BISPECIFIC ANTIBODIES AND METHODS OF USE IN OPHTHALMOLOGY

FIELD OF THE INVENTION

The present invention relates to bispecific antibodies and methods of using the same, for example in ophthalmology.

BACKGROUND

Ocular vascular diseases such as age related macular degeneration (AMD) and diabetic retinopathy (DR) are due to abnormal choroidal or retinal neovascularization, respectively. They are the leading causes of visual loss in industrialized nations. Since the retina consists of well-defined layers of neuronal, glial, and vascular elements, relatively small disturbances such as those seen in vascular proliferation or edema can lead to significant loss of visual function. Inherited retinal degenerations, such as Retinitis Pigmentosa (RP), are also associated with vascular abnormalities, such as arteriolar narrowing and vascular atrophy. They affect as many as 1 in 3,500 individuals and are characterized by progressive night blindness, visual field loss, optic nerve atrophy, arteriolar attenuation, and central loss of vision often progressing to complete blindness.

Ischemic retinopathies are characterized by loss or dysfunction of the retinal vasculature which results in a reduction of blood flow and hypoxia. The retina responds to hypoxia by generating signals to grow new blood vessels, but these new vessels are usually fragile and disorganized. It is the growth of these abnormal new vessels that creates most of the threat to vision since they can leak, lead to hemorrhage or lead to scarring that may end in retinal detachment. Current treatments for ischemic retinopathies seek to halt the growth of the pathological vessels but do not address the underlying ischemia that drives their growth. Furthermore, standard treatment for diabetic retinopathy, an ischemic retinopathy that affects millions, involves destruction of a portion of the retina with a laser in an attempt to stop new vessel growth and preserve central vision. Strategies have been employed to block the function of vascular endothelial growth factor (VEGF), a major promoter of vessel growth. In the short term, anti-VEGF therapy can improve vision, but it does not address the underlying ischemia and in fact may exacerbate this condition as it inhibits all vessel growth, including beneficial

collaterals. There is also the serious concern of systemic exposure of these drugs in elderly and/or diabetic patients where new vessel growth may be required in ischemic brains, hearts or limbs.

Typically for ocular diseases via intravitreal application smaller antibody fragments like Fab or Fab_2 are often used as they have a low serum half-life and the risk of systemic toxicities is lower. However this smaller fragments typically have also lower intravitreal half-lives (e.g. due to the faster diffusion into serum) and have to be dosed typically more often.

Multispecific antibodies with a domain replacement/exchange in one binding arm (CrossMabVH-VL) are described in detail in WO 2009/080252 and Schaefer, W. et al, Proc. Natl. Acad. Sci. USA, 108 (2011) 11187-11191 (which are incorporated as reference herein). They clearly reduce the byproducts caused by the mismatch of a light chain against a first antigen with the wrong heavy chain against the second antigen (compared to approaches without such domain exchange). However their preparation is not completely free of side products. The main side product is based on a Bence-Jones-type interaction. See also Schaefer, W. et al, Proc. Natl. Acad. Sci. USA, 108 (2011) 11187-11191; in Figure S1I of the Supplement).

In WO 2011/117329 bispecific, bivalent anti-VEGF/anti-ANG2 antibodies are reported. Human FcRn-binding modified antibodies and methods of use are reported in WO 2014/177460. Kienast, Y., et al. (Clin. Canc. Res. 19 (2013) 6730-6740) reported Ang-2-VEGF-A CrossMab as a novel bispecific human IgG1 antibody blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and anti-metastatic efficacy.

SUMMARY

The invention provides novel bispecific antibodies and methods of using the same.

Herein is reported a bispecific antibody that specifically binds to two different antigens selected from the group consisting of human ANG2, human VEGF, human IL-1beta and human PDGF-B.

In one embodiment the antibody is not an anti-ANG2/VEGF antibody.

In one embodiment the antibody specifically binds to human IL-1beta and comprises

a) a heavy chain variable domain comprising

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 02, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 04, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 05, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 15,

and

a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19,

or

a) a heavy chain variable domain comprising a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 21, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 22, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 24, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 26; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 27; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 28.

In one embodiment the antibody specifically binds to human PDGF-B and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30, (b) a HVR-H2 comprising

the amino acid sequence of SEQ ID NO: 31, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 35; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

In one embodiment the antibody specifically binds to human ANG2 and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 57, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 58, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 60, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 62; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 63; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 64,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 66, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 67, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 69, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 71; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 72; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 73,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 75, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 76, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 78, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 80; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 81; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 82,

or

d) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 84, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 85, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 87, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 89; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 90; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 91.

In one embodiment the antibody specifically binds to human VEGF and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 93, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 94, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 96, and a light

chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 97; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 98; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 99,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 101, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 102, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 104, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 106; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 107; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 108.

In one embodiment the antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other.

In one preferred embodiment the antibody comprises

i) in the constant domain CL of the first light chain under a) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the first heavy chain under a) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index),

or

ii) in the constant domain CL of the second light chain under b) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the second heavy chain under b) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index).

In one embodiment the antibody is a bivalent, bispecific antibody, comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

In one embodiment the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein

i) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide, or

ii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, or

iii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, or

iv) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366W and the second Fc-

region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366S, L368A, Y407V, or

v) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366S, L368A, Y407V, or

vi) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide, or

vii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, or

viii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, or

ix) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366S, L368A, Y407V, or

x) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366S, L368A, Y407V.

In one embodiment the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein the antibody comprises the combination of mutations

i) I253A, H310A, and H435A, or

ii) H310A, H433A, and Y436A, or
iii) L251D, L314D, and L432D, or
iv) combinations of i) to iii)

in the first Fc-region polypeptide and in the second Fc-region polypeptide.

One aspect as reported herein is a pharmaceutical formulation comprising an antibody as reported herein and optionally a pharmaceutically acceptable carrier.

One aspect as reported herein is the antibody as reported herein for use as a medicament.

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

In one embodiment the medicament is for the treatment of an ocular vascular disease, preferably for the treatment of macular degeneration.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

I. DEFINITIONS

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

"Affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (k_d).

Affinity can be measured by common methods known in the art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

An “affinity matured” antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The terms “anti-IL-1beta antibody” and “an antibody that binds to IL-1beta” refer to an antibody that is capable of binding IL-1beta with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting IL-1beta. In one embodiment, the extent of binding of an anti-IL-1beta antibody to an unrelated, non-IL-1beta protein is less than about 10% of the binding of the antibody to IL-1beta as measured, e.g., by ELISA or surface plasmon resonance. In certain embodiments, an antibody that binds to IL-1beta has a dissociation constant (KD) of $\leq$ 1 μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, or $\leq$ 0.1 nM (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-10} M, e.g., from 10^{-9} M to 10^{-10} M). In certain embodiments, an anti-IL-1beta antibody binds to an epitope of IL-1beta that is conserved among IL-1beta from different species.

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

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab’-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

An “antibody that binds to the same epitope” as a reference antibody refers to an antibody that has interactions with at least the same amino acid residues as the reference antibody. These interactions are e.g. ionic interactions between charged amino acid residues or hydrophobic interactions between hydrophobic amino acid residues.

The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

The term "immunoconjugate" denotes a covalent conjugate between an antibody and a non-antibody moiety. Such a non-antibody moiety can be a detectable label, an effector molecule or a cytotoxic agent.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (e.g., At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu); chemotherapeutic agents or drugs (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or anticancer agents disclosed below.

"Effector functions" refer to those biological activities attributable to the Fc-region of an antibody, which vary with the antibody class. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

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 “Fc-region” herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc-regions and variant Fc-regions. In one embodiment, a human IgG heavy chain Fc-region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) or the C-terminal glycyl-lysine dipeptide (Gly446Lys447) of the Fc-region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc-region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat, 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.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The terms “full length antibody”, “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc-region as defined herein.

The terms "host cell", "host cell line", and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

A “human antibody” is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th ed., Bethesda MD (1991), NIH Publication 91-3242, Vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., *supra.* In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., *supra*.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

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

HVRs herein include

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

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat, 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.);

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

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

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

An "immunoconjugate" is an antibody conjugated to one or more heterologous molecule(s).

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

An "isolated" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S. et al., J. Chromatogr. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

"Isolated nucleic acid encoding an anti-IL-1beta antibody" refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

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

A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the

indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

"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. 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.

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:

100 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.

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.

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.

The term “IL-1beta” as used herein, refers to human IL-1beta. The term encompasses “full-length,” unprocessed IL-1beta as well as any form of IL-1beta that results from processing in the cell. The term also encompasses naturally occurring variants of IL-1beta, e.g., splice variants or allelic variants. The amino acid sequence of human IL-1beta is shown in SEQ ID NO: 92.

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, 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.

The term “variable region” or “variable domain” refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable

regions (HVRs). (See, e.g., Kindt, T.J. et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., N.Y. (2007), page 91) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively (see, e.g., Portolano, S. et al., J. Immunol. 150 (1993) 880-887; Clackson, T. et al., Nature 352 (1991) 624-628).

The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

II. COMPOSITIONS AND METHODS

Herein are reported novel bispecific antibodies specifically binding to different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human PDGF-B), wherein the antibody is not an anti-ANG2/VEGF bispecific antibody. Antibodies of the invention are useful, e.g., for the treatment of ocular vascular diseases, such as macular degeneration.

A. Exemplary Antibodies

A.1 Anti-IL1beta antibodies from which the anti-IL-1beta binding site of the bispecific antibody as reported herein can be derived

Herein four novel anti-human IL-1beta antibodies are provided.

The first anti-IL-1beta antibody is a novel murine anti-human IL-1beta antibody with a VH of SEQ ID NO: 20 and a VL of SEQ ID NO: 25. This antibody is termed IL-1beta-mumAb in the following. This antibody binds to human, cynomolgus, rabbit, rat and murine IL-1beta and inhibits the interaction between IL-1beta and the human IL-1 receptors I and II.

The antibody has the following properties:

binding to human IL-1beta	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
IL-1beta-mumAb	1.12	0.75	0.07

binding to cynomolgus IL-1beta	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
IL-1beta-mumAb	1.15	0.95	0.08

binding to murine IL-1beta	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
IL-1beta-mumAb	2.47	12.2	0.49
Gevokizumab	2.48	5.35	0.22

binding to rat IL-1beta	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
IL-1beta-mumAb	2.04	6.36	0.31
Gevokizumab	2.79	0.20	0.007

5

binding to rabbit IL-1beta	**KD [nM]**
mumAb (SEQ ID NO: 9 and 10)	1.4
Gevokizumab	n.a.

The above data was determined by BIAcore.

origin of IL-1beta	**EC_{50} [ng/mL]**	**EC_{50} (based on MWC 150kDa) [10^{-10} M]**	**example**
human 1	34.02	2.27	4 variant 1
human 2	15.16	1.01	4 variant 2
murine	23.07	1.54	6
cynomolgus	21.27	1.42	5

The above data was determined by ELISA.

Inhibition of binding of IL-1beta to IL-1 receptor I and II:

	IC_{50} [ng/mL]	**IC_{50} (based on MWC 150kDa) [10^{-9} M]**	**example**
IL-1beta receptor I	230.9	1.54	7
IL-1beta receptor II	132.4	0.88	8

The above data was determined by ELISA.

In a stimulation experiment it could be shown that the murine antibody as reported herein can inhibit ICAM-1 expression upon IL-1β stimulation of A549 cells (see Table below).

antibody	**IC_{50} [nM]**
IL-1beta-mumAb	0.7

In the following Table the IC_{50} value for inhibition of ICAM-1 expression upon IL-1beta stimulation of HUVEC cells is shown.

antibody	**IC_{50} [nM]**
IL-1beta-mumAb	16.50

In stimulation experiments it could be shown that the humanized antibodies as reported herein reduce IL-6 expression upon IL-1beta stimulation of A549 cells (see Table below).

antibody	**EC_{50} [nM]**
IL-1beta-mumAb	1.09

In addition, the murine antibody showed stability in stress tests. The binding activity has been determined using surface plasmon resonance (see Table below).

antibody	**relative binding activity**	
	2 weeks at 37 °C pH 7.5	**2 weeks at 40 °C pH 6.0**
IL-1beta-mumAb	103 %	102 %

100 % = sample stored at -80 °C

The same stability can be seen when the high molecular weight content is determined (see Table below).

antibody	high molecular weight fraction		
	start	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
IL-1beta-mumAb	0.97 %	1.24 %	1.00 %

The same stability can be seen in the CE-SDS analysis (see Table below).

antibody	relative area%		
	start	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
IL-1beta-mumAb	91.9 %	86.5 %	90.0 %

The thermal stability of the murine antibody has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

antibody	Tagg [°C]	Tm [°C]
IL-1beta-mumAb	approx. 60	approx. 64

The other three antibodies are humanized variants of the murine anti-IL1-beta antibody H34: huH34-1 (SEQ ID NO: 01 to 05, and 16), huH34-2 (SEQ ID NO: 06 to 10, and 16) and huH34-3 (SEQ ID NO: 11 to 16).

Herein is reported a humanized anti-IL-1beta antibody. This antibody is derived from the murine anti-IL-1beta antibody H34.

Based upon the amino acid sequence of the murine H34 antibody, a corresponding humanized anti-IL-1β antibody was generated (huH34-2). The humanized variant VH is based on the human VBase_VH1_1 and the J-element of the human IGHJ4-01-3 germline (huH34-1). In order to restore affinity one backmutation was introduced at position 48 of framework region 2 (M48I). In framework region 3, 4 positions were backmutated: V67A, M69F, R71V and A93V. In addition, the cysteine in position 52a of HVR-H2 was replaced by a serine. For VL, the humanized variant is based on the human IMGT_hVK_3_11 germline with an IGKJ2-01 J-element. One backmutation was introduced at positions 36 of framework region 2 (Y36S). The amino acid sequence of the humanized VH is shown in SEQ ID NO: 04 and the amino acid sequence of the humanized VL is shown in SEQ ID NO: 06.

Murine anti-IL-1beta antibody H34 contains a cysteine in HVR-H2 (C52a, Kabat numbering) that needs to be removed for development as a therapeutic candidate that can be produced at large scale. Removing this Cys by a C52aS mutation in the murine antibody results in a reduced affinity for IL-1beta by a factor of about 6 to 7 (see Tables below).

binding to human IL-1beta antibody	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
H34	1.85	1.27	0.07
H34 + C52aS mutations	1.60	6.45	0.40
binding to cynomolgus IL-1beta antibody	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
H34	1.99	0.98	0.05
H34 + C52aS mutations	1.43	7.28	0.51

The above data was determined by BIAcore.

For the humanized version of H34 (huH34-2) the loss of affinity upon C52aS mutation is compensated and this antibody has a comparable affinity (and comparable functional potency in cellular assays) as the murine parental antibody H34.

This compensation effect is accountable to the germline sequence that was chosen for humanization and the choice of backmutations within framework IGHJ4-01-3 and IMGT_hVK_3_11. An additional variant was designed based on the same human germline for VH (IGHJ4-01-3) and VL (IMGT_hVK_3_11), respectively. Backmutations described for huH34-2 were omitted from the VH and VL sequence (SEQ ID NO: 7 and 8).

binding to human IL-1beta antibody	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
H34	1.85	1.27	0.07
H34 + C52aS mutations	1.60	6.45	0.40
huH34-1	1.49	15.1	1.02
huH34-2	1.93	1.10	0.06
huH34-2 FAB	1.81	1.11	0.06
huH34-3	1.97	3.02	0.15
Gevokizumab	3.01	0.52	0.02
Canakinumab	2.78	0.52	0.02
binding to cynomolgus IL-1beta antibody	**ka [1/Ms * 10^6]**	**kd [1/s * 10^{-4}]**	**KD [nM]**
H34	1.99	0.98	0.05
H34 + C52aS mutations	1.43	7.28	0.51
huH34-1	1.61	21.2	1.31
huH34-2	2.20	1.18	0.05
huH34-3	2.21	4.91	0.22
Gevokizumab	3.21	0.67	0.02
Canakinumab	2.15	284	13.2

The above data was determined by BIAcore.

In one embodiment the humanized anti-IL-1beta antibody binds to human and cynomolgus IL-1beta.

In the presence of human IL-1beta the binding signal in a surface plasmon resonance experiment increased from Gevokizumab. Thus, antibody-bound IL-1b still binds to IL-1 receptor I. Therefore, the mode of action for Gevokizumab is allosteric inhibition of IL-1RAc binding (allosteric antibody).

For Canakinumab, H34 and mumAb IL-1beta binding to IL-1 receptor I is prevented after antibody binding. Thus, mode of action is receptor blocking for Canakinumab, H34 and mumAb (competitive antibody).

antibody	**IC_{50} in the presence of @ 10 nM IL-1beta [nM]**
Canakinumab	1.6
mumAb	2.5
H34	3.5

In stimulation experiments it could be shown that the humanized antibodies as reported herein have the same activity as the murine parental antibody. In the following Table the IC_{50} values for inhibition of ICAM-1 expression upon IL-1beta stimulation of A549 cells are shown for different antibodies.

antibody	**IC_{50} [nM]**
H34	0.18
huH34-1	> 7
huH34-2	0.23
huH34-3	2.23
Gevokizumab	0.94
Canakinumab	0.31

In the following Table the IC_{50} value for inhibition of ICAM-1 expression upon IL-1beta stimulation of HUVEC cells is shown.

antibody	**IC_{50} [nM]**
H34	0.24
huH34-2	0.30
Canakinumab	9.02

In stimulation experiments it could be shown that the humanized antibodies as reported herein reduce IL-6 expression upon IL-1beta stimulation of A549 cells (see Table below).

antibody	**EC_{50} [nM]**
huH34-1	5.52
huH34-2	0.11
huH34-3	1.09
Gevokizumab	0.11
Canakinumab	0.12

In proliferation inhibition experiments it could be shown that the humanized antibody as reported herein inhibits proliferation of D10 cells (see Table below).

antibody	**IC_{50} [nM]**
huH34-2	0.83
Gevokizumab	3.36
Canakinumab	1.99

In the following Table the IC_{50} values for inhibition of TNFalpha expression upon MSU stimulation of THP1 cells are shown for different antibodies.

antibody	IC_{50} [nM]
H34	0.43
huH34-2	2.38
Canakinumab	0.41

In addition, the humanized antibodies show improved stability compared to the murine H34 parent antibody in stress tests. The binding activity has been determined using surface plasmon resonance (see Table below).

antibody	relative binding activity	
	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
H34	70 %	101 %
huH34-1	96 %	99 %
huH34-2	94 %	99 %
huH34-3	96 %	100 %

100 % = sample stored at -80 °C

The same stability can be seen when the high molecular weight content is determined (see Table below).

antibody	high molecular weight fraction		
	start	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
huH34-1	4.57 %	4.13 %	4.39 %
huH34-2	0.21 %	0.15 %	0.13 %
huH34-3	0.19 %	0.17 %	0.13 %

The same stability can be seen in the CE-SDS analysis (see Table below).

antibody	relative area%		
	start	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
huH34-1	96.1 %	92.9 %	93.8 %
huH34-2	96.4 %	92.5 %	95.2 %
huH34-3	96.0 %	92.1 %	95.1 %

The thermal stability of the different humanized antibodies has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

antibody	Tagg [°C]	Tm [°C]
huH34-1	61.5	69.1
huH34-2	63.0	72.0
huH34-3	63.0	70.6

The high-resolution crystal structure of the huH34-2 Fab-fragment bound to human IL-1 beta showed detailed information on the functional epitope of this antibody. The structure was compared to the structure of the ternary IL-1b signaling complex (human IL-1b bound to the IL-1 receptor 1, IL-1R1, and the IL-1 accessory protein, IL-1RAcP, PDB code 4DEP). It has been found that the epitope of huH34 overlaps with the interaction sites of both IL-1R1 and IL-1RAcP. Thus, the antibody blocks the formation of the IL-1 beta signaling complex at the first step of its assembly, which is the association of IL-1 beta and the IL-1R1.

Antibody 0031 is a bispecific anti-ANG2/IL-1beta antibody comprising as the IL-1beta binding specificity the VH and VL domain of huH34-2.

Antibody 0032 is a bispecific anti-VEGF/IL-1beta antibody comprising as the IL-1beta binding specificity the VH and VL domain of huH34-2.

For the determination of the kinetic binding values the assay as reported in Example 52 was used.

ANG2	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
antibody-0031	1.45E+05	1.15E-03	8	604

VEGF	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
antibody-0032	2.77E+04	<1E-06	<0.1	-

IL-1beta	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
huH34-2 bivalent	2.43E+06	1.15E-04	0.05	101
antibody-0031	2.56€+06	3.02E-04	0.12	38
antibody-0032	2.49E+06	3.05E-04	0.12	38

It has been shown by SPR analysis that all bispecific antibodies have the property of binding to both its antigens simultaneously.

In an ANG2 specific pTie2-ELISA the antibody 0031, is 6 times more active than the anti-ANG2/VEGF antibody reported in WO 2014/09465.

In one embodiment the humanized anti-IL-1beta antibody binds to human and cynomolgus IL-1beta.

In the following Table the IC_{50} value for inhibition of ICAM-1 expression upon IL-1beta stimulation of A549 cells is shown.

antibody	**IC_{50} [ng/mL]**
antibody 0031	103.9
Gevokizumab	204.4

In the following Table the IC_{50} value for inhibition of ICAM-1 expression upon IL-1beta stimulation of HUVEC cells is shown.

antibody	**IC_{50} [ng/mL]**
huH34-2	1.2-0.9
huH34-2 Fab	1.1-2.5
antibody 0031	2.0-5.5
antibody 0032	3.5-6.3

In stimulation experiments it could be shown that the humanized antibodies as reported herein reduce IL-6 expression upon IL-1beta stimulation of A549 cells (see Table below).

antibody	**EC_{50} [ng/mL]**
antibody 0031	17.0
antibody 0032	38.7
Gevokizumab	62.0
Canakinumab	86.4

The thermal stability of the different bispecific antibodies has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

antibody	**Tagg [°C]**	**Tm [°C]**
0031	61	67.5
0032	55	62.5

Antibody huH34-2 is described with the sequences of SEQ ID NO: 06 to 10 and 16 (binding sites, HVRs, VH, VL). The bispecific formats of antibody huH34-2 are described in sequences SEQ ID NO: 102 to 103 and 182 to 189. All of these sequences constitute alone and in combination aspects of the current invention.

In one preferred embodiment the anti-IL-1beta antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07; (b) a HVR-H2 comprising

the amino acid sequence of SEQ ID NO: 08; and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10. In one embodiment the antibody further comprises (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19.

In one preferred embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 06 and SEQ ID NO: 16, respectively, including post-translational modifications of those sequences.

In one preferred embodiment the anti-human IL-1beta antibody specifically binds to human and cynomolgus IL-1beta and comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10, (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17, (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18, and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19. In one preferred embodiment the anti-human IL-1beta antibody has a heavy chain variable domain that has the amino acid sequence of SEQ ID NO: 06 and a light chain variable domain that has the amino acid sequence of SEQ ID NO: 16.

A.2 Anti-PDGF-B antibodies from which the anti-PDGF-B binding site of the bispecific antibody as reported herein can be derived

Herein different novel anti-human PDGF-B antibodies are provided.

The first anti-PDGF-B antibody is a novel murine anti-human PDGF-B antibody with a VH of SEQ ID NO: 29 and a VL of SEQ ID NO: 34. This antibody is termed PDGF-B-mumAb in the following. This antibody binds to human, cynomolgus, rat and murine PDGF-B and inhibits the interaction between PDGF-B and its receptor.

The antibody has the following properties as listed in the Tables below.

	huPDGF-BB (EC50) [ng/mL]	muPDGF-BB (EC50) [ng/mL]	ratPDGF-BB (EC50) [ng/mL]	huPDGF-AA (EC50) [ng/mL]
PDGF-B-mumAb	16.3	8.8	5.124	2.04

	PPII huPDGF-BB::huPDGF-Rß IC50 [ng/ml]
PDGF-B-mumAb	12.16

	yield [mg]	scale [l]	yield [mg/L supernatant]	monomer (analytical SEC) [%]	main peak (CE-SDS/SDS PAGE) [%]
PDGF-B-mumAb	6.4	0.2	32	99	98

	IC_{50} Phospho-Inhibition		**IC_{50} Proliferation 3T3**	
PDGF-B-mumAb	1.7	ng/ml	55.1	ng/ml

	Pericytes				Balb-C3T3	
	Migration EC_{50} [nM]		Proliferation EC_{50} [nM]		Proliferation EC_{50} [nM]	
human PDGF-BB	0.02/0.03/0.03		0.03/0.02/0.019		0.028/0.026/0.031	
	IC50[nM]	% neutralization	IC50[nM]	% neutralization	IC50[nM]	% neutralization
R&D polyclonal reference antibody	1.13/0.78/0.4	100	0.49/0.36/0.41	100	079/0.69/0.65	100
PDGF-B-mumAb	0.08	100	0.21	100	0.179	100

5

	Pericytes		Balb-C3T3
	Fab Migration [nM]	Fab Proliferation [nM]	Fab Proliferation [nM]
PDGF-B-mumAb	0.24	2.17	2.3

	kd [1/s]	**t1/2 [min]**
PDGF-B-mumAb	4.50E-04	26

Fab	**ka [1/Ms]**	**kd [1/s]**	**KD [nM]**	**t1/2 [s]**
PDGF-B-mumAb Fab	4.39E+05	2.01E-03	5	345

	App. KD [nM]	T1/2 [s]		App. KD** [nM]	T1/2 (s)
PDGF-B-mumAb FAB	5	345	mumAb	0.042	2867

The storage stability of the antibody is shown in the Table below (active concentration with reference surface).

	2w/40°C pH 6.0 with reference [%]	2w/37°C pH 7.4 with reference [%]
PDGF-B-mumAb	**103**	**103**

The thermal stability of the antibody has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

antibody	**Tagg [°C]**	**Tm [°C]**
PDGF-B-mumAb	57	62 – 63.5

The other antibodies are human antibodies obtained from a human Ig locus transgenic rabbit. The antibodies have the binding properties as listed in the Table below.

Antibody 0085 is an anti-PDGF-B antibody comprising a VH of SEQ ID NO: 38 and a VL of SEQ ID NO: 43. Antibody 0086 is an anti-PDGF-B antibody comprising a VH of SEQ ID NO: 47 and a VL of SEQ ID NO: 52.

	huPDGF-BB [EC50] [ng/mL]	muPDGF-BB [EC50] [ng/mL]	ratPDGF-BB [EC50] [ng/mL]	cynPDGF-BB [EC50] [ng/mL]	huPDGF-AA [EC50] [ng/mL]	huPDGF-CC [EC50] [ng/mL]
antibody 0085	7.55	24.96	15.96	15.33	n.d.	n.d.
antibody 0086	12.04	14.08	10.57	16.19	n.d.	n.d.

	huPDGF-BB - avidity by SPR [M]	huPDGF-BB - kon by SPR [M-1s-1]	huPDGF-BB - koff by SPR [s-1]	cynPDGF-BB - avidity by SPR [M]	cynPDGF-BB - kon by SPR [M-1s-1]	cynPDGF-BB - koff by SPR [s-1]
antibody 0085	4.76E-11	0.93E6	0.44E-4	4.78E-11	0.94E6	4.52E-5
antibody 0086	2.18E-11	1.04E6	0.23E-4	1.78E-11	1.04E6	1.84E-5

	PPII huPDGF-BB::huPDGF-Rß IC50 [ng/ml]
antibody 0085	17.76
antibody 0086	11.73

In one embodiment the humanized anti-PDGF-B antibody binds to human, rat, mouse and cynomolgus PDGF-B.

	yield [mg]	scale [l]	yield [mg/l supernatant]	monomer (analytical SEC) [%]	main peak (CE-SDS/SDS PAGE) [%]
antibody 0085	22.5	0.25	90	99	96
antibody 0086	20.8	0.25	83	99	96
antibody 0106	12.0	0.3	40	98	99
antibody 0107	10.5	0.3	35	98	99
antibody 0144	37.7	1.5	25.1	>98	>95
antibody 0117	46.3	1	46.3	>98	>95
antibody 0145	21.5	1	21.5	>98	>95
antibody 0146	14.6	0.5	29.2	>98	>95

	IC_{50} Phospho-Inhibition		**IC_{50} Proliferation 3T3**	
antibody 0085	n.d.	n.d.	31.8	ng/ml

antibody 0086	n.d.	n.d.	56.0	ng/ml

In diverse activity assays the antibody show biological activity as can be seen from the data presented in the Tables below.

	Pericytes				Balb-C3T3	
	Migration EC50[nM]		Proliferation EC50[nM]		Proliferation EC50[nM]	
huPDGF-BB	0.03/0.04		0.02/0.03		0.025/0.023	
	IC50(nM)	% neutralization	IC50(nM)	% neutralization	IC50(nM)	% neutralization
R&D polyclonal Antibody	0.56/0.32	100	0.36/0.41	100	0.69/0.65	100
antibody 0085	0.15	90	0.11	100	0.06	100
antibody 0086	0.09	100	0.09	100	0.11	100

	Fab Migration inhibition [nM]	Fab Proliferation inhibition [nM]	Fab Proliferation inhibition [nM]
antibody 0085	0.41	1.02	1.36
antibody 0086	0.14	0.62	1.18

The kinetic binding properties of the different antibodies have been determined using surface plasmon resonance technology (see the following Tables).

	kd (1/s)	**t1/2 [min]**
antibody-0085	1.02E-04	114
antibody-0086	7.93E-05	146

Fab	**ka (1/Ms)**	**kd (1/s)**	**KD* (nM)**	**t1/2 (s)**
antibody-0106	2.02E+05	3.74E-04	2	1853
antibody-0107	1.95E+05	3.62E-04	2	1915

Fab	App. KD* (nM)	T1/2 (s)	**IgG**	App. KD** (nM)	T1/2 (s)
antibody-0106	2	1853	antibody-0085	0.023	8739
antibody-0107	2	1915	antibody-0086	0.030	2461

The storage stability of the antibody 0086 is shown in the Table below (active concentration with reference surface).

	2w/40°C pH 6.0 with reference (%)	2w/37°C pH 7.4 with reference (%)
antibody-0086	99	98

The thermal stability of the antibodies has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

	Tagg [°C]	**Tm [°C]**
antibody-0086	64	64.5-68

Antibody 0144 is a bispecific anti-ANG2/PDGF-B antibody comprising as the PDGF-B binding specificity the VH and VL domain of antibody 0085.

Antibody 0117 is a bispecific anti-VEGF/PDGF-B antibody comprising as the PDGF-B binding specificity the VH and VL domain of antibody 0085.

For the determination of the kinetic binding values the assay as reported in Example 52 was used.

ANG2	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
antibody 0144	9.06E+04	1.55E-03	17	446

VEGF	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
antibody 0117	2.46E+04	<1E-06	<0.1	-

PDGF-BB	**ka [1/Ms]**	**kd [1/s]**	**KD* [nM]**	**t1/2 [s]**
antibody 0106	2.94E+05	2.91E-04	1	40
antibody 0117	7.67E+04	2.45E-04	3	47
antibody 0144	8.31E+04	2.15E-04	3	53

It has been shown by SPR analysis that all bispecific antibodies have the property of binding to both its antigens simultaneously.

The bispecific antibodies show binding and biological activity in cell based assays.

	IC_{50} Proliferation human pericytes		**IC_{50} Proliferation 3T3**	
antibody 0117	0.016	nM	0.019	nM
antibody 0144	0.030	nM	0.020	nM
antibody 0085 FAB	0.012	nM	not det.	

	IC_{50} Phospho-Inhibition human pericytes		**IC_{50} Migration-Inhibition human pericytes**	
antibody 0117	0.055	nM	0.06	nM
antibody 0144	0.055	nM	0.06	nM
antibody 0085 FAB	not det.		0.27	nM

In an ANG2 specific pTie2-ELISA the antibody 0144, is 6 times more active than the anti-ANG2/VEGF antibody reported in WO 2014/09465.

In a VEGF specific reporter assay the antibody 0117 has a similar activity than the anti-ANG2/VEGF antibody reported in WO 2014/09465.

Antibody 0085 is described with the sequences of SEQ ID NO: 38 to 46 (binding sites, HVRs, VH, VL). The bispecific formats of antibody 0085 are described in sequences SEQ ID NO: 129 to 130, 150 to 153, 162 to 165, and 174 to 177. All of these sequences constitute alone and in combination aspects of the current invention.

Antibody 0086 is described with the sequences of SEQ ID NO: 47 to 55 (binding sites, HVRs, VH, VL). The bispecific formats of antibody 0086 are described in sequences SEQ ID NO: 131, 154 to 157, 166 to 169, and 178 to 181. All of these sequences constitute alone and in combination aspects of the current invention.

Antibody 0144 is described with the sequences of SEQ ID NO: 162 to 165 (CrossMab format, 2 heavy chains, 2 light chains).

Antibody 0144 and 0145 were incubated at different pH values for 2 weeks and thereafter their binding to PDGF-BB and ANG2, respectively, have been determined.

antibody 0144	binding to	rel. binding [%]	SD
reference value at start	PDGF-BB	100	
incubation at pH 6		101	4
incubation at pH 7.4		94	6
reference value at start	ANG2	100	
incubation at pH 6		100	1
incubation at pH 7.4		91	4

antibody 0145	binding to	rel. binding [%]	SD
reference value at start	PDGF-BB	100	
incubation at pH 6		99	1
incubation at pH 7.4		95	2
reference value at start	ANG2	100	
incubation at pH 6		99	2
incubation at pH 7.4		94	0

In one preferred embodiment the anti-PDGF-B antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 31; and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33. In one embodiment the antibody further comprises (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 35; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37.

In one embodiment, the antibody comprises ta humanized variant of the VH and VL sequences in SEQ ID NO: 29 and SEQ ID NO: 34, respectively, including post-translational modifications of those sequences.

In one preferred embodiment the anti-PDGF-B antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40; and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42. In one embodiment the antibody further comprises (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46.

In one preferred embodiment the anti-PDGF-B antibody as reported herein comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40; (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 41; (d) a HVR-L1

comprising the amino acid sequence of SEQ ID NO: 43; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 44; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 45. In also one preferred embodiment the anti-PDGF-B antibody as reported herein comprises a VH that has the amino acid sequence of SEQ ID NO: 38 and a VL that has the amino acid sequence of SEQ ID NO: 43. In also one preferred embodiment the anti-PDGF-B antibody is a bispecific antibody.

In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 38 and SEQ ID NO: 43, respectively, including post-translational modifications of those sequences.

In one preferred embodiment the anti-PDGF-B antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49; and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51. In one embodiment the antibody further comprises (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

In one preferred embodiment the anti-PDGF-B antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 50; (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51; (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55. In also one preferred embodiment the anti-PDGF-B antibody as reported herein comprises a VH that has the amino acid sequence of SEQ ID NO: 47 and a VL that has the amino acid sequence of SEQ ID NO: 52. In also one preferred embodiment the anti-PDGF-B antibody is a bispecific antibody.

In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 47 and SEQ ID NO: 52, respectively, including post-translational modifications of those sequences.

A.3 Anti-ANG2 antibodies from which the anti-ANG2 binding site of the bispecific antibody as reported herein can be derived

Human ANG2 binding kinetics:

molecule	k_a (1/Ms)	k_d (1/s)	K_D (nM)*	t1/2 (s)
0009	1.92E+06	0.07565	39	9
0041	3.85E+06	3.17E-03	1	219
0075	2.22E+06	3.10E-02	14	22
0090	2.16E+06	2.53E-03	1	274
0098	1.56E+07	1.58E-04	10*	
0099	2.61E+07	1.10E-04	4*	
0100	2.06E+07	1.67E-04	8*	
0101	1.83E+07	1.20E-04	7*	

*Avidity.

Cynomolgus ANG2 binding kinetics:

molecule	k_a (1/Ms)	k_d (1/s)	K_D (nM)*	t1/2 (s)
0009	1.45E+06	8.81E-02	61	8
0041	2.14E+06	3.60E-03	2	193
0075	1.34E+06	3.25E-02	24	21
0090	2.02E+06	3.08E-03	2	225

The relative biological activities of the antibodies as reported herein are given in the Table below.

molecule	relative biological activity (nM)
0009	72
0041	838
0075	128
0090	706
0098	100

The thermal stability of the different antibodies has been evaluated by determining the aggregation onset temperature (Tagg) and the melting temperature (Tm) (see Table below).

molecule	Tagg [°C]	Tm [°C]
0009	62.2	65.9
0041	63.1	66.0
0075	63.6	67.0
0090	64.0	67.4

In addition, the antibodies show good stability in stress tests. The binding activity has been determined using surface plasmon resonance (see Table below).

molecule	relative binding activity	
	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
0009	99 %	91 %
0041	100 %	101 %
0075	101 %	105 %
0090	112 %	100 %

100 % = sample stored at -80 °C

The same stability can be seen in the CE-SDS analysis (see Table below).

molecule	relative area%		
	start	2 weeks at 37 °C pH 7.4	2 weeks at 40 °C pH 6.0
0009	98.9	98.5	98.8
0041	98.9	98.6	98.6
0090	99.2	98.5	98.2

In one preferred embodiment the anti-ANG2 antibody comprises (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 75; (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 76; and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 78. In one embodiment the antibody further comprises (d) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 80; (e) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 81; and (f) a HVR-L3 comprising the amino acid sequence of SEQ ID NO: 82.

In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 74 and SEQ ID NO: 79, respectively, including post-translational modifications of those sequences.

A.4 Anti-VEGF antibodies from which the anti-VEGF binding site of the bispecific antibody as reported herein can be derived

In one preferred embodiment the anti-VEGF antibody comprises the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 107. The HVRs comprise the CDRs according to Kabat. In one embodiment the antibody further comprises the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 108. The HVRs comprise the CDRs according to Kabat.

In one embodiment, the antibody comprises the HC and LC sequences in SEQ ID NO: 107 and SEQ ID NO: 108, respectively, including post-translational modifications of those sequences.

In one preferred embodiment the anti-VEGF antibody comprises the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 109. The HVRs comprise the CDRs according to Kabat. In one embodiment the antibody further comprises the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 110. The HVRs comprise the CDRs according to Kabat.

In one embodiment, the antibody comprises the HC and LC sequences in SEQ ID NO: 109 and SEQ ID NO: 110, respectively, including post-translational modifications of those sequences.

A.5 Bispecific antibodies

One aspect as reported herein is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

The antibody under a) does not contain a modification as reported under b) and the heavy chain and the light chain under a) are isolated chains.

In the antibody under b)

within the light chain

the variable light chain domain VL is replaced by the variable heavy chain domain VH of said antibody,

and

within the heavy chain

the variable heavy chain domain VH is replaced by the variable light chain domain VL of said antibody.

In one embodiment

i) in the constant domain CL of the first light chain under a) the amino acid at position 124 (numbering according to Kabat) is substituted by a positively charged amino acid, and wherein in the constant domain CH1 of the first heavy chain under a) the amino acid at position 147 or the amino acid at position 213 (numbering according to Kabat EU index) is substituted by a negatively charged amino acid,

or

ii) in the constant domain CL of the second light chain under b) the amino acid at position 124 (numbering according to Kabat) is substituted by a positively charged amino acid, and wherein in the constant domain CH1 of the second heavy chain under b) the amino acid at position 147 or the amino acid at position 213 (numbering according to Kabat EU index) is substituted by a negatively charged amino acid.

In one preferred embodiment

i) in the constant domain CL of the first light chain under a) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the first heavy chain under a) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index),

or

ii) in the constant domain CL of the second light chain under b) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the second heavy chain under b) the

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

In one embodiment in the constant domain CL of the second heavy chain the amino acids at position 124 and 123 are substituted by K (numbering according to Kabat EU index).

In one embodiment in the constant domain CH1 of the second light chain the amino acids at position 147 and 213 are substituted by E (numbering according to EU index of Kabat).

In one preferred embodiment in the constant domain CL of the first light chain the amino acids at position 124 and 123 are substituted by K, and in the constant domain CH1 of the first heavy chain the amino acids at position 147 and 213 are substituted by E (numbering according to Kabat EU index).

In one embodiment in the constant domain CL of the second heavy chain the amino acids at position 124 and 123 are substituted by K, and wherein in the constant domain CH1 of the second light chain the amino acids at position 147 and 213 are substituted by E, and in the variable domain VL of the first light chain the amino acid at position 38 is substituted by K, in the variable domain VH of the first heavy chain the amino acid at position 39 is substituted by E, in the variable domain VL of the second heavy chain the amino acid at position 38 is substituted by K, and in the variable domain VH of the second light chain the amino acid at position 39 is substituted by E (numbering according to Kabat EU index).

One aspect as reported herein is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other, and wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

The antibody under a) does not contain a modification as reported under b) and the heavy chain and the light chain und a) are isolated chains.

In the antibody under b)

within the light chain

the variable light chain domain VL is replaced by the variable heavy chain domain VH of said antibody, and the constant light chain domain CL is replaced by the constant heavy chain domain CH1of said antibody;

and

within the heavy chain

the variable heavy chain domain VH is replaced by the variable light chain domain VL of said antibody, and the constant heavy chain domain CH1 is replaced by the constant light chain domain CL of said antibody.

One aspect as reported herein is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

The antibody under a) does not contain a modification as reported under b) and the heavy chain and the light chain under a) are isolated chains.

In the antibody under b)

within the light chain

the constant light chain domain CL is replaced by the constant heavy chain domain CH1of said antibody;

and within the heavy chain

the constant heavy chain domain CH1 is replaced by the constant light chain domain CL of said antibody.

One aspect as reported herein is a multispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains, and

b) one, two, three or four single chain Fab fragments specifically binding to one to four further antigens (i.e. a second and/or third and/or fourth and/or fifth antigen, preferably specifically binding to one further antigen, i.e. a second antigen),

wherein said single chain Fab fragments under b) are fused to said full length antibody under a) via a peptidic linker at the C- or N- terminus of the heavy or light chain of said full length antibody,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

In one embodiment one or two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of the heavy or light chains of said full length antibody.

In one embodiment one or two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of the heavy chains of said full length antibody.

In one embodiment one or two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of the light chains of said full length antibody.

In one embodiment two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of each heavy or light chain of said full length antibody.

In one embodiment two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of each heavy chain of said full length antibody.

In one embodiment two identical single chain Fab fragments binding to a second antigen are fused to said full length antibody via a peptidic linker at the C-terminus of each light chain of said full length antibody.

One aspect as reported herein is a trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains,

b) a first polypeptide consisting of
   ba) an antibody heavy chain variable domain (VH),
   or
   bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),
   wherein said first polypeptide is fused with the N-terminus of its VH domain via a peptidic linker to the C-terminus of one of the two heavy chains of said full length antibody,

c) a second polypeptide consisting of
   ca) an antibody light chain variable domain (VL),
   or
   cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL),
   wherein said second polypeptide is fused with the N-terminus of the VL domain via a peptidic linker to the C-terminus of the other of the two heavy chains of said full length antibody,

and

wherein the antibody heavy chain variable domain (VH) of the first polypeptide and the antibody light chain variable domain (VL) of the second

polypeptide together form an antigen-binding site specifically binding to a second antigen,

and

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

In one embodiment the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) are linked and stabilized via an interchain disulfide bridge by introduction of a disulfide bond between the following positions:

i) heavy chain variable domain position 44 to light chain variable domain position 100, or
ii) heavy chain variable domain position 105 to light chain variable domain position 43, or
iii) heavy chain variable domain position 101 to light chain variable domain position 100 (numbering always according to Kabat EU index).

Techniques to introduce unnatural disulfide bridges for stabilization are described e.g. in WO 94/029350, Rajagopal, V., et al., Prot. Eng. (1997) 1453-59; Kobayashi, H., et al., Nuclear Medicine & Biology, Vol. 25, (1998) 387-393; or Schmidt, M., et al., Oncogene (1999) 18 1711-1721. In one embodiment the optional disulfide bond between the variable domains of the polypeptides under b) and c) is between heavy chain variable domain position 44 and light chain variable domain position 100. In one embodiment the optional disulfide bond between the variable domains of the polypeptides under b) and c) is between heavy chain variable domain position 105 and light chain variable domain position 43. (numbering always according to EU index of Kabat) In one embodiment a trivalent, bispecific antibody without said optional disulfide stabilization between the variable domains VH and VL of the single chain Fab fragments is preferred.

One aspect as reported herein is a trispecific or tetraspecific antibody, comprising

a) a first light chain and a first heavy chain of a full length antibody which specifically binds to a first antigen, and

b) a second (modified) light chain and a second (modified) heavy chain of a full length antibody which specifically binds to a second antigen, wherein the variable domains VL and VH are replaced by each other, and/or wherein the constant domains CL and CH1 are replaced by each other, and

c) wherein one to four antigen binding peptides which specifically bind to one or two further antigens (i.e. to a third and/or fourth antigen) are fused via a peptidic linker to the C- or N-terminus of the light chains or heavy chains of a) and/or b),

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

The antibody under a) does not contain a modification as reported under b) and the heavy chain and the light chain und a) are isolated chains.

In one embodiment the trispecific or tetraspecific antibody comprises under c) one or two antigen binding peptides which specifically bind to one or two further antigens.

In one embodiment the antigen binding peptides are selected from the group of a scFv fragment and a scFab fragment.

In one embodiment the antigen binding peptides are scFv fragments.

In one embodiment the antigen binding peptides are scFab fragments.

In one embodiment the antigen binding peptides are fused to the C-terminus of the heavy chains of a) and/or b).

In one embodiment the trispecific or tetraspecific antibody comprises under c) one or two antigen binding peptides which specifically bind to one further antigen.

In one embodiment the trispecific or tetraspecific antibody comprises under c) two identical antigen binding peptides which specifically bind to a third antigen. In one preferred embodiment such two identical antigen binding peptides are fused both via the same peptidic linker to the C-terminus of the heavy chains of a) and b). In one preferred embodiment the two identical antigen binding peptides are either a scFv fragment or a scFab fragment.

In one embodiment the trispecific or tetraspecific antibody comprises under c) two antigen binding peptides which specifically bind to a third and a fourth antigen. In one embodiment said two antigen binding peptides are fused both via the same peptide connector to the C-terminus of the heavy chains of a) and b). In one preferred embodiment said two antigen binding peptides are either a scFv fragment or a scFab fragment.

One aspect as reported herein is a bispecific, tetravalent antibody comprising

a) two light chains and two heavy chains of an antibody, which specifically bind to a first antigen (and comprise two Fab fragments),

b) two additional Fab fragments of an antibody, which specifically bind to a second antigen, wherein said additional Fab fragments are fused both via a peptidic linker either to the C- or N-termini of the heavy chains of a),

and

wherein in the Fab fragments the following modifications were performed

i) in both Fab fragments of a), or in both Fab fragments of b), the variable domains VL and VH are replaced by each other, and/or the constant domains CL and CH1 are replaced by each other,

or

ii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,

and

in both Fab fragments of b) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

or

iii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

and

in both Fab fragments of b) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,

or

iv) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and in both Fab fragments of b) the constant domains CL and CH1 are replaced by each other,

or

v) in both Fab fragments of a) the constant domains CL and CH1 are replaced by each other, and in both Fab fragments of b) the variable domains VL and VH are replaced by each other,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

In one embodiment said additional Fab fragments are fused both via a peptidic linker either to the C-termini of the heavy chains of a), or to the N-termini of the heavy chains of a).

In one embodiment said additional Fab fragments are fused both via a peptidic linker either to the C-termini of the heavy chains of a).

In one embodiment said additional Fab fragments are fused both via a peptide connector to the N-termini of the heavy chains of a).

In one embodiment in the Fab fragments the following modifications are performed:

i) in both Fab fragments of a), or in both Fab fragments of b), the variable domains VL and VH are replaced by each other,
and/or
the constant domains CL and CH1 are replaced by each other.

In one embodiment in the Fab fragments the following modifications are performed:

i) in both Fab fragments of a) the variable domains VL and VH are replaced by each other,
and/or
the constant domains CL and CH1 are replaced by each other.

In one embodiment in the Fab fragments the following modifications are performed:

i) in both Fab fragments of a) the constant domains CL and CH1 are replaced by each other.

In one embodiment in the Fab fragments the following modifications are performed:

i) in both Fab fragments of b) the variable domains VL and VH are replaced by each other,
and/or
the constant domains CL and CH1 are replaced by each other.

In one embodiment in the Fab fragments the following modifications are performed:

i) in both Fab fragments of b) the constant domains CL and CH1 are replaced by each other.

One aspect as reported herein is a bispecific, tetravalent antibody comprising:

a) a (modified) heavy chain of a first antibody, which specifically binds to a first antigen and comprises a first VH-CH1 domain pair, wherein to the C-terminus of said heavy chain the N-terminus of a second VH-CH1 domain pair of said first antibody is fused via a peptidic linker,

b) two light chains of said first antibody of a),

c) a (modified) heavy chain of a second antibody, which specifically binds to a second antigen and comprises a first VH-CL domain pair, wherein to the

C-terminus of said heavy chain the N-terminus of a second VH-CL domain pair of said second antibody is fused via a peptidic linker, and

d) two (modified) light chains of said second antibody of c), each comprising a CL-CH1 domain pair,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

One aspect as reported herein is a bispecific antibody comprising

a) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen, and

b) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of the light chain via a peptidic linker,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

The antibody under a) does not contain a modification as reported under b) and the heavy chain and the light chain are isolated chains.

One aspect as reported herein is a bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains, and

b) an Fv fragment specifically binding to a second antigen comprising a VH^2 domain and a VL^2 domain, wherein both domains are connected to each other via a disulfide bridge,

wherein only either the VH^2 domain or the VL^2 domain is fused via a peptidic linker to the heavy or light chain of the full length antibody specifically binding to a first antigen,

wherein the first and the second antigen are different antigens selected from the group consisting of (human) ANG2, (human) VEGF, (human) IL-1beta and (human) PDGF-B.

In the bispecific the heavy chains and the light chains under a) are isolated chains.

In one embodiment the other of the VH^2 domain or the VL^2 domain is not fused via a peptide linker to the heavy or light chain of the full length antibody specifically binding to a first antigen.

In all aspects as reported herein the first light chain comprises a VL domain and a CL domain and the first heavy chain comprises a VH domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain.

In one embodiment of all aspects the antibody as reported herein is a multispecific antibody, which requires heterodimerization of at least two heavy chain polypeptides, and wherein the antibody specifically binds to human IL-1beta and a second non-human IL-1beta antigen.

Several approaches for CH3-modifications in order to support heterodimerization have been described, for example in WO 96/27011, WO 98/050431, EP 1870459, WO 2007/110205, WO 2007/147901, WO 2009/089004, WO 2010/129304, WO 2011/90754, WO 2011/143545, WO 2012/058768, WO 2013/157954, WO 2013/096291, which are herein included by reference. Typically, in the approaches known in the art, the CH3 domain of the first heavy chain and the CH3 domain of the second heavy chain are both engineered in a complementary manner so that the heavy chain comprising one engineered CH3 domain can no longer homodimerize with another heavy chain of the same structure (e.g. a CH3-engineered first heavy chain can no longer homodimerize with another CH3-engineered first heavy chain; and a CH3-engineered second heavy chain can no longer homodimerize with another CH3-engineered second heavy chain). Thereby the heavy chain comprising one engineered CH3 domain is forced to heterodimerize with another heavy chain comprising the CH3 domain, which is engineered in a complementary manner. For this embodiment of the invention, the CH3 domain of the first heavy chain and the CH3 domain of the second heavy chain are engineered in a complementary manner by amino acid substitutions, such that the first heavy chain and the second heavy chain are forced to heterodimerize, whereas the first heavy chain and the second heavy chain can no longer homodimerize (e.g. for steric reasons).

The different approaches for supporting heavy chain heterodimerization known in the art, that were cited and included above, are contemplated as different alternatives used in a multispecific antibody according to the invention, which comprises a "non-crossed Fab region" derived from a first antibody, which specifically binds to a first antigen, and a "crossed Fab region" derived from a second antibody, which specifically binds to a second antigen, in combination with the particular amino acid substitutions described above for the invention.

The CH3 domains of the multispecific antibody as reported herein can be altered by the "knob-into-holes" technology which is described in detail with several examples in e.g. WO 96/027011, Ridgway, J.B., et al., Protein Eng. 9 (1996) 617-621; and Merchant, A.M., et al., Nat. Biotechnol. 16 (1998) 677-681. In this method the interaction surfaces of the two CH3 domains are altered to increase the heterodimerization of both heavy chains containing these two CH3 domains. Each of the two CH3 domains (of the two heavy chains) can be the "knob", while the other is the "hole". The introduction of a disulfide bridge further stabilizes the heterodimers (Merchant, A.M., et al., Nature Biotech. 16 (1998) 677-681; Atwell, S., et al., J. Mol. Biol. 270 (1997) 26-35) and increases the yield.

In one preferred embodiment the multispecific antibody as reported herein comprises a T366W mutation in the CH3 domain of the "knobs chain" and T366S, L368A, Y407V mutations in the CH3 domain of the "hole-chain" (numbering according to Kabat EU index). An additional interchain disulfide bridge between the CH3 domains can also be used (Merchant, A.M., et al., Nature Biotech. 16 (1998) 677-681) e.g. by introducing a Y349C mutation into the CH3 domain of the "knobs chain" and a E356C mutation or a S354C mutation into the CH3 domain of the "hole chain". Thus in a another preferred embodiment, the multispecific antibody as reported herein comprises the Y349C and T366W mutations in one of the two CH3 domains and the E356C, T366S, L368A and Y407V mutations in the other of the two CH3 domains or the multispecific antibody as reported herein comprises the Y349C and T366W mutations in one of the two CH3 domains and the S354C, T366S, L368A and Y407V mutations in the other of the two CH3 domains (the additional Y349C mutation in one CH3 domain and the additional E356C or S354C mutation in the other CH3 domain forming a interchain disulfide bridge) (numbering according to Kabat EU index).

But also other knobs-in-holes technologies as described by EP 1 870 459A1, can be used alternatively or additionally. In one embodiment the multispecific antibody as

reported herein comprises the R409D and K370E mutations in the CH3 domain of the "knobs chain" and the D399K and E357K mutations in the CH3 domain of the "hole-chain" (numbering according to Kabat EU index).

In one embodiment the multispecific antibody as reported herein comprises a T366W mutation in the CH3 domain of the "knobs chain" and the T366S, L368A and Y407V mutations in the CH3 domain of the "hole chain" and additionally the R409D and K370E mutations in the CH3 domain of the "knobs chain" and the D399K and E357K mutations in the CH3 domain of the "hole chain" (numbering according to the Kabat EU index).

In one embodiment the multispecific antibody as reported herein comprises the Y349C and T366W mutations in one of the two CH3 domains and the S354C, T366S, L368A and Y407V mutations in the other of the two CH3 domains, or the multispecific antibody as reported herein comprises the Y349C and T366W mutations in one of the two CH3 domains and the S354C, T366S, L368A and Y407V mutations in the other of the two CH3 domains and additionally the R409D and K370E mutations in the CH3 domain of the "knobs chain" and the D399K and E357K mutations in the CH3 domain of the "hole chain" (numbering according to the Kabat EU index).

Apart from the "knob-into-hole technology" other techniques for modifying the CH3 domains of the heavy chains of a multispecific antibody to enforce heterodimerization are known in the art. These technologies, especially the ones described in WO 96/27011, WO 98/050431, EP 1870459, WO 2007/110205, WO 2007/147901, WO 2009/089004, WO 2010/129304, WO 2011/90754, WO 2011/143545, WO 2012/058768, WO 2013/157954 and WO 2013/096291 are contemplated herein as alternatives to the "knob-into-hole technology" in combination with a multispecific antibody as reported herein.

In one embodiment of a multispecific antibody as reported herein the approach described in EP 1870459 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. This approach is based on the introduction of charged amino acids with opposite charges at specific amino acid positions in the CH3/CH3-domain-interface between both, the first and the second heavy chain.

Accordingly, this embodiment relates to a multispecific antibody as reported herein, wherein in the tertiary structure of the antibody the CH3 domain of the first heavy chain and the CH3 domain of the second heavy chain form an interface that is located between the respective antibody CH3 domains, wherein the respective amino acid sequences of the CH3 domain of the first heavy chain and the CH3 domain of the second heavy chain each comprise a set of amino acids that is located within said interface in the tertiary structure of the antibody, wherein from the set of amino acids that is located in the interface in the CH3 domain of one heavy chain a first amino acid is substituted by a positively charged amino acid and from the set of amino acids that is located in the interface in the CH3 domain of the other heavy chain a second amino acid is substituted by a negatively charged amino acid. The multispecific antibody according to this embodiment is herein also referred to as "CH3(+/-)-engineered multispecific antibody" (wherein the abbreviation "+/-" stands for the oppositely charged amino acids that were introduced in the respective CH3 domains).

In one embodiment of said CH3(+/-)-engineered multispecific antibody as reported herein the positively charged amino acid is selected from K, R and H, and the negatively charged amino acid is selected from E or D.

In one embodiment of said CH3(+/-)-engineered multispecific antibody as reported herein the positively charged amino acid is selected from K and R, and the negatively charged amino acid is selected from E or D.

In one embodiment of said CH3(+/-)-engineered multispecific antibody as reported herein the positively charged amino acid is K, and the negatively charged amino acid is E.

In one embodiment of said CH3(+/-)-engineered multispecific antibody as reported herein in the CH3 domain of one heavy chain the amino acid R at position 409 is substituted by D and the amino acid K at position is substituted by E, and in the CH3 domain of the other heavy chain the amino acid D at position 399 is substituted by K and the amino acid E at position 357 is substituted by K (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein the approach described in WO2013/157953 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. In one

embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid T at position 366 is substituted by K, and in the CH3 domain of the other heavy chain the amino acid L at position 351 is substituted by D (numbering according to Kabat EU index). In another embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid T at position 366 is substituted by K and the amino acid L at position 351 is substituted by K, and in the CH3 domain of the other heavy chain the amino acid L at position 351 is substituted by D (numbering according to Kabat EU index).

In another embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid T at position 366 is substituted by K and the amino acid L at position 351 is substituted by K, and in the CH3 domain of the other heavy chain the amino acid L at position 351 is substituted by D (numbering according to Kabat EU index). Additionally at least one of the following substitutions is comprised in the CH3 domain of the other heavy chain: the amino acid Y at position 349 is substituted by E, the amino acid Y at position 349 is substituted by D and the amino acid L at position 368 is substituted by E (numbering according to Kabat EU index). In one embodiment the amino acid L at position 368 is substituted by E (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein the approach described in WO2012/058768 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. In one embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid L at position 351 is substituted by Y and the amino acid Y at position 407 is substituted by A, and in the CH3 domain of the other heavy chain the amino acid T at position 366 is substituted by A and the amino acid K at position 409 is substituted by F (numbering according to Kabat EU index). In another embodiment, in addition to the aforementioned substitutions, in the CH3 domain of the other heavy chain at least one of the amino acids at positions 411 (originally T), 399 (originally D), 400 (originally S), 405 (originally F), 390 (originally N) and 392 (originally K) is substituted (numbering according to Kabat EU index). Preferred substitutions are:

- substituting the amino acid T at position 411 by an amino acid selected from N, R, Q, K, D, E and W (numbering according to Kabat EU index),

- substituting the amino acid D at position 399 by an amino acid selected from R, W, Y, and K (numbering according to Kabat EU index),
- substituting the amino acid S at position 400 by an amino acid selected from E, D, R and K (numbering according to Kabat EU index),
- substituting the amino acid F at position 405 by an amino acid selected from I, M, T, S, V and W (numbering according to Kabat EU index;
- substituting the amino acid N at position 390 by an amino acid selected from R, K and D (numbering according to Kabat EU index; and
- substituting the amino acid K at position 392 by an amino acid selected from V, M, R, L, F and E (numbering according to Kabat EU index).

In another embodiment of said multispecific antibody as reported herein (engineered according to WO2012/058768), in the CH3 domain of one heavy chain the amino acid L at position 351 is substituted by Y and the amino acid Y at position 407 is substituted by A, and in the CH3 domain of the other heavy chain the amino acid T at position 366 is substituted by V and the amino acid K at position 409 is substituted by F (numbering according to Kabat EU index). In another embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid Y at position 407 is substituted by A, and in the CH3 domain of the other heavy chain the amino acid T at position 366 is substituted by A and the amino acid K at position 409 is substituted by F (numbering according to Kabat EU index). In said last aforementioned embodiment, in the CH3 domain of said other heavy chain the amino acid K at position 392 is substituted by E, the amino acid T at position 411 is substituted by E, the amino acid D at position 399 is substituted by R and the amino acid S at position 400 is substituted by R (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein the approach described in WO 2011/143545 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. In one embodiment of said multispecific antibody as reported herein, amino acid modifications in the CH3 domains of both heavy chains are introduced at positions 368 and/or 409 (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein the approach described in WO 2011/090762 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. WO 2011/090762 relates to amino acid modifications according to the “knob-into-hole”

technology. In one embodiment of said CH3(KiH)-engineered multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid T at position 366 is substituted by W, and in the CH3 domain of the other heavy chain the amino acid Y at position 407 is substituted by A (numbering according to Kabat EU index). In another embodiment of said CH3(KiH)-engineered multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid T at position 366 is substituted by Y, and in the CH3 domain of the other heavy chain the amino acid Y at position 407 is substituted by T (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein, which is of IgG2 isotype, the approach described in WO 2011/090762 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody.

In one embodiment of a multispecific antibody as reported herein, the approach described in WO 2009/089004 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. In one embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid K or N at position 392 is substituted by a negatively charged amino acid (in one preferred embodiment by E or D, in one preferred embodiment by D), and in the CH3 domain of the other heavy chain the amino acid D at position 399 the amino acid E or D at position 356 or the amino acid E at position 357 is substituted by a positively charged amino acid (in one preferred embodiment K or R, in one preferred embodiment by K, in one preferred embodiment the amino acids at positions 399 or 356 are substituted by K) (numbering according to Kabat EU index). In one further embodiment, in addition to the aforementioned substitutions, in the CH3 domain of the one heavy chain the amino acid K or R at position 409 is substituted by a negatively charged amino acid (in one preferred embodiment by E or D, in one preferred embodiment by D) (numbering according to Kabat EU index). In one even further embodiment, in addition to or alternatively to the aforementioned substitutions, in the CH3 domain of the one heavy chain the amino acid K at position 439 and/or the amino acid K at position 370 is substituted independently from each other by a negatively charged amino acid (in one preferred embodiment by E or D, in one preferred embodiment by D) (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein, the approach described in WO 2007/147901 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody. In one embodiment of said multispecific antibody as reported herein, in the CH3 domain of one heavy chain the amino acid K at position 253 is substituted by E, the amino acid D at position 282 is substituted by K and the amino acid K at position 322 is substituted by D, and in the CH3 domain of the other heavy chain the amino acid D at position 239 is substituted by K, the amino acid E at position 240 is substituted by K and the amino acid K at position 292 is substituted by D (numbering according to Kabat EU index).

In one embodiment of a multispecific antibody as reported herein, the approach described in WO 2007/110205 is used to support heterodimerization of the first heavy chain and the second heavy chain of the multispecific antibody

In one embodiment of all aspects and embodiments as reported herein the multispecific antibody is a bispecific antibody or a trispecific antibody. In one preferred embodiment of the invention the multispecific antibody is a bispecific antibody.

In one embodiment of all aspects as reported herein, the antibody is a bivalent or trivalent antibody. In one embodiment the antibody is a bivalent antibody.

In one embodiment of all aspects as reported herein, the multispecific antibody has a constant domain structure of an IgG type antibody. In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG1, or of human subclass IgG1 with the mutations L234A and L235A. In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG2. In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG3. In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG4 or, of human subclass IgG4 with the additional mutation S228P. In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG1 or human subclass IgG4. In one further embodiment of all aspects as reported herein, the multispecific antibody is

characterized in that said multispecific antibody is of human subclass IgG1 with the mutations L234A and L235A (numbering according to Kabat EU index). In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG1 with the mutations L234A, L235A and P329G (numbering according to Kabat EU index). In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG4 with the mutations S228P and L235E (numbering according to Kabat EU index). In one further embodiment of all aspects as reported herein, the multispecific antibody is characterized in that said multispecific antibody is of human subclass IgG4 with the mutations S228P, L235E and P329G (numbering according to Kabat EU index).

In one embodiment of all aspects as reported herein, an antibody comprising a heavy chain including a CH3 domain as specified herein, comprises an additional C-terminal glycine-lysine dipeptide (G446 and K447, numbering according to Kabat EU index). In one embodiment of all aspects as reported herein, an antibody comprising a heavy chain including a CH3 domain, as specified herein, comprises an additional C-terminal glycine residue (G446, numbering according to Kabat EU index).

In one embodiment the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein

i) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide, or

ii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, or

iii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, or

iv) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366S, L368A, Y407V, or

v) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366S, L368A, Y407V, or

vi) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide, or

vii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, or

viii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, or

ix) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366S, L368A, Y407V, or

x) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366S, L368A, Y407V.

In one embodiment the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein the antibody comprises the combination of mutations

i) I253A, H310A, and H435A, or

ii) H310A, H433A, and Y436A, or

iii) L251D, L314D, and L432D, or

iv) combinations of i) to iii)

in the first Fc-region polypeptide and in the second Fc-region polypeptide.

In one embodiment of all aspects as reported herein the antibody as reported herein is an effector silent antibody. In one embodiment of all aspects as reported herein the antibody is an effector silent antibody and does not bind to human FcRn. In one preferred embodiment of all aspects as reported herein is the antibody of the human subclass IgG1 and has the mutations L234A, L235A, P329G, I253A, H310A and H434A in both heavy chains (numbering according to the Kabat index).

In a further aspect, an anti-IL-1beta antibody according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-4 below.

1. Antibody Affinity

Methods for the determination of the KD value are outlined in the Examples below.

When using a BIACORE® surface plasmon resonance assay the KD value can be measured alternatively as follows: An assay using a BIACORE®-2000 or a BIACORE ®-3000 (BIAcore, Inc., Piscataway, NJ) is performed at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). In one embodiment, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (~0.2 µM) before injection at a flow rate of 5 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 µl/min. Association rates (k_a) and dissociation rates (k_d) are calculated using a simple one-to-one Langmuir binding model (BIACORE ® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The

equilibrium dissociation constant (KD) is calculated as the ratio k_d/k_a (see, e.g., Chen, Y. et al., J. Mol. Biol. 293 (1999) 865-881). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the association-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25 °C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in US 4,816,567; and Morrison, S.L. et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro, J.C. and Fransson, J., Front. Biosci. 13 (2008) 1619-1633, and are further described, e.g., in Riechmann, I. et al., Nature 332 (1988) 323-329; Queen, C. et

al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033; US 5, 821,337, US 7,527,791, US 6,982,321, and US 7,087,409; Kashmiri, S.V. et al., Methods 36 (2005) 25-34 (describing specificity determining region (SDR) grafting); Padlan, E.A., Mol. Immunol. 28 (1991) 489-498 (describing "resurfacing"); Dall'Acqua, W.F. et al., Methods 36 (2005) 43-60 (describing "FR shuffling"); and Osbourn, J. et al., Methods 36 (2005) 61-68 and Klimka, A. et al., Br. J. Cancer 83 (2000) 252-260 (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims, M.J. et al., J. Immunol. 151 (1993) 2296-2308; framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter, P. et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Presta, L.G. et al., J. Immunol. 151 (1993) 2623-2632); human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro, J.C. and Fransson, J., Front. Biosci. 13 (2008) 1619-1633); and framework regions derived from screening FR libraries (see, e.g., Baca, M. et al., J. Biol. Chem. 272 (1997) 10678-10684 and Rosok, M.J. et al., J. Biol. Chem. 271 (19969 22611-22618).

3. Multispecific Antibodies

An antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for IL-1beta and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of IL-1beta. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express IL-1beta. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein, C. and Cuello, A.C., Nature 305 (1983) 537-540, WO 93/08829, and Traunecker, A. et al., EMBO J. 10 (1991) 3655-3659), and "knob-in-hole" engineering (see, e.g., US 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004); cross-linking

two or more antibodies or fragments (see, e.g., US 4,676,980, and Brennan, M. et al., Science 229 (1985) 81-83); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny, S.A. et al., J. Immunol. 148 (1992) 1547-1553; using "diabody" technology for making bispecific antibody fragments (see, e.g., Holliger, P. et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448); and using single-chain Fv (sFv) dimers (see, e.g. Gruber, M et al., J. Immunol. 152 (1994) 5368-5374); and preparing trispecific antibodies as described, e.g., in Tutt, A. et al., J. Immunol. 147 (1991) 60-69).

Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576).

The antibody or fragment herein also includes a "Dual Acting Fab" or "DAF" comprising an antigen binding site that binds to IL-1beta as well as another, different antigen (see, US 2008/0069820, for example).

The antibody or fragment herein also includes multispecific antibodies described 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.

4. Antibody Variants

In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a) Substitution, Insertion, and Deletion Variants

In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in the Table under the heading of "preferred substitutions". More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; norleucine	Leu
Leu (L)	norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, P.S., Methods Mol. Biol. 207 (2008) 179-196), and/or residues that contact antigen, with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom, H.R. et al. in Methods in Molecular Biology 178 (2002) 1-37. In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g.,

conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may, for example, be outside of antigen contacting residues in the HVRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham, B.C. and Wells, J.A., Science 244 (1989) 1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

b) Glycosylation variants

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

Where the antibody comprises an Fc-region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a

branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc-region (see, e.g., Wright, A. and Morrison, S.L., TIBTECH 15 (1997) 26-32). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc-region. For example, the amount of fucose in such antibody may be from 1 % to 80 %, from 1 % to 65 %, from 5 % to 65 % or from 20 % to 40 %. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc-region (EU numbering of Fc-region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US 2003/0157108; US 2004/0093621. Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO 2005/053742; WO 2002/031140; Okazaki, A. et al., J. Mol. Biol. 336 (2004) 1239-1249; Yamane-Ohnuki, N. et al., Biotech. Bioeng. 87 (2004) 614-622. Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka, J. et al., Arch. Biochem. Biophys. 249 (1986) 533-545; US 2003/0157108; and WO 2004/056312, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (see, e.g., Yamane-Ohnuki, N. et al., Biotech. Bioeng. 87 (2004) 614-622; Kanda, Y. et al., Biotechnol. Bioeng. 94 (2006) 680-688; and WO 2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc-region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878; US 6,602,684; and US 2005/0123546. Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc-region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087; WO 1998/58964; and WO 1999/22764.

c) Fc-region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc-region of an antibody provided herein, thereby generating an Fc-region variant. The Fc-region variant may comprise a human Fc-region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc-region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half-life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch, J.V. and Kinet, J.P., Annu. Rev. Immunol. 9 (1991) 457-492. Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in US 5,500,362 (see, e.g. Hellstrom, I. et al., Proc. Natl. Acad. Sci. USA 83 (1986) 7059-7063; and Hellstrom, I. et al., Proc. Natl. Acad. Sci. USA 82 (1985) 1499-1502); US 5,821,337 (see Bruggemann, M. et al., J. Exp. Med. 166 (1987) 1351-1361). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral

blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes, R. et al., Proc. Natl. Acad. Sci. USA 95 (1998) 652-656. C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity (see, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402). To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro, H. et al., J. Immunol. Methods 202 (1996) 163-171; Cragg, M.S. et al., Blood 101 (2003) 1045-1052; and Cragg, M.S. and M.J. Glennie, Blood 103 (2004) 2738-2743). FcRn binding and in vivo clearance/half-life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., Int. Immunol. 18 (2006: 1759-1769).

Antibodies with reduced effector function include those with substitution of one or more of Fc-region residues 238, 265, 269, 270, 297, 327 and 329 (US 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US 7,332,581).

Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., US 6,737,056; WO 2004/056312, and Shields, R.L. et al., J. Biol. Chem. 276 (2001) 6591-6604)

In certain embodiments, an antibody variant comprises an Fc-region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc-region (EU numbering of residues).

In some embodiments, alterations are made in the Fc-region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US 6,194,551, WO 99/51642, and Idusogie, E.E. et al., J. Immunol. 164 (2000) 4178-4184.

Antibodies with increased half-lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer, R.L. et al., J. Immunol. 117 (1976) 587-593, and Kim, J.K. et al., J. Immunol. 24 (1994) 2429-2434), are described in US 2005/0014934. Those antibodies comprise an Fc-region with one or more substitutions therein which improve binding of the Fc-region to FcRn. Such Fc variants include those with

substitutions at one or more of Fc-region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc-region residue 434 (US 7,371,826).

See also Duncan, A.R. and Winter, G., Nature 322 (1988) 738-740; US 5,648,260; US 5,624,821; and WO 94/29351 concerning other examples of Fc-region variants.

d) Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc-region. Cysteine engineered antibodies may be generated as described, e.g., in US 7,521,541.

e) Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain additional non-proteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to

the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

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

B. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in US 4,816,567. In one embodiment, isolated nucleic acid encoding an antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making an antibody as reported herein is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., US 5,648,237, US 5,789,199, and US 5,840,523. (See also Charlton, K.A., In: Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized", resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414; and Li, H. et al., Nat. Biotech. 24 (2006) 210-215.

Suitable host cells for the expression of glycosylated antibody 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 PLANTIBODIES™ technology for producing antibodies in transgenic plants)).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in

Graham, F.L. et al., J. Gen Virol. 36 (1977) 59-74); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather, J.P. et al., Annals N.Y. Acad. Sci. 383 (1982) 44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub, G. et al., Proc. Natl. Acad. Sci. USA 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P. and Wu, A.M., Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268.

C. Assays

Antibodies provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art. Exemplary assays are reported in the Examples.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an antibody as reported herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see US 5,208,020, US 5,416,064 and EP 0 425 235 B1); an auristatin such as monomethyl auristatin drug moieties DE and DF (MMAE and MMAF) (see US 5,635,483, US 5,780,588, and US 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see US 5,712,374, US 5,714,586, US 5,739,116, US 5,767,285, US 5,770,701, US 5,770,710, US 5,773,001, and US 5,877,296; Hinman, L.M. et al., Cancer Res. 53 (1993) 3336-3342; and Lode, H.N. et al., Cancer Res. 58 (1998) 2925-2928); an anthracycline such as

daunomycin or doxorubicin (see Kratz, F. et al., Curr. Med. Chem. 13 (2006) 477-523; Jeffrey, S.C., et al., Bioorg. Med. Chem. Lett. 16 (2006) 358-362; Torgov, M.Y., et al., Bioconjug. Chem. 16 (2005) 717-721; Nagy, A., et al., Proc. Natl. Acad. Sci. USA 97 (2000) 829-834; Dubowchik, G.M., et al., Bioorg. & Med. Chem. Letters 12 (2002) 1529-1532; King, H.D., et al., J. Med. Chem. 45 (2002) 4336-4343; and US 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example TC^{99m} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in

Vitetta, E.S. et al., Science 238 (1987) 1098-1104. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triamine pentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari, R.V. et al., Cancer Res. 52 (1992) 127-131; US 5,208,020) may be used.

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

E. Pharmaceutical Formulations

Pharmaceutical formulations of a multispecific 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 dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). 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 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 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. For example, it may be desirable to further provide an anti-ANG2 antibody or an anti-VEGF antibody. Such active ingredients are suitably present in combination in amounts that are effective for the 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, 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 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.

F. Therapeutic Methods and Compositions

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

In one aspect, a multispecific antibody for use as a medicament is provided. In further aspects, a multispecific antibody for use in treating an ocular vascular disease, preferably macular degeneration, is provided. In certain embodiments, a multispecific antibody for use in a method of treatment is provided. In certain embodiments, the invention provides a multispecific antibody for use in a method of treating an individual having an ocular vascular disease, preferably macular degeneration, comprising administering to the individual an effective amount of the multispecific 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 multispecific antibody for use in inhibiting angiogenesis. In certain embodiments, the invention provides a multispecific antibody for use in a method of inhibiting angiogenesis in an individual comprising administering to the individual an effective of the multispecific antibody to inhibit angiogenesis. An "individual" according to any of the above embodiments is preferably a human.

In a further aspect, the invention provides for the use of a multispecific antibody in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of an ocular vascular disease, preferably macular degeneration. In a further embodiment, the medicament is for use in a method of treating an ocular vascular disease, preferably macular degeneration, comprising administering to an individual having an ocular vascular disease, preferably macular degeneration, 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 inhibiting angiogenesis. In a further embodiment, the medicament is for use in a method of inhibiting angiogenesis in an individual comprising administering to the individual an amount effective of the medicament to inhibit angiogenesis. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides a method for treating an ocular vascular disease, preferably macular degeneration. In one embodiment, the method

comprises administering to an individual having such an ocular vascular disease, preferably macular degeneration, an effective amount of a multispecific 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 inhibiting angiogenesis in an individual. In one embodiment, the method comprises administering to the individual an effective amount of a multispecific antibody to inhibit angiogenesis. In one embodiment, an "individual" is a human.

In a further aspect, the invention provides pharmaceutical formulations comprising any of the multispecific antibodies provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the multispecific antibodies provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the multispecific antibodies provided herein and at least one additional therapeutic agent, e.g., as described below.

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 an anti-VEGF antibody or an anti-ANG2 antibody.

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 or agents. In one embodiment, administration of the multispecific antibody and administration of an additional therapeutic agent occur within about one month, or within about one, two or three weeks, or within about one, two, three, four, five, or six days, of each other.

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, 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.

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 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 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.

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 1 µg/kg to 15 mg/kg (e.g. 0.5mg/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 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus,

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 may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

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 multispecific antibody.

III. 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 an anti-IL-1beta antibody.

IV. SPECIFIC EMBODIMENTS

1. A bispecific antibody that specifically binds to two different antigens selected from the group consisting of human ANG2, human VEGF, human IL-1beta and human PDGF-B.

2. A bispecific antibody that specifically binds to i) human ANG2, and ii) human IL-1beta or human PDGF-B.

3. A bispecific antibody that specifically binds to two i) human VEGF, and ii) human IL-1beta or human PDGF-B.

4. A bispecific antibody that specifically binds to human IL-1beta and human PDGF-B.

5. The antibody according to any one of embodiments 1 to 4, wherein the antibody specifically binds to human IL-1beta and comprises

a) a heavy chain variable domain comprising

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 02, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 04, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 05, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 15,

and

a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19,

or

a) a heavy chain variable domain comprising a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 21, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 22, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 24, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 26; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 27; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 28.

6. The antibody according to any one of embodiments 1 to 5, wherein the antibody specifically binds to human IL-1beta and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10,

and

a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19.

7. The antibody according to any one of embodiments 1 to 6, wherein the antibody specifically binds to human PDGF-B and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 31, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino

acid sequence of SEQ ID NO: 35; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

8. The antibody according to any one of embodiments 1 to 7, wherein the antibody specifically binds to human PDGF-B and comprises a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 31, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 35; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37.

9. The antibody according to any one of embodiments 1 to 7, wherein the antibody specifically binds to human PDGF-B and comprises a heavy

chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46.

10. The antibody according to any one of embodiments 1 to 7, wherein the antibody specifically binds to human PDGF-B and comprises a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

11. The antibody according to any one of embodiments 1, 2 and 5 to 10, wherein the antibody specifically binds to human ANG2 and comprises

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 57, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 58, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 60, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 62; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 63; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 64,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 66, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 67, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 69, and a light

chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 71; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 72; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 73,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 75, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 76, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 78, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 80; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 81; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 82,

or

d) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 84, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 85, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 87, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 89; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 90; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 91.

12. The antibody according to any one of embodiments 1, 2 and 5 to 11, wherein the antibody specifically binds to human ANG2 and comprises a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 75, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 76, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 78, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 80; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 81; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 82.

13. The antibody according to any one of embodiments 1, 3 and 5 to 12, wherein the antibody specifically binds to human VEGF and comprises

a) the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 107 and the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 108,

or

b) the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 109 and the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 110.

14. The antibody according to any one of embodiments 1, 3 and 5 to 13, wherein the antibody specifically binds to human VEGF and comprises the HC and LC sequences in SEQ ID NO: 107 and SEQ ID NO: 108, respectively, including post-translational modifications of those sequences.

15. The antibody according to any one of embodiments 1, 3 and 5 to 13, wherein the antibody specifically binds to human VEGF and comprises the HC and LC sequences in SEQ ID NO: 109 and SEQ ID NO: 110, respectively, including post-translational modifications of those sequences.

16. The antibody according to any one of embodiments 1 to 15, wherein the antibody is of the human subclass IgG1 or the human subclass IgG4.

17. The antibody according to any one of embodiments 1 to 16, wherein the antibody is of the human subclass IgG1 with a kappa light chain.

18. The antibody according to any one of embodiments 1 to 17, wherein the antibody is a monoclonal antibody.

19. The antibody according to any one of embodiments 1 to 18, wherein the antibody is a bispecific antibody.

20. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other.

21. The antibody according to embodiment 20, wherein the antibody comprises

i) in the constant domain CL of the first light chain under a) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the first heavy chain under a) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index),

or

ii) in the constant domain CL of the second light chain under b) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the second heavy chain under b) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index).

22. The antibody according to any one of embodiment 20 to 21, wherein the antibody comprises in the constant domain CL of the second heavy chain the amino acids at position 124 and 123 are substituted by K (numbering according to Kabat EU index).

23. The antibody according to any one of embodiment 20 to 22, wherein the antibody comprises in the constant domain CH1 of the second light chain the amino acids at position 147 and 213 are substituted by E (numbering according to EU index of Kabat).

24. The antibody according to any one of embodiment 20 to 23, wherein the antibody comprises in the constant domain CL of the first light chain the amino acids at position 124 and 123 are substituted by K, and in the constant domain CH1 of the first heavy chain the amino acids at position 147 and 213 are substituted by E (numbering according to Kabat EU index).

25. The antibody according to any one of embodiment 20 to 24, wherein the antibody comprises in the constant domain CL of the second heavy chain the amino acids at position 124 and 123 are substituted by K, and wherein in the constant domain CH1 of the second light chain the amino acids at position 147 and 213 are substituted by E, and in the variable domain VL of the first light chain the amino acid at position 38 is substituted by K, in the variable domain VH of the first heavy chain the amino acid at position 39 is substituted by E, in the variable domain VL of the second heavy chain the amino acid at position 38 is substituted by K, and in the variable domain VH of the second light chain the amino acid at position 39 is substituted by E (numbering according to Kabat EU index).

26. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bivalent, bispecific antibody, comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other, and wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

27. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bivalent, bispecific antibody, comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

28. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a multispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains, and

b) one, two, three or four single chain Fab fragments specifically binding to one to four further antigens (i.e. a second and/or third and/or fourth and/or fifth antigen, preferably specifically binding to one further antigen, i.e. a second antigen),

wherein said single chain Fab fragments under b) are fused to said full length antibody under a) via a peptidic linker at the C- or N- terminus of the heavy or light chain of said full length antibody.

29. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains,

b) a first polypeptide consisting of

ba) an antibody heavy chain variable domain (VH),

or

bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),

wherein said first polypeptide is fused with the N-terminus of its VH domain via a peptidic linker to the C-terminus of one of the two heavy chains of said full length antibody,

c) a second polypeptide consisting of

ca) an antibody light chain variable domain (VL),

or

cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL),

wherein said second polypeptide is fused with the N-terminus of the VL domain via a peptidic linker to the C-terminus of the other of the two heavy chains of said full length antibody,

and

wherein the antibody heavy chain variable domain (VH) of the first polypeptide and the antibody light chain variable domain (VL) of the second polypeptide together form an antigen-binding site specifically binding to a second antigen.

30. The antibody according to embodiment 29, wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) are linked and stabilized via an interchain disulfide bridge by introduction of a disulfide bond between the following positions:

i) heavy chain variable domain position 44 to light chain variable domain position 100, or

ii) heavy chain variable domain position 105 to light chain variable domain position 43, or

iii) heavy chain variable domain position 101 to light chain variable domain position 100 (numbering always according to Kabat EU index).

31. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a trispecific or tetraspecific antibody, comprising

a) a first light chain and a first heavy chain of a full length antibody which specifically binds to a first antigen, and

b) a second (modified) light chain and a second (modified) heavy chain of a full length antibody which specifically binds to a second antigen, wherein the variable domains VL and VH are replaced by each other, and/or wherein the constant domains CL and CH1 are replaced by each other, and

c) wherein one to four antigen binding peptides which specifically bind to one or two further antigens (i.e. to a third and/or fourth antigen) are fused

via a peptidic linker to the C- or N-terminus of the light chains or heavy chains of a) and/or b).

32. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bispecific, tetravalent antibody comprising

a) two light chains and two heavy chains of an antibody, which specifically bind to a first antigen (and comprise two Fab fragments),

b) two additional Fab fragments of an antibody, which specifically bind to a second antigen, wherein said additional Fab fragments are fused both via a peptidic linker either to the C- or N-termini of the heavy chains of a),

and

wherein in the Fab fragments the following modifications were performed

i) in both Fab fragments of a), or in both Fab fragments of b), the variable domains VL and VH are replaced by each other, and/or the constant domains CL and CH1 are replaced by each other,

or

ii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,

and

in both Fab fragments of b) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

or

iii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

and

in both Fab fragments of b) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,

or

iv) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and in both Fab fragments of b) the constant domains CL and CH1 are replaced by each other,

or

v) in both Fab fragments of a) the constant domains CL and CH1 are replaced by each other, and in both Fab fragments of b) the variable domains VL and VH are replaced by each other.

33. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bispecific, tetravalent antibody comprising:

a) a (modified) heavy chain of a first antibody, which specifically binds to a first antigen and comprises a first VH-CH1 domain pair, wherein to the C-terminus of said heavy chain the N-terminus of a second VH-CH1 domain pair of said first antibody is fused via a peptidic linker,

b) two light chains of said first antibody of a),

c) a (modified) heavy chain of a second antibody, which specifically binds to a second antigen and comprises a first VH-CL domain pair, wherein to the C-terminus of said heavy chain the N-terminus of a second VH-CL domain pair of said second antibody is fused via a peptidic linker, and

d) two (modified) light chains of said second antibody of c), each comprising a CL-CH1 domain pair.

34. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bispecific antibody comprising

a) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen, and

b) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of the light chain via a peptidic linker.

35. The antibody according to any one of embodiments 1 to 19, wherein the antibody is a bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains, and

b) an Fv fragment specifically binding to a second antigen comprising a VH^2 domain and a VL^2 domain, wherein both domains are connected to each other via a disulfide bridge,

wherein only either the VH^2 domain or the VL^2 domain is fused via a peptidic linker to the heavy or light chain of the full length antibody specifically binding to a first antigen.

36. The antibody according to any one of embodiments 1 to 35, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein

i) the first Fc-region polypeptide is selected from the group comprising
- human IgG1 Fc-region polypeptide,
- human IgG2 Fc-region polypeptide,
- human IgG3 Fc-region polypeptide,
- human IgG4 Fc-region polypeptide,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A,
- human IgG1 Fc-region polypeptide with the mutations Y349C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations S354C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366S, L368A, Y407V,

- human IgG1 Fc-region polypeptide with the mutations P329G,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G,
- human IgG1 Fc-region polypeptide with the mutations P329G, Y349C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations P329G, S354C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366S, L368A, Y407V,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G,
- human IgG4 Fc-region polypeptide with the mutations Y349C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S354C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations P329G,
- human IgG4 Fc-region polypeptide with the mutations P329G, Y349C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations P329G, S354C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366S, L368A, Y407V,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366S, L368A, Y407V,
- human IgG1, IgG2 or IgG4 with the mutations K392D, and
- human IgG3 with the mutation N392D,

and

ii) the second Fc-region polypeptide is selected from the group comprising
- human IgG1 Fc-region polypeptide,
- human IgG2 Fc-region polypeptide,

- human IgG3 Fc-region polypeptide,
- human IgG4 Fc-region polypeptide,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A,
- human IgG1 Fc-region polypeptide with the mutations S354C, T366W,
- human IgG1 Fc-region polypeptide with the mutations Y349C, T366W,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366W,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366W,
- human IgG1 Fc-region polypeptide with the mutations P329G,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G,
- human IgG1 Fc-region polypeptide with the mutations P329G, S354C, T366W,
- human IgG1 Fc-region polypeptide with the mutations P329G, Y349C, T366W,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366W,
- human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366W,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G,
- human IgG4 Fc-region polypeptide with the mutations S354C, T366W,
- human IgG4 Fc-region polypeptide with the mutations Y349C, T366W,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366W,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366W,
- human IgG4 Fc-region polypeptide with the mutations P329G,
- human IgG4 Fc-region polypeptide with the mutations P329G, S354C, T366W,

- human IgG4 Fc-region polypeptide with the mutations P329G, Y349C, T366W,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366W,
- human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366W,
- human IgG1 with the mutations D399K, D356K, and/or E357K, and
- human IgG2, IgG3 or IgG4 with the mutations D399K, E356K, and/or E357K.

37. The antibody according to any one of embodiments 1 to 35, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein

i) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide, or

ii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, or

iii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, or

iv) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366S, L368A, Y407V, or

v) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366S, L368A, Y407V, or

vi) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide, or

vii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, or

viii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, or

ix) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366S, L368A, Y407V, or

x) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366S, L368A, Y407V.

38. The antibody according to any one of embodiments 1 to 37, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein the antibody comprises the combination of mutations

i) I253A, H310A, and H435A, or
ii) H310A, H433A, and Y436A, or
iii) L251D, L314D, and L432D, or
iv) combinations of i) to iii)

in the first Fc-region polypeptide and in the second Fc-region polypeptide.

39. The antibody according to any one of embodiments 1 to 37, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and wherein

a) the first and a second Fc-region polypeptide are both of human IgG1 or human IgG4 subclass (derived from human origin) and comprise one or two of the mutations selected from i) the group I253A, H310A and H435A, or ii) the group H310A, H433A and Y436A, or iii) the group L251D, L314D and L432D (numbering according to Kabat EU index numbering system) in the first Fc-region polypeptide and one or two of the mutations selected from the group comprising the mutations L251D, I253A, H310A, L314D, L432D, H433A, H435A and Y436A (numbering according to Kabat EU index numbering system) in the second Fc-region polypeptide so that all of the mutations in the first and the second Fc-region polypeptide when taken together result in that the mutations i) I253A, H310A and H435A, or ii) H310A, H433A and Y436A, or iii) L251D, L314D and L432D are comprised in the variant (human) IgG class Fc-region,

or

b) the first and a second Fc-region polypeptide both of human IgG1 or human IgG4 subclass (i.e. derived from human origin) and both comprise the mutations I253A/H310A/H435A or H310A/H433A/Y436A or L251D/L314D/L432D or combinations thereof in the Fc-region (numbering according to Kabat EU index numbering system), whereby either all mutations are in the first or the second Fc-region polypeptide, or one or two mutations are in the first Fc-region polypeptide and one or two mutations are in the second Fc-region polypeptide so that all of the mutations in the first and the second Fc-region polypeptide when taken together result in that the mutations i) I253A, H310A and H435A, or ii) H310A, H433A and Y436A, or iii) L251D, L314D and L432D are comprised in the Fc-region,

or

c) the first and a second Fc-region polypeptide both of human IgG1 or human IgG4 subclass (i.e. derived from human origin) and comprise the mutations I253A/H310A/H435A or H310A/H433A/Y436A or L251D/L314D/L432D in the first as well as in the second Fc-region polypeptide (numbering according to Kabat EU index numbering

system), or comprises the combinations of the mutations I253A/H310A/H435A in the first Fc-region polypeptide and the combination of the mutations H310A/H433A/Y436A in the second Fc-region polypeptide (numbering according to Kabat EU index numbering system).

40. The antibody according to any one of embodiments 1 to 37, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and wherein

a) the first variant Fc-region polypeptide is derived from a first parent IgG class Fc-region polypeptide and the second variant Fc-region polypeptide is derived from a second parent IgG class Fc-region polypeptide, whereby the first parent IgG class Fc-region polypeptide is identical to or different from the second parent IgG class Fc-region polypeptide, and

b) the first variant Fc-region polypeptide differs from the second variant Fc-region polypeptide in one or more amino acid residues other than those amino acid residues in which the first parent IgG class Fc-region polypeptide differs from the second parent IgG class Fc-region polypeptide, and

c) the IgG class Fc-region comprising the first variant Fc-region polypeptide and the second variant Fc-region polypeptide has an affinity to a human Fc-receptor that is different than that of an IgG class Fc-region comprising the first parent IgG class Fc-region polypeptide of a) and the second parent IgG class Fc-region polypeptide of a),

wherein either the first Fc-region polypeptide or the second Fc-region polypeptide or both Fc-region polypeptides comprise independently of each other one of the following mutations or combination of mutations:

- T307H, or
- Q311H, or
- E430 H, or
- N434H, or
- T307H and Q311H, or

- T307H and E430H, or
- T307H and N434A, or
- T307H and N434H, or
- T307Q and Q311H, or
- T307Q and E430H, or
- T307Q and N434H, or
- T307H and Q311H and E430H and N434A, or
- T307H and Q311H and E430H and N434H, or
- T307H and Q311H and E430H and N434Y, or
- T307Q and Q311H and E430H and N434A, or
- T307Q and Q311H and E430H and N434H, or
- T307Q and Q311H and E430H and N434Y, or
- T307Q and V308P and N434Y and Y436H, or
- T307H and M252Y and S254T and T256E, or
- T307Q and M252Y and S254T and T256E, or
- Q311H and M252Y and S254T and T256E, or
- E430 H and M252Y and S254T and T256E, or
- N434H and M252Y and S254T and T256E, or
- T307H and Q311H and M252Y and S254T and T256E, or
- T307H and E430H and M252Y and S254T and T256E, or
- T307H and N434A and M252Y and S254T and T256E, or
- T307H and N434H and M252Y and S254T and T256E, or
- T307Q and Q311H and M252Y and S254T and T256E, or
- T307Q and E430H and M252Y and S254T and T256E, or
- T307Q and N434H and M252Y and S254T and T256E, or
- T307H and Q311H and E430H and N434A and M252Y and S254T and T256E, or
- T307H and Q311H and E430H and N434H and M252Y and S254T and T256E, or
- T307H and Q311H and E430H and N434Y and M252Y and S254T and T256E, or
- T307Q and Q311H and E430H and N434A and M252Y and S254T and T256E, or
- T307Q and Q311H and E430H and N434H and M252Y and S254T and T256E, or
- T307Q and Q311H and E430H and N434Y and M252Y and S254T and T256E, or

- T307Q and V308P and N434Y and Y436H and M252Y and S254T and T256E.

41. The antibody according to any one of embodiments 1 to 37, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide,

and wherein the first Fc-region polypeptide comprises the mutations Y349C, T366S, L368A and Y407V (hole-chain) and the second Fc-region polypeptide comprises the mutations S354C and T366W (knob-chain),

and wherein the first Fc-region polypeptide (hole-chain) comprises the mutations

i) I253A or I253G, and
ii) L314A or L314G or L314D,

and wherein the first Fc-region polypeptide and the second Fc-region polypeptide are connected by one or more disulfide bridges,

and wherein the CH3-domain of the first polypeptide and the CH3-domain of the second polypeptide both bind or both do not bind to protein A

(numbering according to the Kabat EU index).

42. The antibody according to embodiment 41, wherein the antibody comprises the mutations

i) I253A or I253G, and
ii) L314A or L314G or L314D, and
iii) T250Q, and/or
iv) T256E or T256A.

43. The antibody according to any one of embodiments 41 to 42, wherein the antibody comprises the mutations

i) I253A or I253G, and
ii) L314A or L314G or L314D, and
iii) optionally a) T250Q, and/or T256E or T256A, and.
iv) a) L251A or L251G or L251D, and/or b) H310A or H310G.

44. The antibody according to any one of embodiments 41 to 43, wherein the antibody comprises the mutation

i) I253A or I253G, and
ii) L314A or L314G or L314D, and
iii) a) T250Q, and/or T256E or T256A, and.
iv) a) L251A or L251G or L251D, and/or b) H310A or H310G.
v) optionally a) T307A or T307H or T307Q or T307P, and/or b) Q311H, and/or c) M252Y, and/or d) S254T.

45. The antibody according to any one of embodiments 41 to 44, wherein the antibody comprises the mutation
i) T250Q, and/or
ii) M252Y, and/or
iii) S254T, and/or
iv) T256E or T256A, and/or
v) T307A or T307H or T307Q or T307P, and/or
vi) Q311H.

46. An antibody according to any one of embodiments 1 to 45 for use as a medicament.

47. An antibody according to any one of embodiments 1 to 45 for use in the treatment of an ocular vascular disease.

48. Use of an antibody according to any one of embodiments 1 to 45 for the treatment of eye diseases, especially of ocular vascular diseases.

49. An antibody according to any one of embodiments 1 to 45 for use in treating an eye disease.

50. An antibody according to any one of embodiments 1 to 45 for use in treating eye diseases, especially ocular vascular diseases.

51. A method of treating an individual having an ocular vascular disease comprising administering to the individual an effective amount of an antibody according to any one of embodiments 1 to 45.

52. A pharmaceutical formulation comprising the antibody according to any one of embodiments 1 to 45.

53. A pharmaceutical formulation comprising the antibody according to any one of embodiments 1 to 45 for use in the treatment of ocular vascular diseases.

54. Use of the antibody according to any one of embodiments 1 to 45 for the manufacture of a medicament for the treatment of ocular vascular diseases.

55. A method of treatment of patient suffering from ocular vascular diseases by administering the antibody according to any one of embodiments 1 to 45 to a patient in the need of such treatment.

56. The pharmaceutical formulation according to any one of embodiments 52 to 53, wherein the antibody is administered via intravitreal application.

57. The administering according to any one of embodiments 55 to 56, wherein the administering is an intravitreal application.

58. A nucleic acid encoding the antibody according to any one of embodiments 1 to 45.

59. A cell comprising one or more nucleic acids encoding the antibody according to any one of embodiments 1 to 45.

60. A method for producing an antibody according to any one of embodiments 1 to 45, wherein the method comprises the following steps:
a) optionally transfecting a mammalian cell with one or more nucleic acids encoding the antibody according to any one of embodiments 1 to 45,
b) cultivating the cell to express the antibody, and
c) recovering the antibody from the cell or the cultivation medium and thereby producing the antibody.

V. EXAMPLES

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

Example 1

Original generation and characterization of murine anti-IL1beta antibody H34

Materials--BALB/c female mice were obtained from Banting and Kingman (Freemont, CA). Complete and Incomplete Freud's Adjuvant (CFA and IFA) were from Difco (Detroit, MI). HB101 was from Hana Biologics, Inc. (Berkeley, CA).

Dulbecco's Phosphate-Buffered Saline (PBS) without calcium and magnesium, and glutamine were from GIBCO Labs (Grand Island, NY). Fetal bovine serum was from Hyclone Labs (Logan, UT) and Hypoxanthine-Aminopterin-Thymidine (HAT) and Hypoxanthine-Thymidine (HT) supplements, and 50% polyethylene glycol (PEG) 1450 was from Bethesda Research Labs (Gaithersburg, MD). Rabbit anti-mouse IgG+A+M peroxidase conjugate, streptavidin peroxidase, mouse Ig isotype identification kit and orthophenylene diamine (OPD) were from Zymed Labs (South San Francisco, CA). Sepharose protein-A and Sephadex G-25 were from Pharmacia (Piscataway, NJ). Pristane (2,6,10,14-tetramethyl pentadecane) was from Aldrich Chem. Co. (Milwaukee, WI). ^{125}I Bolton-Hunter reagent was from New England Nuclear (Boston, MA). All other chemicals were analytical grade from Sigma.

Hybridoma Production--Hybridomas to IL-1beta were produced using the method of Kohler and Milstein, supra as described by Lerner (1981) Yale J. Biol. Med., 54: 347. Twelve week old female BALB/c mice were injected intraperitoneally and in the hind footpads with 5 µg of purified MW 17,500 form of IL-1beta in CFA. Five booster injections in Incomplete Freund's Adjuvant (IFA) were given at 3-4 week intervals. Serum antibody titers were determined periodically by ELISA and after 5 injections a titer was detectable. The animal chosen for fusion received an intravenous (IV) boost of 10 µg of IL-1beta in sterile PBS. The spleen was removed 4 days later and the splenocytes fused with P3X63-Ag8.653 Myeloma cells using 50% PEG 1450. Cells were cultured in 96-well plates ($1*10^6$ cells/well) in HAT medium. Hybridoma supernatants were assayed for anti-IL-1beta activity by solid-phase antigen ELISA, solid-phase antibody RIA with ^{125}IIL-1beta and inhibition of IL-1beta-induced thymocyte proliferation (see below). Hybridomas were cloned by limiting dilution in HAT medium with thymocytes ($5 * 10^5$ /well) at least 3 times.

Antibody production and purification--Monoclonal antibody was produced in ascites by injecting $2 * 10^6$ hybridoma cells intraperitoneally into Pristane-treated mice (Kohler et al., supra). Ascites fluid was collected and antibody purified by sepharose-protein A chromatography (Goding, J. Immunol. Methods 20 (1978) 241).

Monoclonal antibody ILB1-H34 was prepared from the corresponding cell lines as described above.

ELISA of IL-1beta antibody--Vinyl assay plates (Costar) were coated with 50 μL/well of a 5 μg/mL solution of antigen diluted in PBS and incubated overnight at 4 (degree) C. Wells were countercoated using 5% non-fat dry milk/0.05% Thimerosal/PBS one hour at room temperature. The wells were washed with 0.1% bovine serum albumin (BSA)/0.05% Thimerosal/PBS and 50 μL/well of anti-IL-1beta antibodies were incubated for 2 hours at room temperature. Antibody was detected by indirect ELISA using rabbit anti-mouse IgG+A+M peroxidase conjugate and OPD substrate solution. Alternatively, purified monoclonal antibody was biotinylated (Geusdon et al., J. Histochem. Cytochem. 27 (1979) 1131) and detected using streptavidin peroxidase and OPD substrate solution. Isotype of the monoclonal antibodies was identified by indirect ELISA using a mouse Ig isotype identification kit.

Thymocyte proliferation assay--IL-1beta and PHA (10 μg/ml) were added to cultures of C3H/HeJ mouse thymocytes (1 * 10^6 /well) in MEM/5% fetal bovine serum (FBS)/100 μg/ml gentamicin, 2-mercaptoethanol (2 * 10^{-5} M), 25 mM Hepes medium. After 48 hours at 37 °C, 0.5 μCi/well of ^{3}H thymidine was added and the cultures were incubated overnight. The cells were collected on glass fiber filters using a cell harvester and processed for scintillation counting.

Receptor binding assay--The 17,500 form of IL-1beta was labeled using diiodo ^{125}I Bolton-Hunter reagent according to the manufacturer's instructions. One μg of IL-1beta in 10 μL of PBS was reacted with 1 mCi of reagent for 4 hours at 4 °C; 500 μL of PBS/0.2% gelatin was added and labeled IL-1beta was separated from free Bolton-Hunter reagent by chromatography on a 20 * 1 cm column of Sephadex G-25 with PBS/0.2% gelatin. ^{125}IIL-1beta was added to confluent monolayers of BALB/c 3T3 fibroblasts in DMEM/1% BSA/0.1% sodium azide/0.01% Triton X-100 in 24-well culture plates. After 1 hour at 37°C the monolayers were washed extensively in media without labelled IL-1beta. The monolayers were removed using 0.1 N NaOH for gamma counting. Non-specific binding of ^{125}IIL-1beta was measured by incubating in the presence of 200-fold molar excess of unlabeled IL-1beta.

Determination of antibody affinity--Monoclonal antibody affinity was determined from data obtained using an immunoprecipitation radioimmunoassay. Briefly, 5000 cpm/tube of ^{125}IIL-1beta was incubated with dilutions of purified monoclonal antibody in 0.3 ml of 1% non-fat dry milk/0.5% Thimerosal/PBS overnight at 4 °C. Antigen-antibody complexes were precipitated by the addition of 100 μL/tube each

of 10% normal mouse serum/PBS and 4 mg/ml goat anti mouse IgG sera in PBS. After 4 hours at 4 °C, the 1/ml tube of ice-cold 2% polyethylene glycol-6000 was added and the tubes centrifuged at 3000 * g for 20 min. at 4 °C. The supernatants were aspirated and the pellets counted in a gamma counter. Affinity constants were calculated from bound/free ratios at different concentrations of antibody (Berson et al., Clin. Chim. Acta. 22 (1969) 51-69).

Affinity constants were calculated using data obtained from an immunoprecipitation radioimmunoassay (RIA) of ^{125}IIL-1beta binding of different antibody concentrations as described above. The anti-IL-1beta antibody H34 has an affinity of 64 *10^9 L/mol for IL-1beta.

Example 2

Immunization of mice

For immunization of NMRI mice, a RIMMS ("Rapid IMmunization, Multiple Sites") schedule was used.

Example 3

Determination of anti-IL-1beta antibody serum titer

Human recombinant IL-1beta was immobilized on a 96-well NUNC Maxisorb plate at 2.5 µg/ml, 100 µl/well, in PBS, followed by: blocking of the plate with 2% CroteinC in PBS, 200 µl/well; application of serial dilutions of antisera, in duplicates, in 0.5 % CroteinC in PBS, 100 µl/well; detection with HRP-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch) diluted 1:16,000 in 0.5 % CroteinC in PBS, 100 µl/well. For all steps, plates were incubated for 1 hour at 37 °C. Between all steps, plates were washed 3 times with 0.05 % Tween 20 in PBS. Signal was developed by addition of BM Blue POD Substrate soluble (Roche Diagnostics GmbH, Mannheim, Germany), 100 µl/well; and stopped by addition of 1 M HCl, 100 µl/well. Absorbance was read out at 450 nm, against 690 nm as reference. Titer was defined as dilution of antisera resulting in half-maximal signal.

Example 4

Human IL-1beta binding ELISA

Variant 1

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human IL-1beta (Peprotech Cat. No 200-01B) was immobilized at a concentration of 500 ng/mL in 25 µL in PBS on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-IL-1beta antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:2000 (Donkey $F(ab)_2$ anti-rabbit IgG POD, Amersham, NA9340V or sheep IgG anti-mouse IgG POD, Amersham RPN4201). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Mannheim, Germany, Cat. No 11835033001) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Variant 2

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen His-tagged human IL-1beta (Sino Biologics, Cat. No. 10139-H07E) was immobilized at a concentration of 0.25 µg/mL in 25 µL in PBS on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-IL-1beta antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:2000 (Donkey $F(ab)_2$ anti-rabbit IgG POD, Amersham, NA9340V or sheep IgG anti-mouse IgG POD, Amersham RPN4201). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Mannheim, Germany, Cat. No 11835033001) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 5

Cynomolgus IL-1beta binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human IL-1beta (Sino Biologics, Cat. No. 90010CNAE) was immobilized at a concentration of 0.5 µg/mL in 25 µL in PBS on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the

following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-IL-1beta antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:2000 (Donkey $F(ab)_2$ anti-rabbit IgG POD, Amersham, NA9340V or sheep IgG anti-mouse IgG POD, Amersham RPN4201). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Mannheim, Germany, Cat. No 11835033001) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 6

Murine IL-1beta binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen murine IL-1beta (Sino Biologics, Cat. No. 50101-MNAE) was immobilized at a concentration of 0.5 µg/mL in 25 µL in PBS on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-IL-1beta antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:2000 (Donkey $F(ab)_2$ anti-rabbit IgG POD, Amersham, NA9340V or sheep IgG anti-mouse IgG POD, Amersham RPN4201). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Mannheim, Germany, Cat. No 11835033001) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 7

Protein-Protein Interaction Inhibition assay: human IL-1beta:human IL-1 receptor type 1

The protein-protein interaction inhibition analysis of human IL-1beta to the human IL-1 receptor type I was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The human His-tagged IL-1beta protein (Sino Biologics, Cat.No.10139-H07E) was immobilized at a concentration of 1 µg/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate

(Thermo Scientific Cat.No.464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS with dispense and aspiration: 1) blocking step saturating unbound surface (1 hour, 2 % BSA); 2) 12.5 µL anti-IL-1beta antibody in increasing concentrations was incubated with 12.5 µL Fc-tagged human IL-1beta receptor (Sino Biologics, Ca.No10126-H02H) at 300 ng/mL in a volume of 250 µL for 1 hour; 3) detection was achieved using peroxidase-labeled anti huFc antibody (Goat F(ab_2) anti-human FC POD, Jackson, Cat. No 109-036-098). 10-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Cat. No. 11835033001) the optical density was determined at 370 nm. The IC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 8

Protein-Protein Interaction Inhibition assay: human IL-1beta:human IL-1 receptor type 2

The protein-protein interaction inhibition analysis of human IL-1beta to the human IL-1 receptor type II was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The human His-tagged IL-1beta protein (Sino Biologics, Cat.No.10139-H07E) was immobilized at a concentration of 1 µg/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat.No.464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) 12.5 µL anti-IL-1beta antibody in increasing concentrations was incubated with 12.5 µL Fc-tagged human IL-1beta receptor (RnD, Ca.No.663-2R-50) at 30 ng/mL in a volume of 250 µL for 1 hour; 3) detection was achieved using peroxidase-labeled anti-huFc antibody (Goat F(ab_2) anti-human FC POD, Jackson, Cat. No 109-036-098). 10-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 11835033001) the optical density was determined at 370 nm. The IC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 9

Expression of mouse hybridoma H34 murine anti-IL-1beta antibody producing hybridoma

Medium contains following reagents: RPMI (PAN), 20 % foetal calf serum, 2 mM Glutamine (PAN), 1x Sodium pyruvate (PAN), 1x NEAS (PAN)

Pre-thaw the frozen cell-containing-vial by placing the tube in a 37 °C water bath for 60 seconds. With 2 ml pre-warmed (37 °C) medium cells have been quickly resuspended and transferred from the vial into a 10 ml flask (CellStar); already containing 8 ml medium. Centrifuge flask for 5 minutes at 1000 rpm (25 °C). Then discard supernatant und resuspend gently cell-pellet by up-and-down-pipetting in 10 ml pre-warmed (37 °C) medium. Fill the whole solution in a T25-flask and place flask in an incubator (37 °C, 7 % CO_2, 85 % humidity) for 2 days.

Split the cells the next 5 days each 2-3 days, by dilution in new medium, with a density of 1-2 x 10^5 cells/ml. Then start the following days to reduce part of foetal calf serum from 20 % in a first step to 10 %. After two splits in 10 % foetal calf serum, mix medium (RPMI (PAN), 10 % foetal calf serum, 2 mM Glutamine (PAN), 1x Sodium pyruvate (PAN), 1x NEAS (PAN)) 1:1 with Hyclone-ADCF-MAb-medium (Thermo-Scientific) and use this medium for another two splits. Then rise ratio of Hyclone : RPMI-with-10 % foetal calf serum to 3:1 and seed cells with a higher density of 2-3 x 10^5 cells/ml. At least split cells in 100 % Hyclone-medium added by Nutridoma CS (Roche Diagnostics GmbH, Mannheim, Germany).

Then expand volume of cells-solution to 20 ml (T75 flask) for 5 splits. For antibody-production fill 15 ml cells, with a density of 2.0 x 10^6 cells/ml in Celline CL1000 reactor and incubate for 8-9 days (37 °C, 7 % CO_2, 85 % humidity). For harvesting, fill the supernatant in a 50 ml falcon and centrifuge with 4000 rpm 4 times (after each cycle the supernatant will be filled in a new 50 ml falcon). Finally freeze cell-free-supernatant at -20°C.

Example 10

Antibody purification from murine hybridoma

Antibody-containing H34 hybridoma supernatant was filtered and purified by two chromatographic steps. The antibodies were captured by affinity chromatography using HiTrap Protein G (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 was recovered with 25 mM citrate buffer, pH 3.0, and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 9.0. Size exclusion chromatography on Superdex 200™ (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions were concentrated with an Ultrafree -CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Antibody-containing hybridoma supernatant was filtered and purified by two chromatographic steps. The supernatants were mixed with 50 % v/v 2 M glycine, pH 8.6, 600 mM NaCl and were captured by affinity chromatography using HiTrap MabSelectSuRe (GE Healthcare) equilibrated with 1 M glycine, pH 8.6, 300 mM NaCl. Unbound proteins were removed by washing with equilibration buffer, and the antibody was recovered with 100 mM citrate buffer, pH 2.8 and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 8.5. Size exclusion chromatography on Superdex 200™ (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions were concentrated with an Ultrafree -CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Example 11

Transfection and transient expression of the humanized antibodies in HEK cells

Transient expression of antibodies in suspension-adapted HEK293F (FreeStyle 293-F cells; Invitrogen) cells with Transfection Reagent 293-free (Novagen).

Cells have been passaged, by dilution, at least four times (volume 30 ml) after thawing in a 125 ml shake flask (Incubate/Shake at 37 °C, 7 % CO_2, 85 % humidity, 135 rpm).

The cells were expanded to $3x10^5$ cells/ml in 250 ml volume. Three days later, cells have been split and new seeded with a density of $7*10^5$ cells/ml in a 250 ml volume in a 1 liter shake flask. Transfection will be 24 hours later at a cell density around $1.4 – 2.0x10^6$ cells/ml.

Before transfection dilute 250 µg plasmid-DNA (122 µg light and 128 µg heavy chain) in a final volume of 10 ml with pre-heated (water bath; 37 °C) Opti-MEM (Gibco). Mix solution gentle and incubate at room temperature for not longer than 5 min. Then add 333.3 µl 293-free transfection reagent to DNA-OptiMEM-solution. Mix gently and incubate at room temperature for 15-20 minutes. Add whole volume of mixture to 1 L shake flask with 250 ml HEK-cell-culture-volume.

Incubate/Shake at 37 °C, 7 % CO_2, 85 % humidity, 135 rpm for 6 or 7 days.

Harvest supernatant by a first centrifugation-step at 2,000 rpm, 4 °C, for 10 minutes. Then transfer the supernatant in a new centrifugation-flask for a second centrifuge at 4,000 rpm, 4 °C, for 20 minutes. Thereafter the cell-free-supernatant is filtered through a 0.22 µm bottle-top-filter and stored in a freezer (-20 °C).

Example 12
Antibody purification from HEK supernatant

The antibody-containing culture supernatants were filtered and purified by two chromatographic steps. The antibodies 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 was recovered with 50 mM citrate buffer, pH 2.8, and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 9.0. Size exclusion chromatography on Superdex 200™ (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions were concentrated with an Ultrafree -CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Example 13
Analytics of antibody preparations

The protein concentration of antibody preparations was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

Purity and integrity of the antibodies were analyzed by CE-SDS using a LabChip GX II (PerkinElmer) with Protein Express Chip and HT Protein Express Reagents Kit.

Aggregate content of antibody preparations was determined by high-performance SEC using a TSK-GEL QC-PAK GFC 300 using 2 x PBS, pH 7.4 as running buffer or by high-performance SEC using a BioSuite High Resolution SEC, 250 Å, 5 µm analytical size-exclusion column (Waters GmbH) using 200 mM K_2HPO_4/KH_2PO_4, 250 mM KCl, pH 7.0 as running buffer.

Example 14

Preparation of Fab fragment from an antibody and analytics:

12 mg antibody (1 mg/ml in 20 mM Histidine, 140 mM NaCl, pH 6.0) were incubated with 240 µl L-cysteine solution (Merck Millipore; 250 mM in 20 mM Histidine, 140 mM NaCl, pH 6.0) and 327 µl Papain (Roche Life Science; 0.001 U/mg antibody) for 120 min at 37 °C. After cleavage, 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 was used for removal of intact IgG and Fc fragment. Subsequently, flow-through of MabSelectSuRe chromatography was further purified using size exclusion chromatography on Superdex 200™ (GE Healthcare) as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The Fab fragment containing solutions 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 integrity of the Fab-fragments were analyzed by SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Invitrogen) in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Simply Blue Safe Stain (Invitrogen).

Aggregate content of Fab preparations was determined by high-performance SEC using a BioSuite High Resolution SEC, 250 Å, 5 µm analytical size-exclusion column (Waters GmbH) using 200 mM K_2HPO_4/KH_2PO_4, 250 mM KCl, pH 7.0 as running buffer.

Example 15

ICAM-1 expression after IL-1beta stimulation of A549 cells

A549 cells (10,000/well) were gown overnight in RPMI 1640 supplemented with 10% FCS. Thereafter the medium was replaced by Hunger medium supplemented with 0.5 % serum.

The anti-IL-1beta antibody was incubated for 2 hours with IL-1beta at 250 pg/ml and different concentrations of the antibody (1000, 100, 10, 1, 0.1, 0 ng/ml). Thereafter A549 cells were incubated overnight with the IL-1beta/antibody mixture in quadruplicates.

The cells were washed four times with ice cold PBS and thereafter fixed with PFA for 20 minutes. Thereafter the cells were blocked with GSDB, non-permeabilizing. After incubation for 2 hours with anti-ICAM-1 antibody (R&D Systems, 5 µg/ml) the sample was washed four times with PBS. For staining the sample was incubated for 1 hour with anti-mouse antibody-HRP conjugate (Amersham) diluted 1:1000. Afterwards the sample was washed four times with PBS and incubated for 2 hours with ABTS. Absorption was measured at 405 nm with a reference at 495 nm.

Example 16

IL-6 determination after IL-1beta stimulation of A549 cells (Quantikine ELISA, R&D Systems)

A549 cells (10,000/well) were gown overnight in RPMI 1640 supplemented with 10% FCS. Thereafter the medium was replaced by Hunger medium supplemented with 0.5 % serum and the cultivation continued for 96 hours.

The anti-IL-1beta antibody (1 µg/ml) was incubated for 2 hours with IL-1beta at 250 pg/ml. Thereafter A549 cells were incubated overnight with the IL-1beta/antibody mixture in duplicates.

A sample of 100 µl of the cultivation supernatant was taken for further analysis.

A 96 well plate coated with a monoclonal anti-human IL-6 antibody was blocked for 15 min. with assay diluent RD1W. Thereafter the supernatant sample was added and incubated for 2 hours at RT. The wells were washed four times with 200 µl wash buffer each. Thereafter the wells were incubated with polyclonal anti-human

IL-6 antibody conjugated to HRP at RT for two hours. The wells were washed four times with 200 µl wash buffer each. Afterwards the wells were incubated for 20 min. with tetramethyl benzidine and H2O2. The reaction was stopped by the addition of 2 N sulfuric acid after 20 min. Absorption was determined at 450 nm with a reference wavelength of 570 nm.

Example 17

Bioactivity assay

Murine helper T lymphocyte (Th-2) D10.G4.1 line has been used extensively as a reliable and sensitive assay for IL-1 (interleukin-1) bioactivity, since D10 cells will proliferate only minimally to con A in the absence of IL-1 or feeder cells (see Symons, J.A., et al. in Lymphokines and Interferons, a Practical Approach. Clemens, M.J. et al. (Eds): IRL Press. 272 (1987)). The ED_{50} for this effect is typically <12 pg/mL.

35,000 D10.G4.1 T-Cells/well (freshly thawed) are stimulated for 72 hours in IL-1beta (1 ng/ml) containing media (RPMI/2.5µg/ml ConA/10 % FCS).

Readout was determined by CellTiterGlo® Luminescent Cell Viability Assay according to the manufacturer's instructions.

Example 18

ICAM-1 up-regulation on HUVEC cells induced by IL-1beta

HUVEC cells (Lonza, Cat# 00191027) in corresponding media EBM/EGM (Cat# CC-4176) were seeded out in a 96 well culture plate (Costar, Cat#3596) at 40,000 cells/well in EBM+2 % BSA 200 µl/well. Cells were incubated to recover in a 5 %-CO_2 incubator at 37 °C for 24h. Two dilution series 40fold concentrated as finally requested were performed: one with the anti-IL-1b antibody huH34-2 and the other with recombinant human IL-1beta (R&D Systems, Cat#201-LB) in EBM+2 % BSA. The two series were mixed against each other 1:1 and incubated for one hour at RT. 10 µl of this IL-1beta/anti-IL-1beta antibody mix was added to the cells and gently mixed. Incubation was performed in a 5 %-CO_2 incubator at 37 °C for 20 hours. Thereafter all the media was removed from the cells and the cells were washed twice with PBS. After one wash with Cell Dissociation Solution Non-enzymatic 1x (Sigma, Cat# C5914) an incubation with 100 µl of the Cell Dissociation Solution at 37 °C was done. Detachment was checked by observing by microscope every 5 minutes. When 80 % of the cells became globate, cells were

transferred into a FACS-Plate (96 well, 340 µl Storage, PP, V-bottom Plate (Falcon Cat#353263)). The remaining cells were detached from the culture plate with 100 µl PBS+1 % BSA by aspirating 4 times and also added to the FACS-plate. After 5 min. centrifugation by 300xg the supernatant was discarded. Pellets were resuspended in 100 µl PBS+1 % BSA+10 µg/ml human IgG (Sigma; Cat#I2511) AND Incubated for 15 min. at RT. 10 µl of anti-human ICAM-1 Fluorescein conjugate (CD54) (R&D Systems, Cat#BBA20) was added followed by an incubation at 4 °C for 30-45 min. After 5 min. centrifugation by 300xg the supernatant was discarded. The pellet was resuspended in 110 µl PBS+1 % BSA and measured on LSRII.

Example 19

MSU induced TNFalpha production in THP1 cells

THP1 cells (Invitrogen, Cat. No. thp-null) were grown until a density of $1x10^6$ cells/ml in growth medium, RPMI 1640 (Gibco, Cat#A10491) supplemented with 10 % FBS (heat inactivated) and transferred into a Falcon tube. PMA (phorbol myristate acetate, Invitrogen, Cat# tlrl-pma) was added with a final concentration of 300 ng/ml and incubated in the 5 %-CO_2 incubator at 37 °C for 3 hours. Cells were washed once with PBS (5 min. centrifugation at 300xg, supernatant discarded) and resuspended with a density of $1.33x10^6$ cells/ml in Hunger RPMI (Gibco, Cat#31870-025) supplemented with 2mM L-Glutamine and 10 % FBS (heat inactivated). 150 µl/well of the cell suspension was seeded out in a 96 well culture plate (Costar, Cat#3596) at $2x10^5$ cells/well. Overnight incubation was performed in a 5 %-CO_2 incubator at 37 °C. 50 µl of a 4-fold concentrated MSU suspension, (monosodium urate crystals, Invitrogen, Cat# tlrl-msu) final concentration 250 µg/ml in Hunger medium, was added and incubated in the 5 %-CO_2 incubator at 37 °C for 6 hours. Dilution series of the anti-IL-1beta antibodies were performed in growth medium. Supernatants from the THP1 cells were discarded and the wells were washed once with PBS. Then the prepared anti-IL-1b antibody dilution series were added to the wells. Overnight incubation was performed in a 5 %-CO_2 incubator at 37 °C. Supernatants were collected and analyzed by TNFalpha singleplex.

Example 20

Chemical degradation test

Samples were split into three aliquots and re-buffered into 20 mM His/His*HCl, 140 mM NaCl, pH 6.0 or into PBS, respectively, and stored at 40 °C (His/NaCl) or 37 °C (PBS). A control sample was stored at -80 °C.

After incubation ended, samples were analyzed for relative active concentration (BIAcore), aggregation (SEC) and fragmentation (capillary electrophoresis or SDS-PAGE) and compared with the untreated control.

Example 21

Thermal stability

Samples were prepared at a concentration of 1 mg/mL in 20 mM Histidine/Histidine chloride, 140 mM NaCl, pH 6.0, transferred into an optical 384-well plate by centrifugation through a 0.4 µm filter plate and covered with paraffin oil. The hydrodynamic radius was measured repeatedly by dynamic light scattering on a DynaPro Plate Reader (Wyatt) while the samples were heated with a rate of 0.05 °C/min from 25 °C to 80 °C.

Alternatively, samples were transferred into a 10 µL micro-cuvette array and static light scattering data as well as fluorescence data upon excitation with a 266 nm laser were recorded with an Optim1000 instrument (Avacta Inc.), while they were heated at a rate of 0.1 °C/min from 25 °C to 90 °C.

The aggregation onset temperature is defined as the temperature at which the hydrodynamic radius (DLS) or the scattered light intensity (Optim1000) starts to increase.

The melting temperature is defined as the inflection point in fluorescence intensity vs. wavelength graph.

Example 22

Immunization

For immunization of mice (NMRI mice) and rabbits (human Ig locus transgenic rabbits) a RIMMS ("Rapid IMmunization, Multiple Sites") based schedule was used. The antigen was human PDGF-BB (Cell Signaling Tech.).

Example 23

Determination of anti-PDGF-B antibody serum titer

Human recombinant PDGF-B was immobilized on a 96-well NUNC Maxisorb plate at 2.5 µg/ml (mouse) or 1.0 µg/ml (rabbit), 100 µl/well, in PBS, followed by: blocking of the plate with 2% CroteinC in PBS, 200 µl/well; application of serial dilutions of antisera, in duplicates, in 0.5 % CroteinC in PBS, 100 µl/well; detection with HRP-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch) diluted 1:16,000 in 0.5 % CroteinC in PBS for mouse sera or with biotinylated goat anti-human kappa antibody (Southern Biotech) diluted 1:5,000 and HRP-conjugated streptavidin diluted 1:8,000 in 0.5% CroteinC in PBS for rabbit sera, 100 µl/well. For all steps, plates were incubated for 1 hour at 37 °C. Between all steps, plates were washed 3 times with 0.05 % Tween 20 in PBS. Signal was developed by addition of BM Blue POD Substrate soluble (Roche Diagnostics GmbH, Mannheim, Germany), 100 µl/well; and stopped by addition of 1 M HCl, 100 µl/well. Absorbance was read out at 450 nm, against 690 nm as reference. Titer was defined as dilution of antisera resulting in half-maximal signal.

Example 24

B-Cell Cloning from Rabbits

Isolation of rabbit peripheral blood mononuclear cells (PBMC)

Blood samples were taken of three immunized rabbits. EDTA containing whole blood was diluted twofold with 1x PBS (PAA, Pasching, Austria) before density centrifugation using lympholyte mammal (Cedarlane Laboratories, Burlington, Ontario, Canada) according to the specifications of the manufacturer. The PBMCs were washed twice with 1x PBS.

EL-4B5 medium

RPMI 1640 (Pan Biotech, Aidenbach, Germany) supplemented with 10% FCS (Hyclone, Logan, UT, USA), 2 mM Glutamine, 1% penicillin/streptomycin solution (PAA, Pasching, Austria), 2 mM sodium pyruvate, 10 mM HEPES (PAN Biotech, Aidenbach, Germany) and 0.05 mM β-mercaptoethanol (Gibco, Paisley, Scotland) was used.

Coating of plates

Sterile cell culture 6-well plates were coated with either PBGF-BB (2 µg/ml) or a mixture of PDGF-AA and PDGF-CC proteins (1 µg/ml PDGF-AA and PDGF-CC) in carbonate buffer (0.1 M sodium bicarbonate, 34 mM disodium hydrogen carbonate, pH 9.55) overnight at 4 °C. Plates were washed in sterile PBS three times before use.

Depletion of macrophages/monocytes

The PBMCs of half of the blood sample were seeded on sterile 6-well plates (cell culture grade) to deplete macrophages and monocytes through unspecific adhesion.

The remaining 50 % of the PBMCs were seeded in plates that were pre-coated with a mixture of PDGF-AA and PDGF-CC proteins in order to remove B-cells binding to these proteins and to remove macrophages and monocytes in one step.

Each well was filled at maximum with 4 ml medium and up to 6 x 10^6 PBMCs from the immunized rabbit and were allowed to bind for 1 h at 37 °C in the incubator.

The cells in the supernatant (peripheral blood lymphocytes (PBLs)) were used for the antigen panning step.

Enrichment of B cells on the PDGF BB protein

6-well tissue culture plates coated with PDGF-BB protein were seeded with up to 6 x 10^6 PBLs per 4 ml medium and allowed to bind for 1 h at 37 °C in the incubator. Non-adherent cells were removed by carefully washing the wells 1-2 times with 1x PBS. The remaining sticky cells were detached by trypsin for 10 min at 37 °C in the incubator. Trypsination was stopped with EL-4B5 medium. The cells were kept on ice until the immune fluorescence staining.

Immune fluorescence staining and Flow Cytometry

The anti-IgG FITC (AbD Serotec, Düsseldorf, Germany) was used for single cell sorting. For surface staining, cells from the depletion and enrichment step were incubated with the anti-IgG antibody conjugated to FITC in PBS and incubated for 45 min in the dark at 4 °C. After staining the PBMCs were washed two fold with ice cold PBS. Finally the PBMCs were resuspended in ice cold PBS and

immediately subjected to the FACS analyses. Propidium iodide in a concentration of 5 µg/ml (BD Pharmingen, San Diego, CA, USA) was added prior to the FACS analyses to discriminate between dead and live cells.

A Becton Dickinson FACSAria equipped with a computer and the FACSDiva software (BD Biosciences, USA) were used for single cell sort.

B-cell cultivation

Briefly, single sorted rabbit B-cells were incubated in 96-well plates with 200 µl/well EL-4B5 medium containing Pansorbin Cells (1:100,000) (Calbiochem (Merck), Darmstadt, Deutschland), 5 % rabbit thymocyte supernatant (MicroCoat, Bernried, Germany) and gamma-irradiated murine EL-4B5 thymoma cells (2.5×10^4 cells/well) for 7 days at 37 °C in the incubator. The supernatants of the B-cell cultivation were removed for screening and the remaining cells were harvested immediately and were frozen at -80 °C in 100 µl RLT buffer (Qiagen, Hilden, Germany).

Example 25

Hybridoma generation

Cell culture

The mouse myeloma cell line P3x63-Ag8.653 was used fusion partner for the generation of mouse-mouse hybridomas. The cells are thawed about 14 days prior to fusion and cultured in the presence of 8-azaguanine. Every 3 to 4 days the cells are split and adjusted to a concentration of 1-2 x 10^5 cells/m.

Cell fusion

Material:

mouse hybridoma medium (RPMI 1640 (PAN), FBS ultra-low-IgG, 2 mM L-glutamine, 1 mM Na-pyruvate, NEAA, Nutridoma-CS with muIL-6, HAZ (SIGMA, #A9666), adjusted to RT

RPMI 1640 medium (37 °C)

RPMI 1640 medium (4 °C)

PEG (37 °C)

Prior to fusion about the myeloma cells are centrifuged (250 rpm, 7 min.). The cell pellet is resuspended in culture medium. For one spleen about 1-5 * 10^7 cells are required.

For cell fusion a ratio of lymphocytes to myeloma cells of about 5:1 should be used. P3x63-Ag8.653-cells are resuspended in 50 ml RPMI 1640 medium and centrifuged (250 rpm, 7 min.). The supernatant is removed. Thereafter the cells are added to the spleen cells.

During fusion the temperature is adjusted to 37 °C in a water bath.

The PEG solution (37 °C) is added drop wise to the cells.

The fusion mixture is incubated between 15 and 120 minutes at 37 °C in an incubator. Thereafter the fusion mixture is centrifuged (250 rpm, 7 min.) and resuspended in 1200 µl medium resuspended. 100 µl cell suspension was added to 50 ml semi-solid hybridoma-medium, homogenized and each 4 ml added per well of a 6-well plate. After 9-13 days of cultivation single clones are picked.

Example 26

Hybridoma cultivation

About 5x10^6 cells are suspended in 50 ml Hyclone medium. The cultivation mixture is incubated for 96 hours. Thereafter 75 ml Hyclone medium was added. The cultivation was continued for 7 days. If the viability fell below 40 % the cell suspension was filtered through a 0.22 µ filter and the filtrate was used for purification.

Example 27

B-cell cloning

PCR amplification of V-domains

Total RNA was prepared from B-cell lysate (resuspended in RLT buffer - Qiagen - Cat. N° 79216) using the NucleoSpin 8/96 RNA kit (Macherey&Nagel; 740709.4, 740698) according to manufacturer's protocol. RNA was eluted with 60 µl RNAse free water. 6 µl of RNA was used to generate cDNA by reverse transcriptase reaction using the Superscript III First-Strand Synthesis SuperMix (Invitrogen 18080-400) and an oligo dT-primer according to the manufacturer's instructions. All steps were performed on a Hamilton ML Star System. 4 µl of cDNA were used

to amplify the immunoglobulin heavy and light chain variable regions (VH and VL) with the AccuPrime SuperMix (Invitrogen 12344-040) in a final volume of 50 µl using the primers rbHC.up and rbHC.do for the heavy chain, rbLC.up and rbLC.do for the light chain of wild-type rabbit B-cells and BcPCR_FHLC_leader.fw and BcPCR_huCkappa.rev for the light chain of transgenic rabbit B-cells. All forward primers were specific for the signal peptide (of respectively VH and VL) whereas the reverse primers were specific for the constant regions (of respectively VH and VL). The PCR conditions for the RbVH+RbVL were as follows: Hot start at 94°C for 5 min; 35 cycles of 20s at 94°C, 20s at 70°C, 45s at 68 °C, and a final extension at 68°C for 7 min. The PCR conditions for the HuVL were as follows: Hot start at 94°C for 5 min; 40 cycles of 20s at 94°C, 20s at 52°C, 45s at 68 °C, and a final extension at 68°C for 7 min.

rbHC.up SEQ ID NO: 111	AAGCTTGCCACCATGGAGACTGGGCTGCGC TGGCTTC
rbHCf.do SEQ ID NO: 112	CCATTGGTGAGGGTGCCCGAG
rbLC.up SEQ ID NO: 113	AAGCTTGCCACCATGGACAYGAGGGCCCCC ACTC
rbLC.do SEQ ID NO: 114	CAGAGTRCTGCTGAGGTTGTAGGTAC
BcPCR_FHLC_leader.fw SEQ ID NO: 115	ATGGACATGAGGGTCCCCGC
BcPCR_huCkappa.rev SEQ ID NO: 116	GATTTCAACTGCTCATCAGATGGC

8 µl of 50 µl PCR solution were loaded on a 48 E-Gel 2% (Invitrogen G8008-02). Positive PCR reactions were cleaned using the NucleoSpin Extract II kit (Macherey&Nagel; 740609250) according to manufacturer's protocol and eluted in

50 µl elution buffer. All cleaning steps were performed on a Hamilton ML Starlet System.

Example 28

Recombinant expression of rabbit monoclonal bivalent antibodies

For recombinant expression of rabbit monoclonal bivalent antibodies, PCR-products coding for VH or VL were cloned as cDNA into expression vectors by the overhang cloning method (RS Haun et al., BioTechniques 13 (1992) 515-518; MZ Li et al., Nature Methods 4 (2007) 251-256). The expression vectors contained an expression cassette consisting of a 5' CMV promoter including intron A, and a 3' BGH poly adenylation sequence. In addition to the expression cassette, the plasmids contained a pUC-derived origin of replication and a beta-lactamase gene conferring ampicillin resistance for plasmid amplification in E.coli. Three variants of the basic plasmid were used: one plasmid containing the rabbit IgG constant region designed to accept the VH regions; two plasmids containing rabbit or human kappa LC constant region to accept the VL regions.

Linearized expression plasmids coding for the kappa or gamma constant region and VL/VH inserts were amplified by PCR using overlapping primers.

Purified PCR products were incubated with T4 DNA-polymerase, which generated single-strand overhangs. The reaction was stopped by dCTP addition.

In the next step, plasmid and insert were combined and incubated with recA which induced site specific recombination. The recombined plasmids were transformed into E.coli. The next day the grown colonies were picked and tested for correct recombined plasmid by plasmid preparation, restriction analysis and DNA-sequencing.

For antibody expression, the isolated HC and LC plasmids were transiently co-transfected into HEK293 cells and the supernatants were harvested after 1 week.

Example 29

Recombinant expression of rabbit monoclonal monovalent antibodies

For recombinant expression of selected candidates as monoclonal monovalent antibodies rabbit constant regions of all VH chains were converted into human constant regions enclosing the Knob-mutation in the CH3 segment. For VL chains

derived from rabbit wild type B-cells, rabbit C kappa constant regions were converted into human. 4 µl of cDNA of the selected candidates were used to amplify the immunoglobulin heavy and light chain variable regions with the AccuPrime SuperMix (Invitrogen 12344-040) in a final volume of 50 µl with forward primers specific for the signal peptide and reverse primers specific for the CDR3-J region with (at the 3' end) overlap sequence (20 bp) homologous to the human constant regions (respectively of VH and VL). The PCR conditions for the VH and VL chain amplification were as follows: Hot start at 94°C for 5 min; 35 cycles of 20s at 94°C, 20s at 68°C, 45s at 68 °C, and a final extension at 68°C for 7 min.

PCR-products coding for VH or VL were cloned as cDNA into expression vectors by the overhang cloning method (RS Haun et al., BioTechniques 13 (1992) 515-518; MZ Li et al., Nature Methods 4 (2007) 251-256). The expression vectors contained an expression cassette consisting of a 5' CMV promoter including intron A, and a 3' BGH poly adenylation sequence. In addition to the expression cassette, the plasmids contained a pUC-derived origin of replication and a beta-lactamase gene conferring ampicillin resistance for plasmid amplification in E.coli. Two variants of the basic plasmid were used: one plasmid containing the human IgG constant region designed to accept the new amplified VH chain and a second plasmid containing the human kappa LC constant region to accept the VL chain.

Linearized expression plasmids coding for the kappa or gamma constant region and VL/VH inserts were amplified by PCR using overlapping primers.

Purified PCR products were incubated with T4 DNA-polymerase, which generated single-strand overhangs. The reaction was stopped by dCTP addition.

In the next step, plasmid and insert were combined and incubated with recA which induced site specific recombination. The recombined plasmids were transformed into E.coli. The next day the grown colonies were picked and tested for correct recombined plasmid by plasmid preparation, restriction analysis and DNA-sequencing.

Example 30

Human PDGF-BB binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human PDGF-BB (Cell Signaling, Cat.

No. 8921BF) was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5% BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA 9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, Cat. No. 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 31

Cynomolgus PDGF-BB binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human PDGF-BB was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA 9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 32

Murine PDGF-BB binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen murine PDGF-BB (Peprotech 315-18) was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step:

saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA 9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 33

Rat PDGF-BB binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen rat PDGF-BB (R&D, 520-BB) was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA 9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 34

Human PDGF-AA binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human PDGF-AA (Peprotech, Cat. No. AF-100-13A) was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA

9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 35

Human PDGF-CC binding ELISA

The binding analysis was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The antigen human PDGF-CC (Peprotech AF-100-00C) was immobilized at a concentration of 125 ng/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific, Cat. No. 464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS, 0.5 % BSA, 0.05 % Tween with dispense and aspiration: 1) blocking step: saturating unbound surface (1 hour, 2 % BSA); 2) anti-PDGF-BB antibody in increasing concentrations for 1 hour; 3) detection antibody, dilution = 1:3000 (ECL anti-rabbit IgG-POD, NA9340V + ECL anti-human IgG-POD, NA933V or alternatively for murine antibodies ECL anti-mouse IgG-POD; NA 9310V). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The EC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 36

Protein-Protein Interaction Inhibition assay: human PDGF-B:human PDGF-BB receptor

The protein-protein interaction inhibition analysis of human PDGF-BB to the human PDGF-BB receptor was carried out using an enzyme-linked immunosorbent assay (ELISA)-based technology. The human Fc-tagged PDGF-BB receptor protein (RnD, Cat.No.385-PR-100) was immobilized at a concentration of 750 µg/mL in 25 µL in PBS, 0.5 % BSA and 0.05 % Tween on a 384 well microtiter plate (Thermo Scientific Cat.No.464718). Every of the following steps was followed by a washing routine of 3 times 90 µL PBS with dispense and aspiration: 1) blocking step saturating unbound surface (1 hour, 2 % BSA); 2) 15 µL anti-PDGF-BB antibody in increasing concentrations was incubated with 15 µL biotinylated human PDGF-BB (Cell Signaling, 8921BF) at 75 nM in a volume of 30 µL for 1 hour; 3) detection was achieved using peroxidase-labeled

streptavidin (Roche Diagnostics GmbH, Mannheim, Germany, 11089153001). 20-30 min. after adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Piercenet, 34021) the optical density was determined at 370 nm. The IC_{50} was calculated with a four parameter logistic model using GraphPad Prism 6.0 software.

Example 37

Antibody purification from murine hybridoma

Antibody-containing hybridoma supernatant is filtered and purified by two chromatographic steps. The antibodies are captured by affinity chromatography using HiTrap Protein G (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 are removed by washing with equilibration buffer, and the antibody is recovered with 25 mM citrate buffer, pH 3.0, and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 9.0. Size exclusion chromatography on Superdex 200™ (GE Healthcare) is used as second purification step. The size exclusion chromatography is performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions are concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Antibody-containing hybridoma supernatant is filtered and purified by two chromatographic steps. The supernatants are mixed with 50 % v/v 2 M glycine, pH 8.6, 600 mM NaCl and are captured by affinity chromatography using HiTrap MabSelectSuRe (GE Healthcare) equilibrated with 1 M glycine, pH 8.6, 300 mM NaCl. Unbound proteins are removed by washing with equilibration buffer, and the antibody is recovered with 100 mM citrate buffer, pH 2.8 and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 8.5. Size exclusion chromatography on Superdex 200™ (GE Healthcare) is used as second purification step. The size exclusion chromatography is performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions are concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Example 38

Human PDGF-BB Binding Surface Plasmon Resonance Spectroscopy Assay

The binding analysis was carried out using a surface plasmon resonance spectroscopy-based technology applying a BIAcore B4000 system (GE Healthcare). The antigen human PDGF-BB (Cell Signaling, 8921BF) was immobilized at a concentration of 1 µg/mL in PBS, 0.1 % BSA, 0.05 % Tween using amine coupling chemistry on a C1 sensor chip (GE Healthcare, BR-1005-35). Anti-PDGF-BB antibody in increasing concentrations was applied in 10 mM HEPES pH 7.2, 150 mM NaCl. Utilizing a read out time of 180 sec. association phase and 600 sec. dissociation phase the apparent k_a and apparent k_d were calculated. The apparent K_D (avidity) was calculated using the BIAcore T200 v2.0 fitting software.

Example 39

Cynomolgus PDGF-BB Binding Surface Plasmon Resonance Spectroscopy Assay

The binding analysis was carried out using a surface plasmon resonance spectroscopy-based technology applying a BIAcore B4000 system (GE Healthcare). The antigen cynomolgus PDGF-BB was immobilized at a concentration of 1 µg/mL in PBS, 0.1 % BSA, 0.05 % Tween using amine coupling chemistry on a C1 sensor chip (GE Healthcare, BR-1005-35). Anti-PDGF-BB antibody in increasing concentrations was applied in 10 mM HEPES pH 7.2, 150 mM NaCl. Utilizing a read out time of 180 sec. association phase and 600 sec. dissociation phase the apparent k_a and apparent k_d were calculated. The apparent K_D (avidity) was calculated using the BIAcore T200 v2.0 fitting software.

Example 40

Transfection and transient expression of the humanized antibodies in HEK cells

Transient expression of antibodies in suspension-adapted HEK293F (FreeStyle 293-F cells; Invitrogen) cells with Transfection Reagent 293-free (Novagen).

Cells have been passaged, by dilution, at least four times (volume 30 ml) after thawing in a 125 ml shake flask (Incubate/Shake at 37 °C, 7 % CO_2, 85 % humidity, 135 rpm).

The cells were expanded to $3x10^5$ cells/ml in 250 ml volume. Three days later, cells have been split and new seeded with a density of $7*10^5$ cells/ml in a 250 ml volume in a 1 liter shake flask. Transfection will be 24 hours later at a cell density around $1.4 – 2.0x10^6$ cells/ml.

Before transfection dilute 250 µg plasmid-DNA (122 µg light and 128 µg heavy chain) in a final volume of 10 ml with pre-heated (water bath; 37 °C) Opti-MEM (Gibco). Mix solution gentle and incubate at room temperature for not longer than 5 min. Then add 333.3 µl 293-free transfection reagent to DNA-OptiMEM-solution. Mix gently and incubate at room temperature for 15-20 minutes. Add whole volume of mixture to 1 L shake flask with 250 ml HEK-cell-culture-volume.

Incubate/Shake at 37 °C, 7 % CO_2, 85 % humidity, 135 rpm for 6 or 7 days.

Harvest supernatant by a first centrifugation-step at 2,000 rpm, 4 °C, for 10 minutes. Then transfer the supernatant in a new centrifugation-flask for a second centrifuge at 4,000 rpm, 4 °C, for 20 minutes. Thereafter the cell-free-supernatant is filtered through a 0.22 µm bottle-top-filter and stored in a freezer (-20 °C).

Example 41

Preparation of Fab fragment from an antibody and analytics:

5 mg antibody (about 1 mg/ml in 20 mM Histidine, 140 mM NaCl, pH 6.0) were incubated with 90 µl L-cysteine solution (Merck Millipore; 250 mM in 20 mM Histidine, 140 mM NaCl, pH 6.0) and 12 µl Papain (Roche Life Science; 3.2 U/mg antibody) for 120 min at 37 °C. After cleavage, 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 was used for removal of intact IgG and Fc fragment. Subsequently, flow-through of MabSelectSuRe chromatography was further purified using size exclusion chromatography on Superdex 200™ (GE Healthcare) as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The Fab fragment containing solutions 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 integrity of the Fab-fragments were analyzed by SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Invitrogen) in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Simply Blue Safe Stain (Invitrogen).

Aggregate content of Fab preparations was determined by high-performance SEC using a Superdex 200 10/300 GL analytical size-exclusion column (GE Healthcare) using 2 x PBS, pH 7.4 as running buffer.

Example 42

3T3 Cell Proliferation ELISA

For the determination of proliferation inhibition a BrdU colorimetric assay according to the manufacturer's manual was used (Roche Diagnostics GmbH, Mannheim, Germany, # 11 647 229 001). In the assay 5 ng/ml human PDGF-BB and antibody was used.

Example 43

Phospho-PDGF-R beta (Tyr751) sandwich ELISA

10,000 3T3-cells/well were cultivated for 24 h in DMEM-medium supplemented with 10 % Newborn Calf Serum. Thereafter the cells were incubated for 24 h in Hunger medium (DMEM-medium with 0.5 % Newborn Calf Serum).

Prior to addition the antibody (1 µg/ml) was pre-incubated for 2 h in medium containing 5 ng/ml PDGF-BB. The 3T3 cells were incubated for 10 min. in Hunger medium with 5 ng/ml human PDGF-BB and antibody.

Prior to lysis the cells were washed 4 times with PBS. Lysis of the cells was performed in 100 µl lysis buffer.

The lysis solution was incubated overnight at 4 °C in the wells of a rabbit anti-PDGF-R beta antibody coated 96-well plate. Thereafter the wells were washed 4 times with PBS. After incubation for 1 hour with mouse anti-Phospho-PDGF receptor beta antibody the wells are washed 4 times with wash buffer. For detection the wells were incubated for 30 min. with 100 µl TMB substrate solution. The reaction was stopped by addition of 100 µl stop solution. The absorption was determined at 450 nm.

Example 44

Cell Migration Assay

Use of CIM-Plate 16 (ACEA Biosciences Inc., n° 05665817001, pore size membrane 8 µm).

Protocol

Starvation (in CellSystems CSC Serum-free Medium without Growth Factor (b°SF-4ZR-500-S) overnight of Primary Human Retinal Pericyte Cells ((ACBRI 183 CellSystems), immortalized with hTERT) before use (50-70% confluence)

Plate Preparation

Upper Chamber: (50 µl Medium + 100 µl cells suspension)

Coating both sides with Fibronectin (Sigma n°F0895-2mg) 20 µg/ml in PBS:

Add 40 µl of fibronectin solution on the sensor side (bottom side) of each well, incubate the upper-chamber in the hood for 30 minutes. Carefully aspirate the fibronectin solution from the sensor side, avoid touching the electrodes. Turn the UC over such that the well side up and the sensor side is down. Add 50 µl of fibronectin solution in the well and incubate 30 minutes under the hood at RT. Carefully aspirate the solution from inside the well. Add 50 µl CSC Serum-free Medium without Growth Factor in the well.

Lower Chamber: 160 µl/well of your sample to test (1X concentrated)

All dilutions in CellSystems CSC Serum-free Medium without Growth Factor (n°SF-4ZR-500-S); incubate 2 h in the hood the mix of sample/antibody; add 160 µl/well of the sample; assemble the UC and LC using the CIM-Plate16 Assembly Tool; place the CIM-Plate 16 in the incubator at 37 °C for 1 h to equilibrate; place the CIM-Plate 16 on the RTCA DP Analyser; start background measurement by initiating Step 1 in the RTCA software.

Cell Preparation

Detach the cells with cell dissociation buffer and resuspend the cells in CellSystems CSC Serum-free Medium without Growth Factor (n°SF-4ZR-500-S) at $1.5x10^5$ cells/ml; remove the CIM-Plate 16 from the RTCA Analyser and add in

the UC, 15,000 Primary Human Retinal Pericyte Cells ((ACBRI 183 CellSystems)), immortalized with hTERT)/well/100 µl in Basal Medium (Cell Systems CSC Serum-free Medium without Growth Factor n°SF-4ZR-500-S); place back the plate in the RTCA Analyser and start immediately the measurement during 10-20 hours.

Example 45

Cell Proliferation Assay

CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, G3580).

Primary Human Retinal Pericyte Cells ((ACBRI 183 CellSystems)), immortalized with hTERT), 2500 cells/well/100 µl

Media

Growth medium = CSC complete medium, culture boost (ACBRI n° 4Z=-500)

Assay medium = CSC Serum Free medium without growth factors (ACBRI n°SF-4ZR-500-S)

Balbc3T3, 5000 cells/well/100 µl

Media

Growth medium = DMEM (Gibco n°41966), 10 % NBCS

Assay medium = DMEM (Gibco n°41966), 0.4 % NBCS

Proliferation assay

Seed cells in growth medium 24-48 hours before assay. When cells are 80-90 % confluent, harvest the cells with Trypsin solution. Resuspend the cells in growth medium at 0. 25×10^5 cells/ml (or 0.5×10^5 cells/ml). Add 100 µl cell suspension to each well of 96-well plate. Incubate for 24 hours at 37 . Remove growth medium, add 100 µl assay medium to each well. Incubate for 24 hours. Add 100 µl standards or samples to each well (2X concentrated, diluted in assay medium). Incubate for 72 hours at 37 with 5 % CO_2. Add 40 µl of dye solution on cells and incubate at 37 °C. Read at 490 nm at different times (1 h to 8 h).

Example 46

Anti-PDGF-BB antibodies kinetic screening

Binding of anti-PDGF-BB antibodies to human PDGF-BB was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). Around 20 resonance units (RU) of recombinant human PDGF-BB (5 µg/ml; ordering code 220-BB; R&D Systems) were coupled on a Series S C1 chip (GE Healthcare BR-1005-35) at pH 4.0 by using an amine coupling kit supplied by GE Healthcare. Running buffer for immobilization was HBS-N pH 7.4 (10 mM HEPES, 150 mM NaCl, pH 7.4, GE Healthcare). For the following kinetic characterization, running and dilution buffer was HBS-P pH 7.4 (10 mM HEPES, 150 mM NaCl, 0.05 % Surfactant P20, pH 7.4, GE Healthcare). The flow cell was set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice.

Association was measured by injection of the anti-PDGF-BB antibodies in concentrations of 30 nM and 3 nM in solution for 30 sec. at a flow rate of 100 µl/min. The dissociation phase was monitored for up to 600 sec. and triggered by switching from the sample solution to running buffer. The surface was regenerated by washing with two consecutive injections of 0.85 % H_3PO_4 (phosphoric acid) solution for 60 sec. at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a blank surface. Blank injections were also subtracted (= double referencing). For calculation of K_D and other kinetic parameters the Langmuir 1:1 model was used.

Example 47

Anti-PDGF-BB Fabs kinetic characterization

Binding of anti-PDGF-BB Fab samples to human PDGF-BB was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). Around 50 resonance units (RU) of recombinant human PDGF-BB (0.5 µg/ml; ordering code 220-BB; R&D Systems) were coupled on a Series S CM3 chip (GE Healthcare BR-1005-36) at pH 4.0 by using an amine coupling kit supplied by GE Healthcare. Running buffer for Immobilization was HBS-N pH 7.4 (10 mM HEPES, 150 mM NaCl, pH 7.4, GE Healthcare). For the following kinetic characterization, running and dilution buffer was HBS-P pH 7.4 (10 mM HEPES, 150 mM NaCl, 0.05 % Surfactant P20, pH 7.4, GE Healthcare). The flow cell was

set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice.

Association was measured by injection of the anti-PDGF-BB Fabs in various concentrations in solution for 180 sec. at a flow rate of 30 µl/min starting with 300 nM in serial 1:3 dilutions. The dissociation phase was monitored for up to 900 sec. and triggered by switching from the sample solution to running buffer. The surface was regenerated by washing with a 0.85 % H_3PO_4 (phosphoric acid) solution for 60 sec. at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a blank surface. Blank injections were also subtracted (= double referencing). For calculation of KD and other kinetic parameters the Langmuir 1:1 model was used.

Example 48

Preparation of Fab fragment from an antibody and analytics:

12 mg antibody (1 mg/ml in 20 mM Histidine, 140 mM NaCl, pH 6.0) were incubated with 240 µl L-cysteine solution (Merck Millipore; 250 mM in 20 mM Histidine, 140 mM NaCl, pH 6.0) and 327 µl Papain (Roche Life Science; 0.001 U/mg antibody) for 120 min at 37 °C. After cleavage, 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 was used for removal of intact IgG and Fc fragment. Subsequently, flow-through of MabSelectSuRe chromatography was further purified using size exclusion chromatography on Superdex 200™ (GE Healthcare) as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The Fab fragment containing solutions 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 integrity of the Fab-fragments were analyzed by SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Invitrogen) in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Simply Blue Safe Stain (Invitrogen).

Aggregate content of Fab preparations was determined by high-performance SEC using a BioSuite High Resolution SEC, 250 Å, 5 µm analytical size-exclusion column (Waters GmbH) using 200 mM K_2HPO_4/KH_2PO_4, 250 mM KCl, pH 7.0 as running buffer.

Example 49

ANG2 binding kinetics and cross-reactivity of maturated XFabs

Binding of the maturated XFabs to human ANG2-RBD-Fc-region fusion was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). Around 4000 RU of anti-human antibody (10 µg/ml anti-human IgG (Fc) antibody; ordering code BR-1008-39; GE Healthcare) were coupled on a Series S CM5 chip (GE Healthcare BR-1005-30) at pH 5.0 by using an amine coupling kit supplied by the GE Healthcare. HBS-N (10 mM HEPES, 150 mM NaCl pH 7.4, GE Healthcare) was used as running buffer during the immobilization procedure. For the following kinetic characterization, sample and running buffer was HBS-P (10 mM HEPES, 150 mM NaCl pH 7.4, 0.05 % Surfactant P20; GE Healthcare). The flow cell was set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice prior to kinetic characterization.

Human or cynomolgus ANG2-RBD-Fc-region fusion was captured by injecting a 1 µg/ml solution for 30 sec. at a flow rate of 5 µl/min. Association was measured by injection of the XFabs in various concentrations in solution for 90 sec. at a flow rate of 90 µl/min starting with 300 nM in serial 1:3 dilutions. The dissociation phase was monitored for up to 600 sec. and triggered by switching from the sample solution to running buffer. All surfaces were regenerated by 60 sec. washing with a 3 M $MgCl_2$ solution at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from an anti-human IgG antibody (Fc) surface. Blank injections were also subtracted (= double referencing). For calculation of KD and other kinetic parameters the Langmuir 1:1 model was

Example 50

Biological activity

The method determines the capacity of an antibody to inhibit binding of ANG2 to its receptor Tie2. For expression of the Tie2 receptor tyrosine kinase on the cell surface the HEK293 cell line, a human embryonic kidney cell line, was stably

transfected with the expression vector for human Tie2 resulting in the cell line HEK293_Tie2.

The cells are stimulated with ANG2 that binds to the Tie2 receptor and induces the autophosphorylation of the receptor. The binding to Tie2 can be inhibited by addition of an anti-ANG2 antibody as reported herein. The grade of phosphorylation is analyzed by an ELISA. The OD values correlate with the amount of phosphorylated Tie2 and are plotted against the antibody concentrations. The EC_{50} value is determined and reported relative to the reference standard on the same plate as relative biological activity (RBA).

A multi-well plate was coated with antibody against the human Tie2 receptor (100 µl of 10 µg/ml; R&D Systems, Cat# MAB3132; 96 well Maxisorb immuno plate, incubated overnight at room temperature; coated plates were washed three times, volume 250 µl; thereafter incubated with 200 µl blocking 1 – 2 hours at room temperature).

Separately the HEK293_Tie2 cells (40 µl; 5 x 10^6 cells/ml; DMEM/F12) were added to a pre-incubated mixture (80 µl) of dilution series of the antibody in question and ANG2 (R&D Systems, Cat# 623-CF). After 10 minutes the cells were lysed (60 µl lysis buffer added; incubated for 15 min.) and the cell lysate was transferred to the coated plate for the ELISA.

The Tie2 receptor of the lysate binds to the capture anti-Tie2 antibody (100 µl lysate; incubated for 90 min. at RT). The phosphorylated tyrosins on the Tie2 receptor were detected by anti-phosphotyrosine antibody conjugated to biotin (100 µl; 0.3 µg/ml anti-phosphotyrosine antibody, clone 4G10®, biotin conjugate, Upstate, Cat# 16-103; incubated for 60 min. at RT). Biotin residues were bound by the streptavidin-horseradish peroxidase conjugate (100 µl; 100 mU/ml; Roche Diagnostics GmbH, Mannheim, Germany, Cat# 11089153001; incubated for 30 min. at RT). The peroxidase substrate TMB (100 µl; Roche Diagnostics GmbH, Mannheim, Germany, Cat# 11835033001) was added and the optical density was measured after 5-10 min at 450 nm.

Example 51

Production and purification of bispecific antibodies

Transient expression of bispecific antibodies in suspension-adapted HEK293F (FreeStyle 293-F cells; Invitrogen) cells after transfection of DNA with Transfection Reagent 293-free (Novagen).

Cells have been passaged every third or fourth day, by dilution, at least four times (volume 30 ml) after thawing in a 125 ml shake flask (incubate/Shake at 37°C, 7 % CO2, 85% humidity, 135 rpm). The cells were expanded by seeding the cells with a cell density of $3x10^5$ cells/ml in 250 ml medium. Three days later, cells have been split and newly seeded with a density of $2*10^5$ cells/ml in 500 ml medium. Four days later, cells have been split and newly seeded with a density of $7*10^5$ cells/ml in 1 liter medium (incubate/Shake at 37°C, 7 % CO2, 85% humidity, 110 rpm). Transfection was done 24 hours later at a cell density around 1.4 – $2.0x10^6$ cells/ml.

Before transfection 1000 µg plasmid-DNA (2x 250 µg light encoding plasmid DNA and 2x 250 µg heavy chain encoding plasmid DNA) were diluted in a final volume of 40 ml with pre-heated (water bath; 37°C) Opti-MEM (Gibco). The solutions were gently mixed and incubated at room temperature for not longer than 5 min. Then 1333 µl 293-free transfection reagent were added to the DNA-Opti-MEM-solution. The mixture was gently mixed and incubated at room temperature for 15-20 minutes. The whole volume of mixture was carefully added to the 1 liter HEK-cell-culture. The cells were further incubated with shaking at 110 rpm at 37°C, 7 % CO2, 85% humidity, for 7 days.

The supernatant was harvested after 7 days by a first centrifugation-step at 2000 rpm, 4°C, for 10 minutes. Then the supernatant was transferred into a new centrifugation-flask for a second centrifugation-step at 4000 rpm, 4°C, for 20 minutes. The cell-free-supernatant was filtered through a 0.22 µm filter (Millipore) and stored in a freezer (-20°C) until purification-procedure was started.

The antibody-containing culture supernatants were filtered and purified by at least two chromatographic steps. The antibodies were captured by affinity chromatography using CaptureSelect Pre-packed Column IgG-CH1 (life technologies, #494320005) equilibrated with PBS (1 mM KH2PO4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl), pH 7.4. Unbound proteins were removed

by washing with equilibration buffer, and antibodies were recovered with 25 mM citrate buffer, pH 3.0 and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 9.0.

Size exclusion chromatography on Superdex 200™ (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The antibody containing solutions were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

The anti-PDGF-B/ANG2 antibody (clone 0144-0004) was further purified using a hydrophobic interaction chromatography (HIC). Ammonium sulfate was added to final concentration of 1 M to the antibody containing solution. The solution was applied to a Butyl Sepharose 4 Fast Flow (GE Healthcare) column equilibrated in 1 M ammonium sulfate, 35 mM acetate, pH 5.6. The antibody was eluted in a linear gradient (0-100 %) with 35 mM acetate, pH 5.6. Antibody containing fractions were pooled and applied to size exclusion chromatography.

The protein concentration of antibody preparations was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

Purity and integrity of the antibodies were analyzed by CE-SDS using a LabChip GX II (PerkinElmer) with Protein Express Chip and HT Protein Express Reagents Kit.

Aggregate content of antibody preparations was determined by high-performance SEC using a BioSuite High Resolution SEC, 250 Å, 5 µm analytical size-exclusion column (Waters GmbH) using 200 mM K_2HPO_4/KH_2PO_4, 250 mM KCl, pH 7.0 as running buffer.

	antibody	scale [l]	yield [mg]	yield [mg/l supernatant]	Monomer (SE-HPLC) [%]	Monomer (CE-SDS) [%]	columns
anti-PDGF-B/ANG2 antibody	0144	1.5	37.7	25.1	>98	>95	CH1 select, HIC, SEC
anti-PDGF-B/VEGF antibody	0117	1	46.3	46.3	>98	>95	CH1 select, SEC
anti-PDGF-B/ANG2 antibody	0145	1	21.5	21.5	>98	>95	CH1 select/SEC

anti-PDGF-B/ANG2 antibody	0146	0.5	14,6	29.2	>98	>95	CH1 select/SEC
anti-IL-1beta /ANG2 antibody	0031	1	29.2	29.2	>98	>95	CH1 select, SEC
anti-IL-1beta /VEGF antibody	0032	1.5	23.6	15.7	>98	>95	CH1 select, IEX, SEC

Antibody 0144 is a CrossMab antibody comprising an ANG2 binding site of SEQ ID NO: 117 (VH) and SEQ ID NO: 118 (VL) and a PDGF-B binding site of SEQ ID NO: 92 (VH) and SEQ ID NO: 97 (VL).

Antibody 0117 is a CrossMab antibody comprising a VEGF binding site of SEQ ID NO: 119 (VH) and SEQ ID NO: 120 (VL) and a PDGF-B binding site of SEQ ID NO: 92 (VH) and SEQ ID NO: 97 (VL).

Antibody 0145 is a CrossMab antibody comprising an ANG2 binding site of SEQ ID NO: 119 (VH) and SEQ ID NO: 120 (VL) and a PDGF-B binding site of SEQ ID NO: 101 (VH) and SEQ ID NO: 106 (VL).

Antibody 0146 is a CrossMab antibody comprising an ANG2 binding site of SEQ ID NO: 117 (VH) and SEQ ID NO: 118 (VL) and a PDGF-B binding site of SEQ ID NO: 121 (VH) and SEQ ID NO: 122 (VL).

Antibody 0031 is a CrossMab antibody comprising an IL-1beta binding site of SEQ ID NO: 06 (VH) and SEQ ID NO: 16 (VL) and an ANG2 binding site of SEQ ID NO: 140 (VH) and SEQ ID NO: 141 (VL).

Antibody 0032 is a CrossMab antibody comprising a VEGF binding site of SEQ ID NO: 142 (VH) and SEQ ID NO: 143 (VL) and an IL-1beta binding site of SEQ ID NO: 06 (VH) and SEQ ID NO: 16 (VL).

Example 52

Bispecific antibody kinetic characterization

PDGF-BB

Binding of bispecific anti-PDGF-BB/ANG2 antibodies to human PDGF-BB was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). Around 50 resonance units (RU) of recombinant human PDGF-BB (0.5 µg/ml; ordering code 220-BB; R&D Systems) were coupled on a Series S

CM3 chip (GE Healthcare BR-1005-36) at pH 4.0 by using an amine coupling kit supplied by GE Healthcare. Running buffer for Immobilization was HBS-N pH 7.4 (10 mM HEPES, 150 mM NaCl, pH 7.4, GE Healthcare). For the following kinetic characterization, running and dilution buffer was HBS-P pH 7.4 (10 mM HEPES, 150 mM NaCl, 0.05 % Surfactant P20, pH 7.4, GE Healthcare). The flow cell was set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice.

Association was measured by injection of the bispecific antibody in various concentrations in solution for 180 sec. at a flow rate of 30 µl/min starting with 300 nM in serial 1:3 dilutions. The dissociation phase was monitored for up to 900 sec. and triggered by switching from the sample solution to running buffer. The surface was regenerated by washing with a 0.85 % H_3PO_4 (phosphoric acid) solution for 60 sec. at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a blank surface. Blank injections were also subtracted (= double referencing). For calculation of KD and other kinetic parameters the Langmuir 1:1 model was used.

ANG2:

Binding of the bispecific antibody to human ANG2-RBD-mouse Fc-region fusion was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). Around 4000 RU of anti-mouse Fc-region antibody (10 µg/ml anti-mouse (Fc) antibody) were coupled on a Series S CM5 chip (GE Healthcare BR-1005-30) at pH 5.0 by using an amine coupling kit supplied by the GE Healthcare. HBS-N (10 mM HEPES, 150 mM NaCl pH 7.4, GE Healthcare) was used as running buffer during the immobilization procedure. For the following kinetic characterization, sample and running buffer was HBS-P (10 mM HEPES, 150 mM NaCl pH 7.4, 0.05 % Surfactant P20; GE Healthcare). The flow cell was set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice prior to kinetic characterization.

Human ANG2-RBD-murine Fc-region fusion was captured by injecting a 1 µg/ml solution for 30 sec. at a flow rate of 5 µl/min. Association was measured by injection of the bispecific antibody in various concentrations in solution for 90 sec. at a flow rate of 90 µl/min starting with 300 nM in serial 1:3 dilutions. The dissociation phase was monitored for up to 600 sec. and triggered by switching from the sample solution to running buffer. All surfaces were regenerated by 60

sec. washing with a 3 M MgCl2 solution at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from an anti-mouse IgG antibody (Fc) surface. Blank injections were also subtracted (= double referencing). For calculation of KD and other kinetic parameters the Langmuir 1:1 model was

VEGF:

Binding of the bispecific antibody to human VEGF isoform 121 was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). An anti-hexa-histidine antibody was coupled on a CM5 chip (GE Healthcare BR-1005-30) according to the manufacturer's instructions by using an amine coupling kit supplied by the GE Healthcare. HBS-N (10 mM HEPES, 150 mM NaCl pH 7.4, GE Healthcare) was used as running buffer during the immobilization procedure. For the following kinetic characterization, sample and running buffer was HBS-P (10 mM HEPES, 150 mM NaCl pH 7.4, 0.05 % Surfactant P20; GE Healthcare). The flow cell was set to 25 °C - and the sample block set to 12 °C - and primed with running buffer twice prior to kinetic characterization.

Histidine-tag comprising human VEGF isoform 121 was captured by injecting a solution for 30 sec. at a flow rate of 5 µl/min. Association was measured by injection of the bispecific antibody in various concentrations in solution for 90 sec. at a flow rate of 90 µl/min starting with 300 nM in serial 1:3 dilutions. The dissociation phase was monitored for up to 600 sec. and triggered by switching from the sample solution to running buffer. All surfaces were regenerated by 60 sec. washing with a 3 M $MgCl_2$ solution at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from an anti-hexa-histidine antibody surface. Blank injections were also subtracted (= double referencing). For calculation of KD and other kinetic parameters the Langmuir 1:1 model was used.

IL-1beta:

Binding kinetics of anti-IL-1beta antibodies to human IL-1beta were investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). All experiments were performed at 25°C using HBS-P (10 mM His, 140 mM NaCl, 0.05% Tween 20 pH 7.4) as running and dilution buffer. Anti-

human Fc antibodies were immobilized on a Series S CM5 Sensor Chip (GE Healthcare) using standard amine coupling chemistry. Bispecific antibodies were captured on the surface leading to a capturing response of 100 – 200 RU. Human IL-1beta was injected for 90 s with concentrations from 0.74 up to 60 nM (1:3 dilution series) onto the surface (association phase). The dissociation phase was monitored for 600 sec by washing with running buffer. The surface was regenerated by injecting 3 M MgCl2 (for anti-human Fc antibody) or 10 mM Glycine pH 1.5 (for anti-mouse Fc antibody) for 60 sec at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a mock surface. Blank injections were subtracted (double referencing). The derived curves were fitted to a 1:1 Langmuir binding model using the BIAevaluation software.

Example 53

X-ray structure determination

Apo Fab fragment H34

Crystallization screening for Fab fragment H34 was performed at a concentration of 32mg/ml. Crystallization droplets were set up at 21 °C by mixing 0.1 µl of protein solution with 0.1 µl reservoir solution in vapor diffusion sitting drop experiments. Crystals appeared out of various conditions containing PEG as precipitating agent. Crystals used to determine the structure of H34 appeared within 2 days out of 0.1 M HEPES pH 7.0, 20% PEG 4000 and out of 0.1M sodium cacodylate, 15% PEG4000.

Crystals were harvested with dried Paraffin oil as cryoprotectant and then flash-cooled in liquid N_2. Diffraction images were collected with a Pilatus 6M detector at a temperature of 100K at the beam line X10SA of the Swiss Light Source and processed with the XDS package (Kabsch, W. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Cryst. 26 (1993) 795-800). Data from two crystals were merged to yield a 1.64 Å resolution data set in space group P1 and two Fab per crystallographic asymmetric unit (see Table below).

The structure was determined by molecular replacement using the Fab 577 from Roche-internal PDB-ID 1htfr as search model. The Fab was split into constant and variable domains and both used for separate searches in the CCP4 program

PHASER CCP4 (CCP4 (Collaborative Computational Project, N. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D, (1994) 760-763) to account for possible changes in the elbow angle. The model was rebuilt in COOT (Emsley, P., Lohkamp, B., Scott, WG. & Cowtan, K. Features and development of COOT. Acta Crystallogr. D Biol. Crystallogr. 60 (2010) 486-501) and refined with CCP4 program.

Table: Data collection and structure refinement statistics for H34 Fab apo-crystal

Data Collection	
Wavelength (Å)	1.0
Resolution[1] (Å)	48.27 - 1.64 (1.699 - 1.64)
Space group	P1
Unit cell (Å, °)	50.03 69.72 80.58 93.389 95.059 110.195
Total reflections	424699 (40316)
Unique reflections	123149 (12254)
Multiplicity	3.4 (3.3)
Completeness (%)	0.99 (0.99)
Mean I/σ(I)	5.95 (0.59)
Wilson B-factor	28.27
R-merge [2]	0.1151 (1.612)
R-meas	0.1352 (1.908)
CC1/2	0.991 (0.332)
CC*	0.998 (0.706)
Refinement	
Reflections used in refinement	123149 (12068)
Reflections used for R-free	6101 (592)
R-work [3]	0.2005 (0.3964)
R-free [4]	0.2350 (0.4117)
CC(work)	0.959 (0.593)
CC(free)	0.943 (0.586)
Number of non-hydrogen atoms	7574
macromolecules	6622
Protein residues	859
RMS bonds (Å)	0.007
RMS angles (°)	1.09
Ramachandran favored (%)	97
Ramachandran allowed (%)	2.7
Ramachandran outliers (%)	0.23
Rotamer outliers (%)	1.1
Clashscore	1.30
Average B-factor (Å^2)	32.58
macromolecules	31.78
solvent	38.12

All data computed with Phenix.

[1] Values in parentheses refer to the highest resolution bins.

[2] $R_{merge}=\Sigma|I-<I>|/\Sigma I$ where I is intensity.

[3] $R_{work}=\Sigma|F_o-<F_c>|/\Sigma F_o$ where F_o is the observed and F_c is the calculated structure factor amplitude.

[4] R_{free} was calculated based on 5% of the total data omitted during refinement.

Complex Fab fragment H34 with human Il-1beta

Prior to crystallization screening Fab fragment H34 was mixed with IL-1beta (Peprotech) in a molar ratio of 1.2:1. The protein mixture was incubated at 21°C for 2h. Protein concentration used in crystallization experiments was 32mg/ml. Crystallization droplets were set up at 21 °C by mixing 0.1 µl of protein with 0.1 µl reservoir solutions in vapor diffusion sitting drop experiments. Crystals appeared out of 0.1 M Tris pH 8.0, 20% PEG 4000 within 2 days and grew to a final size of 0.15 mm x 0.06 mm x 0.01 mm within 4 days.

Crystals were harvested without addition of cryo-protectant and then flash frozen in liquid N2. Diffraction images were collected with a Pilatus 6M detector at a temperature of 100K at the beam line X10SA of the Swiss Light Source and processed with the XDS package (Kabsch, W. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Cryst. 26 (1993) 795-800 (1993)). Data collection and processing followed the same route as for the H34 apo crystal (see above). Statistics are collected in the Table above. Data from two crystals were merged to obtain a more complete dataset. Molecular replacement was successful using the H34 Fab structure and interleukin-1beta (PDB-ID 1l2h) as search models. Model building and refinement was performed as described above.

Table: Data collection and structure refinement statistics for H34 Fab IL-1beta complex crystal

Data Collection	
Wavelength	1
Resolution range [1]	48.22 - 1.36 (1.409 - 1.36)
Space group	P 1
Unit cell	41.1 48.92 70.36 96.162 101.938 96.035
Total reflections	564817 (55575)
Unique reflections	113220 (11310)
Multiplicity	5.0 (4.9)

Completeness (%)	0.99 (1.00)
Mean I/σ(I)	9.82 (0.78)
Wilson B-factor	17.64
R-merge [2]	0.09016 (2.448)
R-meas	0.1007 (2.744)
CC1/2	0.999 (0.277)
CC*	1 (0.659)
Refinement	
Reflections used in refinement	113220 (10997)
Reflections used for R-free	5663 (564)
R-work	0.1559 (0.3786)
R-free	0.2063 (0.4137)
CC(work)	0.979 (0.659)
CC(free)	0.971 (0.626)
Number of non-hydrogen atoms	5318
macromolecules	4570
Protein residues	576
RMS(bonds)	0.005
RMS(angles)	1.09
Ramachandran favored (%)	98
Ramachandran allowed (%)	1.9
Ramachandran outliers (%)	0
Rotamer outliers (%)	1.3
Clashscore	1.86
Average B-factor	28.68
macromolecules	26.52
solvent	41.85

All data computed with Phenix.

[1] Values in parentheses refer to the highest resolution bins.

[2] $R_{merge}=\Sigma|I-<I>|/\Sigma I$ where I is intensity.

[3] $R_{work}=\Sigma|F_o-<F_c>|/\Sigma F_o$ where F_o is the observed and F_c is the calculated structure factor amplitude.

[4] R_{free} was calculated based on 5% of the total data omitted during refinement.

Example 54

Binding Kinetics and Cross-reactivity of anti-IL-1beta Antibodies

Binding kinetics of anti-IL-1beta antibodies to human, cynomolgus, rat and murine IL-1beta as well as cross-reactivity to human IL-1beta and human IL-1alpha were investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). All experiments were performed at 25°C using HBS-P (10 mM His, 140 mM NaCl, 0.05% Tween 20 pH 7.4) as running and dilution buffer. Anti-human or anti-mouse Fc antibodies were immobilized on a Series S CM5 Sensor

Chip (GE Healthcare) using standard amine coupling chemistry. Anti-IL-1beta antibodies were captured on the surface leading to a capturing response of 100 – 200 RU. IL-1beta molecules were injected for 90 s with concentrations from 0.74 up to 60 nM (1:3 dilution series) onto the surface (association phase). The dissociation phase was monitored for 600 sec by washing with running buffer. Cross-reactivity to human IL-1beta and human IL-1α was determined by a single injection of 100 nM antigen according to the conditions described above. The surface was regenerated by injecting 3 M MgCl2 (for anti-human Fc antibody) or 10 mM Glycine pH 1.5 (for anti-mouse Fc antibody) for 60 sec at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a mock surface. Blank injections were subtracted (double referencing). The derived curves were fitted to a 1:1 Langmuir binding model using the BIAevaluation software.

Example 55

Binding Kinetics of anti-IL-1beta IgG compared to anti-IL-1beta Fab

Binding of anti-IL-1beta IgG and Fab to human IL-1beta was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). All experiments were performed at 25°C using HBS-P (10 mM His, 140 mM NaCl, 0.05% Tween 20 pH 7.4) as running and dilution buffer. Anti-human Fc or anti-human Fab antibodies were immobilized on a Series S CM5 Sensor Chip (GE Healthcare) using standard amine coupling chemistry. Anti-IL-1beta IgG and Fab was captured on the surface leading to a capturing response of approximately 100 and 50 RU, respectively. Human IL-1beta was injected for 90 s with concentrations from 0.74 up to 60 nM (1:3 dilution series) onto the surface (association phase) at a flow rate of 30 µl/min. The dissociation phase was monitored for 600 sec by washing with running buffer. The surface was regenerated by injecting 3 M MgCl2 (for anti-human Fc antibody) or 10 mM Glycine pH 1.5 (for anti-mouse Fc antibody) for 60 sec at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a mock surface. Blank injections were subtracted (double referencing). The derived curves were fitted to a 1:1 Langmuir binding model using the BIAevaluation software.

Example 56

Mode of Action Analysis of anti-IL-1beta Antibodies

Binding inhibition of anti-IL-1beta to human IL-1RI was investigated by surface plasmon resonance using a BIACORE T200 instrument (GE Healthcare). All experiments were performed at 25°C using HBS-P (10 mM His, 140 mM NaCl, 0.05% Tween 20 pH 7.4) as running and dilution buffer. Human IL-1RI was immobilized on a Series S CM5 Sensor Chip (GE Healthcare) using standard amine coupling chemistry. 10 nM of human IL-1beta were pre-incubated with anti-IL-1beta antibodies concentrations from 100 nM down to 0.098 nM (1:2 dilution series). The IL-1beta/anti-IL-1beta antibody mixtures were injected onto the flow cell at 5 µl/min and the binding response (RU) after 60s was used to monitor inhibition. The surface was regenerated by injecting 10 mM NaOH for 60 sec at a flow rate of 5 µl/min. Bulk refractive index differences were corrected by subtracting the response obtained from a mock surface.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Patent Claims

1. A bispecific antibody that specifically binds to human IL-1beta and a second antigen selected from the group consisting of human ANG2, human VEGF and human PDGF-B, comprising

a) a heavy chain variable domain comprising

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 02, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 03, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 05, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 15,

and

a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19,

or

a) a heavy chain variable domain comprising a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 21, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 22, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 24, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 26; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 27; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 28.

2. A bispecific antibody that specifically binds to human PDGF-B and a second antigen selected from the group consisting of human ANG2, human VEGF and human IL-1beta, comprising

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 31, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 35; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

3. The bispecific antibody according to any one of claims 1 or 2 that further specifically binds to human ANG-2 further comprising

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 57, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 58, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 60, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 62; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 63; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 64,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 66, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 67, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 69, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 71; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 72; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 73,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 75, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 76, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 78, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 80; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 81; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 82,

or

d) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 84, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 85, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 87, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 89; (b) a HVR-L2 comprising the amino

acid sequence of SEQ ID NO: 90; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 91.

4. The bispecific antibody according to any one of claims 1 or 2 that further specifically binds to human VEGF and further comprises

a) the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 107 and the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 108,

or

b) the HVR-H1, HVR-H2 and HVR-H3 as contained in the heavy chain variable domain of SEQ ID NO: 109 and the HVR-L1, HVR-L2 and HVR-L3 as contained in the heavy chain variable domain of SEQ ID NO: 110.

5. A bispecific antibody that specifically binds to human IL-1beta and human PDGF-B, comprising

- a first binding site specifically binding to human IL-1beta comprising

a) a heavy chain variable domain comprising

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 02, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 03, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 05, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 07, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 08, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 10, or

(a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 15,

and

a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 19,

or

a) a heavy chain variable domain comprising a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 21, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 22, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 24, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 26; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 27; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 28.

- a second binding site specifically binding to human PDGF-B comprising

a) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 30, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 31, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 33, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 35; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 36; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 37,

or

b) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 39, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 40, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 42, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 46,

or

c) a heavy chain variable domain comprising (a) a HVR-H1 comprising the amino acid sequence of SEQ ID NO: 48, (b) a HVR-H2 comprising the amino acid sequence of SEQ ID NO: 49, and (c) a HVR-H3 comprising the amino acid sequence of SEQ ID NO: 51, and a light chain variable domain comprising (a) a HVR-L1 comprising the amino acid sequence of SEQ ID NO: 53; (b) a HVR-L2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 55.

6. The antibody according to any one of claims 1 to 5, wherein the antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other.

7. The antibody according to claim 6, wherein the antibody comprises

i) in the constant domain CL of the first light chain under a) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein in the constant domain CH1 of the first heavy chain under a) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index),

or

ii) in the constant domain CL of the second light chain under b) the amino acid at position 124 is substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat) (in one preferred embodiment independently by lysine (K) or arginine (R)), and wherein

in the constant domain CH1 of the second heavy chain under b) the amino acid at position 147 or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index).

8. The antibody according to any one of claims 1 to 5, wherein the antibody is a bivalent, bispecific antibody, comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

9. The antibody according to any one of claims 1 to 8, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein

i) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide, or

ii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, or

iii) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, or

iv) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, Y349C, T366S, L368A, Y407V, or

v) the first Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG1 Fc-region polypeptide with the mutations L234A, L235A, P329G, Y349C, T366S, L368A, Y407V, or

vi) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide, or

vii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, or

viii) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, or

ix) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, Y349C, T366S, L368A, Y407V, or

x) the first Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, S354C, T366W and the second Fc-region polypeptide is a human IgG4 Fc-region polypeptide with the mutations S228P, L235E, P329G, Y349C, T366S, L368A, Y407V.

10. The antibody according to any one of claims 1 to 9, wherein the antibody comprises a first Fc-region polypeptide and a second Fc-region polypeptide, and

wherein the antibody comprises the combination of mutations

i) I253A, H310A, and H435A, or

ii) H310A, H433A, and Y436A, or

iii) L251D, L314D, and L432D, or

iv) combinations of i) to iii)

in the first Fc-region polypeptide and in the second Fc-region polypeptide.

11. A pharmaceutical formulation comprising an antibody according to any one of claims 1 to 10 and optionally a pharmaceutically acceptable carrier.

12. The antibody according to any one of claims 1 to 10 for use as a medicament.

13. Use of the antibody according to any one of claims 1 to 10 in the manufacture of a medicament.

14. The use according to any one of claims 12 and 13, wherein the medicament is for the treatment of an ocular vascular disease, preferably for the treatment of macular degeneration.