CX3CR1-BINDING POLYPEPTIDES

FIELD OF THE INVENTION

The present invention relates to CX3CR1-binding polypeptides, in particular polypeptides comprising specific immunoglobulin domains. The invention also relates to nucleic acids encoding such polypeptides; to methods for preparing such polypeptides; to host cells expressing or capable of expressing such polypeptides; to compositions comprising such polypeptides; and to uses of such polypeptides or such compositions, in particular for prophylactic, therapeutic and diagnostic purposes.

BACKGROUND OF THE INVENTION

CX3CR1 is a G-protein coupled integral membrane protein, which is a chemokine receptor. It is predominantly expressed on cell types such as monocytes, dendritic cells and T cells that have been associated with the initiation and progression of atherosclerotic plaques. It is upregulated on monocytes by oxidized lipids and mediates migration of these cells into and survival within plaques. Its unique ligand fractalkine (FKN) is expressed on the surface of vascular endothelial and smooth muscle cells in lesions where it modulates leukocyte adhesion. Fractalkine is also released into the circulation by proteolytic cleavage where it functions as a chemotactic agent.

In humans, a CX3CR1 variant (V249I/T280M) with decreased activity has been shown to be associated with a lower risk of cardiovascular disease (coronary heart disease, cerebrovascular disease or peripheral vascular disease)(McDermott, 2001; *Circ Res* 89:401), coronary artery disease (angiographic evidence of stenosis) (McDermott, 2003; *J. Clin. Invest.* 111:1241), and carotid artery occlusive disease (Ghilardi, 2004; *Stroke* 35:1276). CX3CR1 co-localized with fractalkine which showed enhanced immunostaining by polyclonal antibodies within atherosclerotic plaques (Wong, 2002 *Cardiovasc. Path.* 11:332). No fractalkine staining was observed in non-plaque arterial regions.

Several independent mouse genetic studies have shown a beneficial effect of CX3CR1 deficiency on atherosclerosis. A reduction in lesion area in the aortic arch and thoracic aorta as well as a decrease in monocyte/macrophage accumulation in plaques was seen in two independently derived strains of $CX3CR1^{-/-}$ $apoE^{-/-}$ mice fed a high fat diet (Combadière, 2003; *Circulation*, 107:1009, Lesnik, 2003; *J. Clin. Invest.* 111:333).

This shows that CX3CR1 is involved in cardiovascular diseases and the modulation of its activity could provide promising therapies. There is therefore a need for antagonist molecules against CX3CR1 with beneficial pharmacological properties, which can be used as therapeutic agents to treat diseases, in particular cardiovascular diseases in humans.

Accordingly, one aim of the present disclosure is to provide anti-CX3CR1 antagonist molecules, in particular anti-CX3CR1 antagonist molecules, which have high binding affinity to CX3CR1.

A further aim of the present disclosure is to provide anti-CX3CR1 antagonist molecules, which have high specificity for CX3CR1.

A further aim of the present disclosure is to provide anti-CX3CR1 antagonists, which have potent activity.

A further aim of the present disclosure is to provide anti-CX3CR1 antagonists, which have a favorable bioavailability and half-life.

A further aim of the present disclosure is to provide anti-CX3CR1 antagonists, which have favorable biophysical properties.

Further aims of the present disclosure include combinations of any of the aims set forth above.

SUMMARY OF THE DISCLOSURE

The disclosure provides polypeptides which bind to human CX3CR1 and are capable of blocking the binding of human fractalkine to human CX3CR1. The polypeptide may be an immunoglobulin comprising an antigen-binding domain comprising three complementarity determining regions CDR1, CDR2 and CDR3, wherein said immunoglobulin binds to human CX3CR1 and is capable of blocking the binding of human fractalkine to human CX3CR1. In

one aspect, there is provided a polypeptide comprising an anti-CX3CR1 (anti-CX3 chemokine receptor 1) immunoglobulin single variable domain, wherein said anti-CX3CR1 immunoglobulin single variable domain consists essentially of four framework regions (FR1, FR2, FR3 and FR4) and three complementary determining regions (CDR1, CDR2 and CDR3) and wherein said CDR3 has the amino acid sequence of Asp-Pro-Arg-Arg-Gly-Trp-Asp-Thr-Arg-Tyr (SEQ ID NO: 186) and wherein said polypeptide comprises a CDR1, CDR2 and CDR3 having the amino acid sequences set forth in:

- SEQ ID No: 213, 221 and 186, respectively; or
- SEQ ID No: 141, 162 and 186, respectively; or
- SEQ ID No: 141, 164 and 186, respectively; or
- SEQ ID No: 141, 166 and 186, respectively; or
- SEQ ID No: 141, 167 and 186, respectively; or
- SEQ ID No: 213, 214 and 186, respectively.

The polypeptide may comprise one or more anti-CX3CR1 immunoglobulin single variable domain, wherein said polypeptide is capable of blocking the binding of human fractalkine to human CX3CR1.

A polypeptide of the present disclosure may be characterized by one or more of the following properties:

- Bind with high affinity to human CX3CR1;
- Block the binding of soluble fractalkine to human CX3CR1;
- Inhibit fractalkine induced chemotaxis;
- Inhibit fractalkine induced human CX3CR1 receptor internalization;
- Cross-react with cyno CX3CR1 within 10-fold of E/IC_{50} for human CX3CR1 for binding and functional inhibition.

A polypeptide of the present disclosure maycomprise an anti-CX3CR1 immunoglobulin single variable domain and may further comprise a half-life extending moiety, for example an albumin binding moiety, a polyethylene glycol molecule or a Fc domain. A polypeptide of the present disclosure may comprise two or more anti-CX3CR1 immunoglobulin single variable domains. The two anti-CX3CR1 immunoglobulin single variable domains may be covalently linked by a linker peptide. The two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present disclosure may have the same amino acid sequence. The two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present disclosure may have different amino acid sequences. A polypeptide of the present disclosure may comprise two anti-CX3CR1 immunoglobulin single variable domains and further comprises a half-life extending moiety, for example an albumin binding moiety, a polyethylene glycol molecule or a Fc domain.

A polypeptide of the present disclosure may comprise a first anti-CX3CR1 immunoglobulin single variable domain covalently linked to an albumin binding moiety by a first linker peptide, wherein said albumin binding moiety is further covalently linked to a second anti-CX3CR1 immunoglobulin single variable domain by a second linker peptide.

A polypeptide of the present disclosure may comprise an anti-CX3CR1 immunoglobulin single variable domain covalently linked to a Fc domain by a

linker peptide. Such polypeptide comprising an anti-CX3CR1 immunoglobulin single variable domain covalently linked to a Fc domain by a linker peptide may be provided as a dimer, for example through disulfide bridges.

In a further aspect, there is provided a polypeptide comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain, each comprising CDR1, CDR2 and CDR3 having amino acid sequences set forth in SEQ ID NO's: 141, 164 and 186, or SEQ ID NO's: 141, 162 and 186, or SEQ ID NO's: 213, 214 and 186, or SEQ ID NO's: 213, 221 and 186.

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 225-227 or 257-262.

In a further aspect, there is provided a nucleic acid molecule encoding a polypeptide as defined above.

In a further aspect, there is provided an expression vector comprising a nucleic acid molecule as defined above.

In a further aspect, there is provided a host cell comprising a nucleic acid molecule encoding a polypeptide as defined above, wherein the host cell is capable of expressing the polypeptide.

In a further aspect, there is provided a pharmaceutical composition comprising (i) a polypeptide as defined above, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

In a further aspect, there is provided a method of manufacturing a polypeptide as defined above, comprising the steps of

- culturing a host cell under conditions that allow expression of a polypeptide as defined above,

wherein the host cell is carrying an expression vector comprising a nucleic acid molecule, the nucleic acid molecule comprising a region encoding a polypeptide as defined above, and wherein the host cell is a prokaryotic or a eukaryotic cell.

In a further aspect, there is provided a polypeptide as defined above for use in the treatment, prevention or alleviation of a disease, disorder or condition, in a human being.

In a further aspect, there is provided use of a polypeptide as defined above for the manufacture of a medicament for the treatment, prevention or alleviation of a disease, disorder or condition, in a human being, wherein the disease or disorder is selected from cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulonephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

In a further aspect, there is provided a diagnostic kit comprising a polypeptide as defined above, or the use thereof

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 225.

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 226.

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 227.

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 258.

In a further aspect, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 261.

In a further aspect, there is provided a pharmaceutical composition comprising (i) a polypeptide as defined above, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

The polypeptides of the present disclosure are used for the prevention, treatment, alleviation and/or diagnosis of CX3CR1-associated diseases, disorders or conditions, in particular cardiovascular diseases, such as atherosclerosis.

The present disclosure may provide:

Embodiment 1: An immunoglobulin comprising an antigen-binding domain comprising three complementarity determining regions CDR1, CDR2 and CDR3, wherein said immunoglobulin binds to human CX3CR1 and is capable of blocking the binding of human fractalkine to human CX3CR1.

Embodiment 2: A polypeptide comprising one or more anti-CX3CR1 immunoglobulin single variable domain, wherein said polypeptide is capable of blocking the binding of human fractalkine to human CX3CR1.

Embodiment 3: A polypeptide according to embodiment 2, wherein said anti-CX3CR1 immunoglobulin single variable domain consists essentially of four framework regions (FR1, FR2, FR3 and FR4) and three complementary determining regions (CDR1, CDR2 and CDR3).

Embodiment 4: A polypeptide according to embodiment 3, wherein said anti-CX3CR1 immunoglobulin single variable domain has the structure FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4.

Embodiment 5: A polypeptide according to any one of embodiments 2 to 4, wherein said anti-CX3CR1 immunoglobulin single variable domain is an antibody domain.

Embodiment 6: A polypeptide according to embodiment 5, wherein said anti-CX3CR1 immunoglobulin single variable domain is a VH, VL, VHH, camelized

VH, or VHH that is optimized for stability, potency, manufacturability and/or similarity to human framework regions.

Embodiment 7: A polypeptide according to any one of embodiments 1 to 6, wherein said polypeptide has an affinity to human CX3CR1 at:

a) an EC50 of less than or equal to 10nM, less than or equal to 5nM, less than or equal to 2.5nM or less than or equal to 1nM, as determined by cell binding FACS; or

b) an IC50 of less than or equal to 10nM, less than or equal to 5nM, less than or equal to 2.5nM or less than or equal to 1nM, as determined by competition FACS.

Embodiment 8: A polypeptide according to any one of embodiments 1 to 7, wherein said polypeptide blocks the binding of human fractalkine to human CX3CR1 at an IC50 of less than or equal to 300nM, or less than or equal to 100nM, or less than or equal to 20nM, or less than or equal to 10nM, or less than or equal to 5nM, or less than or equal to 2.5nM or less than or equal to 1nM.

Embodiment 9: A polypeptide according to any one of embodiments 1 to 8, wherein said polypeptide inhibits fractalkine induced chemotaxis mediated by human CX3CR1 at an IC_{50} of less than or equal to 500 nM, or of less than or equal to 100 nM, or of less than or equal to 75 nM, or of less than or equal to 50 nM, or less than or equal to 10 nM or less than or equal to 5nM.

Embodiment 10: A polypeptide according to any one of embodiments 1 to 9, wherein said polypeptide inhibits fractalkine internalization mediated by human CX3CR1 at an IC_{50} of less than or equal to 10 nM, or less than or equal to 5nM or or less than or equal to 1nM.

Embodiment 11: A polypeptide according to any one of embodiments 3 to 10, wherein said CDR3 has the amino acid sequence of Asp-Xaa1-Arg-Arg-Gly-Trp-Xaa2-Xaa3-Xaa4-Xaa5 (SEQ ID NO: 197), wherein:

- Xaa1 is Pro, Ala or Gly;
- Xaa2 is Asp or Asn;

- Xaa3 is Thr or Ser;
- Xaa4 is Arg, Lys, Ala or Gly; and
- Xaa5 is Tyr or Phe.

Embodiment 12: A polypeptide according to any one of embodiments 3 to 11, wherein:

a)

- Xaa1 is Pro, Ala or Gly;
- Xaa2 is Asp or Asn;
- Xaa3 is Thr;
- Xaa4 is Arg or Lys; and
- Xaa5 is Tyr,

and/or

b) wherein said CDR3 is selected from any of SEQ ID No's: 186-190.

Embodiment 13: A polypeptide according to any one of embodiments 3 to 12, wherein said CDR3 has the amino acid sequence of Asp-Pro-Arg-Arg-Gly-Trp-Asp-Thr-Arg-Tyr (SEQ ID NO: 186).

Embodiment 14: A polypeptide according to any one of embodiments 3 to 10, wherein:

i) said CDR1:

a) has the amino acid sequence of SEQ ID NO: 141;

b) has an amino acid sequence that has at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 141;

c) has an amino acid sequence that has 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 141, wherein

- at position 2 the S has been changed into T, or G;
- at position 6 the S has been changed into R;
- at position 7 the N has been changed into T; and/or
- at position 9 the M has been changed into K; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 141-145 and 213;

ii) said CDR2:

a) has the amino acid sequence of SEQ ID NO: 164;

b) has an amino acid sequence that has at least 70% amino acid identity with the amino acid sequence of SEQ ID NO: 164;

c) has an amino acid sequence that has 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 164, wherein

- at position 1 the G has been changed into A, L, V or S;
- at position 3 the N has been changed into D, S, Q, G or T;
- at position 4 the S has been changed into T, K, G or P;
- at position 5 the V has been changed into A;
- at position 6 the G has been changed into D;
- at position 7 the I has been changed into T, or V;
- at position 8 the T has been changed into A; and/or
- at position 9 the K has been changed into R; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 162-175 and 214-221; and

iii) said CDR3:

a) has the amino acid sequence of SEQ ID NO: 186;

b) has an amino acid sequence that has at least 70% amino acid identity the amino acid sequence of SEQ ID NO: 186;

c) has an amino acid sequence that has 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NO: 186, wherein

- at position 2 the P has been changed into A, or G;
- at position 7 the D has been changed into N; and/or
- at position 9 the R has been changed into K; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 186-190.

Embodiment 15: A polypeptide according to any one of embodiments 3 to 10, wherein

i) said CDR1 has the amino acid sequence of SEQ ID NO: 146;

ii) said CDR2 has an amino acid sequence that a) has at least 90% amino acid identity with the amino acid sequence of SEQ ID NO:

176 or b) has the amino acid sequence of SEQ ID NO: 176 or 177; and

iii) said CDR3 has the amino acid sequence of SEQ ID NO: 191.

Embodiment 16: A polypeptide according to any one of embodiments 3 to 10, wherein

i) said CDR1:

a) has the amino acid sequence of SEQ ID NO: 147; or

b) has an amino acid sequence that has 6, 5, 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 147, wherein

- at position 1 the G has been changed into K, R, or A;
- at position 2 the T has been changed into I, P, S or L;
- at position 3 the I has been changed into V, or T;
- at position 4 the F has been changed into L;
- at position 5 the S has been changed into R, or D;
- at position 6 the N has been changed into S, T, or D; and/or
- at position 7 the N has been changed into T, or Y; or

c) has an amino acid sequence selected from any one of SEQ ID NO's: 147-161;

ii) said CDR2:

a) has the amino acid sequence of SEQ ID NO: 179; or

b) has an amino acid sequences that has 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 179, wherein

- at position 3 the S has been changed into T, or G;
- at position 4 the N has been changed into S, or I;
- at position 5 the S has been changed into T;
- at position 6 the G has been changed into Y; and/or
- at position 8 the T has been changed into A; or

c) has an amino acid sequence selected from any one of SEQ ID NO's: 178-185; and

iii) said CDR3:

a) has the amino acid sequence of SEQ ID NO: 192; or

b) has an amino acid sequence that has at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 192; or

c) has an amino acid sequence that has 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein

- at position 2 the A has been changed into G;
- at position 8 the T has been changed into S;
- at position 9 the A has been changed into G; and/or
- at position 10 the Y has been changed into F; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 192-196.

Embodiment 17: A polypeptide according to embodiment 3, wherein the amino acid sequences of said CDR1, CDR2 and CDR3 are set forth in:

- SEQ ID No: 141, 162 and 186, respectively; or
- SEQ ID No: 141, 163 and 187, respectively; or
- SEQ ID No: 141, 164 and 186, respectively; or
- SEQ ID No: 141, 166 and 186, respectively; or
- SEQ ID No: 141, 167 and 186, respectively; or
- SEQ ID No: 141, 167 and 189, respectively; or
- SEQ ID No: 141, 168 and 186, respectively; or
- SEQ ID No: 141, 168 and 187, respectively; or
- SEQ ID No: 141, 169 and 190, respectively; or
- SEQ ID No: 141, 170 and 186, respectively; or
- SEQ ID No: 141, 171 and 186, respectively; or
- SEQ ID No: 141, 174 and 186, respectively; or
- SEQ ID No: 141, 175 and 187, respectively; or
- SEQ ID No: 142, 165 and 188, respectively; or
- SEQ ID No: 142, 173 and 188, respectively; or
- SEQ ID No: 143, 164 and 186, respectively; or
- SEQ ID No: 144, 172 and 187, respectively; or
- SEQ ID No: 145, 172 and 187, respectively; or
- SEQ ID No: 141, 214 and 186, respectively; or
- SEQ ID No: 141, 215 and 186, respectively; or

- SEQ ID No: 141, 216 and 186, respectively; or
- SEQ ID No: 141, 217 and 186, respectively; or
- SEQ ID No: 141, 218 and 186, respectively; or
- SEQ ID No: 141, 219 and 186, respectively; or
- SEQ ID No: 141, 220 and 186, respectively; or
- SEQ ID No: 213, 221 and 186, respectively; or
- SEQ ID No: 213, 214 and 186, respectively.

Embodiment 18: A polypeptide according to embodiment 3, wherein the amino acid sequences of said CDR1, CDR2 and CDR3 are set forth in:

- SEQ ID No: 146, 176 and 191, respectively; or
- SEQ ID No: 146, 177 and 191, respectively.

Embodiment 19: A polypeptide according to embodiment 3, wherein the amino acid sequences of said CDR1, CDR2 and CDR3 are set forth in:

- SEQ ID No: 147, 178 and 192, respectively; or
- SEQ ID No: 147, 179 and 192, respectively; or
- SEQ ID No: 147, 179 and 194, respectively; or
- SEQ ID No: 148, 179 and 193, respectively; or
- SEQ ID No: 149, 179 and 192, respectively; or
- SEQ ID No: 149, 180 and 192, respectively; or
- SEQ ID No: 149, 181 and 192, respectively; or
- SEQ ID No: 149, 183 and 192, respectively; or
- SEQ ID No: 149, 185 and 192, respectively; or
- SEQ ID No: 150, 179 and 194, respectively; or
- SEQ ID No: 150, 182 and 194, respectively; or
- SEQ ID No: 151, 179 and 193, respectively; or
- SEQ ID No: 151, 182 and 194, respectively; or
- SEQ ID No: 151, 184 and 196, respectively; or
- SEQ ID No: 152, 179 and 195, respectively; or
- SEQ ID No: 153, 179 and 194, respectively; or
- SEQ ID No: 154, 182 and 194, respectively; or
- SEQ ID No: 155, 179 and 195, respectively; or

- SEQ ID No: 156, 181 and 192, respectively; or
- SEQ ID No: 157, 179 and 194, respectively; or
- SEQ ID No: 158, 179 and 192, respectively; or
- SEQ ID No: 159, 178 and 192, respectively; or
- SEQ ID No: 160, 179 and 194, respectively; or
- SEQ ID No: 161, 179 and 194, respectively.

Embodiment 20: A polypeptide according to embodiment 3, wherein the amino acid sequences of said CDR1, CDR2 and CDR3 are set forth in: SEQ ID NO's: 141, 164 and 186, respectively, or SEQ ID NO's: 141, 162 and 186, respectively.

Embodiment 21: A polypeptide according to embodiment 3, wherein the amino acid sequences of said CDR1, CDR2 and CDR3 are set forth in: SEQ ID NO's: 213, 214 and 186 respectively, SEQ ID NO's: 213, 221 and 186 respectively, or SEQ ID NO's: 141, 162 and 186 respectively.

Embodiment 22: A polypeptide according to any one of embodiments 2 to 10, wherein said anti-CX3CR1 immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in:

a) the amino acid sequence of SEQ ID NO: 3;
b) amino acid sequences that have at least 90% amino acid identity with the amino acid sequences of SEQ ID NO: 3;
c) amino acid sequences that have 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 3; or
d) an amino acid sequence of any one of SEQ ID NO: 1-48, 121-140 or 222-224.

Embodiment 23: A polypeptide according to any one of embodiments 2 to 10, wherein said anti-CX3CR1 immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in:

a) the amino acid sequence of SEQ ID NO: 49;
b) an amino acid sequence that has at least 95% amino acid identity with the amino acid sequences of SEQ ID NO: 49;

c) an amino acid sequence that has 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 49; or
d) an amino acid sequence of any one of SEQ ID NO: 49-52.

Embodiment 24: A polypeptide according to any one of embodiments 2 to 10, wherein said anti-CX3CR1 immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in:
a) the amino acid sequence of SEQ ID NO: 67;
b) an amino acid sequence that has at least 90% amino acid identity with the amino acid sequences of SEQ ID NO: 67;
c) an amino acid sequence that has 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 67; or
d) an amino acid sequence of any one of SEQ ID NO: 53-120.

Embodiment 25: A polypeptide according to embodiment 2, wherein said anti-CX3CR1 immunoglobulin single variable domain comprises the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3.

Embodiment 26: A polypeptide according to embodiment 2, wherein said anti-CX3CR1 immunoglobulin single variable domain comprises the sequence set forth in any one of SEQ ID NO: 121-140 or SEQ ID NO: 222-224.

Embodiment 27: A polypeptide according to any of one of the embodiments above, which is humanized and/or optimized for stability, potency, manufacturability and/or similarity to human framework regions.

Embodiment 28: A polypeptide according to embodiment 27, which is humanized and/or sequence optimized in one or more of the following positions (according to Kabat numbering): 1, 11, 14, 16, 74, 83, 108.

Embodiment 29: A polypeptide according to embodiment 28, comprising one or more of the following mutations: E1D, S11L, A14P, E16G, A74S, K83R, Q108L.

Embodiment 30: A polypeptide according to any one of embodiments 3-29, in which:

i) FR1 is selected from SEQ ID NO's: 198-204;

ii) FR2 is selected from SEQ ID NO's: 205-208;

iii) FR3 is selected form SEQ ID NO's: 209-210; and/or

iv) FR4 is selected from SEQ ID NO's: 211-212.

Embodiment 31: A polypeptide according to any one of embodiments 3-30, which is humanized and/or sequence optimized in one or more of the following positions (according to Kabat numbering): 52, 53.

Embodiment 32: A polypeptide according to embodiment 31, comprising one or more of the following mutations: N52S, S53T.

Embodiment 33: A polypeptide according to any one of embodiments 3-32, in which CDR2 is selected from SEQ ID NO's: 214-221.

Embodiment 34: A polypeptide according to any one of embodiments 2-33, wherein said anti-CX3CR1 immunoglobulin single variable domain comprises the sequence set forth in any of SEQ ID NO's: 138-140 or 222-224.

Embodiment 35: A polypetide according to any one of embodiments 22 to 26, wherein said VHH domain consists of any one of said amino acid sequences.

Embodiment 36: A polypeptide according to any one of embodiments 2 to 35, wherein said immunoglobulin single variable domain cross-blocks the binding of at least one of the immunoglobulin single variable domains of SEQ ID NO's: 1-120, 121-140 and 222-224 to CX3CR1.

Embodiment 37: A polypeptide according to any one of embodiments 2 to 35, wherein said immunoglobulin single variable domain is cross-blocked from binding to CX3CR1 by at least one of the amino acid sequences of SEQ ID NO's: 1-120, 121-140 and 222-224.

Embodiment 38: A polypeptide according to any one of embodiments 2 to 37, wherein the polypeptide further comprises a half-life extending moiety.

Embodiment 39: A polypeptide according to embodiment 38, wherein said half-life extending moiety is covalently linked to said polypeptide and is selected from the group consisting of an albumin binding moiety, such as an anti-albumin immunoglobulin domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, a recombinant polyethylene glycol molecule, human serum albumin, a fragment of human serum albumin, an albumin binding peptide or a Fc domain.

Embodiment 40: A polypeptide according to embodiment 38 or 39, wherein said half-life extending moiety consists of an anti-albumin immunoglobulin single variable domain.

Embodiment 41: A polypeptide according to embodiment 40, wherein the immunoglobulin single variable domain is selected from a VHH domain, a humanized VHH domain, a camelized VH domain, a domain antibody, a single domain antibody and/or "dAb"s.

Embodiment 42: A polypeptide according to embodiment 41, wherein the anti-albumin immunoglobulin single variable domain is selected from SEQ ID NO's: 230-232.

Embodiment 43: A polypeptide according to any one of embodiment 2 to 39, wherein said polypeptide is linked to an Fc portion (such as a human Fc, for example as set forth in SEQ ID NO: 252), optionally via a suitable linker or hinge region.

Embodiment 44: A polypeptide according to any one of embodiments 2 to 39, wherein said polypeptide is further linked to one or more constant domains (for example, 2 or 3 constant domains that can be used as part of/to form an Fc portion), to an Fc portion and/or to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the

invention and/or may confer the ability to bind to one or more Fc receptors, optionally via a suitable linker or hinge region.

Embodiment 45: A polypeptide according to any one of embodiments 2 to 37, wherein said polypeptide further comprises a second immunoglobulin single variable domain, preferably a second anti-CX3CR1 immunoglobulin single variable domain.

Embodiment 46: A polypeptide according to embodiment 45, wherein said first and said second immunoglobulin single variable domains are covalently linked by a linker peptide.

Embodiment 47: A polypeptide according to embodiment 45 or 46, wherein said second immunoglobulin single variable domains essentially consist of four framework regions (FR1 to FR4) and three complementary determining regions (CDR1 to CDR3).

Embodiment 48: A polypeptide according to any one of embodiments 45 to 47, wherein said first and said second immunoglobulin single variable domains are antibody domains.

Embodiment 49: A polypeptide according to any one of embodiments 45 to 48, wherein said first and second immunoglobulin single variable domains are a VH, VL, VHH, camelized VH, or VHH that is optimized for stability, potency, manufacturability and/or similarity to human framework regions.

Embodiment 50: A polypeptide according to any one of embodiments 45 to 49, wherein said CDR1 to CDR3 of said second immunoglobulin single variable domain are set forth in any one of embodiments 11 to 21.

Embodiment 51: A polypeptide according to any one of embodiments 45 to 50, wherein said first and said second immunoglobulin single variable domains comprise the same CDR3.

Embodiment 52: A polypeptide according to embodiment 51, wherein said CDR3 is set forth in any one of embodiment 11 to 13.

Embodiment 53: A polypeptide according to any one of embodiments 45 to 53, wherein said first and said second immunoglobulin single variable domains comprise the same CDR1, CDR2 and CDR3.

Embodiment 54: A polypeptide according to embodiment 53, wherein said CDR1 to CDR3 are set forth in any one of embodiments 11 to 21.

Embodiment 55: A polypeptide according to any one of embodiments 45 to 54, wherein said first and said second immunoglobulin single variable domains comprise the same VHH domain.

Embodiment 56: A The polypeptide according to any one of embodiments 45 to 55, wherein said VHH domain is set forth in any one of embodiments 22 to 37.

Embodiment 57: A polypeptide comprising a first immunoglobulin single variable domain comprising the CDR1, CDR2 and CDR3 set forth SEQ ID NO's: 141, 164 and 186 or SEQ ID NO's: 141, 162 and 186 and a second immunoglobulin single variable domain as set forth in any one of embodiments 2 to 37.

Such a polypeptide may in particular be a polypeptide according to any of embodiments 45 to 56.

Embodiment 58: A polypeptide according to embodiment 57, wherein said first immunoglobulin single variable domain comprises the CDR1, CDR2 and CDR3 set forth in SEQ ID NO's: 213, 214 and 186, SEQ ID NO's: 213, 221 and 186 or SEQ ID NO's: 141, 162 and 186.

Embodiment 59: A polypeptide according to embodiment 57 or 58, wherein said second immunoglobulin single variable domain comprises the CDR1, CDR2 and CDR3 set forth SEQ ID NO's: 141, 164 and 186 or SEQ ID NO's: 141, 162 and 186.

Embodiment 60: A polypeptide according to embodiment 57 0r 58, wherein said second immunoglobulin single variable domain comprises the CDR1, CDR2 and CDR3 set forth in: SEQ ID NO's: 213, 214 and 186, SEQ ID NO's: 213, 221 and 186 or SEQ ID NO's: 141, 162 and 186.

Embodiment 61: A polypeptide comprising a first immunoglobulin single variable domain, wherein said first immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 and a second immunoglobulin single variable domain according to any one of embodiments 2 to 37.

Such a polypeptide may in particular be a polypeptide according to any of embodiments 45 to 60.

Embodiment 62: A polypeptide according to embodiment 61, wherein said first immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in any one of SEQ ID NO: 121-140 or 222-224.

Embodiment 63: A polypeptide according to embodiment 61 or 62, wherein said second immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3.

Embodiment 64: A polypeptide according to embodiment 63, wherein said second immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in any one of SEQ ID NO: 121-140 or 222-224.

Embodiment 65: A polypeptide according to any one of embodiments 45 to 64, wherein the polypeptide further comprises a half-life extending moiety.

Embodiment 66: A polypeptide according to embodiment 65, wherein said half-life extending moiety is covalently linked to said polypeptide and is selected from the group consisting of an albumin binding moiety, such as an anti-albumin immunoglobulin domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, a recombinant

polyethylene glycol molecule, human serum albumin, a fragment of human serum albumin, an albumin binding peptide or a Fc domain.

Embodiment 67: A polypeptide according to embodiment 66, wherein said half-life extending moiety consists of an anti-albumin immunoglobulin single variable domain.

Embodiment 68: A polypeptide according to embodiment 67, wherein the immunoglobulin single variable domain is selected from a VHH domain, a humanized VHH domain, a camelized VH domain, a domain antibody, a single domain antibody and/or "dAb"s.

Embodiment 69: A polypeptide according to embodiment 68, wherein the anti-albumin immunoglobulin single variable domain is selected from SEQ ID NO's: 230-232.

Embodiment 70: A polypeptide according to any one of embodiments 45 to 64, wherein said polypeptide is linked to an Fc portion (such as a human Fc, for example as set forth in SEQ ID NO: 252), optionally via a suitable linker or hinge region.

Embodiment 71: A polypeptide according to any one of embodiments 45 to 66, wherein said polypeptide is further linked to one or more constant domains (for example, 2 or 3 constant domains that can be used as part of/to form an Fc portion), to an Fc portion and/or to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the invention and/or may confer the ability to bind to one or more Fc receptors, optionally via a suitable linker or hinge region.

Embodiment 72: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 225-227.

Embodiment 73: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 249 or 277-281.

Embodiment 74: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 257-262.

Embodiment 75: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 253 or 254.

Embodiment 76: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 263 or 266.

Embodiment 77: A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 267-276 and 282.

Embodiment 78: A nucleic acid molecule comprising a region encoding a polypeptide according to any one of embodiments 1 to 77.

Embodiment 79: An expression vector comprising a nucleic acid molecule according to embodiment 78.

Embodiment 80: A host cell carrying an expression vector comprising a nucleic acid molecule, said nucleic acid molecule comprising a region encoding a polypeptide according to any one of embodiments 1 to 77, wherein said host cell is capable of expressing a polypeptide according to any one of embodiments 1 to 77, and wherein said host cell is a prokaryotic or a eukaryotic cell.

Embodiment 81: A pharmaceutical composition comprising (i) as the active ingredient, one or more polypeptides according to any one of embodiments 1 to 77, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

Embodiment 82: A method of manufacturing a polypeptide according to any one of embodiments 1 to 77, comprising the steps of

- culturing a host cell under conditions that allow expression of a polypeptide according to any one of embodiments 1 to 77, wherein said host cell carrying an expression vector comprising a nucleic acid molecule, said nucleic acid molecule comprising a region encoding

a polypeptide according to any one of embodiments 1 to 77, and wherein said host cell is a prokaryotic or a eukaryotic cell;

- recovering said polypeptide; and
- purifying said polypeptide.

Embodiment 83: A method of using a polypeptide according to any one of embodiments 1 to 77 for the treatment, prevention or alleviation of a disease, disorder or condition, in particular in a human being.

Embodiment 84: The method of embodiment 83, wherein said disease, disorder or condition is a CX3CR1-associated disease, disorder or condition.

Embodiment 85: The method of embodiment 83, wherein said disease, disorder or condition is atherosclerosis.

Embodiment 86: An injectable pharmaceutical composition comprising the polypeptide according to any one of embodiments 1 to 77, said composition being suitable for intravenous or subcutaneous injection in a human being.

Embodiment 87: A method for preventing and/or treating a disease or disorder that is associated with CX3CR1, wherein said method comprises administering to a subject in need thereof a pharmaceutically active amount of at least one polypeptide according to any one of embodiments 1 to 77.

Embodiment 88: A method of embodiment 85, further comprising administering an additional therapeutic agent selected from the group consisting of a statin, an antiplatelet, an anticoagulant, an antidiabetic and an antihypertensive.

Embodiment 89: A method for inhibiting the binding of CX3CR1 to fractalkine in a mammalian cell, comprising administering to the cell a polypeptide according to any one of embodiments 1 to 77, whereby signaling mediated by the fractalkine is inhibited.

Embodiment 90: A method for detecting and/or quantifying CX3CR1 levels in a biological sample by contacting the sample with a polypeptide according to any one of embodiments 1 to 77 and detecting binding of the polypeptide with CX3CR1.

Embodiment 91: A method for diagnosing an CX3CR1-associated disorder or for determining if a subject has an increased risk of developing an CX3CR1-associated disorder, wherein the method comprises contacting a biological sample from a subject with a polypeptide according to any one of embodiments 1 to 77 and detecting binding of the polypeptide to CX3CR1 to determine the expression or concentration of CX3CR1.

Embodiment 92. A polypeptide according to any one of embodiments 1 to 77 for use in the treatment, prevention or alleviation of a disease, disorder or condition, in a human being.

Embodiment 93. The polypeptide for use according to embodiment 92, wherein the disease, disorder or condition is a CX3CR1-associated disease, disorder or condition.

Embodiment 94. The polypeptide for use according to embodiment 92, wherein the disease, disorder or condition is selected from cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulomephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

Embodiment 95. The polypeptide for use according to embodiment 92, wherein the disease, disorder or condition is atherosclerosis.

Embodiment 96. Use of a polypeptide according to any of embodiments 1 to 77 for the manufacture of a medicament for the treatment, prevention or alleviation of a disease, disorder or condition, in a human being.

Embodiment 97. The method according to embodiment 87, wherein the disease or disorder is selected from cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulonephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

Embodiment 98. The method according to embodiment 87, wherein the disease, disorder or condition is atherosclerosis.

Embodiment 99. A diagnostic kit or diagnostic method comprising a polypeptide according to any one of embodiments 1 to 77, or the use thereof.

Embodiment 100. A diagnostic kit or diagnostic method according to embodiment 99, for the diagnosis of at least one of cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulomephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The above and other aspects and embodiments of the invention will become clear from the further description herein, in which:

a) Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); Lewin, "Genes IV", Oxford University Press, New York, (1990), and Roitt et al., "Immunology" (2nd Ed.), Gower Medical Publishing, London, New York (1989), as well as to the general background art cited herein; Furthermore, unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known *per se*, as will be clear to the skilled person. Reference is for example again made to the standard handbooks, to the general background art referred to above and to the further references cited therein;

b) Unless indicated otherwise, the terms "*immunoglobulin*" and "*immunoglobulin sequence*" - whether used herein to refer to a *heavy chain antibody* or to a *conventional 4-chain antibody* - are used as general terms to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as VHH domains or VH/VL domains, respectively). In addition, the term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "(single) variable domain sequence", "VHH sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation;

c) The term "*domain*" (of a polypeptide or protein) as used herein refers to a folded protein structure which has the ability to retain its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

d) The term "*immunoglobulin domain*" as used herein refers to a globular region of an antibody chain (such as e.g. a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a 2-layer sandwich of about 7 antiparallel beta-strands arranged in two beta-sheets, optionally stabilized by a conserved disulphide bond.

e) The term "*immunoglobulin variable domain*" as used herein means an immunoglobulin domain essentially consisting of four "framework regions" which are referred to in the art and hereinbelow as "framework region 1" or "FR1"; as "framework region 2" or"FR2"; as "framework region 3" or "FR3"; and as "framework region 4" or "FR4", respectively; which framework regions are interrupted by three "complementarity determining regions" or "CDRs", which are referred to in the art and hereinbelow as "complementarity determining region 1"or "CDR1"; as "complementarity determining region 2" or "CDR2"; and as "complementarity determining region 3" or "CDR3", respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. It is the immunoglobulin variable domain(s) that confer specificity to an antibody for the antigen by carrying the antigen-binding site.

f) The terms "*immunoglobulin single variable domain*" and “single variable domain” as used herein mean an immunoglobulin variable domain which is capable of specifically binding to an epitope of the antigen without pairing with an additional variable immunoglobulin domain. One example of immunoglobulin

single variable domains in the meaning of the present invention are "*domain antibodies*", such as the immunoglobulin single variable domains VH and VL (VH domains and VL domains). Another example of immunoglobulin single variable domains are "*VHH domains*" (or simply "VHHs") from camelids, as defined hereinafter.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')2 fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e. by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

f1) "*VHH domains*" , also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (i.e. of "antibodies devoid of light chains"; Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R.: "Naturally occurring antibodies devoid of light chains"; Nature 363, 446-448 (1993)). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains" or "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_L domains" or "VL domains"). VHH domains can specifically bind to an epitope without an additional antigen binding domain (as opposed to VH or VL domains in a conventional 4-chain antibody, in which case the epitope is recognized by a VL

domain together with a VH domain). VHH domains are small, robust and efficient antigen recognition units formed by a single immunoglobulin domain.

In the context of the present invention, the terms VHH domain, VHH, V_HH domain, VHH antibody fragment, VHH antibody, as well as "Nanobody®" and "Nanobody® domain" ("Nanobody" being a trademark of the company Ablynx N.V.; Ghent; Belgium) are used interchangeably and are representatives of immunoglobulin single variable domains (having the structure: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 and specifically binding to an epitope without requiring the presence of a second immunoglobulin variable domain), and which are distinguished from VH domains by the so-called "hallmark residues", as defined in e.g. WO2009/109635, Fig. 1.

The amino acid residues of a VHH domain are numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown e.g. in Figure 2 of Riechmann and Muyldermans, J. Immunol. Methods 231, 25-38 (1999). According to this numbering,

- FR1 comprises the amino acid residues at positions 1-30,
- CDR1 comprises the amino acid residues at positions 31-35,
- FR2 comprises the amino acids at positions 36-49,
- CDR2 comprises the amino acid residues at positions 50-65,
- FR3 comprises the amino acid residues at positions 66-94,
- CDR3 comprises the amino acid residues at positions 95-102, and
- FR4 comprises the amino acid residues at positions 103-113.

However, it should be noted that - as is well known in the art for V_H domains and for VHH domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, generally, the numbering according to

Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence.

Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat and applied to VHH domains as described above will be followed, unless indicated otherwise.

The total number of amino acid residues in a VHH domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

Further structural characteristics and functional properties of VHH domains and polypeptides containing the same can be summarized as follows:

VHH domains (which have been "designed" by nature to functionally bind to an antigen without the presence of, and without any interaction with, a light chain variable domain) can function as a single, relatively small, functional antigen-binding structural unit, domain or polypeptide. This distinguishes the VHH domains from the VH and VL domains of conventional 4-chain antibodies, which by themselves are generally not suited for practical application as single antigen-binding proteins or immunoglobulin single variable domains, but need to be combined in some form or another to provide a functional antigen-binding unit (as in for example conventional antibody fragments such as Fab fragments; in scFv's, which consist of a VH domain covalently linked to a VL domain).

Because of these unique properties, the use of VHH domains - either alone or as part of a larger polypeptide - offers a number of significant advantages over the use of conventional VH and VL domains, scFv's or conventional antibody fragments (such as Fab- or F(ab')2-fragments):

- only a single domain is required to bind an antigen with high affinity and with high selectivity, so that there is no need to have two separate domains present, nor to assure that these two domains are present in the right spacial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv's);
- VHH domains can be expressed from a single gene and require no post-translational folding or modifications;
- VHH domains can easily be engineered into multivalent and multispecific formats (as further discussed herein);
- VHH domains are highly soluble and do not have a tendency to aggregate (as with the mouse-derived antigen-binding domains described by Ward et al., Nature 341: 544-546 (1989));
- VHH domains are highly stable to heat, pH, proteases and other denaturing agents or conditions and, thus, may be prepared, stored or transported without the use of refrigeration equipments, conveying a cost, time and environmental savings;
- VHH domains are easy and relatively cheap to prepare, even on a scale required for production. For example, VHH domains and polypeptides containing the same can be produced using microbial fermentation (e.g. as further described below) and do not require the use of mammalian expression systems, as with for example conventional antibody fragments;
- VHH domains are relatively small (approximately 15 kDa, or 10 times smaller than a conventional IgG) compared to conventional 4-chain antibodies and antigen-binding fragments thereof, and therefore
-- show high(er) penetration into tissues and
-- can be administered in higher doses
than such conventional 4-chain antibodies and antigen-binding fragments thereof;
- VHH domains can show so-called cavity-binding properties (inter alia due to their extended CDR3 loop, compared to conventional VH domains) and can therefore also access targets and epitopes not accessible to conventional 4-chain antibodies and antigen-binding fragments thereof.

Methods of obtaining VHH domains binding to a specific antigen or epitope have been described earlier, e.g. in WO2006/040153 and WO2006/122786. As also described therein in detail, VHH domains derived from camelids can be "humanized" by replacing one or more amino acid residues in the amino acid sequence of the original VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being. A humanized VHH domain can contain one or more fully human framework region sequences, and, in an even more specific embodiment, can contain human framework region sequences derived from DP-29, DP-47, DP-51, or parts thereof, optionally combined with JH sequences, such as JH5.

f2) "*Domain antibodies*", also known as "Dab"s, "Domain Antibodies", and "dAbs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in e.g. Ward, E.S., *et al.*: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli"; Nature 341: 544-546 (1989); Holt, L.J. *et al.*: "Domain antibodies: proteins for therapy"; TRENDS in Biotechnology 21(11): 484-490 (2003); and WO2003/002609.
Domain antibodies essentially correspond to the VH or VL domains of non-camelid mammalians, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, i.e. without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences. Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutical use in humans. As in the case of VHH domains, they are well expressed also in prokaryotic expression systems, providing a significant reduction in overall manufacturing cost.

Domain antibodies, as well as VHH domains, can be subjected to affinity maturation by introducing one or more alterations in the amino acid sequence of one or more CDRs, which alterations result in an improved affinity of the resulting

immunoglobulin single variable domain for its respective antigen, as compared to the respective parent molecule. Affinity-matured immunoglobulin single variable domain molecules of the invention may be prepared by methods known in the art, for example, as described by Marks *et al.*, 1992, Biotechnology 10:779-783, or Barbas, *et al.*, 1994, Proc. Nat. Acad. Sci, USA 91: 3809-3813.; Shier *et al.*, 1995, Gene 169:147-155; Yelton *et al.*, 1995, Immunol. 155: 1994-2004; Jackson *et al.*, 1995, J. Immunol. 154(7):3310-9; and Hawkins *et al.*, 1992, J. Mol. Biol. 226(3): 889 896; KS Johnson and RE Hawkins, "Affinity maturation of antibodies using phage display", Oxford University Press 1996.

f3) Furthermore, it will also be clear to the skilled person that it is possible to "graft" one or more of the CDR's mentioned above onto other "scaffolds", including but not limited to human scaffolds or non-immunoglobulin scaffolds. Suitable scaffolds and techniques for such CDR grafting are known in the art.

g) The terms "*epitope*" and "*antigenic determinant*", which can be used interchangeably, refer to the part of a macromolecule, such as a polypeptide, that is recognized by antigen-binding molecules, such as conventional antibodies or the polypeptides of the invention, and more particularly by the antigen-binding site of said molecules. Epitopes define the minimum binding site for an immunoglobulin, and thus represent the target of specificity of an immunoglobulin.

The part of an antigen-binding molecule (such as a conventional antibody or a polypeptide of the invention) that recognizes the epitope is called a *paratope*.

h) The term "*biparatopic*" (antigen-)binding molecule or "biparatopic" polypeptide as used herein shall mean a polypeptide comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein these two variable domains are capable of binding to two different epitopes of one antigen, which epitopes are not normally bound at the same time by one monospecific immunoglobulin, such as e.g. a conventional antibody or one immunoglobulin single variable domain. Biparatopic polypeptides

can be composed of variable domains which have different epitope specificities, and do not contain mutually complementary variable domain pairs which bind to the same epitope. The two variable domains do therefore not compete with each other for binding to the target.

i) A polypeptide (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain, a polypeptide of the invention, or generally an antigen binding molecule or a fragment thereof) that can "*bind to*" or "*specifically bind to*", that "*has affinity for*" and/or that "*has specificity for*" a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "*against*" or "*directed against*" said epitope, antigen or protein or is a "*binding*" molecule with respect to such epitope, antigen or protein, or is said to be “anti”-epitope, “anti”-antigen or “anti”-protein (e.g anti-CX3CR1).

k) Generally, the term "*specificity*" refers to the number of different types of antigens or epitopes to which a particular antigen-binding molecule or antigen-binding protein (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain, or a polypeptide of the invention) can bind. The specificity of an antigen-binding protein can be determined based on its affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD), is a measure for the binding strength between an epitope and an antigen-binding site on the antigen-binding protein: the lesser the value of the KD, the stronger the binding strength between an epitope and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (KA), which is 1/KD). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known *per se*, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain, or a polypeptide of the invention) and the pertinent antigen. Avidity is related to both the affinity between an epitope and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

l) Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as generally known and agreed upon in the art. When comparing two amino acid sequences, the term "*amino acid difference*" refers to insertions, deletions or substitutions of the indicated number of amino acid residues at a position of the reference sequence, compared to a second sequence. In case of substitution(s), such substitution(s) will preferably be conservative amino acid substitution(s), which means that an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 98/49185, wherein conservative amino acid substitutions preferably are substitutions in which one amino acid within the following groups (i) - (v) is substituted by another amino acid residue within the same group: (i) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (ii) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (iii) polar, positively charged residues: His, Arg and Lys; (iv) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (v) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative amino acid substitutions are as follows:

Ala into Gly or into Ser;

Arg into Lys;

Asn into Gln or into His;

Asp into Glu;

Cys into Ser;

Gln into Asn;

Glu into Asp;

Gly into Ala or into Pro;

His into Asn or into Gln;

Ile into Leu or into Val;

Leu into Ile or into Val;

Lys into Arg, into Gln or into Glu;

Met into Leu, into Tyr or into Ile;

Phe into Met, into Leu or into Tyr;
Ser into Thr;
Thr into Ser;
Trp into Tyr;
Tyr into Trp or into Phe;
Val into Ile or into Leu.

m) A nucleic acid or polypeptide molecule is considered to be "*(in) essentially isolated (form)*" - for example, when compared to its native biological source and/or the reaction medium or cultivation medium from which it has been obtained - when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another nucleic acid, another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. In particular, a nucleic acid or polypeptide molecule is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10- fold, more in particular at least 100-fold, and up to 1000-fold or more. A nucleic acid or polypeptide molecule that is "in essentially isolated form" is preferably essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide-gelelectrophoresis;

n) "*Sequence identity*" between e.g. two immunoglobulin single variable domain sequences indicates the percentage of amino acids that are identical between these two sequences. It may be calculated or determined as described in paragraph f) on pages 49 and 50 of WO08/020079. "*Sequence similarity*" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions.

Target specificity

The polypeptides of the invention have specificity for human CX3CR1. Thus, the polypeptides of the invention preferably bind to human CX3CR1 (SEQ ID NO:

255). In one aspect, the polypeptides of the present invention also bind to cynomolgus CX3CR1 (SEQ ID NO: 256).

Polypeptides of the invention

The invention provides novel pharmaceutically active agents for the prevention, treatment, alleviation and/or diagnosis of CX3CR1 associated diseases, disorders or conditions, such as cardiovascular diseases. In particular, the invention provides polypeptides which bind to human CX3CR1 and are capable of blocking the binding of human fractalkine to human CX3CR1. In one aspect, the polypeptide is an immunoglobulin comprising an antigen-binding domain comprising three complementarity determining regions CDR1, CDR2 and CDR3, wherein said immunoglobulin binds to human CX3CR1 and is capable of blocking the binding of human fractalkine to human CX3CR1. In a further aspect, the polypeptide comprises one or more anti-CX3CR1 immunoglobulin single variable domain, wherein said polypeptide is capable of blocking the binding of human fractalkine to human CX3CR1.

In one aspect, a polypeptide of the present invention is characterized by one or more of the following properties:

- Bind with high affinity to human CX3CR1, for example at an EC_{50} of less than or equal to 10nM, less than or equal to 5nM, less than or equal to 2.5nM or less than or equal to 1nM, as determined by cell binding FACS;
- Block the binding of human fractalkine to human CX3CR1, for example at an IC50 of less than or equal to 300nM, or less than or equal to 100nM, or less than or equal to 20nM, or less than or equal to 10nM, or less than or equal to 5nM, or less than or equal to 2.5nM or less than or equal to 1nM;
- Inhibit fractalkine induced chemotaxis mediated by human CX3CR1, for example at an IC_{50} of less than or equal to 500 nM, or less than or equal to 100 nM, or of less than or equal to 75 nM, or less than or equal to 50 nM, or less than or equal to 10 nM or less than or equal to 5nM; the obtained efficacy of inhibition is more than or equal to 15%, or more than or equal to 50%, or more than or equal to 80%, or more than or equal to 95%;

- Inhibit fractalkine induced internalization mediated by human CX3CR1, for example at an IC_{50} of less than or equal to 10 nM or less than or equal to 5nM;
- Cross-react with cynomolgus CX3CR1, for example within 10-fold of E/IC_{50} for human CX3CR1 for binding and functional inhibition.

In a further aspect, a polypeptide of the present invention further comprises a half-life extending moiety, for example an albumin binding moiety, a polyethylene glycol molecule or a Fc domain. In a further aspect, a polypeptide of the present invention comprises two or more anti-CX3CR1 immunoglobulin single variable domains. In one aspect, the two anti-CX3CR1 immunoglobulin single variable domains are covalently linked by a linker peptide. In one aspect, the two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present invention have the same amino acid sequence. In another aspect, the two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present invention have different amino acid sequences. In one aspect, a polypeptide of the present invention comprises two anti-CX3CR1 immunoglobulin single variable domains and further comprises a half-life extending moiety, for example an albumin binding moiety, a polyethylene glycol molecule or a Fc domain.
In one aspect, a polypeptide of the present invention comprises a first anti-CX3CR1 immunoglobulin single variable domain covalently linked to an albumin binding moiety by a first linker peptide, wherein said albumin binding moiety is further covalently linked to a second anti-CX3CR1 immunoglobulin single variable domain by a second linker peptide.
In one aspect, a polypeptide of the present invention comprises an anti-CX3CR1 immunoglobulin single variable domain covalently linked to a Fc domain by a linker peptide. In one aspect, such polypeptide comprising an anti-CX3CR1 immunoglobulin single variable domain covalently linked to a Fc domain by a linker peptide is provided as a dimer, for example through disulfide bridges.

Polypeptides according to the present invention are obtained as described hereinbelow. In summary, single variable domains of the present invention were

identified from a library expressing single variable domains (VHH) derived from a llama immunized with DNA encoding human CX3CR1. The phage library was panned on hCX3CR1 viral lipoparticles and binding phage were screened for their ability to compete for receptor binding with Alexa-fluor labeled fractalkine (AF-FKN). Representative single variable domains of the present invention are described herein in further details.

In one aspect, an immunoglobulin single variable domain of the present invention consists essentially of four framework regions (FR1, FR2, FR3 and FR4) and three complementary determining regions (CDR1, CDR2 and CDR3). In particular, the immunoglobulin single variable domain has the structure FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. In one aspect, the immunoglobulin single variable domain is an antibody domain.

In one aspect, the CDR3 of a polypeptide of the present invention, in particular a immunoglobulin single domain of the present invention has the amino acid sequence of Asp-Xaa1-Arg-Arg-Gly-Trp-Xaa2-Xaa3-Xaa4-Xaa5 as set forth in SEQ ID NO: 197, wherein:

- Xaa1 is Pro, Ala or Gly;
- Xaa2 is Asp or Asn;
- Xaa3 is Thr or Ser;
- Xaa4 is Arg, Lys, Ala or Gly; and
- Xaa5 is Tyr or Phe.

In one aspect, the CDR3 of a polypeptide of the present invention, in particular a immunoglobulin single domain of the present invention, has the amino acid sequence of Asp-Xaa1-Arg-Arg-Gly-Trp-Xaa2-Xaa3-Xaa4-Xaa5 as set forth in SEQ ID NO: 197, wherein:

- Xaa1 is Pro, Ala or Gly;
- Xaa2 is Asp or Asn;
- Xaa3 is Thr;
- Xaa4 is Arg or Lys; and
- Xaa5 is Tyr.

In one aspect, the CDR3 of a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the amino acid sequence of Asp-Pro-Arg-Arg-Gly-Trp-Asp-Thr-Arg-Tyr as set forth in SEQ ID NO: 186.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3:

- CDR1:
  a) has the amino acid sequence of GSIFSSNAMA (SEQ ID NO: 141); or
  b) has an amino acid sequence that has at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 141; or
  c) has an amino acid sequence that has 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 141, wherein
     - at position 2 the S has been changed into T, or G;
     - at position 6 the S has been changed into R;
     - at position 7 the N has been changed into T; and/or
     - at position 9 the M has been changed into K; or
  d) has an amino acid sequence selected from any one of SEQ ID NO's: 141-145 and 213;
- CDR2:
  a) has the amino acid sequence of GINSVGITK (SEQ ID NO: 164); or
  b) has an amino acid sequence that has at least 70% amino acid identity with the amino acid sequence of SEQ ID NO: 164; or
  c) has an amino acid sequence that has 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 164, wherein
     - at position 1 the G has been changed into A, L, V or S;
     - at position 3 the N has been changed into D, S, Q, G or T;
     - at position 4 the S has been changed into T, K, G or P;
     - at position 5 the V has been changed into A;
     - at position 6 the G has been changed into D;
     - at position 7 the I has been changed into T, or V;

- at position 8 the T has been changed into A; and/or
- at position 9 the K has been changed into R; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 162-175 and 214-221; and

- CDR3:

a) has the amino acid sequence of DPRRGWDTRY (SEQ ID NO: 186); or
b) has an amino acid sequence that has at least 70% amino acid identity the amino acid sequence of SEQ ID NO: 186; or
c) has an amino acid sequence that has 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NO: 186, wherein
   - at position 2 the P has been changed into A, or G;
   - at position 7 the D has been changed into N; and/or
   - at position 9 the R has been changed into K; or
d) has an amino acid sequence selected from any one of SEQ ID NO's: 186-190.

In a further aspect, a polypeptide of the present invention, in particular a immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3, wherein:

- said CDR1 has the amino acid sequence of GRTFSSYAMG (SEQ ID NO: 146);
- said CDR2 has an amino acid sequence that a) has at least 90% amino acid identity with the amino acid sequence of GISGSASRKY (SEQ ID NO: 176) or b) has the amino acid sequence of SEQ ID NO: 176 or 177; and
- said CDR3 has the amino acid sequence of SNSYPKVQFDY (SEQ ID NO: 191).

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3:

- said CDR1:

a) has the amino acid sequence of GTIFSNNAMG (SEQ ID NO: 147); or

b) has an amino acid sequence that has 6, 5, 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 147, wherein
- at position 1 the G has been changed into K, R, or A;
- at position 2 the T has been changed into I, P, S or L;
- at position 3 the I has been changed into V, or T;
- at position 4 the F has been changed into L;
- at position 5 the S has been changed into R, or D;
- at position 6 the N has been changed into S, T, or D; and/or
- at position 7 the N has been changed into T, or Y; or

c) has an amino acid sequence selected from any one of SEQ ID NO's: 147-161;

- said CDR2:

a) has the amino acid sequence of SISNSGSTN (SEQ ID NO: 179); or

b) has an amino acid sequences that has 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 179, wherein
- at position 3 the S has been changed into T, or G;
- at position 4 the N has been changed into S, or I;
- at position 5 the S has been changed into T;
- at position 6 the G has been changed into Y; and/or
- at position 8 the T has been changed into A; or

c) has an amino acid sequence selected from any one of SEQ ID NO's: 178-185; and

- said CDR3:

a) has the amino acid sequence of DARRGWNTAY (SEQ ID NO: 192); or

b) has an amino acid sequence that has at least 80% amino acid identity with the amino acid sequence of SEQ ID NO: 192; or

c) has an amino acid sequence that has 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
- at position 2 the A has been changed into G;
- at position 8 the T has been changed into S;
- at position 9 the A has been changed into G; and/or
- at position 10 the Y has been changed into F; or

d) has an amino acid sequence selected from any one of SEQ ID NO's: 192-196.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3:

- SEQ ID No: 141, 162 and 186, respectively; or
- SEQ ID No: 141, 163 and 187, respectively; or
- SEQ ID No: 141, 164 and 186, respectively; or
- SEQ ID No: 141, 166 and 186, respectively; or
- SEQ ID No: 141, 167 and 186, respectively; or
- SEQ ID No: 141, 167 and 189, respectively; or
- SEQ ID No: 141, 168 and 186, respectively; or
- SEQ ID No: 141, 168 and 187, respectively; or
- SEQ ID No: 141, 169 and 190, respectively; or
- SEQ ID No: 141, 170 and 186, respectively; or
- SEQ ID No: 141, 171 and 186, respectively; or
- SEQ ID No: 141, 174 and 186, respectively; or
- SEQ ID No: 141, 175 and 187, respectively; or
- SEQ ID No: 142, 165 and 188, respectively; or
- SEQ ID No: 142, 173 and 188, respectively; or
- SEQ ID No: 143, 164 and 186, respectively; or
- SEQ ID No: 144, 172 and 187, respectively; or
- SEQ ID No: 145, 172 and 187, respectively; or
- SEQ ID No: 141, 214 and 186, respectively; or
- SEQ ID No: 141, 215 and 186, respectively; or
- SEQ ID No: 141, 216 and 186, respectively; or
- SEQ ID No: 141, 217 and 186, respectively; or
- SEQ ID No: 141, 218 and 186, respectively; or
- SEQ ID No: 141, 219 and 186, respectively; or
- SEQ ID No: 141, 220 and 186, respectively; or
- SEQ ID No: 213, 221 and 186, respectively; or
- SEQ ID No: 213, 214 and 186, respectively.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3:

- SEQ ID No: 146, 176 and 191, respectively; or
- SEQ ID No: 146, 177 and 191, respectively.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the following CDR1, CDR2 and CDR3:

- SEQ ID No: 147, 178 and 192, respectively; or
- SEQ ID No: 147, 179 and 192, respectively; or
- SEQ ID No: 147, 179 and 194, respectively; or
- SEQ ID No: 148, 179 and 193, respectively; or
- SEQ ID No: 149, 179 and 192, respectively; or
- SEQ ID No: 149, 180 and 192, respectively; or
- SEQ ID No: 149, 181 and 192, respectively; or
- SEQ ID No: 149, 183 and 192, respectively; or
- SEQ ID No: 149, 185 and 192, respectively; or
- SEQ ID No: 150, 179 and 194, respectively; or
- SEQ ID No: 150, 182 and 194, respectively; or
- SEQ ID No: 151, 179 and 193, respectively; or
- SEQ ID No: 151, 182 and 194, respectively; or
- SEQ ID No: 151, 184 and 196, respectively; or
- SEQ ID No: 152, 179 and 195, respectively; or
- SEQ ID No: 153, 179 and 194, respectively; or
- SEQ ID No: 154, 182 and 194, respectively; or
- SEQ ID No: 155, 179 and 195, respectively; or
- SEQ ID No: 156, 181 and 192, respectively; or
- SEQ ID No: 157, 179 and 194, respectively; or
- SEQ ID No: 158, 179 and 192, respectively; or
- SEQ ID No: 159, 178 and 192, respectively; or
- SEQ ID No: 160, 179 and 194, respectively; or

- SEQ ID No: 161, 179 and 194, respectively.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the CDR1, CDR2 and CDR3 set forth in:

- SEQ ID NO's: 141, 164 and 186; or
- SEQ ID NO's: 141, 162 and 186.

In a further aspect, a polypeptide of the present invention, in particular an immunoglobuling single domain of the present invention, has the CDR1, CDR2 and CDR3 set forth in:

- SEQ ID NO's: 213, 214 and 186; or
- SEQ ID NO's: 213, 221 and 186; or
- SEQ ID NO's: 141, 162 and 186.

Representative polypeptides of the present invention having the CDRs described above are shown in Tables 1, 2, 3 (representative polypeptides of families 101, 9 and 13, respectively) and 4 (representative polypeptides of optimized variants of family 101.

Table 1: Family 101

Nanobody	SEQ	CDR1*	SEQ CDR1	CDR2*	SEQ CDR2	CDR3*	SEQ CDR3
CX3CR1BII PMP66B02	1	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII PMP54A12	2	GSIFSSNA MA	141	VINSVGIT K	163	DARRGW DTRY	187
CX3CR1BII PMP54A3	3	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP54A4	4	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186

CX3CR1BII PMP54A5	5	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP54A7	6	GTIFSSNA MA	142	GINSVDIT K	165	DPRRGW NTRY	188
CX3CR1BII PMP54B1	7	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP54B2	8	GTIFSSNA MA	142	GINSVDIT K	165	DPRRGW NTRY	188
CX3CR1BII PMP54B3	9	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP54B5	10	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP54D5	11	GSIFSSNA MA	141	LINSVGIT K	167	DGRRGW DTRY	189
CX3CR1BII PMP54D8	12	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP54F6	13	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP54G3	14	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP54H1	15	GTIFSSNA MA	142	GINSVDIT K	165	DPRRGW NTRY	188
CX3CR1BII PMP54H4	16	GSIFSSNA MA	141	VINSVGIT K	163	DARRGW DTRY	187
CX3CR1BII PMP61F10	17	GTIFSSNA MA	142	GINSVDIT K	165	DPRRGW NTRY	188
CX3CR1BII PMP61D1	18	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP61D5	19	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP61E2	20	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP61F11	21	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII	22	GSIFSSNA	141	LINSVGIT	167	DPRRGW	186

PMP61G2		MA		K		DTRY	
CX3CR1BII PMP61G3	23	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP61G4	24	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP61F4	25	GSIFSSNA MA	141	VINTVGIT K	168	DARRGW DTRY	187
CX3CR1BII PMP61A11	26	GSIFSSNA MA	141	VINSVGIT K	163	DARRGW DTRY	187
CX3CR1BII PMP61B2	27	GSIFSSNA MA	141	VINTVGIT K	168	DARRGW DTRY	187
CX3CR1BII PMP61C9	28	GSIFSSNA MA	141	LIDSAGIT K	169	DARRGW NTKY	190
CX3CR1BII PMP65H02	29	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP65E11	30	GSIFSSNA MA	141	GINSVGIA K	170	DPRRGW DTRY	186
CX3CR1BII PMP65E10	31	GSIFSSNA KA	143	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP65E05	32	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP65B11	33	GSIFSSNA MA	141	VINKVGIT K	171	DPRRGW DTRY	186
CX3CR1BII PMP65B07	34	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186
CX3CR1BII PMP65B09	35	GSIFSRNA MA	144	SINSVGIT K	172	DARRGW DTRY	187
CX3CR1BII PMP65H01	36	GGIFSRN AMA	145	SINSVGIT K	172	DARRGW DTRY	187
CX3CR1BII PMP65G07	37	GTIFSSNA MA	142	GINSVDIT R	173	DPRRGW NTRY	188
CX3CR1BII PMP66H08	38	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP66H04	39	GSIFSSNA MA	141	AINSVGIT K	166	DPRRGW DTRY	186

CX3CR1BII PMP66F02	40	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP66E11	41	GSIFSSNA MA	141	AINSVGTT K	174	DPRRGW DTRY	186
CX3CR1BII PMP66D10	42	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP66D08	43	GSIFSSNA MA	141	GINSVGIT K	164	DPRRGW DTRY	186
CX3CR1BII PMP66A04	44	GSIFSSNA MA	141	LINSVGIT K	167	DPRRGW DTRY	186
CX3CR1BII PMP66D04	45	GTIFSSNA MA	142	GINSVDIT K	165	DPRRGW NTRY	188
CX3CR1BII PMP66D02	46	GSIFSSNA MA	141	VINSVGIT K	163	DARRGW DTRY	187
CX3CR1BII PMP66D06	47	GSIFSSNA MA	141	SIDSVGIT K	175	DARRGW DTRY	187
CX3CR1BII PMP66G01	48	GSIFSSNA MA	141	LINSVGIT K	167	DGRRGW DTRY	189

*CDR sequences were determined according to Antibody Engineering, vol 2 by Konetermann & Dübel (Eds.), Springer Verlag Heidelberg Berlin, 2010. The sequence numbers in the table (SEQ) refer to the sequences in the sequence listing of the instant application.

Table 2: Family 9

Nanobody	**SEQ**	**CDR1***	**SEQ CDR1**	**CDR2***	**SEQ CDR2**	**CDR3***	**SEQ CDR3**
CX3CR1BII PMP11H11	49	GRTFSSY AMG	146	GISGSAS RKY	176	SNSYPKV QFDY	191
CX3CR1BII PMP12B6	50	GRTFSSY AMG	146	GISGSAS RKY	176	SNSYPKV QFDY	191
CX3CR1BII PMP12G9	51	GRTFSSY AMG	146	GISGSGS RKY	177	SNSYPKV QFDY	191

CX3CR1BII PMP15G11	52	GRTFSSY AMG	146	GISGSGS RKY	177	SNSYPKV QFDY	191

*CDR sequences were determined according to Antibody Engineering, vol 2 by Konetermann & Dübel (Eds.), Springer Verlag Heidelberg Berlin, 2010. The sequence numbers in the table (SEQ) refer to the sequences in the sequence listing of the instant application.

Table 3: Family 13

Nanobody	**SEQ**	**CDR1***	**SEQ CDR1**	**CDR2***	**SEQ CDR2**	**CDR3***	**SEQ CDR3**
CX3CR1BII PMP18E6	53	GTIFSNNA MG	147	SISSSGST N	178	DARRGW NTAY	192
CX3CR1BII PMP12C2	54	GTIFSNTA MG	148	SISNSGST N	179	DARRGW NSGY	193
CX3CR1BII PMP18A10	55	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18A2	56	GIIFSNNA MG	149	SIGSTYST N	180	DARRGW NTAY	192
CX3CR1BII PMP18A8	57	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP18A9	58	GIIFSNNA MG	149	SISSTYST N	181	DARRGW NTAY	192
CX3CR1BII PMP18B7	59	GTIFRSNA MG	151	SISNSGST N	179	DARRGW NSGY	193
CX3CR1BII PMP18B9	60	GTIFSNNA MG	147	SISSSGST N	178	DARRGW NTAY	192
CX3CR1BII PMP18C6	61	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18C9	62	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18D1	63	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192

CX3CR1BII PMP18D10	64	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18D12	65	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18F1	66	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18F5	67	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18F6	68	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18F9	69	GTIFRTNA MG	152	SISNSGST N	179	DGRRGW NTGY	195
CX3CR1BII PMP18G5	70	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP18H1	71	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18H10	72	KTIFRSNA MG	153	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP18H7	73	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP18H9	74	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP20B3	75	GIIFSNNA MG	149	SIGSTYST N	180	DARRGW NTAY	192
CX3CR1BII PMP20C12	76	GTIFRSNA MG	151	SISNSGST N	179	DARRGW NSGY	193
CX3CR1BII PMP20C3	77	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP20C6	78	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP20D8	79	GTTFRSN AMG	154	SITNSGST N	182	DARRGW NTGY	194
CX3CR1BII PMP20E11	80	RTIFRSNA MG	150	SITNSGST N	182	DARRGW NTGY	194
CX3CR1BII	81	GTIFSNNA	147	SISNSGST	179	DARRGW	194

PMP20E5		MG		N		NTGY	
CX3CR1BII PMP20F3	82	GTIFSNNA MG	147	SISSSGST N	178	DARRGW NTAY	192
CX3CR1BII PMP20F4	83	ATIFRSNA MG	155	SISNSGST N	179	DGRRGW NTGY	195
CX3CR1BII PMP20F5	84	ATIFRSNA MG	155	SISNSGST N	179	DGRRGW NTGY	195
CX3CR1BII PMP21B6	85	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP24A12	86	GIIFSNNA MG	149	SISNSGSA N	183	DARRGW NTAY	192
CX3CR1BII PMP24A6	87	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP24B9	88	GTIFRSNA MG	151	SISISGST N	184	DARRGW NTGF	196
CX3CR1BII PMP24D3	89	GIIFSNNA MG	149	SISSTYST N	181	DARRGW NTAY	192
CX3CR1BII PMP24F7	90	GLIFSNNA MG	156	SISSTYST N	181	DARRGW NTAY	192
CX3CR1BII PMP28B4	91	ATIFRSNA MG	155	SISNSGST N	179	DGRRGW NTGY	195
CX3CR1BII PMP28F1	92	GIIFSNNA MG	149	SIGSTYST N	180	DARRGW NTAY	192
CX3CR1BII PMP28F6	93	GIIFSNNA MG	149	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP28F9	94	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP29A5	95	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP29D5	96	GTIFRSNA MG	151	SISNSGST N	179	DARRGW NSGY	193
CX3CR1BII PMP29E3	97	KTIFRSNA MG	153	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP29E7	98	KTIFRSNA MG	153	SISNSGST N	179	DARRGW NTGY	194

CX3CR1BII PMP29G10	99	GTIFRSNA MG	151	SITNSGST N	182	DARRGW NTGY	194
CX3CR1BII PMP29G7	100	GIIFSNNA MG	149	SITNTGST N	185	DARRGW NTAY	192
CX3CR1BII PMP29H1	101	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP37A8	102	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP37B9	103	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP37C12	104	GSIFRSNA MG	157	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP37C7	105	RTIFSNNA MG	158	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP37D9	106	GTVFSNN AMG	159	SISSSGST N	178	DARRGW NTAY	192
CX3CR1BII PMP37E12	107	KPIFRSNA MG	160	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP41B10	108	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP41B11	109	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192
CX3CR1BII PMP41B8	110	GIIFSNNA MG	149	SIGSTYST N	180	DARRGW NTAY	192
CX3CR1BII PMP41C10	111	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP41F9	112	GIIFSNNA MG	149	SIGSTYST N	180	DARRGW NTAY	192
CX3CR1BII PMP41H10	113	GLTLDDY AMG	161	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP46B5	114	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP46D3	115	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII	116	GIIFSNNA	149	SISSTYST	181	DARRGW	192

PMP46H5		MG		N		NTAY	
CX3CR1BII PMP48B8	117	KTIFRSNA MG	153	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP48D11	118	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP48G8	119	RTIFRSNA MG	150	SISNSGST N	179	DARRGW NTGY	194
CX3CR1BII PMP48H9	120	GTIFSNNA MG	147	SISNSGST N	179	DARRGW NTAY	192

*CDR sequences were determined according to Antibody Engineering, vol 2 by Konetermann & Dübel (Eds.), Springer Verlag Heidelberg Berlin, 2010. The sequence numbers in the table (SEQ) refer to the sequences in the sequence listing of the instant application.

Table 4: Optimized variants

Nanobody	**SEQ**	**CDR1**	**SEQ CDR1**	**CDR2**	**SEQ CDR2**	**CDR3**	**SEQ CDR3**
CX3CR1BII PMP66B02	1	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 043	121	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 045	122	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 047	123	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 048	124	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 049	125	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII	126	GSIFSSNA	141	AINSVGVT	162	DPRRGW	186

050		MA		K		DTRY	
CX3CR1BII 061	127	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 056	128	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 057	129	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 060	130	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186
CX3CR1BII 065	131	GSIFSSNA MA	141	AISSVGVT K	214	DPRRGW DTRY	186
CX3CR1BII 067	132	GSIFSSNA MA	141	AIQSVGVT K	215	DPRRGW DTRY	186
CX3CR1BII 068	133	GSIFSSNA MA	141	AIGSVGVT K	216	DPRRGW DTRY	186
CX3CR1BII 074	134	GSIFSSNA MA	141	AITSVGVT K	217	DPRRGW DTRY	186
CX3CR1BII 118	135	GSIFSSNA MA	141	AINTVGVT K	218	DPRRGW DTRY	186
CX3CR1BII 129	136	GSIFSSNA MA	141	AINGVGV TK	219	DPRRGW DTRY	186
CX3CR1BII 158	137	GSIFSSNA MA	141	AINPVGVT K	220	DPRRGW DTRY	186
CX3CR1BII 306	138	GSIFSSTA MA	213	AISSVGVT K	214	DPRRGW DTRY	186
CX3CR1BII 307	139	GSIFSSTA MA	213	AISTVGVT K	221	DPRRGW DTRY	186
CX3CR1BII 308	140	GSIFSSNA MA	141	AINSVGVT K	162	DPRRGW DTRY	186

*CDR sequences were determined according to Antibody Engineering, vol 2 by Konetermann & Dübel (Eds.), Springer Verlag Heidelberg Berlin, 2010. The sequence numbers in the table (SEQ) refer to the sequences in the sequence listing of the instant application.

In a further aspect, the present invention provides polypeptides having one or more VHH domains.

In one aspect, a VHH domain of the present invention comprises or essentially consists of the sequence set forth in:

a) the amino acid sequence of SEQ ID NO: 3; or
b) amino acid sequences that have at least 90% amino acid identity with the amino acid sequences of SEQ ID NO: 3; or
c) amino acid sequences that have 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 3 or
d) an amino acid sequence of any one of SEQ ID NO: 1-48, or SEQ ID NO: 121-140, or SEQ ID NO: 222-224.

In a further aspect, a VHH domain of the present invention comprises or essentially consists of the sequence set forth in:

a) the amino acid sequence of SEQ ID NO: 49; or
b) an amino acid sequence that has at least 95% amino acid identity with the amino acid sequences of SEQ ID NO: 49; or
c) an amino acid sequence that has 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 49; or
d) an amino acid sequence of any one of SEQ ID NO: 49-52.

In a further aspect, a VHH domain of the present invention comprises or essentially consists of the sequence set forth in:

a) the amino acid sequence of SEQ ID NO: 67; or
b) an amino acid sequence that has at least 90% amino acid identity with the amino acid sequences of SEQ ID NO: 67; or

c) an amino acid sequence that has 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid difference with the amino acid sequences of SEQ ID NO: 67; or

d) an amino acid sequence of any one of SEQ ID NO: 53-120.

In a further aspect, a VHH domain of the present invention comprises or essentially consists of the amino acid sequence set forth in any one of SEQ ID NO: 121-140, or SEQ ID NO: 222-224.

In a further aspect, a VHH domain of the present invention comprises or essentially consists of the amino acid sequence set forth in any one of SEQ ID NO: 138-140.

In a further aspect, a VHH domain of the present invention comprises or essentially consists of the amino acid sequence set forth in any one of SEQ ID NO: 222-224.

Representative VHH domains of the present invention are shown in Table 5 and representative optimized VHH domains of the present invention are shown in Table 6 below:

Table 5: VHH domains

SEQ ID NO: 1-48 are VHH domains of family 101. SEQ ID NO: 49-52 are VHH domains of family 9. SEQ ID NO: 53-120 are VHH domains of family 13.

CX3CR1BII PMP66B02	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	1
CX3CR1BII PMP54A12	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINSVGITKYADSVKGRFTI SGDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW	SEQ ID NO:	2

	DTRYWGQGTQVTVSS		
CX3CR1BII PMP54A3	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	3
CX3CR1BII PMP54A4	EVQLVESGRGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	4
CX3CR1BII PMP54A5	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	5
CX3CR1BII PMP54A7	EVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTQVTVSS	SEQ ID NO:	6
CX3CR1BII PMP54B1	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTAYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	7
CX3CR1BII PMP54B2	EVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTLVTVSS	SEQ ID NO:	8
CX3CR1BII PMP54B3	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	9
CX3CR1BII PMP54B5	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTAYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	10
CX3CR1BII PMP54D5	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPPGKQRDLVALINSVGITKYADSVKGRFT ISSDNAKNTVYLEMNSLKPEDTAVYYCTSDGRRG	SEQ ID NO:	11

	WDTRYWGQGTQVTVSS		
CX3CR1BII PMP54D8	EVQLVESGGGSVQAGGSLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	12
CX3CR1BII PMP54F6	KVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	13
CX3CR1BII PMP54G3	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVALINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	14
CX3CR1BII PMP54H1	EVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITKYADSVKGRFTV SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTQVTVSS	SEQ ID NO:	15
CX3CR1BII PMP54H4	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINSVGITKYADSVKGRFTI SGDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTLVTVSS	SEQ ID NO:	16
CX3CR1BII PMP61F10	KVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTQVTVSS	SEQ ID NO:	17
CX3CR1BII PMP61D1	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAFGKQRDLVALINSVGITKYADSVKGRFTIS RDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWD TRYWGQGTQVTVSS	SEQ ID NO:	18
CX3CR1BII PMP61D5	KVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAFGKQRDLVALINSVGITKYADSVKGRFTIS RDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWD TRYWGQGTQVTVSS	SEQ ID NO:	19
CX3CR1BII PMP61E2	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDMAVYYCTSDPRRG	SEQ ID NO:	20

	WDTRYWGQGTQVTVSS		
CX3CR1BII PMP61F11	KVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQPPGKQRDLVAAINSVGITKYADSVKGRFTI FRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	21
CX3CR1BII PMP61G2	EVQLVKSGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVALINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	22
CX3CR1BII PMP61G3	KVQLVESGGGSMQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMMSLKPEDTAVYYCTSDPRRG WDTRYWGQGTQVTVSS	SEQ ID NO:	23
CX3CR1BII PMP61G4	KVQLVESGGGSVQAGGSLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMMSLKPEDTAVYYCTSDPRRG WDTRYWGQGTQVTVSS	SEQ ID NO:	24
CX3CR1BII PMP61F4	EVQLVESGGGSVQAGASLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINTVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTLVTVSS	SEQ ID NO:	25
CX3CR1BII PMP61A11	EVQLVESRGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINSVGITKYADSVKGRFTI SGDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTQVTVSS	SEQ ID NO:	26
CX3CR1BII PMP61B2	EVQLVESRGGSVQAGASLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINTVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTQVTVSS	SEQ ID NO:	27
CX3CR1BII PMP61C9	EVQLVKSGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQALGKQRDLVALIDSAGITKYADSVKGRFTIS RDNAKNTVYLQMNRLKPEDTAVYYCASDARRGW NTKYWGQGTLVTVSS	SEQ ID NO:	28
CX3CR1BII PMP65H02	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVHLQMNSLKPEDTAVYYCTSDPRRGW	SEQ ID NO:	29

	DTRYWGQGTLVTVSS		
CX3CR1BII PMP65E11	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGIAKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	30
CX3CR1BII PMP65E10	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAKA WYRQAPGKQRDLVAGINSVGITKYADSVKGRFTIS RDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWD TRYWGQGTLVTVSS	SEQ ID NO:	31
CX3CR1BII PMP65E05	KVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	32
CX3CR1BII PMP65B11	EVQLVKSGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINKVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	33
CX3CR1BII PMP65B07	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	34
CX3CR1BII PMP65B09	EVQLVESGGGSVQAGESLRLSCAASGSIFSRNAM AWYRQAPGKQRDLVASINSVGITKYGDSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTLVTVSS	SEQ ID NO:	35
CX3CR1BII PMP65H01	EVQLVESGGGSVQAGESLRLSCAASGGIFSRNAM AWYRQAPGKQRDLVASINSVGITKYGDSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTQVTVSS	SEQ ID NO:	36
CX3CR1BII PMP65G07	EVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITRYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTQVTVSS	SEQ ID NO:	37
CX3CR1BII PMP66H08	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVALINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW	SEQ ID NO:	38

	DTRYWGQGTQVTVSS		
CX3CR1BII PMP66H04	EVQLVESGGGSVQAGGSLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGITKYADSVKGRFTI SRDNAKNTVYLQMMSLKPEDTAVYYCTSDPRRG WDTRYWGQGTLVTVSS	SEQ ID NO:	39
CX3CR1BII PMP66F02	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVALINSVGITKYAGSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	40
CX3CR1BII PMP66E11	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGTTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	41
CX3CR1BII PMP66D10	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQALGKQRDLVALINSVGITKYADSVKGRFTIS RDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWD TRYWGQGTQVTVSS	SEQ ID NO:	42
CX3CR1BII PMP66D08	EVQLMESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAGINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSS	SEQ ID NO:	43
CX3CR1BII PMP66A04	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQALGKQRDLVALINSVGITKYADSVKGRFTIS RDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWD TRYWGQGTLVTVSS	SEQ ID NO:	44
CX3CR1BII PMP66D04	KVQLVESGGGSVQAGESLRLSCAASGTIFSSNAM AWYRQAPGKQRDLVAGINSVDITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW NTRYWGQGTLVTVSS	SEQ ID NO:	45
CX3CR1BII PMP66D02	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAVINSVGITKYADSVKGRFTT SGDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW DTRYWGQGTQVTVSS	SEQ ID NO:	46
CX3CR1BII PMP66D06	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVASIDSVGITKYRDSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDARRGW	SEQ ID NO:	47

	DTRYWGQGTQVTVSS		
CX3CR1BII PMP66G01	EMQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVALINSVGITKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDGRRG WDTRYWGQGTQVTVSS	SEQ ID NO:	48
CX3CR1BII PMP11H11	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAM GWFRQAPGKERAFVAGISGSASRKYYADSVKGRF TVSRDNARNTVYLQMNSLKPEDTAVYYCAASNSY PKVQFDYYGQGTQVTVSS	SEQ ID NO:	49
CX3CR1BII PMP12B6	EVQLVQSGGGLVQAGGSLRLSCVASGRTFSSYAM GWFRQAPGRERAFVAGISGSASRKYYADSVKGRF TVSRDNARNTVYLQMNSLKPEDTAVYYCAASNSY PKVQFDYYGQGTQVTVSS	SEQ ID NO:	50
CX3CR1BII PMP12G9	EVQLVESGGGLVQPGGSLRLSCVASGRTFSSYAM GWFRQAPGKEREFVAGISGSGSRKYYADSVKGRF TISRDNARNTVYLQMNSLKPEDRAVYYCAASNSYP KVQFDYYGQGTQVTVSS	SEQ ID NO:	51
CX3CR1BII PMP15G11	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAM GWFRQAPGKEREFVAGISGSGSRKYYADSVKGRF TISRDNARNTVYLQMNSLKPEDRAVYYCAASNSYP KVQFDYYGQGTQVTVSS	SEQ ID NO:	52
CX3CR1BII PMP18E6	KVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISSSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTLDARRG WNTAYWGQGAQVTVSS	SEQ ID NO:	53
CX3CR1BII PMP12C2	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNTAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNSGYWGQGTQVTVSS	SEQ ID NO:	54
CX3CR1BII PMP18A10	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSAKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	55
CX3CR1BII PMP18A2	EVQLVESGGGVVQPGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASIGSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW	SEQ ID NO:	56

	NTAYWGQGTPVTVSS		
CX3CR1BII PMP18A8	EVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	57
CX3CR1BII PMP18A9	EVQLVESGGGVVQPGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASISSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW NTAYWGQGTPVTVSS	SEQ ID NO:	58
CX3CR1BII PMP18B7	EVQLVESGGGLVQPGGSLRLSCATSGTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNSGYWGQGTQVTVSS	SEQ ID NO:	59
CX3CR1BII PMP18B9	EVQLVESRGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISSSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTLDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	60
CX3CR1BII PMP18C6	EVQLMESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	61
CX3CR1BII PMP18C9	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	62
CX3CR1BII PMP18D1	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKSTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	63
CX3CR1BII PMP18D10	EVQLVESGGGLVQPGGSLGLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	64
CX3CR1BII PMP18D12	EVQLVESGGGLVQPGGSLRLSCTTSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNNLKPEDTGVYYCTLDARRG	SEQ ID NO:	65

	WNTAYWGQGTQVTVSS		
CX3CR1BII PMP18F1	KVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	66
CX3CR1BII PMP18F5	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	67
CX3CR1BII PMP18F6	EVQLVDSGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	68
CX3CR1BII PMP18F9	EVQLVESGGGLVQPGGSLRLSCATSGTIFRTNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTAYLQMNSLKPEDTGVYYCTIDGRRG WNTGYWGQGTQVTVSS	SEQ ID NO:	69
CX3CR1BII PMP18G5	EVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	70
CX3CR1BII PMP18H1	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQALGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	71
CX3CR1BII PMP18H10	EVQLVESGGGLVQPGGSLRLSCATSKTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	72
CX3CR1BII PMP18H7	EVQLVESRGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	73
CX3CR1BII PMP18H9	EVQLVKSGGGLVQPGGSLRLSCTTSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNNLKPEDTGVYYCTLDARRG	SEQ ID NO:	74

	WNTAYWGQGTQVTVSS		
CX3CR1BII PMP20B3	EVQLVESGGGLVQAGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASIGSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW NTAYWGQGTPVTVSS	SEQ ID NO:	75
CX3CR1BII PMP20C12	EVQLVESGGGLVQPGGSLRLSCATSGTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNSGYWGQGTRVTVSS	SEQ ID NO:	76
CX3CR1BII PMP20C3	KVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	77
CX3CR1BII PMP20C6	EVQLVESGGGLVQAGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	78
CX3CR1BII PMP20D8	EVQLVESGGGLVQPGRSLRLSCATSGTTFRSNAM GWYRQGPGKKRDLVASITNSGSTNYADSVKGRFT VSRDNDKNTGYLQMSSLKPEDTGVYYCTLDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	79
CX3CR1BII PMP20E11	EVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQGPGKKRDLVASITNSGSTNYADSVKGRFT VSRDNDRNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	80
CX3CR1BII PMP20E5	KVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQVPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	81
CX3CR1BII PMP20F3	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISSSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTLDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	82
CX3CR1BII PMP20F4	EVQLVESGGGLVQPGGSLRLSCATSATIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTAYLQMNSLKPEDTGVYYCTIDGRRG	SEQ ID NO:	83

	WNTGYWGQGTQVTVSS		
CX3CR1BII PMP20F5	EVQLVESGGGLVQPGGSLRLSCATSATIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRST VSRDNDKNTAYLQMNSLKPEDTGVYYCTIDGRRG WNTGYWGQGTQVTVSS	SEQ ID NO:	84
CX3CR1BII PMP21B6	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDMGVYYCTVDARR GWNTAYWGQGTQVTVSS	SEQ ID NO:	85
CX3CR1BII PMP24A12	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSANYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	86
CX3CR1BII PMP24A6	EVQLVESGGGLVQPGGSLRLSCTTSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSGDNDKNTGYLQMNNLKPEDTGVYYCTLDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	87
CX3CR1BII PMP24B9	EVQLVESGGGLVQPGGSLRLSCATSGTIFRSNAM GWYRQAPGKKRDLVASISISGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGFWGQGTQVTVSS	SEQ ID NO:	88
CX3CR1BII PMP24D3	EVQLVESGGGLVQPGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASISSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW NTAYWGQGTPVTVSS	SEQ ID NO:	89
CX3CR1BII PMP24F7	EVQLMESGGGMVQVGGSLRLSCTASGLIFSNNAM GWYRQGPGKKRDLVASISSTYSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG WNTAYWGQGTPVTVSS	SEQ ID NO:	90
CX3CR1BII PMP28B4	EVQLVESGGGLVQPGGSLRLSCAISATIFRSNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTAYLQMNSLKPEDTGVYYCTIDGRRGW NTGYWGQGTQVTVSS	SEQ ID NO:	91
CX3CR1BII PMP28F1	EMQLVESGGGVVQPGGSLRLSCVTSGIIFSNNAM GWYRQGPGKKRDLVASIGSTYSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG	SEQ ID NO:	92

	WNTAYWGQGTPVTVSS		
CX3CR1BII PMP28F6	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQAPGKKRDLVASISNSGSTNHADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	93
CX3CR1BII PMP28F9	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQVPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	94
CX3CR1BII PMP29A5	EVQLVESRGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	95
CX3CR1BII PMP29D5	KVQLVESGGGLVQPGGSLRLSCATSGTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNSGYWGQGTQVTVSS	SEQ ID NO:	96
CX3CR1BII PMP29E3	EVQLVESEGGLVQPGGSLRLPCATSKTIFRSNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	97
CX3CR1BII PMP29E7	EVQLVESGGGLVQPGGSLRLSCATSKTIFRSNAM GWYRQAPGKKRGLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	98
CX3CR1BII PMP29G10	EVQLMESGGGLVQPGGSLRLSCATSGTIFRSNAM GWYRQGPGKKRDLVASITNSGSTNYADSVKGRFT VSRDNDKNTGYLQMSSLKPEDTGVYYCTLDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	99
CX3CR1BII PMP29G7	EVQLVESGGGLVQPGGSLRLSCATSGIIFSNNAMG WYRQGPGKKRDLVASITNTGSTNYADSVKGRFTV SRDNDRNTVYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	100
CX3CR1BII PMP29H1	EVQLVESGGGLVQAGGSLRLSCTTSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNNLKPEDTGVYYCTLDARRG	SEQ ID NO:	101

	WNTAYWGQGTQVTVSS		
CX3CR1BII PMP37A8	EVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSAKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	102
CX3CR1BII PMP37B9	EVQLVESGGLVQPGGSLRLSCATSGTIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	103
CX3CR1BII PMP37C12	EVQLVESGGGLVQAGGSLRLSCVASGSIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	104
CX3CR1BII PMP37C7	EVQLVESGGGLVQPGGSLRLSCATSRTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	105
CX3CR1BII PMP37D9	EVQLVESGGGLVQPGGSLRLSCATSGTVFSNNAM GWYRQAPGKKRDLVASISSSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTLDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	106
CX3CR1BII PMP37E12	EVQLVESGGGLVQPGGSLRLSCATSKPIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	107
CX3CR1BII PMP41B10	EVQLVESEGGLVQPGGSLRLSCTTSGTIFSNNAMG WYRQAPGKKRDLVASISNSGSTNYADSVKGRFTV SRDNDKNTGYLQMNNLKPEDTGVYYCTLDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	108
CX3CR1BII PMP41B11	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSPKPEDTGVYYCTVDARR GWNTAYWGQGTQVTVSS	SEQ ID NO:	109
CX3CR1BII PMP41B8	EVQLVESEGGVVQPGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASIGSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW	SEQ ID NO:	110

	NTAYWGQGTPVTVSS		
CX3CR1BII PMP41C10	EMQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKSTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	111
CX3CR1BII PMP41F9	EVQLVESGGGVVQPGESLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASIGSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW NTAYWGQGTPVTVSS	SEQ ID NO:	112
CX3CR1BII PMP41H10	KVQLVESGGGLVQPGDSLRLSCAASGLTLDDYAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	113
CX3CR1BII PMP46B5	KVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	114
CX3CR1BII PMP46D3	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQVPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLRMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	115
CX3CR1BII PMP46H5	EVQLVESGGGLVQAGGSLRLSCVTSGIIFSNNAMG WYRQGPGKKRDLVASISSTYSTNYADSVKGRFTV SRDNDKNTGYLQMNSLKPEDTGVYYCTIDARRGW NTAYWGQGTPVTVSS	SEQ ID NO:	116
CX3CR1BII PMP48B8	EVQLVESGGGLVQPGGSLRLSCATSKTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYTDSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	117
CX3CR1BII PMP48D11	KVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTGYWGQGTQVTVSS	SEQ ID NO:	118
CX3CR1BII PMP48G8	EVQLVESGGGLVQPGGSLRLSCATSRTIFRSNAM GWYRQAPGKKRDLVASISNSGSTNYADSVKGRFA VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG	SEQ ID NO:	119

	WNTGYWGQGTQVTVSS		
CX3CR1BII PMP48H9	EVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAM GWYRQAPGKKRDLVASISNSGSTNYADFVKGRFT VSRDNDKNTGYLQMNSLKPEDTGVYYCTVDARRG WNTAYWGQGTQVTVSS	SEQ ID NO:	120

Table 6: Optimized VHH domains

CX3CR1BII 043	EVQLVESGGGSVQPGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	121
CX3CR1BII 045	DVQLVESGGGSVQPGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	122
CX3CR1BII 047	EVQLVESGGGLVQPGESLRLSCAASGSIFSSNAMA WYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTIS RDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	123
CX3CR1BII 048	EVQLVESGGGSVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	124
CX3CR1BII 049	EVQLVESGGGSVQPGESLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	125
CX3CR1BII 050	EVQLVESGGGSVQPGESLRLSCAASGSIFSSNAM AWYRQAPGKRRELVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	126
CX3CR1BII 061	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW	SEQ ID NO:	127

	DTRYWGQGTLVTVSS		
CX3CR1BII 056	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKQRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	128
CX3CR1BII 057	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRELVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	129
CX3CR1BII 060	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKQRELVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	130
CX3CR1BII 065	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAISSVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	131
CX3CR1BII 067	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAIQSVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	132
CX3CR1BII 068	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAIGSVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	133
CX3CR1BII 074	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAITSVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	134
CX3CR1BII 118	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINTVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	135
CX3CR1BII 129	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINGVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW	SEQ ID NO:	136

	DTRYWGQGTLVTVSS		
CX3CR1BII 158	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINPVGVTKYADSVKGRFTI SRDNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	137
CX3CR1BII 306	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	138
CX3CR1BII 307	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISTVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	139
CX3CR1BII 308	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	140
CX3CR1BII 00306 (D1E)	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSTAMA WYRQAPGKRRDLVAAISSVGVTKYADSVKGRFTIS RDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	222
CX3CR1BII 00307 (D1E)	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSTAMA WYRQAPGKRRDLVAAISTVGVTKYADSVKGRFTIS RDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	223
CX3CR1BII 00308 (D1E)	EVQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTI SRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRRGW DTRYWGQGTLVTVSS	SEQ ID NO:	224

In a further aspect, a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, is humanized and/or optimized for stability, potency, manufacturability and/or similarity to human framework regions. For example, the polypeptide is humanized and/or

sequence optimized in one or more of the following positions (according to Kabat numbering): 1, 11, 14, 16, 74, 83, 108. In one aspect, the polypeptide comprises one or more of the following mutations: E1D, S11L, A14P, E16G, A74S, K83R, Q108L.

In one aspect, one or more framework regions of a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, are humanized and/or sequence optimized. In one aspect, a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, comprises framework regions (FR) for example as set forth below:

i) FR1 is selected from any one of SEQ ID NO's: 198-204;
ii) FR2 is selected from any one of SEQ ID NO's: 205-208;
iii) FR3 is selected form any one of SEQ ID NO's: 209-210; and/or
iv) FR4 is selected from any one of SEQ ID NO's: 211-212.

Human immunoglobulin framework region sequences (FR) that can also be used as framework region sequences for the immunoglobulin single variable domains as described above are known in the art. Also known in the art are methods for humanizing framework regions of immunoglobulin single variable domains derived from species other than humans.

In a further aspect, one or more CDR regions of a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, is humanized and/or sequence optimized. In one aspect, a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, is humanized and/or sequence optimized in one or more of the following positions (according to Kabat numbering): 52, 53.

In a further aspect, a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, comprises one or more of the following mutations: N52S, S53T.

In a further aspect, a polypeptide according to the present invention, in particular an immunoglobulin single variable domain of the present invention, comprises a CDR2 selected from any one of SEQ ID NO's: 214-221.

Representative humanized and/or optimized sequences of the present invention are shown in Table 4 and 6 hereinabove and in Table 7 herein below.

Table 7: Sequence optimized variants

Table 7a shows the FR1-CDR1-FR2-CRD2 of the sequence optimized variants, table 7b shows FR3-CDR3-FR4-CDR4 of said variants. The sequence numbers in the tables (SEQ) refer to the sequences in the sequence listing of the instant application.

Table 7a: Sequence optimized variants (FR1-CDR1-FR2-CDR2)

Nano body	SEQ	FR1	SEQ FR1	CDR1	SEQ CDR 1	FR2	SEQ FR2	CDR2	SEQ CDR 2
CX3CR1 BIIPMP6 6B02	1	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162
CX3CR1 BII043	121	EVQLVES GGGSVQ PGESLRL SCAAS	199	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162
CX3CR1 BII045	122	DVQLVES GGGSVQ PGESLRL	200	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162

		SCAAS							
CX3CR1 BII047	123	EVQLVES GGGLVQ PGESLRL SCAAS	201	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162
CX3CR1 BII048	124	EVQLVES GGGSVQ PGGSLRL SCAAS	202	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162
CX3CR1 BII049	125	EVQLVES GGGSVQ PGESLRL SCAAS	199	GSIFS SNAM A	141	WYRQ APGK QRDLV A	206	AINSV GVTK	162
CX3CR1 BII050	126	EVQLVES GGGSVQ PGESLRL SCAAS	199	GSIFS SNAM A	141	WYRQ APGKR RELVA	207	AINSV GVTK	162
CX3CR1 BII061	127	EVQLVES GGGLVQ PGGSLRL SCAAS	203	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162
CX3CR1 BII056	128	EVQLVES GGGLVQ PGGSLRL SCAAS	203	GSIFS SNAM A	141	WYRQ APGK QRDLV A	206	AINSV GVTK	162
CX3CR1 BII057	129	EVQLVES GGGLVQ PGGSLRL SCAAS	203	GSIFS SNAM A	141	WYRQ APGKR RELVA	207	AINSV GVTK	162
CX3CR1 BII060	130	EVQLVES GGGLVQ PGGSLRL SCAAS	203	GSIFS SNAM A	141	WYRQ APGK QRELV A	208	AINSV GVTK	162
CX3CR1 BII065	131	EVQLVES GGGSVQ AGESLRL	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AISSV GVTK	214

		SCAAS							
CX3CR1 BII067	132	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AIQSV GVTK	215
CX3CR1 BII068	133	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AIGSV GVTK	216
CX3CR1 BII074	134	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AITSV GVTK	217
CX3CR1 BII118	135	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINTV GVTK	218
CX3CR1 BII129	136	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AING VGVT K	219
CX3CR1 BII158	137	EVQLVES GGGSVQ AGESLRL SCAAS	198	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINPV GVTK	220
CX3CR1 BII306	138	DVQLVES GGGLVQ PGGSLRL SCAAS	204	GSIFS STAMA	213	WYRQ APGKR RDLVA	205	AISSV GVTK	214
CX3CR1 BII307	139	DVQLVES GGGLVQ PGGSLRL SCAAS	204	GSIFS STAMA	213	WYRQ APGKR RDLVA	205	AISTV GVTK	221
CX3CR1 BII308	140	DVQLVES GGGLVQ PGGSLRL	204	GSIFS SNAM A	141	WYRQ APGKR RDLVA	205	AINSV GVTK	162

		SCAAS							

Table 7b: Sequence optimized variants (FR3-CDR3-FR4)

Nano body	SEQ	FR3	SEQ FR3	CDR3	SEQ CDR3	FR4	SEQ FR4
CX3CR1 BIIPMP6 6B02	1	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTQ VTVSS	211
CX3CR1 BII043	121	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII045	122	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII047	123	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII048	124	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII049	125	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII050	126	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT	210	DPRRGW DTRY	186	WGQGTL VTVSS	212

		AVYYCTS					
CX3CR1 BII061	127	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII056	128	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII057	129	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII060	130	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII065	131	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII067	132	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII068	133	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII074	134	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII118	135	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA	209	DPRRGW DTRY	186	WGQGTL VTVSS	212

		VYYCTS					
CX3CR1 BII129	136	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII158	137	YADSVKGRFTI SRDNAKNTVYL QMNSLKPEDTA VYYCTS	209	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII306	138	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII307	139	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212
CX3CR1 BII308	140	YADSVKGRFTI SRDNSKNTVYL QMNSLRPEDT AVYYCTS	210	DPRRGW DTRY	186	WGQGTL VTVSS	212

In one aspect of the present invention, a polypeptide of the invention can additionally contain modifications such as glycosyl residues, modified amino acid side chains, and the like.

It will be clear to the skilled person that for pharmaceutical uses in humans, the polypeptides of the invention are preferably directed against human CX3CR1, whereas for veterinary purposes, the polypeptides of the invention are preferably directed against CX3CR1 from the species to be treated.

It will also be clear to the skilled person that when used as a therapeutic agent in humans, the immunoglobulin single variable domains comprised in the

polypeptides according to the invention are preferably humanized immunoglobulin single variable domains.

According to the invention, an immunoglobulin single variable domain can be a domain antibody, i.e. VL or VH antibody, and/or VHH domains as described above, and/or any other sort of immunoglobulin single variable domain, for example camelized VH, provided that these immunoglobulin single variable domains are anti-CX3CR1 immunoglobulin single variable domains.

In one aspect of the invention, the immunoglobulin single variable domain essentially consists of either a domain antibody sequence or a VHH domain sequence as described above. In particular, the immunoglobulin single variable domain essentially consists of a VHH domain sequences.

In a further aspect, a polypeptide of the present invention comprises two or more anti-CX3CR1 immunoglobulin single variable domains. In a further aspect, a polypeptide of the present invention comprises two anti-CX3CR1 immunoglobulin single variable domains, for example anti-CX3CR1 VHHs. In one aspect, the two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present invention have the same amino acid sequence. In another aspect, the two anti-CX3CR1 immunoglobulin single variable domains in a polypeptide of the present invention have different amino acid sequences.

According to another embodiment of the invention, the at least two immunoglobulin single variable domains present in a polypeptide of the invention can be linked to each other directly (i.e. without use of a linker) or via a linker. The linker is preferably a linker peptide and will, according to the invention, be selected so as to allow binding of the at least two immunoglobulin single variable domains to CX3CR1, either within one and the same CX3CR1 molecule, or within two different molecules.

Suitable linkers will *inter alia* depend on the epitopes and, specifically, the distance between the epitopes on CX3CR1 to which the immunoglobulin single

variable domains bind, and will be clear to the skilled person based on the disclosure herein, optionally after some limited degree of routine experimentation.

Also, when the two or more anti-CX3CR1 immunoglobulin single variable domains are domain antibodies or VHH domains, they may also be linked to each other via a third domain antibody or VHH domain (in which the two or more immunoglobulin single variable domains may be linked directly to the third domain antibody or VHH domain or via suitable linkers). Such a third domain antibody or VHH domain may for example be a domain antibody or VHH domain that provides for an increased half-life, as further described herein. For example, the latter domain antibody or VHH domain may be a domain antibody or VHH domain that is capable of binding to a (human) serum protein such as (human) serum albumin or (human) transferrin, as further described herein.

Alternatively, the two or more anti-CX3CR1 immunoglobulin single variable domains may be linked in series (either directly or via a suitable linker) and the third (single) domain antibody or VHH domain (which may provide for increased half-life, as decribed above) may be connected directly or via a linker to one of these two or more aforementioned immunoglobulin sequences.

Suitable linkers are described herein in connection with specific polypeptides of the invention and may - for example and without limitation - comprise an amino acid sequence, which amino acid sequence preferably has a length of 5 or more amino acids, 7 or more amino acids, 9 or more amino acids, 11 or more amino acids, 15 or more amino acids or at least 17 amino acids, such as about 20 to 40 amino acids. However, the upper limit is not critical but is chosen for reasons of convenience regarding e.g. biopharmaceutical production of such polypeptides.

The linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. If used for therapeutical purposes, the linker is preferably non-immunogenic in the subject to which the polypeptide of the invention is administered.

One useful group of linker sequences are linkers derived from the hinge region of heavy chain antibodies as described in WO 96/34103 and WO 94/04678.

Other examples are poly-alanine linker sequences such as Ala-Ala-Ala.

Further preferred examples of linker sequences are Gly/Ser linkers of different length such as $(gly_xser_y)_z$ linkers, including $(gly_4ser)_3$, $(gly_4ser)_4$, (gly_4ser), (gly_3ser), gly_3, and $(gly_3ser_2)_3$.

If the polypeptide of the invention is modified by the attachment of a polymer, for example a polyethylene glycol (PEG) moiety, the linker sequence preferably includes an amino acid residue, such as a cysteine or a lysine, allowing such modification, e.g. PEGylation, in the linker region.

Examples of linkers are:

GGGGS (5 GS linker, SEQ ID NO: 233)
SGGSGGS (7GS linker, SEQ ID NO: 234)
GGGGCGGGS (8GS linker, SEQ ID NO: 235)
GGGGSGGGS (9GS linker, SEQ ID NO: 236)
GGGGSGGGGS (10GS linker, SEQ ID NO: 237)
GGGGSGGGGSGGGGS (15GS linker, SEQ ID NO: 238)
GGGGSGGGGSGGGGGGGS (18GS linker, SEQ ID NO: 239)
GGGGSGGGGSGGGGSGGGGS (20GS linker, SEQ ID NO: 240)
GGGGSGGGGSGGGGSGGGGSGGGGS (25GS linker, SEQ ID NO: 241)
GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (30GS linker, SEQ ID NO: 242)
GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (35GS linker, SEQ ID NO: 243)
EPKSCDKTHTCPPCP (G1 hinge linker, SEQ ID NO: 244)
GGGGSGGGSEPKSCDKTHTCPPCP (9GS-G1 hinge linker, SEQ ID NO: 245)
EPKTPKPQPAAA (Llama upper long hinge region, SEQ ID NO: 246)

ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCD TPPPCPRCP (G3 hinge, SEQ ID NO: 247)
AAA (Ala linker, SEQ ID NO: 248)

Furthermore, the linker may also be a poly(ethylene glycol) moiety, as shown in e.g. WO04/081026.

Non-limiting examples of polypeptides comprising or consisting of two or more anti-CX3CR1 immunoglobulin single variable domains are given in Table 8a.

Table 8a: Bivalent anti-CX3CR1 polypeptides

CX3CR1 BII007	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAMG WFRQAPGKERAFVAGISGSASRKYYADSVKGRFTV SRDNARNTVYLQMNSLKPEDTAVYYCAASNSYPKV QFDYYGQGTQVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSKVQLVESGGGLVQPGG SLRLSCATSGTIFSNNAMGWYRQAPGKKRDLVASIS SSGSTNYADSVKGRFTVSRDNDKNTGYLQMNSLKP EDTGVYYCTLDARRGWNTAYWGQGAQVTVSS	SEQ ID NO:	267
CX3CR1 BII009	KVQLVESGGGLVQPGGSLRLSCATSGTIFSNNAMG WYRQAPGKKRDLVASISSSGSTNYADSVKGRFTVS RDNDKNTGYLQMNSLKPEDTGVYYCTLDARRGWNT AYWGQGAQVTVSSGGGGSGGGGSGGGGSGGGG SGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGS LRLSCVASGRTFSSYAMGWFRQAPGKERAFVAGIS GSASRKYYADSVKGRFTVSRDNARNTVYLQMNSLK PEDTAVYYCAASNSYPKVQFDYYGQGTQVTVSS	SEQ ID NO:	268
CX3CR1 BII012	EVQLVESGGGSVQAGGSLRLSCAASGSIFSSNAMA WYRQAPGKQRDLVAGINSVGITKYADSVKGRFTISR DNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWDTR YWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSKVQLVESGGGLVQPGGSLRL SCATSGTIFSNNAMGWYRQAPGKKRDLVASISSSGS	SEQ ID NO:	269

	TNYADSVKGRFTVSRDNDKNTGYLQMNSLKPEDTG VYYCTLDARRGWNTAYWGQGAQVTVSS		
CX3CR1 BII016	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAMA WYRQAPGKQRDLVAVINSVGITKYADSVKGRFTISG DNAKNTVYLQMNSLKPEDTAVYYCTSDARRGWDTR YWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEVQLVESGGGSVQAGESL RLSCAASGSIFSSNAMAWYRQAPGKQRDLVAVINSV GITKYADSVKGRFTISGDNAKNTVYLQMNSLKPEDT AVYYCTSDARRGWDTRYWGQGTQVTVSS	SEQ ID NO:	270
CX3CR1 BII017	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAMA WYRQAPPGKQRDLVALINSVGITKYADSVKGRFTISS DNAKNTVYLEMNSLKPEDTAVYYCTSDGRRGWDTR YWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEVQLVESGGGSVQAGESL RLSCAASGSIFSSNAMAWYRQAPPGKQRDLVALINS VGITKYADSVKGRFTISSDNAKNTVYLEMNSLKPEDT AVYYCTSDGRRGWDTRYWGQGTQVTVSS	SEQ ID NO:	271
CX3CR1 BII018	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAMA WYRQAPGKRRDLVAAINSVGVTKYADSVKGRFTISR DNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGWDTR YWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEVQLVESGGGSVQAGESL RLSCAASGSIFSSNAMAWYRQAPGKRRDLVAAINSV GVTKYADSVKGRFTISRDNAKNTVYLQMNSLKPEDT AVYYCTSDPRRGWDTRYWGQGTQVTVSS	SEQ ID NO:	272
CX3CR1 BII019	EMQLVESGGGSVQAGESLRLSCAASGSIFSSNAMA WYRQAPGKQRDLVALINSVGITKYADSVKGRFTISR DNAKNTVYLQMNSLKPEDTAVYYCTSDGRRGWDTR YWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEMQLVESGGGSVQAGESL RLSCAASGSIFSSNAMAWYRQAPGKQRDLVALINSV GITKYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTA VYYCTSDGRRGWDTRYWGQGTQVTVSS	SEQ ID NO:	273

CX3CR1 BII020	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNAMA WYRQAPGKQRDLVAGINSVGITKYADSVKGRFTISR DNAKNTAYLQMNSLKPEDTAVYYCTSDPRRGWDTR YWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSEVQLVESGGGSVQAGESLRL SCAASGSIFSSNAMAWYRQAPGKQRDLVAGINSVGI TKYADSVKGRFTISRDNAKNTAYLQMNSLKPEDTAV YYCTSDPRRGWDTRYWGQGTLVTVSS	SEQ ID NO:	274
CX3CR1 BII026	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAMG WFRQAPGKERAFVAGISGSASRKYYADSVKGRFTV SRDNARNTVYLQMNSLKPEDTAVYYCAASNSYPKV QFDYYGQGTQVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSEVQLVESGGGSVQAGE SLRLSCAASGSIFSSNAMAWYRQAPGKRRDLVAAIN SVGVTKYADSVKGRFTISRDNAKNTVYLQMNSLKPE DTAVYYCTSDPRRGWDTRYWGQGTQVTVSS	SEQ ID NO:	275
CX3CR1 BII027	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAMG WFRQAPGKERAFVAGISGSASRKYYADSVKGRFTV SRDNARNTVYLQMNSLKPEDTAVYYCAASNSYPKV QFDYYGQGTQVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSEVQLVESGGGSVQAGE SLRLSCAASGSIFSSNAMAWYRQAPGKQRDLVAGIN SVGITKYADSVKGRFTISRDNAKNTAYLQMNSLKPE DTAVYYCTSDPRRGWDTRYWGQGTLVTVSS	SEQ ID NO:	276
CX3CR1 BII006	EVQLVESGGGLVQAGGSLRLSCVASGRTFSSYAMG WFRQAPGKERAFVAGISGSASRKYYADSVKGRFTV SRDNARNTVYLQMNSLKPEDTAVYYCAASNSYPKV QFDYYGQGTLVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGG SLRLSCVASGRTFSSYAMGWFRQAPGKERAFVAGI SGSASRKYYADSVKGRFTVSRDNARNTVYLQMNSL KPEDTAVYYCAASNSYPKVQFDYYGQGTLVTVSS	SEQ ID NO:	282

In another embodiment, the at least two immunoglobulin single variable domains of the polypeptide of the invention are linked to each other via another moiety (optionally via one or two linkers), such as another polypeptide which, in a preferred but non-limiting embodiment, may be a further immunoglobulin single variable domain as already described above. Such moiety may either be essentially inactive or may have a biological effect such as improving the desired properties of the polypeptide or may confer one or more additional desired properties to the polypeptide. For example, and without limitation, the moiety may improve the half-life of the protein or polypeptide, and/or may reduce its immunogenicity or improve any other desired property.

In one aspect, a polypeptide of the invention includes, especially when used as a therapeutic agent, a moiety which extends the half-life of the polypeptide of the invention in serum or other body fluids of a patient. The term "half-life" means the time taken for the serum concentration of the (modified) polypeptide to reduce by 50%, *in vivo*, for example due to degradation of the polypeptide and/or clearance and/or sequestration by natural mechanisms.

According to a further embodiment of the invention, the two immunoglobulin single variable domains may be fused to a serum albumin molecule, such as described e.g. in WO01/79271 and WO03/59934.

Alternatively, such half-life extending moiety can be covalently linked or fused to said polypeptide and may be, without limitation, an Fc portion, an albumin moiety, a fragment of an albumin moiety, an albumin binding moiety, such as an anti-albumin immunoglobulin single variable domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin single variable domain, a polyoxyalkylene molecule, such as a polyethylene glycol molecule, an albumin binding peptide, or hydroxyethyl starch (HES) derivatives.

In another aspect, the polypeptide of the invention comprises a moiety which binds to an antigen found in blood, such as serum albumin, serum immunoglobulins, thyroxine-binding protein, fibrinogen or transferrin, thereby

conferring an increased half-life *in vivo* to the resulting polypeptide of the invention. According to one embodiment, such moiety is an albumin-binding immunoglobulin and, in particular, an albumin-binding immunoglobulin single variable domain such as an albumin-binding VHH domain.

In another embodiment, the polypeptide of the invention comprises a moiety which binds to serum albumin, wherein such moiety is an albumin binding peptide, as described e.g. in international patent publications WO2008/068280 and WO2009/127691.

If intended for use in humans, such albumin-binding immunoglobulin single variable domain (also called anti-albumin immunoglobulin single variable domain) will preferably bind to human serum albumin and will preferably be a humanized albumin-binding VHH domain.

Immunoglobulin single variable domains binding to human serum albumin are known in the art and are described in further detail in e.g. WO2006/122786. A specifically useful albumin binding VHH domain consists of or contains the amino acid sequence as set forth in any one of SEQ ID NO: 230-232:

Table 8b

ALB-1	AVQLVESGGGLVQPGNSLRLSCAASGFTFRSFG MSWVRQAPGKEPEWVSSISGSGSDTLYADSVK GRFTISRDNAKTTLYLQMNSLKPEDTAVYYCTIG GSLSRSSQGTQVTVSS	SEQ ID NO:	230
ALB-11 (humanized ALB-1)	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFG MSWVRQAPGKGLEWVSSISGSGSDTLYADSVK GRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIG GSLSRSSQGTLVTVSS	SEQ ID NO:	231
ALB-2	AVQLVESGGGLVQGGGSLRLACAASERIFDLNL MGWYRQGPGNERELVATCITVGDSTNYADSVK GRFTISMDYTKQTVYLHMNSLRPEDTGLYYCKIR	SEQ ID NO:	232

	RTWHSELWGQGTQVTVSS		

According to one embodiment, a polypeptide of the invention may be linked to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the invention and/or may confer the ability to bind to one or more Fc receptors. For example, for this purpose, and without being limited thereto, the antibody parts may be or may comprise CH2 and/or CH3 domains of an antibody, such as from a heavy chain antibody (as described hereabove) and more preferably from a conventional human 4-chain antibody; specifically, the polypeptide of the invention may be linked to an Fc region, for example from human IgG, from human IgE or from another human Ig. For example, WO 94/04678 describes heavy chain antibodies comprising a Camelid VHH domain or a humanized derivative thereof, in which the Camelidae CH2 and/or CH3 domain have been replaced by human CH2 and/or CH3 domains, so as to provide an immunoglobulin that consists of 2 heavy chains each comprising a - optionally humanized - VHH domain and human CH2 and CH3 domains (but no CH1 domain), which immunoglobulin has the effector function provided by the CH2 and CH3 domains, can function without the presence of any light chains, and has an increased half-life as compared to the corresponding VHH domains without such modification.

In one aspect, a polypeptide of the present invention comprises two anti-CX3CR1 VHHs and a VHH capable of binding to serum albumin. In one aspect, the VHHs are fused using linker peptides. Representative examples of such polypeptides of the present invention are shown hereinbelow.

In one aspect, a polypeptide of the present invention comprises a first anti-CX3CR1 VHH fused to a first linker peptide, which is itself fused to a VHH capable of binding to serum albumin, which is itself fused to a second linker peptide, which is itself fused to a second anti-CX3CR1 VHH. In one aspect, the first or the second linker peptide is a 9GS linker, in one aspect, the first and the

second linker peptide is a 9GS linker. In one aspect, the VHH capable of binding to serum albumin is capable of binding to human serum albumin. In one aspect, the VHH capable of binding to serum albumin has the amino acid sequence set forth in SEQ ID NO: 231. In one aspect, the first and the second anti-CX3CR1 VHH have the same amino acid sequence. In one aspect, the first or the second anti-CX3CR1 VHH has the CDR1, CDR2 and CDR3 set forth in:

- SEQ ID NO's: 213, 214 and 186; or
- SEQ ID NO's: 213, 221 and 186; or
- SEQ ID NO's: 141, 162 and 186.

In one aspect, the first and the second anti-CX3CR1 VHH have the CDR1, CDR2 and CDR3 set forth in:

- SEQ ID NO's: 213, 214 and 186; or
- SEQ ID NO's: 213, 221 and 186; or
- SEQ ID NO's: 141, 162 and 186.

In one aspect, the first or the second anti-CX3CR1 VHH has the amino acid sequence set forth in any one of SEQ ID NO: 138 to 140 or SEQ ID NO: 222 to 224. In one aspect, the first and the second anti-CX3CR1 VHH have the same amino acid sequence, wherein said amino acid sequence is the sequence set forth in any one of SEQ ID NO: 138 to 140 or SEQ ID NO: 222 to 224.

Non-limiting examples of polypeptides of the present invention are the polypeptides of any one of SEQ ID NO: 225 to 227, 249 or 277 to 281.

Table 9

CX3CR1BII 00312	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR	SEQ ID NO:	225

	SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSTAMAWYRQAPGKRR DLVAAISSVGVTKYADSVKGRFTISRDNSKNTVYL QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS		
CX3CR1BII 00313	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISTVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSTAMAWYRQAPGKRR DLVAAISTVGVTKYADSVKGRFTISRDNSKNTVYL QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS	SEQ ID NO:	226
CX3CR1BII 00314	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSNAMAWYRQAPGKR RDLVAAINSVGVTKYADSVKGRFTISRDNSKNTV YLQMNSLRPEDTAVYYCTSDPRRGWDTRYWGQ GTLVTVSS	SEQ ID NO:	227
CX3CR1BII 032	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTQVTVSSGGGGSGGGSEVQL VESGGGSVQAGESLRLSCAASGSIFSSNAMAWY RQAPGKRRDLVAAINSVGVTKYADSVKGRFTISR DNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSSGGGGSGGGSEVQLVES GGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQ APGKGLEWVSSISGSGSDTLYADSVKGRFTISRD NAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQ	SEQ ID NO:	277

	GTLVTVSS		
CX3CR1BII 034	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTQVTVSSGGGGSGGGGSGG GGSGGGGSGGGGSGGGGSGGGGSEVQLVES GGGSVQAGESLRLSCAASGSIFSSNAMAWYRQ APGKRRDLVAAINSVGVTKYADSVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCTSDPRRGWDTR YWGQGTQVTVSSGGGGSGGGSEVQLVESGGG LVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KGLEWVSSISGSGSDTLYADSVKGRFTISRDNAK TTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTL VTVSS	SEQ ID NO:	278
CX3CR1BII 036	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGSV QAGESLRLSCAASGSIFSSNAMAWYRQAPGKRR DLVAAINSVGVTKYADSVKGRFTISRDNAKNTVY LQMNSLKPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS	SEQ ID NO:	249
CX3CR1BII 040	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTQVTVSSGGGGSGGGGSEVQL VESGGGSVQAGESLRLSCAASGSIFSSNAMAWY RQAPGKRRDLVAAINSVGVTKYADSVKGRFTISR DNAKNTVYLQMNSLKPEDTAVYYCTSDPRRGW DTRYWGQGTQVTVSSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSGGGGSEVQLVESGGGL VQPGNSLRLSCAASGFTFSSFGMSWVRQAPGK GLEWVSSISGSGSDTLYADSVKGRFTISRDNAKT TLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLV TVSS	SEQ ID NO:	279

CX3CR1BII 041	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTQVTVSSGGGGSGGGGSGG GGSGGGGSGGGGSGGGGSGGGGSEVQLVES GGGSVQAGESLRLSCAASGSIFSSNAMAWYRQ APGKRRDLVAAINSVGVTKYADSVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCTSDPRRGWDTR YWGQGTQVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSEVQLVESGGGLVQP GNSLRLSCAASGFTFSSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLY LQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVS S	SEQ ID NO:	280
CX3CR1BII 042	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTQVTVSSGGGGSGGGGSGG GGSGGGGSGGGGSGGGGSGGGGSEVQLVES GGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQ APGKGLEWVSSISGSGSDTLYADSVKGRFTISRD NAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQ GTLVTVSSGGGGSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSEVQLVESGGGSVQAGESLRL SCAASGSIFSSNAMAWYRQAPGKRRDLVAAINS VGVTKYADSVKGRFTISRDNAKNTVYLQMNSLKP EDTAVYYCTSDPRRGWDTRYWGQGTQVTVSS	SEQ ID NO:	281

In another aspect, a polypeptide of the present invention comprises an anti-CX3CR1 VHH and a Fc domain. In one aspect, a polypeptide of the present invention comprises an anti-CX3CR1 VHH fused to a linker peptide, which is itself fused to a Fc domain. In one aspect, the linker peptide is a 15GS linker. In one aspect, the Fc domain has the amino acid sequence set forth in SEQ ID NO: 250 or 252. In one aspect, the VHH has the CDR1, CDR2 and CDR3 set forth in:

- SEQ ID NO's: 213, 214 and 186; or
- SEQ ID NO's: 213, 221 and 186; or
- SEQ ID NO's: 141, 162 and 186.

In one aspect, the VHH has the amino acid sequence set forth in any one of SEQ ID NO: 138 to 140 or SEQ ID NO: 222 to 224. In one aspect the polypeptide is in the form of a dimer, for example wherein the dimer is formed by one or more disulfide bridge.

Non-limiting examples of polypeptides of the present invention are the polypeptides of SEQ ID NO: 251, 253 or 254.

Table 10

Mouse Fc domain	PPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVT CVVVAVSEDDPDVQISWFVNNVEVHTAQTQTHR EDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNK DLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTK KQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKN TEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCS VVHEGLHNHHTTKSFSRTPGK	SEQ ID NO:	250
66B02-mFc	EVQLVESGGGSVQAGESLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNAKNTVYLQMNSLKPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGGSGG GGSPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLS PIVTCVVVAVSEDDPDVQISWFVNNVEVHTAQTQ THREDYNSTLRVVSALPIQHQDWMSGKEFKCKV NNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEE MTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELN YKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSY SCSVVHEGLHNHHTTKSFSRTPGK	SEQ ID NO:	251
Human Fc Domain	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSREEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK	SEQ ID NO:	252
306D-hFc	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA	SEQ ID	253

	MAWYRQAPGKRRDLVAAISSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGGSGG GGSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK	NO:	
307D-hFc	DVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISTVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGGSGG GGSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK	SEQ ID NO:	254

A polypeptide of the invention may be modified to improve its properties. In one aspect, a polypeptide of the present invention may be modified to increase its stability upon storage. In one aspect, a polypeptide of the present invention may be modified to facilitate its expression in a particular host system. For example, the first codon of a polypeptide of the present invention may be modified. In one aspect, a polypeptide of the present invention begins with a glutamic acid (glu) as its first amino acid. In another aspect, a polypeptide of the present invention begins with an aspartic acid (asp) as its first amino acid, for example to reduce pyroglutamate formation at the N-terminus during storage and hence increase product stability. In another aspect, a polypeptide of the present invention begins with an alanine (ala) or a valine (val) as its first amino acid, for example to facilitate the expression of the polypeptide in a prokaryotic expression system,

such as *Escherichia coli*. Such modification of a polypeptide according to the present invention are made using techniques known in the art.

Representative examples of polypeptides according to the present invention with a modified first codon are set forth in any one of SEQ ID NO: 257-262 and 263-266 and are shown in Tables 11 and 12 below:

Table 11

CX3CR1BII 00312 (D1A)	AVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSTAMAWYRQAPGKRR DLVAAISSVGVTKYADSVKGRFTISRDNSKNTVYL QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS	SEQ ID NO:	257
CX3CR1BII 00313 (D1A)	AVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISTVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSTAMAWYRQAPGKRR DLVAAISTVGVTKYADSVKGRFTISRDNSKNTVYL QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS	SEQ ID NO:	258
CX3CR1BII 00314 (D1A)	AVQLVESGGGLVQPGGSLRLSCAASGSIFSSNA MAWYRQAPGKRRDLVAAINSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGSEVQL VESGGGLVQPGNSLRLSCAASGFTFSSFGMSW VRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTI	SEQ ID NO:	259

	SRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSGGGGSGGGSEVQLVESGGGLV QPGGSLRLSCAASGSIFSSNAMAWYRQAPGKR RDLVAAINSVGVTKYADSVKGRFTISRDNSKNTV YLQMNSLRPEDTAVYYCTSDPRRGWDTRYWGQ GTLVTVSS		
CX3CR1BII 00312 (D1V)	VQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISSVGVTKYADSVKGRF TISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRR GWDTRYWGQGTLVTVSSGGGGSGGGSEVQLV ESGGGLVQPGNSLRLSCAASGFTFSSFGMSWV RQAPGKGLEWVSSISGSGSDTLYADSVKGRFTIS RDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRS SQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQ PGGSLRLSCAASGSIFSSTAMAWYRQAPGKRRD LVAAISSVGVTKYADSVKGRFTISRDNSKNTVYL QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS	SEQ ID NO:	260
CX3CR1BII 00313 (D1V)	VQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISTVGVTKYADSVKGRF TISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRR GWDTRYWGQGTLVTVSSGGGGSGGGSEVQLV ESGGGLVQPGNSLRLSCAASGFTFSSFGMSWV RQAPGKGLEWVSSISGSGSDTLYADSVKGRFTIS RDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRS SQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQ PGGSLRLSCAASGSIFSSTAMAWYRQAPGKRRD LVAAISTVGVTKYADSVKGRFTISRDNSKNTVYLQ MNSLRPEDTAVYYCTSDPRRGWDTRYWGQGTL VTVSS	SEQ ID NO:	261
CX3CR1BII 00314 (D1V)	VQLVESGGGLVQPGGSLRLSCAASGSIFSSNAM AWYRQAPGKRRDLVAAINSVGVTKYADSVKGRF TISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRR GWDTRYWGQGTLVTVSSGGGGSGGGSEVQLV ESGGGLVQPGNSLRLSCAASGFTFSSFGMSWV RQAPGKGLEWVSSISGSGSDTLYADSVKGRFTIS RDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRS SQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQ PGGSLRLSCAASGSIFSSNAMAWYRQAPGKRRD LVAAINSVGVTKYADSVKGRFTISRDNSKNTVYL	SEQ ID NO:	262

	QMNSLRPEDTAVYYCTSDPRRGWDTRYWGQG TLVTVSS		

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Table 12

306D-hFc (D1A)	AVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISSVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGGSGG GGSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK	SEQ ID NO:	263
307D-hFc (D1A)	AVQLVESGGGLVQPGGSLRLSCAASGSIFSSTA MAWYRQAPGKRRDLVAAISTVGVTKYADSVKGR FTISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPR RGWDTRYWGQGTLVTVSSGGGGSGGGGSGG GGSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK	SEQ ID NO:	264
306D-hFc (D1V)	VQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISSVGVTKYADSVKGRF TISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRR GWDTRYWGQGTLVTVSSGGGGSGGGGSGGG GSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV	SEQ ID NO:	265

	SNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK		
307D-hFc (D1V)	VQLVESGGGLVQPGGSLRLSCAASGSIFSSTAM AWYRQAPGKRRDLVAAISTVGVTKYADSVKGRF TISRDNSKNTVYLQMNSLRPEDTAVYYCTSDPRR GWDTRYWGQGTLVTVSSGGGGSGGGGSGGG GSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK	SEQ ID NO:	266

In one further aspect, a polypeptide of the present invention is characterized by one or more of the following properties:

- Bind with high affinity to human CX3CR1;
- Inhibit binding of soluble fractalkine to human CX3CR1;
- Inhibit fractalkine induced chemotaxis;
- Inhibit fractalkine induced human CX3CR1 receptor internalization;
- Cross-react with cyno CX3CR1 within 10-fold of E/IC_{50} for human CX3CR1 for binding and functional inhibition.

Accordingly, in one aspect, a polypeptide of the present invention has an affinity to human CX3CR1 at an IC50 less than or equal to 10nM, or less than or equal to 5nM, or less than or equal to 2.5nM or less than or equal to 1nM, as determined by competition FACS.

In a further aspect, a polypeptide of the present invention has an affinity to human CX3CR1 at an EC50 of less than or equal to 10nM, or less than or equal to 5nM, or less than or equal to 2.5nM or less than or equal to 1nM, as determined by cell binding FACS.

In a further aspect, a polypeptide of the present invention blocks the binding of human CX3CR1 to human fractalkine at or above 50%, or at or above 60%, or at or above 70%, or at or above 80%, or at or above 90%, or at or above 95% as determined by competition FACS with human fractalkine.

In a further aspect, a polypeptide of the present invention blocks the binding of human fractalkine to human CX3CR1 at an IC50 of less than or equal to 300nM, or less than or equal to 100nM, or less than or equal to 20nM, or less than or equal to 10nM, less than or equal to 5nM, less than or equal to 2.5nM or less than or equal to 1nM as determined by competition FACS with human fractalkine.

In a further aspect, a polypeptide of the present invention inhibits fractalkine induced chemotaxis mediated by human CX3CR1 at or above 10%, or at or above 30%, or at or above 40%, or at or above 50%, or at or above 60%, or at or above 70%, or at or above 80%, or at or above 90%.

In a further aspect, a polypeptide of the present invention inhibits fractalkine induced chemotaxis mediated by human CX3CR1 at an IC_{50} of less than or equal to 500 nM, or of less than or equal to 100 nM, or less than or equal to 75 nM, or less than or equal to 50 nM, or less than or equal to 10 nM or less than or equal to 5nM.

In a further aspect, a polypeptide of the present invention inhibits fractalkine induced human CX3CR1 receptor internalization at an IC_{50} of less than or equal to 10 nM, or less than or equal to 5nM or or less than or equal to 1nM.

According to still another embodiment, a half-life extending modification of a polypeptide of the invention (such modification also reducing immunogenicity of the polypeptide) comprises attachment of a suitable pharmacologically acceptable polymer, such as straight or branched chain poly(ethylene glycol) (PEG) or derivatives thereof (such as methoxypoly(ethylene glycol) or mPEG). Generally, any suitable form of PEGylation can be used, such as the PEGylation used in the art for antibodies and antibody fragments (including but not limited to domain antibodies and scFv's); reference is made, for example, to: Chapman,

Nat. Biotechnol., 54, 531-545 (2002); Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003); Harris and Chess, Nat. Rev. Drug. Discov. 2 (2003); WO 04/060965; and US6,875,841.

Various reagents for PEGylation of polypeptides are also commercially available, for example from Nektar Therapeutics, USA, or NOF Corporation, Japan, such as the Sunbright® EA Series, SH Series, MA Series, CA Series, and ME Series, such as Sunbright® ME-100MA, Sunbright® ME-200MA, and Sunbright® ME-400MA.

Preferably, site-directed PEGylation is used, in particular via a cysteine-residue (see for example Yang et al., Protein Engineering 16, 761-770 (2003)). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a polypeptide of the invention, a polypeptide of the invention may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus and/or PEG may be attached to a linker region that bridges two or more functional domains of a polypeptide of the invention, all using techniques of protein engineering known per se to the skilled person.

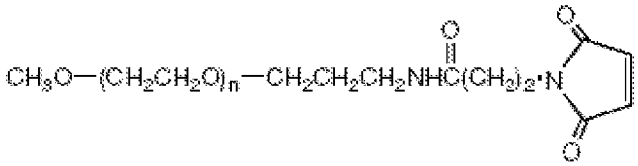
Preferably, for the polypeptides of the invention, a PEG is used with a molecular weight of more than 5 kDa, such as more than 10 kDa and less than 200 kDa, such as less than 100 kDa; for example in the range of 20 kDa to 80 kDa.

With regard to PEGylation, it should be noted that generally, the invention also encompasses any polypeptide of the invention that has been PEGylated at one or more amino acid positions, preferably in such a way that said PEGylation either (1) increases the half-life in vivo; (2) reduces immunogenicity; (3) provides one or more further beneficial properties known per se for PEGylation; (4) does not essentially affect the affinity of the polypeptide for CX3CR1 (e.g. does not reduce said affinity by more than 50 %, and more preferably not by more than 10%, as determined by a suitable assay, such as those described in the Examples below);

and/or (4) does not affect any of the other desired properties of the polypeptides of the invention. Suitable PEG-groups and methods for attaching them, either specifically or non-specifically, will be clear to the skilled person.

According to a specifically preferred embodiment of the invention, a PEGylated polypeptide of the invention includes one PEG moiety of linear PEG having a molecular weight of 40 kDa or 60 kDa, wherein the PEG moiety is attached to the polypeptide in a linker region and, specifially, at a Cys residue, for example at position 5 of a GS8-linker peptide as shown in SEQ ID NO:235.

Preferred examples of PEGylated polypeptides of the invention are PEGylated preferably with one of the PEG reagents as mentioned above, such as "Sunbright® ME-400MA" as shown in the following chemical formula:



which has an average molecular weight of 40 kDa.

Therapeutic uses

In one aspect, the present invention provides a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide for use as a medicament.

In one aspect, the present invention provides the use of a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide for the treatment or prophylaxis of cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulonephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and

demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma, neuropathic pain, inflammatory pain, or cancer.

In another aspect, the present invention provides the use of a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide for the treatment or prophylaxis of atherosclerosis.

In another aspect, the present invention provides the use of a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide for the treatment or prophylaxis of atherosclerosis by preventing and/or reducing the formation of new atherosclerotic lesions or plaques and/or by preventing or slowing progression of existing lesions and plaques.

In another aspect, the present invention provides the use of a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide for the treatment or prophylaxis of atherosclerosis by changing the composition of the plaques to reduce the risk of plaque rupture and atherothrombotic events.

In one aspect, the present invention also provides a method of treating, or reducing the risk of, cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulonephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma, neuropathic pain, inflammatory pain, or cancer, in a person suffering from or at risk of, said disease or condition, wherein the method comprises administering to the person a therapeutically effective amount of a polypeptide according to the present invention or a pharmaceutical composition comprising said polypeptide.

In one aspect, the present invention also provides a method of treating, or reducing the risk of atherosclerosis in a person suffering from or at risk of, said disease or condition, wherein the method comprises administering to the person a therapeutically effective amount of polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide.

In one aspect, the present invention also provides a method of treating, or reducing the risk of atherosclerosis by preventing and/or reducing the formation of new atherosclerotic lesions or plaques and/or by preventing or slowing progression of existing lesions and plaques in a person suffering from or at risk of, said disease or condition, wherein the method comprises administering to the person a therapeutically effective amount of polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide.

In one aspect, the present invention also provides a method of treating, or reducing the risk of atherosclerosis by changing the composition of the plaques so as to reduce the risk of plaque rupture and atherothrombotic events in a person suffering from or at risk of, said disease or condition, wherein the method comprises administering to the person a therapeutically effective amount of a polypeptide of the present invention or a pharmaceutical composition comprising said polypeptide.

In one aspect, a polypeptide of the present invention is indicated for use in the treatment or prophylaxis of a disease or disorder that is associated with CX3CR1.

In one aspect, a polypeptide of the present invention is indicated for use in the treatment or prophylaxis of diseases or conditions in which modulation of activity at the CX3CR1 receptor is desirable. In one aspect, the present invention also provides a method of treating, or reducing the risk of, diseases or conditions in which antagonism of the CX3CR1 receptor is beneficial which comprises administering to a person suffering from or at risk of, said disease or condition, a polypeptide of the present invention.

Prophylaxis is expected to be particularly relevant to the treatment of persons who have suffered a previous episode of, or are otherwise considered to be at increased risk of, the disease or condition in question. Persons at risk of developing a particular disease or condition generally include those having a family history of the disease or condition, or those who have been identified by genetic testing or screening to be particularly susceptible to developing the disease or condition.

In the context of the present invention, the term "prevention, treatment and/or alleviation" not only comprises preventing and/or treating and/or alleviating the disease, but also generally comprises preventing the onset of the disease, slowing or reversing the progress of disease, preventing or slowing the onset of one or more symptoms associated with the disease, reducing and/or alleviating one or more symptoms associated with the disease, reducing the severity and/or the duration of the disease and/or of any symptoms associated therewith and/or preventing a further increase in the severity of the disease and/or of any symptoms associated therewith, preventing, reducing or reversing any physiological damage caused by the disease, and generally any pharmacological action that is beneficial to the patient being treated.

The subject to be treated will be a mammal, and more in particular a human being. As will be clear to the skilled person, the subject to be treated will in particular be a person suffering from, or at risk from, the diseases, disorders or conditions mentioned herein.

It will also be clear to the skilled person that the above methods of treatment of a disease include the preparation of a medicament for the treatment of said disease. Furthermore, it is clear that the polypeptides of the invention can be used as an active ingredient in a medicament or pharmaceutical composition intended for the treatment of the above diseases. Thus, the invention also relates to the use of a polypeptide of the invention in the preparation of a pharmaceutical composition for the prevention, treatment and/or alleviation of any of the diseases, disorders or conditions mentioned hereinabove. The invention further

relates to a polypeptide of the invention for therapeutic or prophylactic use and, specifically, for the prevention, treatment and/or alleviation of any of the diseases, disorders or conditions mentioned hereinabove. The invention further relates to a pharmaceutical composition for the prevention, treatment and/or alleviation of the diseases, disorders or conditions mentioned hereinabove, wherein such composition comprises at least one polypeptide of the invention.

The polypeptides of the invention and/or the compositions comprising the same can be administered to a patient in need thereof in any suitable manner, depending on the specific pharmaceutical formulation or composition to be used. Thus, the polypeptides of the invention and/or the compositions comprising the same can for example be administered intravenously, subcutaneously, intramuscularly, intraperitoneally, transdermally, orally, sublingually (e.g. in the form of a sublingual tablet, spray or drop placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue), (intra-)nasally (e.g. in the form of a nasal spray and/or as an aerosol), topically, by means of a suppository, by inhalation, intravitreally (esp. for the treatment of dry AMD or glaucoma), or any other suitable manner in an effective amount or dose.

The polypeptides of the invention and/or the compositions comprising the same are administered according to a regimen of treatment that is suitable for preventing, treating and/or alleviating the disease, disorder or condition to be prevented, treated or alleviated. The clinician will generally be able to determine a suitable treatment regimen, depending on factors such as the disease, disorder or condition to be prevented, treated or alleviated, the severity of the disease, the severity of the symptoms thereof, the specific polypeptide of the invention to be used, the specific route of administration and pharmaceutical formulation or composition to be used, the age, gender, weight, diet, general condition of the patient, and similar factors well known to the clinician. Generally, the treatment regimen will comprise the administration of one or more polypeptides of the invention, or of one or more compositions comprising the same, in therapeutically and/or prohylactically effective amounts or doses.

Generally, for the prevention, treatment and/or alleviation of the diseases, disorders and conditions mentioned herein and depending on the specific disease, disorder or condition to be treated, the potency of the specific polypeptide of the invention to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the polypeptides of the invention will generally be administered in an amount between 0.005 and 20.0 mg per kilogram of body weight and dose, preferably between 0.05 and 10.0 mg/kg/dose, and more preferably between 0.5 and 10 mg/kg/dose, either continuously (e.g. by infusion) or as single doses (such as e.g. daily, weekly, or monthly doses; cf. below), but can significantly vary, especially, depending on the before-mentioned parameters.

For prophylactic applications, compositions containing the polypeptides of the invention may also be administered in similar or slightly lower dosages. The dosage can also be adjusted by the individual physician in the event of any complication.

Depending on the specific polypeptide of the invention and its specific pharmacokinetic and other properties, it may be administered daily, every second, third, fourth, fifth or sixth day, weekly, monthly, and the like. An administration regimen could include long-term, weekly treatment. By "long-term" is meant at least two weeks and preferably months, or years of duration.

The efficacy of the polypeptides of the invention, and of compositions comprising the same, can be tested using any suitable in vitro assay, cell-based assay, in vivo assay and/or animal model known per se, or any combination thereof, depending on the specific disease involved. Suitable assays and animal models will be clear to the skilled person, and for example include the assays and animal models used in the Examples below.

For pharmaceutical use, the polypeptides of the invention may be formulated as a pharmaceutical preparation comprising (i) at least one polypeptide of the

invention and (ii) at least one pharmaceutically acceptable carrier, diluent, excipient, adjuvant, and/or stabilizer, and (iii) optionally one or more further pharmaceutically active polypeptides and/or compounds. By "pharmaceutically acceptable" is meant that the respective material does not show any biological or otherwise undesirable effects when administered to an individual and does not interact in a deleterious manner with any of the other components of the pharmaceutical composition (such as e.g. the pharmaceutically active ingredient) in which it is contained. Specific examples can be found in standard handbooks, such as e.g. Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990). For example, the polypeptides of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments and other pharmaceutically active proteins. Thus, according to a further embodiment, the invention relates to a pharmaceutical composition or preparation that contains at least one polypeptide of the invention and at least one pharmaceutically acceptable carrier, diluent, excipient, adjuvant and/or stabilizer, and optionally one or more further pharmaceutically active substances.

By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (such as by intravenous, intramuscular, subcutaneous, intrathecal, intracavernosal or intraperitoneal injection or intravenous infusion), for topical administration, for sublingual administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, for transdermal, nasal, intravitreal, rectal or vaginal administration, and the like. Such suitable administration forms - which may be solid, semi-solid or liquid, depending on the manner of administration - as well as methods and carriers for use in the preparation thereof, will be clear to the skilled person.

Pharmaceutical preparations for parenteral administration, such as intravenous, intramuscular, subcutaneous injection or intravenous infusion may for example be sterile solutions, suspensions, dispersions, emulsions, or powders which comprise the active ingredient and which are suitable, optionally after a further dissolution or dilution step, for infusion or injection. Suitable carriers or diluents

for such preparations for example include, without limitation, sterile water and pharmaceutically acceptable aqueous buffers and solutions such as physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution; water oils; glycerol; ethanol; glycols such as propylene glycol, as well as mineral oils, animal oils and vegetable oils, for example peanut oil, soybean oil, as well as suitable mixtures thereof.

Solutions of the active compound or its salts may also contain a preservative to prevent the growth of microorganisms, such as antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal (thiomersal), and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. Other agents delaying absorption, for example, aluminum monostearate and gelatin, may also be added.

In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

Usually, aqueous solutions or suspensions will be preferred. Generally, suitable formulations for therapeutic proteins such as the polypeptides of the invention are buffered protein solutions, such as solutions including the protein in a suitable concentration (such as from 0.001 to 400 mg/ml, preferably from 0.005 to 200 mg/ml, more preferably 0.01 to 200 mg/ml, more preferably 1.0 - 100 mg/ml, such

as 1.0 mg/ml (i.v. administration) or 100 mg/ml (s.c. administration) and an aqueous buffer such as:

- phosphate buffered saline, pH 7.4,
- other phosphate buffers, pH 6.2 to 8.2,
- histidine buffers, pH 5.5 to 7.0,
- succinate buffers, pH 3.2 to 6.6, and
- citrate buffers, pH 2.1 to 6.2,

and, optionally, salts (e.g. NaCl) and/or sugars or polyalcohols (such as trehalose, mannitol, or glycerol) for providing isotonicity of the solution.

Preferred buffered protein solutions are solutions including about 0.05 mg/ml of the polypeptide of the invention dissolved in 25 mM phosphate buffer, pH 6.5, adjusted to isotonicity by adding 220 mM trehalose. In addition, other agents such as a detergent, e.g. 0.02 % Tween-20 or Tween-80, may be included in such solutions. Formulations for subcutaneous application may include significantly higher concentrations of the polypeptide of the invention, such as up to 100 mg/ml or even above 100 mg/ml. However, it will be clear to the person skilled in the art that the ingredients and the amounts thereof as given above do only represent one, preferred option. Alternatives and variations thereof will be immediately apparent to the skilled person, or can easily be conceived starting from the above disclosure.

The polypeptides of the invention may also be administered using suitable depot, slow-release or sustained-release formulations, e.g. suitable for injection, using controlled-release devices for implantation under the skin, and/or using a dosing pump or other devices known per se for the administration of pharmaceutically active substances or principles. In addition, the polypeptides of the invention may be formulated in the form of a gel, cream, spray, drop, patch or film which, if placed on the skin, passes through the skin.

Also, compared to conventional antibodies or antibody fragments, one major advantage of the use of the polypeptides of the invention is that they can also be easily administered via routes other than parenteral administration and can be

easily formulated for such administration. For example, as described in the international application WO2004/041867, such polypeptides may be formulated for oral, intranasal, intrapulmonary and transdermal administration.

According to another embodiment of the invention there is provided a pharmaceutical combination comprising at least one polypeptide of the invention as disclosed herein and at least one other therapeutic agent selected from the group consisting of statins, antiplatelets, anticoagulants, antidiabetics and antihypertensives.

Such pharmaceutical combination may optionally additionally comprise a diluent, excipient, adjuvant and/or stabilizer.

When two or more substances or principles are to be used as part of a combined treatment regimen, they can be administered via the same route of administration or via different routes of administration, at essentially the same time or at different times (e.g. essentially simultaneously, consecutively, or according to an alternating regime). When the substances or principles are to be administered simultaneously via the same route of administration, they may be administered as different pharmaceutical formulations or compositions or part of a combined pharmaceutical formulation or composition. Also, when two or more active substances or principles are to be used as part of a combined treatment regimen, each of the substances or principles may be administered in the same amount and according to the same regimen as used when the compound or principle is used on its own, and such combined use may or may not lead to a synergistic effect. However, when the combined use of the two or more active substances or principles leads to a synergistic effect, it may also be possible to reduce the amount of one, more or all of the substances or principles to be administered, while still achieving the desired therapeutic action. This may for example be useful for avoiding, limiting or reducing any unwanted side-effects that are associated with the use of one or more of the substances or principles when they

are used in their usual amounts, while still obtaining the desired pharmaceutical or therapeutic effect.

Yet, a further embodiment of the invention is a method for treating the diseases and disorders as set out above, comprising administering to an individual, simultaneously, separately or sequentially, an effective amount of at least one polypeptide of the invention and at least one agent selected from the group consisting of a statin, an antiplatelet, an anticoagulant, an antidiabetic and an antihypertensive.

According to a further aspect of the invention, the polypeptide of the invention is prepared to be administered in combination with other drugs used for the treatment of the diseases and disorders set out above, such other drugs being selected from the group consisting of a statin, an antiplatelet, an anticoagulant, an antidiabetic and an antihypertensive.

According to still another aspect of the invention, drugs used for the treatment of the diseases and disorders set out above, such drugs being selected from the group consisting of a statin, an antiplatelet, an anticoagulant, an antidiabetic and an antihypertensive are prepared to be administered in combination with the polypeptide of the invention.

According to a further aspect of the invention, the polypeptide of the invention is used in combination with a device useful for the administration of the polypeptide, such as a syringe, injector pen, or other device.

According to still another embodiment of the invention, there is provided a method of diagnosing a disease, disorder or condition mediated by CX3CR1 dysfunction comprising the steps of:

a) obtaining a sample from a subject, and

b) contacting, in vitro, the sample with a polypeptide of the invention as defined above, and

c) detecting the binding of said polypeptide to said sample, and

d) comparing the binding detected in step (c) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease, disorder or condition characterised by CX3CR1 dysfunction.

According to another embodiment of the invention, there is provided a method of diagnosing a disease, disorder or condition mediated by CX3CR1 dysfunction comprising the steps of:
a) obtaining a sample from a subject, and
b) contacting the sample with a polypeptide of the invention as defined above;
c) determining the amount of CX3CR1 in the sample; and
d) comparing the amount determined in step (c) with a standard, wherein a difference in amount relative to said sample is diagnostic of a disease, disorder or condition characterised by CX3CR1 dysfunction.

The above diagnostic methods can also be used for monitoring the effectiveness of a therapeutic treatment of a subject.

According to another embodiment of the invention, there is provided a kit for diagnosing a disease, disorder or condition mediated by CX3CR1 dysfunction, for use in a method as defined above, such kit comprising at least one polypeptide of the invention and, optionally, one or more media, detection means and/or *in vitro* or *in vivo* imaging agents, and, further optionally, instructions of use. Suitable *in vivo* imaging agents include 99mTc, 111Indium, 123Iodine, and, for magnetic resonance imaging, paramagnetic compounds.

The invention further provides a kit comprising at least one polypeptide of the invention and, additionally, one or more other components selected from the group consisting of other drugs used for the treatment of the diseases and disorders as described above, and devices as described above.

The invention further provides methods of manufacturing a polypeptide of the invention, such methods generally comprising the steps of:

- culturing host cells comprising a nucleic acid capable of encoding a polypeptide of the invention (hereinafter: "nucleic acid of the invention") under conditions that allow expression of the polypeptide of the invention; and,
- recovering or isolating the polypeptide expressed by the host cells from the culture; and
- optionally further purifying and/or modifying and/or formulating the polypeptide of the invention.

A nucleic acid of the invention can be genomic DNA, cDNA or synthetic DNA (such as DNA with a codon usage that has been specifically adapted for expression in the intended host cell or host organism). According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined hereabove.

The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form. The vector may especially be an expression vector, i.e. a vector that can provide for expression of the polypeptide *in vitro* and/or *in vivo* (e.g. in a suitable host cell, host organism and/or expression system). Such expression vector generally comprises at least one nucleic acid of the invention that is operably linked to one or more suitable regulatory element(s), such as promoter(s), enhancer(s), terminator(s), and the like. Specific examples of such regulatory elements and other elements, such as integration factor(s), selection marker(s), signal or leader sequence(s), reporter gene(s), and the like, useful or necessary for expressing polypeptides of the invention, are disclosed e.g. on pp. 131 to 133 of WO2006/040153.

The nucleic acids of the invention can be prepared or obtained in a manner known per se (e.g. by automated DNA synthesis and/or recombinant DNA technology), based on the information on the amino acid sequences for the polypeptides of the invention given herein, and/or can be isolated from a suitable natural source.

According to another embodiment, the invention relates to a host or host cell that expresses or is capable of expressing a polypeptide of the invention; and/or that contains a nucleic acid encoding a polypeptide of the invention. According to a particularly preferred embodiment, said host cells are bacterial cells, yeast cells, fungal cells or mammalian cells.

Suitable bacterial cells include cells from gram-negative bacterial strains such as strains of *Escherichia coli*, *Proteus*, and *Pseudomonas*, and gram-positive bacterial strains such as strains of *Bacillus*, *Streptomyces*, *Staphylococcus*, and *Lactococcus.* Suitable fungal cell include cells from species of *Trichoderma*, *Neurospora*, and *Aspergillus*. Suitable yeast cells include cells from species of *Saccharomyces (*for example *Saccharomyces cerevisiae), Schizosaccharomyces* (for example *Schizosaccharomyces pombe*), *Pichia* (for example *Pichia pastoris* and *Pichia methanolica*), and *Hansenula*.

Suitable mammalian cells include for example CHO cells, BHK cells, HeLa cells, COS cells, NS0 cells, HEK cells, and the like. However, amphibian cells, insect cells, plant cells, and any other cells used in the art for the expression of heterologous proteins can be used as well.

For production on industrial scale, preferred heterologous hosts for the (industrial) production of immunoglobulin single variable domain polypeptides and protein therapeutics containing them include strains of *E. coli*, *Pichia pastoris*, and *S. cerevisiae* that are suitable for large scale expression, production and fermentation, and in particular for large scale (bio-)pharmaceutical expression, production and fermentation.

The choice of the specific expression system would depend in part on the requirement for certain post-translational modifications, more specifically glycosylation. The production of a polypeptide of the invention for which glycosylation is desired or required would necessitate the use of mammalian expression hosts that have the ability to glycosylate the expressed protein. In this respect, it will be clear to the skilled person that the glycosylation pattern obtained

(i.e. the kind, number and position of residues attached) will depend on the cell or cell line that is used for the expression.

Polypeptides of the invention produced in a cell as set out above can be produced either intracellullarly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or they can be produced extracellularly (secreted into the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified.

Further methods and reagents used for the recombinant production of polypeptides, such as suitable expression vectors, transformation or transfection methods, selection markers, methods of induction of protein expression, culture conditions, and the like, are known in the art. Similarly, protein isolation and purification techniques useful in a method of manufacture of a polypeptide of the invention are well known to the skilled person.

Production of the polypeptides of the invention through fermentation in convenient recombinant host organisms such as *E. coli* and yeast is cost-effective, as compared to conventional antibodies which also require expensive mammalian cell culture facilities. Furthermore, achievable levels of expression are high and yields of the polypeptides of the invention are in the range of 1 to 10 g/l (E. coli) and up to 10 g/l (yeast) and more.

EXAMPLES

Generation CHO, Baf/3, Caki and HEK293 cell lines overexpressing human CX3CR1 or cynomolgus CX3CR1

CHO and Baf/3 cells overexpressing human or cynomolgus CX3CR1 were generated using techniques known in the art. Cells expressing human CCR2 or CCR5 were also generated using techniques known in the art.

The cDNA was cloned into pCDNA3.1(+)-neo for human CX3CR1 whereas pcDNA-DEST40-neo was used for mouse CX3CR1.

The amino acid sequences of humanCX3CR1 and cynomolgus CX3CR1 are depicted in SEQ ID NO: 255 and 256, respectively.

To establish Camel Kidney (Caki) cells overexpressing human CX3CR1 or mouse CX3CR1, parental Caki cells were electroporated with pCDNA3.1(+)-neo-hCX3CR1 or pcDNA-DEST40-neo-mCX3CR1, respectively. For all conditions, transfectants were selected by adding 1 mg/mL geneticin (Invitrogen, Carlsbad, CA, USA).

Human Embyonic Kidney (HEK293) cells overexpressing human CX3CR1 or cynomolgus CX3CR1 were generated by lipid-mediated transfection with Fugene (Roche) of pCDNA3.1(+)-neo-hCX3CR1 or cyCX3CR1 plasmids, respectively, in the HEK293 parental cell line. These cells were used as transient transfectants and as such not put under selection. In brief, 2*10E6 cells were seeded per T75 and incubated overnight before transfection. After removal of the culture medium, cells were transfected with the respective plasmids (9 µg) and Fugene (27 µl) according to manufacturer's instructions. 48 hours post transfection, cells were harvested and frozen for further usage.

Example 1: Immunization with CX3CR1 induces a humoral immune response in llama

1.1. Immunizations

After approval of the Ethical Committee (University Antwerp, Belgium, UA2008A1, 2008/096, 2007/068), 9 llamas (designated No. 368, 369, 370, 381, 382, 384, 312, 313 and 314) were immunized.

Six llamas (312, 313, 314, 381, 382 and 384) were immunized with 4 intramuscular injections (2mg/dose at weekly or biweekly intervals) of pVAX1-huCX3CR1 plasmid vector (Invitrogen, Carlsbad, CA, USA). Three llamas (381, 382 and 384) subsequently received 4 subcutaneous injections of human CX3CR1 overexpressing Caki cells which were established as described above. Cells were re-suspended in D-PBS and kept on ice prior to injection.

Three additional llamas (designated No. 368, 369 and 370) were immunized according to standard protocols with 4 subcutaneous injections of human CX3CR1 overexpressing Caki cells which were established as described above. Cells were re-suspended in D-PBS and kept on ice prior to injection. Subsequently, these llamas were administered two injections with recombinant CX3CR1 NT/EC3 fragment coupled to BSA (Table 13). Peptides were ordered at NeoMPS (Polypeptidegroup, Strasbourg, France) and coupled to BSA according to standard protocols.

Table 13 Sequence of peptide fragments used for immunization boost

Fragment	sequence	SEQ ID NO:
CX3CR1-NT	Ac-Met-Asp-Gln-Phe-Pro-Glu-Ser-Val-Thr-Glu-Asn-Phe-Glu-Tyr-Asp-Asp-Leu-Ala-Glu-Ala-Cys-NH2	228
CX3CR1-EC3	Ac-Lys-Leu-Tyr-Asp-Phe-Phe-Pro-Ser-Cys-Asp-Met-Arg-Lys-Asp-Leu-Arg-Leu-NH2	229

The first injection was formulated in Complete Freund's Adjuvant (Difco, Detroit, MI, USA), while the subsequent injection was formulated in Incomplete Freund's Adjuvant (Difco, Detroit, MI, USA).

1.2. Evaluation of induced immune responses in llama

To evaluate the induction of immune responses in the animals against human CX3CR1 by ELISA or FACS, sera were collected from llamas 312,313 and 314 at day 0 (pre-immune), and different time points in the immunization schedule (time of peripheral blood lymphocyte [PBL] collection).

In short, Neutravidin (2µg/ml) was immobilized overnight at 4˚C in a 96-well Maxisorb plate (Nunc, Wiesbaden, Germany). Wells were blocked with a casein solution (1%) in PBS. Subsequently biotinylated recombinant NT fragment (Polypeptide, Strasbourg, France) or biotinylated EC3 fragments of CX3CR1 (Polypeptide, Strasbourg, France) were captured at 2 µg/ml. After addition of serum dilutions, specifically bound immunoglobulins were detected using a horseradish peroxidase (HRP)-conjugated goat anti-llama immunoglobulin (Bethyl Laboratories Inc., Montgomery, TX, USA) and a subsequent enzymatic reaction in the presence of the substrate TMB One (3,3',5,5'-tetramentylbenzidine) (Promega, Mannheim, Germany), showing that a significant antibody-dependent immune response against CX3CR1 was induced after the peptide immunizations.

Additionally, serum titers of cell immunized animals were confirmed by FACS analysis on actively growing human CX3CR1 overexpressing CHO cells. The CX3CR1 serum titer responses for llamas 368, 369 and 370 were determined with serum sampled after 4 cell immunizations (day 49), 4 cell immunizations and 1 peptide boost (day 77) and 4 cell immunizations and 2 peptide boosts (day 81). Cells were harvested and washed before incubation with the serum dilutions. Detection was performed with goat anti-llama IgG (Bethyl, Montgomery, TX, USA) followed by donkey anti-goat coupled with PE (Jackson Laboratories, Suffolk, UK) and read out by analysis on FACSArray (BD Biosciences). A summary of the obtained serum responses as determined by either ELISA or FACS is shown in Table 14 and Table 15.

Table 14 Serum titer analysis for the cell/peptide immunized animals

		ELISA		FACS	
Llama	**Immunogen**	**Recombinant NT**	**Recombinant EC3**	**After cell immunization**	**After peptide boosts**
368	Caki-huCX3CR1 + NT/EC3 peptide	+	+/-	-	-
369	Caki-huCX3CR1 + NT/EC3 peptide	+	+/-	+	+
370	Caki-huCX3CR1 + NT/EC3 peptide	++	++	-	+

Table 15 Serum titer analysis for the DNA/cell immunized animals

		ELISA		FACS	
Llama	**Immunogen**	**Recombinant NT**	**Recombinant EC3**	**After DNA immunization**	**After Cell boosts**
381	DNA + Caki-huCX3CR1	++	+	++	++
382	DNA + Caki-huCX3CR1	+	-	-	-
384	DNA + Caki-huCX3CR1	++	-	-	-

For the DNA only immunized llamas (312, 313 and 314) no serum titer was determined.

Example 2: Cloning of the heavy-chain only antibody fragment repertoires and preparation of phage

Following the final immunogen injection of each subset, immune tissues as the source of B-cells that produce the heavy-chain antibodies were collected from the immunized llamas. For llama 312,313 and 314, two 150-ml blood samples, collected 4 and 8 days after the last antigen injection were collected per animal. For llamas 368, 369 and 370 four 150 ml blood samples were collected, 5 and 7 days after the last cell immunization and additionally 4 and 8 days after the last peptide immunization. Next to those, two lymph node biopsies were taken, 12 days after the last cell immunization and 12 days after the last peptide immunization. For llamas 381, 382 and 384 five 150 ml blood samples were collected, 8 days after the last DNA immunization and additionally 4 days after the first cell boost, 8 and 11 days after the second cell boost and 8 days after the last cell immunization. Next to those, one lymph node biopsy was taken, 8 days after the second cell immunization.

From the blood samples, peripheral blood lymphocytes (PBLs) were prepared using Ficoll-Hypaque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). From the PBLs and the lymph node biopsy (LN), total RNA was extracted, which was used as starting material for RT-PCR to amplify the VHH encoding DNA segments.

For each immunized llama, libraries were constructed by pooling the total RNA isolated from samples originating from a certain subset of the immunization schedule i.e. after one type of immunization antigen, and for some llamas samples from the different animals were pooled into one library (Table 16).

Table 16 Pooling of the different sample for library construction

Library Name	Llama	Sample
368-PBL1+2+LN-V-100209	368	PBL 1 and 2, LN
369+370-PBL1+2+LN-V-100209	369, 370	PBL 1 and 2, LN
368-PBL3+4-V-280909	368	PBL 3 and 4
369-PBL3+4-V-070409	369	PBL 3 and 4

370-PBL3+4-V-070409	370	PBL 3 and 4
381-PBL1-V-180310	381	PBL 1
382-PBL1-V-180310	382	PBL1
384-PBL1-V-180310	384	PBL1
381-PBL1+2+3+4+5+LN-V-280909	381	PBL 1, 2, 3, 4, 5 and LN
382-PBL1+2+3+4+5+LN-V-280909	382	PBL 1, 2, 3, 4, 5 and LN
384-PBL1+2+3+4+5+Ln-V-280909	384	PBL 1, 2, 3, 4, 5 and LN
312+313+314-PBL1+2-V-220210	312, 313 and 314	PBL 1 and 2
312-PBL1+2-V-180310	312	PBL 1 and 2
313-PBL1+2-V-180310	313	PBL 1 and 2
314-PBL1+2-V-180310	314	PBL 1 and 2

In short, the PCR-amplified VHH repertoire was cloned via specific restriction sites into a vector designed to facilitate phage display of the VHH library. The vector was derived from pUC119 and contains the LacZ promoter, a M13 phage gIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multiple cloning site and a hybrid gIII-pelB leader sequence (pAX050). In frame with the VHH coding sequence, the vector encodes a C-terminal c-myc tag and a His6 tag. Phage were prepared according to standard protocols and stored after filter sterilization at 4ºC or at -80°C in 20 % glycerol for further use.

Example 3: Selection of CX3CR1 specific VHHs via phage display

VHH repertoires obtained from all llamas and cloned as phage library were used in different selection strategies, applying a multiplicity of selection conditions. Variables include i) the presentation form of the CX3CR1 protein (on different cell backgrounds or on liposomes/VLPs), ii) the antigen presentation method (In solution when using cells or coated onto plates when using VLPs), iii) the antigen

concentration iv) the orthologue used (human or cynomolgus) v) the number of selection rounds and vi) different elution methods (non-specific via trypsin or specific via the ligand Fractalkine). All solid coated phase selections were done in Maxisorp 96-well plates (Nunc, Wiesbaden, Germany).

Selections were performed as follows: CX3CR1 antigen preparations for solid and solution phase selection formats were presented as described above at multiple concentrations. After 2h incubation with the phage libraries followed by extensive washing, bound phages were eluted with trypsin (1 mg/mL) for 15 minutes. When trypsin was used for phage elution, the protease activity was immediately neutralized by applying 0.8 mM protease inhibitor ABSF. As control, selections without antigen were performed in parallel.

Phage outputs were used to infect *E. coli* which were then in turn used to prepare phage for the next selection round (phage rescue) After the second round selection the phage outputs were used to infect *E. coli* which were then plated on agar plates (LB+carb+glucose$^{2\%}$) for analysis of individual VHH clones. In order to screen a selection output for specific binders, single colonies were picked from the agar plates and grown in 1 mL 96-deep-well plates. LacZ-controlled VHH expression was induced by adding IPTG (1mM final) in the absence of glucose. Periplasmic extracts (in a volume of ~ 80 uL) were prepared according to standard protocols.

Example 4: Screening of periplasmic extracts in CX3CR1-Fraktalkine competition FACS assay

Periplasmic extracts were screened in a human CX3CR1/human Fractalkine FACS competition assay to assess the blocking capacity of the expressed VHHs. Human CX3CR1 was presented on CHO cells overexpressing CX3CR1. Both a setup using cells harvested from an actively growing culture and a setup using frozen cells was used. As a detection reagent labeled fractalkine was used (R&D Systems, Minneapolis, MN, USA) labeled with alexa647 (A647-Fractalkine) at a degree of labeling of 1. To setup the assay, first a titration series of the labeled

fractalkine was performed on the CHO-huCX3CR1 cells in order to determine the EC50 value for binding. Initially screening was performed at a higher concentration of fractalkine (3 nM) to increase the assay robustness. To increase the sensitivity of the screening to a maximum, the EC30 concentration (1 nM) was chosen for subsequent screening. In brief 50 µl of periplasmic extract was added to 6 nM labeled fractalkine (50µl) and 200 000 CHO-huCX3CR1 cells. After one hour incubation at 4 C, cells were washed three times before read out was performed on a FACS Array (Becton Dickinson). First a gate was set on the intact cells as determined from the scatter profile. Next, dead cells were gated out by their fluorescence profile from the PI stain (Sigma, St Louis, US). The fluorescence profile from the alexa647 label was determined for each sample and used for calculation of blocking capacity. As controls, conditions were taken along where there was no VHH present in the peri extract or a known irrelevant VHH and samples were included where excess cold fractalkine was included. For each sample the percentage block was determined using the control samples to determine the assay window.

From this screening, VHHs were selected and sequence analysis revealed 120 unique VHHs belonging to 3 different B-cell lineages. The total number of variants found for each B-cell lineage is depicted in Table 17.

Table 17 Selection parameters used for the identification of the humanCX3CR1 specific VHH B-cell lineages

B-cell lineage	Representative VHH ID	# variants	libraries
9	CX3CR1BII11H11	4	368-PBL1+2+LN-V-100209
13	CX3CR1BII18E06	68	368-PBL1+2+LN-V-100209 368-PBL3+4-V-280909
101	CX3CR1BII66B02	48	312+313+314-PBL1+2-V-220210 314-PBL1+2-V-180310

An overview of the selection procedure and performance during initial screening is given for all VHHs in Table 18.

Table 18 Selection conditions and primary screening result for the huCX3CR1 specific VHH

VHH ID	Family	Library	Selections				% block
			first round		second round		
CX3CR1BII PMP11H11	9	368-PBL1+2+LN-V-100209	BA/F3_hCX3CR1	total (trypsin)	CHO-K1_hCX3CR1	total (trypsin)	99.0
CX3CR1BII PMP18E6	13	368-PBL3+4-V-280909	BA/F3_hCX3CR1	total (trypsin)	CHO-K1_hCX3CR1	total (trypsin)	53.1
CX3CR1BII PMP54A12	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	93.8
CX3CR1BII PMP54A3	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	90.8
CX3CR1BII PMP54A4	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	86.6
CX3CR1BII PMP54A5	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	92.5
CX3CR1BII PMP54A7	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	total (trypsin)	VLPs-hCX3CR1 (10U)	total (trypsin)	68.9
CX3CR1BII PMP54B1	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	92.1
CX3CR1BII PMP54B2	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	65.3
CX3CR1BII PMP54B3	101	312+313+314-PBL1+2-	VLPs-hCX3CR1	hFrac (2 µM)	VLPs-hCX3CR1	hFrac (2 µM)	90.1

		V-220210	(10U)		(10U)		
CX3CR1BII PMP54B5	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	92.6
CX3CR1BII PMP54D5	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	87.8
CX3CR1BII PMP54D8	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	total (trypsin)	VLPs-hCX3CR1 (10U)	total (trypsin)	64.1
CX3CR1BII PMP54F6	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	96.6
CX3CR1BII PMP54G3	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	74.7
CX3CR1BII PMP54H1	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	74.6
CX3CR1BII PMP54H4	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	96.0
CX3CR1BII PMP61F10	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	73.5
CX3CR1BII PMP61D1	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	68.4
CX3CR1BII PMP61D5	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	94.9
CX3CR1BII PMP61E2	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	70.3
CX3CR1BII PMP61F11	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	96.5
CX3CR1BII PMP61G2	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	82.0
CX3CR1BII	101	314-	VLPs-	hFrac (2	VLPs-	hFrac (2	92.1

PMP61G3		PBL1+2-V-180310	hCX3CR1 (10U)	μM)	hCX3CR1 (10U)	μM)	
CX3CR1BII PMP61G4	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	94.5
CX3CR1BII PMP61F4	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	94.4
CX3CR1BII PMP61A11	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	78.0
CX3CR1BII PMP61B2	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	94.5
CX3CR1BII PMP61C9	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	total (trypsin)	VLPs-hCX3CR1 (10U)	total (trypsin)	69.4
CX3CR1BII PMP65H02	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65E11	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65E10	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65E05	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65B11	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65B07	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65B09	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A
CX3CR1BII PMP65H01	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	VLPs-hCX3CR1 (10U)	hFrac (2 μM)	#N/A

CX3CR1BII PMP65G07	101	312+313+314-PBL1+2-V-220210	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66H08	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66H04	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66F02	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66E11	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66D10	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66D08	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66B02	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66A04	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66D04	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66D02	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66D06	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A
CX3CR1BII PMP66G01	101	314-PBL1+2-V-180310	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	VLPs-hCX3CR1 (10U)	hFrac (2 µM)	#N/A

The amino acid sequences of all obtained unique VHHs are shown in the Sequence Listing and above (CDRs and framework regions were indicated).

Example 5: Characterization of purified VHHs

Inhibitory anti-CX3CR1 VHHs selected from the screening described in Example 4 were further purified and characterized. Selected VHHs were expressed in *E. coli* TG1 as c-myc, His6-tagged proteins. Expression was induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37˚C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets. These extracts were used as starting material and VHHs were purified via IMAC and size exclusion chromatography (SEC) resulting in 95% purity as assessed via SDS-PAGE.

Inhibition by anti-CX3CR1 VHHs of human Fractalkine binding to human CX3CR1 expressed on the BA/F3 cells

The blocking capacity towards the ligand fractalkine of the VHHs was evaluated in a human CX3CR1 competition FACS as outlined in Example 4. Either CHO-huCX3CR1 cells, BA/F3-huCX3CR1 cells or transiently transfected HEK293T cells were used. The amount of labeled ligand used in the different competition setups was also varied. The IC_{50} values for VHHs blocking the interaction of human fractalkine to human CX3CR1 are depicted in Table 19.

Table 19 Potency and efficacy of the VHH in a ligand competition FACS

VHH ID	Family	Cell line	IC50	% block	Repeats
11H11	9	CHO-huCX3CR1	1.7 E-8	100	4
18E06	13	CHO-huCX3CR1	1.8 E-9	33	4
54A12	101	CHO-huCX3CR1	2.1 E-9	104	2

54D08	101	CHO-huCX3CR1	1.5 E-8	101	2
54A07	101	CHO-huCX3CR1	1.1 E-8	78	2
54D05	101	CHO-huCX3CR1	2.7 E-9	102	2
54B03	101	CHO-huCX3CR1	2.5 E-8	108	1
54G03	101	CHO-huCX3CR1	5.6 E-8	107	1
11H11	9	BA/F3-huCX3CR1	8.1 E-9	100	3
18E06	13	BA/F3-huCX3CR1	2.8 E-9	71	3
54A12	101	BA/F3-huCX3CR1	4.0 E-9	100	4
54D08	101	BA/F3-huCX3CR1	3.8 E-8	99	1
54A07	101	BA/F3-huCX3CR1	1.5 E-8	81	4
54D05	101	BA/F3-huCX3CR1	5.5 E-9	99	1
54B03	101	BA/F3-huCX3CR1	3.3 E-8	99	1
54G03	101	BA/F3-huCX3CR1	9.8 E-8	98	1
54A12	101	HEK293-huCX3CR1	6.8 E-9	96	5
54D08	101	HEK293-huCX3CR1	8.4 E-8	95	2
54A07	101	HEK293-huCX3CR1	2.3 E-8	52	2
54D05	101	HEK293-huCX3CR1	5.3 E-9	94	5
54B03	101	HEK293-huCX3CR1	6.7 E-8	92	2
54G03	101	HEK293-huCX3CR1	2.7E-7	89	2
61E02	101	HEK293-huCX3CR1	8.2 E-8	98	2
61B04	101	HEK293-huCX3CR1	5.7 E-8	97	2
61B02	101	HEK293-huCX3CR1	1.0 E-8	94	2
54H01	101	HEK293-huCX3CR1	1.0 E-8	64	2
54A04	101	HEK293-huCX3CR1	4.9 E-8	100	2
61F11	101	HEK293-huCX3CR1	4.6 E-8	96	2

61G03	101	HEK293-huCX3CR1	6.0 E-8	96	2
61G04	101	HEK293-huCX3CR1	4.2 E-8	96	2
66B02	101	HEK293-huCX3CR1	2.5 E-9	102	2
66G01	101	HEK293-huCX3CR1	1.4 E-8	99	2

Inhibition by anti-CX3CR1 VHHs of human Fractalkine induced chemotaxis of BA/F3 cells overexpressing human CX3CR1

To evaluate inhibition of Fractalkine induced chemotaxis, a chemotaxis assay was setup using the ChemoTx disposable chamber with 5 µm poresize (Neuroprobe, Gaithersburg, US). Cells were harvested from an actively growing culture and washed before use in assay medium, RPMI (Gibco, Carlsbad, US) supplemented with 0.1% BSA. The bottom chamber was filled with 320 pM human Fractalkine in a total volume of 300 µl. Upon application of the membrane, 0.13E6 cells were deposited on top of the membrane in a total volume of 70 µl. Chemotaxis was allowed for 3 hours at 37°C in a humidified chamber with CO2. After this incubation period, the membrane was removed and cells in the bottom chamber were resuspended. The amount of ATP present in the wells was determined using the CellTiter-Glo kit (Promega, Madison WI, US). Read out was performed on an Envision (Perkin Elmer, Massachusetts, US) with the standard settings for luminescence read out. Titration series were performed in triplicate and each plate contained control samples in triplicate as well. As control, a sample without VHH was included as well as a sample where no human Fractalkine was added to the bottom chamber. A summary of the results is shown in Table 20.

Table 20 Potency and efficacy of the VHH in blocking the fractalkine induced chemotaxis

VHH ID	Fam	IC50	% block	Repeats

11H11	9	2 E-7	89	5
18E06	13	NA	17	4
54A12	101	8 E-8	84	6
54D08	101	NA	33	2
54A07	101	5 E-8	45	4
54D05	101	7 E-8	81	4
54B03	101	1 E-7	73	1
54G03	101	NA	40	1
66B02	101	2 E-8	87	2
66G01	101	4 E-7	54	2

Evaluation of the cross reactivity of the anti-CX3CR1 VHHs against cynomolgus CX3CR1

Initially, a FACS based binding setup was used to evaluate the cynomolgus cross reactivity. For this, the VHHs were incubated with the respective cells for 30 minutes at 4°C followed by three wash steps and subsequently incubated with the detection reagents. As detection, a mouse anti-cmyc antibody (Serotec, MCA2200) followed by a goat anti-mouse antibody coupled to PE (Jackson 115-116-071) was used, each incubation for 30 minutes at 4°C, followed by three wash steps. Results of the assay are shown in Table 21.

Table 21 EC50 value for binding of the respective VHH on human CX3CR1 or on cynomolgus CX3CR1

VHH ID	Family	EC50 (M) Human	EC50 (M) Cynomolgus	ratio	Repeats
11H11	9	8.0 E-10	NA	NA	2

18E06	13	1.5 E-9	1.4 E-8	9	2
54A12	101	3.5 E-8	1.1 E-7	3.3	1
54A07	101	5.4 E-7	3.0 E-8	0.1	1
54D05	101	8.4 E-10	5.0 E-8	59.6	1

For later identified VHHs, a human Fractalkine competition FACS was set up using human or cynomolgus CX3CR1 expressed in HEK293T cells. Both the human and the cynomolgus receptor was transiently transfected in HEK293T cells and transfections were matched by the binding of the labeled ligand, human fractalkine. The competition was evaluated using the EC30 concentration of fractalkine and as such obtained IC50 values are a good estimate of the Ki value, a measure for affinity (Table 22). The experiment was performed as described in Example 4. The ratio of the IC50 values on cynomolgus monkey and human CX3CR1 was used to evaluate potential differences in affinity for CX3CR1 in both species.

Table 22 Efficacy and potency of VHHs in ligand competition FACS towards human and cynomolgus CX3CR1

VHH ID	Family	Human		Cynomolgus		ratio	Repeats
		IC50 (M)	% block	IC50 (M)	% block		
54A12	101	6.8 E-9	96	6.7 E-8	94	9.85	5
54D08	101	8.4 E-8	95	7.2 E-8	91	0.85	2
54A07	101	2.3 E-8	52	2.8 E-7	69	12.3	2
54D05	101	5.3 E-9	94	6.4 E-8	91	12.25	5
54B03	101	6.7 E-8	92	4.7 E-7	95	7.05	2
54G03	101	2.7E-7	89	4.7 E-7	89	1.74	2
61E02	101	8.2 E-8	98	4.6 E-8	96	0.55	2

61B04	101	5.7 E-8	97	8.7 E-8	93	1.53	2
61B02	101	1.0 E-8	94	4.5 E-8	92	4.37	2
54H01	101	1.0 E-8	64	6.8 E-8	92	6.48	2
54A04	101	4.9 E-8	100	3.1 E-8	91	0.64	2
61F11	101	4.6 E-8	96	1.6 E-7	95	3.58	2
61G03	101	6.0 E-8	96	3.0 E-7	89	5.02	2
61G04	101	4.2 E-8	96	2.0 E-7	100	4.8	2
66B02	101	2.5 E-9	102	1.9 E-8	97	7.5	2
66G01	101	1.4 E-8	99	1.2 E-7	100	8.29	2

Binding of the anti-human CX3CR1 VHHs to human CCR2, human CCR5 or mouse CX3CR1

Specificity for the huCX3CR1 receptor was evaluated by performing a FACS binding experiment on CHO-K1 parental cells or CHO cells expressing huCCR2, huCCR5 or msCX3CR1. The VHHs were incubated with the respective cell lines for 30 minutes at 4°C followed by three wash steps and subsequently incubated with the detection reagents. As detection, a mouse anti-cmyc antibody (Serotec, MCA2200) followed by a goat anti-mouse antibody coupled to PE (Jackson 115-116-071) was used, each incubation for 30 minutes at 4°C, followed by three wash steps. For each cell line a quality control with receptor-specific antibody was included. In addition, the highest concentration of each VHH was also incubated with CHO cells expressing huCX3CR1 as a positive control. No binding to msCX3CR1, huCCR2 or huCCR5 could be observed.

Determination of the epitope bin

A competitive binding experiment was setup in order to determine whether the VHHs bind overlapping epitopes on CX3CR1. For this, the VHH 66B02 labeled with alexa647 was used in a competition FACS on the BA/F3 cells expressing huCX3CR1. Representative VHHs from the three functional families were used

as competitors for the binding of the labeled 66B02. The obtained IC50 values are shown in Table 23.

Table 23 Competition FACS based epitope binning

VHH ID	family	IC50 (M)	% block
11H11	9	4.9E-09	100
18E06	13	2.3E-09	100
66B02	101	1.5E-09	100

As a complete inhibition of 66B02 binding could be obtained by all representative VHHs from the different ligand blocking families, it can be concluded that all functional families bind in close enough proximity of each other such that they compete with binding of 66B02.

Example 6: Formatting of VHHs to bivalency

Construction of bivalents

In order to increase potency and/or efficacy from a selection of the obtained VHHs, bivalent molecules were constructed by genetic engineering. Two VHHs were genetically linked together with a 35GS linker in between the two building blocks and subsequently expressed in *E.coli* as described above for the monovalent VHHs. Different bivalent constructs were made as listed in Table 24.

Table 24 Representative bivalent formats

Construct ID	VHH identity	Family	Linker	VHH identity	Family
CX3CR1BII007	CX3CR1BII11H11	9	35GS	CX3CR1BII18E6	13

CX3CR1BII009	CX3CR1BII18E6	13	35GS	CX3CR1BII11H11	9
CX3CR1BII012	CX3CR1BII54D08	101	35GS	CX3CR1BII18E06	101
CX3CR1BII016	CX3CR1BII54A12	101	35GS	CX3CR1BII54A12	101
CX3CR1BII017	CX3CR1BII54D5	101	35GS	CX3CR1BII54D5	101
CX3CR1BII018	CX3CR1BII66B02	101	35GS	CX3CR1BII66B02	101
CX3CR1BII019	CX3CR1BII66G01	101	35GS	CX3CR1BII66G01	101
CX3CR1BII020	CX3CR1BII54B5	101	35GS	CX3CR1BII54B5	101
CX3CR1BII026	CX3CR1BII11H11	9	35GS	CX3CR1BII66B02	101
CX3CR1BII027	CX3CR1BII11H11	9	35GS	CX3CR1BII54B5	101

Inhibition by anti-CX3CR1 VHHs of human Fractalkine binding to human CX3CR1 expressed on the BA/F3 cells

The inhibition of ligand binding to human CX3CR1 was investigated for the different formats as described in Example 4. For this characterization the BA/F3-huCX3CR1 cell line was used showing stable expression of the human CX3CR1 receptor. The alexa647 labeled ligand fractalkine was used at its EC30 concentration and thereby obtained IC50 values are reflective of the Ki values. An overview of the obtained data is shown in Table 25.

Table 25 Potency of bivalent formats in ligand competition

Construct ID	Cell line	IC50 (M)	% block	Repeats
CX3CR1BII007	CHO-huCX3CR1	3.8 E-10	100	2
CX3CR1BII009	CHO-huCX3CR1	7.0 E-10	91	2
CX3CR1BII012	CHO-huCX3CR1	8.0 E-10	93	1
CX3CR1BII016	HEK293-huCX3CR1	1.9 E-10	102	2

CX3CR1BII017	HEK293-huCX3CR1	3.1 E-10	99	2
CX3CR1BII018	HEK293-huCX3CR1	3.0 E-10	102	2
CX3CR1BII019	HEK293-huCX3CR1	2.9 E-10	100	2
CX3CR1BII020	HEK293-huCX3CR1	2.2 E-10	102	2
CX3CR1BII026	BA/F3-huCX3CR1	7.0 E-10	100	3
CX3CR1BII027	BA/F3-huCX3CR1	6.7 E-10	100	3

Inhibition by anti-CX3CR1 VHHs of human Fractalkine induced chemotaxis of BA/F3 cells overexpressing human CX3CR1

Similar to what was described for the monovalent anti-CX3CR1 VHHs, the inhibition of fractalkine induced chemotaxis on the BA/F3-huCX3CR1 cells was evaluated for the bivalent constructs. An identical assay setup was used as described above and the obtained results are summarized in Table 26.

Table 26 Inhibition of fractalkine induced chemotaxis by bivalent VHH constructs

Construct ID	Cell line	IC50 (M)	% block	Repeats
CX3CR1BII007	BA/F3-huCX3CR1	4 E-9	101	5
CX3CR1BII009	BA/F3-huCX3CR1	2 E-8	79	5
CX3CR1BII012	BA/F3-huCX3CR1	4 E-9	78	1
CX3CR1BII016	BA/F3-huCX3CR1	2 E-9	88	3
CX3CR1BII017	BA/F3-huCX3CR1	3 E-9	89	3
CX3CR1BII018	BA/F3-huCX3CR1	6 E-10	98	6
CX3CR1BII019	BA/F3-huCX3CR1	2 E-9	85	3
CX3CR1BII020	BA/F3-huCX3CR1	2 E-9	85	3

CX3CR1BII026	BA/F3-huCX3CR1	3 E-10	98	1
CX3CR1BII027	BA/F3-huCX3CR1	9 E-10	98	1

Evaluation of the cross reactivity of the anti-CX3CR1 VHHs against cynomolgus CX3CR1

Also for the bivalent constructs the cross reactivity towards cynomolgus CX3CR1 was evaluated and compared with the human reactivity. As described earlier, either a binding setup (Table 27) or a ligand competition setup (Table 28) were applied using transient transfected HEK293T cells. Batches of transient transfected cells were matched by their receptor expression level.

Table 27 Binding of bivalent constructs to human or cynomolgus CX3CR1

Construct ID	Cell line	EC50 (M) Human	EC50 (M) Cynomolgus	ratio	Repeats
CX3CR1BII007	HEK293T	3.1 E-10	4.8 E-8	154	2
CX3CR1BII009	HEK293T	2.0 E-9	6.8 E-9	3.3	2
CX3CR1BII012	HEK293T	5.6 E-11	6.3 E-11	1.1	1

Table 28 Ligand competition of bivalent constructs on human or cynomolgus CX3CR1

Construct ID	Cell line	Human		Cynomolgus		ratio	Repeats
		IC50 (M)	% block	IC50 (M)	% block		
CX3CR1BII016	HEK293T	1.9 E-10	102	6.9 E-10	98	3.67	2

CX3CR1BII017	HEK293T	3.1 E-10	99	1.6 E-9	95	5.34	2
CX3CR1BII018	HEK293T	3.0 E-11	102	1.0 E-10	97	3.33	2
CX3CR1BII019	HEK293T	2.9 E-10	100	8.2 E-10	96	2.86	2
CX3CR1BII020	HEK293T	2.2 E-10	102	3.2 E-10	97	1.47	2

Example 7: Exploration of linker length and half life extension

Evaluation of the linker length and positioning of the alb11 VHH

As the linker length used in a bivalent format can impact drastically on the obtained potency, different linker lengths were evaluated.

In addition, Alb11, a Nanobody binding to human serum albumin was included to increase the in vivo half-life of the formatted molecules (WO 06/122787). Different formats were made including variations on the linker lengths used, but also the positioning of the different composing VHHs. A summary of the explored formats is shown in Table 29.

Table 29 Exploration of half life extension and linker length

Construct ID	VHH identity	Linker	VHH identity	Linker	VHH identity
CX3CR1BII032	CX3CR1BII66B02	9GS	CX3CR1BII66B02	9GS	Alb11
CX3CR1BII034	CX3CR1BII66B02	35GS	CX3CR1BII66B02	9GS	Alb11
CX3CR1BII036	CX3CR1BII66B02	9GS	Alb11	9GS	CX3CR1BII66B02
CX3CR1BII040	CX3CR1BII66B02	9GS	CX3CR1BII66B02	35GS	Alb11

CX3CR1BII041	CX3CR1BII66B02	35GS	CX3CR1BII66B02	35GS	Alb11
CX3CR1BII042	CX3CR1BII66B02	35GS	Alb11	35GS	CX3CR1BII66B02

Coding sequences for the formatted VHH were cloned into an in-house constructed plasmid allowing expression in *Pichia pastoris* and secretion into the cultivation medium. The expression vector was derived from pPICZa (Invitrogen) and contained the AOX1 promotor for tightly regulated, methanol induced expression, a restance gene for Zeocin™, a multicloning site and the α-factor secretion signal. Upon transformation expression cultures were grown and VHH expression was induced by addition of methanol and allowed to continue for 48 hours at 30ºC.

The potency of these different formats was evaluated using the ligand competition assay as described above. Seeing that the ligand concentration used is below the EC50 value, the obtained IC50 values are equivalent to the Ki values. The obtained Ki for the different formats is summarized in Table 30.

Table 30 Potency of half life extended formats in ligand competition

Construct ID	Cell line	IC50 (M)	% block	Repeats
CX3CR1BII032	BA/F3-huCX3CR1	5.8 E-10	99	2
CX3CR1BII034	BA/F3-huCX3CR1	5.2 E-10	99	2
CX3CR1BII036	BA/F3-huCX3CR1	5.6 E-10	99	2
CX3CR1BII040	BA/F3-huCX3CR1	5.9 E-10	104	1
CX3CR1BII041	BA/F3-huCX3CR1	6.4 E-10	102	1
CX3CR1BII042	BA/F3-huCX3CR1	8.9 E-10	100	1

Impact of human serum albumin on the potency

The binding of human serum albumin (HSA) to the alb11 VHH could impact on the potency of the format and therefore ligand competition was repeated in presence of HSA. Briefly, to allow the binding of HSA to the alb11 VHH, the constructs under evaluation and fractalkine were pre-incubated with HSA for 30 minutes before addition to the cells. Also the cells were resuspended in FACS buffer supplemented with HSA. The final concentration HSA used was a 50 fold excess above the highest VHH concentration used. Subsequently, competition was allowed for 2 hours and further processing was as described in Example 4.

Construct ID	Cell line	IC50 (M)	% block	Repeats
CX3CR1BII032	BA/F3-huCX3CR1	1.4 E-9	100	2
CX3CR1BII034	BA/F3-huCX3CR1	1.3 E-9	100	2
CX3CR1BII036	BA/F3-huCX3CR1	1.4 E-9	100	2

The potential interference of HSA was also evaluated in an adapted chemotaxis setup, including HSA in the different compartments of the assay. The concentration HSA used was again a 50 fold excess over the highest concentration of construct used and constructs were loaded with HSA for 30 minutes before start of the assay. The assay buffer was also supplemented with HSA such that HSA is present during the entire span of the experiment. As described above, the disposable ChemoTx chamber with 5 µm poresize (Neuroprobe, Gaithersburg, MD, USA) was used. Cells were harvested from an actively growing culture and washed before use in assay medium, RPMI (Gibco, Carlsbad, US) supplemented with 0.1% BSA and 62.5µM HSA (Sigma, A8763). The bottom chamber was filled with 320 pM human Fractalkine in a total volume of 300 µl. Upon application of the membrane, 0.13E6 cells were deposited on top of the membrane in a total volume of 70 µl. Chemotaxis was allowed for 3 hours at 37 C in a humidified chamber with CO2. After this incubation period, the membrane was removed and cells in the bottom chamber were resuspended.

The amount of ATP present in well was determined using the CellTiter-Glo kit (Promega, Madison WI, USA). Read out was performed on an Envision (Perkin Elmer, Waltham, MA, USA) with the standard factory settings for luminescence read out. Titration series were performed in triplicate and each plate contained control samples in triplicate as well. As control, a sample without VHH was included as well as a sample where no human Fractalkine was added to the bottom chamber. The obtained IC50 values are listed in Table 31.

Table 31 Fractalkine induced chemotaxis in the presence of HSA

Construct ID	Cell line	IC50 (M)	% block	Repeats
CX3CR1BII032	BA/F3-huCX3CR1	6E-10	98	2
CX3CR1BII034	BA/F3-huCX3CR1	9E-10	100	2
CX3CR1BII036	BA/F3-huCX3CR1	6E-10	102	2

Inhibition of Fractalkine internalization by the formatted bivalent half-life extended polypeptides

Additional functional assays were performed to demonstrate the antagonist activity of the bivalent half-life extended polypeptides. The polypeptides were evaluated for their ability to inhibit the internalization of A647-Fractalkine in CHO huCX3CR1 cells. Briefly, 1E4 cells/well were plated in black clear bottom, 96 well plates (BD, Franklin Lakes, NJ, USA) and grown overnight. The cells were washed once and then equilibrated in assay buffer (HBSS with calcium and magnesium (Gibco) supplemented with 10 mM HEPES and 0.1% BSA). The formatted polypeptide constructs were added and the plates were incubated for 15 minutes at 37 C. A647-Fractalkine was then added at a final concentration of 8 nM and the cells were incubated for 60 minutes at 37 C. The media was removed and the cells were fixed for 10 minutes with 3.7% formaldehyde solution (Polysciences, Warrington, PA, USA). The cells were rinsed once with PBS and the nuclei were labeled with Hoechst dye (Life Technologies, Grand Island, NY,

USA). To quantitate the internalized labeled Fractalkine, the cells were imaged using the BD Pathway bioimaging system. Image segmentation was performed by identifying the labeled cell nucleus and drawing a 3 pixel ring around that mask. Mean A647 intensity was measured in the cytoplasmic ring. The formatted polypeptides potently inhibited Fractalkine internalization as summarized in Table 32:

Table 32 Inhibition of A647-Fractalkine Internalization

Construct ID	Cell line	IC50 (M)	Repeats
CX3CR1BII032	CHO-huCX3CR1	4.0 E-10	5
CX3CR1BII034	CHO-huCX3CR1	7.4 E-10	3
CX3CR1BII036	CHO-huCX3CR1	4.9 E-10	8
CX3CR1BII040	CHO-huCX3CR1	1.0 E-9	3
CX3CR1BII041	CHO-huCX3CR1	1.1 E-9	2
CX3CR1BII042	CHO-huCX3CR1	8.5 E-10	2

An anti-CX3CR1 formatted bivalent half-life extended polypeptide is devoid of agonist activity

In order to confirm that a bivalent anti-CX3CR1 half-life extended polypeptide did not have agonist activity, CX3CR1BII036 was evaluated for induction of calcium influx in the CHO huCX3CR1 cells. Fractalkine mediated increases in cytosolic calcium levels in these cells in a CX3CR1 dependent manner and CX3CR1BII036 inhibited this response.

The CHO huCX3CR1 cells were plated at 5E4 cells/well in black clear bottom, 96 well plates (BD) and grown overnight. The cells were incubated with Calcium-4 dye/2 mM probenicid (Molecular Devices, Sunnyvale, CA, USA) in HBSS supplemented with 20 mM HEPES for 60 minutes at 37 ^{o}C. For demonstrating polypeptide antagonism, CX3CR1BII036 was preincubated with the cells for 15

minutes prior to the addition of Fractalkine at its EC80 value. Calcium mobilization was monitored on a FLIPR Tetra system (Molecular Devices) as per the manufacturer's instructions. For determining agonism, there was no preincubation with the polypeptide and instead, CX3CR1BII036 was used in place of Fractalkine stimulation. While CX3CR1BII036 inhibited Fractalkine mediated calcium influx with an IC50 of 1.3 nM, no increase in cytosolic calcium levels were observed when the polypeptide alone was added at concentrations up to 1 μM.

Example 8: Exploration of half life extension formats using mouse Fc

To investigate alternative half-life extension modalities, the 66B02 VHH domain was produced as a fusion protein with a mouse IgG2 Fc domain (66B02-mFc). An aspartic acid to alanine mutation (D265A) was incorporated in the CH2 domain to abrogate potential Fc-mediated effector function in this construct (Baudino, J. Immunol., 181, 6664-6669 (2008)). 66B02-mFc was expressed in HEK293T cells or NS0 cells and purified by Protein A affinity chromatography followed by ion exchange chromatography. This molecule was tested for activity utilizing the assay formats described in Example 7. The results are summarized in Table 33:

Table 33 66B02-mFc Activity

Assay	Cell line	IC50 (M)	Repeats
Ligand competition	BA/F3-huCX3CR1	5.7 E-10	2
Chemotaxis	BA/F3-huCX3CR1	8.9 E-10	3
Ligand internalization	CHO-huCX3CR1	5.2 E-10	5
Calcium influx	CHO-huCX3CR1	9.8 E-10	5

While 66B02-mFc potently inhibited Fractalkine mediated CX3CR1 activation, it did not display agonist activity. No increase in cytosolic calcium levels was observed with treatment with up to 1 μM of this molecule.

Example 9: Inhibition of plaque progression in a mouse atherosclerosis model bivalent half-life extended polypeptides

Generation of human CX3CR1 knock-in Apo E $^{-/-}$ mice

Given the lack of cross reactivity of the identified VHHs for mouse CX3CR1 (Example 5), a human CX3CR1 knock-in mouse line (hu CX3CR1 KI) was generated at TaconicArtemis (Koeln, Germany) to enable testing of these molecules in mouse disease models. A strategy was employed that allowed the expression of the human chemokine receptor under the control of the corresponding mouse promoter while disrupting the expression of the endogenous mouse protein. Briefly, a targeting vector was constructed where the mouse CX3CR1 coding region in exon 2 was replaced with the complete human CX3CR1 open reading frame and flanked by selection markers and loxP sites. The targeting vector was introduced into mouse ES cells and clones that had successfully undergone homologous recombination were used to generate chimeric mice. These mice were bred to highly efficient Flp-deleter mice to achieve removal of the selection marker and germline transmission. The resulting hu CX3CR1 KI mice in a C57BL/6 background were then crossed to Apo E $^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, Maine, USA) to generate hu CX3CR1 KI Apo E$^{-/-}$ mice. The Apo E$^{-/-}$ mouse model provides a robust method to elicit extensive atherosclerotic plaque formation that is grossly similar to the human disease with respect to the site-specific localization of plaque formation, histological composition, and the known risk factors (cholesterol, inflammation, hypertension, etc).

Evalulation of the anti-CX3CR1 bivalent half-life extended polypeptides in the mouse Apo E$^{-/-}$ atherosclerosis model

Female hu CX3CR1KI ApoE $^{-/-}$ mice were fed a high fat/high cholesterol diet containing 1.5% cholesterol for 16 weeks beginning at four weeks of age. After 10

weeks, the animals were administered by i.p. injection vehicle (20 mM NaCitrate pH 6.0, 115 mM NaCl), 10 mg/kg 66B02-mFc once or twice per week or 30 mg/kg CX3CR1BII036 twice per week for 6 weeks. The animals were anesthetized by gas anaesthesia and perfused with 0.9% saline. The descending aorta to the ileac bifurcation was carefully removed and fixed in formalin. It was then opened longitudinally, and stained with Sudan IV for 15 minutes, followed by 70% methanol for 2 minutes. The vessels were washed under running water and covered with PBS. The tissues were photographed with a digital camera using SPOT Advanced software (SPOT Imaging Solutions, Sterling Heights, MI, USA). The percentage of lipid staining was determined with image analysis software (Image-Pro Plus, MediaCybernetics, Rockville, MD, USA) and expressed as a percentage positive staining of the vessel. The results from this study are summarized in Table 34:

Table 34 Quantification of Plaque Size in the Descending Aorta in female hu CX3CR1 KI Apo E $^{-/-}$ Mice

Group	Dose	# Animals	% Plaque Area	% Reduction in Plaque Area
Control (10 weeks)	N/A	6	3.4	N/A
Control (16 weeks)	Vehicle	17	14.8	N/A
66B02-mFc	10 mg/kg (1xweek)	17	13.0	16
66B02-mFc	10 mg/kg (2x/week)	17	10.3	39 ($p<0.05$)
CX3CR1BII036	30 mg/kg (2x/week)	17	10.1	41 ($p<0.05$)

Both 66B02-mFc and CX3CR1BII036 significantly inhibited plaque progression when dosed twice weekly. This correlated with coverage as plasma levels of these molecules could be confirmed to be maintained throughout the study. For once weekly dosing of 66B02-mFc, detectable plasma levels were not maintained and this correlated with the lack of significant efficacy observed after 6 weeks of treatment. Neither molecule significantly affected plasma cholesterol or triglyceride levels.

Example 10: Sequence optimization of the parental VHH

In general, during VHH sequence optimization, parental wild type VHH sequences are mutated to yield VHH sequences that are more identical to human VH3-JH germline consensus sequences. Specific amino acids in the framework regions that differ between the VHH and the human VH3-JH germline consensus are altered to the human counterpart in such a way that the protein structure, activity and stability are kept intact. To investigate this, all sequence optimization variants were compared with the parental VHH in three different assays: (i) determination of the melting temperature (Tm) in a Thermal Shift Assay (TSA), (ii) analysis of in vitro potency in fractalkine competition FACS, and for some constructs (iii) analysis of in vitro potency in the fractalkine induced chemotaxis assay.

Mutation of framework residues

For sequence optimization, the following mutations were investigated: E1D, S11L, A14P, E16G, R44Q, D46E, A74S, K83R and Q108L. The individual mutants that were generated in the parental sequence of CX3CR1BII66B02 are depicted in Table 35:

Table 35 Investigated mutations during sequence optimization of 66B02

Clone number	Mutations introduced
C100CX3CR1BII043	A14P,A74S,K83R,Q108L
C100CX3CR1BII045	E1D,A14P,A74S,K83R,Q108L
C100CX3CR1BII047	S11L,A14P,A74S,K83R,Q108L
C100CX3CR1BII048	A14P,E16G,A74S,K83R,Q108L
C100CX3CR1BII049	A14P,R44Q,A74S,K83R,Q108L
C100CX3CR1BII050	A14P,D46E,A74S,K83R,Q108L
C100CX3CR1BII061	S11L,A14P,E16G,A74S,K83R,Q108L
C100CX3CR1BII056	S11L,A14P,E16G,R44Q,A74S,K83R,Q108L
C100CX3CR1BII057	S11L,A14P,E16G,D46E,A74S,K83R,Q108L
C100CX3CR1BII060	S11L,A14P,E16G,R44Q,D46E,A74S,K83R,Q108L

All constructs were cloned in an *E.* coli expression vector, and expressed in *E. coli* as myc/His-tagged proteins in a culture volume of 0.25L to 0.5L TB medium. Expression was induced by addition of 1mM IPTG and allowed to continue for 4 hours at 37°C and 250 rpm. Cells were pelleted, and periplasmic extracts were prepared by freeze-thawing and resuspension in dPBS. These extracts were used as starting material for immobilized metal affinity chromatography (IMAC) using Histrap FF crude columns (GE healthcare). Nanobodies were eluted from the column with 250mM imidazole and subsequently desalted towards dPBS. The purity and integrity of Nanobodies was verified by reducing SDS-PAGE.

As summarized in Table 36, A14P, A74S, K83R and Q108L mutations had no clear effect on potency as determined from competition FACS. Similarly, the additional mutations E1D, S11L and E16G did not affect potency. The introduction of either R44Q or D46E on the other hand resulted in a significant

drop in potency that was even more pronounced if both mutations were introduced.

Table 36 Potency of sequence optimization constructs determined by ligand competition FACS

Clone number	IC50	% block	Tm at pH7
CX3CR1BII66B02	2.6E-09	101.0	65.66
C100CX3CR1BII043	2.2E-09	101	66.49
C100CX3CR1BII045	2.2E-09	101.2	66.07
C100CX3CR1BII047	2.3E-09	101.2	66.49
C100CX3CR1BII048	1.9E-09	101.2	67.74
C100CX3CR1BII049	1.8E-08	101.2	66.07
C100CX3CR1BII050	1.7E-08	101.1	71.90
C100CX3CR1BII061	1.4E-09	98.9	68.57
C100CX3CR1BII056	1.6E-08	101.1	68.57
C100CX3CR1BII057	1.4E-08	99.4	74.39
C100CX3CR1BII060	1.9E-07	98.4	74.81

Also the melting temperature, predictive for the stability of the VHH, was evaluated. Most individual mutations had limited to no effect except for the D46E mutation which raised the melting temperature by approximately 6°C. The introduction of the combined mutations also enhanced the thermal stability, cfr 057 and 060.

Due to the major effects on the potency in ligand competition FACS, the mutations R44Q and D46E were not included in the final sequence.

Mutation of CDR residues

Based on the in silico analysis of the parental sequence, a glycosylation site was predicted at position 52. Therefore two libraries were constructed; one for position 52 and one for position 53, which was designed to include all possible amino acids at the respective position. The libraries were screened as periplasmic extracts in a ligand competition FACS. First, a dilution series was made of periplasmic material from the parental sequence and three dilutions were selected for further screening. A first dilution point (two fold) was chosen to give full block of the ligand interaction whereas the other two dilution points (128 and 512 fold) should result in 70 % and 40% block respectively. Upon production of the periplasmic extracts from the library, all samples were split in two and one of them was subjected to a heat treatment. Both the non-treated and the heat treated samples were subsequently analyzed in the ligand competition FACS at the three dilution points. The impact of the mutation could be estimated by comparing the obtained blockage with that from the parental sequence. The analysis of the heat treated samples provides a measure for a potential impact on stability of the mutation.

Based upon the initial screening results, seven mutations were selected for further characterization. The obtained potency in ligand competition FACS is shown in Table 37.

Table 37 Removal glycosylation site at position 52

Construct	IC50 (M)	% block	Tm at pH7
C100CX3CR1BII66B02	2.5E-09	98.0	65.66
CX3CR1BII66B02 (N52S, Q108L)	1.7E-09	98.0	66.07
CX3CR1BII66B02 (N52Q, Q108L)	2.1E-09	97.9	59.83
CX3CR1BII66B02 (N52G, Q108L)	1.1E-09	98.0	59.83
CX3CR1BII66B02 (N52T, Q108L)	2.8E-09	98.0	66.07
CX3CR1BII66B02 (S53T, Q108L)	1.3E-09	98.1	66.07
CX3CR1BII66B02 (S53G, Q108L)	1.2E-09	98.3	64.83

CX3CR1BII66B02 (S53P, Q108L)	8.0E-10	98.2	66.91

From this analysis, sequence alignment with the human reference sequence and based upon an *in silico* T cell epitope recognition prediction program, it was decided to include the mutations N52S and S53T in the sequence.

Because of stability reasons an additional library was made for position 32.The ligand competition screening was set up in a similar fashion as described above. Again three dilutions of the periplasmic extracts were screened and the obtained % block was compared with that obtained for the parental sequence. Upon analysis of the various mutants, the substitution of N32T was chosen and included in the final sequence optimized variant.

Example 11: Analysis of the optimized variants

In a final characterization round the constructs listed in Table 38 were characterized.

Table 38 Sequence optimized variants of the lead VHH 66B02

Clone number	Mutation introduced	SEQ ID NO:
CX3CR1BII00306	CX3CR1BII66B02(E1D,S11L,A14P,E16G,N32T,N52S,A74S,K86R,Q113L)	138
CX3CR1BII00307	CX3CR1BII66B02(E1D,S11L,A14P,E16G,N32T,N52S,S53T,A74S,K86R,Q113L)	139
CX3CR1BII00308	CX3CR1BII66B02(E1D,S11L,A14P,E16G,A74S,K86R,Q113L)	140
CX3CR1BII00312	CX3CR1BII66B02(E1D,S11L,A14P,E16G,N32T,N52S,A74S,K86R,Q113L)-9GS-Alb11-9GS-CX3CR1BII66B02(S11L,A14P,E16G,N32T,N52S,A74S,K86R,Q113L)	225

CX3CR1BII00313	CX3CR1BII66B02(E1D,S11L,A14P,E16G,N32T,N52S,S53T,A74S,K86R,Q113L)-9GS-Alb11-9GS-CX3CR1BII66B02(S11L,A14P,E16G,N32T,N52S,S53T,A74S,K86R,Q113L)	226
CX3CR1BII00314	CX3CR1BII66B02(E1D,S11L,A14P,E16G,A74S,K86R,Q113L)-9GS-Alb11-9GS-CX3CR1BII66B02(S11L,A14P,E16G,A74S,K86R,Q113L)	227

A competition FACS experiment was performed as described above as well as a determination of the melting temperature. The obtained values are represented in Table 39.

Table 39 Competition FACS and Tm of the sequence optimized variants

Construct	IC50 (M)	% block	Tm at pH7
C100CX3CR1BII66B02	2.5E-09	98.0	65.05
CX3CR1BII00306	1.7E-09	97.0	68.54
CX3CR1BII00307	1.9E-09	97.0	68.13
CX3CR1BII00308	1.6E-09	97.0	68.13
CX3CR1BII00312	4.6E-10	100.0	59.37
CX3CR1BII00313	4.0E-10	100.0	58.88
CX3CR1BII00314	6.5E-10	100.0	58.40

These constructs were also characterized in fractalkine induced chemotaxis as described above (Table 40).

Table 40 Ligand induced chemotaxis with sequence optimized variants

Construct	IC50 (M)	% block	n
C100CX3CR1BII66B02	3.6E-08	91	3
CX3CR1BII00306	6.3E-08	95	2
CX3CR1BII00307	6.3E-08	100	2
CX3CR1BII00308	4.4E-08	89	2
CX3CR1BII00312	2.7E-09	99	3
CX3CR1BII00313	2.7E-09	99	3
CX3CR1BII00314	3.6E-09	100	3

Selected constructs were evaluated for inhibition of A647-Fractalkine induced internalization in CHO huCX3CR1 cells. The results are summarized in Table 41:

Table 41 Ligand induced internalization with sequence optimized variants

Construct	IC50 (M)	n
CX3CR1BII00312	5.5 E-10	1
CX3CR1BII00313	3.3E-10	6

A sequence optimized anti-CX3CR1 half-life extended polypeptide is devoid of agonist activity

In order to confirm that sequence optimized anti-CX3CR1 half-life extended polypeptide does not have agonist activity, CX3CR1BII00313 was evaluated for induction of calcium influx in the CHO huCX3CR1 cells. While preincubation with CX3CR1BII00313 inhibited Fractalkine-mediated calcium influx with an IC50 of 1.3 nM, no increase in cytosolic calcium levels were observed when the polypeptide alone was added at concentrations up to 1 μM.

Example 12: Exploration of half life extension formats using human Fc

To investigate additional half-life extension modalities, the CX3CR1BII00306 and CX3CR1BII00307 sequence optimized VHH domains were produced as fusion proteins with a human IgG1 Fc domain (306D-hFc and 307D-hFc). Two mutations were incorporated in the CH2 domain to abrogate potential Fc-mediated effector function in this construct. 306D-hFc and 307D-hFc were expressed in HEK293T cells or NS0 cells and purified by Protein A affinity chromatography followed by ion exchange chromatography. These molecules were tested for functional activity utilizing the assay formats described in Example 7. The results are summarized in Table 42:

Table 42 Activity of hFc Fusion Proteins

Assay	Cell line	306D-hFc IC50	306D-hFc Repeats	307D-hFc IC50	307D-hFc Repeats
Ligand competition	BA/F3-huCX3CR1	6.9 E-10	2	7.0 E-10	2
Chemotaxis	BA/F3-huCX3CR1	2.9 E-9	2	3.0 E-9	3
Ligand internalization	CHO-huCX3CR1	4.8 E-10	3	3.7 E-10	3
Calcium influx	CHO-huCX3CR1	1.3 E-9	3	3.2 E-9	3

While these molecules potently inhibited Fractalkine mediated CX3CR1 activation, they did not display agonist activity. No increases in cytosolic calcium levels were observed with treatment with up to 1 μM of these Nanobodies alone.

Example 13: Inhibition of plaque progression in a mouse atherosclerosis model by a sequence optimized anti-CX3CR1 Nanobody

Female hu CX3CR1KI ApoE $^{-/-}$ mice were fed a high fat/high cholesterol diet containing 1.5% cholesterol for 16 weeks beginning at four weeks of age. After 10

weeks, the animals were administered by i.p. injection vehicle (20 mM NaCitrate pH 6.0, 115 mM NaCl), 30 mg/kg CX3CR1BII00313 once or twice per week or 30 mg/kg CX3CR1BII036 twice per week for 6 weeks. The animals were sacrificed and the percentage of plaque area in the descending aorta was quantitated as described above. The results from this study are summarized in Table 43:

Table 43 Quantification of Plaque Size in the Descending Aorta in female hu CX3CR1 KI Apo E $^{-/-}$ Mice

Group	Dose	# Animals	% Plaque Area	% Reduction in Plaque Area
Control (10 weeks)	N/A	6	2.1	N/A
Control (16 weeks)	Vehicle	18	12.0	N/A
CX3CR1BII00313	30 mg/kg (1xweek)	17	10.7	13
CX3CR1BII00313	30 mg/kg (2x/week)	18	5.9	62 ($p<0.01$)
CX3CR1BII036	30 mg/kg (2x/week)	17	6.8	52 ($p<0.01$)

Both CX3CR1BII00313 and CX3CR1BII036 significantly inhibited plaque progression when dosed twice weekly. This correlated with coverage as plasma levels of these molecules could be confirmed to be maintained throughout the study. For once weekly dosing of CX3CR1BII00313, detectable plasma levels were not maintained and this correlated with the lack of significant efficacy observed after 6 weeks of treatment. Neither molecule significantly affected plasma cholesterol or triglyceride levels.

Example 14: Nanobody binding to primary human and cynomolgus monkey CD14+ cells in whole blood

Competition FACS with formatted sequence optimized anti-CX3CR1 Nanobody

To confirm binding of the formatted sequence optimized anti-CX3CR1 Nanobody to human primary cells, CX3CR1BII00313 was demonstrated to compete for the binding of A647 labeled CX3CR1BII018 (A647-018) to CD14+ cells in a competition FACS assay in whole blood. Briefly, a mouse anti-human CD14 antibody conjugated with eFluor 450 (eBioscience, San Diego, CA, USA) was diluted 1:10 in EDTA treated whole blood from a healthy human donor. 40 μl/well was added to 96 well polystyrene round bottom plate followed by 10 μl/well of CX3CR1BII00313 diluted in Stain Buffer with BSA (BD Pharmingen) at a final concentration ranging from 100 nM to 0.002 pM and the samples were incubated for 20 minutes at room temperature. 10 μl/well of A647-018 in Stain Buffer was then added to yield a final concentration of 1 nM (the EC80 of A647-018 binding) and the samples were incubated for an additional 20 minutes at room temperature. 220 μl/well of 1-Step Fix/Lyse solution (eBioscience) was then added. After a 10 minute room temperature incubation the cells were pelleted, washed twice in Stain buffer and resuspended in this buffer. The samples were analyzed on a BD LSR II flow cytometer. The median fluorescence intensity for AlexaFluor 647 was quantified for the gate CD14 positive cell population. CX3CR1BII00313 potently inhibited the binding of A647-018 to CD14 positive cells in human blood with an IC50 of 0.35 nM (n=8).

To confirm binding of the formatted sequence optimized anti-CX3CR1 Nanobody to cynomolgus monkey primary cells, CX3CR1BII00313 was demonstrated to compete for the binding of A647 labeled CX3CR1BII018 (A647-018) to CD14+ cells in a competition FACS assay in cynomolgus monkey whole blood. The method used was analogous to that outlined above except the final concentration of A647-018 was 3 nM (the EC80 of A647-018 binding) and ACK lysing buffer (Life Technologies) was used instead of the 1-Step Fix/Lyse solution. The cells were resuspended in Stain buffer supplemented with 1% formaldehyde prior to analysis. CX3CR1BII00313 potently inhibited the binding of A647-018 to CD14 positive cells in cynomolgus monkey blood with an IC50 of 0.43 nM (n=4).

Example 15: Pharmacokinetics (PK) in cynomolgus monkeys

A pharmacokinetic study was conducted in naïve male cynomolgus monkeys (*Macaca fascicularis*) 2 -5 years of age with a body weight range between 2.4 – 3.5 kg. The monkeys were divided into four treatment groups. Group 1 (n=3) received 0.2 mg/kg of CX3CR1BII00313 i.v.; Group 2 (n=3) received 2 mg/kg of CX3CR1BII00313 i.v.; Group 3 (n=3) received 2 mg/kg CX3CR1BII00313 s.c. and Group 4 (n=3) received 5 mg/kg CX3CR1BII00313 i.v. CX3CR1BII00313 was administered as a 2 mg/ml solution in citrate buffer (20 mM sodium citrate/115 mM sodium chloride, pH 6.0). Blood samples were collected over 6 weeks from a peripheral vein into serum separator tubes for PK analysis.

Serum samples were analyzed using a MSD (Meso Scale Discovery) format. Briefly, a biotinylated anti-Nanobody antibody was bound to a MSD standard streptavidin plate (Meso Scale Discovery, Rockville, MD, USA). The plates were washed with 0.05% Tween 20 in phosphate buffered saline and blocked with 5% w/v of SeraCare BSA (SeraCare Life Sciences, Milford, MA, USA) prior to incubation with serum samples. CX3CR1BII00313 was detected utilizing a sulfo-labeled anti-Nanobody Nanobody and the plates were analyzed on a Sector Imager 2400 (Meso Scale Discovery). Varying concentrations of CX3CR1BII0313 from 5000 to 0.5 ng/ml in 5% monkey serum were used as standards. Target engagement was assessed by monitoring levels of free CX3CR1 on CD14+ gated monocytes. This assay was analogous to the competition FACS assay summarized in Example 14 except no additional CX3CR1BII00313 was added. Serum samples were also monitored for the presence of primate anti-human antibodies (PAHA) as they may impact assessment of PK and free CX3CR1.

ForteBio RED96 was used for detection of PAHA. Briefly, biotinylated CX3CR1BII0313 was captured over streptavidin sensors. Pooled naïve monkey serum was then used as a negative control to calculate cut-off value (defined as two fold above the average binding signal of naïve sera). All serum samples were diluted 20 fold in buffer and the PAHA response was determined to be positive if the binding signal was greater than the cut-off value.

Data for time points following detection of PAHA were excluded from PK/PD analysis. The PK data are summarized in Table 44 below.

Table 44 Pharmacokinetic parameters of CX3CR1BII00313

Dose (mg/kg)	CL (mL/day/kg)	$T_{1/2}$ (day)	MRT (day)	Dose normalized AUC(0-14d) (nM·d)	F%
IV 0.2	113	1	1	**	
IV 2.0	9 ± 1	9 ± 2	8 ± 2	56530	
IV 5.0	**	**	**	58604	
SC 2.0					54

**insufficient data for characterization of terminal phase

Clearance and half-life at 2.0 mg/kg i.v. were 9.4 mL/d/kg and 9.6 days, respectively. At 0.2 mg/kg i.v., clearance was substantially higher (113 mL/d/kg) consistent with saturable target-mediated disposition (TMD) pharmacokinetics. Dose-adjusted $AUC_{(0\text{-}14d)}$ was comparable between the 2 and 5 mg/kg i.v. doses suggesting saturation of TMD at the 2 mg/kg dose. Exposure at 2 weeks following either i.v. or s.c. Nanobody administration was > 70 nM and bioavailability after s.c. administration was 54 %. Free receptor tracked with exposure with greater than 90 % target coverage maintained at exposures > 10 nM.

Claims

1. A polypeptide comprising an anti-CX3CR1 (anti-CX3 chemokine receptor 1) immunoglobulin single variable domain, wherein said anti-CX3CR1 immunoglobulin single variable domain consists essentially of four framework regions (FR1, FR2, FR3 and FR4) and three complementary determining regions (CDR1, CDR2 and CDR3) and wherein said CDR3 has the amino acid sequence of Asp-Pro-Arg-Arg-Gly-Trp-Asp-Thr-Arg-Tyr (SEQ ID NO: 186) and wherein said polypeptide comprises a CDR1, CDR2 and CDR3 having the amino acid sequences set forth in:

- SEQ ID No: 213, 221 and 186, respectively; or
- SEQ ID No: 141, 162 and 186, respectively; or
- SEQ ID No: 141, 164 and 186, respectively; or
- SEQ ID No: 141, 166 and 186, respectively; or
- SEQ ID No: 141, 167 and 186, respectively; or

- SEQ ID No: 213, 214 and 186, respectively.

2. The polypeptide according to claim 1, wherein the polypeptide comprises a CDR1, CDR2 and CDR3 having amino acid sequences set forth in: SEQ ID NO's: 141, 164 and 186, respectively, SEQ ID NO's: 141, 162 and 186, respectively, SEQ ID NO's: 213, 214 and 186 respectively, or SEQ ID NO's: 213, 221 and 186 respectively.

3. The polypeptide according to any one of claims 1 to 2, wherein said anti-CX3CR1 immunoglobulin single variable domain is a VHH domain comprising the sequence set forth in:

a) an amino acid sequence of any one of SEQ ID NO: 1, 3-5, 7, 9, 10, 13, 19, 21, 23, 24, 121-130, 138-140 or 222-224.

4. The polypeptide according to claim 1, wherein the polypeptide further comprises a half-life extending moiety.

5. The polypeptide according to claim 4, wherein said half-life extending moiety is covalently linked to said polypeptide and is selected from the group consisting of an albumin binding moiety, such as an anti-albumin immunoglobulin domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, a recombinant polyethylene glycol molecule, human serum albumin, a fragment of human serum albumin, an albumin binding peptide or a Fc domain.

6. The polypeptide according to claim 4 or 5, wherein said half-life extending moiety consists of an anti-albumin immunoglobulin single variable domain.

7. The polypeptide according to claim 6, wherein the immunoglobulin single variable domain is selected from a VHH domain, a humanized VHH domain, a camelized VH domain, a domain antibody, a single domain antibody and/or "dAb"s.

8. The polypeptide according to claim 7, wherein the anti-albumin immunoglobulin single variable domain comprises a sequence selected from any one of SEQ ID NO's: 230-232.

9. The polypeptide according to claim 1, wherein said polypeptide further comprises a second immunoglobulin single variable domain.

10. The polypeptide according to claim 9 wherein said second immunoglobulin single variable domain comprises a second anti-CX3CR1 immunoglobulin single variable domain.

11. The polypeptide according to claim 9, wherein said second immunoglobulin single variable domain comprises CDR1, CDR2 and CDR3 amino acid sequences set forth in

- SEQ ID No: 213, 221 and 186, respectively; or
- SEQ ID No: 141, 162 and 186, respectively; or

- SEQ ID No: 141, 164 and 186, respectively; or
- SEQ ID No: 141, 166 and 186, respectively; or
- SEQ ID No: 141, 167 and 186, respectively; or
- SEQ ID No: 213, 214 and 186, respectively.

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12. The polypeptide according to claim 11, wherein said first and said second immunoglobulin single variable domains comprise the same CDR1, CDR2 and CDR3.

13. The polypeptide according to claim 12, wherein said first and second immunoglobulin single variable domains comprise a VHH domain having an amino acid sequence set forth in

a) an amino acid sequence of any one of SEQ ID NO: 1, 3-5, 7, 9, 10, 13, 19, 21, 23, 24, 121-130 or 222-224.

14. A polypeptide comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain, each comprising CDR1, CDR2 and CDR3 having amino acid sequences set forth in SEQ ID NO's: 141, 164 and 186, or SEQ ID NO's: 141, 162 and 186, or SEQ ID NO's: 213, 214 and 186, or SEQ ID NO's: 213, 221 and 186.

15. A polypeptide comprising the amino acid sequence of any one of SEQ ID NO: 225-227 or 257-262.

16. A nucleic acid molecule encoding a polypeptide according to any one of claims 1 to 15.

17. An expression vector comprising a nucleic acid molecule according to claim 16.

18. A host cell comprising a nucleic acid molecule encoding a polypeptide according to any one of claims 1 to 15, wherein said host cell is capable of expressing said polypeptide.

19. A pharmaceutical composition comprising (i) a polypeptide according to any one of claims 1 to 15, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

20. The pharmaceutical composition according to claim 16, wherein said pharmaceutical composition is suitable for intravenous or subcutaneous injection in a human being.

21. A method of manufacturing a polypeptide according to any one of claims 1 to 15, comprising the steps of

- culturing a host cell under conditions that allow expression of a polypeptide according to any one of claims 1 to 15,
  wherein said host cell is carrying an expression vector comprising a nucleic acid molecule, said nucleic acid molecule comprising a region encoding a polypeptide according to any one of claims 1 to 15, and
  wherein said host cell is a prokaryotic or a eukaryotic cell.

22. The method according to claim 21, further comprising the steps of

- recovering said polypeptide; and
- purifying said polypeptide.

23. A polypeptide according to any of claims 1 to 15 for use in the treatment, prevention or alleviation of a disease, disorder or condition, in a human being.

24. The polypeptide for use according to claim 23, wherein the disease, disorder or condition is a CX3CR1-associated disease, disorder or condition.

25. The polypeptide for use according to claim 24, wherein the disease, disorder or condition is selected from cardio- and cerebrovascular atherosclerotic

disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulomephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

26. The polypeptide for use according to claim 25, wherein the disease, disorder or condition is atherosclerosis.

27. Use of a polypeptide according to any of claims 1 to 15 for the manufacture of a medicament for the treatment, prevention or alleviation of a disease, disorder or condition, in a human being, wherein the disease or disorder is selected from cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulonephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

28. The use according to claim 27, wherein the disease or disorder is atherosclerosis.

29. A diagnostic kit comprising a polypeptide according to any one of claims 1 to 15, or the use thereof.

30. A diagnostic kit according to claim 29, for the diagnosis of at least one of cardio- and cerebrovascular atherosclerotic disorders, peripheral artery disease, restenosis, diabetic nephropathy, glomerulomephritis, human crescentic glomerulonephritis, IgA nephropathy, membranous nephropathy, lupus nephritis, vasculitis including Henoch-Schonlein purpura and Wegener's granulomatosis, rheumatoid arthritis, osteoarthritis, allograft rejection, systemic sclerosis, neurodegenerative disorders and demyelinating disease, multiple sclerosis (MS), Alzheimer's disease, pulmonary diseases such as COPD, asthma,neuropathic pain, inflammatory pain, or cancer.

31. A polypeptide comprising the amino acid sequence of SEQ ID NO: 225.

32. A polypeptide comprising the amino acid sequence of SEQ ID NO: 226.

33. A polypeptide comprising the amino acid sequence of SEQ ID NO: 227.

34. A polypeptide comprising the amino acid sequence of SEQ ID NO: 258.

35. A polypeptide comprising the amino acid sequence of SEQ ID NO: 261.

36. A pharmaceutical composition comprising (i) a polypeptide according to claim 31, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

37. A pharmaceutical composition comprising (i) a polypeptide according to claim 32, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

38. A pharmaceutical composition comprising (i) a polypeptide according to claim 33, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

39. A pharmaceutical composition comprising (i) a polypeptide according to claim 34, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

40. A pharmaceutical composition comprising (i) a polypeptide according to claim 35, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.