Method for producing monomeric and multimeric molecules and uses thereof

Herein is reported a method for the production of monomeric and multimeric molecules as fusion polypeptide with an immunoglobulin Fc-region and uses thereof, such as a therapeutic agent, in assays, chromatography columns, or generation of disease animal models.

Background of the Invention

An immunoglobulin contains in general two light polypeptide chains and two heavy polypeptide chains. Each of the heavy and light polypeptide chains comprises a variable region (generally the amino terminal portion of the polypeptide chain) which contains a binding domain that is able to interact with an antigen. Each of the heavy and light polypeptide chains also comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the immunoglobulin e.g. to cells bearing an Fc gamma receptor (FcγR), such as phagocytic cells, or to cells bearing the neonatal Fc-receptor (FcRn) also known as Brambell receptor, and also mediates the binding to some factors including factors of the classical complement system such as component (C1q).

Hulett and Hogarth (Hulett, M.D. and Hogarth, P.M., Adv. Immunol. 57 (1994) 1-127) reported that the extracellular receptors for the Fc part of immunoglobulins of class G are a family of transmembrane glycoproteins comprising three different receptor types having different binding specificity: FcγRI, FcγRII, and FcγRIII. Receptors of type I interact with non-complexed IgG, whereas receptors of type II and III interact preferably with complexed IgG.

Certain immunoglobulin-based cytokine fusion proteins are commercially available (e.g. from Biomol). FC-region fusions are reported generally in "Therapeutic monoclonal antibodies – from bench to clinic" (Wiley, Edited by Zhiqiang An, 2009).

In WO 01/03737 immunoglobulin fusion proteins are reported. Improved circulating half-life and efficacy of an antibody-interleukin 2 immunocytokine based on reduced intracellular proteolysis is reported by Gillies, S.D., et al. (Clin. Cancer Res. 8 (2002) 210-216). Dumont, J.A., et al. (Biodrugs 20 (2006) 151-160) report monomeric Fc fusions. High level expression and secretion of Fc-X fusion

proteins in mammalian cells is reported by Lo, K-M., et al. (Prot. Eng. 11 (1998) 495-500). In WO 00/40615 the expression and export of anti-obesity proteins as Fc fusion proteins is reported.

Summary of the Invention

It has been found that polypeptides, which are biologically active as n-mer and form non-defined aggregates/multimers upon expression, can be obtained in soluble form by expressing these polypeptides as fusion polypeptide with an Fc-region that does not substantially bind to an Fc-receptor. Using a fusion polypeptide for the expression of the polypeptide increases the obtainable yield of the polypeptide either in form of the fusion polypeptide or as isolated polypeptide. It has further been found that the polypeptide remains in a defined n-mer form after cleavage and removal of the Fc-region.

There is reported herein a method for the production of a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region comprising the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula I

$$(B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m)_u \qquad \text{(formula I)}$$

wherein

B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC denotes a heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein u=1 or 2,

wherein FC does not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) optionally cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

One aspect as reported herein is a method for the production of a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region and comprises the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s) (:),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

There is reported herein a fusion polypeptide according to formula I

$$(B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m)_u \qquad \text{(formula I)}$$

wherein B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC denotes a heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein u=1 or 2,

wherein FC does not substantially bind to an Fc-receptor.

One aspect as reported herein is a fusion polypeptide according to formula II

Bn-CSo-Is-CSp-FC1-CSq-It-CSr-Bm : FC2 (formula II)

wherein

B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC1 denotes a first heavy chain Fc-region polypeptide,

FC2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein the first FC and the second FC are covalently linked by one or more disulfide bond(s),

wherein FC1 and FC2 do not substantially bind to an Fc-receptor.

There is reported herein an n-mer polypeptide obtained by cleavage of a fusion polypeptide as reported herein obtained by a method according to the method as reported herein.

In one embodiment B is selected from the group of group of cytokines like IL17, IL18 and IL33, of cytokine receptors like IL18R and IL33R, of TNF family members like TNF, TWEAK and TL1a.

In one embodiment FC is a variant of a heavy chain polypeptide selected from the group of human IgG1 heavy chain polypeptide, human IgG2 heavy chain polypeptide, human IgG3 heavy chain polypeptide, human IgG4 heavy chain polypeptide, murine IgG1 heavy chain polypeptide, murine IgG2 heavy chain polypeptide, murine IgG2a heavy chain polypeptide, murine IgG3 heavy chain polypeptide, rabbit IgG heavy chain polypeptide. This includes all naturally occurring allotypes.

In one embodiment the heavy chain Fc-region polypeptide has an amino acid mutation at one or more of position 234, 235, 236, 237, 238, 239, 253, 254, 265, 266, 267, 268, 269, 270, 288, 297, 298, 299, 307, 311, 327, 328, 329, 330, 331, 332, 434, and 435. In one embodiment the one or more of the Fc-receptors is an Fc gamma receptor.

In one embodiment the human IgG1 heavy chain polypeptide has a mutation at one or more of amino acid positions 233, 234, 235, 236, 265, 297, 329, and 331.

In one embodiment the human IgG1 heavy chain polypeptide has one or more of the amino acid mutations E233P, L234A, L235A, L235E, ΔG236, D265A, N297A, N297D, P329A, P329G, and P331S.

In one embodiment the human IgG1 heavy chain polypeptide has the amino acid mutations L234A and L235A and one or more of E233P, L235E, ΔG236, D265A, N297A, N297D, P329A, P329G, and P331S.

In one embodiment the human IgG1 heavy chain polypeptide has the amino acid mutations L234A and L235A and P329A or P329G.

In one embodiment the human IgG2 heavy chain polypeptide has a mutation at one or more of amino acid positions 233, 234, 235, 236, 265, and 329.

In one embodiment the human IgG4 heavy chain polypeptide has a mutation at one or more of amino acid positions 228, 235, 265, and 329.

In one embodiment the human IgG4 heavy chain polypeptide has one or more of the mutations S228P, L235E, D265A, P329A, and P329G.

In one embodiment the human IgG4 heavy chain polypeptide has the mutations S228P and L235E and P329A or P329G.

In one embodiment the heavy chain Fc-region polypeptide has an amino acid mutation at one or more of position 248, 250, 251, 252, 253, 254, 255, 256, 257, 272, 285, 288, 290, 291, 308, 309, 310, 311, 314, 385, 386, 387, 428, 433, 434, 435, and 436. In one embodiment the one or more of the Fc-receptors is an FcRn.

In one embodiment the human IgG heavy chain polypeptide has a mutation at one or more of the amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439, and/or 447.

In one embodiment the human IgG heavy chain polypeptide that has a reduced binding to FcRn has one or more amino acid alterations at the amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439, and/or 447.

In one embodiment the human IgG heavy chain polypeptide that has a reduced binding to FcRn has the amino acid mutations I253A, H310A, and H435A.

In one embodiment u=2 and the first FC comprises the mutation T366W and optionally the mutation S354C and the second FC comprises the mutations T366S, L368A and Y407V and optionally the mutation Y349C.

In one embodiment u=2 and

a) n=1 and m=0 and B fused to the first FC and B fused to the second FC are identical, or

b) n=1 and m=0 and B fused to the first FC and B fused to the second FC are different, or

c) n=0 and m=1 and B fused to the first FC and B fused to the second FC are identical, or

d) n=0 and m=1 and B fused to the first FC and B fused to the second FC are different.

In one embodiment u=1 and the fusion polypeptide comprises a second disulfide-linked FC.

In one embodiment u=1 and the fusion polypeptide comprises a second disulfide-linked FC, wherein one FC comprises the mutation T366W and optionally the mutation S354C and the other FC comprises the mutations T366S, L368A and Y407V and optionally the mutation Y349C.

In one embodiment the method as reported herein is for the production of a polypeptide that is biologically active as 1-mer (monomer) and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region and comprises the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 1-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s) (:),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) optionally cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as 1-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

In one embodiment the method as reported herein is for the production of a polypeptide that is biologically active as 2-mer (dimer) and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region and comprises the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 2-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s) (:),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) optionally cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as 2-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

In one embodiment the method as reported herein is for the production of a polypeptide that is biologically active as 3-mer (trimer) and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region and comprises the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s) (:),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) optionally cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

In one embodiment the intervening amino acid sequence is selected from a first group comprising $(G_3S)_3$, $(G_3S)_4$, $(G_3S)_5$, $(G_3S)_6$, $(G_4S)_3$, $(G_4S)_4$, $(G_4S)_5$, $(G_5S)_2$, $(G_5S)_3$, $(G_5S)_4$, and any combination thereof or from a second group comprising Arg-tag, Avi-tag, His-Avi-tag, His-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag, calmodulin-binding-peptide, cellulose-binding-domain, chitin-binding-domain, GST-tag, or MBP-tag, or from combinations of two elements of these group.

In one embodiment the cleavage site is selected from IgA-protease protease cleavage site, Granzyme B protease cleavage site, Tev protease cleavage site, PreScission protease cleavage site, Thrombin cleavage site, Factor10a protease site, Ides protease cleavage site, Enterokinase cleavage site, or a SUMO protease (Ulp1 (Ubl-specific protease 1) from Saccharomyces cerevisiae) cleavage site.

In one embodiment the fusion polypeptide does not comprise an additional protease cleavage site but an inherent protease cleavage site, such as e.g. a papain cleavage site, a pepsin cleavage site, or an Ides protease cleavage site.

In one embodiment the C-terminal lysine residue in the FC-region(s) has been deleted. This reduces the amount of protease cleavage side-products (removal of artificial protease cleavage site).

There is reported herein the use of an immobilized fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein as affinity chromatography ligand. One aspect as reported herein is the use of an immobilized fusion polypeptide as reported herein as affinity chromatography ligand.

There is reported herein the use of an immobilized fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein in an immunoassay. One aspect as reported herein is the use of an immobilized fusion polypeptide as reported herein in an immunoassay.

In one embodiment the fusion polypeptide or the n-mer polypeptide is bound to a solid phase.

There is reported herein a pharmaceutical composition comprising a fusion polypeptide as reported herein or an n-mer polypeptide as reported herein. One aspect as reported herein is a pharmaceutical composition comprising a fusion polypeptide as reported herein.

There is reported herein the use of a fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein in the manufacture of a medicament.

There is reported herein the use of a fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein as immunogen. One aspect as reported herein is a fusion polypeptide as reported herein for use as an immunogen.

There is reported herein the use of a fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein for obtaining an animal model of a disease by applying the fusion polypeptide as reported herein or the n-mer polypeptide as reported herein to an experimental animal. One aspect as reported herein is the use of a fusion polypeptide as reported herein for obtaining an animal model of a disease by applying the fusion polypeptide as reported herein to an experimental animal.

There is reported herein the use of a fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein for selecting an antibody that specifically binds to B or an n-mer of B. One aspect as reported herein is the use of a fusion polypeptide as reported herein

for selecting an antibody that specifically binds to B or a 2-mer or 3-mer of B.

There is reported herein the use of a fusion polypeptide as reported herein or of an n-mer polypeptide as reported herein for producing a disulfide-linked 2-mer of B. One aspect as reported herein is the use of a fusion polypeptide as reported herein for producing a disulfide-linked 2-mer of B.

Detailed Description of the Invention

Description of the Figures

Figure 1 Plasmid map of Fc-receptor fusion polypeptide expression plasmid.

Figure 2 Analytical SDS-PAGE gel of papain cleavage of Fc-receptor fusion polypeptide.

Figure 3 12% Bis Tris Gel + / -DTT; Cleavage of FcgammaRIIIaV158-Avi-Fc LALA P239G with PreScission protease (lane 4, 8) respectively IgA protease (lane 3, 7): unspecific cleavage with PP can be seen (lane 2, 6).

Figure 4 Separation and quantification of different glycosylation forms of an anti-Her antibody (wild-type, at top) and a glycoengineered anti-Her antibody.

Figure 5 Comparison of affinity column using FcgammaRIIIaV158 and Fc tagged FcgammaRIIIaV158.

Figure 6 BIAcore sensogram of the response of the FcgammaRIIIaV158-Fc LALA P329G fusion polypeptide display more than 100 Response units in comparison to the FcgammaRIIIaV158 with 40 RU; FcgRIIIa V 158_008 denotes the non-cleaved fusion polypeptide, FcgRIIIa V 158_007 denotes a shortened non-functional variant of the FcgRIIIa (=control), FcgRIIIa V 158_jf323 denotes the intact HisAvi-tag comprising functional variant of FcgRIIIa.

Figure 7 Sensograms of Fcgamma receptor V158-Fc LALA P329G fusion polypeptide (Figure 7a), Fcgamma receptor V158 (Figure 7b), cleaved Fcgamma receptor V158-Fc LALA P329G fusion polypeptide (Figure 7c), non-functional Fcgamma receptor V158-Fc LALA P329G fusion polypeptide (Figure 7d) (=control).

Figure 8 Plasmid maps of Fc-TWEAK fusion polypeptide expression plasmids.

Figure 9 Plasmid maps of Fc-IL17A fusion polypeptide expression plasmids.

Figure 10 Titration curves of human TNF alpha variants.

Figure 11 Scheme of the different dimeric Fc IL17 fusion polypeptide setups and states.

Figure 12 Trimeric Fc-region-human TWEAK fusion polypeptide.

Figure 13 Both full-length (1-270) and short (112 – 270) forms of IL-33 are functionally active; IgG-Fc can be added instead of rest (1-112) of IL-33 to get a functionally active IL-33; acts as a monomer; monomeric IgG1-Fc_IL-33.

Figure 14 Trimeric human TNF alpha as IgG1-Fc-KiH_Precision Protease site_shTNFa fusion polypeptide.

Figure 15 Plasmid map of Fc-shTNFalpha fusion polypeptide (knob) expression plasmid.

Figure 16 Plasmid map human IgG1 Fc-region (hole) expression plasmid.

Figure 17 BIAcore data of monomeric IL18R-Fc-fusion polypeptide as reported herein compared to dimeric IL18R-Fc-fusion polypeptide.

Definitions

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

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

The term "CH2 domain" denotes the part of an antibody heavy chain polypeptide that extends approximately from EU position 231 to EU position 340 (EU numbering system according to Kabat). In one embodiment a CH2 domain has the amino acid sequence of SEQ ID NO: 01 (APELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQESTYRWSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAK). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native Fc-region. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, Mol. Immunol. 22 (1985) 161-206.

The term "CH3 domain" denotes the part of an antibody heavy chain polypeptide that extends approximately from EU position 341 to EU position 446. In one embodiment the CH3 domain has the amino acid sequence of SEQ ID NO: 02 (GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPG).

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

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

The formation of the dimeric Fc-region comprising two identical or non-identical antibody heavy chain fragments is mediated by the non-covalent dimerization of the comprised CH3 domains (for involved amino acid residues see e.g. Dall'Acqua, Biochem. 37 (1998) 9266-9273). The Fc-region is covalently stabilized by the formation of disulfide bonds in the hinge region (see e.g. Huber, et al., Nature 264 (1976) 415-420; Thies, et al., J. Mol. Biol. 293 (1999) 67-79). The introduction of amino acid residue changes within the CH3 domain in order to disrupt the dimerization of CH3-CH3 domain interactions do not adversely affect the FcRn binding due to the locations of the CH3-CH3-domain dimerization involved residues are located on the inner interface of the CH3 domain, whereas the residues involved in Fc-region-FcRn interaction are located on the outside of the CH2-CH3 domain.

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

The effector function eliciting receptors are the Fc-receptor types (and sub-types) FcγRI, FcγRII and FcγRIII. The effector functions associated with an IgG1 isotype can be reduced by introducing specific amino acid changes in the lower hinge region, such as L234A and/or L235A, that are involved in FcγR and C1q binding.

Also certain amino acid residues, especially located in the CH2 and/or CH3 domain, are associated with the circulating half-life of an antibody molecule or an Fc-region fusion polypeptide in the blood stream. The circulatory half-life is determined by the binding of the Fc-region to the neonatal Fc-receptor (FcRn).

The numbering of the amino acid residues in the constant region of an antibody is made according to the EU index of Kabat (Kabat et al. 1991, Sequences of Proteins of immunological Interest, U.S. Department of Public Health, Bethesda, Md.).

The term "Fc-region of human origin" denotes the C-terminal region of an immunoglobulin heavy chain of human origin that contains at least a part of the hinge region, the CH2 domain and the CH3 domain. In one embodiment, a human IgG heavy chain Fc-region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc-region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc-region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat, 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.

The term "FcRn binding portion of an Fc-region" denotes the part of an antibody heavy chain polypeptide that extends approximately from EU position 243 to EU position 261 and approximately from EU position 275 to EU position 293 and approximately from EU position 302 to EU position 319 and approximately from EU position 336 to EU position 348 and approximately from EU position 367 to EU position 393 and EU position 408 and approximately from EU position 424 to EU position 440. In one embodiment one or more of the following amino acid residues according to the EU numbering of Kabat are altered F243, P244, P245 P, K246, P247, K248, D249, T250, L251, M252, I253, S254, R255, T256, P257, E258, V259, T260, C261, F275, N276, W277, Y278, V279, D280, V282, E283, V284, H285, N286, A287, K288, T289, K290, P291, R292, E293, V302, V303, S304, V305, L306, T307, V308, L309, H310, Q311, D312, W313, L314, N315, G316, K317, E318, Y319, I336, S337, K338, A339, K340, G341, Q342, P343, R344, E345, P346, Q347, V348, C367, V369, F372, Y373, P374, S375, D376, I377, A378, V379, E380, W381, E382, S383, N384, G385, Q386, P387, E388, N389, Y391, T393, S408, S424, C425, S426, V427, M428, H429, E430, A431, L432, H433, N434, H435, Y436, T437, Q438, K439, and S440 (EU numbering).

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

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 03).

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

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 04).

A polypeptide chain of a variant human Fc-region of the IgG1 isotype with T366S, L368A and Y407V mutations has the following amino acid sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 05).

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

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 06).

A polypeptide chain of a variant human Fc-region of the IgG1 isotype with a L234A, L235A and T366S, L368A and Y407V mutations has the following amino acid sequence:

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 07).

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

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 08).

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

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 09).

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

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 10).

A polypeptide chain of a variant human Fc-region of the IgG1 isotype with a P239G and T366S, L368A and Y407V mutation has the following amino acid sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 11).

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

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 12).

A polypeptide chain of a variant human Fc-region of the IgG1 isotype with a L234A, L235A, P329G and T366S, L368A and Y407V mutation has the following amino acid sequence:

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 13).

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

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 14).

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

ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN
VFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 15).

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

ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN
VFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 16).

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

ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKGLGSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN
VFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 17).

The term "Fc-receptor", short "FcR", denotes a receptor that binds to an Fc-region. In one embodiment the FcR is a native sequence human FcR. Moreover, in one embodiment the FcR is an FcR which binds an IgG antibody (an Fc gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms thereof. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol 9 (1991) 457-492, Capel, et al., Immunomethods 4 (1994) 25-34, de Haas, et al., J. Lab. Clin. Med. 126 (1995) 330-341. Other FcRs are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is

responsible for the transfer of maternal IgGs to the fetus (see e.g. Guyer, et al., J. Immunol. 117 (1976) 587; Kim, et al., J. Immunol. 24 (1994) 249).

The term "Fc gamma receptor", short "FcγR" or "FcgammaR", denote any member of the family of proteins that bind the IgG antibody Fc-region and is encoded by an FcγR gene. In humans this family includes but is not limited to FcγRI (CD64), including isoforms FcγRIA, FcγRIB, and FcγRIC, FcγRII (CD32), including isoforms FcγRIIA (including allotypes H131 and R131), FcγRIIB (including FcγRIIB-1 and FcγRIIB-2), and FcγRIIc, and FcγRIII (CD16), including isoforms FcγRIIIA (including allotypes V158 and F158; Swiss-Prot entry P08637; N-terminus-
MRTEDLPKAVVFLEPQWYRVLEKDSVTLKCQGAYSPEDNSTQWFHNESLI
SSQASSYFIDAATVDDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPRWVF
KEEDPIHLRCHSWKNTALHKVTYLQNGKGRKYFHHNSDFYIPKATLKDSG
SYFCRGLVGSKNVSSETVNITITQGLAVSTISSFFPPGYQ-C-terminus; SEQ ID NO: 18) and FcγRIIIb (including allotypes FcγRIIB-NA1 and FcγRIIB-NA2) (see e.g. Jefferis et al., Immunol. Lett. 82 (2002) 57-65, entirely incorporated by reference), as well as FcγR isoforms or allotypes. An FcγR may be from any organism, including but not limited to humans, mice, rats, rabbits, and monkeys. Mouse FcγRs include but are not limited to FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIII-2 (CD16-2), as well as or FcγR isoforms or allotypes. The Fc-region-FcγR interaction involved amino acid residues are 234-239 (lower hinge region), 265-269 (B/C loop), 297-299 (D/E loop), and 327-332 (F/G) loop (Sondermann, et al., Nature 406 (2000) 267-273). Amino acid mutations that result in a decreased binding/affinity for the FcγRI, FcγRIIA, FcγRIIB, and/or FcγRIIIA include N297A (concomitantly with a decreased immunogenicity and prolonged half-life binding/affinity) (Routledge, et al., Transplantation 60 (1995) 847; Friend et al., Transplantation 68 (1999) 1632; Shields et al., J. Biol. Chem. 276 (1995) 6591), residues 233-236 (Ward and Ghetie, Ther. Immunol. 2 (1995) 77; Armour et al., Eur. J. Immunol. 29 (1999) 2613). Some exemplary amino acid substitutions are described in US 7,355,008 and US 7,381,408.

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

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

The amino acid residues of the FcRn that are conserved across species are the histidine residues at position 310 and 435 in the Fc-region. These residues are responsible for the pH dependence of the Fc-region FcRn interaction (see, e.g., Victor, G., et al., Nature Biotechnol. 15 (1997) 637-640); Dall'Acqua, W. F., et al. J. Immunol. 169 (2002) 5171-5180). Fc-region mutations that attenuate interaction with FcRn can reduce antibody half-life.

The term "hinge region" denotes the part of an antibody heavy chain polypeptide that joins the CH1 domain and the CH2 domain, e. g. from about position 216 to position about 230 according to the EU number system of Kabat. The hinge region is normally a dimeric molecule consisting of two polypeptides with identical amino acid sequence. The hinge region generally comprises about 25 amino acid residues and is flexible allowing the antigen binding regions to move independently. The hinge region can be subdivided into three domains: the upper, the middle, and the lower hinge domain (Roux, et al., J. Immunol. 161 (1998) 4083).

The hinge region is normally a dimeric molecule consisting of two polypeptides with identical amino acid sequence. The hinge region generally comprises about 25 amino acid residues and is flexible allowing the antigen binding regions to move independently. The hinge region can be subdivided into three domains: the upper, the middle, and the lower hinge domain (see e.g. Roux, et al., J. Immunol. 161 (1998) 4083).

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

The term “wild-type Fc-region” denotes an amino acid sequence identical to the amino acid sequence of an Fc-region found in nature. Wild-type human Fc-regions

include a native human IgG1 Fc-region (non-A and A allotypes), native human IgG2 Fc-region, native human IgG3 Fc-region, and native human IgG4 Fc-region as well as naturally occurring variants thereof.

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

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

The term "polypeptide" denotes a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as "proteins". A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "amino acid sequence tag" denotes a sequence of amino acid residues connected to each other via peptide bonds that has specific binding properties. In one embodiment the amino acid sequence tag is an affinity or purification tag. In one embodiment the amino acid sequence tag is selected from the group comprising Arg-tag, His-tag, Avi-tag, His-Avi-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag, calmodulin-binding-peptide, cellulose-binding-domain, chitin-binding-domain, GST-tag, and MBP-tag. In one embodiment the amino acid sequence tag is selected from the group comprising SEQ ID NO: 19 (RRRRR), SEQ ID NO: 20 (RRRRRR), SEQ ID NO: 21 (Avi-tag), SEQ ID NO: 22 (His-Avi-tag), SEQ ID NO: 23 (HHHHHH), SEQ ID NO: 24 (KDHLIHNVHKEFHAHAHNK), SEQ ID NO: 25 (DYKDDDDK), SEQ ID NO: 26 (DYKDHDGDYKDHDIDYKDDDDK), SEQ ID NO: 27 (AWRHPQFGG),

SEQ ID NO: 28 (WSHPQFEK), SEQ ID NO: 29 (MDVEAWLGAR), SEQ ID NO: 30 (MDVEAWLGARVPLVET), SEQ ID NO: 31 (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP), SEQ ID NO: 32 (EQKLISEEDL), SEQ ID NO: 33 (KETAAAKFERQHMDS), SEQ ID NO: 34 (KRRWKKNFIAVSAANRFKKISSSGAL), SEQ ID NO: 35 (cellulose binding domain), SEQ ID NO: 36 (cellulose binding domain), SEQ ID NO: 37 (TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEP SNVPALWQLQ), SEQ ID NO: 38 (GST-tag), and SEQ ID NO: 39 (MBP-tag).

The term "enzymatic cleavage site" denotes a sequence of amino acid residues connected to each other via peptides bonds that can specifically be cleaved by a protease. In one embodiment the protease is IgA-protease, Granzyme B, Tev protease, PreScission protease, Thrombin, Faktor10a, Ides protease, or Enterokinase.

The term "IgA-protease" denotes a protease derived from Neisseria gonorrhoeae with a recognition site comprising one of the following sequences wherein "↓" denotes the position of the cleaved bond:

Pro-Ala-Pro ↓ Ser-Pro	(SEQ ID NO: 40),
Pro-Pro ↓ Ser-Pro	(SEQ ID NO: 41),
Pro-Pro ↓ Ala-Pro	(SEQ ID NO: 42),
Pro-Pro ↓ Thr-Pro	(SEQ ID NO: 43),
Pro-Pro ↓ Gly-Pro	(SEQ ID NO: 44),
Pro-Arg-Pro-Pro ↓ Thr-Pro	(SEQ ID NO: 45),
Val-Val-Ala-Pro-Pro ↓ Ala-Pro	(SEQ ID NO: 46),
Val-Val-Ala-Pro-Pro ↓ Ser-Pro	(SEQ ID NO: 47),
Val-Val-Ala-Pro-Pro ↓ Thr-Pro	(SEQ ID NO: 48),
Val-Val-Ala-Pro-Pro ↓ Gly-Pro	(SEQ ID NO: 49),
Pro-Arg-Pro-Pro ↓ Thr-Pro	(SEQ ID NO: 50),
Ala-Pro-Pro-Ala ↓ Ala-Pro	(SEQ ID NO: 51),
Pro-Arg-Pro-Pro ↓ Ala-Pro	(SEQ ID NO: 52),
Pro-Arg-Pro-Pro ↓ Ser-Pro	(SEQ ID NO: 53),
Pro-Arg-Pro-Pro ↓ Gly-Pro	(SEQ ID NO: 54).

The term "linker" or "peptidic linker" as used within this application denotes peptide linkers of natural and/or synthetic origin. They consist of a linear amino acid chain wherein the 20 naturally occurring amino acids are the monomeric building blocks. The chain has a length of from 1 to 50 amino acids, preferred

between 1 and 28 amino acids, especially preferred between 3 and 25 amino acids. The linker may contain repetitive amino acid sequences or sequences of naturally occurring polypeptides, such as polypeptides with a hinge-function. The linker has the function to ensure that a peptide conjugated to an anti-CD4 antibody can perform its biological activity by allowing the peptide to fold correctly and to be presented properly. Preferably the linker is a "synthetic peptidic linker" that is designated to be rich in glycine, glutamine, and/or serine residues. These residues are arranged e.g. in small repetitive units of up to five amino acids, such as GGGGS, QQQQG, or SSSSG. This small repetitive unit may be repeated for two to five times to form a multimeric unit. At the amino- and/or carboxy-terminal ends of the multimeric unit up to six additional arbitrary, naturally occurring amino acids may be added. Other synthetic peptidic linkers are composed of a single amino acid that is repeated between 10 to 20 times and may comprise at the amino- and/or carboxy-terminal end up to six additional arbitrary, naturally occurring amino acids. All peptidic linkers can be encoded by a nucleic acid molecule and therefore can be recombinantly expressed.

Fusion polypeptide as reported herein

Often the expression of polypeptides as Fc-region fusion polypeptide results in the formation of multimeric aggregates of non-defined composition. Also expressing the Fc-region fusion polypeptide is difficult due to steric hindrance between the two chains.

It has been found that polypeptides, which are biologically active as n-mer and form non-defined aggregates/multimers upon expression, can be obtained in soluble form by expressing these polypeptides as fusion polypeptide with an Fc-region that does not substantially bind to an Fc-receptor. Using a fusion polypeptide for the expression of the polypeptide increases the obtainable yield of the polypeptide either in form of the fusion polypeptide or as isolated polypeptide. It has further been found that the polypeptide remains in a defined n-mer form after cleavage and removal of the Fc-region. Especially suited are Fc-regions that do not substantially bind to an Fc-receptor.

The term "do not substantially bind to an Fc-receptor" denotes that the Fc-region comprised in the fusion polypeptide does not bind to an Fc-receptor to such an extent that aggregates are formed.

The polypeptide can be fused to the Fc-region either directly, or via a peptidic linker, or via a protease cleavage site, or via a peptidic linker combined with a protease cleavage site.

It has been found that an asymmetric dimeric fusion polypeptide can be obtained which comprises an enzymatic cleavage site in one fusion polypeptide whereas the second fusion polypeptide does not comprise an enzymatic cleavage site. Thus, in one embodiment the dimeric fusion polypeptide comprises two identical fusion polypeptides except that only one fusion polypeptide comprises an enzymatic cleavage site between the biologically active polypeptide and the Fc-region.

By expressing the biologically active polypeptide as fusion polypeptide as reported herein the obtainable expression yield can be increased or the large scale production of the biologically active polypeptide can be achieved at all.

Likewise it is possible by using a fusion polypeptide as reported herein to improve the pharmacokinetic properties of the biologically active polypeptide.

Likewise it is possible by using a fusion polypeptide as reported herein to provide soluble cytokines and cytokine receptors.

It is possible to provide cytokine receptors in biologically active form by using a fusion polypeptide as reported herein. These cytokine receptors are not biologically active in cleaved form.

By using the fusion polypeptide as reported herein it is possible to provide functional, biologically active and soluble membrane receptors and/or domains.

By using a dimeric fusion polypeptide as reported herein it is possible to force the disulfide based dimerization of cytokines prior to the cleavage of the fusion polypeptide and liberation of the dimeric cytokine.

Likewise it is possible by using the dimeric fusion polypeptide as reported herein to provide heterodimeric, disulfide-linked cytokines by forced disulfide linkage.

One aspect as reported herein is a fusion polypeptide according to formula I

$$(B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m)_u \qquad \text{(formula I)}$$

wherein B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC denotes a heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein u=1 or 2,

wherein FC does not substantially bind to an Fc-receptor.

One aspect as reported herein is a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein the first FC and the second FC are covalently linked by one or more disulfide bond(s),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor.

The polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region can be from any species including but not limited to human, mouse, rat, rabbit, and monkey.

In one embodiment the polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region (=B in formula I or formula II) is selected from the group of IL17, IL18, IL33, IL18R, IL33R, TNF, TWEAK, and TL1a.

In one embodiment FC is a variant of a heavy chain polypeptide selected from the group of human IgG1 heavy chain polypeptide, human IgG2 heavy chain polypeptide, human IgG3 heavy chain polypeptide, human IgG4 heavy chain polypeptide, murine IgG1 heavy chain polypeptide, murine IgG2 heavy chain polypeptide, murine IgG2a heavy chain polypeptide, murine IgG3 heavy chain polypeptide, rabbit IgG heavy chain polypeptide.

The Fc-region comprised in the fusion polypeptide as reported herein shall not substantially bind to an Fc-receptor.

In one embodiment the fusion polypeptide possesses substantially no effector functions, which make it a desirable candidate for applications in which certain effector functions are unnecessary or deleterious. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of effector functions. For example, Fc-receptor (FcR) binding assays can be conducted to ensure that the fusion polypeptide lacks FcγR binding (hence likely lacking ADCC activity). The primary cells for mediating ADCC, NK cells, express FcγRIII 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 U.S. Patent No. 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); U.S. Patent No. 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. 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).

The affinities and binding properties of an Fc-region for its ligand, may be determined by a variety of in vitro assay methods (biochemical or immunological based assays) known in the art for determining Fc-region/FcR interactions, i.e., specific binding of an Fc-region to an FcγR including but not limited to, equilibrium methods (e.g. enzyme-linked immuno absorbent assay (ELISA) or radioimmunoassay (RIA)), or kinetics (e.g. BIACORE® analysis), and other methods such as indirect binding assays, competitive inhibition assays, fluorescence resonance energy transfer (FRET), gel electrophoresis and chromatography (e.g., gel filtration). These and other methods may utilize a label on one or more of the components being examined and/or employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels. A detailed description of binding affinities and kinetics can be found in Paul, W.E., ed., Fundamental Immunology, 4th Ed., Lippincott-Raven, Philadelphia (1999).

In one embodiment FC is, or FC^1 and FC^2 independently of each other are either an Fc-region of human origin of the subclass IgG4 or an Fc-region of human origin of the subclass IgG1, IgG2, or IgG3, which is modified in such a way that no Fcγ receptor (e.g. FcγRIIIa) binding. In one embodiment FC is, or FC^1 and FC^2 independently of each other are an Fc-region of human origin, especially either from human IgG4 subclass or a mutated Fc-region from human IgG1 subclass. In one embodiment FC is, or FC^1 and FC^2 independently of each other are of human IgG1 subclass with mutations L234A and L235A. In one embodiment FC is, or FC^1 and FC^2 independently of each other are of human IgG4 subclass with mutation S228P. While IgG4 shows reduced Fcγ receptor (FcγRIIIa) binding, antibodies of other IgG subclasses show strong binding. However Pro238, Asp265, Asp270,

Asn297 (loss of Fc carbohydrate), Pro329, Leu234, Leu235, Gly236, Gly237, Ile253, Ser254, Lys288, Thr307, Gln311, Asn434, or/and His435 are residues which, if altered, provide also reduced Fcγ receptor binding (Shields, R.L., et al., J. Biol. Chem. 276 (2001) 6591-6604; Lund, J., et al., FASEB J. 9 (1995) 115-119; Morgan, A., et al., Immunology 86 (1995) 319-324; EP 0 307 434). In one embodiment FC is, or FC^1 and FC^2 independently of each other are in regard to Fcγ receptor binding of IgG4 subclass, or of IgG1 or IgG2 subclass, with a mutation in L234, L235, and/or D265, and/or contains the PVA236 mutation. In one embodiment FC comprises, or FC^1 and FC^2 independently of each other comprise one or more of the mutations S228P, L234A, L235A, L235E, and/or PVA236 (PVA236 means that the amino acid sequence ELLG (given in one letter amino acid code) from amino acid position 233 to 236 of IgG1 or EFLG of IgG4 is replaced by PVA). In one embodiment the mutations are S228P for IgG4, and L234A and L235A for IgG1.

Fc-region binding sites are known in the state of the art and described e.g. by Lukas, T.J., et al., J. Immunol. 127 (1981) 2555-2560; Brunhouse, R. and Cebra, J.J., Mol. Immunol. 16 (1979) 907-917; Burton, D.R., et al., Nature 288 (1980) 338-344; Thommesen, J.E., et al., Mol. Immunol. 37 (2000) 995-1004; Idusogie, E.E., et al., J. Immunol. 164 (2000) 4178-4184; Hezareh, M., et al., J. Virol. 75 (2001) 12161-12168; Morgan, A., et al., Immunology 86 (1995) 319-324; and EP 0 307 434. Fc-region binding sites are, e.g., characterized by the amino acids L234, L235, D270, N297, E318, K320, K322, P331, and P329 (numbering according to EU index of Kabat).

Fc-regions with reduced effector function include those with substitution of one or more of Fc-region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DA" Fc mutant with substitution of residue 265 and the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

Certain Fc-region 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 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 Patent No. 6,194,551, WO 99/51642, and Idusogie, E.E. et al., J. Immunol. 164 (2000) 4178-4184.

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.

In one embodiment the heavy chain Fc-region polypeptide has an amino acid mutation at one or more of position 234, 235, 236, 237, 238, 239, 253, 254, 265, 266, 267, 268, 269, 270, 288, 297, 298, 299, 307, 311, 327, 328, 329, 330, 331, 332, 434, and 435. In one embodiment the one or more of the Fc-receptors is an Fc gamma receptor.

In one embodiment the human IgG1 heavy chain polypeptide has a mutation at one or more of amino acid positions 233, 234, 235, 236, 265, 297, 329, and 331.

In one embodiment the human IgG1 heavy chain polypeptide has one or more of the amino acid mutations E233P, L234A, L235A, L235E, ΔG236, D265A, N297A, N297D, P329A, P329G, and P331S.

In one embodiment the human IgG1 heavy chain polypeptide has the amino acid mutations L234A and L235A and one or more of E233P, L235E, ΔG236, D265A, N297A, N297D, P329A, P329G, and P331S.

In one embodiment the human IgG1 heavy chain polypeptide has the amino acid mutations L234A and L235A and P329A or P329G.

In one embodiment the human IgG2 heavy chain polypeptide has a mutation at one or more of amino acid positions 233, 234, 235, 236, 265, and 329.

In one embodiment the human IgG4 heavy chain polypeptide has a mutation at one or more of amino acid positions 228, 235, 265, and 329.

In one embodiment the human IgG4 heavy chain polypeptide has one or more of the mutations S228P, L235E, P329A, and P329G.

In one embodiment the human IgG4 heavy chain polypeptide has the mutations S228P and L235E and P329A or P329G.

In one embodiment the heavy chain Fc-region polypeptide has an amino acid mutation at one or more of position 248, 250, 251, 252, 253, 254, 255, 256, 257,

272, 285, 288, 290, 291, 308, 309, 310, 311, 314, 385, 386, 387, 428, 433, 434, 435, and 436. In one embodiment the one or more of the Fc-receptors is an FcRn.

In one embodiment the human IgG heavy chain polypeptide has a mutation at one or more of the amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439, and/or 447.

In one embodiment the human IgG heavy chain polypeptide that has a reduced binding to FcRn has one or more amino acid alterations at the amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439, and/or 447.

The fusion polypeptide can comprise between the biologically active polypeptide and the Fc-region a linker polypeptide. This linker polypeptide can be used to adjust the distance between the biologically active polypeptide and the Fc-region to allow both regions to function in the intended way.

In one embodiment the linker polypeptide is selected from the group comprising $(G_3S)_3$, $(G_3S)_4$, $(G_3S)_5$, $(G_3S)_6$, $(G_4S)_3$, $(G_4S)_4$, $(G_4S)_5$, $(G_5S)_2$, $(G_5S)_3$, $(G_5S)_4$, and any combination thereof.

Additionally, the fusion polypeptide can comprise between the biologically active polypeptide and the Fc-region a tag, e.g. suitable for affinity purification or immobilization.

In one embodiment the tag is selected from a the group comprising Arg-tag, Avi-tag, His-Avi-tag, His-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag, calmodulin-binding-peptide, cellulose-binding-domain, chitin-binding-domain, GST-tag, MBP-tag, streptavidin or avidin, biotin, lectin, polysaccharide, steroid, steroid binding protein, hormone, and hormone receptor.

The linker polypeptide and the tag can be combined in an intervening amino acid sequence that is located between the biologically active polypeptide and the Fc-region.

In one embodiment the intervening amino acid sequence is selected from a first group comprising $(G_3S)_3$, $(G_3S)_4$, $(G_3S)_5$, $(G_3S)_6$, $(G_4S)_3$, $(G_4S)_4$, $(G_4S)_5$, $(G_5S)_2$, $(G_5S)_3$, $(G_5S)_4$, and any combination thereof, or from a second group comprising Arg-tag, Avi-tag, His-Avi-tag, His-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag, calmodulin-binding-peptide, cellulose-binding-domain,

chitin-binding-domain, GST-tag, or MBP-tag, or from combinations of two elements of these group.

The intervening amino acid sequence can be located either before or after a cleavage site in the fusion polypeptide.

In one embodiment the cleavage site is an enzymatic cleavage site. In one embodiment the enzymatic cleavage site is selected from the group comprising IgA-protease protease cleavage site, Granzyme B protease cleavage site, Tev protease cleavage site, PreScission protease cleavage site, Thrombin cleavage site, Factor10a protease site, Ides protease cleavage site, SUMO protease (Ulp1 (Ubl-specific protease 1) from Saccharomyces cerevisiae) cleavage site and Enterokinase cleavage site. In one embodiment the cleavage site is selected from the group of IgA protease cleavage site, PreScission protease cleavage site, granzyme B cleavage site, and Ides protease cleavage site.

In one embodiment the fusion polypeptide comprises an inherent cleavage site of the protease papain, or the protease pepsin, or the Ides protease.

One aspect as reported herein is a dimeric fusion polypeptide comprising two fusion polypeptides as reported herein.

One aspect as reported herein is a dimeric polypeptide comprising one fusion polypeptide as reported herein and an Fc-region.

As the fusion polypeptide as reported herein comprises an Fc-region which in turn comprises an immunoglobulin hinge region the dimeric fusion polypeptide comprises one or more disulfide bridges covalently linking the first fusion polypeptide with the second fusion polypeptide.

As the fusion polypeptide as reported herein comprises an Fc-region which in turn comprises an immunoglobulin hinge region the dimeric polypeptide comprises one or more disulfide bridges covalently linking the fusion polypeptide with the Fc-region.

The dimeric fusion polypeptide can be a homodimeric fusion polypeptide or a heterodimeric fusion polypeptide.

One aspect as reported herein is a fusion polypeptide according to formula I

$$(B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m)_u \qquad \text{(formula I)}$$

wherein B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC denotes a heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein u=2,

wherein FC does not substantially bind to an Fc-receptor.

One aspect as reported herein is a dimeric polypeptide comprising a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 1-mer, 2-mer, or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor.

As the dimeric polypeptide can be formed by the combination of two different polypeptides, or a polypeptide and a fusion polypeptide as reported herein, or two different fusion polypeptides as reported herein a mechanism to ensure the heterodimerization has to be used.

In one embodiment the first FC comprises the mutation T366W and optionally the mutation S354C and the second FC comprises the mutations T366S, L368A and Y407V and optionally the mutation Y349C.

In one embodiment the fusion polypeptide is characterized in that

a) B of the first and second fusion polypeptide are identical, or

b) B of the first fusion polypeptide and B of the second fusion polypeptide are different.

In one embodiment the C-terminal lysine residue in the FC-region(s) has been deleted. This results in the deletion of a putative enzymatic cleavage site.

Applications of the fusion polypeptide as reported herein

One aspect as reported herein is the use of an immobilized fusion polypeptide as reported herein or an immobilized dimeric fusion polypeptide as reported herein as affinity chromatography ligand.

In one embodiment the fusion polypeptide or the dimeric fusion polypeptide is bound to a solid phase. In one embodiment the solid phase is a chromatography material. In one embodiment the fusion polypeptide or the dimeric fusion polypeptide is biotinylated and the solid phase is derivatized with streptavidin.

In one embodiment the fusion polypeptide or the dimeric fusion polypeptide comprises a cleavage site between the Fc-receptor and the Fc-region. In one embodiment the fusion polypeptide or the dimeric fusion polypeptide is cleaved prior to biotinylation.

In one embodiment the fusion polypeptide or the dimeric fusion polypeptide comprises an immobilization tag between the biologically active polypeptide and the cleavage site. In one embodiment the immobilization tag is a His-Avi-tag or an Avi-tag.

Also reported is an affinity chromatography column that comprises a matrix and matrix bound chromatographical functional groups, characterized in that the matrix bound chromatographical functional group comprises a fusion polypeptide or a dimeric fusion polypeptide as reported herein.

In one embodiment the fusion polypeptide or the dimeric fusion polypeptide comprises a cleavage site between the biologically active polypeptide and the Fc-region. In one embodiment the fusion polypeptide or the dimeric fusion polypeptide is cleaved prior to biotinylation.

In one embodiment the fusion polypeptide or the dimeric fusion polypeptide comprises an immobilization tag between the biologically active polypeptide and the cleavage site. In one embodiment the immobilization tag is a His-Avi-tag or an Avi-tag.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for the production of a 1-meric (monomeric), 2-meric (dimeric), or 3-meric (trimeric) biologically active polypeptide, whereby the 1-meric (monomeric), 2-meric (dimeric), or 3-meric (trimeric) biologically active polypeptide is obtained after the enzymatic cleavage of the fusion polypeptide, and whereby the 1-meric (monomeric), 2-meric (dimeric), or 3-meric (trimeric) biologically active polypeptide remains in the 1-meric (monomeric), 2-meric (dimeric), or 3-meric (trimeric) form after the cleavage.

One aspect as reported herein is the use of a dimeric fusion polypeptide of formula III

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : B_n\text{-}I_s\text{-}FC^2\text{-}I_t\text{-}B_m$$

(formula III)

wherein

B denotes a polypeptide that is biologically active as 2-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

for the production of bulky (sterically hindering) dimeric cytokines.

It has been found that an asymmetric dimeric fusion polypeptide can be obtained which comprises an enzymatic cleavage site in one fusion polypeptide whereas the second fusion polypeptide does not comprise an enzymatic cleavage site. Thus, in one embodiment the dimeric fusion polypeptide comprises two identical fusion polypeptides except that only one fusion polypeptide comprises an enzymatic cleavage site between the biologically active polypeptide and the Fc-region.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for increasing the in vivo half-life of a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for providing a soluble cytokine or cytokine receptor.

It is possible to provide cytokine receptors in biologically active form by using a fusion polypeptide as reported herein. These cytokine receptors are not biologically active in cleaved form.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for providing functional, biologically active and soluble membrane receptors and/or membrane receptor domains.

One aspect as reported herein is the use of a dimeric fusion polypeptide as reported herein for providing disulfide-linked dimeric cytokines, whereby the disulfide-bond is formed prior to the enzymatic cleavage of the fusion polypeptide.

By using a dimeric fusion polypeptide as reported herein it is possible to force the disulfide based dimerization of cytokines prior to the cleavage of the fusion polypeptide and liberation of the dimeric cytokine.

One aspect as reported herein is the use of a dimeric fusion polypeptide as reported herein for producing a heterodimeric, disulfide-linked cytokine.

Likewise it is possible by using the dimeric fusion polypeptide as reported herein to provide heterodimeric, disulfide-linked cytokines by forced disulfide linkage.

Recombinant Methods

One aspect as reported herein is a method for the production of a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region comprising the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide as reported herein,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) optionally cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

Methods and techniques known to a person skilled in the art, which are useful for carrying out the current invention, are described e.g. in Ausubel, F.M., ed., Current

Protocols in Molecular Biology, Volumes I to III (1997), Wiley and Sons; Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

The nucleic acid encoding the fusion polypeptide as reported herein or the dimeric fusion polypeptide as reported herein can be expressed in a host cell. After recombinant expression the fusion polypeptide or the dimeric fusion polypeptide can be purified by methods known to a person skilled in the art. These methods are well established and widespread used for immunoglobulin purification and are employed either alone or in combination. Such methods are, for example, affinity chromatography using microbial-derived proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange chromatography), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and preparative electrophoretic methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102).

Expression cassettes comprise a promoter, a DNA segment encoding a secretion signal sequence, the structural gene, and a terminator/polyadenylation signal. The elements are assembled in an operatively linked form either on one plasmid encoding all required different fusion polypeptides, or on two or more plasmids each encoding one fusion polypeptide. For the expression of the structural genes the plasmid(s) is (are) introduced into a suitable host cell. Proteins are produced in mammalian cells such as CHO cells, NS0 cells, Sp2/0 cells, COS cells, HEK cells, K562 cells, BHK cells, PER.C6® cells, and the like. In one embodiment the fusion polypeptide is expressed in a CHO cell, or a BHK cell, or a HEK cell. The regulatory elements of the plasmid have to be selected in a way that they are functional in the selected host cell. The expressed fusion polypeptides are functionally assembled.

An "expression plasmid" is a nucleic acid providing all required elements for the expression of the comprised structural gene(s) in a host cell. Typically, an expression plasmid comprises a prokaryotic plasmid propagation unit, e.g. for E. coli, comprising an origin of replication, and a selectable marker, an eukaryotic

selection marker, and one or more expression cassettes for the expression of the structural gene(s) of interest each comprising a promoter, a structural gene, and a transcription terminator including a polyadenylation signal. Gene expression is usually placed under the control of a promoter, and such a structural gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

Antibodies may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding a fusion polypeptide described herein or a dimeric fusion polypeptide described herein is provided. In one 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 a first fusion polypeptide and an amino acid sequence comprising a second fusion polypeptide, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising a first fusion polypeptide and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising a second fusion polypeptide. 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 a fusion polypeptide or a dimeric fusion polypeptide is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the fusion polypeptide or dimeric fusion polypeptide, as provided above, under conditions suitable for expression of the fusion polypeptide or dimeric fusion polypeptide, and optionally recovering the fusion polypeptide or the dimeric fusion polypeptide from the host cell (or host cell culture medium).

For recombinant production of a fusion polypeptide as reported herein or a dimeric fusion polypeptide as reported herein, nucleic acid encoding a fusion polypeptide or a dimeric fusion polypeptide, 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 fusion polypeptide).

Suitable host cells for cloning or expression of fusion polypeptide-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, fusion polypeptides 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 fusion polypeptide or the dimeric fusion polypeptide may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for fusion polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of a fusion polypeptide 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 fusion polypeptide 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 Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants)).

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

Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the fusion polypeptides or dimeric fusion polypeptides provided herein is useful for detecting the presence of cytokines or cytokine receptors in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection.

In one embodiment, a fusion polypeptide as reported herein or a dimeric fusion polypeptide as reported herein for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of a cytokine or a cytokine receptor in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with a fusion polypeptide as reported herein or a dimeric fusion polypeptide as reported herein under conditions permissive for binding of the fusion polypeptide or the dimeric fusion polypeptide to a cytokine or cytokine receptor, and detecting whether a complex is formed between the fusion polypeptide or the dimeric fusion polypeptide and a cytokine or a cytokine receptor. Such method may be an in vitro or in vivo method.

In certain embodiments, labeled fusion polypeptides or labeled dimeric fusion polypeptides are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme,

saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Pharmaceutical compositions

Pharmaceutical compositions of a fusion polypeptide as reported herein or a dimeric fusion polypeptide as reported herein are prepared by mixing such fusion polypeptide 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. 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 fusion polypeptide, 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.

Therapeutic Methods and Compositions

Any of the fusion polypeptides as reported herein or the dimeric fusion polypeptides as reported herein may be used in therapeutic methods.

In one aspect, a fusion polypeptide or a dimeric fusion polypeptide for use as a medicament is provided. In certain embodiments, a fusion polypeptide or a dimeric fusion polypeptide for use in a method of treatment is provided. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent. 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 fusion polypeptide or a dimeric fusion polypeptide in the manufacture or preparation of a medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides pharmaceutical compositions comprising any of the fusion polypeptides as reported herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical composition comprises any of the fusion polypeptides as reported herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises any of the fusion polypeptides as reported herein and at least one additional therapeutic agent.

Fusion polypeptides of the invention can be used either alone or in combination with other agents in a therapy. For instance, a fusion polypeptide of the invention may be co-administered with at least one additional therapeutic agent.

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 fusion polypeptide of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant.

A fusion polypeptide of the invention or a dimeric fusion polypeptide 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.

Fusion polypeptides of the invention or dimeric fusion polypeptides 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 fusion polypeptide or the dimeric fusion polypeptide 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 fusion polypeptide or the dimeric fusion polypeptide 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 a fusion polypeptide of the invention or the dimeric fusion polypeptide 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 fusion polypeptide or dimeric fusion polypeptide, the severity and course of the disease, whether the fusion polypeptide or the dimeric fusion polypeptide is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the fusion polypeptide or the dimeric fusion polypeptide, and the discretion of the attending physician. The fusion polypeptide or the dimeric fusion polypeptide 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 fusion polypeptide or dimeric fusion polypeptide 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 fusion polypeptide or the dimeric fusion polypeptide 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 fusion polypeptide or the dimeric fusion polypeptide). 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 fusion polypeptide or a dimeric fusion polypeptide.

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 a fusion polypeptide of the invention or a dimeric fusion polypeptide 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 a fusion polypeptide of the invention or a dimeric fusion polypeptide of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

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

The following examples, figures and sequences are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Examples

Example 1

Generation of the expression plasmids for expression of Fc-receptor containing fusion polypeptide

a) Generation of the expression plasmid for an FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide

The FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide encoding gene was assembled by fusing chemically synthesized DNA fragments coding i) for a murine immunoglobulin heavy chain signal sequence (MGWSCIILFLVATATGVHS: SEQ ID NO: 55), ii) a human Fc gamma receptor IIIa V158 from amino acid residues 2-193 (i.e. excluding the starting methionine), and iii) a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3) with the mutations L234A, L235A and P329G.

The expression plasmid for the transient expression of an FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide in HEK293 cells comprised besides the FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide encoding gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,

- a soluble human Fc gamma receptor III V158 polypeptide from amino acid position 2-193 of the wild-type human Fc gamma receptor III V158 protein,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3, LALA P329G), and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide is:

GMRTEDLPKA VVFLEPQWYR VLEKDSVTLK CQGAYSPEDN STQWFHNESL
ISSQASSYFI DAATVDDSGE YRCQTNLSTL SDPVQLEVHI GWLLLQAPRW
VFKEEDPIHL RCHSWKNTAL HKVTYLQNGK GRKYFHHNSD FYIPKATLKD
SGSYFCRGLV GSKNVSSETV NITITQGLAV STISSFFPPG YQGLNDIFEA
QKIEWHELVV APPAPEDKTH TCPPCPAPEA AGGPSVFLFP PKPKDTLMIS
RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS
VLTVLHQDWL NGKEYKCKVS NKALGAPIEK TISKAKGQPR EPQVYTLPPS
RDELTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF
FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK

(SEQ ID NO: 56).

The following fusion polypeptides can be obtained analogously:

- FcgammaRIIa-LR(H131)-Avi-IgA Protease-Fc LALA P239G fusion polypeptide:

QAAAPPKAVL KLEPPWINVL QEDSVTLTCQ GARSPESDSI QWFHNGNLIP
THTQPSYRFK ANNNDSGEYT CQTGQTSLSD PVHLTVLSEW LVLQTPHLEF
QEGETIMLRC HSWKDKPLVK VTFFQNGKSQ KFSHLDPTFS IPQANHSHSG
DYHCTGNIGY TLFSSKPVTI TVQVPSMGSS SPMGIGLNDI FEAQKIEWHE
LVVAPPAPED KTHTCPPCPA PEAAGGPSVF LFPPKPKDTL MISRTPEVTC
VVVDVSHEDP EVKFNWYVDG VEVHNAKTKP REEQYNSTYR VVSVLTVLHQ
DWLNGKEYKC KVSNKALGAP IEKTISKAKG QPREPQVYTL PPSRDELTKN
QVSLTCLVKG FYPSDIAVEW ESNGQPENNY KTTPPVLDSD GSFFLYSKLT
VDKSRWQQGN VFSCSVMHEA LHNHYTQKSL SLSPGK

(SEQ ID NO: 57).

- FcgammaRIIb-Avi-IgA Protease-Fc LALA P239G fusion polypeptide:

APPKAVLKLE PQWINVLQED SVTLTCRGTH SPESDSIQWF HNGNLIPTHT
QPSYRFKANN NDSGEYTCQT GQTSLSDPVH LTVLSEWLVL QTPHLEFQEG
ETIVLRCHSW KDKPLVKVTF FQNGKSKKFS RSDPNFSIPQ ANHSHSGDYH
CTGNIGYTLY SSKPVTITVQ APGLNDIFEA QKIEWHELVV APPAPEDKTH
TCPPCPAPEA AGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK
FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS

NKALGAPIEK TISKAKGQPR EPQVYTLPPS RDELTKNQVS LTCLVKGFYP
SDIAVEWESN GQPENNYKTT PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS
CSVMHEALHN HYTQKSLSLS PGK

(SEQ ID NO: 58).

- FcgammaRIIIb-Avi-IgA Protease-Fc LALA P239G fusion polypeptide:

GMRTEDLPKA VVFLEPQWYS VLEKDSVTLK CQGAYSPEDN STQWFHNESL
ISSQASSYFI DAATVNDSGE YRCQTNLSTL SDPVQLEVHI GWLLLQAPRW
VFKEEDPIHL RCHSWKNTAL HKVTYLQNGK DRKYFHHNSD FHIPKATLKD
SGSYFCRGLV GSKNVSSETV NITITQGLAV STISSFSPPG YQGLNDIFEA
QKIEWHELVV APPAPEDKTH TCPPCPAPEA AGGPSVFLFP PKPKDTLMIS
RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS
VLTVLHQDWL NGKEYKCKVS NKALGAPIEK TISKAKGQPR EPQVYTLPPS
RDELTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF
FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK

(SEQ ID NO: 59).

- minimal FcgammaRIIIa-Avi-Fc LALA p239G fusion polypeptide (without protease cleavage site):

GWLLLQAPRW VFKEEDPIHL RCHSWKNTAL HKVTYLQNGK GRKYFHHNSD
FYIPKATLKD SGSYFCRGLV GSKNVSSETV NITITQGLAV STISSFFPPG
YQGLNDIFEA QKIEWHELED KTHTCPPCPA PEAAGGPSVF LFPPKPKDTL
MISRTPEVTC VVVDVSHEDP EVKFNWYVDG VEVHNAKTKP REEQYNSTYR
VVSVLTVLHQ DWLNGKEYKC KVSNKALGAP IEKTISKAKG QPREPQVYTL
PPSRDELTKN QVSLTCLVKG FYPSDIAVEW ESNGQPENNY KTTPPVLDSD
GSFFLYSKLT VDKSRWQQGN VFSCSVMHEA LHNHYTQKSL SLSPGK

(SEQ ID NO: 60).

c) Generation of the "knob-into-hole" expression plasmids for dimeric Fc-receptor fusion polypeptide

The expression plasmid for the transient expression of the Fc-receptor Fc-region fusion polypeptide (hole) in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with hole mutations T366S, L368A, Y407V, and Y349C within the human gamma-1 heavy chain constant region.

The expression plasmid for the transient expression of the Fc-receptor Fc-region fusion polypeptide (knob) in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence

coding for the Fc-region with knob mutations T366W and S354C within the human gamma-1 heavy chain constant region.

The expression plasmid for the transient expression of the Fc-receptor Fc-region fusion polypeptide (knob/hole) in HEK293 comprised besides the fusion polypeptide (knob/hole) expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the fusion polypeptide (knob/hole) encoding gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3) with the hole mutations T366S, L368A, Y407V, and Y349C or the knob mutations T366W and S354C within the human gamma-1 heavy chain constant region, and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

Example 2

Transient expression, purification and analytical characterization of the FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G fusion polypeptide

The fusion polypeptides were obtained by transient transfection of HEK293 cells (human embryonic kidney cell line 293-derived) cultivated in F17 Medium (Invitrogen Corp.). For transfection "293-Free" Transfection Reagent (Novagen) was used. The knob-into-hole fusion polypeptide pairs were expressed from two different plasmids using an equimolar plasmid ratio upon transfection. Transfections were performed as specified in the manufacturer's instructions. Fusion polypeptide-containing cell culture supernatants were harvested seven days after transfection. Supernatants were stored at reduced temperature until purification.

General information regarding the recombinant expression of human immunoglobulins in e.g. HEK293 cells is given in: Meissner, P., et al., Biotechnol. Bioeng. 75 (2001) 197-203.

The fusion polypeptide-containing culture supernatants were filtered and purified by two chromatographic steps. The fusion polypeptides 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 fusion polypeptide was recovered with 0.05 M citrate buffer, pH 3, immediately after elution neutralized to pH 6.5 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 2 mM MOPS buffer, 0.125 M NaCl, pH 7.2. The eluted fusion polypeptides were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Four different FcgammaRIIIa-Fc fusion polypeptides were purified according to this protocol:

a) FcgammaRIIIaV158-Avi-Fc LALA P239G (without cleavage site)
b) minimal FcgammaRIIIaV158-Avi-Fc LALA P239G (without cleavage site)
c) FcgammaRIIIaV158-Avi-Prescission Protease(PP)-Fc LALA P239G
d) FcgammaRIIIaV158-Avi-IgA Protease-Fc LALA P239G

The protein concentrations of the fusion polypeptides were determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and proper dimer formation of fusion polypeptides were analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Coomassie brilliant blue. Aggregate content of the fusion polypeptide preparations was determined by high-performance SEC using a Superdex 200™ analytical size-exclusion column (GE Healthcare). The integrity of the amino acid backbone of reduced fusion polypeptide was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

Example 3

Cleavage of Fc-receptor containing fusion polypeptide by Papain

FcgammaRIIIa-Fc fusion polypeptides which do not comprise an enzymatic cleavage site can be cleaved by Papain. FcgammaRIIIa-Fc fusion polypeptide was cleaved by adding cysteine and 0.1 mU / mg fusion polypeptide Papain (from Carica papaya, Roche Diagnostics GmbH) at 37°C for 1h. Subsequent purification was done as described in Example 2. An analytical SDS-PAGE gel is shown in Figure 2.

Example 4

Cleavage of Fc-receptor containing fusion polypeptide by Ides protease

Cleavage of the FcgammaRIIIaV158-Avi-Fc LALA P239G fusion polypeptide with Ides protease is very inefficient and therefore in this case not useful.

Example 5

Cleavage of Fc-receptor containing fusion polypeptide by PreScission protease

After dialysis against 50 mM Tris, 150 NaCl, 1 mM EDTA, 1 mM DTT pH 7.4 FcgammaRIIIa-(PP)-Fc fusion polypeptide was cleaved by adding between 1-15 U PreScission protease (GE Healthcare)/100µg fusion polypeptide at room temperature overnight. Only part of the protein could be cleaved. On the other hand unspecific cleavage by PreScission protease of receptor without PP cleavage site was observed.

Example 6

Cleavage of Fc-receptor containing fusion polypeptide by IgA protease

After dialysis against 50 mM Tris pH 8 using a Slide-a-lyzer dialysis cassette FcgammaRIIIa-Fc fusion polypeptide was cleaved by adding IgA Protease (Roche Diagnostics GmbH) at a ratio of w(protease)/w(fusion polypeptide) 1:100 at 21°C overnight. Cleavage was controlled by analytical size exclusion chromatography (SEC, Superdex 75; GE Healthcare). After cleavage, the FcgammaRIIIa receptor was separated from IgA protease by preparative size exclusion chromatography on Superdex 75™ (GE Healthcare) and from Fc-Tag by HiTrap MabSelectSuRe (GE Healthcare) column. An analytical SDS-Page gel is shown in Figure 3.

Table: Yield of fermentation and purification of different FcgammaRIIIa V158 comprising fusion polypeptides.

fusion polypeptide	molecular weight [kDa]	yield after capturing [mg/l supernatant]	monomer content (SEC) [%]	yield after SEC [mg/l supernatant]	yield after cleavage and further purification [mg/l supernatant]
FcgammaRIIIa V158-HisAvi (20l)	25.4	3.5	70	1.4	-
FcgammaRIIIa V158-Avi-Fc LALA P329G (0.5l)	39	14	70	3 (not active)	
FcgammaRIIIa V158-Avi-Fc LALA P329G (0.5l)	49	80	95	78	21 (without Avi-tag)
FcgammaRIIIa V158-Avi-PP-Fc LALA P329G (0.5l)	50	24	50	not determined	not determined
FcgammaRIIIa V158-Avi-IgAP-Fc LALA P329G (9.2l)	50	46	90	36	16 (with Avi-tag)

Example 7

Preparation of FcgammaRIIIaV158 affinity column

An affinity column with FcgammaRIIIaV158 was prepared by in vitro biotinylation of the Avi-tag and subsequent coupling to Streptavidin sepharose. This can be done with the intact fusion polypeptide as well as with the receptor after having cleaved off the Fc-region. It is a very quick and efficient method for preparing an affinity column for analytical and preparative purposes.

Biotinylation of receptor

A soluble extracellular domain of FcgammaRIIIaV158 with Avi Tag expressed in HEK293 cells was biotinylated after purification according to the following protocol: between 1.2 and 12 mg FcgammaRIIIaV158 or between 2.4 and 24 mg

FcgammaRIIIaV158 Fc-region fusion polypeptide tagged in 2 mM MOPS, 125 mM NaCl pH 7.2, 0.02% Tween, and 1 tablet Complete protease inhibitor (Roche) in 3 ml PBS were biotinylated using the biotinylation kit from Avidity according to the manufacturer instructions. Biotinylation reaction was done at room temperature overnight. The modified polypeptide was dialyzed against 20 mM sodium phosphate buffer, 150 mM NaCl pH 7.5 at 4°C overnight to remove excess biotin.

Coupling to streptavidin sepharose

1 g streptavidin sepharose (GE Healthcare) was added to the biotinylated and dialyzed receptor, incubated for 2 hours while shaking and finally filled in a 1 ml XK column (GE Healthcare).

Example 8

Chromatography methods using an Fc-receptor obtained by expressing an Fc-receptor containing fusion polypeptide

General conditions:

Equilibration buffer A:	20 mM Citric acid/ 150 mM NaCl pH 6.0
Elution buffer B:	20 mM Citric acid/ 150 mM NaCl pH 3.0
Elution:	5 min 100% A
	in 60 min to 100% B,
	0.1 min 100% B,
	6 min 100% A
Sample amount:	50µg or more

Separation of fucosylated and afucosylated antibodies

Chromatography of antibodies on FcgammaRIIIa column allows to quantitate the completely fucosylated and the afucosylated fraction of the antibody. The afucosylated fraction of the antibody is correlated to ADCC of the antibody preparation.

In Figure 4 separation and quantification of different glycosylation forms of an anti-Her antibody (wild-type, at top) and a glycoengineered anti-Her antibody on a Fc-FcgammaRIIIa column is shown. Time of analysis could be shortened by modifying the gradient while retaining resolution.

Comparison of affinity column using FcgammaRIIIaV158 and Fc tagged FcgammaRIIIaV158

After coupling both receptor constructs in equimolar amounts, the affinity columns behave equal in separating completely fucosylated and afucosylated antibodies (Figure 5: lower curve: FcgammaRIIIaV158; upper curve: FcgammaRIIIaV158-Fc).

Example 9

FcγRIIIaV158-Avi-IgA Protease-Fc LALA P329G - IgG interaction measurement

The BIAcore® system is well established for the study of molecule interactions. It allows a continuous real-time monitoring of ligand/analyte bindings and thus the determination of association rate constants (k_a), dissociation rate constants (k_d), and equilibrium constants (K_D). Changes in the refractive index indicate mass changes on the surface caused by the interaction of immobilized ligand with analyte injected in solution. If molecules bind immobilized ligands on the surface the mass increases, in case of dissociation the mass decreases.

For the activity determination of the FcgammaRIIIaV158-Fc LALA P329G fusion polypeptide a direct binding assay was used.

Around 400 resonance units (RU) of the capturing system (20 µg/ml human Fab capture Kit GE Healthcare, 28-9583-25) were coupled onto a CM5 chip (GE Healthcare BR1005-30) at pH 5.0 using an amine coupling kit supplied by GE. The sample and system buffer was HBS-P+ pH 7.4 (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05 % (v/v) Surfactant P20). The flow cell was set to 25 °C and sample block to 12 °C. An antibody was captured by injecting a 50 nM solution for 360 sec. at a flow of 10 µl/min. Binding was measured by injection of 50 nM of FcgammaRIIIa fusion polypeptide for 180 sec. at a flow of 50 µl/min for association and 360 sec. for dissociation. The surface was regenerated by 60 sec. washing with glycine pH 2.1 solution at a flow rate of 20 µl/min. For the activity evaluation of the constructs the signal heights and dissociation behavior have been compared.

As shown in Figure 6 the response of the FcgammaRIIIaV158-Fc LALA P329G fusion polypeptide display more than 100 Response units in comparison to the FcgammaRIIIaV158 with 40 RU.

Example 10

FcγRIIIaV158-Avi-IgA Protease-Fc LALA P329G fusion polypeptide IgG kinetic interaction measurement before and after cleavage

For the activity determination of the cleaved FcgammaRIIIaV158-Fc LALA P329G fusion polypeptide a direct binding assay was used.

Around 400 resonance units (RU) of the capturing system (20 µg/ml human Fab capture Kit GE Healthcare, 28-9583-25) were coupled onto a CM5 chip (GE Healthcare BR1005-30) at pH 5.0 using an amine coupling kit supplied by GE. The sample and system buffer was HBS-P+ pH 7.4 (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05 % (v/v) Surfactant P20). The flow cell was set to 25 °C and sample block to 12 °C. An antibody was captured by injecting a 50 nM solution for 80 sec. at a flow of 10 µl/min.

Different concentrations of antibodies ranging from 0 to 250 nM (1:2 dilutions) were passed with a flow rate of 30 µl/min through the flow cells to measure the association at 25 °C for 120 sec. The dissociation phase was monitored for 420 sec. by switching from the sample solution to running buffer. The surface was regenerated by 60 sec. washing with glycine pH 2.1 solution at a flow rate of 20 µl/min.

Bulk refractive index differences were corrected for by subtracting the response obtained from a surface without captured FcγRIIIaV158. Blank Buffer injections are also subtracted (=double referencing).

The equilibrium dissociation constant (K_D), defined as k_a/k_d, was determined by analyzing the sensogram curves obtained with several different concentrations, using BIAevaluation software package. The fitting of the data followed a suitable binding model. In Figure 7 the sensograms of Fcgamma receptor V158-Fc LALA P329G fusion polypeptide (Figure 7a), Fcgamma receptor V158 (Figure 7b), cleaved Fcgamma receptor V158-Fc LALA P329G fusion polypeptide (Figure 7c) is shown.

Example 11

Generation of the expression plasmids for the Fc-TWEAK fusion polypeptide

a) Generation of the expression plasmid for the Fc-TWEAK fusion polypeptide

The Fc-TWEAK fusion gene was assembled by fusing chemically synthesized DNA fragments coding i) a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3; for exemplary sequences see SEQ ID NO: 03 to 17) wherein the human gamma-1 heavy chain constant region was truncated (removal of the last natural lysine amino acid residue), ii) a glycine-serine linker consisting of a Gly_3Ser and a Gly_4Ser repeat (C-terminus of heavy chain-LSPG--GGGSGGGGS--TWEAK), iii) a PreScission™ protease cleavage site (GLEVLFQGP; SEQ ID NO: 61) and iv) a TWEAK polypeptide from amino acid residues 106-249 (i.e. excluding the intracellular and transmembrane domains and a cleavage site) of a human TWEAK wild-type protein.

The expression plasmid for the transient expression of an Fc-TWEAK fusion polypeptide in HEK293 cells comprised besides the Fc-TWEAK expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the Fc-TWEAK fusion gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3),
- a TWEAK polypeptide from amino acid position 106-249 of the wild-type TWEAK protein, and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature Fc-TWEAK-fusion polypeptide is shown in SEQ ID NO: 62:

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGS GGGGSGLEVL FQGPAIAAHY

EVHPRPGQDG AQAGVDGTVS GWEEARINSS SPLRYNRQIG EFIVTRAGLY
YLYCQVHFDE GKAVYLKLDL LVDGVLALRC LEEFSATAAS SLGPQLRLCQ
VSGLLALRPG SSLRIRTLPW AHLKAAPFLT YFGLFQVH.

b) Generation of the "knob-into-hole" expression plasmids for the dimeric Fc-TWEAK (hole) / Fc (knob) fusion polypeptide

The expression plasmid for the transient expression of the Fc (hole) polypeptide in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with hole mutations T366S, L368A, Y407V, Y349C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region.

The amino acid sequence of the mature Fc (hole) polypeptide is shown in SEQ ID NO: 63:

DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVCT LPPSRDELTK NQVSLSCAVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLVSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGK.

The expression plasmid for the transient expression of the Fc-TWEAK (knob) fusion polypeptide in HEK293 comprised besides the Fc (knob) expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the Fc-TWEAK (knob) fusion gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3) with the knob mutations T366W and S354C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region,
- a linker sequence of the type Gly_3Ser-Gly_4Ser and a PreScission™ protease cleavage site (GLEVLFQGP, SEQ ID NO: 61),
- a TWEAK polypeptide from amino acid position 106-249 of the wild-type TWEAK protein, and

- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature Fc-TWEAK (knob) fusion polypeptide is shown in SEQ ID NO: 64:

```
DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPCRDELTK NQVSLWCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGS GGGGSGLEVL FQGPAIAAHY
EVHPRPGQDG AQAGVDGTVS GWEEARINSS SPLRYNRQIG EFIVTRAGLY
YLYCQVHFDE GKAVYLKLDL LVDGVLALRC LEEFSATAAS SLGPQLRLCQ
VSGLLALRPG SSLRIRTLPW AHLKAAPFLT YFGLFQVH.
```

Example 12

Generation of the expression plasmids for the Fc-IL17A fusion polypeptide

a) Generation of the expression plasmid for the Fc-IL17A fusion polypeptide

The Fc-IL17A fusion gene was assembled by fusing chemically synthesized DNA fragments coding i) a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3; for exemplary sequences see SEQ ID NO: 03 to 17) wherein the human gamma-1 heavy chain constant region was truncated (removal of the last natural lysine amino acid residue), iia) a glycine-serine linker consisting of two Gly4Ser repeats plus two Gly3Ser repeats (C-terminus of heavy chain-LSP-GGGGSGGGGSGGGSGGGS-IL17A), or iib) a glycine-serine linker consisting of two Gly_4Ser repeats plus a IgA protease cleavage site (C-terminus of heavy chain-LSP-GGGGSGGGGSGSVVAPPA-IL17A) and iii) a IL17A polypeptide from amino acid residues 24-155 (i.e. excluding the starting methionine and the homologous signal peptide) of a cynomolgus IL17A wild-type protein.

The expression plasmid for the transient expression of an Fc-IL17A fusion polypeptide in HEK293 cells comprised besides the Fc-IL17A expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the Fc-IL17A fusion gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),

- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3),
- a glycine-serine linker consisting of two Gly_4Ser repeats plus two Gly_3Ser repeats or a glycine-serine linker consisting of two Gly_4Ser repeats plus a IgA protease cleavage site,
- an IL17A polypeptide from amino acid position 24-155 of the wild-type IL17A protein, and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature Fc-IL17A-fusion polypeptide is shown in SEQ ID NO: 65:

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGG SGGGGSGGGS GGGSGIAIPR
NSGCPNSEDK NFPRTVMVNL NIHNRNTSTN PKRSSDYYNR STSPWNLHRN
EDPERYPSVI WEAKCRHLGC VKADGNVDYH MNSVPIQQEI LVLRREPRHC,

or

SEQ ID NO: 66:

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGG SGGGGSGSVV APPAGIAIPR
NSGCPNSEDK NFPRTVMVNL NIHNRNTSTN PKRSSDYYNR STSPWNLHRN
EDPERYPSVI WEAKCRHLGC VKADGNVDYH MNSVPIQQEI LVLRREPRHC.

<u>b) Generation of the "knob-into-hole" expression plasmids for the dimeric Fc-IL17A (knob) / Fc-IL17A (hole) fusion polypeptide</u>

The expression plasmid for the transient expression of the IL17A-Fc (hole) fusion polypeptide in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with hole mutations T366S, L368A, Y407V, Y349C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region.

The amino acid sequence of the mature IL17A-Fc (hole) fusion polypeptide is shown in SEQ ID NO: 67:

DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVCT LPPSRDELTK NQVSLSCAVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLVSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGG SGGGGSGSVV APPAGIAIPR
NSGCPNSEDK NFPRTVMVNL NIHNRNTSTN PKRSSDYYNR STSPWNLHRN
EDPERYPSVI WEAKCRHLGC VKADGNVDYH MNSVPIQQEI LVLRREPRHC
PNSFRLEKIL VSVGCTCVTP IVHHVA.

The expression plasmid for the transient expression of the Fc-IL17A (knob) fusion polypeptide in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with the knob mutations T366W and S354C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region.

The amino acid sequence of the mature Fc-IL17A (knob) fusion polypeptide is shown in SEQ ID NO: 68:

DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPCRDELTK NQVSLWCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGG SGGGGSGGGS GGGSGIAIPR
NSGCPNSEDK NFPRTVMVNL NIHNRNTSTN PKRSSDYYNR STSPWNLHRN
EDPERYPSVI WEAKCRHLGC VKADGNVDYH MNSVPIQQEI LVLRREPRHC
PNSFRLEKIL VSVGCTCVTP IVHHVA.

Example 13

Transient expression of the Fc-fusion polypeptides

The Fc-fusion polypeptides were generated by transient transfection of HEK293 cells (human embryonic kidney cell line 293-derived) cultivated in F17 Medium (Invitrogen Corp.). For transfection "293-Free" Transfection Reagent (Novagen) was used. The knob-into-hole Fc-fusion polypeptides were expressed from two different plasmids using an equimolar plasmid ratio upon transfection. Transfections were performed as specified in the manufacturer's instructions. Fc-fusion polypeptide-containing cell culture supernatants were harvested seven days after transfection. Supernatants were stored at reduced temperature until purification.

General information regarding the recombinant expression of human immunoglobulins in e.g. HEK293 cells is given in: Meissner, P., et al., Biotechnol. Bioeng. 75 (2001) 197-203.

Example 14

Purification, processing and analytical characterization of the trimeric TWEAK-Fc fusion polypeptides

The Fc-fusion polypeptide-containing culture supernatants were 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, 600 mM NaCl. Unbound proteins were removed by washing with equilibration buffer, and the fusion polypeptide was recovered with 0.1 M citrate buffer, pH 3.0 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 2 x PBS (2 mM KH_2PO_4, 20 mM Na_2HPO_4, 274 mM NaCl, 5.4 mM KCl), pH 7.4. The eluted Fc-fusion polypeptides 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 concentration of the Fc-fusion polypeptides 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 of Fc-fusion polypeptides was analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Coomassie brilliant blue. Aggregate content and proper trimer formation of the Fc-fusion polypeptide was determined by high-performance SEC using a Superdex 200™ analytical size-exclusion column (GE Healthcare) coupled to a SEC-MALLS detector (Wyatt). The integrity of the amino acid backbone of the reduced Fc fusion polypeptide was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

To obtain trimeric TWEAK without Fc-fusion, the trimeric Fc-fusion polypeptide was incubated with PreScission® protease overnight followed by affinity chromatography using a MabSelectSuRe (GE Healthcare) column in flow-through

mode for removal of non-cleaved Fc-fusion polypeptides as well as free Fc-part. Flow-through fractions were further purified by affinity chromatography in the flow through mode using Glutathione Sepharose 4B (GE Healthcare) for removal of the PreScission® protease. Flow-through fractions containing trimeric TWEAK were further purified by Size exclusion chromatography on Superdex 200™ (GE Healthcare). The size exclusion chromatography was performed in 2 x PBS (2 mM KH_2PO_4, 20 mM Na_2HPO_4, 274 mM NaCl, 5.4 mM KCl), pH 7.4. The eluted trimeric TWEAK containing fractions were concentrated with an Ultrafree - CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Determination of the protein concentrations, purity, aggregate content, trimer formation and integrity of the amino acid backbone was performed with the methods described above.

Example 15

Purification, processing and analytical characterization of the dimeric Fc-IL17 fusion polypeptides

The Fc-fusion polypeptide-containing culture supernatants were filtered and purified by two chromatographic steps. The Fc-fusion polypeptides 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 fusion polypeptide was recovered with 0.1 M 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 eluted Fc-fusion polypeptides 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 Fc-fusion polypeptides were determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and proper dimer formation of Fc-fusion polypeptides were analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Coomassie brilliant blue. Aggregate content of the Fc-fusion polypeptide

preparations was determined by high-performance SEC using a Superdex 200™ analytical size-exclusion column (GE Healthcare). The integrity of the amino acid backbone of the reduced Fc fusion polypeptide was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

For activation, the Fc-fusion polypeptide was formulated in 1 M Tris, pH 7.5 and incubated with IgA protease. After cleavage, size exclusion chromatography on Superdex 200™ (GE Healthcare) was used for removal of the IgA protease. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The eluted Fc-fusion polypeptides were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Determination of the protein concentrations, purity, aggregate content, cleavage efficiency and integrity of the amino acid backbone was performed with the methods described above.

The results are shown in the following Table and in Figure 11.

Table.

	long connector	short connector	short connector, cleaved
concentration	5.5 mg/ml	5.7 mg/ml	5.8 mg/ml
amount	53.3 mg	68 mg (105.5 mg)	28.4 mg
% monomer (SEC)	>98	>98	>96%

Example 16

Generation of the expression plasmids for expression of IL18-receptor containing fusion polypeptide

a) Generation of the expression plasmid for an IL18R-Fc fusion polypeptide

The IL18R-Fc fusion polypeptide encoding gene was assembled by fusing chemically synthesized DNA fragments coding i) for a murine immunoglobulin heavy chain signal sequence (MGWSCIILFLVATATGVHS: SEQ ID NO: 55), ii) a human IL18R (excluding the starting methionine), and iii) a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3).

The expression plasmid for the transient expression of an IL18R-Fc fusion polypeptide in HEK293 cells comprised besides the IL18R-Fc fusion polypeptide expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the IL18R-Fc fusion polypeptide encoding gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a soluble human IL18R-Fc,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3), and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature IL18R-Fc fusion polypeptide is shown in SEQ ID NO: 70:

SKSCIHRSQI HVVEGEPFYL KPCGISAPVH RNETATMRWF KGSASHEYRE
LNNRSSPRVT FHDHTLEFWP VEMEDEGTYI SQVGNDRRNW TLNVTKRNKH
SCFSDKLVTS RDVEVNKSLH ITCKNPNYEE LIQDTWLYKN CKEISKTPRI
LKDAEFGDEG YYSCVFSVHH NGTRYNITKT VNITVIEGRS KVTPAILGPK
CEKVGVELGK DVELNCSASL NKDDLFYWSI RKEDSSDPNV QEDRKETTTW
ISEGKLHASK ILRFQKITEN YLNVLYNCTV ANEEAIDTKS FVLVRKEIPD
IPGHVFTGLE PKSCDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT
PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL
TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRD
ELTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL
YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K.

c) Generation of the "knob-into-hole" expression plasmids for monomeric IL18R fusion polypeptide

The expression plasmid for the transient expression of the IL18R-Fc fusion polypeptide (knob) in HEK293 cells was derived from the expression vector described above under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with knob mutations T366W and S354C within the human gamma-1 heavy chain constant region.

The expression plasmid for the transient expression of the Fc-region polypeptide (hole) in HEK293 cells was derived from the expression vector described above

under item a). It differentiated therefrom in the DNA sequence coding for the Fc-region with hole mutations T366S, L368A, Y407V, and Y349C within the human gamma-1 heavy chain constant region.

The expression plasmid for the transient expression of the IL18R-Fc fusion polypeptide (knob/hole) in HEK293 comprised besides the fusion polypeptide (knob/hole) expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the fusion polypeptide (knob/hole) encoding gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3) with the hole mutations T366S, L368A, Y407V, and Y349C or the knob mutations T366W and S354C within the human gamma-1 heavy chain constant region, and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

Example 17

Transient expression, purification and analytical characterization of the IL18R-Fc fusion polypeptide

The fusion polypeptides were obtained by transient transfection of HEK293 cells (human embryonic kidney cell line 293-derived) cultivated in F17 Medium (Invitrogen Corp.). For transfection "293-Free" Transfection Reagent (Novagen) was used. The knob-into-hole fusion polypeptide pairs were expressed from two different plasmids using an equimolar plasmid ratio upon transfection. Transfections were performed as specified in the manufacturer's instructions. Fusion polypeptide-containing cell culture supernatants were harvested seven days after transfection. Supernatants were stored at reduced temperature until purification.

General information regarding the recombinant expression of human immunoglobulins in e.g. HEK293 cells is given in: Meissner, P., et al., Biotechnol. Bioeng. 75 (2001) 197-203.

The fusion polypeptide-containing culture supernatants were filtered and purified by two chromatographic steps. The fusion polypeptides 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 fusion polypeptide was recovered with 0.05 M citrate buffer, pH 3, immediately after elution neutralized to pH 6.5 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 2 mM MOPS buffer, 0.125 M NaCl, pH 7.2. The eluted fusion polypeptides 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 fusion polypeptides were determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and proper dimer formation of fusion polypeptides were analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Coomassie brilliant blue. Aggregate content of the fusion polypeptide preparations was determined by high-performance SEC using a Superdex 200™ analytical size-exclusion column (GE Healthcare). The integrity of the amino acid backbone of the reduced fusion polypeptide was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

The respective SPR-sensograms are shown in Figure 17 showing the differences between monomer and dimer (avidity effects).

Example 18

Generation of the expression plasmids for the Fc-region-precision protease cleavage site-shTNFalpha fusion polypeptide

a) Generation of the expression plasmid for the Fc-shTNFalpha fusion polypeptide

The Fc-PP-shTNFalpha fusion gene was assembled by fusing chemically synthesized DNA fragments coding i) a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3; for exemplary sequences see SEQ ID NO: 03 to 17) wherein the human gamma-1 heavy chain constant region was truncated (removal of the last natural lysine amino acid residue), ii) a glycine-serine linker consisting of a Gly_3Ser and a Gly_4Ser repeat (C-terminus of heavy chain-LSPG--GGGSGGGGS--TWEAK), iii) a PreScission™ protease cleavage site (GLEVLFQGP, SEQ ID NO: 61) and iv) a shTNFalpha polypeptide (excluding the intracellular and transmembrane domains and a cleavage site) of a human TNFalpha wild-type protein.

The expression plasmid for the transient expression of an Fc-PP-shTNFalpha fusion polypeptide in HEK293 cells comprised besides the Fc-PP-shTNFalpha expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the Fc-PP-shTNFalpha fusion gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3),
- an shTNFalpha polypeptide, and
- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature Fc-PP-shTNFalpha-fusion polypeptide is shown in SEQ ID NO: 71:

```
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGS GGGGSGLEVL FQGPVRSSSR
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TPSDKPVAHV VANPQAEGQL QWLNRRANAL LANGVELRDN QLVVPSEGLY
LIYSQVLFKG QGCPSTHVLL THTISRIAVS YQTKVNLLSA IKSPCQRETP
EGAEAKPWYE PIYLGGVFQL EKGDRLSAEI NRPDYLDFAE SGQVYFGIIA
L.

b) Generation of the "knob-into-hole" expression plasmids for the dimeric Fc (hole) / Fc-PP-shTNFalpha (knob) fusion polypeptide

The expression plasmid for the transient expression of the Fc-region polypeptide (hole) in HEK293 cells was derived from the expression vector described above. It differentiated therefrom in the DNA sequence coding for the Fc-region with hole mutations T366S, L368A, Y407V, Y349C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region.

The amino acid sequence of the mature Fc-region (hole) polypeptide is shown in SEQ ID NO: 69:

DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVCT LPPSRDELTK NQVSLSCAVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLVSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGK.

The expression plasmid for the transient expression of the Fc-PP-shTNFalpha (knob) fusion polypeptide in HEK293 comprised besides the Fc (knob) expression cassette an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. In detail, the transcription unit of the Fc-PP-shTNFalpha (knob) fusion gene comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- a human Fc-gamma-1-heavy chain constant region (hinge-CH2-CH3) with the knob mutations T366W and S354C and the Fc effector functions reducing mutations L234A and L235A within the human gamma-1 heavy chain constant region,
- a linker sequence of the type Gly_3Ser-Gly_4Ser and a PreScission™ protease cleavage site (GLEVLFQGP),
- a PP-shTNFalpha polypeptide, and

- the bovine growth hormone polyadenylation sequence (BGH poly A signal sequence).

The amino acid sequence of the mature Fc-PP-shTNFalpha (knob) fusion polypeptide is shown in SEQ ID NO: 72:

DKTHTCPPCP APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPCRDELTK NQVSLWCLVK
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPGGGGS GGGGSGLEVL FQGPVRSSSR
TPSDKPVAHV VANPQAEGQL QWLNRRANAL LANGVELRDN QLVVPSEGLY
LIYSQVLFKG QGCPSTHVLL THTISRIAVS YQTKVNLLSA IKSPCQRETP
EGAEAKPWYE PIYLGGVFQL EKGDRLSAEI NRPDYLDFAE SGQVYFGIIA
L.

Example 19

Purification, processing and analytical characterization of the trimeric Fc-PP-shTNFalpha fusion polypeptides

The Fc-fusion polypeptide-containing culture supernatants were 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, 600 mM NaCl. Unbound proteins were removed by washing with equilibration buffer, and the fusion polypeptide was recovered with 0.1 M citrate buffer, pH 3.0 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 2 x PBS (2 mM KH_2PO_4, 20 mM Na_2HPO_4, 274 mM NaCl, 5.4 mM KCl), pH 7.4. The eluted Fc-fusion polypeptides 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 concentration of the Fc-fusion polypeptides 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 of Fc-fusion polypeptides was analyzed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1. 4-dithiotreitol) and staining with Coomassie brilliant blue. Aggregate content and proper trimer formation of the Fc-fusion polypeptide was determined by high-performance SEC using a Superdex 200™ analytical size-

exclusion column (GE Healthcare) coupled to a SEC-MALLS detector (Wyatt). The integrity of the amino acid backbone of reduced Fc fusion polypeptide was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

To obtain trimeric PP-shTNFalpha without Fc-fusion, the trimeric Fc-fusion polypeptide was incubated with PreScission® protease overnight followed by affinity chromatography using a MabSelectSuRe (GE Healthcare) column in flow-through mode for removal of non-cleaved Fc-fusion polypeptides as well as free Fc-part. Flow-through fractions were further purified by affinity chromatography in the flow through mode using Glutathione Sepharose 4B (GE Healthcare) for removal of the PreScission® protease. Flow-through fractions containing trimeric shTNFalpha were further purified by Size exclusion chromatography on Superdex 200™ (GE Healthcare). The size exclusion chromatography was performed in 2 x PBS (2 mM KH_2PO_4, 20 mM Na_2HPO_4, 274 mM NaCl, 5.4 mM KCl), pH 7.4. The eluted trimeric shTNFalpha containing fractions were concentrated with an Ultrafree-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

Determination of the protein concentrations, purity, aggregate content, trimer formation and integrity of the amino acid backbone was performed with the methods described above.

TNF alpha as IgG1-LALA-Fc-KiH_PreScission-Protease-site_shTNFa fusion polypeptide was obtained by transient expression in HEK 293 cells at 283 mg/l. Cleavage by PreScission protease resulted in 15 mg per 100 mg fusion polypeptide (total from 283 mg = 43 mg). Functional assessment in A375 assay (see Example 20).

Example 20

Titration of human TNF alpha

A-375 cells were seeded with $2x10^4$ cells per well in 200µl per well in 96 well F cell culture plate (Costar 3596). The cells were cultured overnight at 37 °C, 5 % CO_2 before medium was removed.

The following cytokines were titrated with 100µl per well (50-0 nM)

- rhTNF-a (R&D Systems 210-TA/CF),

- hTNFalpha-Fc fusion polypeptide as reported herein, and
- cleaved hTNFalpha-Fc fusion polypeptide.

The cells were incubated for 24 hours at 37 °C, 5 % CO_2. The supernatant was transferred into 96 well RB plate and stored at -20 °C. The cytokine profile was analyzed using hIL-8 (BD 558277) CBA Flex Set.

sample	EC50 [nM]
hTNF-a R&D	0.005
hTNF-a-hFc	0.078
cleaved hTNF-a	0.009

The titration curves are shown in Figure 10.

Patent Claims

1. A method for the production of a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region and comprises the following steps

a) cultivating a cell comprising a nucleic acid encoding a fusion polypeptide according to formula II

$$B_n\text{-}CS_o\text{-}I_s\text{-}CS_p\text{-}FC^1\text{-}CS_q\text{-}I_t\text{-}CS_r\text{-}B_m : FC^2 \qquad \text{(formula II)}$$

wherein

B denotes a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC^1 denotes a first heavy chain Fc-region polypeptide,

FC^2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein FC^1 and FC^2 are covalently linked by one or more disulfide bond(s) (:),

wherein FC^1 and FC^2 do not substantially bind to an Fc-receptor,

b) recovering the fusion polypeptide from the cell or the cultivation medium,

c) cleaving the fusion polypeptide with a protease,

and thereby producing a polypeptide that is biologically active as 2-mer or 3-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region.

2. A fusion polypeptide according to formula II

Bn-CSo-Is-CSp-FC1-CSq-It-CSr-Bm : FC2 (formula II)

wherein

B denotes a polypeptide that is biologically active as n-mer and forms non-defined aggregates/multimers upon expression in the absence of a fused Fc-region,

FC1 denotes a first heavy chain Fc-region polypeptide,

FC2 denotes a second heavy chain Fc-region polypeptide,

CS denotes a cleavage site, and

I denotes an intervening amino acid sequence,

wherein n=1 and m=0, or n=0 and m=1,

wherein if n=1 then o=0 or 1, and if o=0 then p=0 or 1, and if o=1 then p=0, and s=0 or 1, and q=0, and t=0, and r=0,

wherein if m=1 then q=0 or 1, and if q=0 then r=0 or 1, and if q=1 then r=0, and t=0 or 1, and o=0, and s=0, and p=0,

wherein the first FC and the second FC are covalently linked by one or more disulfide bond(s),

wherein FC1 and FC2 do not substantially bind to an Fc-receptor.

3. The method according to claim 1 or the fusion polypeptide according to claim 2, characterized in that B is selected from the group of IL17, TWEAK, TNF and other TNF family members, TL1a, IL18, IL18R, IL33, and IL33R.

4. The method according to claim 1 or 3 or the fusion polypeptide according to claim 2 or 3, characterized in that FC is a variant of a heavy chain polypeptide selected from the group of human IgG1 heavy chain polypeptide, human IgG2 heavy chain polypeptide, human IgG3 heavy chain polypeptide, human IgG4 heavy chain polypeptide, murine IgG1 heavy chain polypeptide, murine IgG2 heavy chain polypeptide, murine IgG3 heavy chain polypeptide, rabbit IgG heavy chain polypeptide.

5. The method according to any one of claim 1, 3 or 4 or the fusion polypeptide according to any one of claim 2, 3 or 4, characterized in that FC is selected from human IgG1 heavy chain polypeptide with the mutations L234A, L235A and P329G, human IgG4 heavy chain polypeptide with the mutation S228P, L235E and P329G.

6. The method according to any one of claim 1, 3, 4 or 5 or the fusion polypeptide according to any one of claim 2, 3, 4 or 5, characterized in that the first FC comprises the mutation T366W and the second FC comprises the mutations T366S, L368A and Y407V.

7. The method according to claim 6 or the fusion polypeptide according to claim 6, characterized in that the first FC comprises the mutation T366W and the mutation S354C.

8. The method according to claim 6 or 7 or the fusion polypeptide according to claim 6 or 7, characterized in that the second FC comprises the mutations T366S, L368A and Y407V and the mutation Y349C.

9. The method according to any one of claims 6 to 8 or the fusion polypeptide according to any one of claims 6 to 8, characterized in that

a) n=1 and m=0 and B fused to the first FC and B fused to the second FC are identical,

b) n=1 and m=0 and B fused to the first FC and B fused to the second FC are different,

c) n=0 and m=1 and B fused to the first FC and B fused to the second FC are identical,

d) n=0 and m=1 and B fused to the first FC and B fused to the second FC are different.

10. Use of an immobilized fusion polypeptide according to any one of claims 2 and 3 to 9 as affinity chromatography ligand.

11. Use of an immobilized fusion polypeptide according to any one of claims 2 and 3 to 9 in an immunoassay.

12. The use according to any one of claims 10 to 11, characterized in that the fusion polypeptide or the n-mer polypeptide is bound to a solid phase.

13. Pharmaceutical composition comprising a fusion polypeptide according to any one of claims 2 and 3 to 9.

14. A fusion polypeptide according to any one of claims 2 and 3 to 9 for use as an immunogen.

15. Use of a fusion polypeptide according to any one of claims 2 and 3 to 9 for obtaining an animal model of a disease by applying the fusion polypeptide according to

any one of claims 2 and 3 to 9 to an experimental animal.

16. Use of a fusion polypeptide according to any one of claims 2 and 3 to 9 for selecting an antibody that specifically binds to B or a 2-mer or 3-mer of B.

17. Use of a fusion polypeptide according to any one of claims 2 and 3 to 9 for producing a disulfide-linked 2-mer of B.