences 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.

In certain embodiments the Fab fragments forming part of the bispecific antigen binding molecules 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 can be used in the bispecific antigen binding molecules of the invention. Non-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 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 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 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); Queen et al., *Proc Natl Acad Sci USA* 86, 10029-10033 (1989); US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Jones et al., *Nature* 321, 522-525 (1986); Morrison et al., *Proc Natl Acad Sci* 81, 6851-6855 (1984); Morrison and Oi, *Adv Immunol* 44, 65-92 (1988); Verhoeyen et

al., *Science* 239, 1534-1536 (1988); Padlan, *Molec Immun* 31(3), 169-217 (1994); Kashmiri et al., *Methods* 36, 25-34 (2005) (describing SDR (a-CDR) 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* 83, 252-260 (2000) (describing the “guided selection” approach to FR shuffling). Human antibodies and human variable regions 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 variable regions can form part of and be derived from human monoclonal antibodies made by the hybridoma method (see e.g. *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Human antibodies and human variable regions may also 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 (see e.g. Lonberg, *Nat Biotech* 23, 1117-1125 (2005)). Human antibodies and human variable regions may also be generated by isolating Fv clone variable region sequences selected from human-derived phage display libraries (see e.g., Hoogenboom et al. in *Methods in Molecular Biology* 178, 1-37 (O’Brien et al., ed., Human Press, Totowa, NJ, 2001); and McCafferty et al., *Nature* 348, 552-554; Clackson et al., *Nature* 352, 624-628 (1991)). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments.

In certain embodiments, the Fab fragments useful in the present invention are engineered to have enhanced binding affinity according to, for example, the methods disclosed in U.S. Pat. Appl. Publ. No. 2004/0132066, the entire contents of which are hereby incorporated by reference. The ability of the bispecific antigen binding molecule of the invention 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 technique (analyzed on a BIACORE T100 system) (Liljebblad, et al., *Glyco J* 17, 323-329 (2000)), and traditional binding assays (Heeley, *Endocr Res* 28, 217-229 (2002)). Competition assays may be used to identify an antibody, antibody fragment, antigen binding domain or variable domain that competes with a reference antibody for binding to a particular antigen. 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). In an exemplary competition assay, immobilized antigen is incubated in a solution comprising a first labeled antibody that binds to the antigen and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to the antigen. The second antibody may be present in a hybridoma supernatant. As a control, immobilized antigen 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 the antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen 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 the antigen. See Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

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 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 bispecific antigen binding molecule binds. For example, for affinity chromatography purification of bispecific antigen binding molecules of the invention, a matrix with protein A or protein G may be used. Sequential Protein A or G affinity chromatography and size exclusion chromatography can be used to isolate a bispecific antigen binding molecule essentially as described in the Examples. The purity of the 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. For example, the heavy chain fusion proteins expressed as described in the Examples were shown to be intact and properly assembled as demonstrated by reducing SDS-PAGE (see e.g. Figure 3). Three bands were resolved at approximately Mr 25,000, Mr 50,000 and Mr 75,000, corresponding to the predicted molecular weights of the bispecific antigen binding molecule light chains, heavy chain and heavy chain/Fab heavy chain fusion protein, respectively.

Assays

Bispecific antigen binding molecules provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

5 Affinity assays

The affinity of the bispecific antigen binding molecule for an Fc receptor or a target antigen can be determined in accordance with the methods set forth in the Examples by surface plasmon resonance (SPR), using standard instrumentation such as a BIAcore instrument (GE Healthcare), and receptors or target proteins such as may be obtained by recombinant expression.

10 Alternatively, binding of bispecific antigen binding molecules for different receptors or target antigens may be evaluated using cell lines expressing the particular receptor or target antigen, for example by flow cytometry (FACS). A specific illustrative and exemplary embodiment for measuring binding affinity is described in the following and in the Examples below.

According to one embodiment, K_D is measured by surface plasmon resonance using a
15 BIACORE® T100 machine (GE Healthcare) at 25 °C.

To analyze the interaction between the Fc-portion and Fc receptors, His-tagged recombinant Fc-receptor is captured by an anti-Penta His antibody (Qiagen) immobilized on CM5 chips and the bispecific constructs are used as analytes. Briefly, carboxymethylated dextran biosensor chips (CM5, GE Healthcare) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide
20 hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Anti Penta-His antibody is diluted with 10 mM sodium acetate, pH 5.0, to 40 µg/ml before injection at a flow rate of 5 µl/min to achieve approximately 6500 response units (RU) of coupled protein. Following the injection of the ligand, 1 M ethanolamine is injected to block unreacted groups. Subsequently the Fc-receptor is captured for 60 s at 4 or 10 nM. For kinetic
25 measurements, four-fold serial dilutions of the bispecific construct (range between 500 nM and 4000 nM) are injected in HBS-EP (GE Healthcare, 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05 % Surfactant P20, pH 7.4) at 25 °C at a flow rate of 30 µl/min for 120 s.

To determine the affinity to the target antigen, bispecific constructs are captured by an anti human Fab specific antibody (GE Healthcare) that is immobilized on an activated CM5-sensor
30 chip surface as described for the anti Penta-His antibody. The final amount of coupled protein is is approximately 12000 RU. The bispecific constructs are captured for 90 s at 300 nM. The target antigens are passed through the flow cells for 180 s at a concentration range from 250 to 1000 nM with a flowrate of 30 µl/min. The dissociation is monitored for 180 s.

Bulk refractive index differences are corrected for by subtracting the response obtained on reference flow cell. The steady state response was used to derive the dissociation constant K_D by non-linear curve fitting of the Langmuir binding isotherm. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® T100 Evaluation Software version 1.1.1) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_D) is calculated as the ratio k_{off}/k_{on} . See, e.g., Chen et al., J Mol Biol 293, 865-881 (1999).

Activity assays

Biological activity of the bispecific antigen binding molecules of the invention can be measured by various assays known in the art, including those described in the Examples. Biological activities may for example include the induction of proliferation of T cells, the induction of signaling in T cells, the induction of expression of activation markers in T cells, the induction of cytokine secretion by T cells, the inhibition of signaling in target cells such as tumor cells or cells of the tumor stroma, the inhibition of proliferation of target cells, the induction of lysis of target cells, and the induction of tumor regression and/or the improvement of survival.

Compositions, Formulations, and Routes of Administration

In a further aspect, the invention provides pharmaceutical compositions comprising any of the 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 the bispecific antigen binding molecules provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises any of the 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 a bispecific antigen binding molecule of the invention in a form suitable for administration in vivo, the method comprising (a) obtaining a bispecific antigen binding molecule according to the invention, and (b) formulating the bispecific antigen binding molecule with at least one pharmaceutically acceptable carrier, whereby a preparation of bispecific antigen binding molecule is formulated for administration in vivo.

Pharmaceutical compositions of the present invention comprise a therapeutically effective amount of one or more bispecific antigen binding molecule dissolved or dispersed in a 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 at least one 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 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, 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 therapeutic or pharmaceutical compositions is contemplated.

The composition may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. Bispecific antigen binding molecules of the present invention (and any additional therapeutic agent) can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrasplenically, intrarenally, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, by inhalation (e.g. aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (e.g. liposomes), or by other method or any combination of the forgoing 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,

incorporated herein by reference). Parenteral administration, in particular intravenous injection, is most commonly used for administering polypeptide molecules such as the bispecific antigen binding molecules of the invention.

Parenteral compositions include those designed for administration by injection, e.g. 5 subcutaneous, intradermal, intralesional, intravenous, intraarterial intramuscular, intrathecal or intraperitoneal injection. For injection, the bispecific antigen binding molecules of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain 10 formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the 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 bispecific antigen binding molecules of 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. 15 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 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 20 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 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 25 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; 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; 30 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; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other

carbohydrates including glucose, mannose, or dextrans; 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 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 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 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 monostearate, gelatin or combinations thereof.

In addition to the compositions described previously, the 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 bispecific antigen binding 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 bispecific antigen binding molecules of the invention may be manufactured by means of conventional mixing, dissolving, 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 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 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, 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.

15 **Therapeutic Methods and Compositions**

Any of the bispecific antigen binding molecules provided herein may be used in therapeutic methods. Bispecific antigen binding molecules of the invention can be used for example in the treatment of cancers.

For use in therapeutic methods, bispecific antigen binding molecules of the invention would be formulated, dosed, and administered in a fashion consistent with good 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, bispecific antigen binding molecules of the invention for use as a medicament are provided. In further aspects, bispecific antigen binding molecules of the invention for use in treating a disease are provided. In certain embodiments, bispecific antigen binding molecules of the invention for use in a method of treatment are provided. In one embodiment, the invention provides a bispecific antigen binding molecule as described herein for use in the treatment of a disease in an individual in need thereof. In certain embodiments, the invention provides a 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 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. An "individual" according to any of the above embodiments is a mammal, preferably a human.

5 In a further aspect, the invention provides for the use of a 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 administering to an individual having the disease a therapeutically effective amount of the
10 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. An "individual" according to any of the above embodiments may be a mammal, preferably a human.

15 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 a bispecific antigen binding molecule of the invention. In one embodiment a composition is administered to said individual, comprising the bispecific antigen binding molecule of the invention in a pharmaceutically acceptable form. In certain embodiments the
20 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.

25 In certain embodiments the disease to be treated is a proliferative disorder, particularly cancer. Non-limiting examples of cancers include bladder cancer, brain cancer, head and neck cancer, pancreatic cancer, lung cancer, breast cancer, ovarian cancer, uterine cancer, cervical cancer, endometrial cancer, esophageal cancer, colon cancer, colorectal cancer, rectal cancer, gastric cancer, prostate cancer, blood cancer, skin cancer, squamous cell carcinoma, bone cancer, and
30 kidney cancer. Other cell proliferation disorders that can be treated using a 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, 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 renal cell cancer, skin cancer, lung cancer, colorectal cancer, breast cancer, brain cancer, head and neck cancer. A skilled artisan readily recognizes that in many cases the bispecific antigen binding molecule may not provide a cure but may only provide partial benefit. In some embodiments, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of 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 a bispecific antigen binding molecule of the invention is administered to a cell. In other embodiments, a therapeutically effective amount of a bispecific antigen binding molecule of the invention is administered to an individual for the treatment of disease.

For the prevention or treatment of disease, the appropriate dosage of a 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 bispecific antigen binding molecule, the severity and course of the disease, whether the bispecific antigen binding molecule is administered for preventive or therapeutic purposes, previous or concurrent therapeutic interventions, the patient's clinical history and response to the 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 schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

The 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 $\mu\text{g}/\text{kg}$ to 15 mg/kg (e.g. 0.1 mg/kg – 10 mg/kg) of bispecific antigen binding 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 $\mu\text{g}/\text{kg}$ 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 symptoms occurs. One exemplary dosage of the 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
5 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 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
10 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,
15 based on the numbers described above. 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 bispecific antigen binding molecule). An initial higher loading dose, followed by one or more lower doses
20 may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The 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 bispecific antigen binding molecules of the invention, or pharmaceutical compositions thereof,
25 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
30 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.

5 Dosage amount and interval may be adjusted individually to provide plasma levels of the 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 example, by HPLC.

10 In cases of local administration or selective uptake, the effective local concentration of the 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 bispecific antigen binding molecules described herein will generally provide therapeutic benefit without causing substantial toxicity. Toxicity and therapeutic efficacy of a 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 the ratio LD₅₀/ED₅₀. Bispecific antigen binding molecules that exhibit large therapeutic indices are preferred. In one embodiment, the 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 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. 1, incorporated herein by reference in its entirety).

25 The attending physician for patients treated with 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 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.

Other Agents and Treatments

The bispecific antigen binding molecules of the invention may be administered in combination with one or more other agents in therapy. For instance, a 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, 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, 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 bispecific antigen binding molecule used, the type of disorder or treatment, and other factors discussed above. The 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 more therapeutic agents are included in the same or separate compositions), and separate administration, in which case, administration of the bispecific antigen binding molecule of the invention can occur prior to, simultaneously, and/or following, administration of the additional

therapeutic agent and/or adjuvant. Bispecific antigen binding molecules of the invention can also be used in combination with radiation therapy.

Articles of Manufacture

5 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 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
10 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 a bispecific antigen binding molecule of the invention. The label or package insert indicates that the
15 composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a 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
20 invention may further comprise a package insert indicating that the compositions can be 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
25 standpoint, including other buffers, diluents, filters, needles, and syringes.

Examples

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

30 **General methods**

Recombinant DNA Techniques

Standard methods were used to manipulate DNA as described in Sambrook et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturers' instructions. General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A. et al., (1991) Sequences of Proteins of Immunological Interest, 5th ed., NIH Publication No. 91-3242.

DNA Sequencing

DNA sequences were determined by double strand sequencing.

10

Gene Synthesis

Desired gene segments where required were either generated by PCR using appropriate templates or were synthesized by Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR products by automated gene synthesis. In cases where no exact gene sequence was available, oligonucleotide primers were designed based on sequences from closest homologues and the genes were isolated by RT-PCR from RNA originating from the appropriate tissue. The gene segments flanked by singular restriction endonuclease cleavage sites were cloned into standard cloning / sequencing vectors. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. Gene segments were designed with suitable restriction sites to allow sub-cloning into the respective expression vectors. All constructs were designed with a 5'-end DNA sequence coding for a leader peptide which targets proteins for secretion in eukaryotic cells. SEQ ID NOs 74-76 give exemplary leader peptides.

25

Isolation of primary human pan T cells from PBMCs

Peripheral blood mononuclear cells (PBMCs) were prepared by Histopaque density centrifugation from enriched lymphocyte preparations (buffy coats) obtained from local blood banks or from fresh blood from healthy human donors. Briefly, blood was diluted with sterile PBS and carefully layered over a Histopaque gradient (Sigma, H8889). After centrifugation for 30 minutes at 450 x g at room temperature (brake switched off), part of the plasma above the PBMC containing interphase was discarded. The PBMCs were transferred into new 50 ml Falcon tubes and tubes were filled up with PBS to a total volume of 50 ml. The mixture was

centrifuged at room temperature for 10 minutes at 400 x g (brake switched on). The supernatant was discarded and the PBMC pellet washed twice with sterile PBS (centrifugation steps at 4°C for 10 minutes at 350 x g). The resulting PBMC population was counted automatically (ViCell) and stored in RPMI1640 medium, containing 10% FCS and 1% L-alanyl-L-glutamine
5 (Biochrom, K0302) at 37°C, 5% CO₂ in the incubator until assay start.

T cell enrichment from PBMCs was performed using the Pan T Cell Isolation Kit II (Miltenyi Biotec #130-091-156), according to the manufacturer's instructions. Briefly, the cell pellets were diluted in 40 µl cold buffer per 10 million cells (PBS with 0.5% BSA, 2 mM EDTA, sterile filtered) and incubated with 10 µl Biotin-Antibody Cocktail per 10 million cells for 10 min at
10 4°C. 30 µl cold buffer and 20 µl Anti-Biotin magnetic beads per 10 million cells were added, and the mixture incubated for another 15 min at 4°C. Cells were washed by adding 10-20x the current volume and a subsequent centrifugation step at 300 x g for 10 min. Up to 100 million cells were resuspended in 500 µl buffer. Magnetic separation of unlabeled human pan T cells was performed using LS columns (Miltenyi Biotec #130-042-401) according to the
15 manufacturer's instructions. The resulting T cell population was counted automatically (ViCell) and stored in AIM-V medium at 37°C, 5% CO₂ in the incubator until assay start (not longer than 24 h).

Isolation of primary human naive T cells from PBMCs

20 Peripheral blood mononuclear cells (PBMCs) were prepared by Histopaque density centrifugation from enriched lymphocyte preparations (buffy coats) obtained from local blood banks or from fresh blood from healthy human donors. T-cell enrichment from PBMCs was performed using the Naive CD8⁺ T cell isolation Kit from Miltenyi Biotec (#130-093-244), according to the manufacturer's instructions, but skipping the last isolation step of CD8⁺ T cells (also see
25 description for the isolation of primary human pan T cells).

Isolation of primary cynomolgus PBMCs from heparinized blood

Peripheral blood mononuclear cells (PBMCs) were prepared by density centrifugation from fresh blood from healthy cynomolgus donors, as follows: Heparinized blood was diluted 1:3 with
30 sterile PBS, and Lymphoprep medium (Axon Lab #1114545) was diluted to 90% with sterile PBS. Two volumes of the diluted blood were layered over one volume of the diluted density gradient and the PBMC fraction was separated by centrifugation for 30 min at 520 x g, without brake, at room temperature. The PBMC band was transferred into a fresh 50 ml Falcon tube and

washed with sterile PBS by centrifugation for 10 min at 400 x g at 4°C. One low-speed centrifugation was performed to remove the platelets (15 min at 150 x g, 4°C), and the resulting PBMC population was automatically counted (ViCell) and immediately used for further assays.

5 Target cells

For the assessment of MCSP-targeting bispecific antigen binding molecules, the following tumor cell lines were used: the human melanoma cell line WM266-4 (ATCC #CRL-1676), derived from a metastatic site of a malignant melanoma and expressing high levels of human MCSP; and the human melanoma cell line MV-3 (a kind gift from The Radboud University Nijmegen
10 Medical Centre), expressing medium levels of human MCSP.

For the assessment of CEA-targeting bispecific antigen binding molecules, the following tumor cell lines were used: the human gastric cancer cell line MKN45 (DSMZ #ACC 409), expressing very high levels of human CEA; the human female Caucasian colon adenocarcinoma cell line LS-174T (ECACC #87060401), expressing medium to low levels of human CEA; the human
15 epithelioid pancreatic carcinoma cell line Panc-1 (ATCC #CRL-1469), expressing (very) low levels of human CEA; and a murine colon carcinoma cell line MC38-huCEA, that was engineered in-house to stably express human CEA.

In addition, a human T cell leukaemia cell line, Jurkat (ATCC #TIB-152), was used to assess binding of different bispecific constructs to human CD3 on cells.

20

Example 1

Preparation, purification and characterization of bispecific antigen binding molecules

The heavy and light chain variable region DNA sequences were subcloned in frame with either the constant heavy chain or the constant light chain pre-inserted into the respective recipient
25 mammalian expression vector. The antibody expression was driven by an MPSV promoter and a synthetic polyA signal sequence is located at the 3' end of the CDS. In addition each vector contained an EBV OriP sequence.

The molecules were produced by co-transfecting HEK293 EBNA cells with the mammalian expression vectors. Exponentially growing HEK293 EBNA cells were transfected using the
30 calcium phosphate method. Alternatively, HEK293 EBNA cells growing in suspension were transfected using polyethylenimine (PEI). For preparation of "1+1 IgG Crossfab" constructs, cells were transfected with the corresponding expression vectors in a 1:1:1:1 ratio ("vector second heavy chain" : "vector first light chain" : "vector light chain Crossfab" : "vector first

heavy chain-heavy chain Crossfab”). For preparation of “2+1 IgG Crossfab” constructs cells were transfected with the corresponding expression vectors in a 1:2:1:1 ratio (“vector second heavy chain” : “vector light chain” : “vector first heavy chain-heavy chain Crossfab” : “vector light chain Crossfab”). For preparation of the “2+1 IgG Crossfab (N-terminal), linked light chain”
5 construct, cells were transfected with the corresponding expression vectors in a 1:1:1:1 ratio (“vector heavy chain” : “vector light chain” : “vector heavy chain (CrossFab-Fab-Fc)” : “vector linked light chain”). For preparation of the “1+1 CrossMab” construct, cells were transfected with the corresponding expression vectors in a 1:1:1:1 ratio (“vector first heavy chain” : “vector second heavy chain” : “vector first light chain” : “vector second light chain”).

10 For transfection using calcium phosphate cells were grown as adherent monolayer cultures in T-flasks using DMEM culture medium supplemented with 10 % (v/v) FCS, and transfected when they were between 50 and 80 % confluent. For the transfection of a T150 flask, 15 million cells were seeded 24 hours before transfection in 25 ml DMEM culture medium supplemented with FCS (at 10% v/v final), and cells were placed at 37°C in an incubator with a 5% CO₂ atmosphere
15 overnight. For each T150 flask to be transfected, a solution of DNA, CaCl₂ and water was prepared by mixing 94 µg total plasmid vector DNA divided in the corresponding ratio, water to a final volume of 469 µl and 469 µl of a 1 M CaCl₂ solution. To this solution, 938 µl of a 50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄ solution at pH 7.05 were added, mixed immediately for 10 s and left to stand at room temperature for 20 s. The suspension was diluted with 10 ml of
20 DMEM supplemented with 2 % (v/v) FCS, and added to the T150 in place of the existing medium. Subsequently, additional 13 ml of transfection medium were added. The cells were incubated at 37°C, 5% CO₂ for about 17 to 20 hours, then medium was replaced with 25 ml DMEM, 10 % FCS. The conditioned culture medium was harvested approximately 7 days post-media exchange by centrifugation for 15 min at 210 x g, sterile filtered (0.22 µm filter),
25 supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

For transfection using polyethylenimine (PEI) HEK293 EBNA cells were cultivated in suspension in serum free CD CHO culture medium. For the production in 500 ml shake flasks, 400 million HEK293 EBNA cells were seeded 24 hours before transfection. For transfection cells were centrifuged for 5 min at 210 x g, and supernatant was replaced by 20 ml pre-warmed
30 CD CHO medium. Expression vectors were mixed in 20 ml CD CHO medium to a final amount of 200 µg DNA. After addition of 540 µl PEI, the mixture was vortexed for 15 s and subsequently incubated for 10 min at room temperature. Afterwards cells were mixed with the DNA/PEI solution, transferred to a 500 ml shake flask and incubated for 3 hours at 37°C in an

incubator with a 5% CO₂ atmosphere. After the incubation time 160 ml F17 medium was added and cells were cultivated for 24 hours. One day after transfection 1 mM valproic acid and 7% Feed 1 (Lonza) were added. After a cultivation of 7 days, supernatant was collected for purification by centrifugation for 15 min at 210 x g, the solution was sterile filtered (0.22 µm filter), supplemented with sodium azide to a final concentration of 0.01 % w/v, and kept at 4°C.

The secreted proteins were purified from cell culture supernatants by Protein A affinity chromatography, followed by a size exclusion chromatography step.

For affinity chromatography supernatant was loaded on a HiTrap ProteinA HP column (CV = 5 ml, GE Healthcare) equilibrated with 25 ml 20 mM sodium phosphate, 20 mM sodium citrate, pH 7.5 or 40 ml 20 mM sodium phosphate, 20 mM sodium citrate, 0.5 M sodium chloride, pH 7.5. Unbound protein was removed by washing with at least ten column volumes 20 mM sodium phosphate, 20 mM sodium citrate, 0.5 M sodium chloride pH 7.5, followed by an additional wash step using six column volumes 10 mM sodium phosphate, 20 mM sodium citrate, 0.5 M sodium chloride pH 5.45. Subsequently, the column was washed with 20 ml 10 mM MES, 100 mM sodium chloride, pH 5.0, and target protein was eluted in six column volumes 20 mM sodium citrate, 100 mM sodium chloride, 100 mM glycine, pH 3.0. Alternatively, target protein was eluted using a gradient over 20 column volumes from 20 mM sodium citrate, 0.5 M sodium chloride, pH 7.5 to 20 mM sodium citrate, 0.5 M sodium chloride, pH 2.5. The protein solution was neutralized by adding 1/10 of 0.5 M sodium phosphate, pH 8. The target protein was concentrated and filtrated prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 25 mM potassium phosphate, 125 mM sodium chloride, 100 mM glycine solution of pH 6.7. For the purification of 1+1 IgG Crossfab the column was equilibrated with 20 mM histidine, 140 mM sodium chloride solution of pH 6.0.

The protein concentration of purified protein samples 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. Purity and molecular weight of the bispecific constructs were analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiothreitol) and staining with Coomassie (SimpleBlue™ SafeStain from Invitrogen), using the NuPAGE® Pre-Cast gel system (Invitrogen, USA) according to the manufacturer's instructions (4-12% Tris-Acetate gels or 4-12% Bis-Tris). Alternatively, purity and molecular weight of molecules were analyzed by CE-SDS analyses in the presence and absence of a reducing agent, using the Caliper LabChip GXII system (Caliper Lifescience) according to the manufacturer's instructions.

The aggregate content of the protein samples was analyzed using a Superdex 200 10/300GL analytical size-exclusion chromatography column (GE Healthcare) in 2 mM MOPS, 150 mM NaCl, 0.02% (w/v) NaN₃, pH 7.3 running buffer at 25°C. Alternatively, the aggregate content of antibody samples was analyzed using a TSKgel G3000 SW XL analytical size-exclusion column
5 (Tosoh) in 25 mM K₂HPO₄, 125 mM NaCl, 200 mM L-arginine monohydrochloride, 0.02% (w/v) NaN₃, pH 6.7 running buffer at 25°C.

The “2+1 IgG Crossfab (C-terminal)” construct (see SEQ ID NOs 11, 12, 13 and 14) was prepared using plasmid vectors having a genomic gene organization, including intron sequences, a CMV promoter and a polyadenylation signal from the gene of bovine growth hormone. The
10 bispecific construct was transiently expressed in HEK293F cells by simultaneous transfection of required plasmids via lipofection using different plasmid ratios. Cell were grown in F17 medium. Supernatant was collected 5-7 days after transfection. Harvested cell-culture supernatant was sterile filtrated through a 0.2 µm pore-size membrane (Millipore) prior to purification. For purification, the bispecific molecule was captured on a MabSelectSure resin (GE Healthcare),
15 washed with 1x PBS and eluted with 20 mM sodium-citrate at pH 3.0. The molecule was further purified by size exclusion chromatography using a Superdex™ 200 GL (Amersham Bioscience) column equilibrated with 20 mM histidine, 140 mM NaCl, pH 6.0. Characterization (antibody integrity assessment) of the bispecific molecule was done using Capillary electrophoresis (CE-SDS) analysis, using microfluidic Labchip technology (Caliper). 5 µl of protein solution was
20 prepared for CE-SDS analysis using the HT Protein Express Reagent Kit according to the manufacturer’s instructions and analysed on a LabChip GXII system using a HT Protein Express Chip.

Figures 2-5 show the results of the SDS PAGE and analytical size exclusion chromatography and Table 2 shows the yields, aggregate content after Protein A and final monomer content of the
25 preparations of the different bispecific constructs. Importantly, the bispecific IgG Crossfab constructs of the present invention showed a 10 to 20 fold reduced aggregate content after Protein A affinity chromatography as compared to corresponding bispecific constructs comprising a single chain Fab fragment instead of a Crossfab fragment (data not shown).

Figure 13 shows the result of the CE-SDS analyses of the anti-CD3/anti-MCSP bispecific “2+1
30 IgG Crossfab (N-terminal), linked light chain” construct (see SEQ ID NOs 1, 4, 5 and 85). 2 µg sample was used for analyses. Figure 14 shows the result of the analytical size exclusion chromatography of the final product (20 µg sample injected).

Figure 20 shows the results of the CE-SDS analyses of the 1+1 IgG Crossfab (N-terminal); VL/VH exchange (LC007/V9), the 1+1 CrossMab; CL/CH1 exchange (LC007/V9), the 2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (LC007/V9), the 2+1 IgG Crossfab (N-terminal); VL/VH exchange (M4-3 ML2/V9), the 2+1 IgG Crossfab (N-terminal); CL/CH1 exchange (M4-3 ML2/V9), and the 2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (CH1A1A/V9), and Table 2 shows the yields, aggregate content after Protein A and final monomer content of the preparations of the different bispecific constructs.

TABLE 2. Yields, aggregate content after Protein A and final and monomer content.

Construct	Yield [mg/l]	Aggregates after Protein A [%]	HMW [%]	LMW [%]	Monomer [%]
2+1 IgG Crossfab (N-terminal); VL/VH exchange (MCSP (LC007)/huCD3)	12.8	2.2	0	0	100
2+1 IgG Crossfab (N-terminal); VL/VH exchange (MCSP (LC007)/cyCD3)	3.2	5.7	0.4	0	99.6
1+1 IgG Crossfab (N-terminal); VL/VH exchange (MCSP (LC007)/huCD3)	9.8	0	0	0	100
2+1 IgG Crossfab (N-terminal), inverted; VL/VH exchange (CEA/huCD3)	0.34	13.04	4.4	0	95.6
2+1 IgG Crossfab (C-terminal); CL/CH1 exchange (c-Met/Her3)	15	14			
2+1 IgG Crossfab (N-terminal), linked light chain; VL/VH exchange (MCSP (LC007)/huCD3)	0.54	40	1.4	0	98.6
1+1 IgG Crossfab (N-terminal); VL/VH exchange (MCSP (LC007)/huCD3)	6.61	8.5	0	0	100
1+1 CrossMab; CL/CH1 exchange (MCSP (LC007)/huCD3)	6.91	10.5	1.3	1.7	97
2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (MCSP (LC007)/huCD3)	9.45	6.1	0.8	0	99.2
2+1 IgG Crossfab (N-terminal); VL/VH exchange (MCSP (M4-3 ML2)/huCD3)	36.6	0	9.5	35.3	55.2
2+1 IgG Crossfab (N-terminal); CL/CH1 exchange (MCSP(M4-3 ML2)/huCD3)	2.62	12	2.8	0	97.2

2+1 IgG Crossfab (N-terminal), inverted; CL/CH1 exchange (CEA/huCD3)	12.7	43	0	0	100
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Example 2

Simultaneous binding of bispecific constructs to both target antigens

5 Simultaneous binding to of the “2+1 IgG Crossfab (N-terminal)” construct (SEQ ID NOs 1, 3, 4, 5) to human MCSP and human CD3 ϵ was analyzed by surface plasmon resonance (Figure 6).

All surface plasmon resonance (SPR) experiments are performed on a Biacore T100 at 25°C with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, Biacore, Freiburg/Germany).

10 Analysis of simultaneous binding of the bispecific construct to the tumor antigen and the human CD3 ϵ was performed by direct coupling of 1650 resonance units (RU) of biotinylated D3 domain of MCSP on a sensor chip SA using the standard coupling procedure. The assay setup is shown in Figure 6A.

The “2+1 IgG Crossfab (N-terminal)” construct was captured for 60 s at 200 nM. Human
15 CD3 γ (G₄S)₅CD3 ϵ -AcTev-Fc(knob)-Avi/Fc(hole) was subsequently passed at a concentration of 2000 nM and a flow rate of 40 μ l/min for 60 s. Bulk refractive index differences were corrected for by subtracting the response obtained on a reference flow cell where the recombinant CD3 ϵ was flown over a surface with immobilized D3 domain of MCSP without captured bispecific construct.

20 As shown in Figure 6B, the construct was able to bind the tumor antigen and the CD3 simultaneously. The binding level (RU) after injection of human CD3 ϵ was higher than the binding level achieved after injection of the construct alone reflecting that both tumor antigen and the human CD3 ϵ are bound to the bispecific construct.

25 Example 3

T cell activation by bispecific constructs in the presence and absence of target cells

Cytokine release

The purified “2+1 IgG Crossfab (N-terminal)” construct (SEQ ID NOs 1, 3, 4, 5) and the
30 “(scFv)₂” molecule, both targeting human MCSP and human CD3, were analyzed for their ability to induce T cell-mediated *de novo* secretion of cytokines in the presence or absence of tumor target cells.

Briefly, 280 μ l whole blood from a healthy donor were plated per well of a deep-well 96-well plate. 30 000 Colo-38 tumor target cells, expressing human MCSP, as well as the two bispecific constructs and IgG controls were added at 1 nM final concentration. The cells were incubated for 24 h at 37°C, 5% CO₂ and then centrifuged for 5 min at 350 x g. The supernatant was transferred
5 into a new deep-well 96-well-plate for the subsequent analysis. The CBA analysis was performed according to manufacturer's instructions for FACS CantoII, using the combination of the following CBA Flex Sets: human granzyme B (BD #560304), human IFN- γ Flex Set (BD #558269), human TNF Flex Set (BD #558273), human IL-10 Flex Set (BD #558274), human IL-6 Flex Set (BD #558276), human IL-4 Flex Set (BD #558272), human IL-2 Flex Set (BD
10 #558270).

Figure 7 shows the levels of the different cytokine measured in the supernatant. The main cytokine secreted in the presence of Colo-38 tumor cells was IL-6, followed by IFN- γ . In addition, also the levels of granzyme B strongly increased upon activation of T cells in the presence of target cells. In general, both bispecific constructs induced high levels of cytokine
15 secretion in the presence of target cells (Figure 7, A and B), but not in the absence of target cells (Figure 7, C and D). There was no significant secretion of Th2 cytokines (IL-10 and IL-4) upon activation of T cells in the presence (or absence) of target cells.

Expression of surface activation markers

20 In another experiment, purified "2+1 IgG Crossfab (N-terminal)" (SEQ ID NOs 4, 5, 6, 7), targeting cynomolgus CD3 and human MCSP, was analyzed for its potential to up-regulate the surface activation marker CD25 on CD8⁺ T cells in the presence of tumor target cells. Briefly, human MCSP-expressing MV-3 tumor target cells were harvested with Cell Dissociation Buffer, washed and resuspendend in DMEM containing 2% FCS and 1% GlutaMax. 30 000 cells per
25 well were plated in a round-bottom 96-well plate and the respective antibody dilution was added at the indicated concentrations (Figure 8). The bispecific construct and the different IgG controls were adjusted to the same molarity. Cynomolgus PBMC effector cells, isolated from blood of two healthy animals, were added to obtain a final E:T ratio of 3:1. After incubation for 43 h at 37°C, 5% CO₂, the cells were centrifuged at 350 x g for 5 min and washed twice with PBS,
30 containing 0.1% BSA. Surface staining for CD8 (Miltenyi Biotech #130-080-601) and CD25 (BD #557138) was performed according to the supplier's suggestions. Cells were washed twice with 150 μ l/well PBS containing 0.1% BSA and fixed for 15 min at 4°C, using 100 μ l/well

fixation buffer (BD #554655). After centrifugation, the samples were resuspended in 200 μ l/well PBS with 0.1% BSA and analyzed using a FACS CantoII machine (Software FACS Diva).

As depicted in Figure 8, the bispecific construct induces concentration-dependent up-regulation of CD25 on CD8⁺ T cells only in the presence of target cells. The anti cyno CD3 IgG (clone FN-18) is also able to induce up-regulation of CD25 on CD8⁺ T cells, without being crosslinked to tumor target cells (see data obtained with cyno Nestor). There is no hyperactivation of cyno T cells with the maximal concentration of the bispecific construct (in the absence of target cells).

In another experiment, the CD3-MCSP “2+1 IgG Crossfab (N-terminal), linked light chain” (see SEQ ID NOs 1, 4, 5 and 85) was compared to the CD3-MCSP “2+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4 and 5) for its potential to up-regulate the early activation marker CD69 or the late activation marker CD25 on CD8⁺ T cells in the presence of tumor target cells. Primary human PBMCs (isolated as described above) were incubated with the indicated concentrations of bispecific constructs for at least 22 h in the presence or absence of MCSP-positive Colo38 target cells. Briefly, 0.3 million primary human PBMCs were plated per well of a flat-bottom 96-well plate, containing the MCSP-positive target cells (or medium). The final effector to target cell (E:T) ratio was 10:1. The cells were incubated with the indicated concentration of the bispecific constructs and controls for the indicated incubation times at 37°C, 5% CO₂. The effector cells were stained for CD8, and CD69 or CD25 and analyzed by FACS CantoII.

Figure 19 shows the result of this experiment. There were no significant differences detected for CD69 (A) or CD25 up-regulation (B) between the two 2+1 IgG Crossfab molecules (with or without the linked light chain).

In yet another experiment, the CD3-MCSP “2+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4, 5) and “1+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4, 86) constructs were compared to a bispecific CD3-MCSP IgG-like construct having one antigen binding arm replaced by a Crossfab fragment (“1+1 CrossMab”; see SEQ ID NOs 4, 87, 88, 89) for their potential to up-regulate CD69 or CD25 on CD4⁺ or CD8⁺ T cells in the presence of tumor target cells. The assay was performed as described above, in the presence or absence of human MCSP expressing MV-3 tumor cells, with an incubation time of 24 h.

As shown in Figure 21, the “1+1 IgG Crossfab (N-terminal)” and “2+1 IgG Crossfab (N-terminal)” constructs induced more pronounced upregulation of activation markers than the “1+1 CrossMab” molecule.

Example 4

Re-directed T cell cytotoxicity mediated by cross-linked bispecific constructs targeting CD3 on T cells and MCSP on tumor cells (LDH release assay)

Bispecific constructs were analyzed for their potential to induce T cell-mediated apoptosis in tumor target cells upon crosslinkage of the construct via binding of the antigen binding moieties
5 to their respective target antigens on cells.

In one experiment purified “2+1 IgG Crossfab (N-terminal)” construct (SEQ ID NOs 1, 3, 4, 5), targeting human CD3 and human MCSP, and the corresponding “(scFv)₂” molecule were compared. Briefly, huMCSP-expressing MDA-MB-435 human melanoma target cells were harvested with Cell Dissociation Buffer, washed and resuspended in AIM-V medium
10 (Invitrogen # 12055-091). 30 000 cells per well were plated in a round-bottom 96-well plate and the respective dilution of the construct was added at the indicated concentration. All constructs and controls were adjusted to the same molarity. Human pan T effector cells were added to obtain a final E:T ratio of 5:1. As a positive control for the activation of human pan T cells, 1 µg/ml PHA-M (Sigma #L8902) was used. For normalization, maximal lysis of the target cells (=
15 100%) was determined by incubation of the target cells with a final concentration of 1% Triton X-100. Minimal lysis (= 0%) refers to target cells co-incubated with effector cells, but without any construct or antibody. After an overnight incubation of 20 h at 37°C, 5% CO₂, LDH release of apoptotic/necrotic target cells into the supernatant was measured with the LDH detection kit (Roche Applied Science, #11 644 793 001), according to the manufacturer’s instructions.

20 As depicted in Figure 9, the “2+1 IgG Crossfab (N-terminal)” construct induces apoptosis in target cells comparable to the “(scFv)₂” molecule.

In a further experiment the purified “2+1 IgG Crossfab (N-terminal)” (SEQ ID NOs 1, 3, 4, 5), the “1+1 IgG Crossfab (N-terminal)” (SEQ ID NOs 1, 2, 3, 4) and the “(scFv)₂” molecule were analyzed for their potential to induce T cell-mediated apoptosis in tumor target cells upon
25 crosslinkage of the construct via binding of both target antigens, CD3 and MCSP, on cells. huMCSP-expressing MDA-MB-435 human melanoma cells were used as target cells, the E:T ratio was 5:1, and the incubation time 20 h. The results are shown in Figure 10. The “2+1 IgG Crossfab (N-terminal)” construct induces apoptosis in target cells comparably to the “(scFv)₂” molecule. The comparison of the mono- and bivalent “IgG Crossfab (N-terminal)” formats
30 shows that the bivalent one is more potent in this assay.

In yet another experiment, purified “2+1 IgG Crossfab (N-terminal)” (SEQ ID NOs 4, 5, 6, 7) targeting cynomolgus CD3 and human MCSP, and the corresponding “(scFv)₂” construct were compared, using MCSP-expressing human melanoma cell line (MV-3) as target cells. Briefly,

MV-3 cells were harvested with Cell Dissociation Buffer, washed and resuspended in DMEM containing 2% FCS and 1% GlutaMax. 30 000 cells per well were plated in a round-bottom 96-well plate and the respective dilution of construct or reference IgG was added at the concentrations indicated. The bispecific construct and the different IgG controls were adjusted to the same molarity. Cynomolgus PBMC effector cells, isolated from blood of healthy cynomolgus, were added to obtain a final E:T ratio of 10:1. After incubation for 26 h at 37°C, 5% CO₂, LDH release of apoptotic/necrotic target cells into the supernatant was measured with the LDH detection kit (Roche Applied Science, #11 644 793 001), according to the manufacturer's instructions.

10 As depicted in Figure 11, the "2+1 IgG Crossfab (N-terminal)" construct is more potent in terms of EC₅₀ than the "(scFv)₂" molecule.

In another set of experiments, the CD3-MCSP "2+1 IgG Crossfab (N-terminal), linked light chain" (see SEQ ID NOs 1, 4, 5 and 85) was compared to the CD3-MCSP "2+1 IgG Crossfab (N-terminal)" (see SEQ ID NOs 1, 3, 4 and 5). Briefly, target cells (human Colo-38, human MV-3 or WM266-4 melanoma cells) were harvested with Cell Dissociation Buffer on the day of the assay (or with trypsin one day before the assay was started), washed and resuspended in the appropriate cell culture medium (RPMI1640, including 2% FCS and 1% Glutamax). 20 000 - 30 000 cells per well were plated in a flat-bottom 96-well plate and the respective antibody dilution was added as indicated (triplicates). PBMCs as effector cells were added to obtain a final effector-to-target cell (E:T) ratio of 10:1. All constructs and controls were adjusted to the same molarity, incubation time was 22 h. Detection of LDH release and normalization was done as described above.

Figure 15 to 18 show the result of four assays performed with MV-3 melanoma cells (Figure 15), Colo-38 cells (Figure 16 and 17) or WM266-4 cells (Figure 18). As shown in Figure 15, the construct with the linked light chain was less potent compared to the one without the linked light chain in the assay with MV-3 cells as target cells. As shown in Figure 16 and 17, the construct with the linked light chain was more potent compared to the one without the linked light chain in the assays with high MCSP expressing Colo-38 cells as target cells. Finally, as shown in Figure 18, there was no significant difference between the two constructs when high MCSP-expressing WM266-4 cells were used as target cells.

In another experiment, two CEA-targeting "2+1 IgG Crossfab (N-terminal), inverted" constructs were compared, wherein in the Crossfab fragment either the V regions (VL/VH, see SEQ ID NOs 3, 8, 9, 10) or the C regions (CL/CH1, see SEQ ID NOs 9, 10, 87, 95) were exchanged. The

assay was performed as described above, using human PBMCs as effector cells and human CEA-expressing target cells. Target cells (MKN-45 or LS-174T tumor cells) were harvested with trypsin-EDTA (LuBiosciences #25300-096), washed and resuspended in RPMI1640 (Invitrogen #42404042), including 1% Glutamax (LuBiosciences #35050087) and 2% FCS. 30 000 cells per well were plated in a round-bottom 96-well plate and the bispecific constructs were added at the indicated concentrations. All constructs and controls were adjusted to the same molarity. Human PBMC effector cells were added to obtain a final E:T ratio of 10:1, incubation time was 28 h. EC50 values were calculated using the GraphPad Prism 5 software.

As shown in Figure 22, the construct with the CL/CH1 exchange shows slightly better activity on both target cell lines than the construct with the VL/VH exchange. Calculated EC50 values were 115 and 243 pM on MKN-45 cells, and 673 and 955 pM on LS-174T cells, for the CL/CH1-exchange construct and the VL/VH-exchange construct, respectively.

Similarly, two MCSP-targeting “2+1 IgG Crossfab (N-terminal)” constructs were compared, wherein in the Crossfab fragment either the V regions (VL/VH, see SEQ ID NOs 3, 91, 92, 93) or the C regions (CL/CH1, see SEQ ID NOs 87, 91, 93, 94) were exchanged. The assay was performed as described above, using human PBMCs as effector cells and human MCSP-expressing target cells. Target cells (WM266-4) were harvested with Cell Dissociation Buffer (LuBiosciences #13151014), washed and resuspended in RPMI1640 (Invitrogen #42404042), including 1% Glutamax (LuBiosciences #35050087) and 2% FCS. 30 000 cells per well were plated in a round-bottom 96-well plate and the constructs were added at the indicated concentrations. All constructs and controls were adjusted to the same molarity. Human PBMC effector cells were added to obtain a final E:T ratio of 10:1, incubation time was 26 h. EC50 values were calculated using the GraphPad Prism 5 software.

As depicted in Figure 23, the two constructs show comparable activity, the construct with the CL/CH1 exchange having a slightly lower EC50 value (12.9 pM for the CL/CH1-exchange construct, compared to 16.8 pM for the VL/VH-exchange construct).

Figure 24 shows the result of a similar assay, performed with human MCSP-expressing MV-3 target cells. Again, both constructs show comparable activity, the construct with the CL/CH1 exchange having a slightly lower EC50 value (approximately 11.7 pM for the CL/CH1-exchange construct, compared to approximately 82.2 pM for the VL/VH-exchange construct). Exact EC50 values could not be calculated, since the killing curves did not reach a plateau at high concentrations of the compounds.

In a further experiment, the CD3-MCSP “2+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4, 5) and “1+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4, 86) constructs were compared to the CD3-MCSP “1+1 CrossMab” (see SEQ ID NOs 4, 87, 88, 89). The assay was performed as described above, using human PBMCs as effector cells and WM266-4 or MV-3 target cells (E:T ratio = 10:1) and an incubation time of 21 h.

As shown in Figure 25, the “2+1 IgG Crossfab (N-terminal)” construct is the most potent molecule in this assay, followed by the “1+1 IgG Crossfab (N-terminal)” and the “1+1 CrossMab”. This ranking is even more pronounced with MV-3 cells, expressing medium levels of MCSP, compared to high MCSP expressing WM266-4 cells. The calculated EC50 values on MV-3 cells were 9.2, 40.9 and 88.4 pM, on WM266-4 cells 33.1, 28.4 and 53.9 pM, for the “2+1 IgG Crossfab (N-terminal)”, the “1+1 IgG Crossfab (N-terminal)” and the “1+1 CrossMab”, respectively.

Example 5

15 Binding of bispecific constructs to the respective target antigen on cells

Binding of the different bispecific constructs to CD3 on Jurkat (ATCC #TIB-152) cells, and the respective tumor antigen MCSP on WM266-4 cells or CEA on LS174-T cells, was determined by FACS. Briefly, cells were harvested, counted and checked for viability. 0.15 – 0.2 million cells per well were plated in a round-bottom 96-well plate and incubated with the indicated concentration of the bispecific constructs and controls for 30 min at 4°C. For a better comparison, the constructs were normalized to same molarity. Cells were washed with PBS containing 0.1% BSA once. After incubation with a FITC- or PE-conjugated secondary antibody for 30 min at 4°C, bound constructs were detected using a FACSCantoII (Software FACS Diva). A FITC- or PE-conjugated AffiniPure F(ab')₂ Fragment goat anti-human IgG Fcγ Fragment Specific (Jackson Immuno Research Lab # 109-096-098 / working solution 1:20, or #109-116-170 / working solution 1:80, respectively) was used. Unless otherwise indicated, cells were fixed with 100 μl/well fixation buffer (BD #554655) for 15 min at 4°C in the dark, centrifuged for 6 min at 400 x g and kept in 200 μl/well PBS containing 0.1% BSA until analysis. EC50 values were calculated using the GraphPad Prism 5 software.

30 Figure 26 shows the binding of CD3/CEA “2+1 IgG Crossfab (N-terminal), inverted” bispecific constructs with either a VL/VH (see SEQ ID NOs 3, 8, 9, 10) or a CL/CH1 exchange (see SEQ ID NOs 9, 10, 87, 95) in the Crossfab fragment to human CD3, expressed by Jurkat cells, or to human CEA, expressed by LS-174T cells. As a control, the equivalent maximum concentration

of the corresponding IgGs and the background staining due to the labeled 2ndary antibody (goat anti-human FITC-conjugated AffiniPure F(ab')₂ Fragment, Fcγ Fragment-specific, Jackson Immuno Research Lab # 109-096-098) were assessed as well. Both constructs show good binding to human CEA, as well as to human CD3 on cells. The calculated EC₅₀ values were 4.6 and 3.9 nM (CD3), and 9.3 and 6.7 nM (CEA) for the “2+1 IgG Crossfab (N-terminal), inverted (VL/VH)” and the “2+1 IgG Crossfab (N-terminal), inverted (CL/CH1)” constructs, respectively. Figure 27 shows the binding of CD3/MCSP “2+1 IgG Crossfab (N-terminal)” (see SEQ ID NOs 1, 3, 4, 5) and “2+1 IgG Crossfab (N-terminal), inverted” (see SEQ ID NOs 4, 87, 89, 90) constructs to human CD3, expressed by Jurkat cells, or to human MCSP, expressed by WM266-4 cells. While binding of both construct to MCSP on cells was comparably good, the binding of the “inverted” construct to CD3 was reduced compared to the other construct. The calculated EC₅₀ values were 6.1 and 1.66 nM (CD3), and 0.57 and 0.95 nM (MCSP) for the “2+1 IgG Crossfab (N-terminal), inverted” and the “2+1 IgG Crossfab (N-terminal)” constructs, respectively.

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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.

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Claims

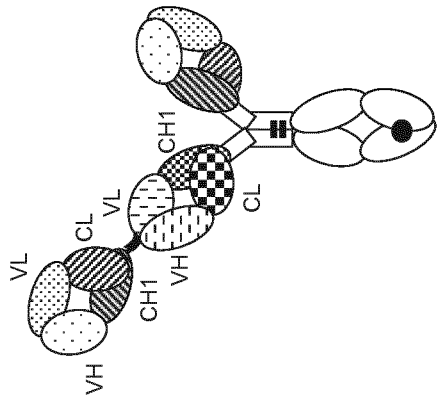
1. A bispecific antigen binding molecule, comprising a first Fab fragment which specifically binds to a first antigen, a second Fab fragment which specifically binds to a second antigen,
5 and an Fc domain composed of a first and a second subunit capable of stable association;
wherein
 - a) the bispecific antigen binding molecule provides monovalent binding to the first and/or the second antigen,
 - b) the first Fab fragment, the second Fab fragment and the first Fc domain subunit are fused
10 to each other, and
 - c) in the first and/or the second Fab fragment one of the following replacements is made: (i) the variable domains VL and VH are replaced by each other, (ii) the constant domains CL and CH1 are replaced by each other, or (iii) both the variable and constant domains VL-CL and VH-CH1 are replaced by each other,
15 provided that not the same replacement is made in the first and the second Fab fragment.
2. The bispecific antigen binding molecule of claim 1, wherein the first Fab fragment is fused at its C-terminus to the N-terminus of the second Fab fragment, which is in turn fused at its C-terminus to the N-terminus of the first Fc domain subunit.
3. The bispecific antigen binding molecule of claim 2, wherein the first Fab fragment is fused
20 at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the second Fab fragment, which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit.
4. The bispecific antigen binding molecule of claim 1, wherein the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fab fragment, which is in turn fused at
25 its C-terminus to the N-terminus of the first Fc domain subunit.
5. The bispecific antigen binding molecule of claim 4, wherein the second Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the heavy chain of the first Fab fragment, which is in turn fused at the C-terminus of its heavy chain to the N-terminus of the first Fc domain subunit.
- 30 6. The bispecific antigen binding molecule of claim 3 or 5, wherein additionally the Fab light chain of the first Fab fragment and the Fab light chain of the second Fab fragment are fused to each other, optionally via a peptide linker.

7. The bispecific antigen binding molecule of claim 1, wherein the second Fab fragment is fused at its C-terminus to the N-terminus of the first Fc domain subunit, which is in turn fused at its C-terminus to the N-terminus of the first Fab fragment.
8. The bispecific antigen binding molecule of any one of the preceding claims, wherein the replacement is made in the first Fab fragment.
9. The bispecific antigen binding molecule of any one of the preceding claims, wherein the replacement is a replacement of the variable domains VL and VH by each other.
10. The bispecific antigen binding molecule of any one of the preceding claims, wherein the replacement is a replacement of the constant domains CL and CH1 by each other.
- 10 11. The bispecific antigen binding molecule of any one of the preceding claims, essentially consisting of the first Fab fragment, the second Fab fragment, the Fc domain, and optionally one or more peptide linkers.
12. The bispecific antigen binding molecule of any one of claims 1 to 10, comprising a third Fab fragment which specifically binds to the first or the second antigen.
- 15 13. The bispecific antigen binding molecule of claim 12, wherein the third Fab fragment is fused to the second Fc domain subunit.
14. The bispecific antigen binding molecule of claim 12 or 13, wherein the third Fab fragment is fused at its C-terminus to the N-terminus of the second Fc domain subunit.
15. The bispecific antigen binding molecule of claim 14, wherein the third Fab fragment is fused at the C-terminus of its heavy chain to the N-terminus of the second Fc domain subunit.
- 20 16. The bispecific antigen binding molecule of any one of claims 12 to 15, wherein the third Fab fragment specifically binds to the second antigen.
17. The bispecific antigen binding molecule of any one of claims 12 to 16, wherein the second Fab fragment, the third Fab fragment and the Fc domain are part of an immunoglobulin molecule.
- 25 18. The bispecific antigen binding molecule of claim 17, wherein the immunoglobulin molecule is an IgG class immunoglobulin molecule.
19. The bispecific antigen binding molecule of claim 17 or 18, wherein the immunoglobulin molecule is an IgG1 or IgG4 subclass immunoglobulin molecule.
- 30 20. The bispecific antigen binding molecule of any one of claims 17 to 19, wherein the immunoglobulin molecule is a human immunoglobulin molecule.
21. The bispecific antigen binding molecule of any one of claims 12 to 20, essentially consisting of a first Fab fragment which specifically binds to the first antigen, an immunoglobulin

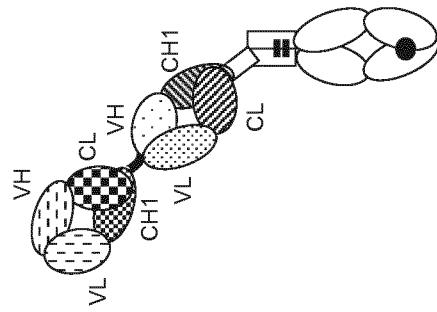
molecule which specifically binds to the second antigen, and optionally one or more peptide linkers.

22. The bispecific antigen binding molecule of any one of the preceding claims, wherein the same replacement is made in Fab fragments that specifically bind to the same antigen.
- 5 23. The bispecific antigen binding molecule of any one of the preceding claims, providing monovalent binding to the first antigen.
24. The bispecific antigen binding molecule of any one of the preceding claims, wherein a replacement is made only in the first Fab fragment.
25. The bispecific antigen binding molecule of any one of the preceding claims, not comprising
10 a single chain Fab fragment.
26. The bispecific antigen binding molecule of any one of the preceding claims, wherein the Fc domain comprises a modification promoting the association of the first and second Fc domain subunit.
27. The bispecific antigen binding molecule of claim 26, wherein in the CH3 domain of the first
15 subunit of the Fc domain 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 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,
20 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.
28. The bispecific antigen binding molecule of any one of the preceding claims, wherein the Fc domain is an IgG Fc domain.
29. The bispecific antigen binding molecule of any one of the preceding claims, wherein the Fc
25 domain is an IgG1 or IgG4 Fc domain.
30. The bispecific antigen binding molecule of any one of the preceding claims, wherein the Fc domain is human.
31. The bispecific antigen binding molecule of any one of the preceding claims, wherein the Fc domain is engineered to have altered binding affinity to an Fc receptor and/or altered
30 effector function, as compared to a non-engineered Fc domain.
32. An isolated polynucleotide encoding the bispecific antigen binding molecule of any one of claims 1 to 31 or a fragment thereof.

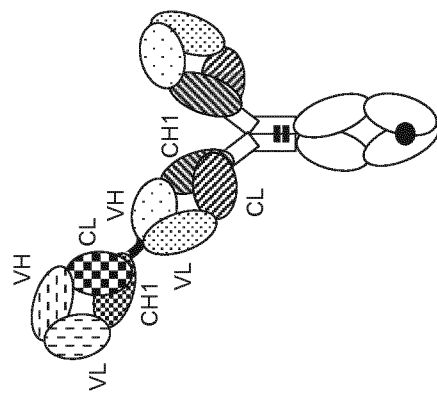
33. An expression vector comprising the isolated polynucleotide of claim 32.
34. A host cell comprising the isolated polynucleotide of claim 32 or the expression vector of claim 33.
- 5 35. A method for producing the bispecific antigen binding molecule of any one of claims 1 to 31, comprising the steps of a) culturing the host cell of claim 30 under conditions suitable for the expression of the bispecific antigen binding molecule and b) recovering the bispecific antigen binding molecule.
36. A pharmaceutical composition comprising the bispecific antigen binding molecule of any one of claims 1 to 31 and a pharmaceutically acceptable carrier.
- 10 37. The bispecific antigen binding molecule of any one of claims 1 to 31 or the pharmaceutical composition of claim 36 for use as a medicament.
38. The bispecific antigen binding molecule of any one of claims 1 to 31 or the pharmaceutical composition of claim 36 for use in the treatment of a disease in an individual in need thereof.
- 15 39. The bispecific antigen binding molecule or the pharmaceutical composition of claim 38, wherein the disease is cancer.
40. Use of the bispecific antigen binding molecule of any one of claims 1 to 31 for the manufacture of a medicament for the treatment of a disease in an individual in need thereof.
- 20 41. A method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the bispecific antigen binding molecule of any one of claims 1 to 31 in a pharmaceutically acceptable form.
42. The use of claim 40 or the method of claim 41, wherein said disease is cancer.
43. The invention as described hereinbefore.



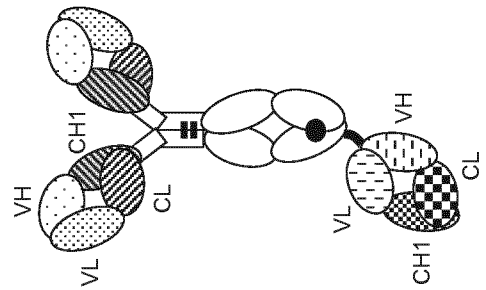
A



B



C



D

Figure 1

Figure 2

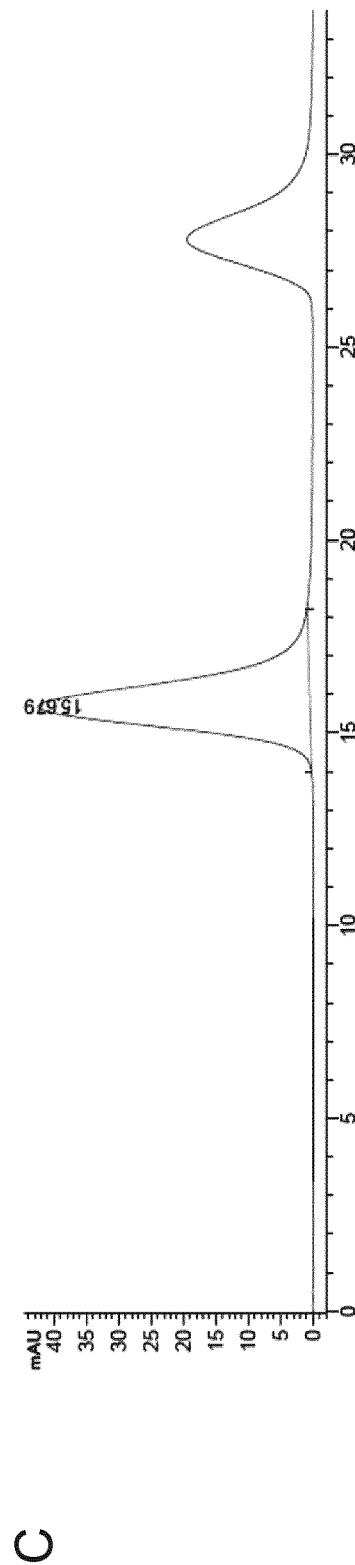
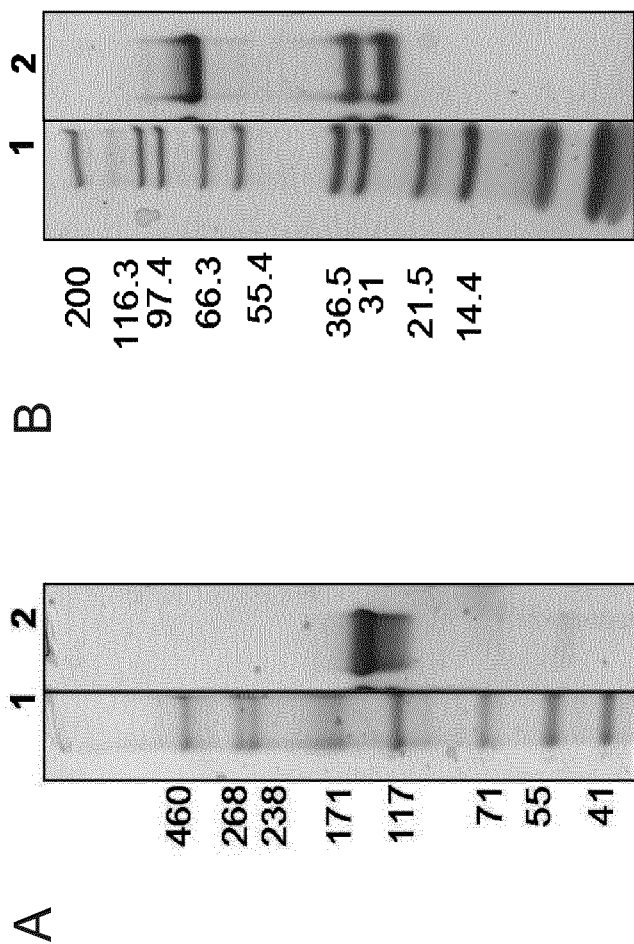


Figure 3

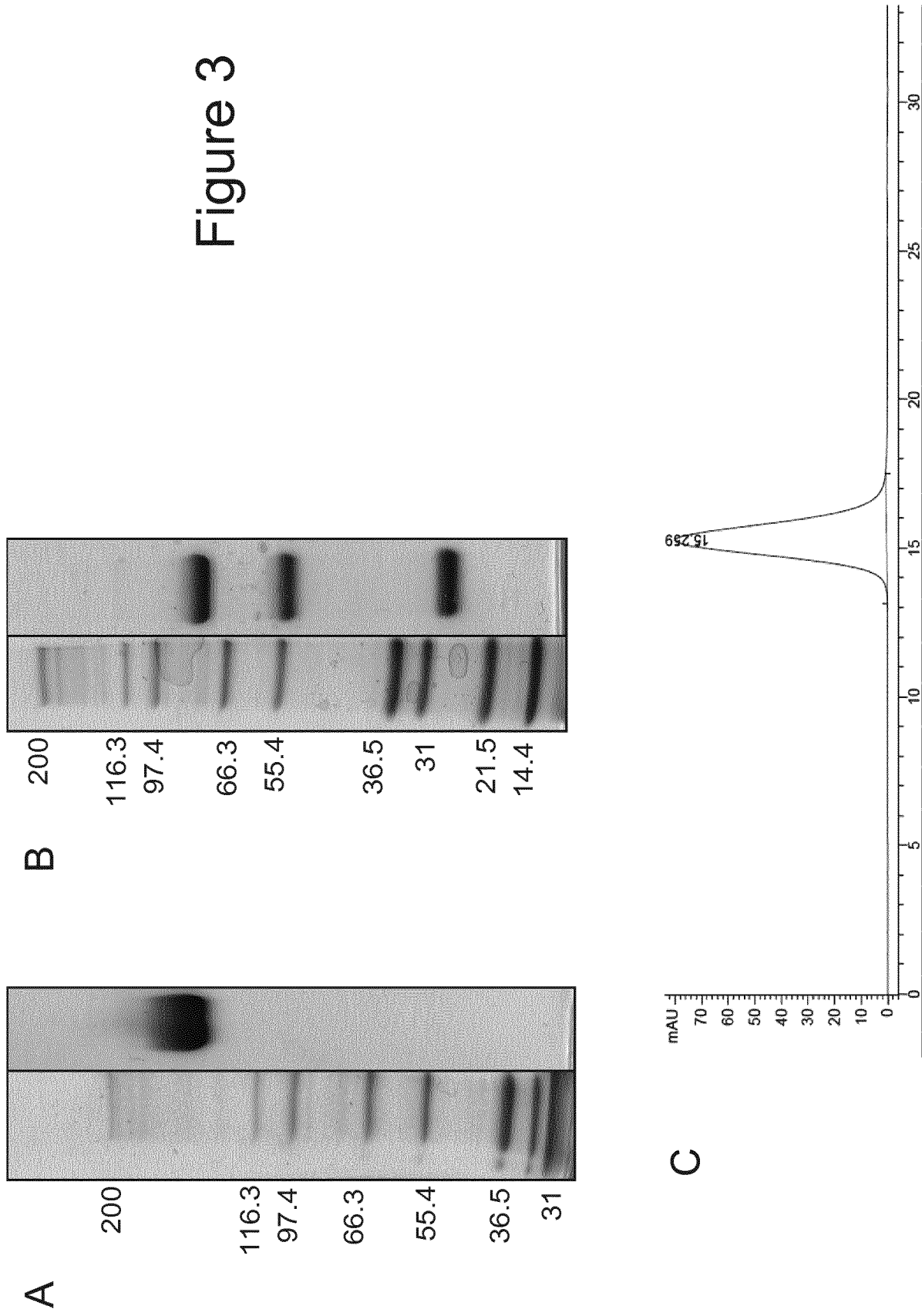
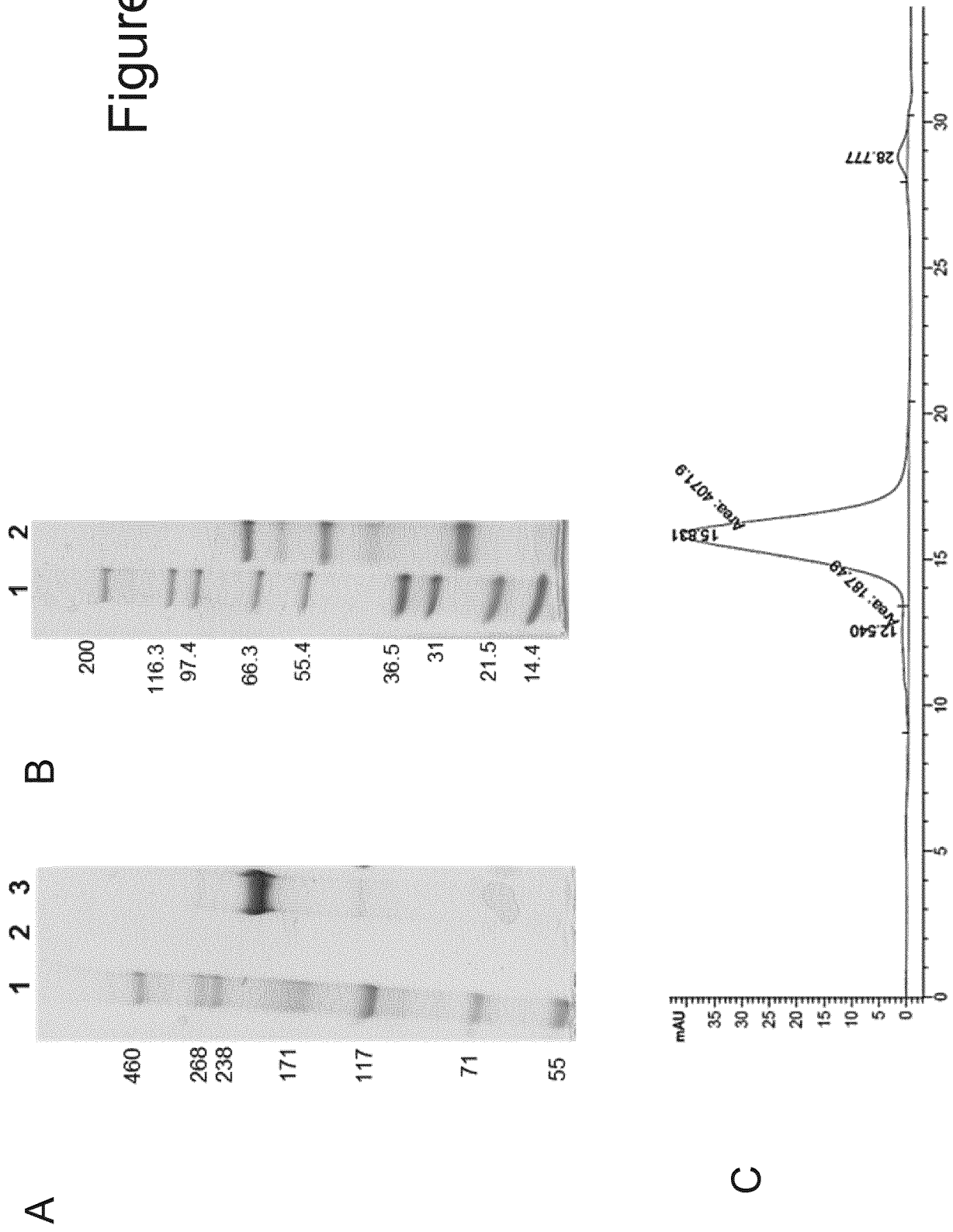
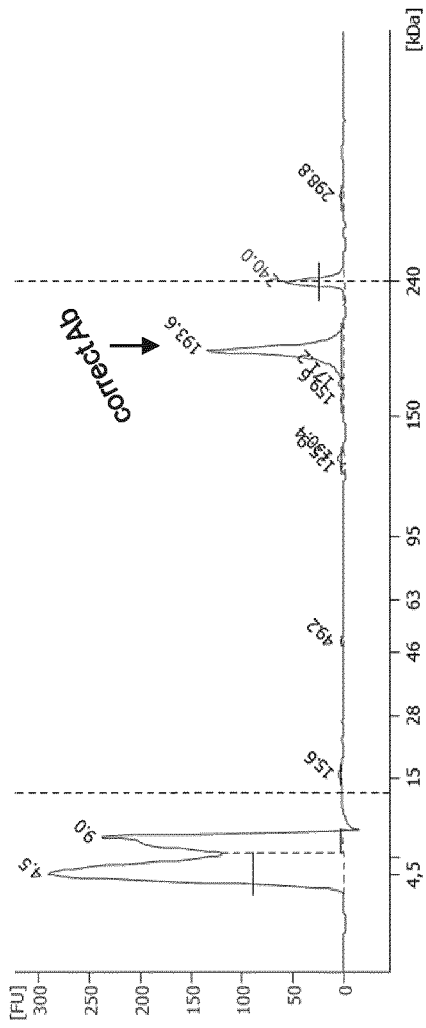


Figure 4



A



B

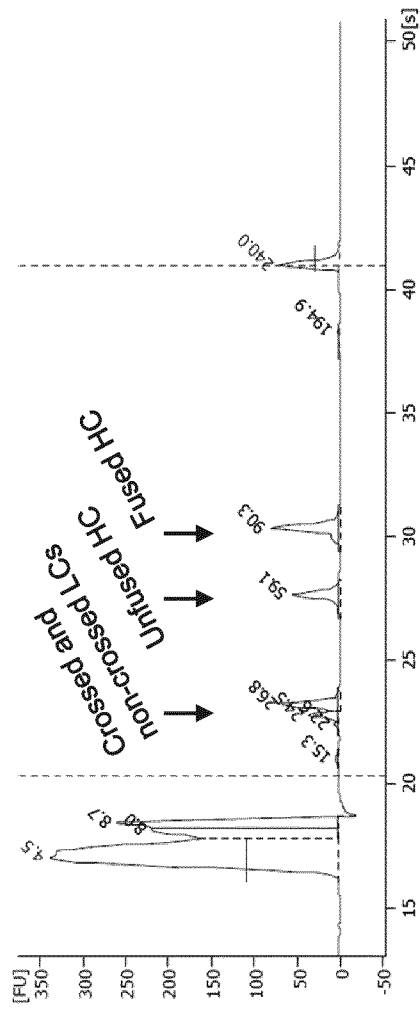
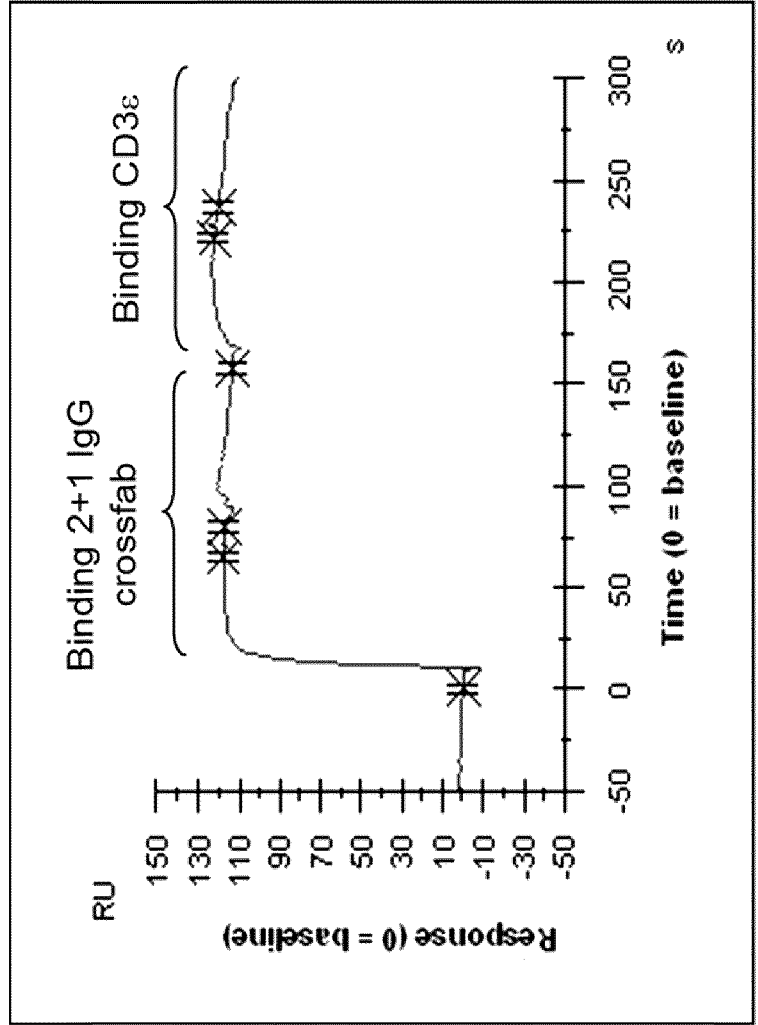
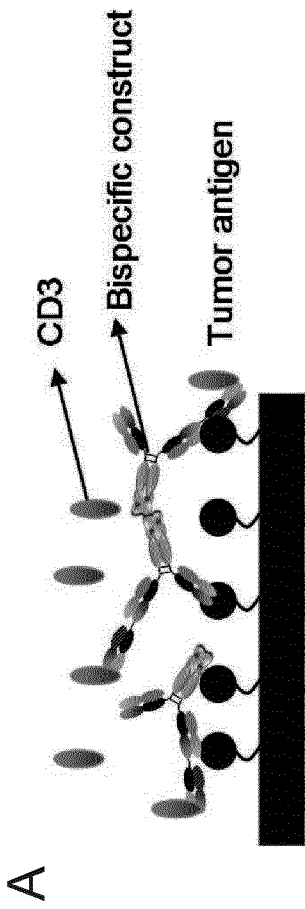


Figure 5

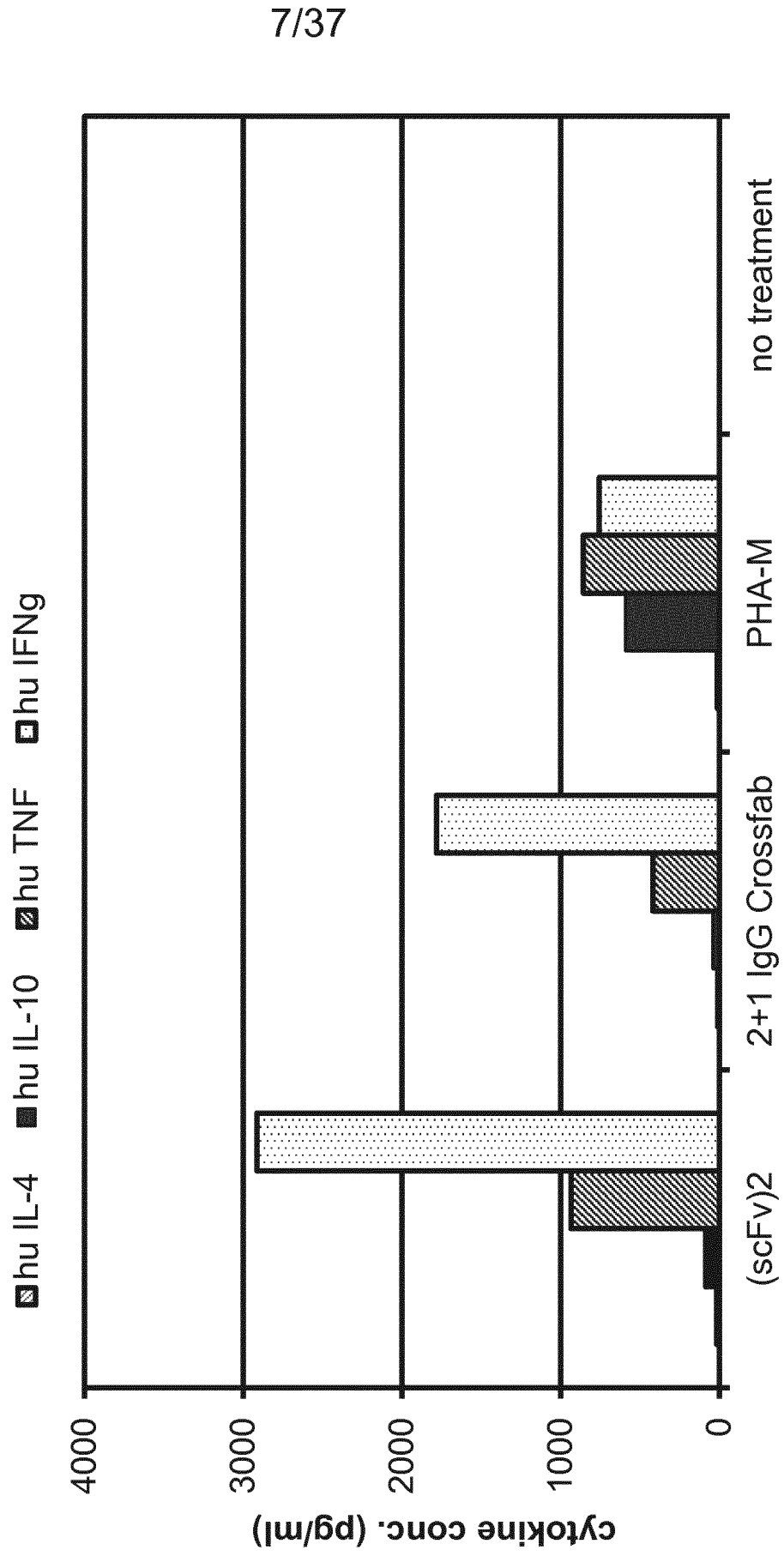
Figure 6



B

Figure 7

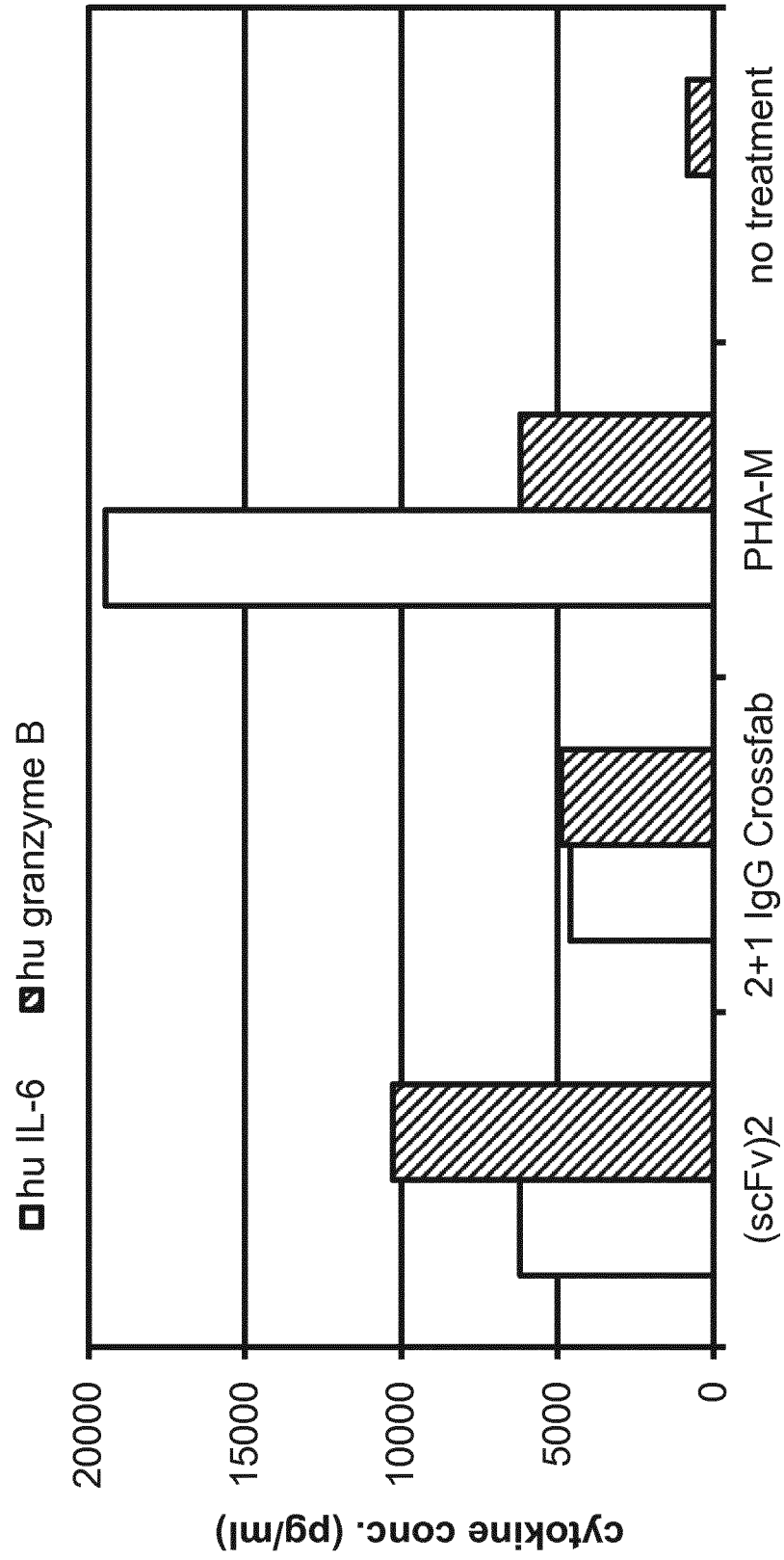
A



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Figure 7

B



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Figure 7

C

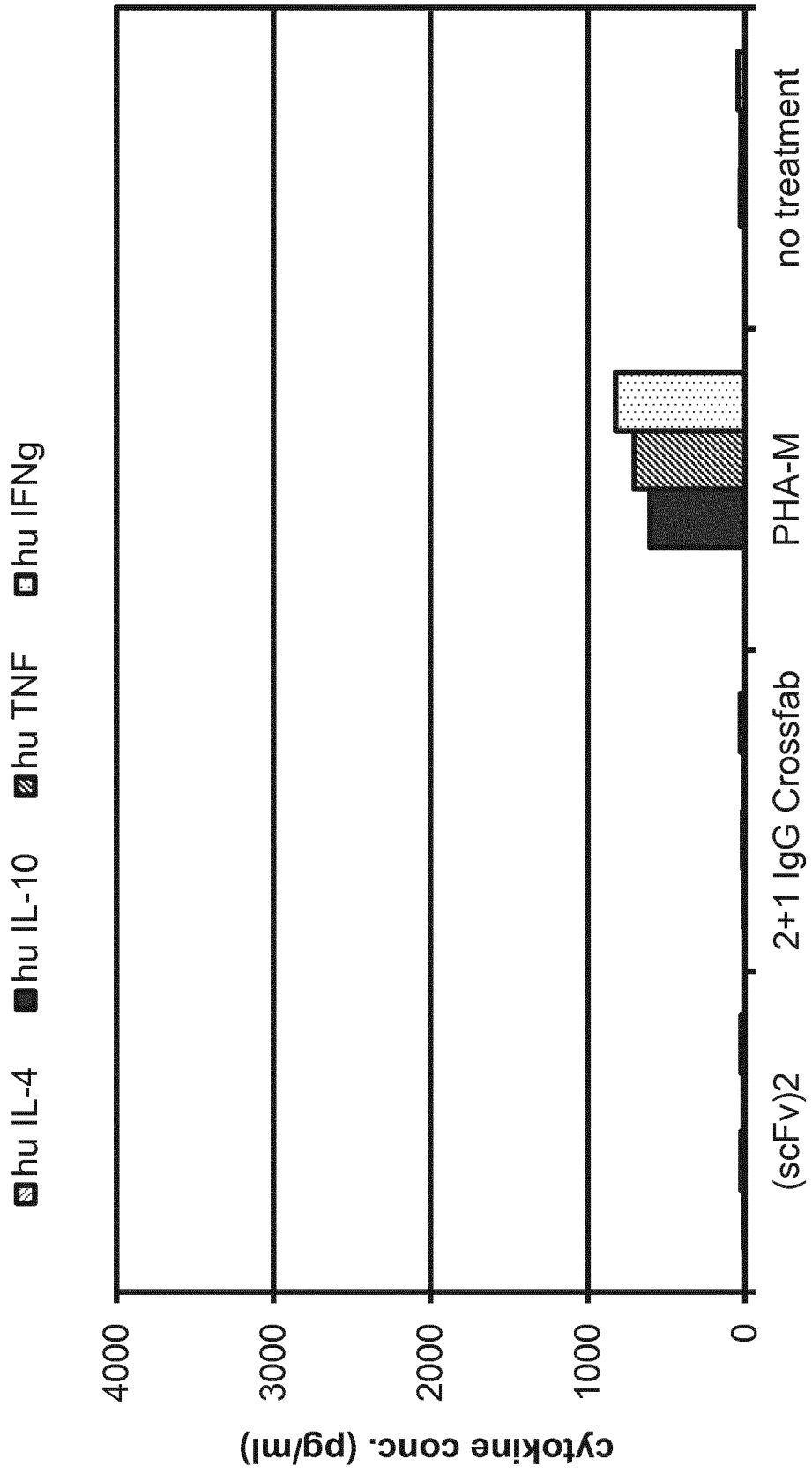


Figure 7

D

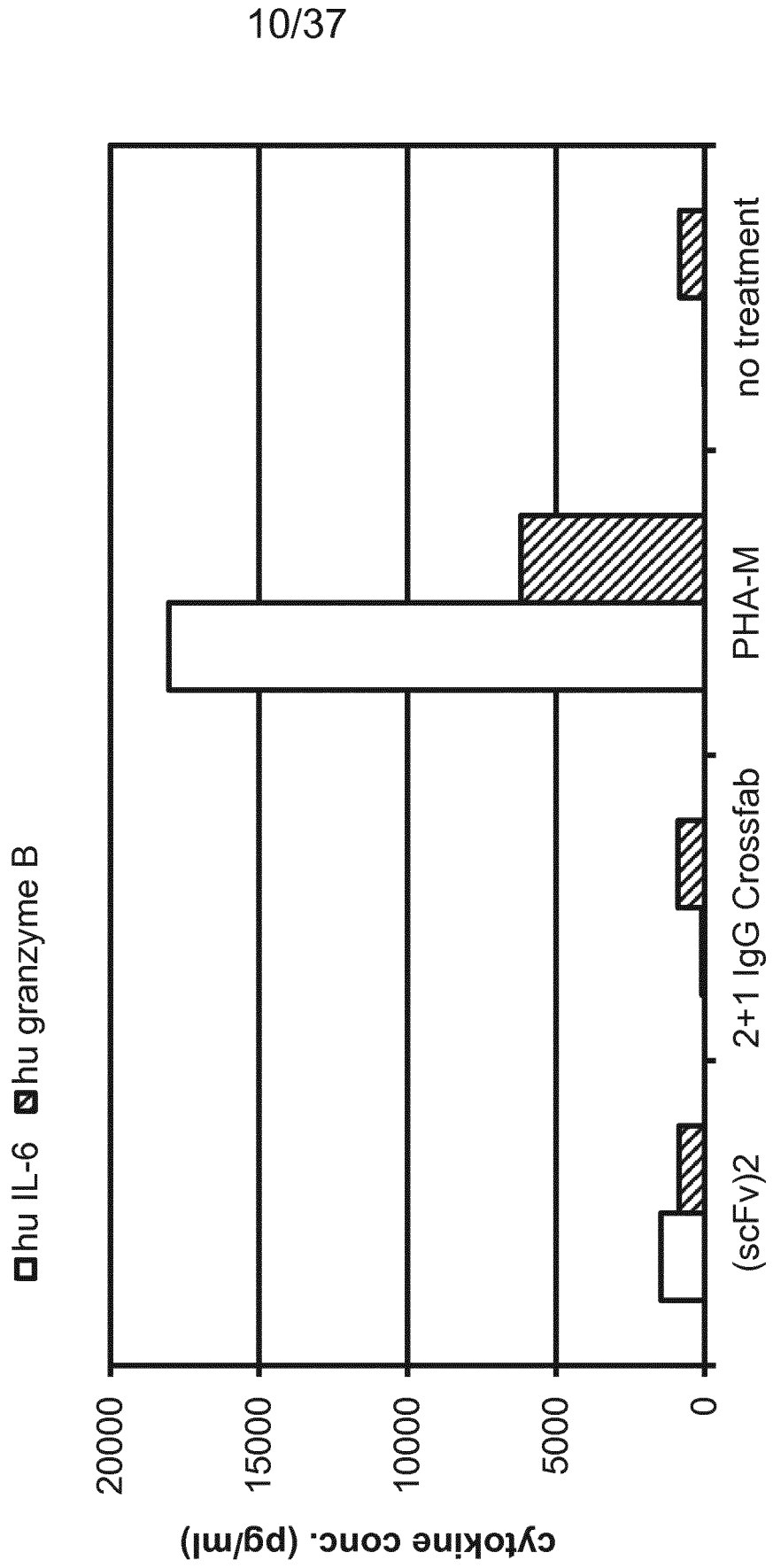
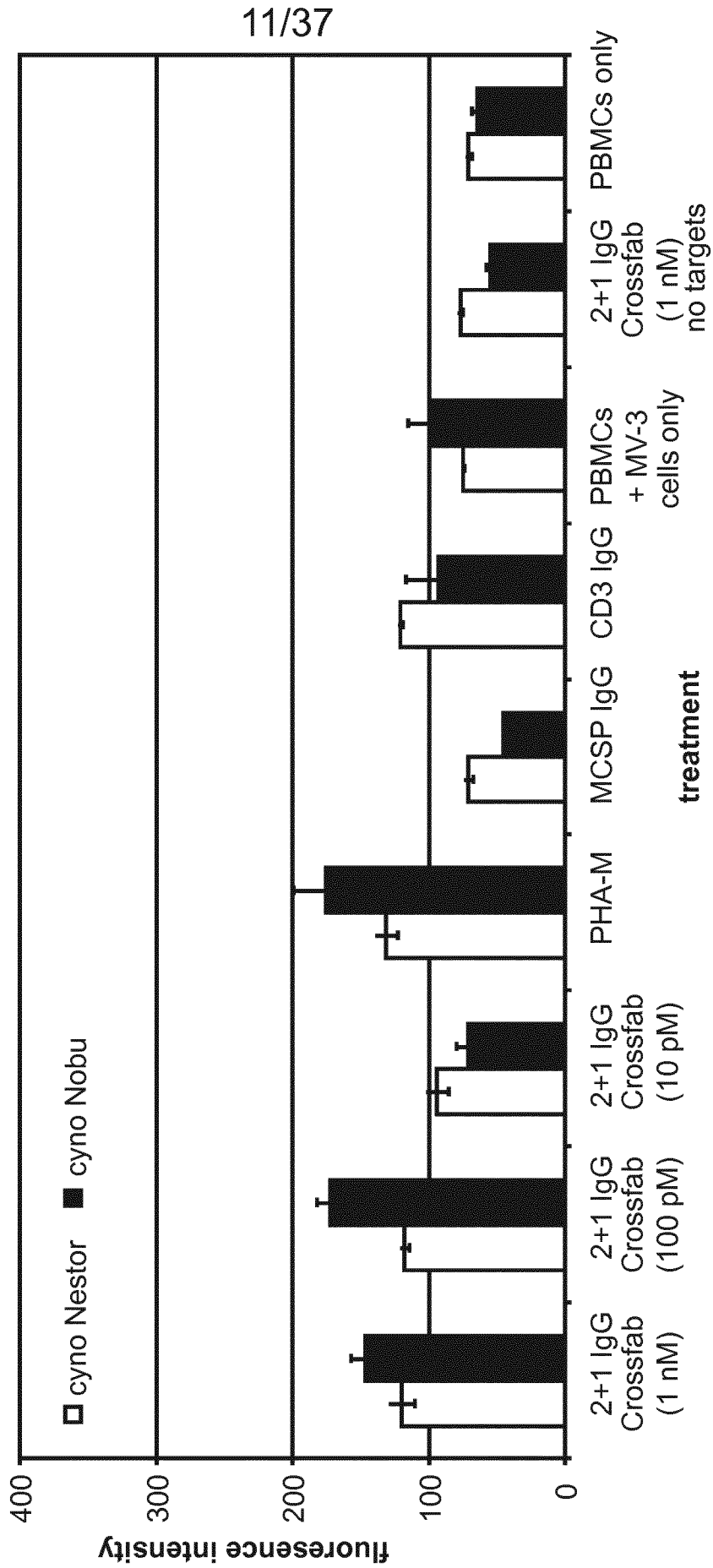


Figure 8



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Figure 9

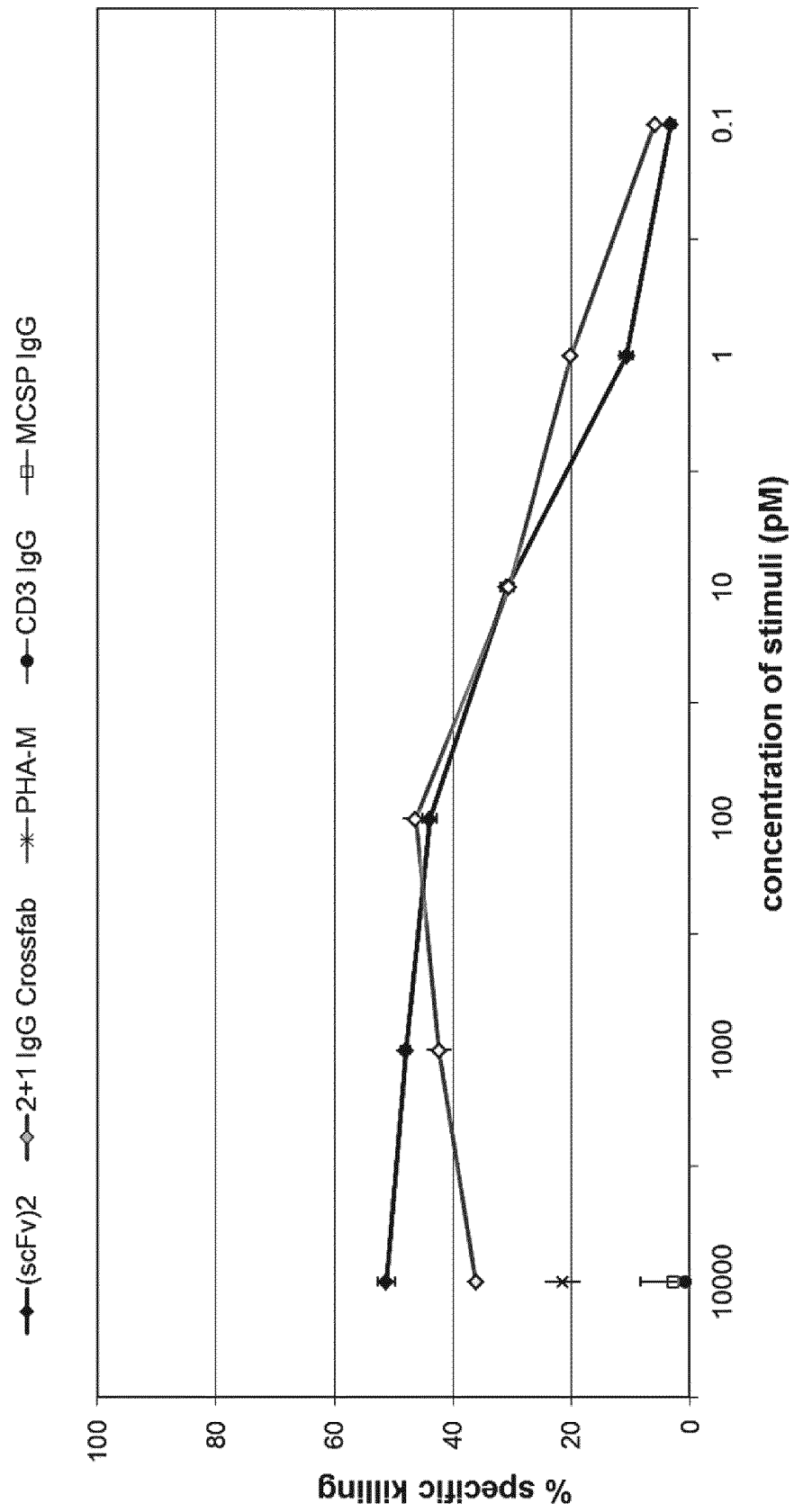
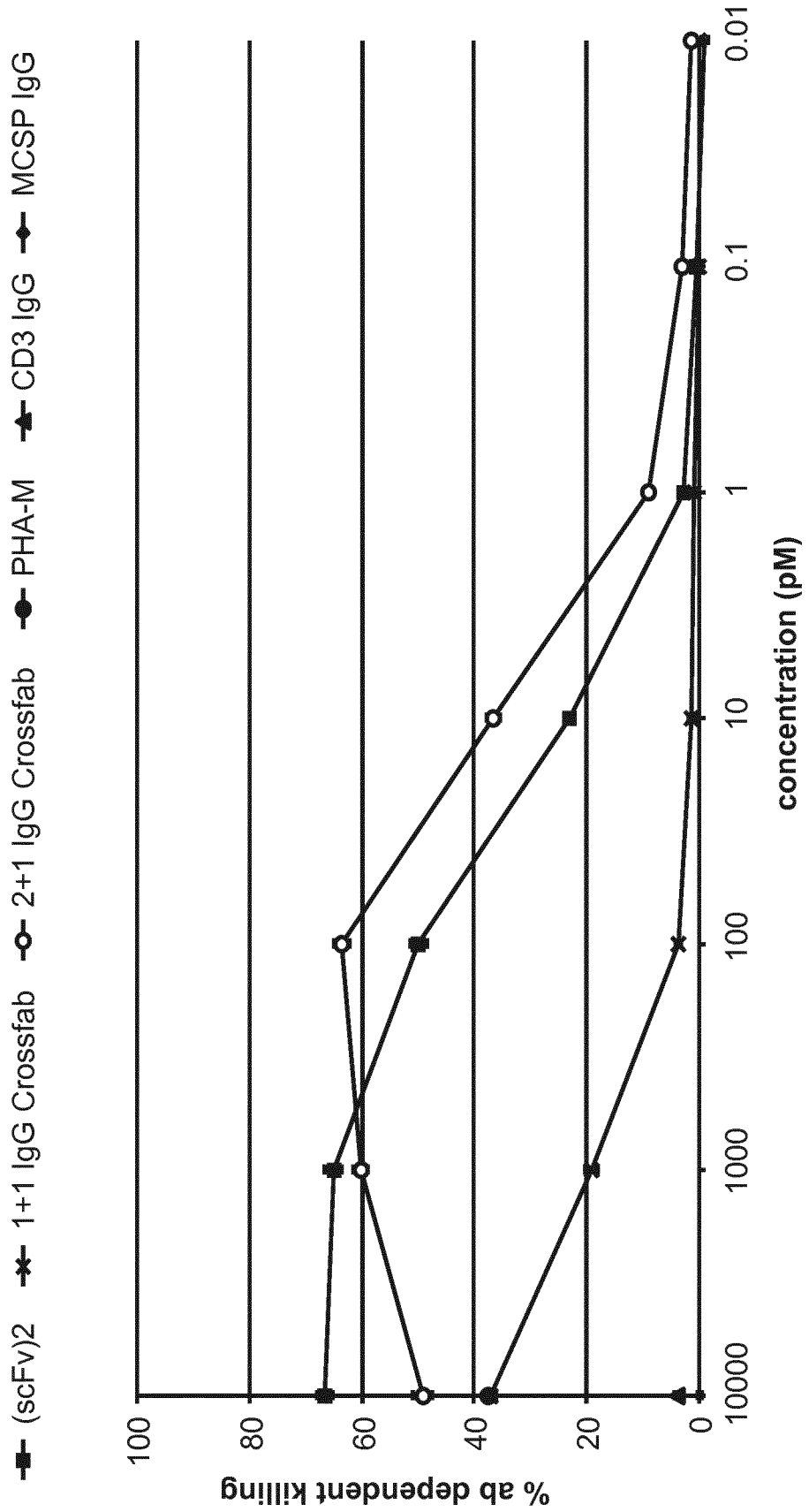
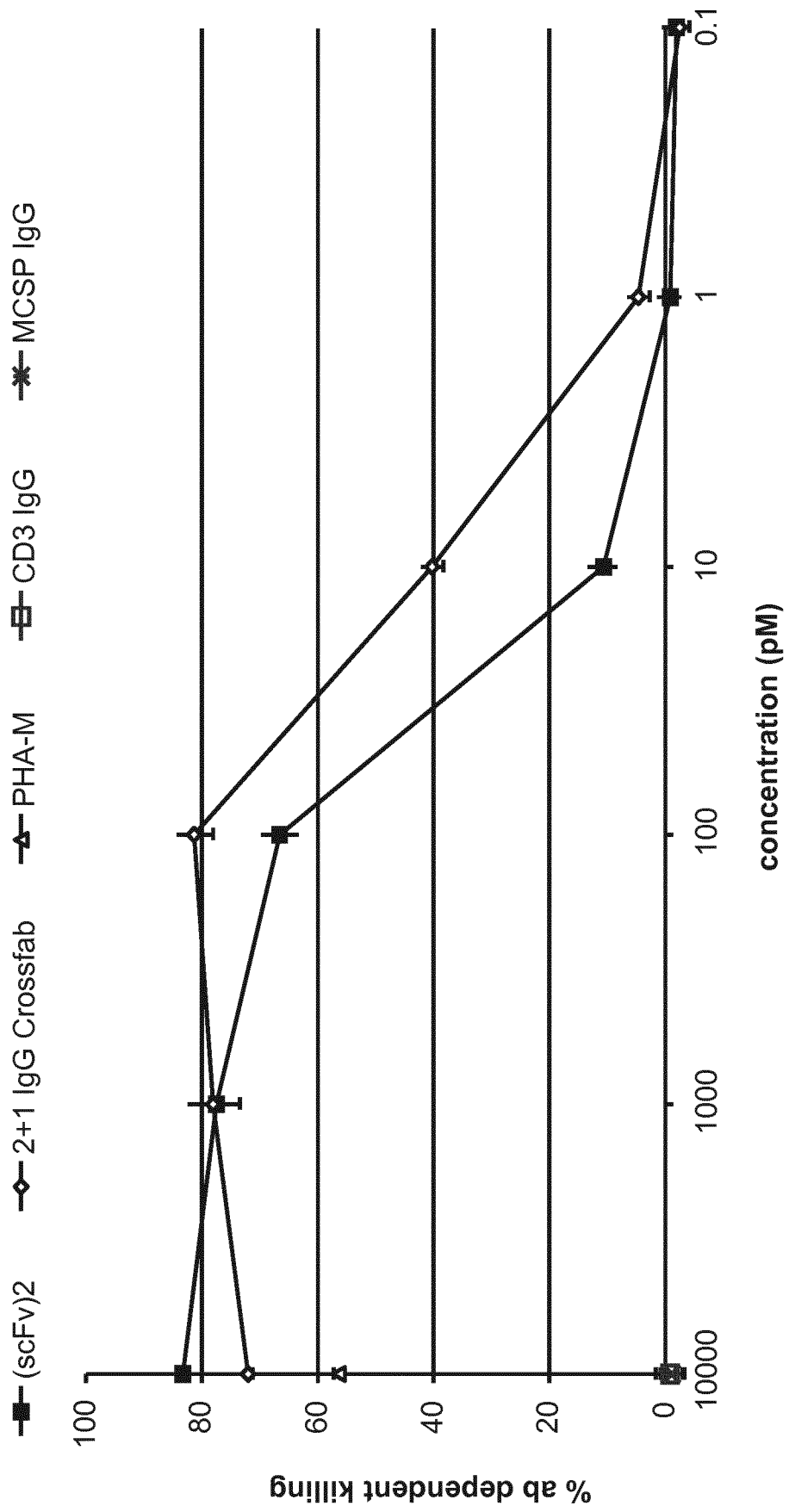


Figure 10



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Figure 11



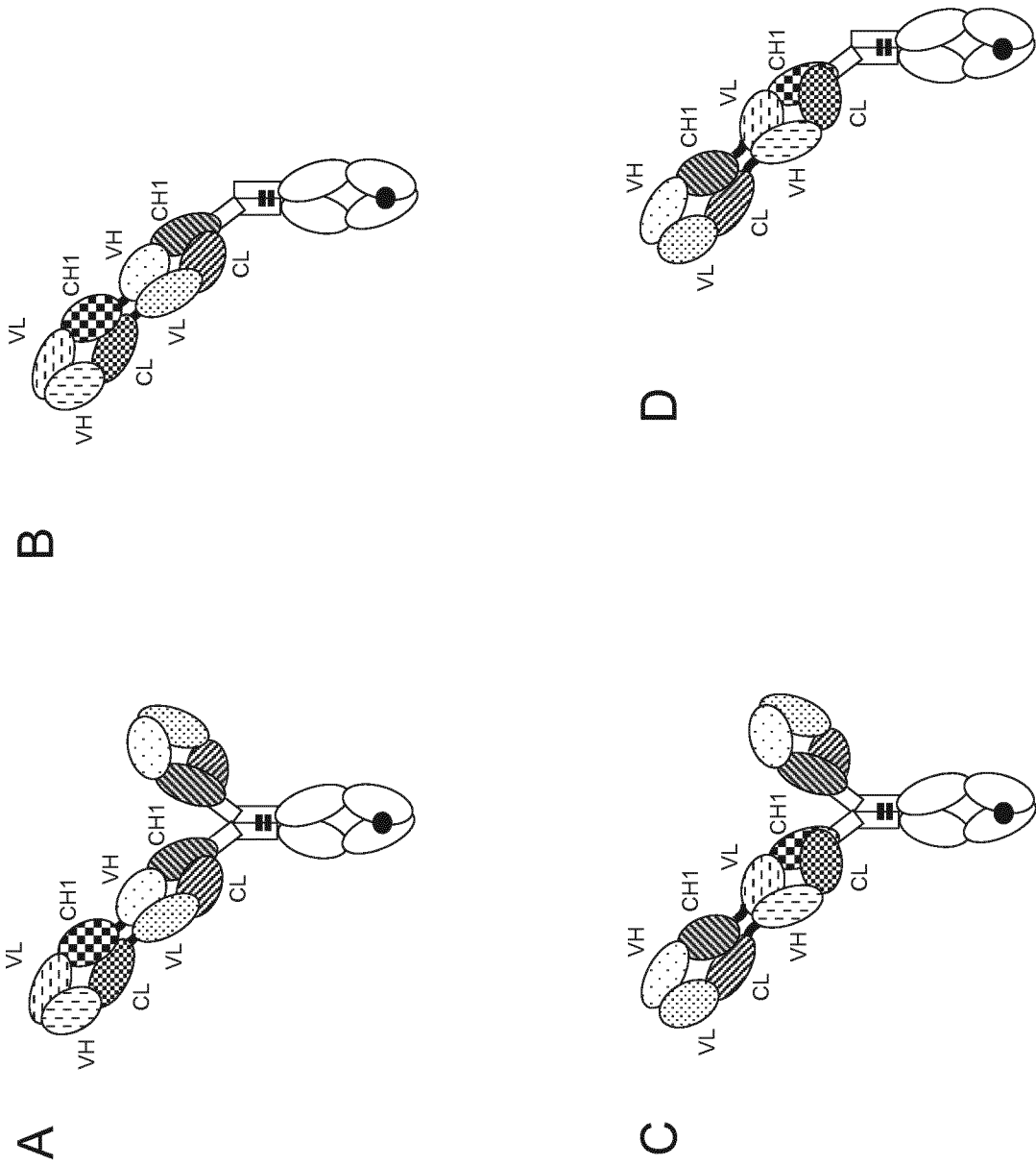


Figure 12

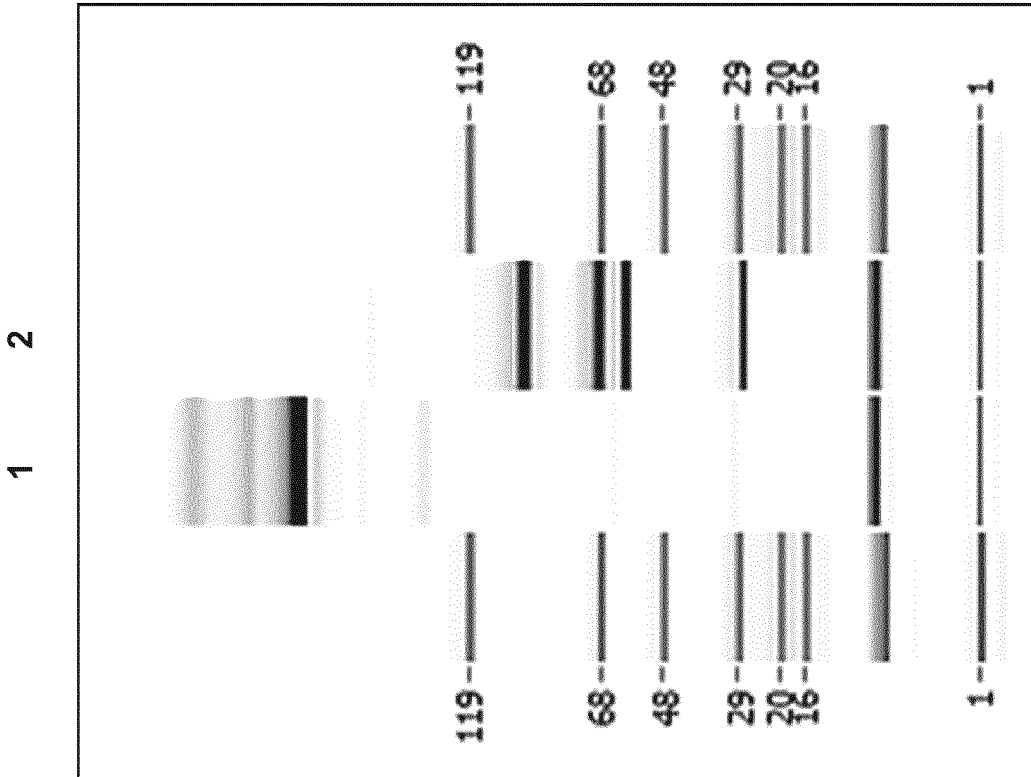
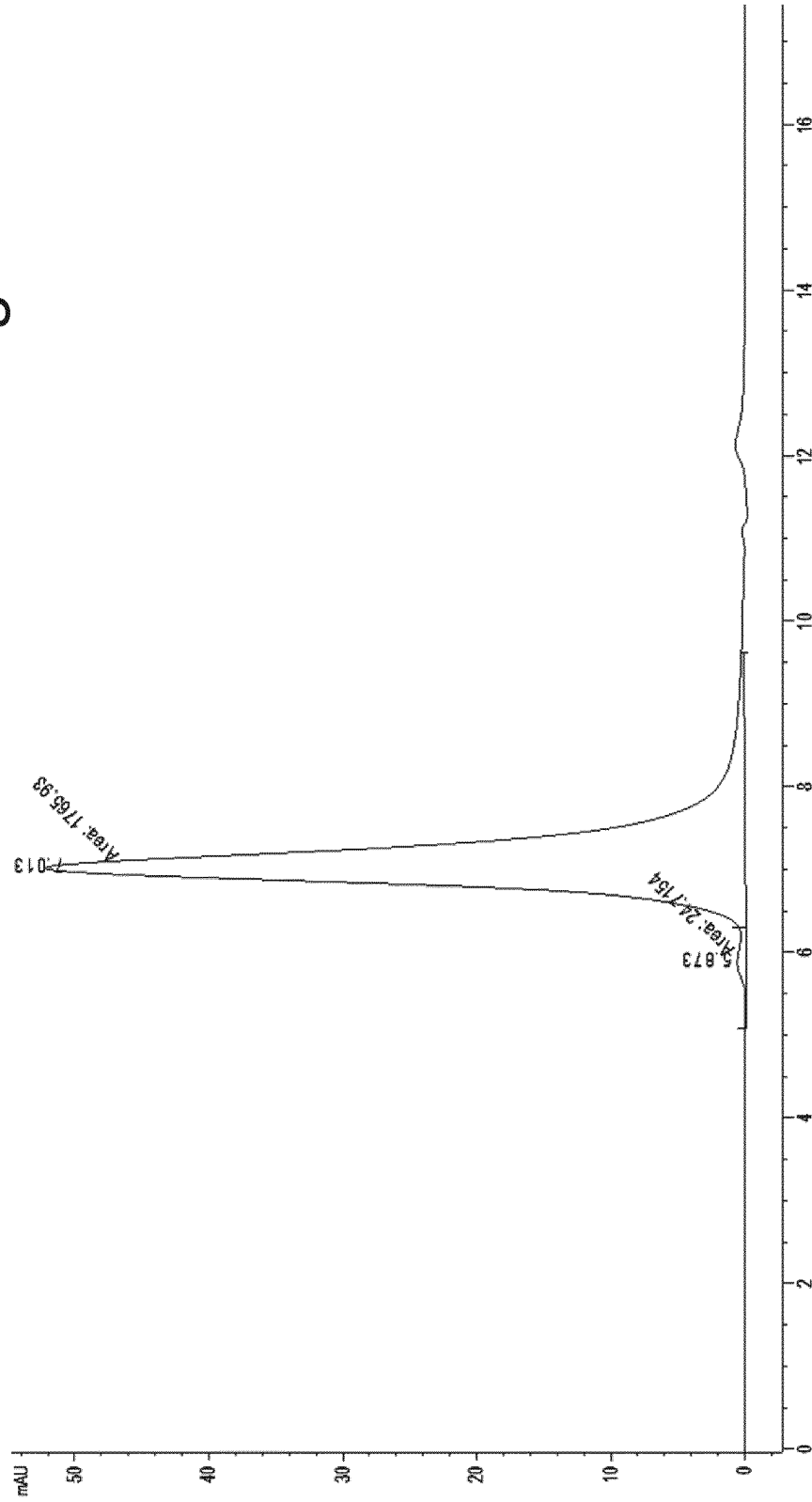


Figure 13

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Figure 14



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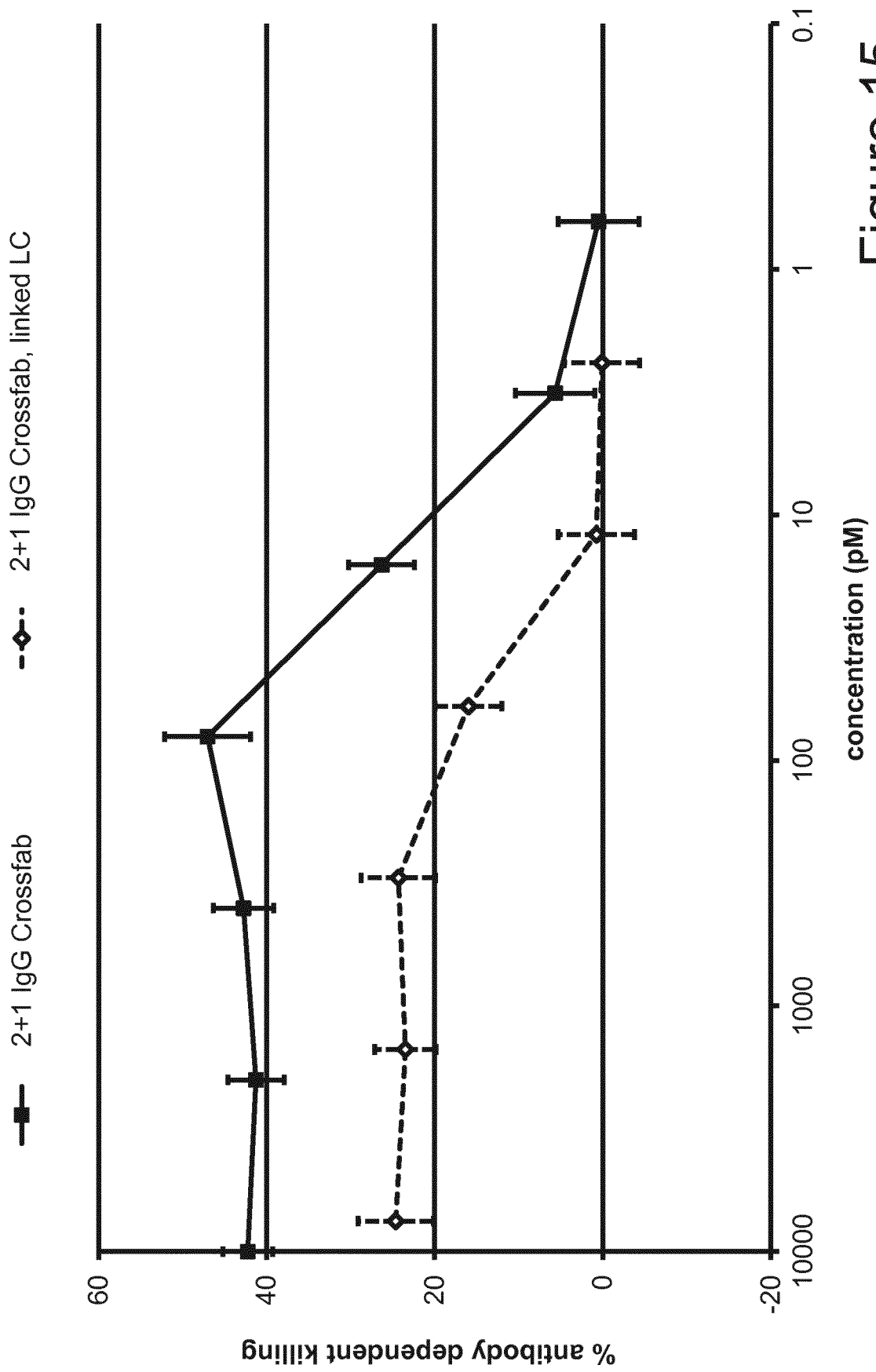


Figure 15

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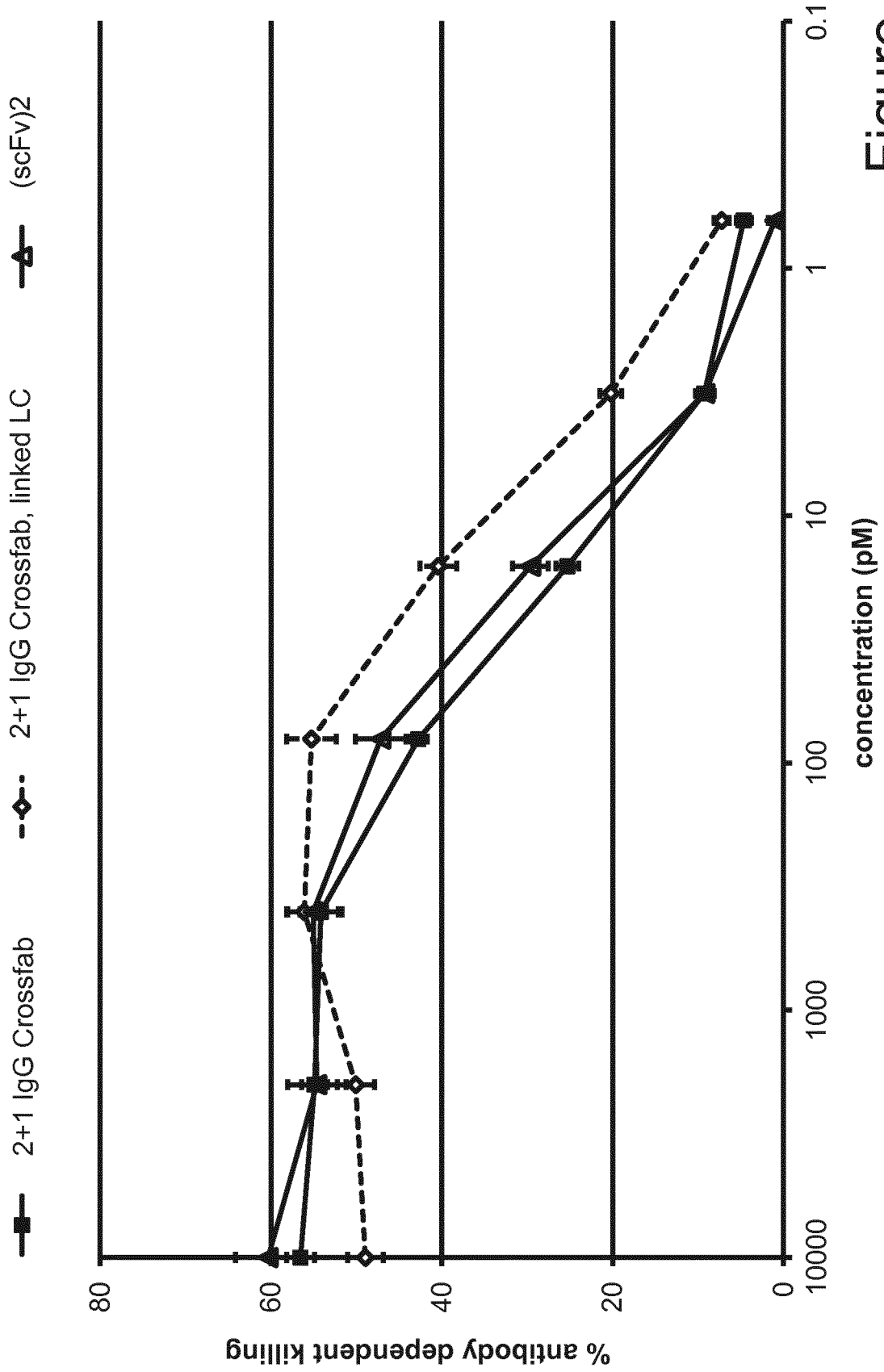


Figure 16

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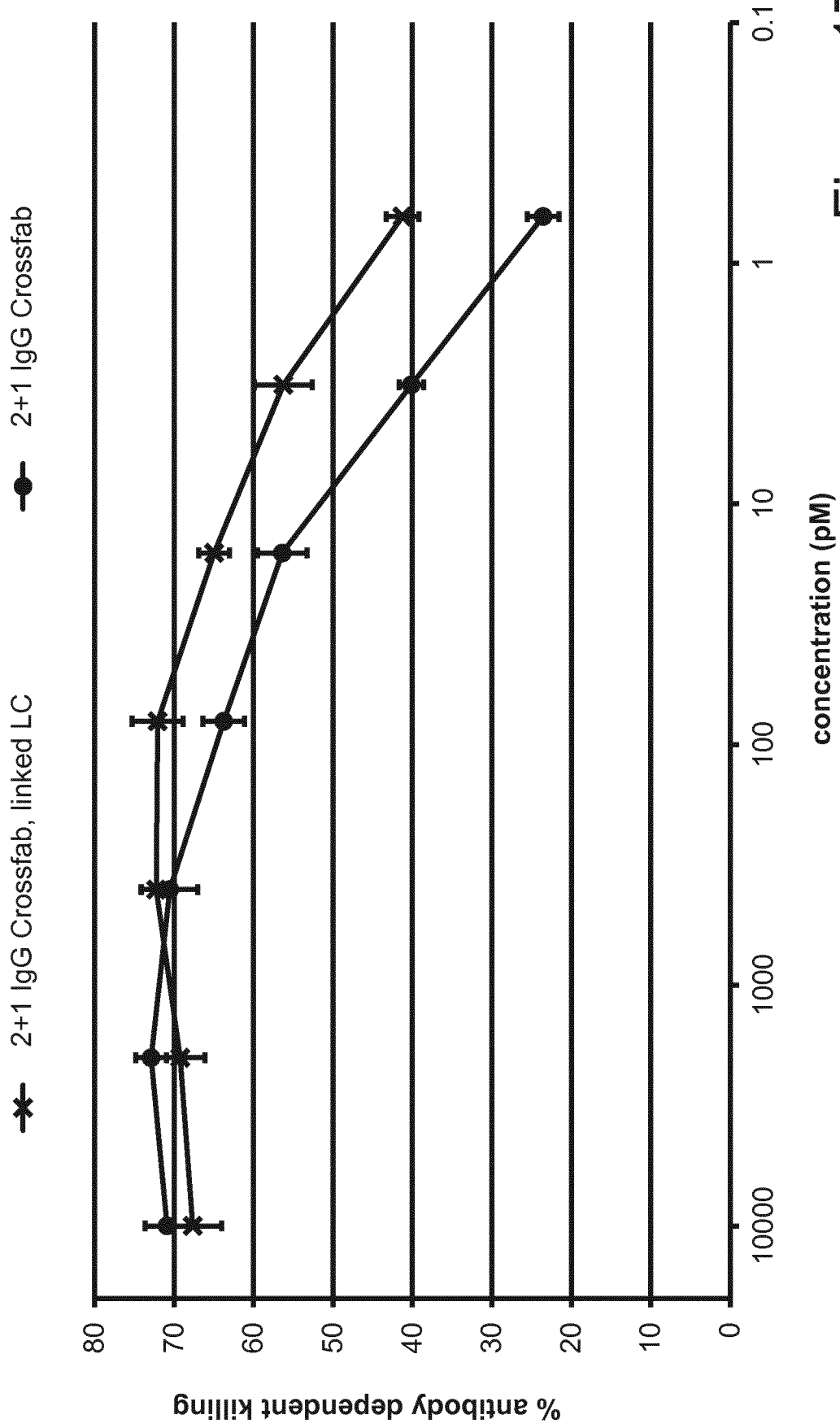


Figure 17

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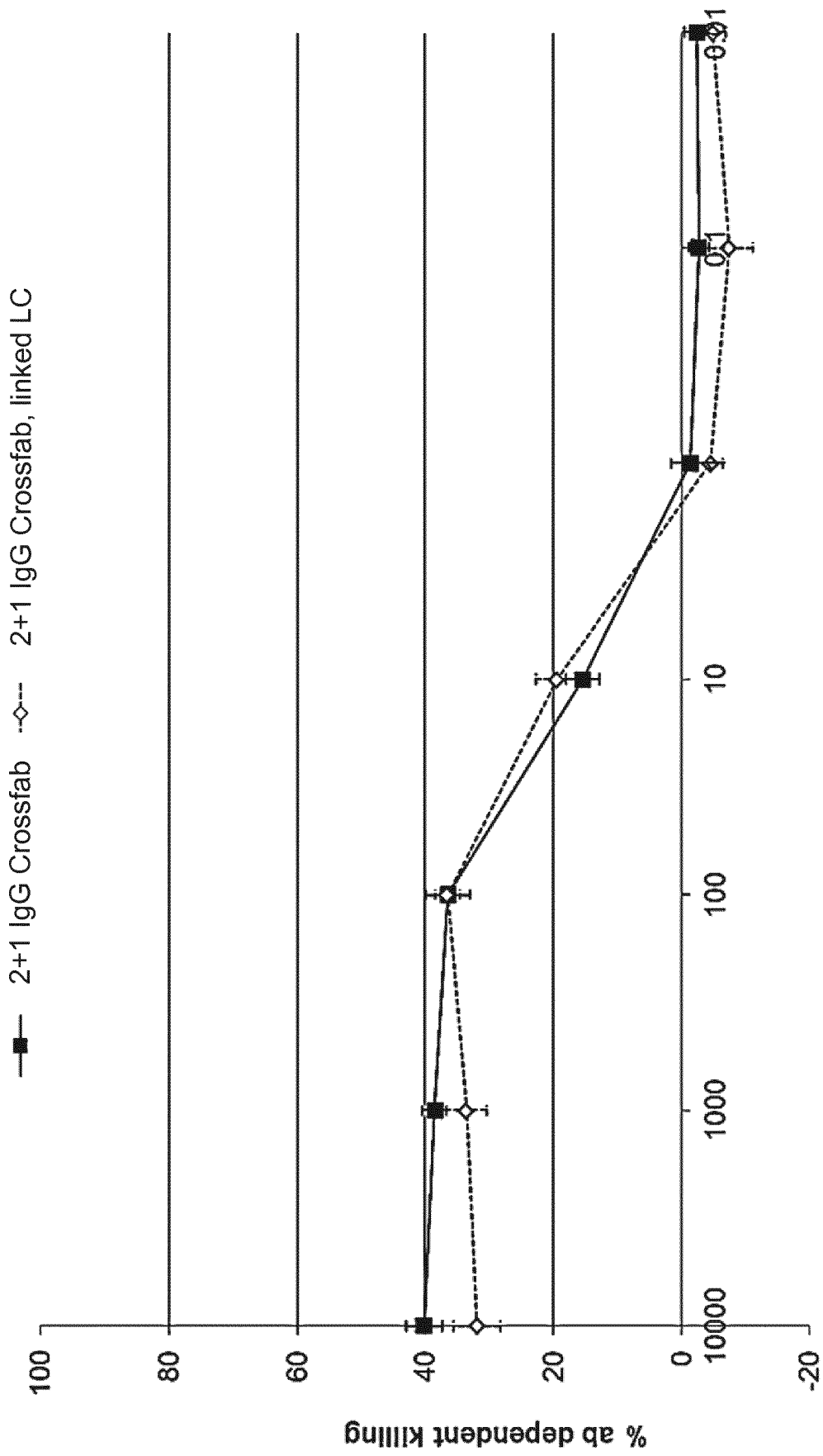


Figure 18

final antibody concentration (pM)

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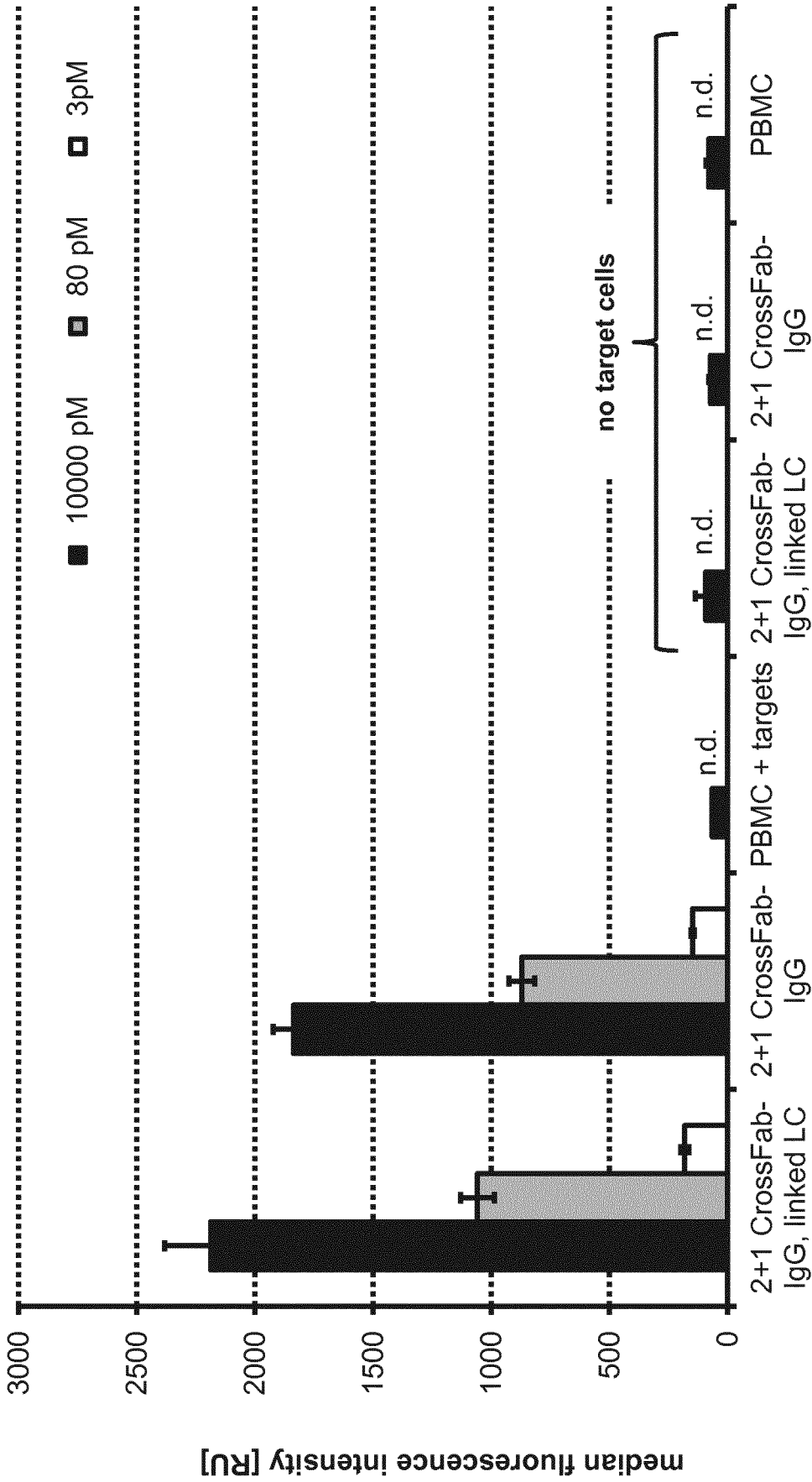


Figure 19A

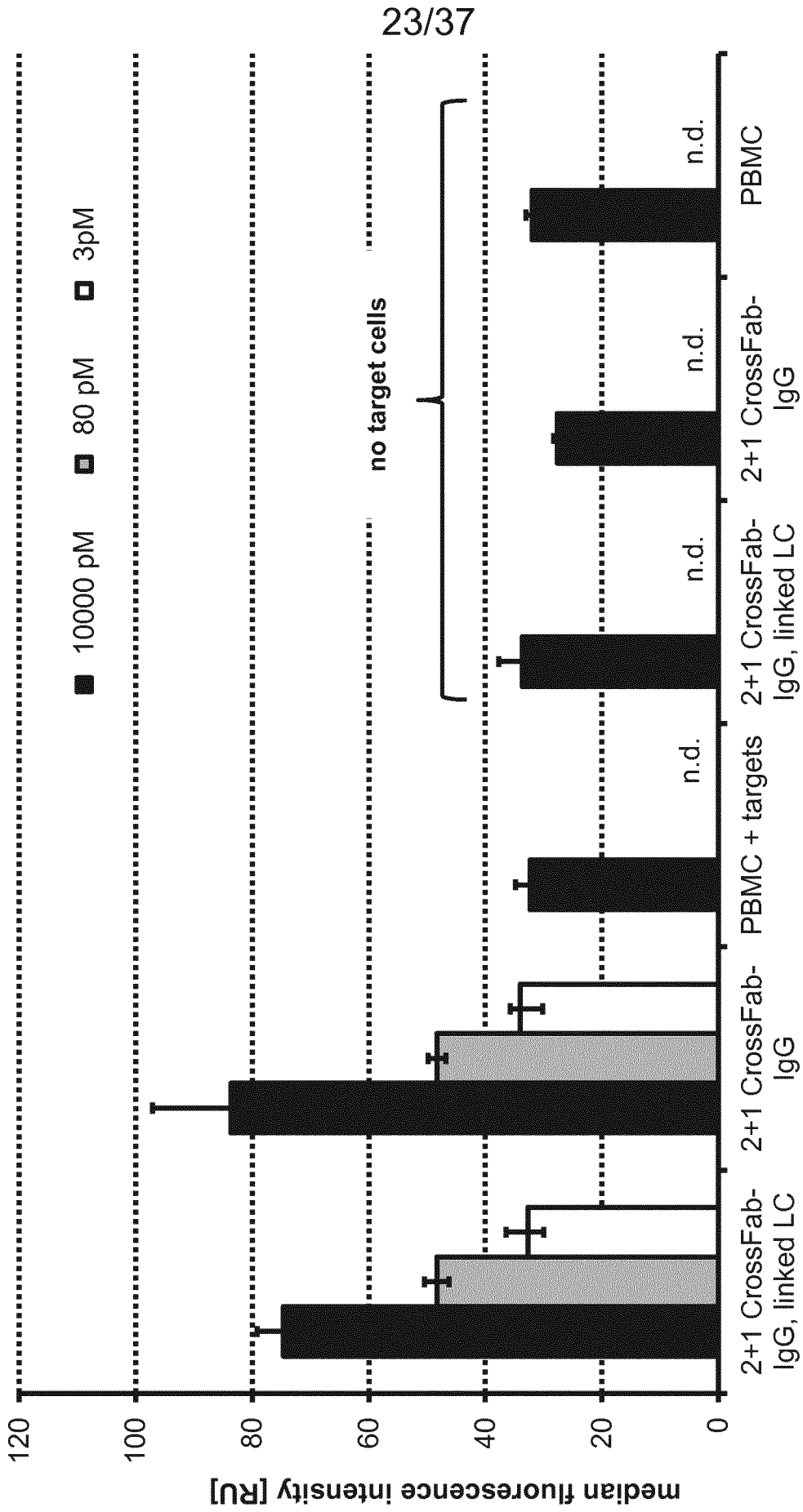
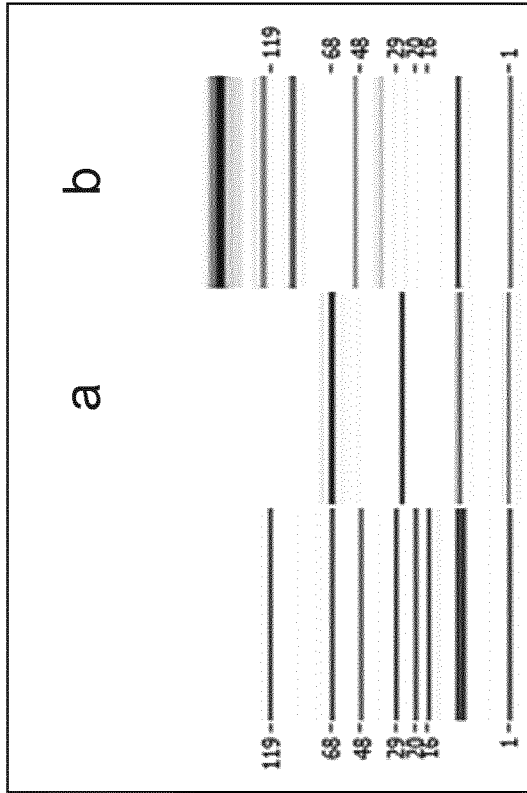
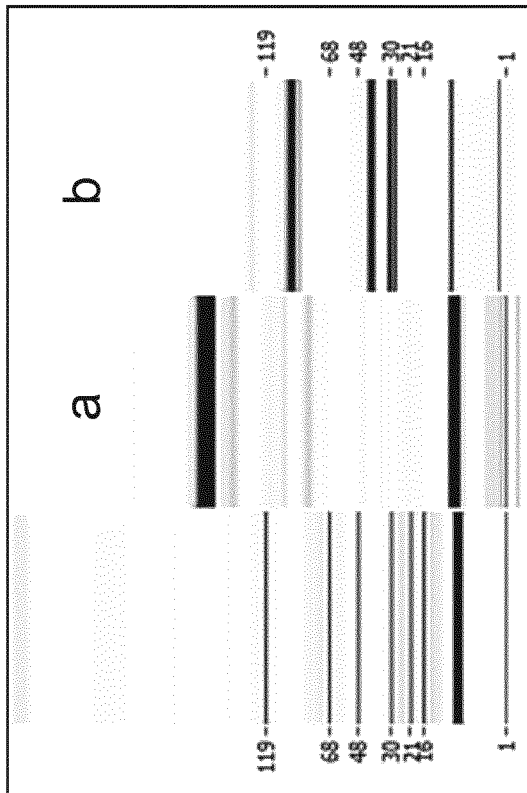


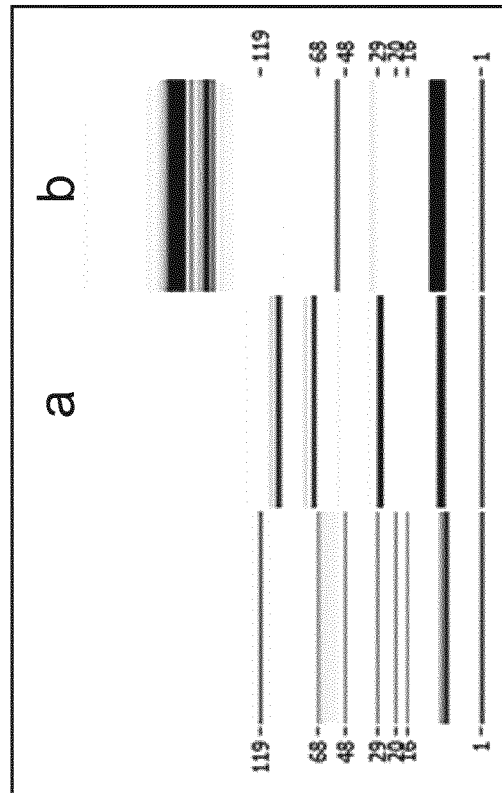
Figure 19B



B



A



C

Figure 20

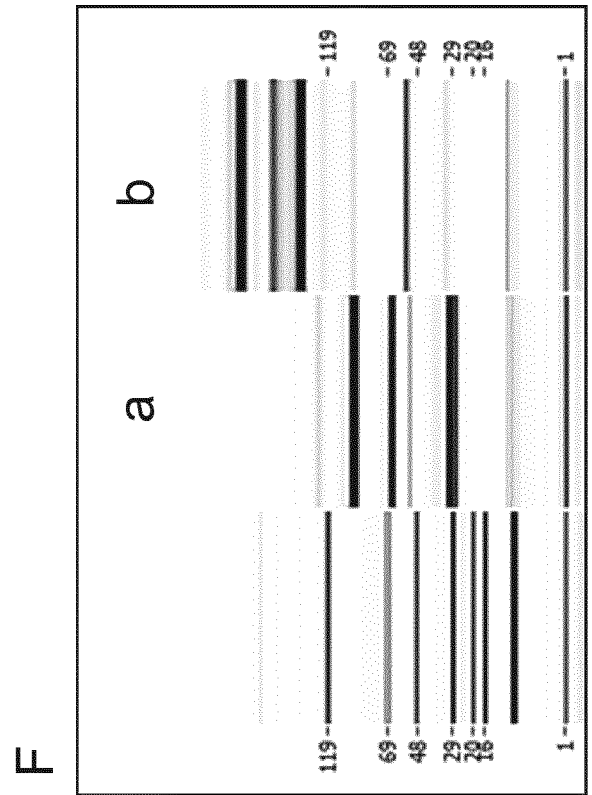
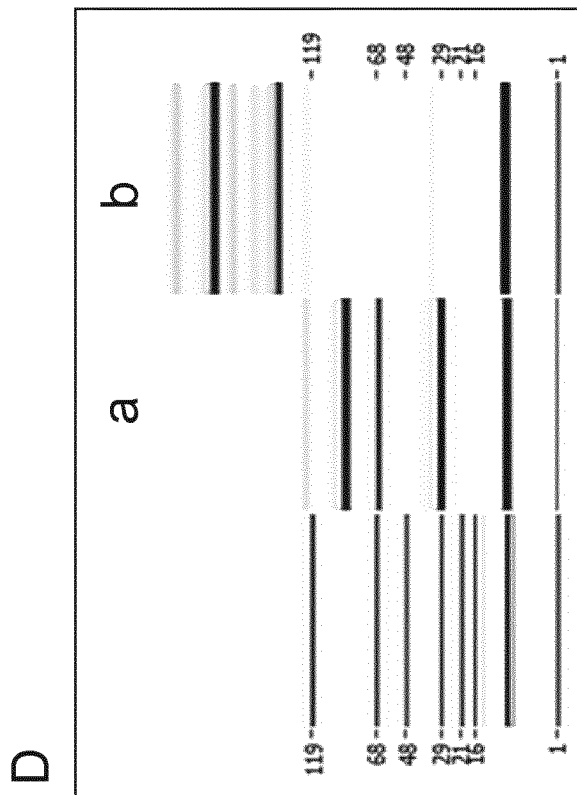
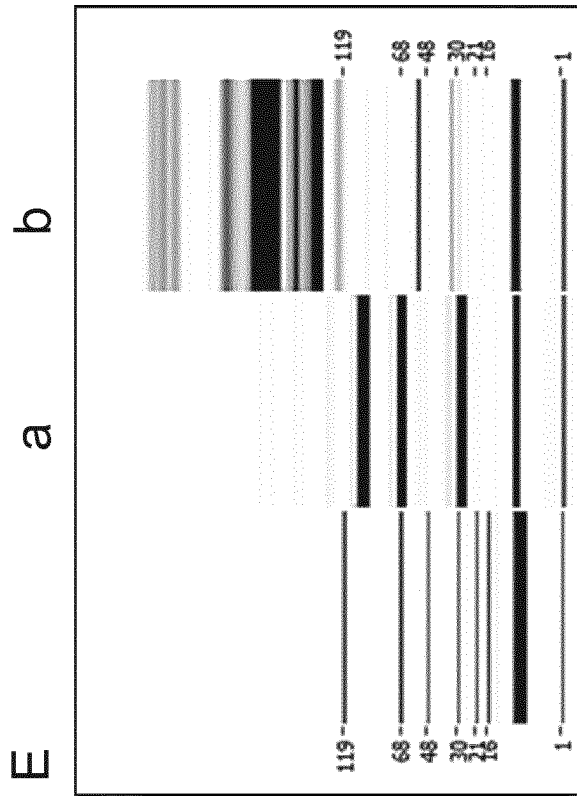


Figure 20

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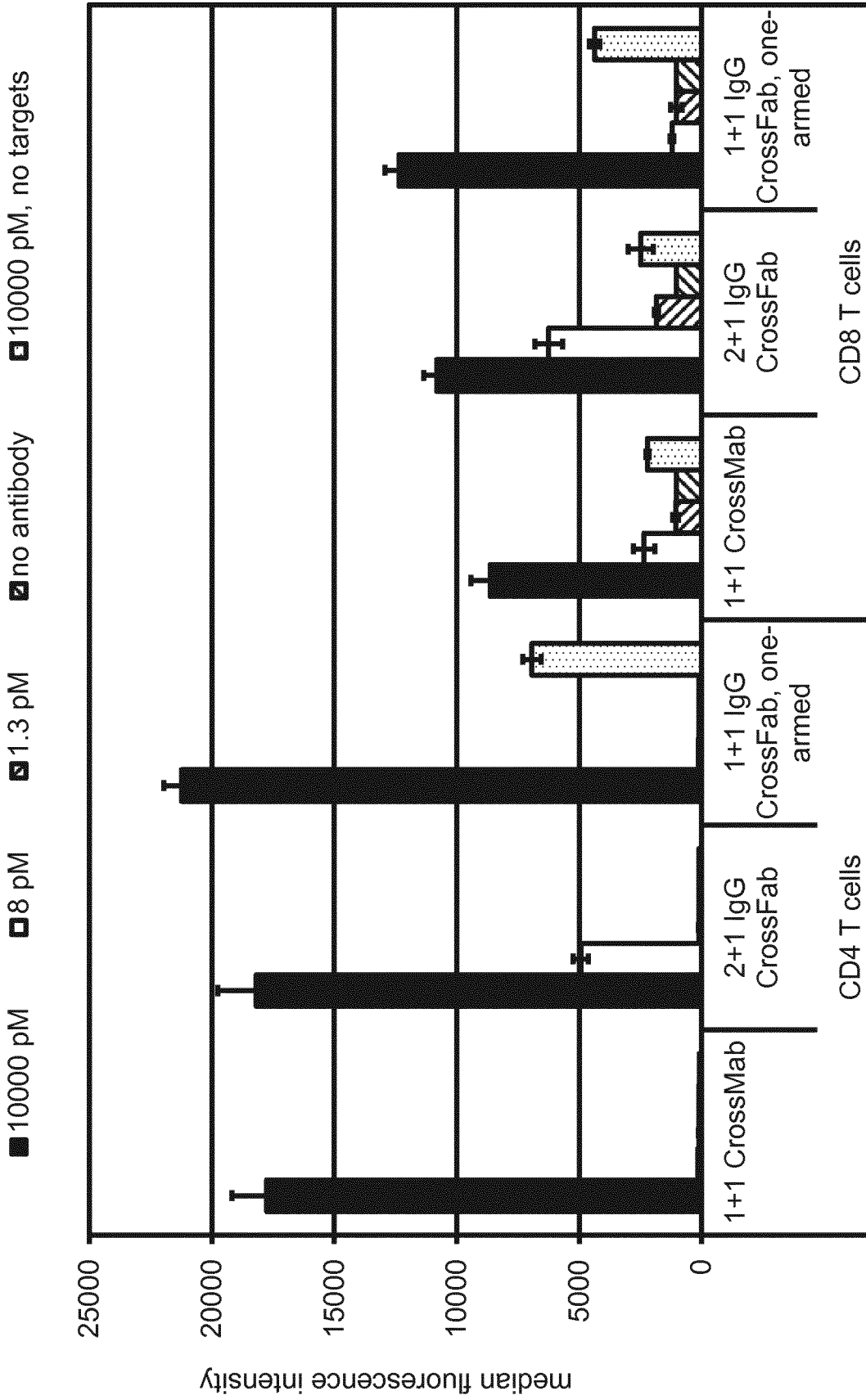


Figure 21A

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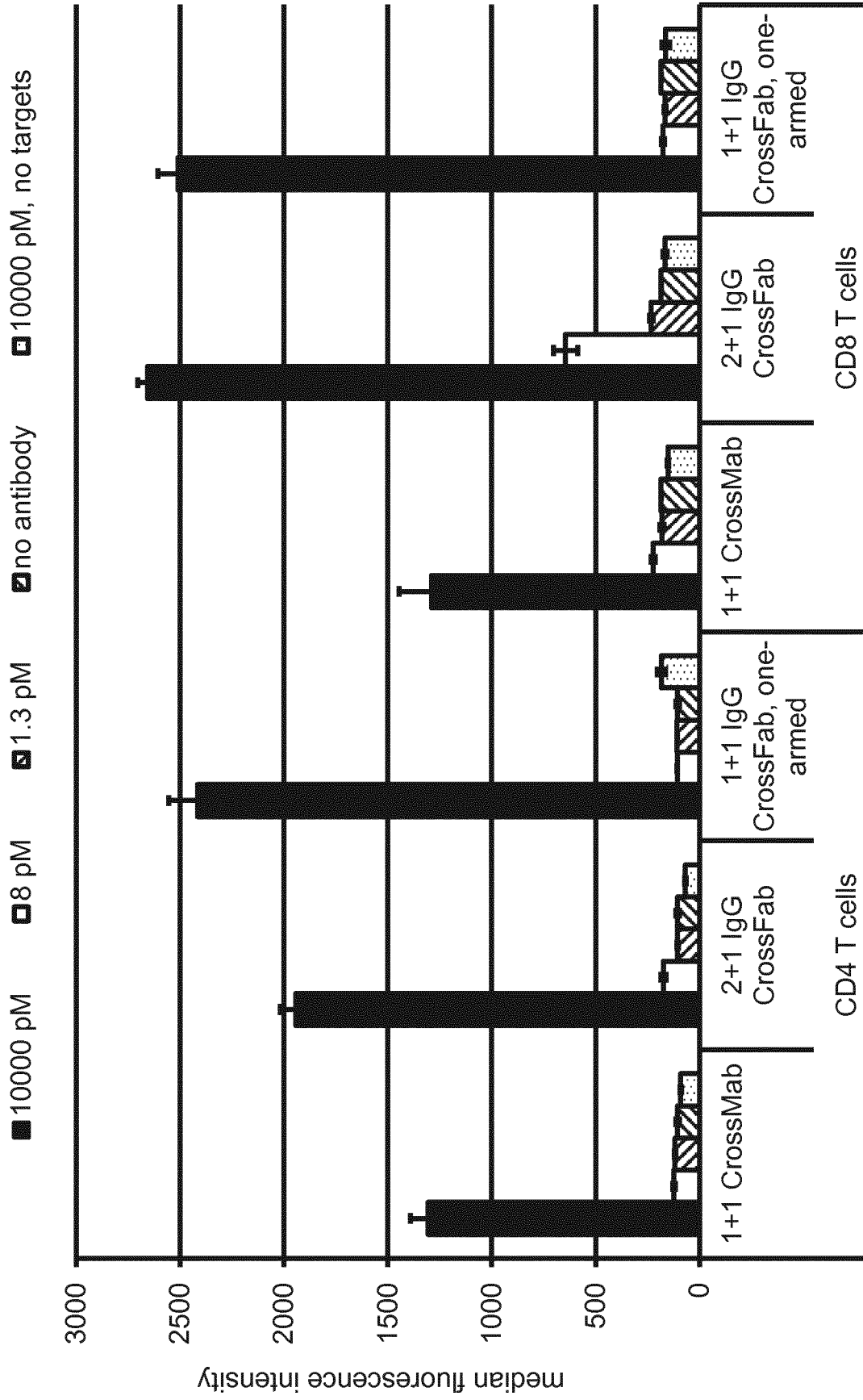


Figure 21B

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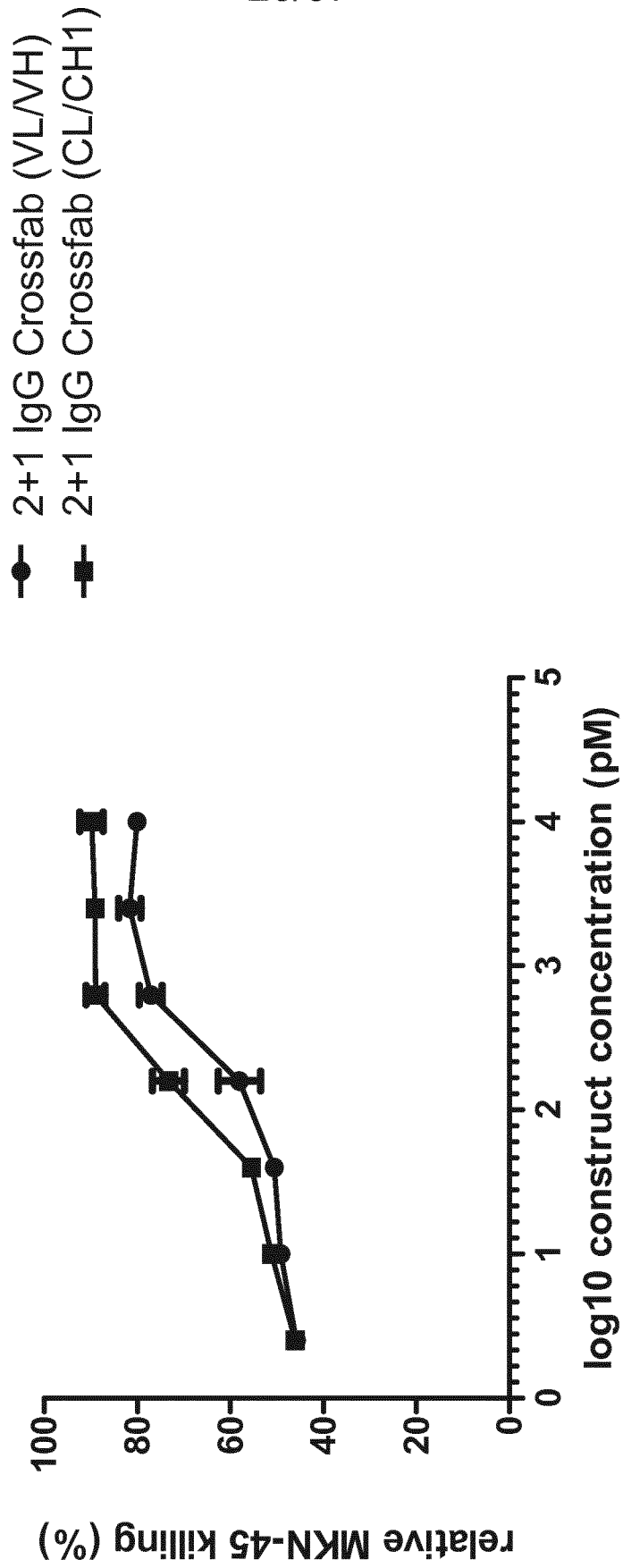


Figure 22A

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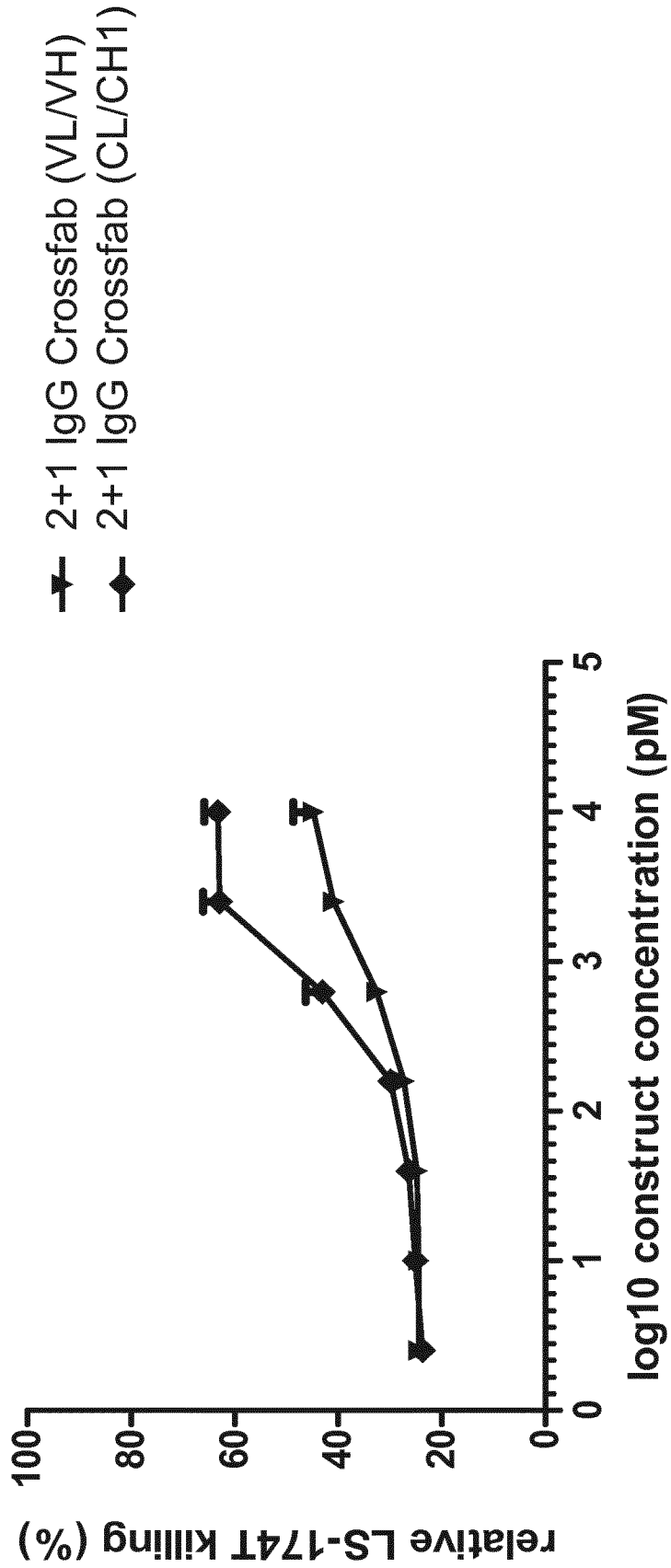


Figure 22B

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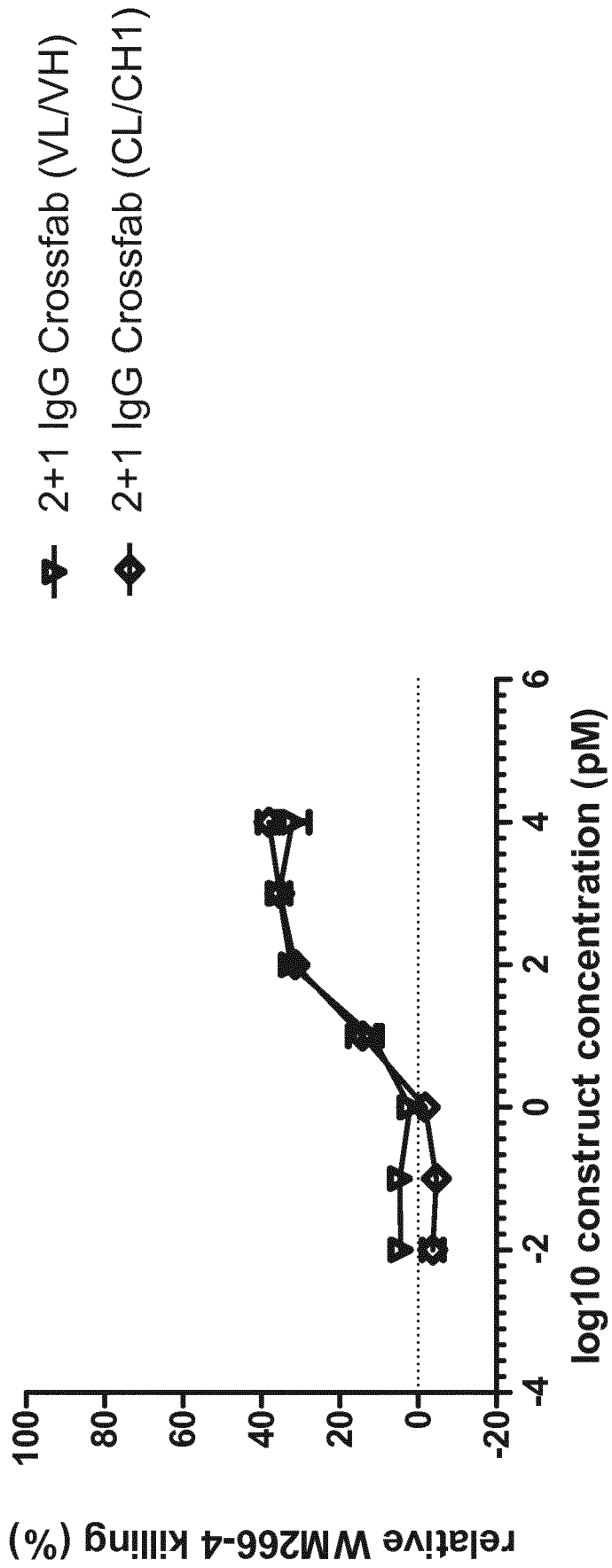


Figure 23

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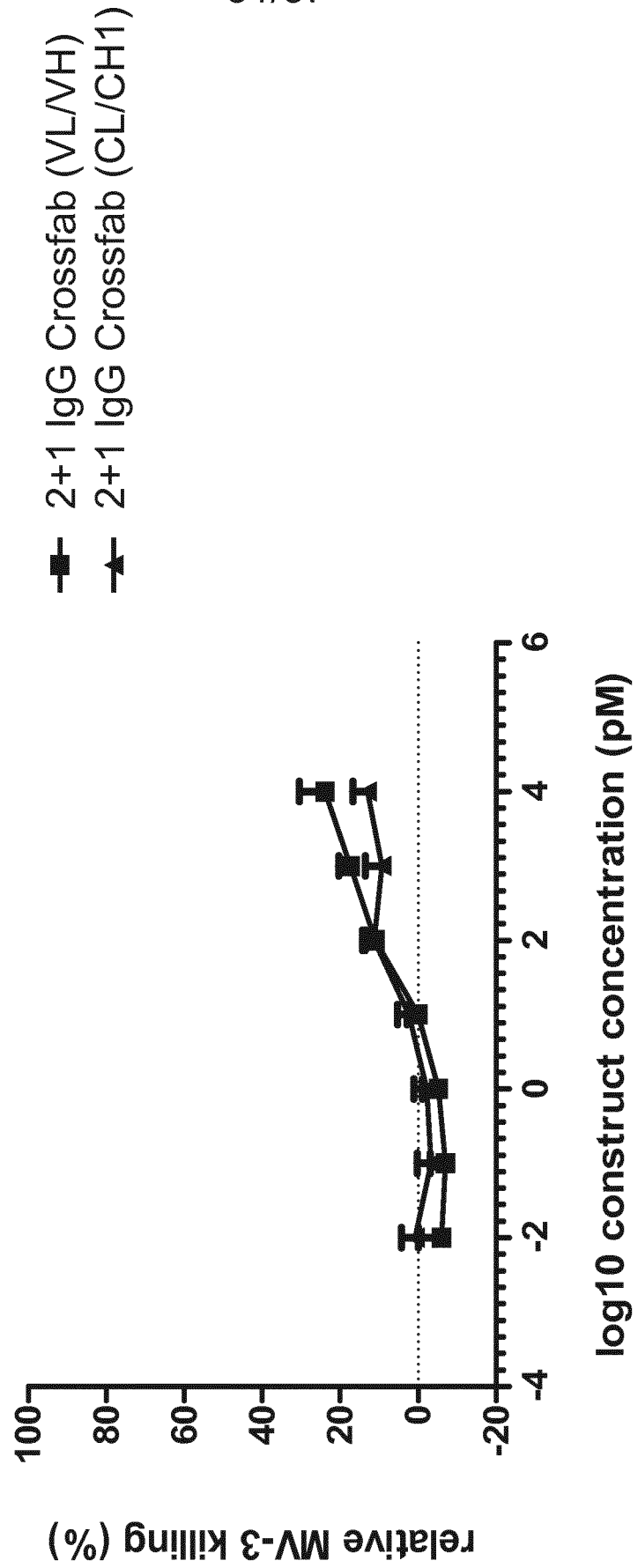


Figure 24

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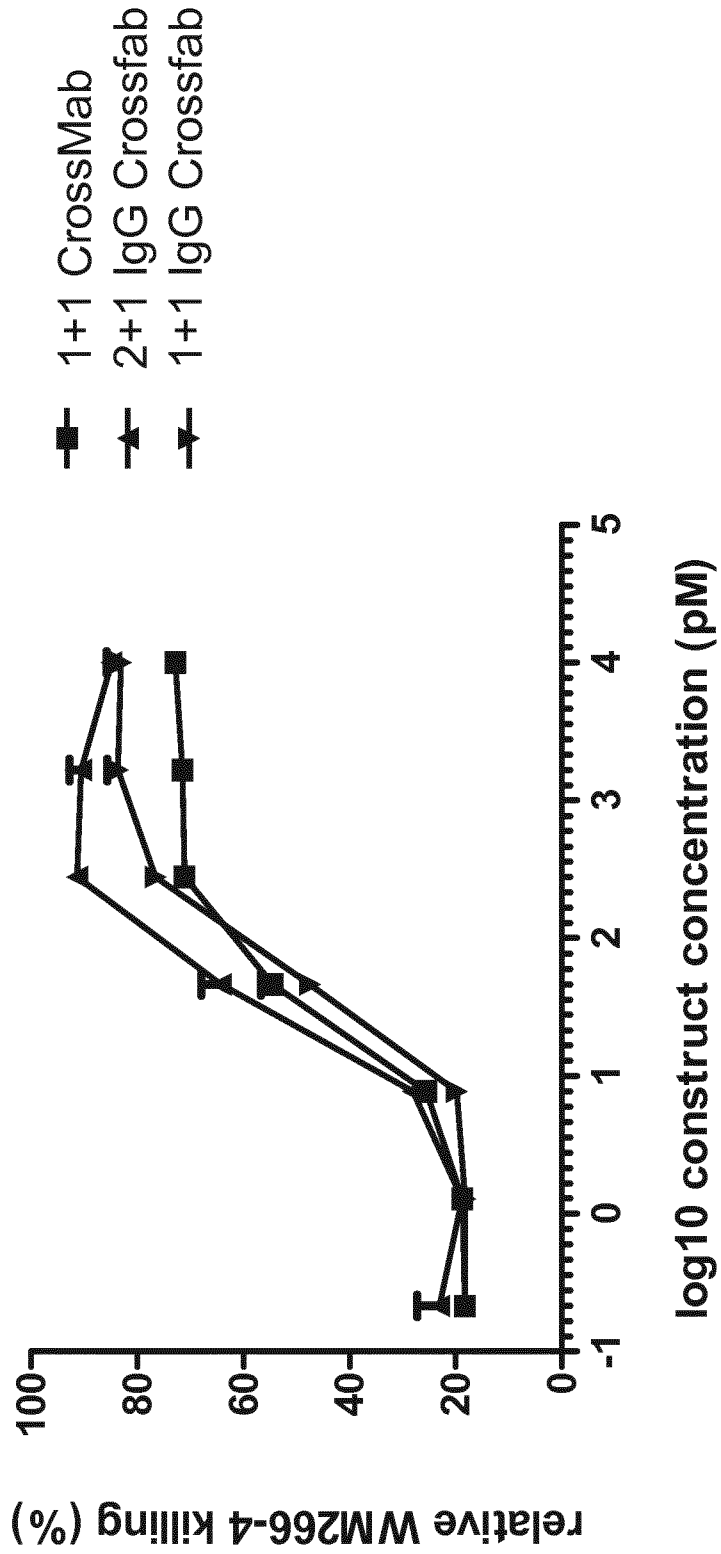


Figure 25A

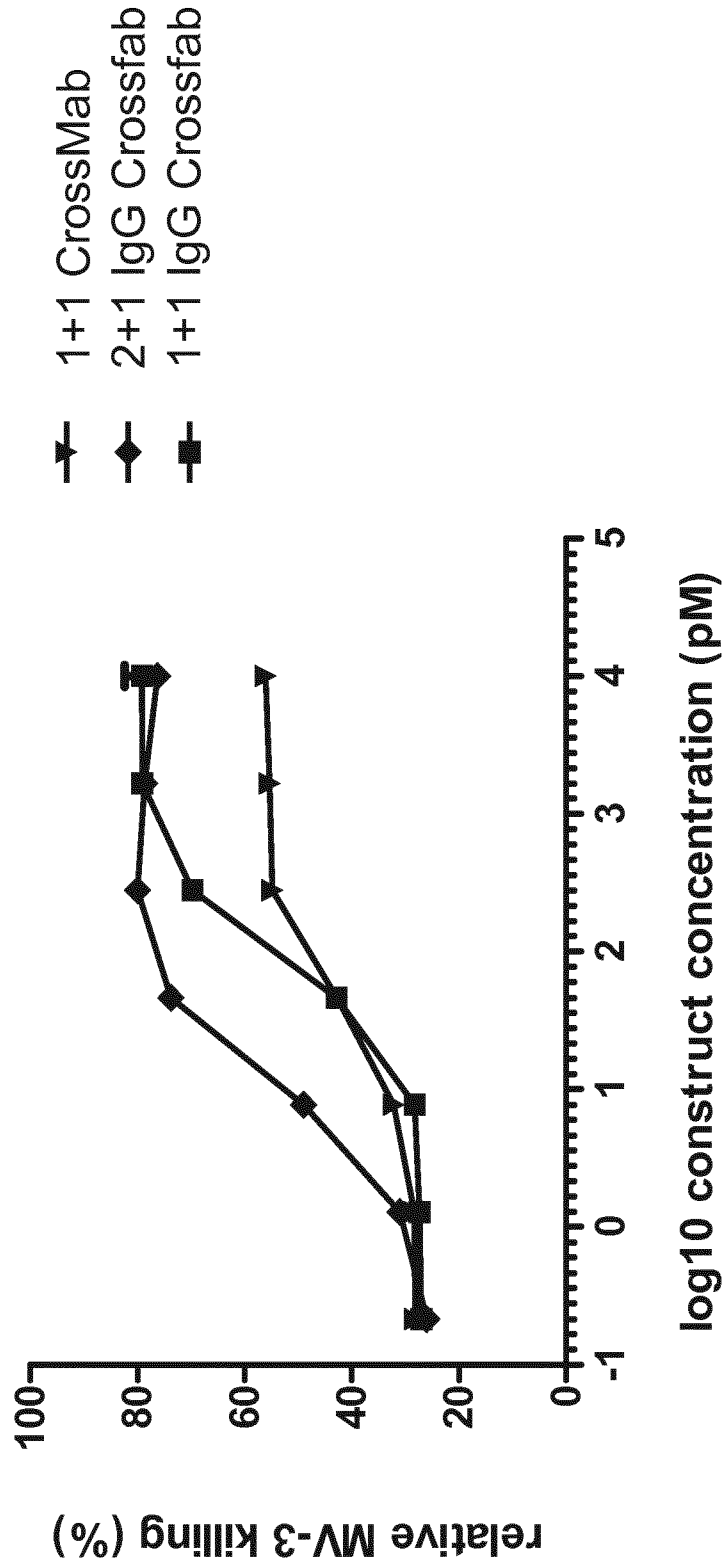


Figure 25B

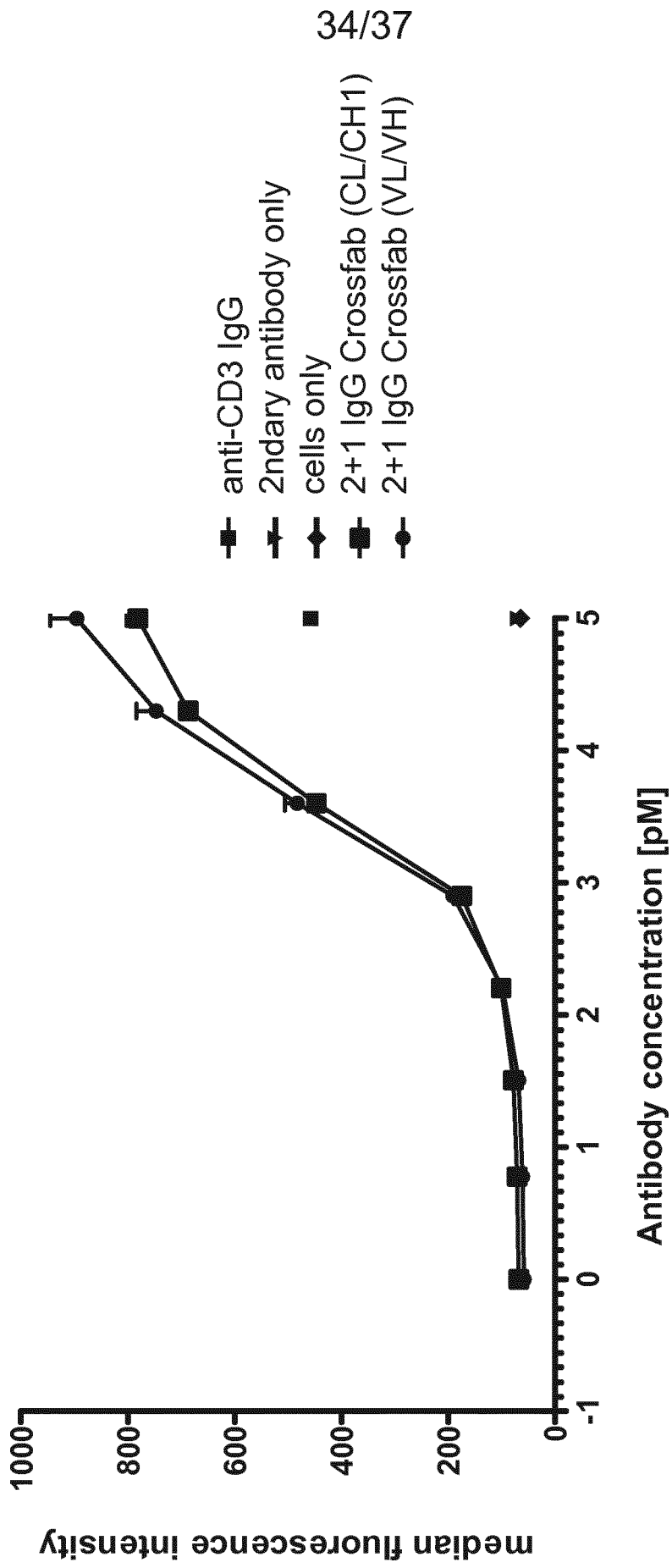


Figure 26A

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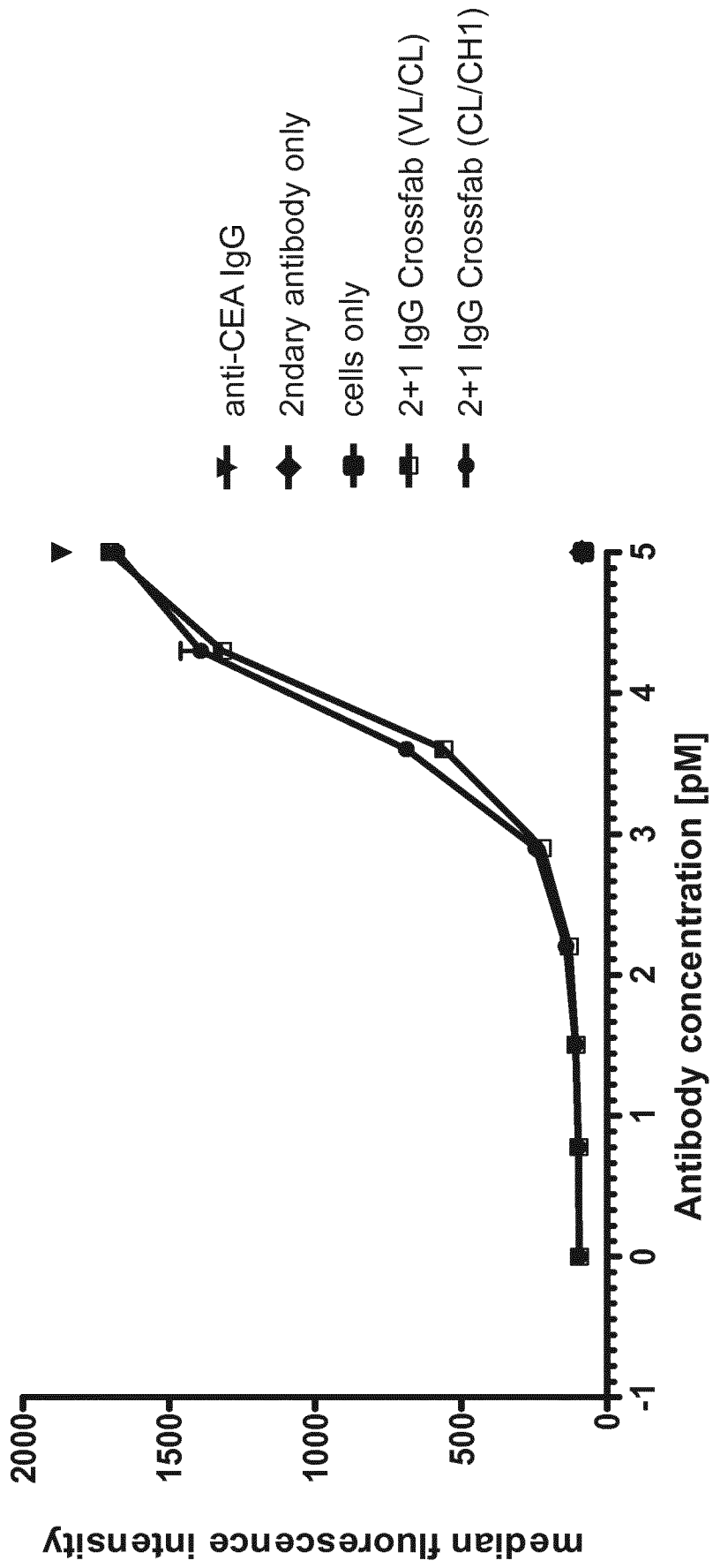


Figure 26B

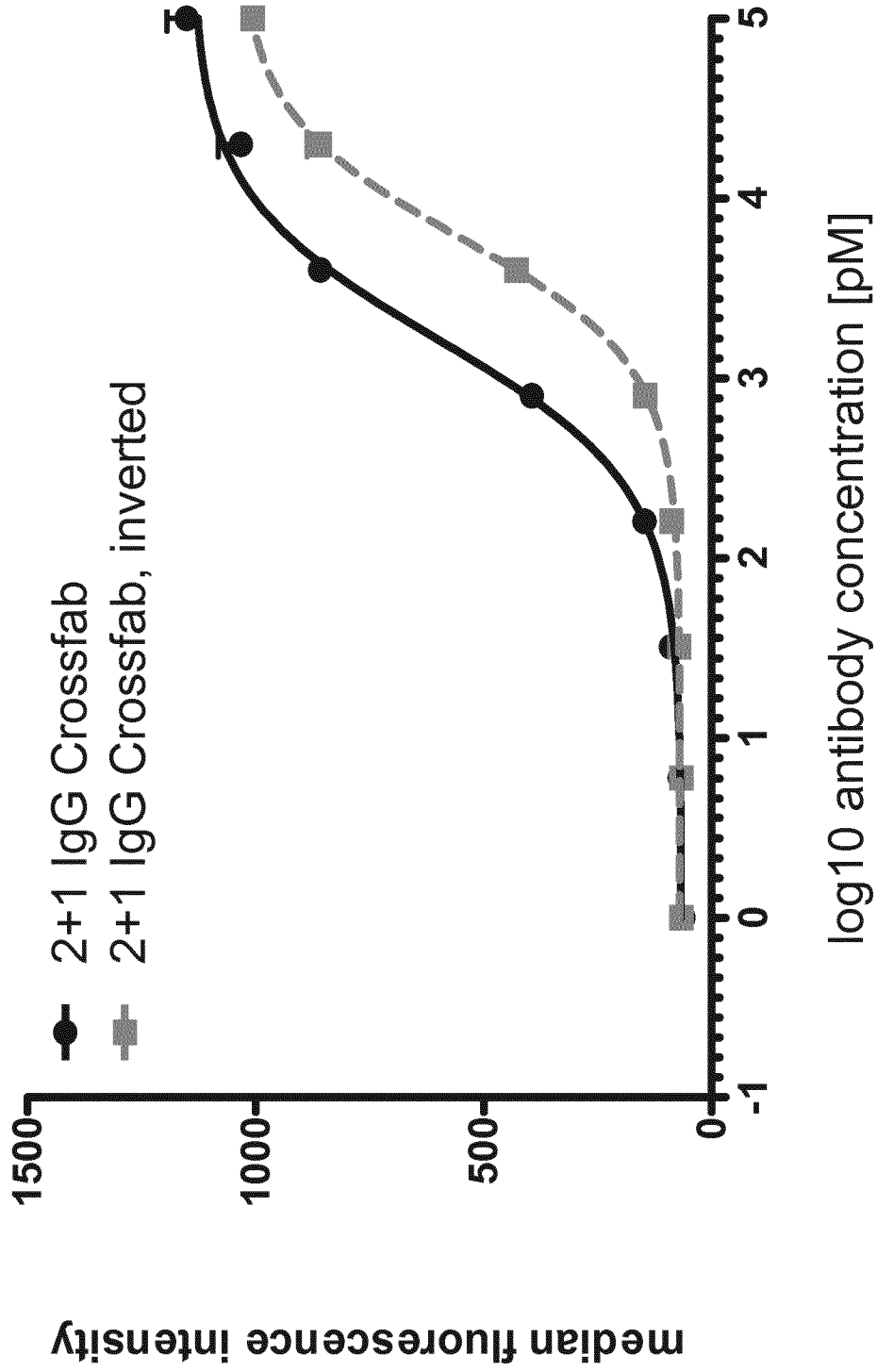


Figure 27A

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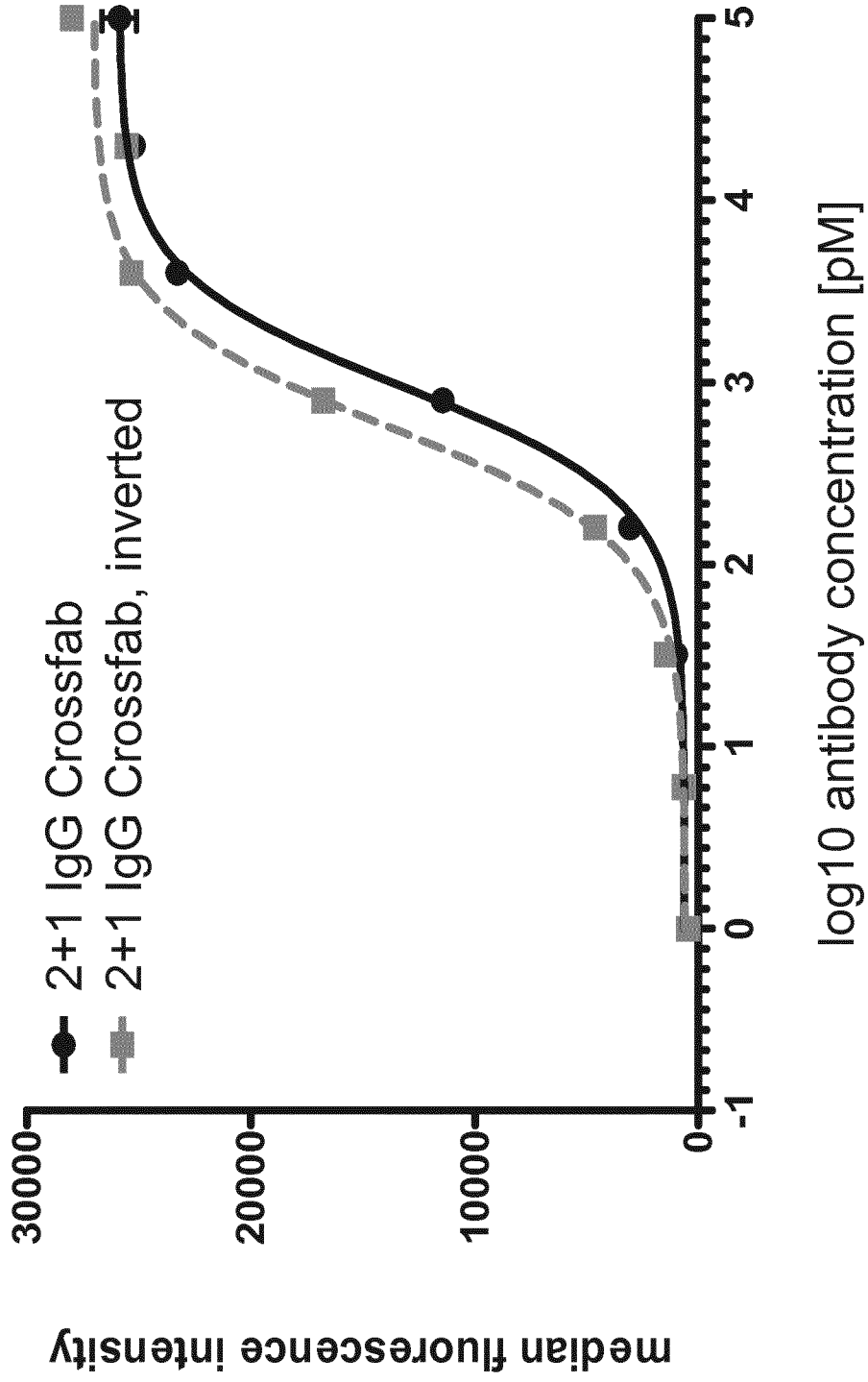


Figure 27B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/066213

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/30 A61K39/395 A61P35/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C07K A61K A61P
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/018386 A1 (MEDIMMUNE LLC [US]; WU HERREN [US]; GAO CHANGSHOU [US]; HAY CARL [US];) 5 February 2009 (2009-02-05) the whole document in particular abstract paragraphs [0002] - [0024], [0266] - [0271] claims 1-139; figures 1-40; examples 1-36 -----	1-43
X	WO 2009/080253 A1 (HOFFMANN LA ROCHE [CH]; KLEIN CHRISTIAN [DE]; SCHAEFER WOLFGANG [DE]) 2 July 2009 (2009-07-02) the whole document in particular abstract page 3, line 9 - page 14, line 20 claims 1-10; figures 1-15; examples 1-4 ----- -/--	1-43

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search 26 November 2012	Date of mailing of the international search report 06/12/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Ferreira, Roger

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/066213

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/080254 A1 (HOFFMANN LA ROCHE [CH]; KLEIN CHRISTIAN [DE]; SCHAEFER WOLFGANG [DE]) 2 July 2009 (2009-07-02) the whole document in particular abstract page 3, line 8 - page 12, line 25 claims 1-7; figures 1-10; examples -----	1-43
X	WO 2009/080251 A1 (HOFFMANN LA ROCHE [CH]; KLEIN CHRISTIAN [DE]; SCHAEFER WOLFGANG [DE]) 2 July 2009 (2009-07-02) the whole document in particular abstract page 3, line 9 - page 15, line 21 claims 1-10; figures 1-31; examples 1-4 -----	1-43
X	WO 2010/115551 A1 (ROCHE GLYCART AG [CH]; BOSENMAIER BIRGIT [DE]; BRINKMANN ULRICH [DE];) 14 October 2010 (2010-10-14) the whole document in particular abstract page 5, line 15 - page 45, line 22 claims 1-13; figures 1-10; examples 1-16 -----	1-43
X	WO 2010/115589 A1 (ROCHE GLYCART AG [CH]; BRINKMANN ULRICH [DE]; CROASDALE REBECCA [DE];) 14 October 2010 (2010-10-14) the whole document in particular abstract page 2, line 25 - page 19, line 23 claims 1-14; figures 1-10; examples 1-6 -----	1-43
X	WO 2010/115552 A1 (ROCHE GLYCART AG [CH]; BOSENMAIER BIRGIT [DE]; BRINKMAN ULRICH [DE];) 14 October 2010 (2010-10-14) the whole document in particular abstract page 4, line 27 - page 50, line 17 claims 1-15; figures 1-22; examples 1-19 -----	1-43
X	WO 2010/136172 A1 (HOFFMANN LA ROCHE [CH]; CROASDALE REBECCA [DE]; KLEIN CHRISTIAN [CH];) 2 December 2010 (2010-12-02) the whole document in particular abstract page 3, line 10 - page 26, line 28 claims 1-17; figures 1-11; examples 1-3 -----	1-43
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/066213

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/145792 A1 (HOFFMANN LA ROCHE [CH]; IMHOF-JUNG SABINE [DE]; KLEIN CHRISTIAN [CH];) 23 December 2010 (2010-12-23) the whole document in particular abstract page 3, line 20 - page 22, line 31 claims 1-13; figures 1-7; examples 1-3 -----	1-43
A	W. SCHAEFER ET AL: "Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 108, no. 27, 5 July 2011 (2011-07-05) , pages 11187-11192, XP055003817, ISSN: 0027-8424, DOI: 10.1073/pnas.1019002108 the whole document -----	1-43
A	CHAN L A ET AL: "Variable region domain exchange in human IgGs promotes antibody complex formation with accompanying structural changes and altered effector functions", MOLECULAR IMMUNOLOGY, PERGAMON, GB, vol. 41, no. 5, 1 July 2004 (2004-07-01), pages 527-538, XP002519620, ISSN: 0161-5890, DOI: 10.1016/J.MOLIMM.2004.03.034 the whole document -----	1-43
A	WO 2007/073499 A2 (MEDIMMUNE INC [US]; MICROMET AG [DE]; KINCH MICHAEL S [US]; ROFF SHANN) 28 June 2007 (2007-06-28) the whole document -----	1-43
A	WOLF ET AL: "BiTEs: bispecific antibody constructs with unique anti-tumor activity", DRUG DISCOVERY TODAY, ELSEVIER, RAHWAY, NJ, US, vol. 10, no. 18, 15 September 2005 (2005-09-15), pages 1237-1244, XP005103829, ISSN: 1359-6446, DOI: 10.1016/S1359-6446(05)03554-3 the whole document ----- -/--	1-43

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/066213

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DIRK NAGORSEN ET AL: "Immunomodulatory therapy of cancer with T cell-engaging BiTE antibody blinatumomab", EXPERIMENTAL CELL RESEARCH, ACADEMIC PRESS, US, vol. 317, no. 9, 10 March 2011 (2011-03-10), pages 1255-1260, XP028205663, ISSN: 0014-4827, DOI: 10.1016/J.YEXCR.2011.03.010 [retrieved on 2011-03-16] the whole document	1-43
A	BOSCH J J ET AL: "MCSP/CD3-bispecific single-chain antibody construct engages CD4+ and CD8+ T cells for lysis of MCSP-expressing human uveal melanoma cells", INTERNET CITATION, 21 April 2010 (2010-04-21), XP002685799, Retrieved from the Internet: URL:http://www.abstractsonline.com/Plan/ViewAbstract.aspx?mID=2521&sKey=ef81a5fa-b15c-4c4e-aa57-870b6479e274&cKey=a695d122-de20-451f-aeba-daf2b2a898f1&mKey={0591FA3B-AFEF-49D2-8E65-55F41EE8117E} [retrieved on 2012-10-23] abstract	1-43
A	WO 2007/075270 A2 (IBC PHARMACEUTICALS INC [US]; CHANG CHIEN-HSING [US]; GOLDENBERG DAVID) 5 July 2007 (2007-07-05) the whole document	1-43
A	WO 2008/119567 A2 (MICROMET AG [DE]; EBERT EVELYN [DE]; MEIER PETRA [DE]; SRISKANDARAJAH) 9 October 2008 (2008-10-09) cited in the application the whole document	1-43
A	OSHIMI ET AL: "Increased lysis of patient CD10-positive leukemic cells by T cells coated with anti-CD3 Fab' antibody cross-linked to anti-CD10 Fab' antibody.", BLOOD, vol. 77, no. 5, 1 March 1991 (1991-03-01), pages 1044-1049, XP055041891, ISSN: 0006-4971 the whole document	1-43
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/066213

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
 on paper
 in electronic form
 - b. (time)
 in the international application as filed
 together with the international application in electronic form
 subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

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