



(86) **Date de dépôt PCT/PCT Filing Date:** 2013/06/25
 (87) **Date publication PCT/PCT Publication Date:** 2014/01/03
 (85) **Entrée phase nationale/National Entry:** 2014/10/28
 (86) **N° demande PCT/PCT Application No.:** EP 2013/063258
 (87) **N° publication PCT/PCT Publication No.:** 2014/001324
 (30) **Priorité/Priority:** 2012/06/27 (EP12173875.1)

(51) **Cl.Int./Int.Cl. C07K 16/00** (2006.01),
C07K 16/46 (2006.01), **C12N 9/16** (2006.01),
C12N 9/52 (2006.01), **C12N 9/80** (2006.01),
C12P 21/06 (2006.01)

(71) **Demandeur/Applicant:**
 F. HOFFMANN-LA ROCHE AG, CH

(72) **Inventeurs/Inventors:**
 FENN, SEBASTIAN, DE;
 KOPETZKI, ERHARD, DE;
 TIEFENTHALER, GEORG, DE

(74) **Agent:** GOWLING LAFLEUR HENDERSON LLP

(54) **Titre : METHODE DE SELECTION ET DE PRODUCTION D'ENTITES DE CIBLAGE PERSONNALISEES FORTEMENT
 SELECTIVES ET MULTI-SPECIFIQUES RENFERMANT AU MOINS DEUX ENTITES DE LIAISON DIFFERENTES ET LEURS
 UTILISATIONS**

(54) **Title: METHOD FOR SELECTION AND PRODUCTION OF TAILOR-MADE HIGHLY SELECTIVE AND MULTI-SPECIFIC
 TARGETING ENTITIES CONTAINING AT LEAST TWO DIFFERENT BINDING ENTITIES AND USES THEREOF**

(57) **Abrégé/Abstract:**

Herein is reported a method for producing a bispecific antibody comprising the step of incubating (i) an antibody Fab fragment or a sc Fv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01), (ii) a one-armed antibody comprising a full length antibody heavy chain, a full length antibody light chain, and an Fc-heavy chain, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains that thereof forms an antigen binding site, whereby the full length antibody heavy chain and the Fc-heavy chain are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the Fc-heavy chain has an oligoglycine amino acid sequence at its N-terminus, and (iii) a Sortase A enzyme.

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

REVISED VERSION

(19) World Intellectual Property
Organization
International Bureau



WIPO | PCT



(10) International Publication Number
WO 2014/001324 A9

(43) International Publication Date
3 January 2014 (03.01.2014)

(51) International Patent Classification:

C07K 16/00 (2006.01) *C12N 9/80* (2006.01)
C12N 9/16 (2006.01) *C12P 21/06* (2006.01)
C12N 9/52 (2006.01) *C07K 16/46* (2006.01)

(21) International Application Number:

PCT/EP2013/063258

(22) International Filing Date:

25 June 2013 (25.06.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

12173875.1 27 June 2012 (27.06.2012) EP

(71) Applicant (for all designated States except US): **HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacher Strasse 124, CH-4070 Basel (CH).

(71) Applicant (for US only): **HOFFMANN-LA ROCHE INC.** [US/US]; 340 Kingsland Street, Nutley, New Jersey 07110 (US).

(72) Inventors: **FENN, Sebastian**; Hans-Graessel-Weg 12, 81375 Muenchen (DE). **KOPETZKI, Erhard**; Kastnerhofstrasse 21, 82377 Penzberg (DE). **TIEFENTHALER, Georg**; Oberriedern 4, 82404 Sindelsdorf (DE).

(74) Agents: **BURGER, Alexander** et al.; Roche Diagnostics GmbH, Patent Department (LPP.....6164), P.O.Box 11 52, 82372 Penzberg (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(88) Date of publication of the revised international search report:

19 June 2014

(15) Information about Correction:

see Notice of 19 June 2014

(54) Title: METHOD FOR SELECTION AND PRODUCTION OF TAILOR-MADE HIGHLY SELECTIVE AND MULTI-SPECIFIC TARGETING ENTITIES CONTAINING AT LEAST TWO DIFFERENT BINDING ENTITIES AND USES THEREOF

(57) Abstract: Herein is reported a method for producing a bispecific antibody comprising the step of incubating (i) an antibody Fab fragment or a sc Fv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01), (ii) a one-armed antibody comprising a full length antibody heavy chain, a full length antibody light chain, and an Fc-heavy chain, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains that thereof forms an antigen binding site, whereby the full length antibody heavy chain and the Fc-heavy chain are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the Fc-heavy chain has an oligoglycine amino acid sequence at its N-terminus, and (iii) a Sortase A enzyme.



WO 2014/001324 A9

Method for selection and production of tailor-made highly selective and multi-specific targeting entities containing at least two different binding entities and uses thereof

Herein is reported a method for selecting and producing multispecific entities by using a transpeptidase, such as Sortase A, wherein the specificities can be chosen independently of each other and the use of this method for the generation of novel tailor-made multispecific antibodies.

5 **Background of the Invention**

Monoclonal antibodies have a great therapeutic potential and play an important role in today's medical portfolio. During the last decade, a significant trend in the pharmaceutical industry has been the development of monoclonal antibodies (mAbs) and antibody Fc-fusion polypeptides (crystallizable fragment-fusion polypeptides) as therapeutic agents across diverse clinical settings including oncology, chronic inflammatory diseases, transplantation, infectious diseases, cardiovascular medicine, or ophthalmologic diseases (Carter, J.P., Nature Reviews Immunology 6 (2006) 343-357; Chan, A.C. and Carter, J.P., Nature Reviews Immunology 10 (2010) 301-316).

15 The clinical efficiency of a therapeutic antibody relies mainly on two functionalities: i) the target-specific binding mediated by the Fv-domain, and ii) the immune-mediated effector function such as ADCC (antibody-dependent cell-mediated cytotoxicity), CDC (complement-dependent cytotoxicity), and ADCP (antibody-dependent cellular phagocytosis) which are mediated by the antibody Fc-region. The Fc-region of an immunoglobulin of the IgG class comprises the hinge region and two constant domains (CH2 and CH3). The Fc-region also interacts with the neonatal FcRn receptor and thereby determines the half-life of the antibody in vivo. The hinge region is the region at which the arms of an antibody molecule form a Y-like structure enabling flexibility in the molecule at this point. The IgG subclass/subclasses differ in the number of disulfide bonds and the length of the hinge region.

The effector functions associated with the Fc-region of an antibody vary with the class and subclass of the antibody and include e.g. binding of the antibody via its Fc-region to a specific Fc receptor (FcR) on a cell which triggers various biological

- 2 -

responses (see e.g. Jiang, X.-R., et al., *Nature Reviews Drug Discovery* 10 (2011) 101-110; Presta, L.G., *Current Opinion in Immunology* 20 (2008) 460-470).

5 The hinge region of an antibody or of an Fc-region comprising fusion polypeptide or conjugate is involved in at least a part of the antibody's functions such as antigen binding and Fc-region-mediated antibody effector functions. Whereas antigen binding (especially bivalent avid antibody binding) depends on the flexibility, length and spatial orientation of a particular/native hinge region the Fc-region mediated effector functions are dependent on the class and subclass of the antibody. The functional monovalency observed for some human IgG4 antibodies in comparison with the bivalency for the other IgG antibodies is another example showing the involvement of the Fc-region region in antigen binding properties (Salfeld, J.G., *Nature Biotechnology* 12 (2007) 1369-1372; Presta, L.G., *Current Opinion in Immunology* 20 (2008) 460-470).

10

Levary, D.A., et al., report protein-protein fusion catalyzed by Sortase A (*PLOS ONE* 6 (2011)). Engineering of an anti-epidermal growth factor receptor antibody to single chain format and labeling by sortase A-mediated protein ligation is reported by Madej, M.P., et al. (*Biotechnol. Bioeng.* 109 (2012) 1461-1470). Ta, H.T., et al., report enzymatic single-chain antibody tagging as a universal approach to targeted molecular imaging and cell homing in cardiovascular diseases (*Cir. Res.* 109 (2011) 365-373). Popp, M., et al., report making and breaking peptide bonds – protein engineering using sortase (*Angew. Chem. Int. Ed. Eng.* 50 (2011) 5024-5032). In WO 2010/087994 methods for ligation and uses thereof are reported.

15

20

Summary of the Invention

Herein is reported a method for providing tailor-made, highly specific therapeutic molecules for the treatment of a disease, such as cancer, in a patient in need of a treatment, whereby the therapeutic molecule is adapted to the characteristics of the disease of the patient and/or to the genotype/phenotype of the patient.

25

Such adaptation is achieved by making a tailor-made molecule taking into account the genotype/phenotype of the disease harboring/affected cells of the patient.

30 In a first step the genotype/phenotype of the cells (e.g. the presence and number/quantity of disease-specific cell surface antigens) that are intended to be targeted with the therapeutic molecule is determined. This can be achieved, e.g. by cell imaging techniques such as immunohistochemical staining (IHC,

- 3 -

immunohistochemistry) of patient's cells derived e.g. from blood and/or biopsied material using fluorescently labeled monospecific (therapeutic or diagnostic) antibodies. Alternatively the genotype/phenotype of the cells can be analyzed after staining with labeled therapeutic or diagnostic antibodies using FACS-based methods. In vivo imaging techniques including optical imaging, molecular imaging, fluorescence imaging, bioluminescence Imaging, MRI, PET, SPECT, CT, and intravital microscopy may be used also for determination of the genotype/phenotype of disease-related cells of a patient. Depending on the determined genotype/phenotype of the disease-related cells of a patient a tailor-made combination of targeting/binding entities can be/is chosen and are combined in a therapeutic molecule. Such a therapeutic molecule may be for example a bispecific antibody.

Such tailor-made therapeutic molecules i) will be highly specific, ii) will have a good efficacy, and iii) will induce less side effects compared to conventionally chosen therapeutics. This can be achieved by endowing the therapeutic molecule with improved targeting and/or improved tailor-made delivery properties, e.g. for a therapeutic payload to its intended site of action.

The improved delivery of the therapeutic molecule to its site of action, such as e.g. a cancer cell, can be achieved by a higher/increased selectivity and/or specificity of the targeted therapeutic molecule compared to conventionally chosen therapeutic molecules. The therapeutic molecule comprises at least two entities that specifically bind to different antigens (e.g. two different surface markers) or to different epitopes on the same antigen (e.g. two different epitopes on the same surface marker).

The increased selectivity and/or specificity of the tailor-made therapeutic molecule can be achieved by the simultaneous binding of both targeting entities to their respective targets/epitopes, i.e. it is achieved by avidity effects. Especially suited is the combination of two binding entities having a low to medium affinity for their respective targets/epitopes. Additionally, off-target binding is greatly reduced or can even be eliminated completely.

It has been found that tailor-made bispecific targeting and binding molecules can be provided using an enzymatic conjugation reaction between a first binding entity, such as a darpin domain based binding entity, an anticalin domain based binding entity, a T-cell receptor fragment like scTCR domain based binding entity, a camel

- 4 -

VH domain based binding entity, a tenth fibronectin 3 domain based binding entity, a tenascin domain based binding entity, a cadherin domain based binding entity, an ICAM domain based binding entity, a titin domain based binding entity, a GCSF-R domain based binding entity, a cytokine receptor domain based binding entity, a glycosidase inhibitor domain based binding entity, a superoxide dismutase domain based binding entity, or an antibody fragment (Fab or scFv fragment), comprising the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue) in its C-terminal amino acid sequence region and an one-armed antibody fragment (OA-Fc), which comprises a full length antibody heavy chain paired with the cognate full length light chain and an antibody heavy chain Fc-region polypeptide with an oligoglycine G_m (m = 2, or 3, or 4, or 5) at its N-terminus, using the enzyme Sortase A.

It has been found that with the method as reported herein it is possible to tailor-make e.g. bispecific antibodies specifically directed to two surface markers found on the surface of a cell, such as a cancer cell. As the binding specificities are individually provided by the starting components it is possible to tailor-make a multispecific targeting and binding molecule simply by determining the surface markers present on a cell, e.g. on a cancer cell, and conjugating the respective antibody fragments that specifically bind to these surface markers or their respective ligands by an enzymatic procedure. As the enzymatic conjugation is performed by the enzyme Sortase A the resulting bispecific antibody is characterized by the presence of the amino acid sequence LPX1TG ((SEQ ID NO: 01, wherein X1 can be any amino acid residue).

One aspect as reported herein is a method for producing a multispecific binding molecule comprising the step of incubating

- (i) a first binding entity comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue),
- (ii) an antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain and an antibody heavy chain Fc-region polypeptide,

whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site,

- 5 -

whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and

5 whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus,

and

(iii) a Sortase A enzyme

and thereby producing the multispecific binding molecule.

10 One aspect as reported herein is a method for producing a multispecific binding molecule comprising the following steps

(i) determining the cell surface makers present in a cell containing sample and i) selecting thereof at least a first cell surface marker and a second cell surface marker, or ii) selecting thereof a multitude of cell surface markers corresponding to the number of binding specificities of the multispecific binding molecule,

15 (ii) incubating (a) a first binding entity, which specifically binds to the first cell surface marker or its ligand, and which comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), (b) an antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site, which specifically binds to the second cell surface marker or its ligand, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, and (c) a Sortase A enzyme

25 and thereby producing the multispecific binding molecule.

- 6 -

One aspect as reported herein is a method for the selection of at least two binding entities from a collection/library of binding entities which are assembled in a single multispecific binding molecule by incubating (a) a first binding entity, which specifically binds to a first epitope or antigen, and which comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), (b) an antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site, which specifically binds to a second epitope or antigen, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, and (c) a Sortase A enzyme for use as a therapeutic agent. Such an agent has improved targeting/delivery properties.

One aspect as reported herein is a method for producing a bispecific antibody comprising the step of incubating

- (i) an antibody Fab fragment or a scFv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue),
- (ii) an one-armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide,

whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site,

whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and

whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus,

- 7 -

and

(iii) a Sortase A enzyme

and thereby producing the bispecific antibody.

One aspect as reported herein is a method for producing a bispecific antibody
5 comprising the following steps

(i) determining the cell surface makers present in a cell containing sample
and selecting thereof at least a first cell surface marker and a second cell
surface marker,

10 (ii) incubating (a) an antibody Fab fragment or a scFv antibody comprising
within the 20 C-terminal amino acid residues the amino acid sequence
LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue),
whereby the Fab fragment or scFv antibody specifically binds to the first
cell surface marker or its ligand, (b) an one-armed antibody fragment
15 comprising a full length antibody heavy chain, a full length antibody light
chain, and an antibody heavy chain Fc-region polypeptide, whereby the
full length antibody heavy chain and the full length antibody light chain
are cognate antibody chains complementary to each other and the pair of
variable domains (VH and VL) thereof forms an antigen binding site that
20 specifically binds to the second cell surface marker or its ligand, whereby
the full length antibody heavy chain and the antibody heavy chain Fc-
region polypeptide are covalently linked to each other via one or more
disulfide bonds forming an antibody hinge region, and whereby the
antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m =
2, or 3, or 4, or 5) amino acid sequence at its N-terminus, and (c) a Sortase
25 A enzyme

and thereby producing the bispecific antibody.

One aspect as reported herein is a method for determining a combination of binding
entities for a multispecific binding molecule comprising the following steps

30 (i) determining the binding specificity and/or selectivity and/or affinity and/or
effector function and/or in vivo half-life of a multitude of multispecific
binding molecules whereby in the multitude of multispecific binding
molecules each (possible) combination of binding entities is comprised,

- 8 -

and

- 5 (ii) choosing the multispecific binding molecule with suitable binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

One aspect as reported herein is a method for determining a combination of antigen binding sites comprising the following steps

- 10 (i) determining the binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life of a multitude of bispecific antibodies prepared by combining (a) each member of a first multitude of antibody Fab fragments or scFv antibody fragments whereby each member comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), whereby the Fab fragment or scFv antibody specifically binds to a first epitope or antigen, with (b) each member of a multitude of one-armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to a second epitope or antigen, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m ($m = 2, \text{ or } 3, \text{ or } 4, \text{ or } 5$) amino acid sequence at its N-terminus, and (c) a Sortase A enzyme

and

- 30 (ii) choosing the bispecific antibody with suitable binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

In one embodiment the binding entities are independently of each other selected from a darpin domain based binding entity, an anticalin domain based binding

- 9 -

entity, a T-cell receptor fragment like scTCR domain based binding entity, a camel VH domain based binding entity, a tenth fibronectin 3 domain based binding entity, a tenascin domain based binding entity, a cadherin domain based binding entity, an ICAM domain based binding entity, a titin domain based binding entity, a GCSF-R domain based binding entity, a cytokine receptor domain based binding entity, a glycosidase inhibitor domain based binding entity, a superoxide dismutase domain based binding entity, or antibody fragments like Fab or scFv fragments.

5
10 In one embodiment of all aspects the multispecific binding molecule is a bispecific antibody, and/or the first binding entity is an antibody Fab fragment or a scFv antibody.

In one embodiment the combining is characterized by incubating the antibody Fab fragment or a scFv antibody fragment and the antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, with a Sortase A enzyme.

15 In one embodiment the Fab fragment or scFv antibody fragment comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue).

20 In one embodiment the full length antibody heavy chain and the full length antibody light chain of the one-armed antibody fragment are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second surface marker, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus.

25 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises within the 20 C-terminal amino acid residues the amino acid sequence G_nSLPX1TG (SEQ ID NO: 02, wherein X1 can be any amino acid residue, with n = 1, 2 or 3).

30 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises within the 20 C-terminal amino acid residues the amino acid sequence GSLPX1TGGSGS (SEQ ID NO: 03, wherein X1 can be any amino acid residue).

- 10 -

In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises within the 20 C-terminal amino acid residues the amino acid sequence GGGSLPX1TGGSGS (SEQ ID NO: 04, wherein X1 can be any amino acid residue).

5 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence X2GSLPX1TGGSGS (SEQ ID NO: 05, wherein X1 can be any amino acid residue) within the 20 C-terminal amino acid residues whereby X2 can be any amino acid residue except G.

10 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence G_n SLPX1TGGSGSX3 (SEQ ID NO: 06, wherein X1 can be any amino acid residue, with $n=1, 2$ or 3) within the 20 C-terminal amino acid residues, whereby X3 is an amino acid sequence tag.

15 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence X2GSLPX1TGGSGSX3 (SEQ ID NO: 07, wherein X1 can be any amino acid residue) within the 20 C-terminal amino acid residues, whereby X2 can be any amino acid residue except G, and whereby X3 is an amino acid sequence tag.

In one embodiment of all aspects the antibody heavy chain Fc-region polypeptide comprises two glycine residues at its N-terminus.

20 In one embodiment of all aspects the one-armed antibody fragment comprises the amino acid sequence GGCPX4C (SEQ ID NO: 08) at the N-terminus of its heavy chain, whereby X4 is either S or P.

In one embodiment of all aspects X1 is E.

25 One aspect as reported herein is a multispecific binding molecule obtained by a method as reported herein.

One aspect is a multispecific binding molecule comprising the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue) in one of its heavy chains.

30 In one embodiment the multispecific binding molecule comprises the amino acid sequence G_n SLPX1TG (SEQ ID NO: 02, wherein X1 can be any amino acid residue, with $n = 1, 2$ or 3) in one of its heavy chains.

- 11 -

In one embodiment the multispecific binding molecule comprises the amino acid sequence G_n SLPX1TGGCPX4C (SEQ ID NO: 09, wherein X1 can be any amino acid residue, wherein X4 can be S or P, with $n = 1, 2$ or 3) in one of its heavy chains.

5 In one embodiment the multispecific binding molecule comprises the amino acid sequence X2GSLPX1TGGCPX4C (SEQ ID NO: 10, wherein X1 can be any amino acid residue, wherein X4 can be S or P) in one of its heavy chains, whereby X2 can be any amino acid residue except G.

10 One aspect as reported herein is a bispecific antibody obtained by a method as reported herein.

One aspect is a bispecific antibody comprising the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue) in one of its heavy chains.

15 In one embodiment the bispecific antibody comprises the amino acid sequence G_n SLPX1TG (SEQ ID NO: 02, wherein X1 can be any amino acid residue, with $n=1, 2$ or 3) in one of its heavy chains.

In one embodiment the bispecific antibody comprises the amino acid sequence G_n SLPX1TGGCPX4C (SEQ ID NO: 09, wherein X1 can be any amino acid residue, wherein X4 can be S or P, with $n = 1, 2$ or 3) in one of its heavy chains.

20 In one embodiment the bispecific antibody comprises the amino acid sequence X2GSLPX1TGGCPX4C (SEQ ID NO: 10, wherein X1 can be any amino acid residue, wherein X4 can be S or P) in one of its heavy chains, whereby X2 can be any amino acid residue except G.

25 One aspect as reported herein is a pharmaceutical formulation comprising a multispecific binding molecule as reported herein.

One aspect as reported herein is the use of a multispecific binding molecule as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of cancer.

- 12 -

One aspect as reported herein is a method of treating an individual having cancer comprising administering to the individual an effective amount of a multispecific binding molecule as reported herein.

5 One aspect as reported herein is a method for destroying cancer cells in an individual comprising administering to the individual an effective amount of a multispecific binding molecule as reported herein.

One aspect as reported herein is a pharmaceutical formulation comprising a bispecific antibody as reported herein.

10 One aspect as reported herein is the use of a bispecific antibody as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of cancer.

One aspect as reported herein is a method of treating an individual having cancer comprising administering to the individual an effective amount of a bispecific antibody as reported herein.

15 One aspect as reported herein is a method for destroying cancer cells in an individual comprising administering to the individual an effective amount of a bispecific antibody as reported herein. In one embodiment of all aspects as reported herein the Fc-region is a human Fc-region or a variant thereof.

20 In one embodiment the human antibody Fc-region is of human IgG1 subclass, or of human IgG2 subclass, or of human IgG3 subclass, or of human IgG4 subclass.

In one embodiment the antibody Fc-region is a human antibody Fc-region of the human IgG1 subclass, or of the human IgG4 subclass.

25 In one embodiment the human antibody Fc-region comprises a mutation of the naturally occurring amino acid residue at least at one of the following amino acid positions 228, 233, 234, 235, 236, 237, 297, 318, 320, 322, 329, and/or 331 to a different residue, wherein the residues in the antibody Fc-region are numbered according to the EU index of Kabat.

30 In one embodiment the human antibody Fc-region comprises a mutation of the naturally occurring amino acid residue at position 329 and at least one further mutation of at least one amino acid residue selected from the group comprising

- 13 -

amino acid residues at position 228, 233, 234, 235, 236, 237, 297, 318, 320, 322 and 331 to a different residue, wherein the residues in the Fc-region are numbered according to the EU index of Kabat. The change of these specific amino acid residues results in an altering of the effector function of the Fc-region compared to the non-modified (wild-type) Fc-region.

5

In one embodiment the human antibody Fc-region has a reduced affinity to the human FcγRIIIA, and/or FcγRIIA, and/or FcγRI compared to a conjugate comprising the corresponding wild-type IgG Fc-region.

In one embodiment the amino acid residue at position 329 in the human antibody Fc-region is substituted with glycine, or arginine, or an amino acid residue large enough to destroy the proline sandwich within the Fc-region.

10

In one embodiment the mutation in the human antibody Fc-region of the naturally occurring amino acid residue is at least one of S228P, E233P, L234A, L235A, L235E, N297A, N297D, P329G, and/or P331S.

In one embodiment the mutation is L234A and L235A if the antibody Fc-region is of human IgG1 subclass, or S228P and L235E if the antibody Fc-region is of human IgG4 subclass.

15

In one embodiment the antibody Fc-region comprises the mutation P329G.

In one embodiment the antibody Fc-region comprises the mutation T366W in the first heavy chain Fc-region polypeptide and the mutations T366S, L368A and Y407V in the second heavy chain Fc-region polypeptide, wherein the numbering is according to the EU index of Kabat.

20

In one embodiment the antibody Fc-region comprises the mutation S354C in the first heavy chain Fc-region polypeptide and the mutation Y349C in the second heavy chain Fc-region polypeptide.

25

Detailed Description of embodiments of the Invention

I. DEFINITIONS

In the present specification and claims the numbering of the residues in an immunoglobulin heavy chain Fc-region is that of the EU index of Kabat (Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health

30

- 14 -

Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91-3242, expressly incorporated herein by reference).

5 The term "alteration" denotes the mutation, addition, or deletion of one or more amino acid residues in a parent amino acid sequence, e.g. of an antibody or fusion polypeptide comprising at least an FcRn binding portion of an Fc-region, to obtain a variant antibody or fusion polypeptide.

10 The term "amino acid mutation" denotes a modification in the amino acid sequence of a parent amino acid sequence. Exemplary modifications include amino acid substitutions, insertions, and/or deletions. In one embodiment the amino acid mutation is a substitution. The term "amino acid mutations at the position" denotes the substitution or deletion of the specified residue, or the insertion of at least one amino acid residue adjacent the specified residue. The term "insertion adjacent to a specified residue" denotes the insertion within one to two residues thereof. The insertion may be N-terminal or C-terminal to the specified residue.

15 The term "amino acid substitution" denotes the replacement of at least one amino acid residue in a predetermined parent amino acid sequence with a different "replacement" amino acid residue. The replacement residue or residues may be a "naturally occurring amino acid residue" (i.e. encoded by the genetic code) and selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine
20 (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). In one embodiment the replacement residue is not cysteine. Substitution with one or more non-naturally
25 occurring amino acid residues is also encompassed by the definition of an amino acid substitution herein. A "non-naturally occurring amino acid residue" denotes a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include norleucine,
30 ornithine, norvaline, homoserine, aib and other amino acid residue analogues such as those described in Ellman, et al., Meth. Enzym. 202 (1991) 301-336. To generate such non-naturally occurring amino acid residues, the procedures of Noren, et al. (Science 244 (1989) 182) and/or Ellman, et al. (supra) can be used. Briefly, these procedures involve chemically activating a suppressor tRNA with a
35 non-naturally occurring amino acid residue followed by in vitro transcription and

- 15 -

translation of the RNA. Non-naturally occurring amino acids can also be incorporated into peptides via chemical peptide synthesis and subsequent fusion of these peptides with recombinantly produced polypeptides, such as antibodies or antibody fragments.

5 The term “amino acid insertion” denotes the incorporation of at least one additional amino acid residue into a predetermined parent amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, the present application contemplates larger “peptide insertions”, e.g. insertion of about three to about five or even up to about ten amino acid residues. The inserted
10 residue(s) may be naturally occurring or non-naturally occurring as defined above.

The term "amino acid deletion" denotes the removal of at least one amino acid residue at a predetermined position in an amino acid sequence.

Within this application whenever an amino acid alteration is mentioned it is a deliberated amino acid alteration and not a random amino acid modification.

15 The term “amino acid sequence tag” denotes a sequence of amino acid residues connected to each other via peptide bonds that has specific binding properties. In one embodiment the amino acid sequence tag is an affinity or purification tag. In one embodiment the amino acid sequence tag is selected from Arg-tag, His-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag,
20 calmodulin-binding-peptide, cellulose-binding-domain, chitin-binding-domain, GST-tag, or MBP-tag. In one embodiment the amino acid sequence tag is selected from SEQ ID NO: 11 (RRRRR), or SEQ ID NO: 12 (RRRRRR), or SEQ ID NO: 13 (HHHHHH), or SEQ ID NO: 14 (KDHLIHNVHKEFHAAHANK), or SEQ ID NO: 15 (DYKDDDDK), or SEQ ID NO: 16
25 (DYKDHDGDYKDHDIDYKDDDDK), or SEQ ID NO: 17 (AWRHPQFGG), or SEQ ID NO: 18 (WSHPQFEK), or SEQ ID NO: 19 (MDVEAWLGAR), or SEQ ID NO: 20 (MDVEAWLGARVPLVET), or SEQ ID NO: 21 (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP), or SEQ ID NO: 22 (EQKLISEEDL), or SEQ ID NO: 23 (KETAAAKFERQHMD), or SEQ
30 ID NO: 24 (KRRWKKNFIAVSAANRFKKISSSGAL), or SEQ ID NO: 25 (cellulose binding domain), or SEQ ID NO: 26 (cellulose binding domain), or SEQ ID NO: 27 (TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEP SNVPALWQLQ), or SEQ ID NO: 28 (GST-tag), or SEQ ID NO: 29 (MBP-tag).

- 16 -

The term "antibody fragment" denotes a molecule other than a full length antibody that comprises a portion of a full length 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 multispecific antibodies formed from antibody fragments.

Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, F(ab')₂, Fv, and scFv fragments, and other fragments described below. For a review of certain antibody fragments, see Hudson, P.J., et al., Nat. Med. 9 (2003) 129-134. For a review of scFv fragments, see, e.g., Plueckthun, A., In: The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore (eds.), Springer-Verlag, New York (1994), pp. 269-315; see also WO 93/16185; US 5,571,894 and US 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 US 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 0 404 097; WO 1993/01161; Hudson, P.J., et al., Nat. Med. 9 (2003) 129-134; and Holliger, P., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448. Triabodies and tetrabodies are also described in Hudson, P.J., et al., Nat. Med. 9 (2003) 129-134).

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.

The term "bispecific antibody" denotes an antigen binding molecule that can specifically bind to a first antigen or epitope and to a second antigen or epitope, whereby the first antigen or epitope are different from the second antigen or epitope.

- 17 -

Bispecific antibody formats are described e.g. in WO 2009/080251, WO 2009/080252, WO 2009/080253, WO 2009/080254, WO 2010/112193, WO 2010/115589, WO 2010/136172, WO 2010/145792, and WO 2010/145793.

5 The term “antibody-dependent cell-mediated cytotoxicity”, short “ADCC”, denotes a cell-mediated reaction in which non-antigen specific cytotoxic cells that express FcRs (e.g. natural killer cells (NK cells), neutrophils, and macrophages) recognize a target cell by binding to immunoglobulin Fc-region and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express Fc γ RIII only, whereas monocytes express Fc γ RI, Fc γ RII and Fc γ RIII. FcR
10 expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9 (1991) 457-492.

The term “antibody-dependent cellular phagocytosis”, short “ADCP”, denotes a process by which antibody-coated cells are internalized, either in whole or in part, by phagocytic immune cells (e.g. macrophages, neutrophils, or dendritic cells) that
15 bind to an immunoglobulin Fc-region.

The term “binding to an Fc receptor” denotes the binding of an Fc-region to an Fc receptor in, for example, a BIAcore^(R) assay (Pharmacia Biosensor AB, Uppsala, Sweden).

20 In the BIAcore^(R) assay the Fc receptor is bound to a surface and binding of the analyte, e.g. an Fc-region comprising fusion polypeptide or an antibody, is measured by surface plasmon resonance (SPR). The affinity of the binding is defined by the terms k_a (association constant; rate constant for the association of the Fc-region fusion polypeptide or conjugate to form an Fc-region/Fc receptor complex), k_d (dissociation constant; rate constant for the dissociation of the
25 Fc-region fusion polypeptide or conjugate from an Fc-region/Fc receptor complex), and KD (k_d/k_a). Alternatively, the binding signal of a SPR sensorgram can be compared directly to the response signal of a reference, with respect to the resonance signal height and the dissociation behaviors.

30 The term “C1q” denotes a polypeptide that includes a binding site for the Fc-region of an immunoglobulin. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway. Human C1q can be purchased commercially from, e.g. Quidel, San Diego, Calif.

- 18 -

The term "CH2 domain" denotes the part of an antibody heavy chain polypeptide that extends approximately from EU position 231 to EU position 340 (EU numbering system according to Kabat). In one embodiment a CH2 domain has the amino acid sequence of

5 APELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDG
 VEVHNAKTKPREEQESTYRWSVLTVLHQDWLNGKEYKCKVSNKALPAPI
 EKTISKAK (SEQ ID NO: 30). The CH2 domain is unique in that it is not closely
 paired with another domain. Rather, two N-linked branched carbohydrate chains
 are interposed between the two CH2 domains of an intact native Fc-region. It has
 10 been speculated that the carbohydrate may provide a substitute for the domain-
 domain pairing and help stabilize the CH2 domain. Burton, Mol. Immunol. 22
 (1985) 161-206.

The term "CH3 domain" denotes the part of an antibody heavy chain polypeptide that extends approximately from EU position 341 to EU position 446. In one
 15 embodiment the CH3 domain has the amino acid sequence of
 GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
 KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSL
 SLSPG (SEQ ID NO: 31).

The term "class" of an antibody denotes the type of constant domain or constant
 20 region possessed by its heavy chain. There are five major classes of antibodies in
 humans: 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.

25 The term "complement-dependent cytotoxicity", short "CDC", denotes a
 mechanism for inducing cell death in which an Fc-region of a target-bound
 Fc-region fusion polypeptide or conjugate activates a series of enzymatic reactions
 culminating in the formation of holes in the target cell membrane. Typically,
 antigen-antibody complexes such as those on antibody-coated target cells bind and
 30 activate complement component C1q which in turn activates the complement
 cascade leading to target cell death. Activation of complement may also result in
 deposition of complement components on the target cell surface that facilitate
 ADCC or ADCP by binding complement receptors (e.g., CR3) on leukocytes.

- 19 -

The term “effector function” denotes those biological activities attributable to the Fc-region of an antibody, which vary with the antibody subclass. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis (ADCP); down regulation of cell surface receptors (e.g. B-cell receptor); and B-cell activation. Such function can be effected by, for example, binding of an Fc-region to an Fc receptor on an immune cell with phagocytic or lytic activity, or by binding of an Fc-region to components of the complement system.

10 An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term “reduced effector function” denotes a reduction of a specific effector function associated with a molecule, like for example ADCC or CDC, in comparison to a control molecule (for example a polypeptide with a wild-type Fc-region) by at least 20 %. The term “strongly reduced effector function” denotes a reduction of a specific effector function associated with a molecule, like for example ADCC or CDC, in comparison to a control molecule by at least 50 %.

20 The term “Fc-region” denotes the C-terminal region of an immunoglobulin. The Fc-region is a dimeric molecule comprising two disulfide-linked antibody heavy chain fragments (heavy chain Fc-region polypeptide chains). An Fc-region can be generated by papain digestion, or IdeS digestion, or trypsin digestion of an intact (full length) antibody or can be produced recombinantly.

25 The Fc-region obtainable from a full length antibody or immunoglobulin comprises at least residues 226 (Cys) to the C-terminus of the full length heavy chain and, thus, comprises a part of the hinge region and two or three constant domains, i.e. a CH2 domain, a CH3 domain, and an additional/extra CH4 domain on IgE and IgM class antibodies. It is known from US 5,648,260 and US 5,624,821 that the modification of defined amino acid residues in the Fc-region results in phenotypic effects.

30 The formation of the dimeric Fc-region comprising two identical or non-identical antibody heavy chain fragments is mediated by the non-covalent dimerization of the comprised CH3 domains (for involved amino acid residues see e.g. Dall'Acqua, Biochem. 37 (1998) 9266-9273). The Fc-region is covalently stabilized by the

- 20 -

5 formation of disulfide bonds in the hinge region (see e.g. Huber, et al., Nature 264 (1976) 415-420; Thies, et al., J. Mol. Biol. 293 (1999) 67-79). The introduction of amino acid residue changes within the CH3 domain in order to disrupt the dimerization of CH3-CH3 domain interactions do not adversely affect the neonatal Fc receptor (FcRn) binding due to the location of the CH3-CH3-domain dimerization involved residues are located on the inner interface of the CH3 domain, whereas the residues involved in Fc-region-FcRn interaction are located on the outside of the CH2-CH3 domain.

10 The residues associated with effector functions of an Fc-region are located in the hinge region, the CH2, and/or the CH3 domain as determined for a full length antibody molecule. The Fc-region associated/mediated functions are:

- (i) antibody-dependent cellular cytotoxicity (ADCC),
- (ii) complement (C1q) binding, activation and complement-dependent cytotoxicity (CDC),
- 15 (iii) phagocytosis/clearance of antigen-antibody complexes,
- (iv) cytokine release in some instances, and
- (v) half-life/clearance rate of antibody and antigen-antibody complexes.

20 The Fc-region associated effector functions are initiated by the interaction of the Fc-region with effector function specific molecules or receptors. Mostly antibodies of the IgG1 subclass can effect receptor activation, whereas antibodies of the IgG2 and IgG4 subclasses do not have effector function or have limited effector function.

25 The effector function eliciting receptors are the Fc receptor types (and sub-types) Fc γ RI, Fc γ RII and Fc γ RIII. The effector functions associated with an IgG1 subclass can be reduced by introducing specific amino acid changes in the lower hinge region, such as L234A and/or L235A, which are involved in Fc γ R and C1q binding. Also certain amino acid residues, especially located in the CH2 and/or CH3 domain, are associated with the circulating half-life of an antibody molecule or an Fc-region fusion polypeptide in the blood stream. The circulatory half-life is determined by the binding of the Fc-region to the neonatal Fc receptor (FcRn).

30 The sialyl residues present on the Fc-region glycostructure are involved in anti-inflammatory mediated activity of the Fc-region (see e.g. Anthony, R.M., et al., Science 320 (2008) 373-376).

- 21 -

The numbering of the amino acid residues in the constant region of an antibody is made according to the EU index of Kabat (Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91 3242).

5 The term “human Fc-region” denotes the C-terminal region of an immunoglobulin heavy chain of human origin that contains at least a part of the hinge region, the CH2 domain and the CH3 domain. In one embodiment, a human IgG antibody heavy chain Fc-region extends from about Glu216, or from about Cys226, or from about Pro230, to the carboxyl-terminus of the heavy chain. However, the
10 C-terminal lysine (Lys447) of the antibody Fc-region may or may not be present.

The term “variant Fc-region” denotes an amino acid sequence which differs from that of a “native” or “wild-type” Fc-region amino acid sequence by virtue of at least one “amino acid alteration/mutation”. In one embodiment the variant Fc-region has at least one amino acid mutation compared to a native Fc-region or
15 to the Fc-region of a parent polypeptide, e.g. from about one to about ten amino acid mutations, and in one embodiment from about one to about five amino acid mutations in a native Fc-region or in the Fc-region of the parent polypeptide. In one embodiment the (variant) Fc-region has at least about 80 % homology with a wild-type Fc-region and/or with an Fc-region of a parent polypeptide, and in one
20 embodiment the variant Fc-region has least about 90 % homology, in one embodiment the variant Fc-region has at least about 95 % homology.

The variant Fc-regions as reported herein are defined by the amino acid alterations that are contained. Thus, for example, the term P329G denotes a variant Fc-region with the mutation of proline to glycine at amino acid position 329 relative to the
25 parent (wild-type) Fc-region. The identity of the wild-type amino acid may be unspecified, in which case the aforementioned variant is referred to as 329G. For all positions discussed in the present invention, numbering is according to the EU index. The EU index or EU index as in Kabat or EU numbering scheme refers to the numbering of the EU antibody (Edelman, et al., Proc. Natl. Acad. Sci. USA 63 (1969) 78-85, hereby entirely incorporated by reference.) The alteration can be an
30 addition, deletion, or mutation. The term “mutation” denotes a change to naturally occurring amino acids as well as a change to non-naturally occurring amino acids, see e.g. US 6,586,207, WO 98/48032, WO 03/073238, US 2004/0214988, WO 2005/35727, WO 2005/74524, Chin, J.W., et al., J. Am. Chem. Soc. 124 (2002) 9026-9027; Chin, J.W. and Schultz, P.G., ChemBioChem 11 (2002) 1135-
35

- 22 -

1137; Chin, J.W., et al., PICAS United States of America 99 (2002) 11020-11024; and, Wang, L. and Schultz, P.G., Chem. (2002) 1-10 (all entirely incorporated by reference herein).

5 A polypeptide chain of a wild-type human Fc-region of the IgG1 subclass has the following amino acid sequence:

CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSV
 10 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 32).

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with the mutations L234A, L235A has the following amino acid sequence:

CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 15 NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSS
 VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 33).

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a T366S, 368A, and Y407V mutation has the following amino acid sequence:

20 CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSSV
 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 34).

25 A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a T366W mutation has the following amino acid sequence:

CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDI
 30 AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSV
 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 35).

- 23 -

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a L234A, L235A and T366S, 368A, and Y407V mutation has the following amino acid sequence:

5 CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPISRDELTKNQVSLSCAVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 36).

10 A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a L234A, L235A and T366W mutation has the following amino acid sequence:

CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
 15 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 37).

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a P329G mutation has the following amino acid sequence:

20 CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 38).

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a L234A, L235A and P329G mutation has the following amino acid sequence:

25 CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 39).

- 24 -

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a P239G and T366S, 368A, and Y407V mutation has the following amino acid sequence:

5 CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALGAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV
 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 40).

10 A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a P329G and T366W mutation has the following amino acid sequence:

15 CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
 YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
 MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 41).

A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a L234A, L235A, P329G and T366S, 368A, and Y407V mutation has the following amino acid sequence:

20 CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 NKALGAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 42).

25 A polypeptide chain of a variant human Fc-region of the IgG1 subclass with a L234A, L235A, P329G and T366W mutation has the following amino acid sequence:

30 CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 NKALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 43).

- 25 -

A polypeptide chain of a wild-type human Fc-region of the IgG4 subclass has the following amino acid sequence:

5 CPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNW
YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVM
HEALHNHYTQKSLSLGLGK (SEQ ID NO: 44).

A polypeptide chain of a variant human Fc-region of the IgG4 subclass with a S228P and L235E mutation has the following amino acid sequence:

10 CPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNW
YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVM
HEALHNHYTQKSLSLGLGK (SEQ ID NO: 45).

15 A polypeptide chain of a variant human Fc-region of the IgG4 subclass with a S228P, L235E and P329G mutation has the following amino acid sequence:

20 CPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNW
YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
GLGSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVM
HEALHNHYTQKSLSLGLGK (SEQ ID NO: 46).

The term “Fc receptor”, short “FcR”, denotes a receptor that binds to an Fc-region. In one embodiment the FcR is a native sequence human FcR. Moreover, in one embodiment the FcR is an FcR which binds an IgG antibody (an Fc gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms thereof. FcγRII receptors include FcγRIIA (an “activating receptor”) and FcγRIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see e.g. Daëron, M., Annu. Rev. Immunol. 15 (1997) 203-234). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol. 9

25

30

- 26 -

(1991) 457-492, Capel, et al., *Immunomethods* 4 (1994) 25-34, de Haas, et al., *J. Lab. Clin. Med.* 126 (1995) 330-341. Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (see e.g. Guyer, et al., *J. Immunol.* 117 (1976) 587; Kim, et al., *J. Immunol.* 24 (1994) 249).

The term “Fc gamma receptor”, short “Fc γ R”, denotes any member of the family of proteins that bind the IgG antibody Fc-region and is encoded by an Fc γ R gene. In humans this family includes but is not limited to Fc γ RI (CD64), including isoforms Fc γ RIA, Fc γ RIB, and Fc γ RIC, Fc γ RII (CD32), including isoforms Fc γ RIIA (including allotypes H131 and R131), Fc γ RIIB (including Fc γ RIIB-1 and Fc γ RIIB-2), and Fc γ RIIC, and Fc γ RIII (CD16), including isoforms Fc γ RIIIA (including allotypes V158 and F158) and Fc γ RIIIB (including allotypes Fc γ RIIB-NA1 and Fc γ RIIB-NA2) (see e.g. Jefferis, et al., *Immunol. Lett.* 82 (2002) 57-65, entirely incorporated by reference), as well as any undiscovered human Fc γ Rs or Fc γ R isoforms or allotypes. An Fc γ R may be from any organism, including but not limited to humans, mice, rats, rabbits, and monkeys. Mouse Fc γ Rs include but are not limited to Fc γ RI (CD64), Fc γ RII (CD32), Fc γ RIII (CD16), and Fc γ RIII-2 (CD16-2), as well as any undiscovered mouse Fc γ Rs or Fc γ R isoforms or allotypes. The Fc-region-Fc γ R interaction involved amino acid residues are 234-239 (lower hinge region), 265-269 (B/C loop), 297-299 (D/E loop), and 327-332 (F/G) loop (Sondermann, et al., *Nature* 406 (2000) 267-273). Amino acid mutations that result in a decreased binding/affinity for the Fc γ RI, Fc γ RIIA, Fc γ RIIB, and/or Fc γ RIIIA include N297A (concomitantly with a decreased immunogenicity and prolonged half-life binding/affinity) (Routledge, et al., *Transplantation* 60 (1995) 847; Friend, et al., *Transplantation* 68 (1999) 1632; Shields, et al., *J. Biol. Chem.* 276 (2001) 6591-6604), residues 233-236 (Ward and Ghetie, *Ther. Immunol.* 2 (1995) 77; Armour, et al., *Eur. J. Immunol.* 29 (1999) 2613-2624). Some exemplary amino acid substitutions are described in US 7,355,008 and US 7,381,408.

The term “neonatal Fc Receptor”, short “FcRn”, denotes a protein that binds the IgG antibody Fc-region and is encoded at least in part by an FcRn gene. The FcRn may be from any organism, including but not limited to humans, mice, rats, rabbits, and monkeys. As is known in the art, the functional FcRn protein comprises two polypeptides, often referred to as the heavy chain and light chain. The light chain is beta-2-microglobulin and the heavy chain is encoded by the FcRn gene. Unless

- 27 -

otherwise noted herein, FcRn or an FcRn protein refers to the complex of FcRn heavy chain with beta-2-microglobulin. The interacting amino acid residues of the Fc-region with the FcRn are near the junction of the CH2 and CH3 domains. The Fc-region-FcRn contact residues are all within a single IgG heavy chain. The involved amino acid residues are 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 (all in the CH2 domain) and amino acid residues 385-387, 428, and 433-436 (all in the CH3 domain). Amino acid mutations that result in an increased binding/affinity for the FcRn include T256A, T307A, E380A, and N434A (Shields, et al., J. Biol. Chem. 276 (2001) 6591-6604).

10 The term “full length antibody” denotes an antibody that has a structure and amino acid sequence substantially identical to a native antibody structure as well as polypeptides that comprise the Fc-region as reported herein.

The term “full length antibody heavy chain” denotes a polypeptide comprising in N- to C-terminal direction an antibody variable domain, a first constant domain, an antibody heavy chain hinge region, a second constant domain, and a third constant domain.

The term “antibody heavy chain Fc-region” denotes a polypeptide comprising an antibody heavy chain hinge region, a first constant domain, and a second constant domain.

20 The term “full length antibody light chain” denotes a polypeptide comprising in N- to C-terminal direction an antibody variable domain and a constant domain.

The term "hinge region" denotes the part of an antibody heavy chain polypeptide that joins in a wild-type antibody heavy chain the CH1 domain and the CH2 domain, e. g. from about position 216 to about position 230 according to the EU number system of Kabat, or from about position 226 to about position 230 according to the EU number system of Kabat. The hinge regions of other IgG subclasses can be determined by aligning with the hinge-region cysteine residues of the IgG1 subclass sequence.

30 The hinge region is normally a dimeric molecule consisting of two polypeptides with identical amino acid sequence. The hinge region generally comprises about 25 amino acid residues and is flexible allowing the antigen binding regions to move independently. The hinge region can be subdivided into three domains: the upper,

- 28 -

the middle, and the lower hinge domain (see e.g. Roux, et al., J. Immunol. 161 (1998) 4083).

5 The term “lower hinge region” of an Fc-region denotes the stretch of amino acid residues immediately C-terminal to the hinge region, i.e. residues 233 to 239 of the Fc-region according to the EU numbering of Kabat.

10 The term “wild-type Fc-region” denotes an amino acid sequence identical to the amino acid sequence of an Fc-region found in nature. Wild-type human Fc-regions include a native human IgG1 Fc-region (non-A and A allotypes), native human IgG2 Fc-region, native human IgG3 Fc-region, and native human IgG4 Fc-region as well as naturally occurring variants thereof.

15 The term “individual” or “subject” denotes 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). In certain embodiments, the individual or subject is a human.

20 “Percent (%) amino acid sequence identity” with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code.

25

30

- 29 -

The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

5 In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

10 $100 \text{ times the fraction } X/Y$

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

20 The term "pharmaceutical formulation" 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.

25 A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, 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.

30 The term "phenotype of a patient" denotes the composition of cell surface receptors in a kind of cells from a patient. The composition can be a qualitative as well as a quantitative composition. The cells for which the genotype is determined/given can be a single cell or a sample comprising multiple cells.

The term "position" denotes the location of an amino acid residue in the amino acid sequence of a polypeptide. Positions may be numbered sequentially, or according

- 30 -

to an established format, for example the EU index of Kabat for antibody numbering.

5 The term “altered” FcR binding affinity or ADCC activity denotes a polypeptide that has either enhanced or diminished FcR binding activity and/or ADCC activity compared to a parent polypeptide (e.g. a polypeptide comprising a wild-type Fc-region). The variant polypeptide which “has increased binding” to an FcR binds at least one FcR with lower dissociation constant (i.e. better/higher affinity) than the parent or wild-type polypeptide. The polypeptide variant which “has decreased binding” to an FcR, binds at least one FcR with higher dissociation constant (i.e. worse/lower affinity) than the parent or a wild-type polypeptide. Such variants 10 which display decreased binding to an FcR may possess little or no appreciable binding to an FcR, e.g., 0 – 20 % binding to the FcR compared to a wild-type or parent IgG Fc-region.

15 The polypeptide which binds an FcR with “reduced affinity” in comparison with a parent or wild-type polypeptide, is a polypeptide which binds any one or more of the above identified FcRs with (substantially) reduced binding affinity compared to the parent polypeptide, when the amounts of polypeptide variant and parent polypeptide in the binding assay are (essentially) about the same. For example, the polypeptide variant with reduced FcR binding affinity may display from about 1.15 20 fold to about 100 fold, e.g. from about 1.2 fold to about 50 fold reduction in FcR binding affinity compared to the parent polypeptide, where FcR binding affinity is determined.

25 The polypeptide comprising a variant Fc-region which “mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells less effectively” than a parent polypeptide is one which in vitro or in vivo is (substantially) less effective at mediating ADCC, when the amounts of variant polypeptide and parent polypeptide used in the assay are (essentially) about the same. Generally, such variants will be identified using the in vitro ADCC assay as disclosed herein, but other assays or methods for determining ADCC activity, e.g. 30 in an animal model etc., are contemplated. In one embodiment the variant is from about 1.5 fold to about 100 fold, e.g. from about two fold to about fifty fold, less effective at mediating ADCC than the parent, e.g. in the in vitro assay disclosed herein.

- 31 -

The term “receptor” denotes a polypeptide capable of binding at least one ligand. In one embodiment the receptor is a cell-surface receptor having an extracellular ligand-binding domain and, optionally, other domains (e.g. transmembrane domain, intracellular domain and/or membrane anchor). The receptor to be evaluated in the
5 assay described herein may be an intact receptor or a fragment or derivative thereof (e.g. a fusion protein comprising the binding domain of the receptor fused to one or more heterologous polypeptides). Moreover, the receptor to be evaluated for its binding properties may be present in a cell or isolated and optionally coated on an assay plate or some other solid phase.

10 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 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,
15 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, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

20 **II. Tailor-made multispecific binding molecules**

In most cell based diseases the targeting of the disease-related cells via antibody based binding of receptor molecules is one promising approach. However, the expression level of clinically relevant surface receptors (=target) varies from patient to patient and efficacy of standardized antibody based drugs is thus very
25 different. This applies specifically for bi- and multispecific binding molecules whose mode of action is to target two different epitopes/receptors simultaneously.

One promising approach is to design a drug (here a bi- or multispecific binding molecule) specifically for the particular/individual situation of the respective patient.

30 Each cell from an individual is different in view of the expressed cell surface molecules, such as receptors, in number and kind. This is especially true for cancer cells and non-cancer cells. Thus, a cell can be characterized by the cell surface molecules presented.

- 32 -

Based on expression profile data of clinically relevant surface receptors on disease-associated cells of a patient a series of binding entities (for example Fab fragments) are specifically chosen from a library and combined to a multispecific binding molecule as the patient specific drug. These selected binding molecules are specifically chosen with respect to the respective disease-associated cell such as e.g. a tumor cell based e.g. on the expression level of surface receptors and, thus, the need and phenotype of the individual patient.

Such a characterization can be effected by in vitro and in vivo based cell imaging techniques. In vivo imaging techniques include e.g. optical imaging, molecular imaging, fluorescence imaging, bioluminescence imaging, MRI, PET, SPECT, CT, and intravital microscopy. In vitro imaging techniques include e.g. immunohistochemical staining of patient cells with e.g. fluorescently labeled antibodies recognizing specific cell surface markers and analysis of the fluorescence signals by microscopy. Alternatively the genotype/phenotype of the cells can be analyzed after staining with labeled therapeutic or diagnostic antibodies using FACS-based methods.

In one embodiment the genotype/phenotype of patient-derived cells is determined by a FACS-based method. In one embodiment the cell surface markers are determined by using fluorescently labeled diagnostic or therapeutic antibodies. In one embodiment fluorescently labeled therapeutic antibodies are used.

Certain diseases can be correlated with a change in the number of specific cell surface molecules or with occurrence of a new cell surface molecule.

Individuals affected by such a disease will display within certain ranges a disease and/or an individual-specific cell surface marker pattern.

This has to be taken into consideration in order to provide to such an individual a tailor-made, targeted therapeutic.

A number of therapeutic antibodies directed against cell surface molecules and their ligands are known which can be used for the selection and construction of tailor-made multi-specific targeting entities, such as Rituxan/MabThera/Rituximab, 2H7/Ocrelizumab, Zevalin/Ibrizumomab, Arzerra/Ofatumumab (CD20), HLL2/Epratuzumab, Inotuzomab (CD22), Zenapax/Daclizumab, Simulect/Basiliximab (CD25), Herceptin/Trastuzumab, Pertuzumab (Her2/ERBB2), Mylotarg/Gemtuzumab (CD33), Raptiva/Efalizumab (Cd11a),

- 33 -

5 Erbitux/Cetuximab (EGFR, epidermal growth factor receptor), IMC-1121B (VEGF receptor 2), Tysabri/Natalizumab (α 4-subunit of α 4 β 1 and α 4 β 7 integrins), ReoPro/Abciximab (gpIIb-gpIIa and α v β 3-integrin), Orthoclone OKT3/Muromonab-CD3 (CD3), Benlysta/Belimumab (BAFF), Tolerx/Oteliximab (CD3), Soliris/Eculizumab (C5 complement protein), Actemra/Tocilizumab (IL-6R), Panorex/Edrecolomab (EpCAM, epithelial cell adhesion molecule), CEA-CAM5/Labetuzumab (CD66/CEA, carcinoembryonic antigen), CT-11 (PD-1, programmed death-1 T-cell inhibitory receptor, CD-d279), H224G11 (c-Met receptor), SAR3419 (CD19), IMC-A12/Cixutumumab (IGF-1R, insulin-like growth factor 1 receptor), MEDI-575 (PDGF-R, platelet-derived growth factor receptor), CP-675, 206/Tremelimumab (cytotoxic T lymphocyte antigen 4), RO5323441 (placenta growth factor or PGF), HGS1012/Mapatumumab (TRAIL-R1), SGN-70 (CD70), Vedotin(SGN-35)/Brentuximab (CD30), and ARH460-16-2 (CD44).

15 For the determination of the cell surface markers present in a sample of e.g. a patient, different methods are known. One exemplary method is based on fluorescence activated cell sorting (FACS), in particular, the analysis of specifically stained and sorted cell populations. In this method the phenotyping of the sample (cell population) is achieved by analyzing individual cells with respect to the presented cell surface markers using fluorescently labeled antibodies directed against these markers optionally including the statistical distribution of surface markers in the cell population. It is especially suitable to use therapeutic antibodies that have been labeled with a fluorescent label for this purpose as therewith it is ensured that the later tailor-made multispecific binding molecule will bind to the same epitope as the diagnostic antibody. The multispecific binding molecules/bispecific antibodies as reported herein can be used in the preparation of medicaments for the treatment of e.g. an oncologic disease, a cardiovascular disease, an infectious disease, an inflammatory disease, an autoimmune disease, a metabolic (e.g., endocrine) disease, or a neurological (e.g. neurodegenerative) disease. Exemplary non-limiting examples of these diseases are Alzheimer's disease, non-Hodgkin's lymphomas, B-cell acute and chronic lymphoid leukemias, Burkitt lymphoma, Hodgkin's lymphoma, hairy cell leukemia, acute and chronic myeloid leukemias, T-cell lymphomas and leukemias, multiple myeloma, glioma, Waldenstrom's macroglobulinemia, carcinomas (such as carcinomas of the oral cavity, gastrointestinal tract, colon, stomach, pulmonary tract, lung, breast, ovary, prostate, uterus, endometrium, cervix, urinary bladder, pancreas, bone, liver, gall

bladder, kidney, skin, and testes), melanomas, sarcomas, gliomas, and skin cancers, acute idiopathic thrombocytopenic purpura, chronic idiopathic thrombocytopenic purpura, dermatomyositis, Sydenham's chorea, myasthenia gravis, systemic lupus erythematosus, lupus nephritis, rheumatic fever, polyglandular syndromes, bullous pemphigoid, diabetes mellitus, Henoch-Schonlein purpura, post-streptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, rheumatoid arthritis, multiple sclerosis, sarcoidosis, ulcerative colitis, erythema multiforme, IgA nephropathy, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, Sjogren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, scleroderma, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pemphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis/polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis, psoriasis, or fibrosing alveolitis.

A number of cell surface markers and their ligands are known. For example cancer cells have been reported to express at least one of the following cell surface markers and or ligands, including but not limited to, carbonic anhydrase IX, alpha-fetoprotein, alpha-actinin-4, A3 (antigen specific for A33 antibody), ART-4, B7, Ba-733, BAGE, BrE3-antigen, CA125, CAMEL, CAP-1, CASP-8/m, CCCL19, CCCL21, CD1, CD1a, CD2, CD3, CD4, CDS, CD8, CD1-1A, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CDC27, CDK-4/m, CDKN2A, CXCR4, CXCR7, CXCL12, HIF-1-alpha, colon-specific antigen-p (CSAp), CEA (CEACAM5), CEACAM6, c-met, DAM, EGFR, EGFRvIII, EGP-1, EGP-2, ELF2-M, Ep-CAM, Flt-1, Flt-3, folate receptor, G250 antigen, GAGE, GROB, HLA-DR, HM1.24, human chorionic gonadotropin (HCG) and its subunits, HER2/neu, HMGB-1, hypoxia inducible factor (HIF-1), HSP70-2M, HST-2or 1a, IGF-1R, IFN-gamma, IFN-alpha, IFN-beta, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, insulin-like growth factor-1 (IGF-1), KC4-antigen, KS-1-antigen, KS1-4, Le-Y, LDR/FUT, macrophage migration inhibitory factor (MIF), MAGE, MAGE-3, MART-1, MART-2, NY-ESO-1, TRAG-3, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5, MUM-1/2, MUM-3, NCA66, NCA95, NCA90, pancreatic cancer mucin, placental growth factor, p53, PLAGL2, prostatic acid phosphatase, PSA, PRAME,

- 35 -

PSMA, P1GF, ILGF, ILGF-1R, IL-6, IL-25, RS5, RANTES, T101, SAGE, S100, survivin, survivin-2B, TAC, TAG-72, tenascin, TRAIL receptors, TNF-alpha, Tn-antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, VEGFR, ED-B fibronectin, WT-1, 17-1A-antigen, complement factors C3, C3a, C3b, C5a, C5, an angiogenesis marker, bcl-2, bcl-6, Kras, cMET, an oncogene marker and an oncogene product (see, e.g., Sensi, et al., Clin. Cancer Res. 12 (2006) 5023-5032; Parmiani, et al, J. Immunol. 178 (2007) 1975-1979; Novellino, et al., Cancer Immunol. Immunother. 54 (2005) 187-207).

Thus, antibodies recognizing specific cell surface receptors including their ligands can be used for specific and selective targeting and binding to a number/multitude of cell surface markers that are associated with a disease. A cell surface marker is a polypeptide located on the surface of a cell (e.g. a disease-related cell) that is e.g. associated with signaling event or ligand binding.

In one embodiment, for the treatment of cancer/tumors multispecific binding molecules/bispecific antibodies are used that target tumor-associated antigens, such as those reported in Herberman, "Immunodiagnosis of Cancer", in Fleisher (ed.), "The Clinical Biochemistry of Cancer", page 347 (American Association of Clinical Chemists (1979)) and in US 4,150,149; US 4,361,544; and US 4,444,744.

Reports on tumor associated antigens (TAAs) include Mizukami, et al., (Nature Med. 11 (2005) 992-997); Hatfield, et al., (Curr. Cancer Drug Targets 5 (2005) 229-248); Vallbohmer, et al., (J Clin. Oncol. 23 (2005) 3536-3544); and Ren, et al., (Ann. Surg. 242 (2005) 55-63), each incorporated herein by reference with respect to the TAAs identified.

Where the disease involves a lymphoma, leukemia or autoimmune disorder, targeted antigens may be selected from the group consisting of CD4, CD5, CD8, CD14, CD15, CD19, CD20, CD21, CD22, CD23, CD25, CD33, CD37, CD38, CD40, CD40L, CD46, CD54, CD67, CD74, CD79a, CD80, CD126, CD138, CD154, CXCR4, B7, MUC1 or Ia, HM1.24, HLA-DR, tenascin, VEGF, P1GF, ED-B fibronectin, an oncogene, an oncogene product (e.g., c-met or PLAGL2), CD66a-d, necrosis antigens, IL-2, T101, TAG, IL-6, MIF, TRAIL-R1 (DR4) and TRAIL-R2 (DR5).

A number of bispecific antibodies are known directed against two different targets, such as BCMA/CD3, different antigens of the HER family in combination (EGFR, HER2, HER3), CD19/CD3, IL17RA/IL7R, IL-6/IL-23, IL-1-beta/IL-8, IL-6 or

- 36 -

IL-6R/ IL-21 or IL-21R, first specificity directed to a glycoepitope of an antigen selected from the group consisting of Lewis x-, Lewis b- and Lewis y-structures, Globo H-structures, KH1, Tn-antigen, TF-antigen and carbohydrate structures of Mucins, CD44, glycolipids and glycosphingolipids, such as Gg3, Gb3, GD3, GD2, Gb5, Gm1, Gm2, sialyltetraosylceramide and a second specificity directed to an ErbB receptor tyrosine kinase selected from the group consisting of EGFR, HER2, HER3 and HER4, GD2 in combination with a second antigen binding site is associated with an immunological cell chosen from the group consisting of T-lymphocytes NK cell, B-lymphocytes, dendritic cells, monocytes, macrophages, neutrophils, mesenchymal stem cells, neural stem cells, ANG2/VEGF, VEGF/PDGFR-beta, Vascular Endothelial Growth Factor (VEGF) acceptor 2/CD3, PSMA/CD3, EPCAM/CD3, combinations of an antigen is selected from a group consisting of VEGFR-1, VEGFR-2, VEGFR-3, FLT3, c-FMS/CSF1R, RET, c-Met, EGFR, Her2/neu, HER3, HER4, IGFR, PDGFR, c-KIT, BCR, integrin and MMPs with a water-soluble ligand is selected from the group consisting of VEGF, EGF, PIGF, PDGF, HGF, and angiopoietin, ERBB-3/C-MET, ERBB-2/C-MET, EGF receptor 1/CD3, EGFR/HER3, PSCA/CD3, C-MET/CD3, ENDOSIALIN/CD3, EPCAM/CD3, IGF-1R/CD3, FAPALPHA/CD3, EGFR/IGF-1R, IL 17A/F, EGF receptor 1/CD3, and CD19/CD16.

Thus, it has been found that by using a modular approach as reported herein tailor-made bispecific therapeutic antibodies can be provided. These antibodies are tailor-made with respect to cell surface molecules actually present on the cells of an individual in need of a treatment or with respect to ligands interacting with such a cell surface molecule. By determining the cell surface molecule status of an individual a tailor-made combination of therapeutic targets can be chosen.

With this tailor-made generation of bispecific therapeutics by combining 2 single therapeutic molecules for simultaneous targeting and binding to two different epitopes an additive/synergistic effect can be expected in comparison to the single therapeutic molecules.

By using already available monospecific therapeutic binding entities, such as those derived from therapeutic antibodies, a fast and easy production of the required multispecific binding molecule can be achieved.

These avidity engineered binding molecules/antibodies can bind to two or more cell surface markers present on a single cell. This binding is only avid if all/both

- 37 -

binding entities simultaneously bind to the cell. For this purpose medium to low affine antibodies are especially suited. This allows also on the other hand to exclude less specific combinations of binding specificities during a screening process.

5 Selected patient specific multispecific binding molecules can be tested in various cellular in vitro assays/cell samples for relevant criteria (for example optimal binding/binding partners, optimal linker length etc.):

- determining the phosphorylation status of phospho tyrosine kinases

- determining c-Jun N-terminal kinase (JNK) inhibition

10 - determining molecule induced apoptosis

- binding assay performed with monospecific vs. multispecific binding molecule

- determining of proliferation inhibition

15 With such an approach the generation of tailor-made and, thus, highly efficient therapeutic molecules is possible. These molecules will have reduced side effects by improved targeting/delivery (e.g. payload for tumor cells) and improved targeting to target cell is based on higher selectivity and specificity of targeting component (comprising at least two binding molecules).

20 The higher selectivity and specificity of multispecific binding molecule is due to simultaneous binding (avidity) by the combination of two „low affinity“ binders, which reduces possible „off-target“ bindings.

Methods as reported herein

One aspect as reported herein is a method for producing a bispecific antibody comprising the step of incubating

25 (i) an antibody Fab fragment or a scFv antibody comprising within the C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue),

(ii) an antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide,

- 38 -

whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site,

5 whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and

whereby the antibody heavy chain Fc-region has an oligoglycine amino acid sequence at its N-terminus,

and

10 (iii) a Sortase A enzyme

and thereby producing the bispecific antibody.

One aspect as reported herein is a method for producing a bispecific antibody comprising the following steps

15 (i) determining surface makers present on the surface of a cell in a sample and selecting thereof a first surface marker and a second surface marker,

20 (ii) incubating (a) an antibody Fab fragment or a scFv antibody fragment comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), whereby the Fab fragment or scFv specifically binds to the first surface marker, (b) an antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second surface marker, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region has an oligoglycine amino acid sequence at its N-terminus, and (c) a Sortase A enzyme

25

30

and thereby producing the bispecific antibody.

- 39 -

One aspect as reported herein is a method for determining a combination of antigen binding sites comprising the following steps

- 5 (i) determining the binding specificity and/or affinity and/or effector function and/or in vivo half-life of a multitude of bispecific antibodies prepared by combining each member of a first multitude of antibody Fab fragments or scFv antibody fragments with each member of a second multitude of antibody fragments comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide,

10 whereby the first multitude specifically binds to a first cell surface molecule and the second multitude specifically binds to a second cell surface molecule,

and

- (ii) choosing the bispecific antibody with suitable binding specificity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

15 In one embodiment the combining is characterized by incubating the antibody Fab fragment or a scFv antibody fragment and the antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, with a Sortase A enzyme.

20 In one embodiment the Fab fragment or scFv antibody fragment comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue).

25 In one embodiment the full length antibody heavy chain and the full length antibody light chain of the one-armed antibody fragment are cognate antibody chains and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second surface marker, the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and the antibody heavy chain Fc-region polypeptide has an oligoglycine amino acid sequence at its N-terminus.

30 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises within the 20 C-terminal amino acid residues the amino acid sequence

- 40 -

G_n SLPX1TG (SEQ ID NO:02, wherein X1 can be any amino acid residue, with $n=1, 2$ or 3).

5 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises within the 20 C-terminal amino acid residues the amino acid sequence GSLPX1TGGSGS (SEQ ID NO: 03, wherein X1 can be any amino acid residue).

In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence X2GSLPX1TGGSGS (SEQ ID NO: 05, wherein X1 can be any amino acid residue, whereby X2 can be any amino acid residue except G).

10 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence G_n SLPX1TGGSGSX3 (SEQ ID NO: 06, wherein X1 can be any amino acid residue, with $n=1, 2$ or 3) within the 20 C-terminal amino acid residues, whereby X3 is an amino acid sequence tag.

15 In one embodiment of all aspects the antibody Fab fragment or the scFv antibody comprises the amino acid sequence X2GSLPX1TGGSGSX3 (SEQ ID NO: 07, wherein X1 can be any amino acid residue) within the 20 C-terminal amino acid residues whereby X2 can be any amino acid residue except G and X3 is an amino acid sequence tag.

20 In one embodiment of all aspects the antibody heavy chain Fc-region polypeptide comprises two glycine residues at its N-terminus.

In one embodiment of all aspects the one armed antibody Fc-region comprises the amino acid sequence GGCPX4C (SEQ ID NO: 08) at the N-terminus of its heavy chain Fc-region polypeptide, whereby X4 is either S or P.

In one embodiment of all aspects X1 is E.

25 One aspect as reported herein is a multispecific binding molecule/bispecific antibody obtained by a method as reported herein.

One aspect is a multispecific binding molecule/bispecific antibody comprising the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue) in one of its heavy chains.

- 41 -

In one embodiment the multispecific binding molecule/bispecific antibody comprises the amino acid sequence G_n SLPX1TG (SEQ ID NO: 02, wherein X1 can be any amino acid residue, with $n=1, 2$ or 3) in one of its heavy chains.

5 In one embodiment the multispecific binding molecule/bispecific antibody comprises the amino acid sequence G_n SLPX1TGGCPX4C (SEQ ID NO: 09, wherein X1 can be any amino acid residue, wherein X4 can be S or P, with $n=1, 2$ or 3) in one of its heavy chains.

10 In one embodiment the multispecific binding molecule/bispecific antibody comprises the amino acid sequence X2GSLPX1TGGCPX4C (SEQ ID NO: 10, wherein X1 can be any amino acid residue, wherein X4 can be S or P) in one of its heavy chains, whereby X2 can be any amino acid residue except G.

In one embodiment X1 is E.

One aspect as reported herein is a pharmaceutical formulation comprising an antibody/multispecific binding molecule as reported herein.

15 One aspect as reported herein is the use of a bispecific antibody/multispecific binding molecule as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of cancer.

20 One aspect as reported herein is a method of treating an individual having cancer comprising administering to the individual an effective amount of a bispecific antibody/multispecific binding molecule as reported herein.

One aspect as reported herein is a method for destroying cancer cells in an individual comprising administering to the individual an effective amount of a bispecific antibody/multispecific binding molecule as reported herein.

25 In one embodiment of all aspects as reported herein the Fc-region is a human Fc-region, or a variant thereof.

In one embodiment the human Fc-region is of the human IgG1 subclass, or of the human IgG2 subclass, or of the human IgG3 subclass, or of the human IgG4 subclass. In one embodiment the Fc-region is a human Fc-region of the human IgG1 subclass or of the human IgG4 subclass.

- 42 -

5 In one embodiment the human Fc-region comprises a mutation of the naturally occurring amino acid residue at least at one of the following amino acid positions 228, 233, 234, 235, 236, 237, 297, 318, 320, 322, 329, and/or 331 to a different residue, wherein the residues in the Fc-region are numbered according to the EU index of Kabat.

10 In one embodiment the human Fc-region comprises a mutation of the naturally occurring amino acid residue at position 329 and at least one further mutation of at least one amino acid selected from the group comprising amino acid residues at position 228, 233, 234, 235, 236, 237, 297, 318, 320, 322 and 331 to a different residue, wherein the residues in the Fc-region are numbered according to the EU index of Kabat. The change of these specific amino acid residues results in an altering of the effector function of the Fc-region compared to the non-modified (wild-type) Fc-region.

15 In one embodiment the human Fc-region has a reduced affinity to the human FcγRIIIA and/or FcγRIIA and/or FcγRI compared to a conjugate comprising the corresponding wild-type IgG Fc-region.

In one embodiment the amino acid residue at position 329 in the human Fc-region is substituted with glycine, or arginine, or an amino acid residue large enough to destroy the proline sandwich within the Fc-region.

20 In one embodiment the mutation of the naturally occurring amino acid residue is S228P, E233P, L234A, L235A, L235E, N297A, N297D, P329G, and/or P331S. In one embodiment the mutation is L234A and L235A if the Fc-region is of human IgG1 subclass or S228P and L235E if the Fc-region is of human IgG4 subclass. In one embodiment the Fc-region comprises the mutation P329G.

25 By the combination of two mutations at defined positions in the Fc-region a complete reduction of the Fc-region associated effector function can be achieved.

The selection of an effector function eliciting Fc-region is dependent on the intended use of the multispecific binding molecules/bispecific antibody.

30 If the desired use is the functional neutralization of a soluble target a non-effector function eliciting subclass or variant should be selected.

If the desired use is the removal of a (soluble) target an effector function eliciting subclass or variant should be selected.

- 43 -

If the desired use is the antagonization of a cell-bound target a non-effector function eliciting subclass or variant should be selected.

If the desired use is the removal of a target presenting cell an effector function eliciting subclass or variant should be selected.

- 5 The circulating half-life of an antibody or antibody Fc-region conjugate can be influenced by modulating the Fc-region-FcRn interaction.

The minimization or even removal of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) can be achieved by so called hinge-region amino acid changes/substitutions.

- 10 The minimization or even removal of the activation of the classical complement cascade can be achieved by so called hinge-region amino acid changes/substitutions.

- 15 An increase of the circulatory half-life of an antibody or antibody Fc-region conjugate can be achieved by increased binding to the neonatal Fc receptor and results in an improved efficacy, a reduced dose or frequency of administration, or an improved delivery to the target. A reduction of the circulatory half-life of an antibody or antibody Fc-region conjugate can be achieved by reduced binding to the neonatal Fc receptor and results in a reduced whole body exposure or an improved target-to-non-target binding ratio.

- 20 Generally, the method as reported herein is applicable to the production of antibody Fc-region conjugates comprising either a wild-type Fc-region or an altered/variant Fc-region.

In one embodiment the Fc-region is a human Fc-region.

- 25 In one embodiment the Fc-region is “conceptual” and, while it does not physically exist, the antibody engineer may decide upon a variant Fc-region to be used.

In one embodiment the nucleic acid encoding the Fc-region part of the antibody Fc-region conjugate is altered to generate a variant nucleic acid sequence encoding the variant Fc-region part of the antibody Fc-region conjugate.

- 30 The nucleic acid encoding the amino acid sequence of the Fc-region part of the antibody Fc-region conjugate can be prepared by a variety of methods known in the

art. These methods include, but are not limited to, preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding the polypeptides of the antibody Fc-region conjugate.

- 5 The Fc-region interacts with a number of receptors or ligands including but not limited to Fc receptors (e.g. Fc γ RI, Fc γ RIIA, Fc γ RIIIA), the complement protein C1q, and other molecules such as proteins A and G. These interactions are essential for a variety of effector functions and downstream signaling events including, but not limited to, antibody dependent cell-mediated cytotoxicity (ADCC), antibody
10 dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC).

In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has at least one or more of the following properties: reduced or ablated effector function (ADCC and/or CDC and/or ADCP), reduced or ablated
15 binding to Fc receptors, reduced or ablated binding to C1q, or reduced or ablated toxicity.

In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) comprises a wild-type Fc-region that has at least two amino acid mutations, additions, or deletions.

- 20 In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has a reduced affinity to a human Fc receptor (Fc γ R) and/or a human complement receptor compared to an antibody or antibody Fc-region conjugate comprising a wild-type human Fc-region.

25 In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) comprises an Fc-region that has a reduced affinity to a human Fc receptor (Fc γ R) and/or human complement receptor compared to an antibody or antibody Fc-region conjugate comprising a wild-type human Fc-region.

30 In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has reduced affinity to at least one of Fc γ RI, Fc γ RII, and/or Fc γ RIIIA. In one embodiment the affinity to Fc γ RI and Fc γ RIIIA is reduced. In one embodiment the affinity to Fc γ RI, Fc γ RII and Fc γ RIIIA is reduced.

In one embodiment the affinity to Fc γ RI, Fc γ RIIIA and C1q is reduced.

- 45 -

In one embodiment the affinity to FcγRI, FcγRII, FcγRIIIA and C1q is reduced.

5 In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has a reduced ADCC compared to an antibody or antibody Fc conjugate comprising a wild-type Fc-region. In one embodiment the ADCC is reduced by at least 20 % compared to the ADCC induced by an Fc-region fusion polypeptide or conjugate comprising a wild-type Fc-region.

10 In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has an ADCC and CDC induced by the Fc-region that is decreased or ablated compared to an antibody Fc-region conjugate comprising a wild-type Fc-region.

In one embodiment the antibody Fc-region conjugate (as produced with the method as reported herein) has a decreased ADCC, CDC, and ADCP compared to an OA-Fc-region conjugate comprising a wild-type Fc-region.

15 In one embodiment the antibody Fc-region conjugate comprises at least one amino acid substitution in the Fc-region that is selected from the group comprising S228P, E233P, L234A, L235A, L235E, N297A, N297D, P329G, and P331S.

In one embodiment the wild-type Fc-region is a human IgG1 Fc-region or a human IgG4 Fc-region.

20 In one embodiment the antibody Fc-region comprises besides a mutation of the amino acid residue proline at position 329 at least one further addition, mutation, or deletion of an amino acid residue in the Fc-region that is correlated with increased stability of the antibody Fc-region conjugate.

25 In one embodiment the further addition, mutation, or deletion of an amino acid residue in the Fc-region is at position 228 and/or 235 of the Fc-region if the Fc-region is of IgG4 subclass. In one embodiment the amino acid residue serine at position 228 and/or the amino acid residue leucine at position 235 is/are substituted by another amino acid. In one embodiment the antibody Fc-region conjugate comprises a proline residue at position 228 (mutation of the serine residue to a proline residue). In one embodiment the antibody Fc-region conjugate comprises a glutamic acid residue at position 235 (mutation of the leucine residue to a glutamic acid residue).

30

- 46 -

In one embodiment the Fc-region comprises three amino acid mutations. In one embodiment the three amino acid mutations are P329G, S228P and L235E mutation (P329G / SPLE).

5 In one embodiment the further addition, mutation, or deletion of an amino acid residue in the Fc-region is at position 234 and/or 235 of the Fc-region if the Fc-region is of IgG1 subclass. In one embodiment the amino acid residue leucine at position 234 and/or the amino acid residue leucine at position 235 is/are mutated to another amino acid.

10 In one embodiment the Fc-region comprises an amino acid mutation at position 234, wherein the leucine amino acid residue is mutated to an alanine amino acid residue.

In one embodiment the Fc-region comprises an amino acid mutation at position 235, wherein the leucine amino acid residue is mutated to an alanine amino acid residue.

15 In one embodiment the Fc-region comprises an amino acid mutation at position 329, wherein the proline amino acid residue is mutated to a glycine amino acid residue, an amino acid mutation at position 234, wherein the leucine amino acid residue is mutated to an alanine amino acid residue, and an amino acid mutation at position 235, wherein the leucine amino acid residue is mutated to an alanine amino acid residue.
20

Fc-region variants with increased affinity for FcRn have longer serum half-lives, and such molecules will have useful applications in methods of treating mammals where long systemic half-life of the administered antibody Fc-region conjugate is desired, e.g., to treat a chronic disease or disorder.

25 Antibody Fc-region conjugates with decreased FcRn binding affinity have shorter serum half-lives, and such molecules will have useful applications in methods of treating mammals where a shorter systemic half-life of the administered antibody Fc-region conjugate is desired, e.g. to avoid toxic side effects or for in vivo diagnostic imaging applications. Fc-region fusion polypeptides or conjugates with
30 decreased FcRn binding affinity are less likely to cross the placenta, and thus may be utilized in the treatment of diseases or disorders in pregnant women.

- 47 -

Fc-regions with altered binding affinity for FcRn is in one embodiment an Fc-region with an amino acid alteration at one or more of the amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439, and/or 447.

The Fc-region is in one embodiment an Fc-region with one or more amino acid alterations at the amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439, and/or 447.

Fc-regions which display increased binding to FcRn comprise in one embodiment one or more amino acid alterations at the amino acid positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, and/or 434.

In one embodiment the Fc-region is an Fc-region of the IgG1 subclass and comprises the amino acid mutations P329G, and/or L234A and L235A.

In one embodiment the Fc-region is an Fc-region of the IgG4 subclass and comprises the amino acid mutations P329G, and/or S228P and L235E.

In one embodiment the antibody Fc-region comprises the mutation T366W in the first heavy chain Fc-region polypeptide and the mutations T366S, L368A and Y407V in the second heavy chain Fc-region polypeptide, wherein the numbering is according to the EU index of Kabat.

In one embodiment the antibody Fc-region comprises the mutation S354C in the first heavy chain Fc-region polypeptide and the mutation Y349C in the second heavy chain Fc-region polypeptide.

Enzymatic conjugation using Sortase A

A bispecific antibody comprising a one-armed antibody (OA-Fc) and one or more antigen binding domains can be obtained by using the enzyme Sortase A.

Many gram-positive bacteria use sortase to covalently anchor a variety of surface proteins including virulence factors to their cell wall (peptidoglycan). Sortases are extracellular membrane associated enzymes. The wild-type *Staphylococcus aureus* Sortase A (SrtA) is a polypeptide of 206 amino acids with an N-terminal membrane-spanning region. In a first step, sortase A recognizes substrate proteins

- 48 -

that contain a LPX1TG amino acid sequence motif and cleaves the amide bond between the Thr and Gly by means of an active-site Cys. This peptide cleaving reaction results in a sortase A thioester intermediate. In a second step the thioester acyl-enzyme intermediate is resolved by nucleophilic attack of an amino group of oligoglycine containing second substrate polypeptide (corresponding to the pentaglycine unit of peptidoglycan in *S. aureus*) leading to a covalently linked cell wall protein and the regeneration of sortase A. In the absence of oligoglycine nucleophiles, the acyl-enzyme intermediate is hydrolyzed by a water molecule.

Sortase-mediated ligation/conjugation has begun to be applied for a variety of protein engineering and bioconjugation purposes. This new technique enables the introduction of natural and unnatural functionalities into LPX1TG-tagged recombinant or chemically synthesized polypeptides. Examples include the covalent attachment of oligoglycine derivatized polymers (e.g. PEG), fluorophores, vitamins (e.g. biotin and folate) lipids, carbohydrates, nucleic acids, synthetic peptides and proteins (e.g. GFP) (Tsukiji, S. and Nagamune, T., *ChemBioChem* 10 (2009) 787-798; Popp, M.W.-L. and Ploegh, H.L., *Angew. Chem. Int. Ed.* 50 (2011) 5024-5032).

It has been shown that a triglycine and even a diglycine motif of the amino component is sufficient for the SrtA-mediated ligation step (Clancy, K.W., et al., *Peptide Science* 94 (2010) 385-396).

For the enzymatic conjugation a soluble truncated sortase A lacking the membrane-spanning region (SrtA; amino acid residues 60-206 of *Staphylococcus aureus* SrtA) can be used (Ton-That, H., et al., *Proc. Natl. Acad. Sci. USA* 96 (1999) 12424-12429; Ilangovan, H., et al., *Proc. Natl. Acad. Sci. USA* 98 (2001) 6056-6061). The truncated soluble sortase A variant can be produced in *E.coli*.

An antibody Fc-region comprising an oligoglycine at least at one of its N-termini (G_m , $m=2$, or 3, or 4, or 5) can be expressed and purified from the supernatant of eukaryotic cells (e.g. HEK293 cells, CHO cells).

A binding entity (e.g. a single chain antigen binding polypeptide such as a scFv, a scFab, or a darpin, or a multi chain antigen binding polypeptide such as a dsFv or a Fab) comprising the SrtA recognition motif at the C-terminus of one polypeptide chain can be expressed and purified from the supernatant of eukaryotic cells (e.g. HEK293 cells, CHO cells).

- 49 -

One aspect as reported herein is an bispecific antibody that is obtained by conjugating an antigen binding polypeptide/domain (e.g. scFv or Fab) to an one-armed antibody variant (OA-Fc) using the enzyme Sortase A, wherein a sortase recognition sequence is located at the C-terminus of the single chain antigen binding polypeptide (e.g. scFv, scFab or darpin) or the C-terminus of one polypeptide chain of the multi chain antigen binding complex (e.g. dsFv or Fab), and wherein a double or triple glycine motif is located at the N-terminus of the Fc-chain of the one-armed antibody variant (OA-Fc-Gm; m=2 or 3). An one-armed antibody Fab or scFv conjugate comprising an antibody Fab fragment (OA-Fc~Fab) or a scFv antibody fragment (OA-Fc~scFv) and an one-armed antibody (OA-Fc) can be obtained in high yield in an enzymatic conjugation by using (i) a polypeptide comprising the amino acid sequence G_nSLPX1TG (SEQ ID NO:02, wherein X1 can be any amino acid residue, with n=1, 2 or 3) in its C-terminal region, (ii) an heavy chain Fc-region polypeptide comprising an oligoglycine at its N-terminus, and (iii) the enzyme Sortase A.

With this combination of reagents

- i) the reverse reaction recognizing the LPX1TG amino acid sequence within the product conjugate as substrate, and/or
- ii) the generation of a dead-end hydrolysis polypeptide fragment (polypeptide with without/cleaved LPX1TG recognition sequence generated through cleavage of the thioacyl-binding entity Sortase A intermediate by water instead by the G_m-antibody Fc-region nucleophile)

that is normally occurring at increased reaction times can be reduced or even eliminated.

Different combinations of C-terminal and N-terminal amino acid sequence combinations have been tested.

In more detail, as an exemplary binding entity an antibody Fab fragment was used and as exemplary antibody Fc-region a one armed antibody Fc-region (OA-Fc-region = a pair of a full length antibody heavy chain and its cognate light chain and an heavy chain antibody Fc-region polypeptide) was used. Three different sequences at the C-terminus of the antibody Fab fragment VH-CH1 heavy chain and at the N-terminus of the OA-Fc-region respectively were conjugated using the exemplary transpeptidase Sortase A. Nine different conjugates were obtained. The

- 50 -

progress/efficiency of the coupling reaction was determined at different time points. To this end aliquots of the transpeptidation reactions were analyzed by SDS-PAGE. The efficiency of ligation was estimated densitometrically from the gel. The results are given in the following Table 1.

5

Table 1.

One armed antibody Fc-region (OA-Fc-region) (→)	GGGDKTHTCPPC	GGHTCPPC	GGCPPC
Fab VH-CH1 heavy chain (↓)			
KSCGGGSLPETGGSGSHHHHHH	approx. 54 %	approx. 62 %	approx. 73 %
KSCGSLPETGGSGSHHHHHH	approx. 56 %	approx. 56 %	approx. 73 %
KSCLPETGGSGSHHHHHH	approx. 52 %	approx. 54 %	approx. 54 %

In one embodiment the Fab antibody fragment or scFv antibody fragment comprises the amino acid sequence GSLPX1TGGSGS (SEQ ID NO: 03, wherein X1 can be any amino acid residue) within the 20 C-terminal amino acid residues.

10

In one embodiment the Fab antibody fragment or scFv antibody fragment comprises the amino acid sequence X2GSLPX1TGGSGS (SEQ ID NO: 05, wherein X1 can be any amino acid residue, whereby X2 can be any amino acid residue except G).

15

In one embodiment the Fab antibody fragment or scFv antibody fragment comprises the amino acid sequence G_nSLPX1TGGSGSX3 (SEQ ID NO: 06, wherein X1 can be any amino acid residue, with n=1, 2 or 3) within the 20 C-terminal amino acid residues, whereby X3 is an amino acid sequence tag.

20

In one embodiment the Fab antibody fragment or scFv antibody fragment comprises the amino acid sequence X2GSLPX1TGGSGSX3 (SEQ ID NO: 07, wherein X1 can be any amino acid residue, with n=1, 2 or 3) within the 20 C-terminal amino acid residues whereby X2 can be any amino acid residue except G and X3 is an amino acid sequence tag.

The “Combimatrix” approach

It is desirable to combine a first binding entity, such as an antibody Fab fragment, with another specific binding entity, such as a second antibody Fab fragment or a one-armed antibody fragment comprising a full length heavy chain and its cognate light chain and a disulfide linked heavy chain Fc-region polypeptide. In addition it is possible to screen, whether a first binding entity shows better properties when linking it to a number of different other binding entities. Using a so-called Combimatrix approach, a multitude of combinations of binding entities can be addressed in an easy way. It has to be pointed out that the second binding entities can either bind to different targets/epitopes/antigens, or can bind to the same antigen but to different epitopes, or can bind to the same epitope but be different variants of a single binding entity (e.g. humanization candidates).

In this scenario, an automated platform can perform the tasks to pipette, purify and combine the binding entities and their reactions or derivatives. Any platform that uses e.g. 96-well plates or other high throughput formats is suitable, such as an Eppendorf epMotion 5075vac pipetting robot.

First, cloning of the binding entity encoding constructs is performed. The plasmids with the binding entity encoding nucleic acids are usually obtained by gene synthesis, whereby the C-terminal region of one encoded binding entity contains a sortase-motive and a His-tag and one N-terminal region of the respective other binding entity comprises an oligoglycine motif, or by cloning of the variable domains via B-cell PCR and sequence- and ligation-independent cloning (SLIC) into an appropriate vector containing necessary elements like the constant region, a sortase motive and a His-tag respectively. The plasmids are individually transferred into a separate well of a multi-well plate (a whole plate can be loaded). Thereafter, the plasmids are digested with a restriction enzyme mix that cuts out the binding entity-coding region. It is desirable to design all gene synthesis in a way that only one restriction enzyme mix is needed for all plasmids. Afterwards, an optional cleaning step yields purified DNA fragments. These fragments are ligated into a plasmid backbone that had been cut out of an acceptor vector with the same restriction mix as mentioned above. Alternatively, the cloning procedure can be performed by a SLIC-mediated cloning step (see e.g. PCT/EP2012/076155). After ligation, the automated platforms transfers all ligation mixes into a further multi-well plate with competent E. coli cells (e.g. Top10 Multi Shot, Invitrogen) and a transformation reaction is performed. The cells are cultivated to the desired density.

- 52 -

From an aliquot of the cultivation mixture glycerol stocks can be obtained. From the culture plasmid is isolated (e.g. using a plasmid isolation mini kit (e.g. NucleoSpin 96 Plasmid, Macherey& Nagel)). Plasmid identity is checked by digesting an aliquot with an appropriate restriction mix and polyacrylamide gel electrophoresis (e.g. E-Gel 48, Invitrogen). Afterwards a new plate can be loaded with an aliquot of the plasmid for performing a control sequencing reaction.

In the next step the binding entities are expressed. Therefore, HEK cells are seeded onto a multi-well plate (e.g. a 48-well-plate) or small shaker flasks and are transfected with the isolated plasmids (containing the binding entity-coding region in an appropriate backbone vector). Transfected HEK cells are cultivated for several days and harvested (e.g. by filtrating through a 1.2 μm and a 0.22 μm filter plate by using a vacuum station). Titers can be monitored by performing e.g. an ELISA.

The binding entities can be linked to the each other using a sortase-mediated transpeptidation reaction. The first binding entity, the second binding entity, and the sortase reaction mix can be combined in a multi-well format. After incubation at 37°C for 4-72 h (e.g. 16 hours), the conjugates can be harvested by using a negative His-tag selection procedure (the mixture is applied onto e.g. His MultiTrap HP plates (GE Healthcare) and filtrated, whereby all molecules that still have a His-tag are bound on the chromatography column, whereas the conjugates are found in the filtrate; with the filtrate a buffer exchange should be made, e.g. by applying the conjugate onto an ultrafiltration membrane or by using a plate containing an affinity medium that is specific for one of the binding entities.

The multispecific binding molecules can be made using the Combimatrix approach, see Table below).

	1	2	3	4	5	6	7	8	9	10	11
A	1A	2A	3A	4A	5A	6A	7A	8A	9A	10A	11A
B	1B
C	1C
D	1D
E	1E
F	1F
G	1G	10G	11G

- 53 -

In the first row of a multi-well plate different first binding entities comprising a C-terminal Sortase motif of equal molar concentrations are pipetted into each well (excluding first well of the first row), designated in arabic numbers (e.g. 1 to 11). In the first column of the same plate, different second binding entities comprising an oligoglycine in the N-terminal region of equal molar concentrations are pipetted into each well (excluding first well of the first column), designated in letters (e.g. A to G). Thereafter all first binding entities of the first row are combined with all second binding entities of the first column (e.g. resulting in 77 combinations in a 96-well plate), designated by a combination of number and letter (e.g. 1A to 11G). To all combinations Sortase in an appropriate buffer is added. After the enzymatic conjugation has been performed, an optional purification step can be performed. The multispecific binding molecules are then ready for evaluation in cell-based assays.

III. RECOMBINANT METHODS

The ligation components of an OA-Fc-region conjugate, in particular, the one-armed antibody variant (OA-Fc-Gm) and the single chain antigen binding polypeptide (e.g. scFv, scFab or darpin) or the multi chain antigen binding complex (e.g. dsFv or Fab) may be produced using recombinant methods and compositions, see e.g. US 4,816,567.

In one aspect a method of making an OA-Fc~polypeptide conjugate is provided, wherein the method comprises (i) culturing a first host cell comprising a nucleic acid encoding the one-armed antibody variant (OA-Fc-Gm) part of the conjugate under conditions suitable for expression/secretion of the one-armed antibody variant (OA-Fc-Gm) and optionally recovering the OA-Fc-Gm part from the host cell (or host cell culture medium) and (ii) culturing a second host cell comprising a nucleic acid encoding the polypeptide part of the conjugate under conditions suitable for expression/secretion of the polypeptide and optionally recovering the polypeptide part from the host cell (or host cell culture medium) and (iii) conjugating the recombinantly produced parts of the OA-Fc~polypeptide conjugate enzymatically using Sortase A mediated transpeptidation.

For recombinant production of the OA-Fc-Gm part of the OA-Fc~polypeptide conjugate and the polypeptide part, a nucleic acid encoding the OA-Fc-Gm part and the polypeptide part of the OA-Fc~polypeptide conjugate, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or

- 54 -

expression/secretion in a host cell. Such nucleic acid may be readily isolated and/or produced using conventional procedures.

Suitable host cells for cloning or expression/secretion of polypeptide-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, polypeptides may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed (see, e.g., US 5,648,237, US 5,789,199, and US 5,840,523, Charlton, *Methods in Molecular Biology* 248 (2003) 245-254 (B.K.C. Lo, (ed.), Humana Press, Totowa, NJ), describing expression of antibody fragments in *E. coli*). After expression, the polypeptide may be isolated from the bacterial cell paste in a soluble fraction or may be isolated from the insoluble fraction so called inclusion bodies which can be solubilized and refolded to bioactive forms. Thereafter the polypeptide can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeasts are suitable cloning or expression hosts for polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been “humanized”, resulting in the production of a polypeptide with a partially or fully human glycosylation pattern (see e.g. Gerngross, *Nat. Biotech.* 22 (2004) 1409-1414, and Li, et al., *Nat. Biotech.* 24 (2006) 210-215).

Suitable host cells for the expression of glycosylated polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures can also be utilized as hosts (see, e.g., US 5,959,177, US 6,040,498, US 6,420,548, US 7,125,978, and US 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 the COS-7 cell line (monkey kidney CV1 cell transformed by SV40); the HEK293 cell line (human embryonic kidney) BHK cell line (baby hamster kidney); the TM4 mouse sertoli cell line (TM4 cells as described, e.g., in Mather, *Biol. Reprod.* 23 (1980) 243-251); the CV1 cell line (monkey kidney cell); the VERO-76 cell line (African green monkey kidney cell); the HELA cell line (human cervical carcinoma cell); the MDCK cell line (canine

- 55 -

kidney cell); the BRL-3A cell line (buffalo rat liver cell); the W138 cell line (human lung cell); the HepG2 cell line (human liver cell); the MMT 060562 cell line (mouse mammary tumor cell); the TRI cell line, as described, e.g., in Mather, et al., *Annals N.Y. Acad. Sci.* 383 (1982) 44-68; the MRC5 cell line; and FS4 cells-
5 line. Other useful mammalian host cell lines include the CHO cell line (Chinese hamster ovary cell), including DHFR negative CHO cell lines (Urlaub, et al., *Proc. Natl. Acad. Sci. USA* 77 (1980) 4216), and myeloma cell lines such as Y0, NS0 and Sp2/0 cell line. For a review of certain mammalian host cell lines suitable for polypeptide production, see, e.g., Yazaki, and Wu, *Methods in Molecular Biology, Antibody Engineering* 248 (2004) 255-268 (B.K.C. Lo, (ed.), Humana Press, Totowa, NJ).

IV. Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the bispecific antibodies provided herein is useful for detecting the presence of one or both antigens in a biological sample. The term
15 “detecting” as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as biopsies of cancer cells.

In one embodiment, a bispecific antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of
20 cancer cells in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with a bispecific antibody as described herein under conditions permissive for binding of the bispecific antibody to its antigen or antigens, and detecting whether a complex is formed between the bispecific antibody and its antigen or antigens. Such method may be an *in vitro* or
25 *in vivo* method.

Exemplary disorders that may be diagnosed using an antibody of the invention include cancer.

In certain embodiments, labeled bispecific antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as
30 fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{131}I , fluorophores such as rare earth chelates or fluorescein and its derivatives,

- 56 -

rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (US 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

10 V. Pharmaceutical Formulations

Pharmaceutical formulations of a bispecific antibody as described herein are prepared by mixing such antibody having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.), (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyl dimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as poly (vinylpyrrolidone); 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). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rhuPH20 (HYLENEX[®], Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rhuPH20, are described in US 2005/0260186 and US 2006/0104968. In one aspect, a sHASEGP

- 57 -

is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US 6,267,958. Aqueous antibody formulations include those described in US 6,171,586 and
5 WO 2006/044908, the latter formulations including a histidine-acetate buffer.

The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the
10 purpose intended.

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-(methyl methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example,
15 liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.) (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic
20 polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules.

The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

25 **VI. Therapeutic Methods and Compositions**

Any of the bispecific antibodies provided herein may be used in therapeutic methods.

In one aspect, a bispecific antibody for use as a medicament is provided. In further aspects, a bispecific antibody for use in treating cancer is provided. In certain
30 embodiments, a bispecific antibody for use in a method of treatment is provided. In certain embodiments, the invention provides a bispecific antibody for use in a method of treating an individual having cancer comprising administering to the

- 58 -

individual an effective amount of the bispecific antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. In further embodiments, the invention provides a bispecific antibody for use in removing/killing/lysing cancer cells. In certain embodiments, the invention provides a bispecific antibody for use in a method of removing/killing/lysing cancer cells in an individual comprising administering to the individual an effective amount of the bispecific antibody to remove/kill/lyse cancer cells. An “individual” according to any of the above embodiments can be a human.

10 In a further aspect, the invention provides for the use of a bispecific antibody in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of cancer. In a further embodiment, the medicament is for use in a method of treating cancer comprising administering to an individual having cancer an effective amount of the medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. In a further embodiment, the medicament is for removing/killing/lysing cancer cells. In a further embodiment, the medicament is for use in a method of removing/killing/lysing cancer cells in an individual comprising administering to the individual an amount effective of the medicament to remove/kill/lyse cancer cells. An “individual” according to any of the above embodiments may be a human.

In a further aspect, the invention provides a method for treating cancer. In one embodiment, the method comprises administering to an individual having cancer an effective amount of a bispecific antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, as described below. An “individual” according to any of the above embodiments may be a human.

In a further aspect, the invention provides a method for removing/killing/lysing cancer cells in an individual. In one embodiment, the method comprises administering to the individual an effective amount of the bispecific antibody to remove/kill/lyse cancer cells. In one embodiment, an “individual” is a human.

In a further aspect, the invention provides pharmaceutical formulations comprising any of the bispecific antibodies provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises

- 59 -

any of the bispecific antibodies provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the bispecific antibodies provided herein and at least one additional therapeutic agent, e.g., as described below.

5 Antibodies of the invention can be used either alone or in combination with other agents in a therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent. In certain embodiments, an additional therapeutic agent is a cytotoxic agent or a chemotherapeutic agent.

10 Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antibodies of
15 the invention can also be used in combination with radiation therapy.

An antibody of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial,
20 intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

25 Antibodies 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
30 administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the

- 60 -

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.

5 For the prevention or treatment of disease, the appropriate dosage of an antibody 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 type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody 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 10 1 $\mu\text{g}/\text{kg}$ to 15 mg/kg (e.g. 0.5 mg/kg - 10 mg/kg) of antibody 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 15 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 antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, 20 one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses 25 may be administered. An exemplary dosing regimen comprises administering [[add exemplary dosing regimen, if known, e.g., “an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody”]]. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

30 It is understood that any of the above formulations or therapeutic methods may be carried out using an immunoconjugate of the invention in place of or in addition to a bispecific antibody.

VII. Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

It is understood that any of the above articles of manufacture may include an immunoconjugate of the invention in place of or in addition to a bispecific antibody.

Description of the sequence listing:

30	SEQ ID NO: 01 to 07 and 66 to 67	Sortase motifs
	SEQ ID NO: 08	Fc-region nucleophile
	SEQ ID NO: 09 to 10	Sortase motif remainders in the conjugate
	SEQ ID NO: 11 to 29	Amino acid sequence tag
	SEQ ID NO: 30	Human CH2 domain

- 62 -

SEQ ID NO: 31 Human CH3 domain
SEQ ID NO: 32 to 46 Exemplary wild-type and variant antibody heavy chain Fc-region polypeptides
SEQ ID NO: 47 to 65 Sequences used in the examples.

5 **Examples**

The following examples 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.

10 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.

Materials and Methods

Recombinant DNA techniques

15 Standard methods were used to manipulate DNA as described in Sambrook, J., 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 manufacturer's instructions.

Gene synthesis

20 Desired gene segments were prepared by chemical synthesis at Genart GmbH (Regensburg, Germany). The synthesized gene fragments were cloned into an E. coli plasmid for propagation/amplification. The DNA sequence of the subcloned gene fragments were verified by DNA sequencing.

Protein determination

25 The protein concentration of purified polypeptides was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence of the polypeptide.

Example 1**Generation of the expression plasmids**Description of the basic/standard mammalian expression plasmid

Desired proteins were expressed by transient transfection of human embryonic kidney cells (HEK 293). For the expression of a desired gene/protein (e.g. full length antibody heavy chain, full length antibody light chain, or an Fc-chain containing an oligoglycine at its N-terminus) a transcription unit comprising the following functional elements was used:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence (SS),
- a gene/protein to be expressed (e.g. full length antibody heavy chain), and
- the bovine growth hormone polyadenylation sequence (BGH pA).

Beside the expression unit/cassette including the desired gene to be expressed the basic/standard mammalian expression plasmid contains

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a beta-lactamase gene which confers ampicillin resistance in E. coli.

Expression plasmids coding for the following polypeptides/proteins were constructed:

- Pertuzumab heavy chain variable domain combined with a human heavy chain constant region of the subclass IgG1 containing a T366W mutation:

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEW
 VADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYY
 CARNLGPSFYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAL
 GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
 SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV
 FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
 TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
 SKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESN

- 64 -

GQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK (SEQ ID NO: 47).

- Pertuzumab light chain variable domain combined with a human kappa light chain constant region:

5 DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIY
SASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFG
QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW
KVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEEKHKVYACEV
THQGLSSPVTKSFNRGEC (SEQ ID NO: 48).

- 10 - Trastuzumab heavy chain variable domain combined with a human heavy chain constant region of the subclass IgG1 containing a T366S, L368A, and Y407V mutation:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV
ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
15 SRWGGDGFYAMDYWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTA
ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN
AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
20 KTISKAKGQPREPQVCTLPSPRDELTKNQLVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK (SEQ ID NO: 49).

- Trastuzumab light chain variable domain combined with a human kappa light chain constant region:

25 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLI
YSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTF
GQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ
WKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEEKHKVYACE
VTHQGLSSPVTKSFNRGEC (SEQ ID NO: 50).

- 30 - antibody Fab fragment comprising a Pertuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal GGGSLPETGGSGSHHHHHH amino acid sequence:

- 65 -

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEW
 VADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYY
 CARNLGPSFYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAL
 GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
 5 SLGTQTYICNVNHKPSNTKVDKKVEPKSCGGGSLPETGGSGSHHHHHH
 (SEQ ID NO: 51).

- antibody Fab fragment comprising a Pertuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal GSLPETGGSGSHHHHHH sequence:

10 EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEW
 VADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYY
 CARNLGPSFYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAL
 GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
 15 SLGTQTYICNVNHKPSNTKVDKKVEPKSCGSLPETGGSGSHHHHHH
 (SEQ ID NO: 52).

- antibody Fab fragment comprising a Pertuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal LPETGGSGSHHHHHH sequence:

20 EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEW
 VADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYY
 CARNLGPSFYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAL
 GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
 25 SLGTQTYICNVNHKPSNTKVDKKVEPKSCLPETGGSGSHHHHHH (SEQ
 ID NO: 53).

- 25 - antibody Fab fragment comprising a Trastuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal GGGSLPETGGSGSHHHHHH sequence:

30 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV
 ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
 SRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTA
 ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
 SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGGGSLPETGGSGSHHHH
 HH (SEQ ID NO: 54).

- 66 -

- antibody Fab fragment comprising a Trastuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal GSLPETGGSGSHHHHHH sequence:

5 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV
 ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
 SRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTA
 ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
 SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSLPETGGSGSHHHHHH
 (SEQ ID NO: 55).

- 10 - antibody Fab fragment comprising a Trastuzumab heavy chain variable domain and a human heavy chain constant region 1 (CH1) of the subclass IgG1 containing a C-terminal LPETGGSGSHHHHHH sequence:

15 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV
 ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
 SRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTA
 ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
 SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCLPETGGSGSHHHHHH
 (SEQ ID NO: 56).

- 20 - heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366S, L368A, and Y407V mutation containing an N-terminal GGGDKTHTCPPC sequence:

25 GGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
 SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
 WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKN
 QVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKL
 TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID
 NO: 57).

- heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366S, L368A, and Y407V mutation containing an N-terminal GGHTCPPC sequence:

30 GGHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
 EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
 EYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSC

- 67 -

AVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 58).

- heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366S, L368A, and Y407V mutation containing an N-terminal GGCPPC sequence:

5 GGCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVCTLPISRDELTKNQVSLSCA
VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 59).

- 10 - heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366W mutation containing an N-terminal GGGDKTHTCPPC sequence:

GGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTK
15 NQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID
NO: 60).

- heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366W mutations containing an N-terminal GGHTCPPC sequence:

20 GGHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLW
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 61).

- 25 - heavy chain Fc-region polypeptide (human IgG1(CH2-CH3)) with T366W mutation containing an N-terminal GGCPPC sequence:

GGCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCL
30 VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 62).

Example 2**Transient expression, purification and analytical characterization**

5 The antibody chains were generated by transient transfection of HEK293 cells (human embryonic kidney cell line 293-derived) cultivated in F17 Medium (Invitrogen Corp.). For transfection "293-Fectin" Transfection Reagent (Invitrogen) was used. The antibody chains were expressed from three different plasmids, coding for a full length heavy chain (either Pertuzumab-knob, or Trastuzumab-hole), a corresponding full length light chain, and a heavy chain Fc-region polypeptide containing one of the N-terminal oligoglycine sequences either as knob, or as hole variant. The three plasmids were used at an equimolar plasmid ratio upon transfection. Transfections were performed as specified in the manufacturer's instructions. Antibody Fc-region-containing cell culture supernatants were harvested seven days after transfection. Supernatants were stored frozen until purification.

15 The antibody Fc-region-containing culture supernatants were filtered and purified by two chromatographic steps. The antibody Fc-regions were captured by affinity chromatography using HiTrap MabSelectSuRe (GE Healthcare) equilibrated with PBS (1 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 137 mM NaCl, 2.7 mM KCl), pH 7.4. Unbound proteins were removed by washing with equilibration buffer, and the antibody Fc-region was recovered with 0.1 M citrate buffer, pH 3.0. Immediately after elution the solution was neutralized to pH 6.0 with 1 M Tris-base, pH 9.0. Size exclusion chromatography on Superdex 200TM (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 40 mM Tris-HCl buffer, 0.15 M NaCl, pH 7.5. The eluted antibody Fc-regions were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

30 The protein concentrations of the antibody Fc-regions were 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 proper antibody Fc-region formation were analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiothreitol) and staining with Coomassie brilliant blue.

Example 3**Transient expression, purification and analytical characterization of antibody Fab fragments containing the C-terminal LPX1TG motif**

5 The antibody Fab fragments were generated by transient transfection of HEK293 cells (human embryonic kidney cell line 293-derived) cultivated in F17 Medium (Invitrogen Corp.). For transfection "293-Fectin" Transfection Reagent (Invitrogen) was used. The antibody Fab fragments were expressed from two different plasmids, coding for a full length light chain (either Pertuzumab, or Trastuzumab) and a corresponding truncated heavy chain containing one of the C-terminal LPX1TG
10 sequences. The two plasmids were used at an equimolar plasmid ratio upon transfection. Transfections were performed as specified in the manufacturer's instructions. Fab fragment-containing cell culture supernatants were harvested seven days after transfection. Supernatants were stored frozen until purification.

15 The Fab fragment containing culture supernatants were filtered and purified by two chromatographic steps. The Fab fragments were captured by affinity chromatography using HisTrap HP Ni-NTA columns (GE Healthcare) equilibrated with PBS and 20mM Imidazole (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 20mM Imidazole), pH 7.4. Unbound proteins were removed by washing with equilibration buffer. The histidine-tagged protein was eluted with a
20 20 mM to 400 mM linear imidazole gradient in PBS (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 400 mM Imidazole) in 10 column volumes. Size exclusion chromatography on Superdex 200TM (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 40 mM Tris-HCl buffer, 0.15 M NaCl, pH 7.5. The Fab fragments
25 were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

The protein concentrations of the Fab fragments were 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 proper Fab formation were
30 analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiothreitol) and staining with Coomassie brilliant blue.

Example 4**Sortase A mediated ligation of antibody Fc-region and binding entity (Fab fragment)**

For the sortase-mediated transpeptidation reaction, N-terminally truncated Staphylococcus aureus Sortase A was used (Δ_{1-59}). The reaction was performed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (Sortase-buffer). In the reaction, a Fab fragment bearing a sortase motif (LPETG) at its C-terminus of the VH-CH1-heavy chain including no or 2 different connecting short amino acid sequences between the C-terminal end of the VH-CH1 heavy chain (...KSC) and the N-terminus of the sortase motif (LPETGGSGSHHHHHH, SEQ ID NO: 63, GSLPETGGSGSHHHHHH, SEQ ID NO: 64, and GGGSLPETGGSGSHHHHHH, SEQ ID NO: 65) and a one-armed antibody bearing an oligoglycine motif and three different hinge sequences (GGCPPC, SEQ ID NO: 8 with X4 = P, GGHTCPPC, SEQ ID NO: 66, and GGGDKTHTCPPC, SEQ ID NO: 67, respectively) at its N-terminus of the heavy chain Fc-region polypeptide were linked, resulting in the antibody Fc-region conjugate. To perform the reaction, all reagents were brought in solution in sortase buffer. In a first step, the antibody Fc-region and the antibody Fab fragment were mixed, and the reaction was started by the following addition of Sortase A and 5 mM CaCl₂. The components were mixed by pipetting and incubated at 37 °C for 72h. Subsequently, the reaction was stopped by freezing of the reaction mixture and storage at -20°C until analysis.

Molar ratio Fab:One-armed antibody:sortase = 20:4:1

Results

Three different sequences at the C-terminus of the Fab and at the N-terminus of the antibody respectively were conjugated by Sortase A to obtain nine different combinations of antibody Fc-region conjugates. The efficiency of the coupling reaction was evaluated at different time points. To this end aliquots of the transpeptidation reactions were analyzed by SDS-PAGE. The efficiency of ligation was estimated densitometrically from the SDS PAGE gel. Results after 72h of reaction are depicted in Table 2 for the respective sequences.

Table 2: Conjugation of Fab fragments with one-armed antibodies

One armed antibody Fc-region (OA-Fc-region) (→)	GGGDKTHTCPPC	GGHTCPPC	GGCPPC
Fab VH-CH1 heavy chain (↓)			
KSCGGGSLPETGGSGSHHHHHH	approx. 54 %	approx. 62 %	approx. 73 %
KSCGSLPETGGSGSHHHHHH	approx. 56 %	approx. 56 %	approx. 73 %
KSCLPETGGSGSHHHHHH	approx. 52 %	approx. 54 %	approx. 54 %

- 72 -

Patent Claims

1. A method for producing a bispecific antibody comprising the step of incubating
 - (i) an antibody Fab fragment or a scFv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue),
 - (ii) an one-armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide,whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site,
whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and
whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m ($m = 2, \text{ or } 3, \text{ or } 4, \text{ or } 5$) amino acid sequence at its N-terminus,
and
 - (iii) a Sortase A enzymeand thereby producing the bispecific antibody.
2. A method for producing a bispecific antibody comprising the following steps
 - (i) determining the cell surface makers present in a cell containing sample and selecting thereof at least a first cell surface marker and a second cell surface marker,
 - (ii) incubating (a) an antibody Fab fragment or a scFv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), whereby the Fab fragment or scFv antibody specifically binds to the first

- 73 -

5 cell surface marker or its ligand, (b) an one-armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second cell surface marker or its ligand, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m ($m = 2, \text{ or } 3, \text{ or } 4, \text{ or } 5$) amino acid sequence at its N-terminus, and (c) a Sortase A enzyme

and thereby producing the bispecific antibody.

- 15 3. A method for determining a combination of antigen binding sites comprising the following steps
- 20 (i) determining the binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life of a multitude of bispecific antibodies prepared by combining (a) each member of a first multitude of antibody Fab fragments or scFv antibody fragments whereby each member comprises within the 20 C-terminal amino acid residues the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue), whereby the Fab fragment or scFv antibody specifically binds to a first epitope or antigen, with (b) each member of a multitude of one-armed antibody fragments comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to a second epitope or antigen, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m ($m = 2, \text{ or } 3,$
- 25
- 30
- 35

- 74 -

or 4, or 5) amino acid sequence at its N-terminus, and (c) a Sortase A enzyme

and

5 (ii) choosing the bispecific antibody with suitable binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

4. A bispecific antibody obtained by a method according to claim 1 or claim 2.
5. A bispecific antibody comprising the amino acid sequence LPX1TG (SEQ ID NO: 01, wherein X1 can be any amino acid residue) in one of its heavy chains.
- 10
6. The method according to any one of claims 1 to 3 or the antibody according to any one of claims 4 or 5, characterized in that Fc-region comprises a mutation of the naturally occurring amino acid residue at position 329 and at least one further mutation of at least one amino acid residue selected from the group comprising amino acid residues at position 228, 233, 234, 235, 236, 237, 297, 318, 320, 322 and 331 to a different residue, wherein the residues in the Fc-region are numbered according to the EU index of Kabat. The change of these specific amino acid residues results in an altering of the effector function of the Fc-region compared to the non-modified (wild-type) Fc-region.
- 15
- 20
7. A pharmaceutical formulation comprising a bispecific antibody according to claim 4 or claim 5.
8. Use of a bispecific antibody according to claim 4 or claim 5 in the manufacture of a medicament.

25