The present invention relates to amino acid sequences that are directed against (as defined herein) human cellular receptors for viruses and/or bacteria such as e.g. Nanobodies specifically recognizing hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hS-R-B1, hClaudin-1, hClaudin-6 and hClaudin-9, as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such amino acid sequences. Said amino acid sequences may be used to prevent human cell entry of HIV, HCV, adenoviruses, hantavirus, herpesvirus, echo-virus 1 and others.
The present invention relates to amino acid sequences that are directed against (as defined herein) human cellular receptors for viruses and/or bacteria such as e.g. Nanobodies specifically recognizing hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9. as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such amino acid sequences (also referred to herein as "amino acid sequences of the invention", “compounds of the invention”, and “polypeptides of the invention”, respectively). Said amino acid sequences may be used to prevent human cell entry of HIV, HCV, adenoviruses, hantavirus, herpesvirus, echo-virus 1 and others.

The invention also relates to nucleic acids encoding such amino acid sequences and polypeptides (also referred to herein as “nucleic acids of the invention" or “nucleotide sequences of the invention”); to methods for preparing such amino acid sequences and polypeptides; to host cells expressing or capable of expressing such amino acid sequences or polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such amino acid sequences, polypeptides, nucleic acids and/or host cells; and to uses of such amino acid sequences or polypeptides, nucleic acids, host cells and/or compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein.

Other aspects, embodiments, advantages and applications of the invention will become clear from the further description herein.

When searching for their favorite host tissues, animal viruses and bacteria frequently attach to cell-surface receptors that have key roles in normal cell physiology. The receptors mentioned herein are examples of physiologically important receptors that have been usurped by nonenveloped and enveloped viruses for attachment, virus-cell fusion and/or virus entry into mammalian cell or partial virus entry into mammalian cell.

The polypeptides and compositions of the present invention can generally be used to modulate, and in particular inhibit and/or prevent attachment, virus/bacterium-cell fusion and/or virus/bacterium entry into mammalian cell or partial virus/bacterium entry into mammalian cell.
As such, the polypeptides and compositions of the present invention can be used for the prevention and treatment (as defined herein) of viruses and/or bacteria induced diseases and disorders. Generally, "viruses and/or bacteria induced diseases and disorders" can be defined as diseases and disorders that can be prevented and/or treated, respectively, by suitably administering to a subject in need thereof (i.e. having the disease or disorder or at least one symptom thereof and/or at risk of attracting or developing the disease or disorder) of either a polypeptide or composition of the invention (and in particular, of a pharmaceutically active amount thereof). Examples of such viruses and/or bacteria induced diseases and disorders will be clear to the skilled person based on the disclosure herein.

Thus, without being limited thereto, the amino acid sequences and polypeptides of the invention can for example be used to prevent and/or to treat all diseases and disorders that are currently being prevented or treated with active principles that can modulate virus/bacterium attachment, virus/bacterium-cell fusion and/or virus/bacterium entry into mammalian cell or partial virus/bacterium entry into mammalian cell. It is also envisaged that the polypeptides of the invention can be used to prevent and/or to treat all diseases and disorders for which treatment with such active principles is currently being developed, has been proposed, or will be proposed or developed in future. In addition, it is envisaged that, because of their favourable properties as further described herein, the polypeptides of the present invention may be used for the prevention and treatment of other diseases and disorders than those for which these known active principles are being used or will be proposed or developed; and/or that the polypeptides of the present invention may provide new methods and regimens for treating the diseases and disorders described herein.

Generally, it is an object of the invention to provide pharmaceutically active agents, as well as compositions comprising the same, that can be used in the diagnosis, prevention and/or treatment of viruses and/or bacteria induced diseases and disorders and of the further diseases and disorders mentioned herein; and to provide methods for the diagnosis, prevention and/or treatment of such diseases and disorders that involve the administration and/or use of such agents and compositions.

In particular, it is an object of the invention to provide such pharmaceutically active agents, compositions and/or methods that have certain advantages compared to the agents, compositions and/or methods that are currently used and/or known in the art. These advantages will become clear from the further description below.
More in particular, it is an object of the invention to provide therapeutic proteins that can be used as pharmacologically active agents, as well as compositions comprising the same, for the diagnosis, prevention and/or treatment of viruses and/or bacteria induced diseases and disorders and of the further diseases and disorders mentioned herein; and to provide methods for the diagnosis, prevention and/or treatment of such diseases and disorders that involve the administration and/or the use of such therapeutic proteins and compositions.

Accordingly, it is a specific object of the present invention to provide amino acid sequences that are directed against (as defined herein) human cellular receptors for viruses and/or bacteria such as e.g. Nanobodies specifically recognizing hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hOaudin-6 and hClauctin-9 (also referred to herein as “Targets of the invention” or each of these referred to as “one of the Targets of the invention” or “Target of the invention”), in particular against Targets of the invention from a warm-blooded animal, more in particular against Targets of the invention from a mammal, and especially against human Targets of the invention; and to provide proteins and polypeptides comprising or essentially consisting of at least one such amino acid sequence.

In particular, it is a specific object of the present invention to provide such amino acid sequences and such proteins and/or polypeptides that are suitable for prophylactic, therapeutic and/or diagnostic use in a warm-blooded animal, and in particular in a mammal, and more in particular in a human being.

More in particular, it is a specific object of the present invention to provide such amino acid sequences and such proteins and/or polypeptides that can be used for the prevention, treatment, alleviation and/or diagnosis of one or more diseases, disorders or conditions associated with Targets of the invention and/or mediated by Targets of the invention (such as the diseases, disorders and conditions mentioned herein) in a warm-blooded animal, in particular in a mammal, and more in particular in a human being.

It is also a specific object of the invention to provide such amino acid sequences and such proteins and/or polypeptides that can be used in the preparation of pharmaceutical or veterinary compositions for the prevention and/or treatment of one or more diseases, disorders or conditions associated with and/or mediated by Targets of the invention (such as the diseases, disorders and conditions mentioned herein) in a warm-blooded animal, in particular in a mammal, and more in particular in a human being.
In the invention, generally, these objects are achieved by the use of the amino acid sequences, proteins, polypeptides and compositions that are described herein.

In general, the invention provides amino acid sequences that are directed against (as defined herein) and/or can specifically bind (as defined herein) to Targets of the invention; as well as compounds and constructs, and in particular proteins and polypeptides, that comprise at least one such amino acid sequence.

More in particular, the invention provides amino acid sequences that can bind to Targets of the invention with an affinity (suitably measured and/or expressed as a K₀-value (actual or apparent), a Kₐ-value (actual or apparent), a kₚ-rate and/or or a kᵟᵢ-rate, or alternatively as an IC₅₀ value, as further described herein) that is as defined herein; as well as compounds and constructs, and in particular proteins and polypeptides, that comprise at least one such amino acid sequence.

In particular, amino acid sequences and polypeptides of the invention are preferably such that they:

- bind to Targets of the invention with a dissociation constant (Kₐ) of 10⁻⁵ to 10¹² moles/liter or less, and preferably 10⁻⁷ to 10⁻¹² moles/liter or less and more preferably 10⁻⁸ to 10⁻¹⁰ moles/liter (i.e. with an association constant (Kₐ) of 10⁵ to 10¹² liter/moles or more, and preferably 10⁷ to 10¹⁰ liter/moles or more and more preferably 10⁸ to 10¹² liter/moles);

and/or such that they:

- bind to Targets of the invention with a kₚ-rate of between 10² M⁻¹V¹ to about 10⁷ M⁻¹S⁻¹ preferably between 10³ M⁻¹S⁻¹ and 10⁷ M⁻¹V¹, more preferably between 10⁴ M⁻¹V¹ and 10⁷ M⁻¹V¹, such as between 10⁵ M⁻¹V¹ and 10⁷ M⁻¹V¹;

and/or such that they:

- bind to Targets of the invention with a kᵟᵢ-rate between 1 s⁻¹ (t₁/₂=0.69 s) and 10⁶ s⁻¹ (providing a near irreversible complex with a t½ of multiple days), preferably between 10² s⁻¹ and 10⁶ s⁻¹, more preferably between 10³ s⁻¹ and 10⁶ s⁻¹, such as between 10³ s⁻¹ and 10⁶ s⁻¹.

 Preferably, a monovalent amino acid sequence of the invention (or a polypeptide that contains only one amino acid sequence of the invention) is preferably such that it will bind to Targets of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.
Some preferred 1C50 values for binding of the amino acid sequences or polypeptides of the invention to Targets of the invention will become clear from the further description and examples herein.

For binding to Targets of the invention, an amino acid sequence of the invention will usually contain within its amino acid sequence one or more amino acid residues or one or more stretches of amino acid residues (i.e. with each "stretch" comprising two or amino acid residues that are adjacent to each other or in close proximity to each other, i.e. in the primary or tertiary structure of the amino acid sequence) via which the amino acid sequence of the invention can bind to Targets of the invention, which amino acid residues or stretches of amino acid residues thus form the "site" for binding to Targets of the invention (also referred to herein as the "antigen binding site").

The amino acid sequences provided by the invention are preferably in essentially isolated form (as defined herein), or form part of a protein or polypeptide of the invention (as defined herein), which may comprise or essentially consist of one or more amino acid sequences of the invention and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more amino acid sequences of the invention may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further amino acid sequences that can serve as a binding unit (i.e. against one or more other targets than Targets of the invention), so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. Such a protein or polypeptide may also be in essentially isolated form (as defined herein).

The amino acid sequences and polypeptides of the invention as such preferably essentially consist of a single amino acid chain that is not linked via disulphide bridges to any other amino acid sequence or chain (but that may or may not contain one or more intramolecular disulphide bridges. For example, it is known that Nanobodies – as described herein - may sometimes contain a disulphide bridge between CDR3 and CDR1 or FR2).

However, it should be noted that one or more amino acid sequences of the invention may be linked to each other and/or to other amino acid sequences (e.g. via disulphide bridges) to provide peptide constructs that may also be useful in the invention (for example Fab' fragments, F(ab')2 fragments, ScFv constructs, 'diabodies' and other multispecific constructs. Reference is for example made to the review by HolHger and Hudson. Nat Biotechnol. 2005 Sep; 23(9): 1126-36).
Generally, when an amino acid sequence of the invention (or a compound, construct or polypeptide comprising the same) is intended for administration to a subject (for example for therapeutic and/or diagnostic purposes as described herein), it is preferably either an amino acid sequence that does not occur naturally in said subject; or, when it does occur naturally in said subject, in essentially isolated form (as defined herein).

It will also be clear to the skilled person that for pharmaceutical use, the amino acid sequences of the invention (as well as compounds, constructs and polypeptides comprising the same) are preferably directed against human Targets of the invention; whereas for veterinary purposes, the amino acid sequences and polypeptides of the invention are preferably directed against Targets of the invention from the species to be treated, or at least cross-reactive with Targets of the invention from the species to be treated.

Furthermore, an amino acid sequence of the invention may optionally, and in addition to the at least one binding site for binding against Targets of the invention, contain one or more further binding sites for binding against other antigens, proteins or targets. The efficacy of the amino acid sequences and 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 or disorder involved. Suitable assays and animal models will be clear to the skilled person, and for example include the expression of the receptors in Xenopus oocytes, after which the coupling of many Targets of the invention to ion channels allows the activation or inhibition of these Targets of the invention to be monitored in oocytes via voltage clamping techniques. Heterologous Targets of the invention can be functionally expressed in the oocyte by injecting exogenous. Targets-encoding mRNA into the oocyte and then allowing the oocyte's endogenous cellular machinery to translate and insert the receptors into the plasma membrane (see, e.g., Houamed et al, Science 252:1318-21, 1991: Dahmen et al, J. Neurochem. 58:1 176-79, 1992). Following functional expression of receptors, the ability of ligands to induce transmembrane conductance changes can be observed via a two-electrode voltage clamp system (Dahmen et al., supra), which can detect either a depolarization or hyperpolarization of the membrane potential. Other suitable assays are e.g. described in the example section.

Also, according to the invention, amino acid sequences and polypeptides that are directed against Targets of the invention from a first species of warm-blooded animal may or may not show cross-reactivity with Targets of the invention from one or more other species
of warm-blooded animal. For example, amino acid sequences and polypeptides directed against human Targets of the invention may or may not show cross reactivity with Targets of the invention from one or more other species of primates (such as, without limitation, monkeys from the genus Macaca (such as, and in particular, cynomolgus monkeys [Macaca fascicularis] and/or rhesus monkeys [Macaca mulatto]) and baboon (Papio ursinus)) and/or that have been raised against a refolded Target of the invention form a further aspect of the invention.

More generally, amino acid sequences and polypeptides of the invention that are cross-reactive with Targets of the invention from multiple species of mammal will usually be advantageous for use in veterinary applications, since it will allow the same amino acid sequence or polypeptide to be used across multiple species. Thus, it is also encompassed within the scope of the invention that amino acid sequences and polypeptides directed against Targets of the invention from one species of animal (such as amino acid sequences and polypeptides against human Targets of the invention) can be used in the treatment of another species of animal, as long as the use of the amino acid sequences and/or polypeptides provide the desired effects in the species to be treated.

The present invention is in its broadest sense also not particularly limited to or defined by a specific antigenic determinant, epitope, part, domain, subunit or confirmation (where applicable) of Targets of the invention against which the amino acid sequences and polypeptides of the invention are directed. For example, amino acid sequences of the invention may be raised by suitably immunizing a mammal (such as a Camelid) with Targets of the invention that have been expressed in a suitable expression system or that has been isolated from a suitable cell or cell fraction. In particular, amino acid sequences of the invention may be raised against Targets of the invention (or suitable parts or fragments thereof) that have been refolded (for example using the techniques described in the review by Kiefer. Biochim. Biophys. Acta, 1610 (2003), 57-62), and amino acid sequences, Nanobodies and polypeptides that are directed against and/or that have been raised against a refolded Target of the invention form a further aspect of the invention.
The amino acid sequences and polypeptides of the invention may generally be
directed against any desired Target of the invention, and may in particular be directed against
Targets of the invention that have at least one extracellular loop or domain. Examples of such Targets of the invention will be clear to the skilled person based on the prior art cited herein.

According to a specific aspect of the invention, an amino acid sequence or polypeptide of the invention may be directed against (as defined herein) the Targets of the invention that are expressed on the surface of a cell and/or against at least one extracellular region, domain, loop or other extracellular epitope of one of the Targets of the invention. For example, such amino acid sequences may be raised by suitably immunizing a mammal (such as a Camelid) with a cell or cell fraction that has one of the Targets of the invention expressed.

In particular, according to this aspect, an amino acid sequence or polypeptide of the invention is directed against (as defined herein) at least one extracellular region, domain, loop or other extracellular epitope of one of the Targets of the invention, e.g. hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, and is preferably further such that said amino acid sequence or polypeptide of the invention is capable of modulating (as defined herein) said Target of the invention, e.g. hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9. More in particular, according to this aspect, an amino acid sequence or polypeptide of the invention is directed against (as defined herein) at least one extracellular region, domain, loop or other extracellular epitope of a Target of the invention, e.g. hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9; and is preferably further such that said amino acid sequence or polypeptide of the invention is capable of (fully or partially) blocking said Targets of the invention, e.g. hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.

According to this aspect of the invention, the amino acid sequence or polypeptide of the invention may be directed against any suitable extracellular part, region, domain, loop or other extracellular epitope of one of the Targets of the invention, e.g. hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.
alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, but is preferably directed against one of the extracellular parts of the transmembrane domains or more preferably against one of the extracellular loops that link the transmembrane domains.

The amino acid sequence of such suitable extracellular parts, regions, domains, loops or epitopes may be derived by Kyte-Doolittle analysis of the amino acid sequence of the pertinent Target of the invention, e.g. hCD4, hCXCR4, hCCCR5, 1VTLR4, human alphaV integrin, human betaB integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9; by aligning the Targets of the invention belonging to the same (sub)families and identifying the various transmembrane domains and extracellular parts, regions, domain or loops: by TMAP-analysis; or by any suitable combination thereof. The invention also relates to amino acid sequences and polypeptides (as further defined herein) that are directed against and/or have been raised against such extracellular parts, regions, domains, loops or epitopes (and/or that are directed against and/or have been raised against suitable parts or fragments of such extracellular parts, regions, domains, loops or epitopes and/or against synthetic or semi-synthetic peptides that are derived from or based on such extracellular parts, regions, domains, loops or epitopes).

In particular, amino acid sequences and polypeptides of the invention are preferably such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of one of the Targets of the invention (as described herein) or against a peptide derived therefrom with a dissociation constant ($K_D$) of $10^{-5}$ to $10^{-12}$ moles/liter or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter or less and more preferably $10^{-5}$ to $10^{-12}$ moles/liter (i.e. with an association constant ($K_A$) of $10^5$ to $10^{12}$ liter/moles or more, and preferably $10^7$ to $10^8$ to $10^{12}$ liter/moles);

and/or such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of one of the Targets of the invention (as described herein) or against a peptide derived therefrom with a $k_{on}$-rate of between $10^2$ M$^{-1}$s$^{-1}$ to about $10^7$ M$^{-1}$V$^1$, preferably between $10^3$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$V$^1$, more preferably between $10^4$ M$^{-1}$V$^1$ and $10^7$ M$^{-1}$V$^1$, such as between $10^5$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$;

and/or such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of one of the Targets of the invention (as described herein) or against a peptide derived
therefrom with a $k_{\text{off}}$ rate between $1 \text{s}^{-1}$ ($t_{1/2} \approx 0.69 \text{s}$) and $10^{-6} \text{s}^{-1}$ (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between $10^{-2} \text{s}^{-1}$ and $10^{-6} \text{s}^{-1}$, more preferably between $10^{-3} \text{s}^{-1}$ and $10^{-6} \text{s}^{-1}$, such as between $10^{-4} \text{s}^{-1}$ and $10^{-6} \text{s}^{-1}$.

Preferably, a monovalent amino acid sequence of the invention (or a polypeptide that contains only one amino acid sequence of the invention) is preferably such that it will bind to an extracellular part, region, domain, loop or other extracellular epitope of one of the Targets of the invention (as described herein) with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Also, according to this aspect, any multivalent or multispecific (as defined herein) polypeptides of the invention may also be suitably directed against two or more different extracellular parts, regions, domains, loops or other extracellular epitopes on the same antigen, for example against two different extracellular loops, against two different extracellular parts of the transmembrane domains or against one extracellular loops and one extracellular loop. Such multivalent or raultispecific polypeptides of the invention may also have (or be engineered and/or selected for) increased avidity and/or improved selectivity for one of the desired Targets of the invention, and/or for any other desired property or combination of desired properties that may be obtained by the use of such multivalent or multispecific polypeptides.

Generally, it is expected that amino acid sequences and polypeptides of the invention that are directed against an extracellular loop or domain of one of the Targets of the invention (or against a small peptide derived therefrom or based thereon), and/or that have been screened against, selected using and/or raised against an extracellular loop or domain of one of the Targets of the invention (or against a small peptide derived therefrom or based thereon) will also be able to bind (and in particular, to specifically bind, as defined herein) to such an extracellular loop or domain (or peptide derived therefrom) that forms part of one of the Targets of the invention (or at least one subunit thereof) that is present on the surface of a cell. Thus, such (peptides derived from) an extracellular loop or domain may find particular use in methods for generating amino acid sequences and polypeptides of the invention (as defined herein); and such methods and uses form further aspects of the invention; as do amino acid sequences. Nanobodies and polypeptides of the invention that are directed against or raised against such an extracellular loop, domain or peptide derived therefrom.

For example, such a method may comprises the following:
a) a step of suitably immunizing a Camelid with a suitable antigen that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or with a suitable peptide derived therefrom or based thereon, such that an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s) is raised. The antigen may be any suitable antigen that is capable of raising an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s); such as, for example and without limitation, whole cells that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation, cell wall fragments thereof or any other suitable preparation derived from such cells, vesicles that have the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface, a subunit or fragment of a subunit of one of the Targets of the invention, that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or a synthetic or semi-synthetic peptide that comprises and/or is based on (the amino acid sequence of) the desired extracellular part, region, domain, loop or other extracellular epitope(s), more preferably, whole cells (e.g. HEK293) that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation; and

b) a step of selection for binding for the desired extracellular part, region, domain, loop or other extracellular epitope(s) using cell membranes preparation of different (than the one using in immunization) and several cell types overexpressing one of the Targets of the invention. This may for example be performed by selecting from a set, a collection or a library of cells that express heavy chain antibodies on their surface (e.g. B-cells obtained from a suitably immunized Camelid) and using a cell membranes preparation of e.g. a first type of cells such as e.g. CHO for a first round selection and e.g. a second type of cells such as e.g. COS-7 cells for a second round selection; by selecting from a (naïve or immune) library of VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and e.g. a second type of cell such as e.g. COS-7 cell for a second round selection; or by selecting from a (naïve or immune) library of nucleic acid sequences that encode VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and
e.g. a second type of cells such as e.g. COS-7 cell for a second round selection; which may all be performed in a manner known per se; and optionally washing only mildly with a buffer such as PBS without detergents; and which method may optionally further comprise one or more other suitable steps known per se, such as, for example and without limitation, a step of affinity maturation, a step of expressing the desired amino acid sequence, a step of screening for binding and/or for activity against the desired antigen (in this case, against one of the Targets of the invention), a step of determining the desired amino acid sequence or nucleotide sequence, a step of introducing one or more humanizing substitutions (e.g. as further described herein), a step of formatting in a suitable multivalent and/or multispecific format, a step of screening for the desired biological and/or physiological properties (i.e. using a suitable assay, such as those described herein); and/or any suitable combination of one or more of such steps, in any suitable order.

Such methods and the amino acid sequences obtained via such methods, as well as proteins and polypeptides comprising or essentially consisting of the same, form further aspects of this invention.

The amino acid sequences, Nanobodies and polypeptides of the invention may also bind to one of the Targets of the invention at the same site as the endogenous agonist (i.e. at an orthosteric site).

The amino acid sequences, Nanobodies and polypeptides of the invention may also bind to one of the Targets of the invention in such a way that they block adhesion and/or fusion of a virus and/or bacteria.

The amino acid sequences, Nanobodies and polypeptides of the invention may also bind to one of the Targets of the invention in such a way that they mediate allosteric modulation (e.g. bind to one of the Targets of the invention at an allosteric site).

The amino acid sequences, Nanobodies and polypeptides of the invention may also bind to one of the Targets of the invention in such a way that they inhibit or enhance the assembly of one of the Targets of the invention functional homodimers or heterodimers and thus prevent adhesion and/or fusion of a vims and/or bacteria.

More specifically, the amino acid sequences and polypeptides according to the present invention may neutralize a virus (as defined herein) and/or modulate, reduce and/or inhibit the infectivity of a vims (as defined herein). Accordingly, the amino acid sequences and polypeptides of the present invention that neutralize a virus (as defined herein) and/or
modulate, reduce and/or inhibit the infectivity of a virus (as defined herein) in the pre-entry phase of viral infection (i.e. before and/or during viral entry in a target host cell has taken place), are said herein to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

The invention also relates to the use of an one amino acid sequence, Nanobody, compound or polypeptide of the invention in the preparation of a composition (such as, without limitation, a pharmaceutical composition or preparation as further described herein) for modulating viral entry and/or for modulating the biological pathways that are mediated by the Target of the invention, either in vitro (e.g. in an in vitro or cellular assay) or in vivo (e.g. in an a single cell or multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from a viral disease.

In the context of the present invention, "modulating" or "to modulate" generally means either reducing, preventing or inhibiting viral entry and/or reducing, preventing or inhibiting the biological pathways that are mediated by a Target of the invention and/or its ligand. as measured using a suitable in vitro, cellular or in vivo assay (such as those mentioned herein). In particular, "modulating" or "to modulate" may mean either reducing, preventing or inhibiting viral entry and/or reducing, preventing or inhibiting the biological pathways that are mediated by the Target of the invention and/or its ligand as measured using a suitable in vitro, cellular or in vivo assay (such as those mentioned herein), by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to normal (i.e. naturally occurring) viral entry and/or normal (i.e. naturally occurring) biological pathways that are mediated by the Target of the invention and/or its ligand in the same assay under the same conditions but without the presence of the amino acid sequence. Nanobody, compound or polypeptide of the invention.

As will be clear to the skilled person, "modulating" may also involve effecting a change (which may either be an increase or a decrease) in binding specificity and/or selectivity of the Target of the invention for one or more of its binding partners; and/or effecting a change (which may either be an increase or a decrease) in the sensitivity of a Target of the invention for one or more conditions in the medium or surroundings in which an Target of the invention is present (such as pH, ion strength, the presence of co-factors, etc.), compared to the same conditions but without the presence of the amino acid sequence,
Nanobody, compound or polypeptide of the invention. As will be clear to the skilled person, this may again be determined in any suitable manner and/or using any suitable assay known per se, such as the assays described herein or in the prior art cited herein.

"Modulating" may also mean effecting a change with respect to one or more biological or physiological mechanisms, effects, responses, functions, pathways or activities in which the Target of the invention (or in which its binding partners or pathway(s)) are involved. Again, as will be clear to the skilled person this may be determined in any suitable manner and/or using any suitable (in vitro and usually cellular or in assay) assay known per se, such as the assays described herein or in the prior art cited herein. In particular, with respect to one or more biological or physiological mechanisms, effects, responses, functions, pathways or activities in which the Target of the invention and/or its ligand is involved, effecting a change can mean a change by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to the biological or physiological mechanisms, effects, responses, functions, pathways or activities in the same assay under the same conditions but without the presence of the amino acid sequence. Nanobody, compound or polypeptide of the invention.

Modulating may be reversible or irreversible, but for pharmaceutical and pharmacological purposes will usually be in a reversible manner.

 Accordingly, the present invention also relates to amino acid sequences and polypeptides that can be used to modulate, and in particular to inhibit and/or to prevent the viral-mediated biological pathways in which a Target of the invention is involved. In particular, the amino acid sequences and polypeptides of the present invention can be used to neutralize a virus (as defined herein) and/or to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein).

More specifically, the amino acid sequences and polypeptides according to the present invention may neutralize a virus (as defined herein) and/or modulate, reduce and/or inhibit the infectivity of a virus (as defined herein) in the pre-entry phase of viral infection (i.e. before and/or during viral entry in a target host cell has taken place) and/or in the post-entry phase of viral infection (i.e. after viral entry in a target host cell has taken place). Accordingly, the amino acid sequences and polypeptides of the present invention that neutralize a virus (as defined herein) and/or modulate, reduce and/or inhibit the infectivity of a virus (as defined herein) in the pre-entry phase of viral infection (i.e. before and/or during viral entry in a target host cell has taken place), are said herein to modulate and in particular
inhibit and/or prevent viral entry (as further defined herein) in a target host cell. The amino acid sequences and polypeptides according to the present invention may neutralize virus by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to virus neutralization under the same conditions but without the presence of the amino acid sequence, Nanobody, compound or polypeptide of the invention. The neutralization may be increased by at least 2 times, preferably at least 3 times, such as at least 5 times or at least 10 times, for example by at least 15 times, at least 20 times, at least 30 times, at least 50 times, or 100 times or more, compared to virus neutralization under the same conditions but without the presence of the amino acid sequence. Nanobody, compound or polypeptide of the invention. The amino acid sequences and polypeptides according to the present invention may modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to viral entry in a target host under the same conditions but without the presence of the amino acid sequence, Nanobody, compound or polypeptide of the invention.

In a specific aspect, the present invention relates to multivalent (such as bivalent, biparatopic, bispecific, trivalent, triparatopic, trispecific, as further defined herein) amino acid sequences and polypeptides that modulate, and in particular to inhibit and/or to prevent the viral-mediated biological pathways a Target of the invention is involved. In particular, the multivalent (preferably bivalent, more preferably trivalent) amino acid sequences and polypeptides of the present invention can neutralize a virus (as defined herein), modulate, reduce and/or inhibit the infectivity of a virus (as defined herein), and/or modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell. In one aspect, these multivalent (preferably bivalent, more preferably trivalent) amino acid sequences and polypeptides are directed against hCXCR4 and show increased in vitro and/or in vivo neutralization of HIV (as e.g. measured by HIV infection assay such as described herein) compared to the corresponding monovalent amino acid sequence. The neutralization may be increased by at least 2 times, preferably at least 3 times, such as at least 5 times or at least 10 times, for example by at least 15 times, at least 20 times, at least 30 times, at least 50 times, or 100 times or more, compared to the neutralization in the same assay under the same conditions by the corresponding monovalent amino acid sequence. In another aspect, these multivalent (preferably bivalent, more preferably trivalent) amino acid sequences and
polypeptides are directed against hCD4 and show increased in vitro and/or in vivo neutralization of HIV (as e.g. measured by a HIV infection assay as described herein) compared to the corresponding monovalent amino acid sequence. The neutralization may be increased by at least 2 times, preferably at least 3 times, such as at least 5 times or at least 10 times, for example by at least 15 times, at least 20 times, at least 30 times, at least 50 times, or 100 times or more, compared to the neutralization in the same assay under the same conditions by the corresponding monovalent amino acid sequence.

In yet another aspect, these multivalent (preferably bivalent, more preferably trivalent) amino acid sequences and polypeptides are directed against hCXCR4 and show increased competition with CXCL12 for binding hCXCR4 compared to the corresponding monovalent amino acid sequence. The competition may be increased by at least 2 times, preferably at least 3 times, such as at least 5 times or at least 10 times, for example by at least 15 times, at least 20 times, at least 30 times, at least 50 times, or 100 times or more, compared to the competition in the same assay under the same conditions by the corresponding monovalent amino acid sequence.

In yet another aspect, these multivalent (preferably bivalent, more preferably trivalent) amino acid sequences and polypeptides are directed against hCD4 and show increased competition with gp120 for binding hCD4 compared to the corresponding monovalent amino acid sequence. The competition may be increased by at least 2 times, preferably at least 3 times, such as at least 5 times or at least 10 times, for example by at least 15 times, at least 20 times, at least 30 times, at least 50 times, or 100 times or more, compared to the competition in the same assay under the same conditions by the corresponding monovalent amino acid sequence.

Accordingly, the amino acid sequences and (multivalent) polypeptides of the present invention can modulate and in particular inhibit and/or prevent viral entry in a target host cell by specifically binding to a Target of the invention; preferably, the amino acid sequences and polypeptides of the present invention can modulate and in particular inhibit and/or prevent viral entry in a target host cell by binding to a Target of the invention, such that virion attachment to a target host cell is modulated, inhibited and/or prevented (for instance by modulating and/or inhibiting and/or preventing the interaction between an envelope protein of a virus and the Target of the invention on a target host cell and/or the interaction between the an envelope protein of a virus and a target host cell or by competing with an envelope protein for binding to the Target of the invention or said target host cell). In a preferred
aspect, viral entry in a target host cell is modulated and in particular inhibited and/or prevented by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to prevent viral entry in a target host under the same conditions but without the presence of the amino acid sequence, Nanobody, compound or polypeptide of the invention.

A further aspect of the invention relates to a multispecific polypeptide of the invention (as defined herein) that comprises at least one amino acid sequence of the invention (such as a Nanobody) against one of the Targets of the invention. Such multispecific proteins may further be as described herein.

In a preferred embodiment, the amino acid sequence or polypeptide of the invention is a "monoclonal" amino acid sequence or polypeptide, by which is meant that at least each of the one or more amino acid sequences directed against the Target of the invention that are present in said protein or polypeptide (and preferably all of the immunoglobulin sequences that are present in said protein or polypeptide) are "monoclonal" as commonly understood by the skilled person. In this respect, it should however be noted that, as further described herein, the present invention explicitly covers multivalent or multispecific proteins that comprise two or more immunoglobulin sequences (and in particular monoclonal immunoglobulin sequences) that are directed against different parts, regions, domains, loops or epitopes of the same Target of the invention, and in particular against different extracellular parts, regions, domains, loops or epitopes of the same Target of the invention.

It is also within the scope of the invention that, where applicable, an amino acid sequence of the invention can bind to two or more antigenic determinants, epitopes, parts, domains, subunits or confirmations of Targets of the invention. In such a case, the antigenic determinants, epitopes, parts, domains or subunits of Targets of the invention to which the amino acid sequences and/or polypeptides of the invention bind may be essentially the same (for example, if Targets of the invention contain repeated structural motifs or occurs in a multimeric form) or may be different (and in the latter case, the amino acid sequences and polypeptides of the invention may bind to such different antigenic determinants, epitopes, parts, domains, subunits of Targets of the invention with an affinity and/or specificity which may be the same or different). Also, for example, when Targets of the invention exists in an activated conformation and in an inactive conformation, the amino acid sequences and polypeptides of the invention may bind to either one of these confirmation, or may bind to both these confirmations (i.e. with an affinity and/or specificity which may be the same or
different). Also, for example, the amino acid sequences and polypeptides of the invention may bind to a conformation of Targets of the invention in which it is bound to a pertinent ligand, may bind to a conformation of Targets of the invention in which it not bound to a pertinent ligand, or may bind to both such conformations (again with an affinity and/or specificity which may be the same or different).

As further described herein, a polypeptide of the invention may be bivalent and/or multivalent (as defined herein) and contain two or more amino acid sequences of the invention that are directed against a Target of the invention. Generally, such polypeptides will bind to the Target of the invention with increased avidity compared to a single amino acid sequence of the invention. It has also been observed that such polypeptides show (synergistically) increased binding, competition, and/or in vitro and/or in vivo neutralization of the virus.

Such a polypeptide may for example comprise two amino acid sequences of the invention that are directed against the same antigenic determinant, epitope, part, domain, subunit or conformation (where applicable) of a Target of the invention (which may or may not be an interaction site); or such a polypeptide may be biparatopic and/or multiparatopic (as defined herein) and comprise at least one "first" amino acid sequence of the invention that is directed against a first antigenic determinant, epitope, part, domain, subunit or conformation (where applicable) of a Target of the invention (which may or may not be an interaction site); and at least one "second" amino acid sequence of the invention that is directed against a second antigenic determinant, epitope, part, domain, subunit or conformation (where applicable) of said Target of the invention, wherein said second antigenic determinant, epitope, part, domain, subunit or conformation is different from the first (and again may or may not be an interaction site). Preferably, in such "bi- and/or multiparatopic" polypeptides of the invention, at least one amino acid sequence of the invention is directed against an interaction site (as defined herein), although the invention in its broadest sense is not limited thereto.

It is thus also within the scope of the invention that, where applicable, a polypeptide of the invention can bind to two or more antigenic determinants, epitopes, parts, domains, subunits or conformations of a Target of the invention. In such a case, the antigenic determinants, epitopes, parts, domains or subunits of said Target of the invention to which the amino acid sequences and/or polypeptides of the invention bind may be essentially the same (for example, if a Target of the invention contains repeated structural motifs or occurs in a
multimeric form) or may be different (and in the latter case, the amino acid sequences and polypeptides of the invention are said to be “bi- and/or multiparatopic” and may bind to such different antigenic determinants, epitopes, parts, domains, subunits of said Target of the invention with an affinity and/or specificity which may be the same or different).

Accordingly, bi- or multiparatopic polypeptides of the present invention are directed against and/or specifically bind to at least two epitopes of a Target of the invention, and are for example (but not limited to) polypeptides that are directed against and/or can specifically bind to three or even more epitopes of the same Target of the invention.

For example, and generally, a bivalent polypeptide of the invention may comprise two amino acid sequences of the invention directed against an antigenic determinant, epitope, part or domain of the Target of the invention which may be suitably linked, for example via a suitable linker as further described herein. Preferably, such a bivalent polypeptide of the invention is further such that, when it binds to the Target of the invention, it is capable of simultaneously binding to both antigenic determinants, epitopes, parts or domains (i.e. via the two amino acid sequences of the invention capable of binding to said antigenic determinants, epitopes, parts or domains). Examples of such bivalent polypeptides of the invention will become clear from the further description herein. Also, a trivalent polypeptide of the invention may comprise three amino acid sequences of the invention directed against an antigenic determinant, epitope, part or domain of the viral envelope protein, and generally multivalent polypeptides of the invention may contain at least two amino acid sequences of the invention directed against an antigenic determinants, epitopes, parts or domains of the viral envelope protein. Generally, such bivalent, trivalent and multivalent polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bivalent, trivalent and multivalent polypeptides of the invention (for example, these bivalent, trivalent and multivalent polypeptides of the invention preferably comprise single variable domains and more preferably Nanobodies).

In one aspect of the invention, the amino acid sequences and (in particular) polypeptides of the invention are capable of binding to two or more antigenic determinants, epitopes, parts, domains of an envelope protein of a virus which are essentially the same. In this context, the amino acid sequences and polypeptides of the invention are also referred to as "multivalent (monospecific)" (such as e.g. "bivalent (monospecific)" or "trivalent (monospecific)", etc.) amino acid sequences and polypeptides. The multivalent amino acid
sequences and polypeptides of the invention can be directed against any antigenic determinants, epitopes, parts, and/or domains of the envelope protein of a virus.

In a preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bivalent and are directed against the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding to hCXCR4.

Generally, such a bivalent polypeptide of the invention may contain two amino acid sequences of the invention that are capable of binding to the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding to hCXCR4. Generally, such bivalent polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bivalent polypeptides of the invention (for example, these bivalent polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind the CXCL12 binding site; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bivalent and are at least capable, upon binding to hCXCR4, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bivalent and are directed against the gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4.

Generally, such a bivalent polypeptide of the invention will contain two amino acid sequences of the invention that are capable of binding to the gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4. Generally, such bivalent polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bivalent polypeptides of the invention (for example, these bivalent polypeptides of the invention may comprise suitable linkers: are preferably such that they can simultaneously bind the gp120 binding site; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bivalent and are at least capable, upon binding to hCD4, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the
infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In a preferred aspect, the amino acid sequences and (in particular) polypeptides of the invention are capable of binding to two or more different antigenic determinants, epitopes, parts, domains of a Target of the invention. In this context, the amino acid sequences and polypeptides of the invention are also referred to as "multiparatopic" (such as e.g. "biparatopic" or "triparatopic", etc.) amino acid sequences and polypeptides. The multiparatopic amino acid sequences and polypeptides of the invention can be directed against any antigenic determinants, epitopes, parts, and/or domains of the Target of the invention.

For example, and generally, a biparatopic polypeptide of the invention may comprise at least one amino acid sequence of the invention directed against a First antigenic determinant, epitope, part or domain of the Target of the invention and at least one amino acid sequence of the invention directed against a second antigenic determinant, epitope, part or domain of the Target of the invention different from the first antigenic determinant, epitope, part or domain (in which said amino acid sequences may be suitably linked, for example via a suitable linker as further described herein). Preferably, such a biparatopic polypeptide of the invention is further such that, when it binds to the Target of the invention, it is capable of simultaneously binding to the first antigenic determinant, epitope, part or domain (i.e. via the at least one amino acid sequence of the invention capable of binding to said first antigenic determinant, epitope, part or domain) and binding to said second antigenic determinant, epitope, part or domain (i.e. via the at least one amino acid sequence of the invention capable of binding to said second antigenic determinant, epitope, part or domain). Examples of such biparatopic polypeptides of the invention will become clear from the further description herein. Also, a triparatopic polypeptide of the invention may comprise at least one further amino acid sequence of the invention directed against a third antigenic determinant, epitope, part or domain of the Target of the invention (different from both the first and second antigenic determinant, epitope, part or domain), and generally multiparatopic polypeptides of the invention may contain at least two amino acid sequences of the invention directed against at least two different antigenic determinants, epitopes, parts or domains of the Target of the invention. Generally, such biparatopic, triparatopic and multiparatopic polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these biparatopic, triparatopic and
multiparatopic polypeptides of the invention (for example, these biparatopic, triparatopic and
multiparatopic polypeptides of the invention preferably comprise single variable domains and
more preferably Nanobodies).

In a preferred, but non-limiting aspect, the amino acid sequences and (in particular)
polypeptides of the invention are biparatopic (or multiparatopic) and are directed against the
CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding to
hCXCR4, as well as against at least one other antigenic determinant, epitope, part or domain
on hCXCR4.

Generally, such a biparatopic (or multiparatopic) polypeptide of the invention will
contain at least one amino acid sequence of the invention that is capable of binding to the
CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding to
hCXCR4, as well as at least one further amino acid sequence of the invention that is capable
of binding to at least one other antigenic determinant, epitope, part or domain on hCXCR4.
Generally, such biparatopic (or multiparatopic) polypeptides of the invention may be as
further described herein, and the various preferred aspects of the invention as described
herein also apply to these biparatopic (or multiparatopic) polypeptides of the invention (for
example, these biparatopic and multiparatopic polypeptides of the invention may comprise
suitable linkers; are preferably such that they can simultaneously bind the CXCL12 binding
site and the at least one other antigenic determinant, epitope, part or domain on hCXCR4; and
preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in
particular) polypeptides of the invention are biparatopic (or multiparatopic) and are at least
capable, upon binding to hCXCR4, to neutralize a virus (as defined herein); to modulate,
reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in
particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and
polypeptides of the invention are biparatopic (or multiparatopic) and are directed against the
gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4, as
well as against at least one other antigenic determinant on hCD4.

Generally, such a biparatopic (or multiparatopic) polypeptide of the invention will
contain at least one amino acid sequence of the invention that is capable of binding to the
gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4, as
well as at least one further amino acid sequence of the invention that is capable of binding to
at least one other antigenic determinant, epitope, part or domain on hCD4. Generally, such biparatopic (or multiparatopic) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these biparatopic (or multiparatopic) polypeptides of the invention (for example, these biparatopic and multiparatopic polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind the gpl20 binding site and the at least one other antigenic determinant, epitope, part or domain on hCD4; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are biparatopic (or multiparatopic) and are at least capable, upon binding to hCD4, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are biparatopic with both paratopes directed against the CXCL12 binding site on hCXCR4.

Generally, such a biparatopic (or multiparatopic) polypeptide of the invention will contain two amino acid sequences of the invention that are capable of binding to the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding to hCXCR4.

Generally, such biparatopic (or multiparatopic) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these biparatopic (or multiparatopic) polypeptides of the invention (for example, these biparatopic and multiparatopic polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind the CXCL12 binding site on hCXCR4; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are biparatopic with both paratopes directed against the gpl20 binding site on hCD4.

Generally, such a biparatopic (or multiparatopic) polypeptide of the invention will contain two amino acid sequences of the invention that are capable of binding to the gpl20 binding site on hCD4 and/or capable of competing with gpl20 for binding to hCD4.

Generally, such biparatopic (or multiparatopic) polypeptides of the invention may be as
further described herein, and the various preferred aspects of the invention as described herein also apply to these biparatopic (or multiparatopic) polypeptides of the invention (for example, these biparatopic and multiparatopic polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind the gp120 binding site on hCD4; and preferably comprise single variable domains and more preferably Nanobodies).

Also, the polypeptides of the present invention may also be directed against and/or can specifically bind to at least one particular Target of the invention and at least one further epitope of another Target of the invention, which is different from said at least one particular Target of the invention. Thus, the polypeptides according to the invention may be directed against and/or may specifically bind to at least two (or even more) epitopes of at least two different Targets of the invention. Also, said at least one further epitope may or may not be involved in viral entry in a target host cell.

Generally, bi-, and multivalent (as defined herein), bi-, and multispecific (as defined herein) and bi-, and multiparatopic (as defined herein) polypeptides according to the invention may be useful for the prevention and/or treatment of viral diseases by specifically binding to at least one epitope of a Target of the invention and at least one further epitope (which may or may not be different from said at least one epitope) of the Target of the invention.

Preferably, bi-, and multiparatopic polypeptides (as defined herein) according to the invention may be useful for the prevention and/or treatment of viral diseases by specifically binding to at least two (or even more) epitopes (which may be the same or different) on the same Target of the invention.

Alternatively, the polypeptides of the present invention may be directed against and/or can specifically bind to at least one epitope of a Target of the invention and at least one further epitope of another target, which is different from said Target of the invention and which is for instance a further Target of the invention.

In a preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are bispecific (or multispecific) and are directed against an antigenic determinant, epitope, part or domain on hCXCR4 as well as against an antigenic determinant, epitope, part or domain on hCD4. Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCXCR4, as well at least one amino acid sequence of the invention that is capable of binding to the
antigenic determinant, epitope, part or domain on hCD4. Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are bispecific (or multispecific) and are directed against the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding hCXCR4 as well as against an antigenic determinant, epitope, part or domain on hCD4.

Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding hCXCR4, as well at least one amino acid sequence of the invention that is capable of binding to an antigenic determinant, epitope, part or domain on hCD4. Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.
In another preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are bispecific (or multispecific) and are directed against an antigenic determinant, epitope, part or domain on hCXCR4, as well as against the gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4.

Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to an antigenic determinant, epitope, part or domain on hCXCR4, as well at least one amino acid sequence of the invention that is capable of binding to the the gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4. Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding hCXCR4 as well as against the gp120 binding site on hCD4 and/or capable of competing with gp120 for binding to hCD4.

Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for
example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are bispecific (or multispecific) and are directed against an antigenic determinant, epitope, part or domain on hCXCR4 as well as against an antigenic determinant, epitope, part or domain on hCCR5. Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCXCR4, as well at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCCR5. Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are bispecific (or multispecific) and are directed against an antigenic determinant, epitope, part or domain on hCD4 as well as against an antigenic determinant, epitope, part or domain on hCCR5. Generally, such a bispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain
on hCD4, as well at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCCR5. Generally, such bispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these bispecific (or multispecific) polypeptides of the invention (for example, these bispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are bispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

In another preferred, but non-limiting aspect, the amino acid sequences and polypeptides of the invention are trispecific (or multispecific) and are directed against an antigenic determinant, epitope, part or domain on hCXCR4, against an antigenic determinant, epitope, part or domain on hCD4, as well as against an antigenic determinant, epitope, part or domain on hCCR5. Generally, such a trispecific (or multispecific) polypeptide of the invention will contain at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCXCR4, at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCD4, as well at least one amino acid sequence of the invention that is capable of binding to the antigenic determinant, epitope, part or domain on hCCR5. Generally, such trispecific (or multispecific) polypeptides of the invention may be as further described herein, and the various preferred aspects of the invention as described herein also apply to these trispecific (or multispecific) polypeptides of the invention (for example, these trispecific and multispecific polypeptides of the invention may comprise suitable linkers; are preferably such that they can simultaneously bind both binding sites; and preferably comprise single variable domains and more preferably Nanobodies).

In another preferred, but non-limiting aspect, the amino acid sequences and (in particular) polypeptides of the invention are trispecific (or multispecific) and are at least capable, upon binding, to neutralize a virus (as defined herein); to modulate, reduce and/or
inhibit the infectivity of a virus (as defined herein); and/or to modulate and in particular inhibit and/or prevent viral entry (as further defined herein) in a target host cell.

It is also expected that the amino acid sequences and polypeptides of the invention will generally bind to all naturally occurring or synthetic analogs, variants, mutants, alleles, parts and fragments of Targets of the invention; or at least to those analogs, variants, mutants, alleles, parts and fragments of Targets of the invention that contain one or more antigenic determinants or epitopes that are essentially the same as the antigenic determinant(s) or epitope(s) to which the amino acid sequences and polypeptides of the invention bind in Targets of the invention (e.g. in wild-type Targets of the invention). Again, in such a case, the amino acid sequences and polypeptides of the invention may bind to such analogs, variants, mutants, alleles, parts and fragments with an affinity and/or specificity that are the same as, or that are different from (i.e. higher than or lower than), the affinity and specificity with which the amino acid sequences of the invention bind to (wild-type) Targets of the invention. It is also included within the scope of the invention that the amino acid sequences and polypeptides of the invention bind to some analogs, variants, mutants, alleles, parts and fragments of Targets of the invention, but not to others.

When Targets of the invention exists in a monomelic form and in one or more multimeric forms, it is within the scope of the invention that the amino acid sequences and polypeptides of the invention only bind to Targets of the invention in monomeric form, only bind to Targets of the invention in multimeric form, or bind to both the monomeric and the multimeric form. Again, in such a case, the amino acid sequences and polypeptides of the invention may bind to the monomeric form with an affinity and/or specificity that are the same as, or that are different from (i.e. higher than or lower than), the affinity and specificity with which the amino acid sequences of the invention bind to the multimeric form.

Also, when Targets of the invention can associate with other proteins or polypeptides to form protein complexes (e.g. with multiple subunits), it is within the scope of the invention that the amino acid sequences and polypeptides of the invention bind to Targets of the invention in its non-associated state, bind to Targets of the invention in its associated state, or bind to both. In all these cases, the amino acid sequences and polypeptides of the invention may bind to such multimers or associated protein complexes with an affinity and/or specificity that may be the same as or different from (i.e. higher than or lower than) the affinity and/or specificity with which the amino acid sequences and polypeptides of the invention bind to Targets of the invention in its monomeric and non-associated state.
Also, as will be clear to the skilled person, proteins or polypeptides that contain two or more amino acid sequences directed against Targets of the invention may bind with higher avidity to Targets of the invention than the corresponding monomeric amino acid sequence(s). For example, and without limitation, proteins or polypeptides that contain two or more amino acid sequences directed against different epitopes of Targets of the invention may (and usually will) bind with higher avidity than each of the different monomers, and proteins or polypeptides that contain two or more amino acid sequences directed against Targets of the invention may (and usually will) bind also with higher avidity to a multimer of Targets of the invention.

Generally, amino acid sequences and polypeptides of the invention will at least bind to those forms of Targets of the invention (including monomeric, multimeric and associated forms) that are the most relevant from a biological and/or therapeutic point of view, as will be clear to the skilled person.

It is also within the scope of the invention to use parts, fragments, analogs, mutants, variants, alleles and/or derivatives of the amino acid sequences and polypeptides of the invention, and/or to use proteins or polypeptides comprising or essentially consisting of one or more of such parts, fragments, analogs, mutants, variants, alleles and/or derivatives, as long as these are suitable for the uses envisaged herein. Such parts, fragments, analogs, mutants, variants, alleles and/or derivatives will usually contain (at least part of) a functional antigen-binding site for binding against Targets of the invention; and more preferably will be capable of specific binding to Targets of the invention, and even more preferably capable of binding to Targets of the invention with an affinity (suitably measured and/or expressed as a $K_{D}$-value (actual or apparent), a $K_{A}$-value (actual or apparent), a $k_{on}$-rate and/or a $k_{off}$-rate, or alternatively as an IC$_{50}$ value, as further described herein) that is as defined herein. Some non-limiting examples of such parts, fragments, analogs, mutants, variants, alleles, derivatives, proteins and/or polypeptides will become clear from the further description herein. Additional fragments or polypeptides of the invention may also be provided by suitably combining (i.e. by linking or genetic fusion) one or more (smaller) parts or fragments as described herein.

In one specific, but non-limiting aspect of the invention, which will be further described herein, such analogs, mutants, variants, alleles, derivatives have an increased half-life in serum (as further described herein) compared to the amino acid sequence from which they have been derived. For example, an amino acid sequence of the invention may be linked
(chemically or otherwise) to one or more groups or moieties that extend the half-life (such as PEG), so as to provide a derivative of an amino acid sequence of the invention with increased half-life.

In one specific, but non-limiting aspect, the amino acid sequence of the invention may be an amino acid sequence that comprises an immunoglobulin fold or may be an amino acid sequence that, under suitable conditions (such as physiological conditions) is capable of forming an immunoglobulin fold (i.e. by folding). Reference is inter alia made to the review by Halaby et al., J. (1999) Protein Eng, 12, 563-71. Preferably, when properly folded so as to form an immunoglobulin fold, such an amino acid sequence is capable of specific binding (as defined herein) to Targets of the invention; and more preferably capable of binding to Targets of the invention with an affinity (suitably measured and/or expressed as a $K_\text{D}$-value (actual or apparent), a $K_\text{A}$-value (actual or apparent), a $k_\text{on}$-rate and/or a karate, or alternatively as an $IC_{50}$ value, as further described herein) that is as defined herein. Also, parts, fragments, analogs, mutants, variants, alleles and/or derivatives of such amino acid sequences are preferably such that they comprise an immunoglobulin fold or are capable for forming, under suitable conditions, an immunoglobulin fold.

In particular, but without limitation, the amino acid sequences of the invention may be amino acid sequences that essentially consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively); or any suitable fragment of such an amino acid sequence (which will then usually contain at least some of the amino acid residues that form at least one of the CDR’s, as further described herein).

The amino acid sequences of the invention may in particular be an immunoglobulin sequence or a suitable fragment thereof, and more in particular be an immunoglobulin variable domain sequence or a suitable fragment thereof, such as light chain variable domain sequence (e.g. a $V_L$-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g. a $V_i$-sequence) or a suitable fragment thereof. When the amino acid sequence of the invention is a heavy chain variable domain sequence, it may be a heavy chain variable domain sequence that is derived from a conventional four-chain antibody (such as, without limitation, a $V_H$ sequence that is derived from a human antibody) or be a so-called $V_{\text{HH}}$-sequence (as defined herein) that is derived from a so-called "heavy chain antibody" (as defined herein).
However, it should be noted that the invention is not limited as to the origin of the amino acid sequence of the invention (or of the nucleotide sequence of the invention used to express it), nor as to the way that the amino acid sequence or nucleotide sequence of the invention is (or has been) generated or obtained. Thus, the amino acid sequences of the invention may be naturally occurring amino acid sequences (from any suitable species) or synthetic or semi-synthetic amino acid sequences. In a specific but non-limiting aspect of the invention, the amino acid sequence is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence, including but not limited to "humanized" (as defined herein) immunoglobulin sequences (such as partially or fully humanized mouse or rabbit immunoglobulin sequences, and in particular partially or fully humanized V_{H\text{H}} sequences or Nanobodies), "camelized" (as defined herein) immunoglobulin sequences, as well as immunoglobulin sequences that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences). CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person: or any suitable combination of any of the foregoing. Reference is for example made to the standard handbooks, as well as to the further description and prior art mentioned herein.

Similarly, the nucleotide sequences of the invention may be naturally occurring nucleotide sequences or synthetic or semi-synthetic sequences, and may for example be sequences that are isolated by PCR from a suitable naturally occurring template (e.g. DNA or RNA isolated from a cell), nucleotide sequences that have been isolated from a library (and in particular, an expression library), nucleotide sequences that have been prepared by introducing mutations into a naturally occurring nucleotide sequence (using any suitable technique known per se, such as mismatch PCR), nucleotide sequence that have been prepared by PCR using overlapping primers, or nucleotide sequences that have been prepared using techniques for DNA synthesis known per se.

The amino acid sequence of the invention may in particular be a domain antibody (or an amino acid sequence that is suitable for use as a domain antibody), a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" (or an amino acid sequence that is suitable for use as a dAb) or a Nanobody (as defined herein, and including but not limited to a V_{HH} sequence); other single variable
domains, or any suitable fragment of any one thereof. For a general description of (single) domain antibodies, reference is also made to the prior art cited above, as well as to EP 0 368 684. For the term "dAb's", reference is for example made to Ward et al. (Nature 1989 Oct 12; 341 (6242): 544-6), to Holt et al., Trends Biotechnol., 2003, 21(1 1):484-490; as well as to for example WO 06/030220, WO 06/003388 and other published patent applications of Domantis Ltd. It should also be noted that, although less preferred in the context of the present invention because they are not of mammalian origin, single domain antibodies or single variable domains can be derived from certain species of shark (for example, the so-called "IgNAR domains", see for example WO 05/18629).

In particular, the amino acid sequence of the invention may be a Nanobody (as defined herein) or a suitable fragment thereof. [Note: Nanobody®, Nanobodies® and Nanoclone® are registered trademarks of Ablynx N.K.] Such Nanobodies directed against Targets of the invention will also be referred to herein as “Nanobodies of the invention”.

For a general description of Nanobodies, reference is made to the further description below, as well as to the prior art cited herein. In this respect, it should however be noted that this description and the prior art mainly described Nanobodies of the so-called “V_{H}3 class” (i.e. Nanobodies with a high degree of sequence homology to human germline sequences of the V_{H}3 class such as DP-47, DP-5 1 or DP-29), which Nanobodies form a preferred aspect of this invention. It should however be noted that the invention in its broadest sense generally covers any type of Nanobody directed against Targets of the invention, and for example also covers the Nanobodies belonging to the so-called “V_{H}4 class” (i.e. Nanobodies with a high degree of sequence homology to human germline sequences of the V_{H}4 class such as DP-78), as for example described in WO 07/1 18670.

Generally, Nanobodies (in particular V_{HH} sequences and partially humanized Nanobodies) can in particular be characterized by the presence of one or more "Hallmark residues" (as described herein) in one or more of the framework sequences (again as further described herein).

Thus, generally, a Nanobody can be defined as an amino acid sequence with the (general) structure

\[ \text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]
in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to
CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which
one or more of the Hallmark residues are as further defined herein.

In particular, a Nanobody can be an amino acid sequence with the (general) structure

\[ \text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to
CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the
framework sequences are as further defined herein.

More in particular, a Nanobody can be an amino acid sequence with the (general)
structure

\[ \text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to
CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:
i) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83,
84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark
residues mentioned in Table A-3 below;

and in which:
ii) said amino acid sequence has at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO"s: 1 to 22, in which for the purposes of
determining the degree of amino acid identity, the amino acid residues that form the
CDR sequences (indicated with X in the sequences of SEQ ID N(Ts: 1 to 22) are
disregarded.

In these Nanobodies, the CDR sequences are generally as further defined herein.

Thus, the invention also relates to such Nanobodies that can bind to (as defined
herein) and/or are directed against Targets of the invention, to suitable fragments thereof, as
well as to polypeptides that comprise or essentially consist of one or more of such
Nanobodies and/or suitable fragments.

SEQ ID NO’s 238 to 253 give the amino acid sequences of a number of \(V_{H_1}\)
sequences that have been raised against human CXCR4 (Table 1).
In particular, the invention in some specific aspects provides:

- amino acid sequences that are directed against (as defined herein) a hCXCR4 and that have at least 80%, preferably at least 85%, such as 90% or 95% or more sequence identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239. These amino acid sequences may further be such that they neutralize binding of the cognate ligand to hCXCR4; and/or compete with the cognate ligand for binding to hCXCR4; and/or are directed against an interaction site (as defined herein) on hCXCR4 (such as the ligand binding site);

- amino acid sequences that cross-block (as defined herein) the binding of at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239 to hCXCR4 and/or that compete with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239 for binding to hCXCR4. Again, these amino acid sequences may further be such that they neutralize binding of the cognate ligand to hCXCR4; and/or compete with the cognate ligand for binding to hCXCR4; and/or are directed against an interaction site (as defined herein) on hCXCR4 (such as the ligand binding site);

which amino acid sequences may be as further described herein (and may for example be Nanobodies); as well as polypeptides of the invention that comprise one or more of such amino acid sequences (which may be as further described herein, and may for example be bispecific and/or biparatopic polypeptides as described herein), and nucleic acid sequences that encode such amino acid sequences and polypeptides. Such amino acid sequences and polypeptides do not include any naturally occurring ligands.

SEQ ID NO's 308 to 311 give the amino acid sequences of a number of VαH sequences that have been raised against human CD4 (Table 3).

In particular, the invention in some specific aspects provides:

- amino acid sequences that are directed against (as defined herein) a hCD4 and that have at least 80%, preferably at least 85%, such as 90% or 95% or more sequence identity with at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably 311. These amino acid sequences may further be such that they neutralize binding of the cognate ligand to hCD4; and/or compete with the cognate ligand for binding to hCD4; and/or are directed against an interaction site (as defined herein) on hCD4 (such as the ligand binding site);
amino acid sequences that cross-block (as defined herein) the binding of at least one of the amino acid sequences of SEQ ID NO’s: 308 to 311, more preferably 311 to hCD4 and/or that compete with at least one of the amino acid sequences of SEQ ID NCTs: 308 to 311, more preferably 311 for binding to hCD4. Again, these amino acid sequences may further be such that they neutralize binding of the cognate ligand to hCD4; and/or compete with the cognate ligand for binding to hCD4; and/or are directed against an interaction site (as defined herein) on hCD4 (such as the ligand binding site); which amino acid sequences may be as further described herein (and may for example be Nanobodies); as well as polypeptides of the invention that comprise one or more of such amino acid sequences (which may be as further described herein, and may for example be bispecific and/or biparatopic polypeptides as described herein), and nucleic acid sequences that encode such amino acid sequences and polypeptides. Such amino acid sequences and polypeptides do not include any naturally occurring ligands.
Table 1: Nanobodies directed against human CXCR4 (SEQ ID NO: 254)

<table>
<thead>
<tr>
<th>SEQ ID NO: X, where X =</th>
<th>Name</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>238</td>
<td>238C1</td>
<td>EVQLVESGGGLVQGTGSLRSLCAASGFTFSSYAMSWVRQAPGERKLEWVSGIKSSGD</td>
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<td>238D2</td>
<td>STRYAGSVDKRTTIRSDNAKYNLQLMQSLKPEDEVTAYCAYKRSVSGRGTVYNYQDDYR</td>
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<tr>
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<td>239G3</td>
<td>RSGVSDVYDSVKDRFTISRNNAKNTLYLQMSSNLKPEDEVTAYCAASAGSGLARK</td>
</tr>
<tr>
<td>240</td>
<td>237B5</td>
<td>KVQLVESGGGLVQPGSLRSLCAASGFFSITHYAMSWVRQAPKCL5WASIAWNGG</td>
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<tr>
<td></td>
<td>237B6</td>
<td>STDYAYSVKGRTI5IRDNAKNTLYLQMSSNLKSEDTAAYCARDQGFFYSCTYYTR</td>
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<tr>
<td></td>
<td>237A5</td>
<td>QYGRGQGTQTVVSS</td>
</tr>
<tr>
<td></td>
<td>237D2</td>
<td>EVDLYFSDVYQPGSLRSLCAASGFFSITHYAMSWVRQAPKCL5WASIAWNGG</td>
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<td>237E4</td>
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<td>237G2</td>
<td>QYGRGQGTQTVVSS</td>
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<td>237D4</td>
<td>EVDLYFSDVYQPGSLRSLCAASGFFSITHYAMSWVRQAPKCL5WASIAWNGG</td>
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<td>237F1</td>
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<tr>
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Table 1.1: CDR sequences of Nanobodies directed against human CXCR4 (SEQ ID NO: 254)

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<tr>
<th>SEQ ID NO: X, where X =</th>
<th>Name</th>
<th>CDR X, where X =</th>
<th>Amino acid sequence</th>
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<td>142</td>
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<td>1</td>
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<tr>
<td>152</td>
<td>237A6</td>
<td>1</td>
<td>IKAMS</td>
</tr>
<tr>
<td>153</td>
<td>237D1</td>
<td>1</td>
<td>VNDMG</td>
</tr>
<tr>
<td>154</td>
<td>237E1</td>
<td>1</td>
<td>YYTIG</td>
</tr>
<tr>
<td>155</td>
<td>237G7</td>
<td>1</td>
<td>YHAIY</td>
</tr>
<tr>
<td>156</td>
<td>238C4</td>
<td>1</td>
<td>NYAMG</td>
</tr>
<tr>
<td>157</td>
<td>237C1</td>
<td>1</td>
<td>INAMG</td>
</tr>
<tr>
<td>174</td>
<td>238C1, 238D2</td>
<td>2</td>
<td>GIKSSGCDSTRYAGSVKG</td>
</tr>
<tr>
<td>175</td>
<td>238D4, 238C3</td>
<td>2</td>
<td>ATRSGVRKSGVAIYGDSVK</td>
</tr>
<tr>
<td>176</td>
<td>237B5</td>
<td>2</td>
<td>TIKPSGYTTNYANAVKG</td>
</tr>
<tr>
<td>178</td>
<td>238B10</td>
<td>2</td>
<td>YHRWSDGANLYADSVKG</td>
</tr>
<tr>
<td>179</td>
<td>238C5, 238C2, 238xH5, 238C3, 238D6, 238E6</td>
<td>2</td>
<td>CISGSGGSTDYADSVK</td>
</tr>
<tr>
<td>180</td>
<td>238F7</td>
<td>2</td>
<td>YHRWSDGANLYADSVKG</td>
</tr>
<tr>
<td>181</td>
<td>238H2</td>
<td>2</td>
<td>STINSTRGTYADSVKG</td>
</tr>
<tr>
<td>182</td>
<td>237D4</td>
<td>2</td>
<td>AILSWNGGSSADYADSVKG</td>
</tr>
</tbody>
</table>
Table 3: Nanobodies directed against human CD4 (SEQ ID NO: 268)

<table>
<thead>
<tr>
<th>SEQ ID NO: X, where X =</th>
<th>Name</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>308</td>
<td>01B6</td>
<td>EVQLVESGGGLVQPSLGSLGSHLCAASGFTTSGYVWYVRQAPGKQLE&lt;br&gt;WSAIESFGGAGGYPDYSVKCRTISRDNYKNTYVILQMNLSLKEA&lt;br&gt;TYCASSLTAHTHEYDYWGQGQTQTVSS</td>
</tr>
<tr>
<td>309</td>
<td>01E2</td>
<td>EVQLVESGGGLVQPSLGSLGSHLCAASGFTTSGYVWYVRQAPGKQLE&lt;br&gt;WSAIESFGGAGGYPDYSVKCRTISRDNYKNTYVILQMNLSLKEA&lt;br&gt;TYCASSLTAHTHEYDYWGQGQTQTVSS</td>
</tr>
<tr>
<td>310</td>
<td>01H12</td>
<td>EVQLVESGGGLVQPSLGSLGSHLCAASGFTTSGYVWYVRQAPGKQLE&lt;br&gt;WSAIESFGGAGGYPDYSVKCRTISRDNYKNTYVILQMNLSLKEA&lt;br&gt;TYCASSLTAHTHEYDYWGQGQTQTVSS</td>
</tr>
<tr>
<td>311</td>
<td>03F:1</td>
<td>EVQLVESGGGLVQPSLGSLGSHLCAASGFTTSGYVWYVRQAPGKQLE&lt;br&gt;WSAIESFGGAGGYPDYSVKCRTISRDNYKNTYVILQMNLSLKEA&lt;br&gt;TYCASSLTAHTHEYDYWGQGQTQTVSS</td>
</tr>
</tbody>
</table>
Accordingly, some particularly preferred Nanobodies of the invention are Nanobodies which can bind (as further defined herein) to and/or are directed against human CXCR4 and which:

i) have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded. In this respect, reference is also made to Table A-I, which lists the framework 1 sequences (SEQ ID NO's: 126 to 141), framework 2 sequences (SEQ ID NO's: 158 to 173), framework 3 sequences (SEQ ID NO's: 190 to 205) and framework 4 sequences (SEQ ID NO's: 222 to 237) of the Nanobodies of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 (with respect to the amino acid residues at positions 1 to 4 and 27 to 30 of the framework 1 sequences, reference is also made to the comments made below. Thus, for determining the degree of amino acid identity, these residues are preferably disregarded);

and in which:

ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 below.

Table 3.1: CDR sequences of Nanobodies directed against human CD4 (SEQ ID NO: 268)

<table>
<thead>
<tr>
<th>SEQ ID NO: X, where X =</th>
<th>Name</th>
<th>CDR X, where X =</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>284 01B6 1</td>
<td>GYWMY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>285 01E2 1</td>
<td>SYSMG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>286 01H12 1</td>
<td>FNAMG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>287 03F11 1</td>
<td>VMG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>292 01B6 2</td>
<td>AISFGGGSTYPSVKG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>293 01E2 2</td>
<td>AISWGGGTYPSVKG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>294 01H12 2</td>
<td>TIAUGATKYADSVKG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>295 03F11 2</td>
<td>AVRWSSTGIYQYADVSKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 01B6 3</td>
<td>SLTATHYEDY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>301 01E2 3</td>
<td>DRWWRPGALQMDY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>302 01H12 3</td>
<td>RVFDLENFY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>303 03F11 3</td>
<td>DTYNSKPARMDDYDF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Accordingly, some particularly preferred Nanobodies of the invention are Nanobodies which can bind (as further defined herein) to and/or are directed against human CD4 and which:

iii) have at least 80% amino acid identity with at least one of the amino acid sequences of

SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. In which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded. In this respect, reference is also made to Table A-1, which lists the framework 1 sequences (SEQ ID NO's: 280 to 283), framework 2 sequences (SEQ ID NO's: 288 to 291), framework 3 sequences (SEQ ID NO's: 296 to 299) and framework 4 sequences (SEQ ID NO's: 304 to 307) of the Nanobodies of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311 (with respect to the amino acid residues at positions 1 to 4 and 27 to 30 of the framework 1 sequences, reference is also made to the comments made below. Thus, for determining the degree of amino acid identity, these residues are preferably disregarded);

and in which:

preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 below.

In these Nanobodies, the CDR sequences are generally as further defined herein.

Again, such Nanobodies may be derived in any suitable manner and from any suitable source, and may for example be naturally occurring $V_{HH}$ sequences (i.e. from a suitable species of Camelid) or synthetic or semi-synthetic amino acid sequences, including but not limited to "humanized" (as defined herein) Nanobodies, ‘camelized’ (as defined herein) immunoglobulin sequences (and in particular camelized heavy chain variable domain sequences), as well as Nanobodies that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing as further described herein. Also, when a Nanobody comprises a $V_{HH}$ sequence, said Nanobody may be suitably humanized, as further described herein, so as to provide one or more further (partially or fully) humanized Nanobodies of the invention. Similarly, when a Nanobody comprises a synthetic or semi-
synthetic sequence (such as a partially humanized sequence), said Nanobody may optionally be further suitably humanized, again as described herein, again so as to provide one or more further (partially or fully) humanized Nanobodies of the invention.

In particular, humanized Nanobodies may be amino acid sequences that are as generally defined for Nanobodies in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Some preferred, but non-limiting humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring $V_{HH}$ sequence with the corresponding framework sequence of one or more closely related human $V_H$ sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said $V_{HH}$ sequence (in any manner known per se, as further described herein) and the resulting humanized $V_{HH}$ sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) a Nanobody may be partially humanized or fully humanized.

Some particularly preferred humanized Nanobodies of the invention are humanized variants of the Nanobodies of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239.

Thus, some other preferred Nanobodies of the invention are Nanobodies which can bind (as further defined herein) to human CXCR4 and which:

i) are a humanized variant of one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239; and/or

ii) have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; and in which:
preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 below.

Some other preferred Nanobodies of the invention are Nanobodies which can bind (as further defined herein) to human CD4 and which:

i) are a humanized variant of one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311; and/or

ii) have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

i) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabul numbering are chosen from the Hallmark residues mentioned in Table A-3 below.

According to another specific aspect of the invention, the invention provides a number of stretches of amino acid residues (i.e. small peptides) that are particularly suited for binding to Targets of the invention. These stretches of amino acid residues may be present in, and/or may be incorporated into, an amino acid sequence of the invention, in particular in such a way that they form (part of) the antigen binding site of an amino acid sequence of the invention. As these stretches of amino acid residues were first generated as CDR sequences of heavy chain antibodies or $V_{H}$ sequences that were raised against Targets of the invention (or may be based on and/or derived from such CDR sequences, as further described herein), they will also generally be referred to herein as "CDR sequences" (i.e. as CDR1 sequences. CDR2 sequences and CDR3 sequences, respectively). It should however be noted that the invention in its broadest sense is not limited to a specific structural role or function that these stretches of amino acid residues may have in an amino acid sequence of the invention, as long as these stretches of amino acid residues allow the amino acid sequence of the invention to bind to Targets of the invention. Thus, generally, the invention in its broadest sense comprises any amino acid sequence that is capable of binding to Targets of the invention and that comprises one or more CDR sequences as described herein, and in particular a suitable combination of two or more such CDR sequences, that are suitably linked to each other via one or more further amino acid sequences, such that the entire amino acid sequence forms a
binding domain and/or binding unit that is capable of binding to Targets of the invention. It should however also be noted that the presence of only one such CDR sequence in an amino acid sequence of the invention may by itself already be sufficient to provide an amino acid sequence of the invention that is capable of binding to Targets of the invention; reference is for example again made to the so-called "Expedite fragments" described in WO 03/050531.

Thus, in another specific, but non-limiting aspect, the amino acid sequence of the invention may be an amino acid sequence that comprises at least one amino acid sequence that is chosen from the group consisting of the CDR1 sequences, CDR2 sequences and CDR3 sequences that are described herein (or any suitable combination thereof). In particular, an amino acid sequence of the invention may be an amino acid sequence that comprises at least one antigen binding site, wherein said antigen binding site comprises at least one amino acid sequence that is chosen from the group consisting of the CDR1 sequences, CDR2 sequences and CDR3 sequences that are described herein (or any suitable combination thereof).

Generally, in this aspect of the invention, the amino acid sequence of the invention may be any amino acid sequence that comprises at least one stretch of amino acid residues, in which said stretch of amino acid residues has an amino acid sequence that corresponds to the sequence of at least one of the CDR sequences described herein. Such an amino acid sequence may or may not comprise an immunoglobulin fold. For example, and without limitation, such an amino acid sequence may be a suitable fragment of an immunoglobulin sequence that comprises at least one such CDR sequence, but that is not large enough to form a (complete) immunoglobulin fold (reference is for example again made to the "Expedite fragments" described in WO 03/050531). Alternatively, such an amino acid sequence may be a suitable "protein scaffold" that comprises least one stretch of amino acid residues that corresponds to such a CDR sequence (i.e. as part of its antigen binding site). Suitable scaffolds for presenting amino acid sequences will be clear to the skilled person, and for example comprise, without limitation, to binding scaffolds based on or derived from immunoglobulins (i.e. other than the immunoglobulin sequences already described herein), protein scaffolds derived from protein A domains (such as Affibodies™), tandemstat, fibronectin, lipocalin, CTLA-4, T-cell receptors, designed ankyrin repeats, avimers and PDZ domains (Binz et al., Nat. Biotech 2005, Vol 23:1257), and binding moieties based on DNA or RNA including but not limited to DNA or RNA aptamers (Ulrich et al., Comb Chem High Throughput Screen 2006 9(8):61 9-32).
Again, any amino acid sequence of the invention that comprises one or more of these CDR sequences is preferably such that it can specifically bind (as defined herein) to Targets of the invention, and more in particular such that it can bind to Targets of the invention with an affinity (suitably measured and/or expressed as a $K_D$-value (actual or apparent), a $K_A^-$ value (actual or apparent), a $k_{on}$-rate and/or a $k_{off}$ rate, or alternatively as an IC$_{50}$ value, as further described herein), that is as defined herein.

More in particular, the amino acid sequences according to this aspect of the invention may be any amino acid sequence that comprises at least one antigen binding site, wherein said antigen binding site comprises at least two amino acid sequences that are chosen from the group consisting of the CDR1 sequences described herein, the CDR2 sequences described herein and the CDR3 sequences described herein, such that (i) when the first amino acid sequence is chosen from the CDR1 sequences described herein, the second amino acid sequence is chosen from the CDR2 sequences described herein or the CDR3 sequences described herein; (ii) when the first amino acid sequence is chosen from the CDR2 sequences described herein, the second amino acid sequence is chosen from the CDR1 sequences described herein or the CDR3 sequences described herein; or (iii) when the first amino acid sequence is chosen from the CDR3 sequences described herein, the second amino acid sequence is chosen from the CDR1 sequences described herein or the CDR3 sequences described herein.

Even more in particular, the amino acid sequences of the invention may be amino acid sequences that comprise at least one antigen binding site, wherein said antigen binding site comprises at least three amino acid sequences that are chosen from the group consisting of the CDR1 sequences described herein, the CDR2 sequences described herein and the CDR3 sequences described herein, such that the first amino acid sequence is chosen from the CDR1 sequences described herein, the second amino acid sequence is chosen from the CDR2 sequences described herein, and the third amino acid sequence is chosen from the CDR3 sequences described herein. Preferred combinations of CDR1, CDR2 and CDR3 sequences will become clear from the further description herein. As will be clear to the skilled person, such an amino acid sequence is preferably an immunoglobulin sequence (as further described herein), but it may for example also be any other amino acid sequence that comprises a suitable scaffold for presenting said CDR sequences.
Thus, in one specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against hCXCR4, that comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
d) the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
g) the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;
or any suitable combination thereof.

When an amino acid sequence of the invention contains one or more amino acid sequences according to b) and/or c):

i) any amino acid substitution in such an amino acid sequence according to b) and/or c) is preferably, and compared to the corresponding amino acid sequence according to a), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to b) and/or c) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to a);

and/or

iii) the amino acid sequence according to b) and/or c) may be an amino acid sequence that is derived from an amino acid sequence according to a) by means of affinity maturation using one or more techniques of affinity maturation known per se.
Similarly, when an amino acid sequence of the invention contains one or more amino acid sequences according to e) and/or f):

i) any amino acid substitution in such an amino acid sequence according to e) and/or f) is preferably, and compared to the corresponding amino acid sequence according to d), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to e) and/or f) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to d);

and/or

iii) the amino acid sequence according to e) and/or f) may be an amino acid sequence that is derived from an amino acid sequence according to d) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Also, similarly, when an amino acid sequence of the invention contains one or more amino acid sequences according to h) and/or i):

i) any amino acid substitution in such an amino acid sequence according to h) and/or i) is preferably, and compared to the corresponding amino acid sequence according to g), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to h) and/or i) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to g);

and/or

iii) the amino acid sequence according to h) and/or i) may be an amino acid sequence that is derived from an amino acid sequence according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last preceding paragraphs also generally apply to any amino acid sequences of the invention that comprise one or more amino acid sequences according to b), c), e), f), h) or i), respectively.

In this specific aspect, the amino acid sequence preferably comprises one or more stretches of amino acid residues chosen from the group consisting of:

i) the amino acid sequences of SEQ ID NO’s: 142 to 157. more preferably 142 to 143:
ii) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175; and

iii) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207; or any suitable combination thereof.

Also, preferably, in such an amino acid sequence, at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against hCXCR4.

In a more specific, but again non-limiting aspect, the invention relates to an amino acid sequence directed against hCXCR4 that comprises two or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

such that (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), d), e) or f).
In this specific aspect, the amino acid sequence preferably comprises two or more stretches of amino acid residues chosen from the group consisting of:

i) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

ii) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

and

iii) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207: such that, (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175 or of SEQ ID NO's: 206 to 22L more preferably 206 to 207; (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143 or of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143 or of SEQ ID NO's: 174 to 189, more preferably 174 to 175.

Also, in such an amino acid sequence, the at least two stretches of amino acid residues again preferably form part of the antigen binding site for binding against hCXCR4.

In an even more specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against hCXCR4, that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

d) the second stretch of amino acid residues is chosen from the group consisting of:

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175:
and the third stretch of amino acid residues is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

Preferably, in this specific aspect, the first stretch of amino acid residues is chosen
from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 157, more
preferably 142 to 143; the second stretch of amino acid residues is chosen from the group
consisting of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to
175; and the third stretch of amino acid residues is chosen from the group consisting of the
amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

Again, preferably, in such an amino acid sequence, the at least three stretches of
amino acid residues forms part of the antigen binding site for binding against hCXCR4.

Preferred combinations of such stretches of amino acid sequences will become clear
from the further disclosure herein.

Preferably, in such amino acid sequences the CDR sequences have at least 70% amino
acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino
acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid
identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID
NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239. This degree of
amino acid identity can for example be determined by determining the degree of amino acid
identity (in a manner described herein) between said amino acid sequence and one or more of
the sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID
NO: 239, in which the amino acid residues that form the framework regions are disregarded.
Also, such amino acid sequences of the invention can be as further described herein.

Also, such amino acid sequences are preferably such that they can specifically bind
(as defined herein) to hCXCR4; and more in particular bind to hCXCR4 with an affinity
(suitably measured and/or expressed as a $K_D$-value (actual or apparent), a $K_A$-value (actual or
apparent), a $k^\text{on}$-rate and/or a karate, or alternatively as an $IC_{50}$ value, as further described
herein) that is as defined herein.
When the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

and/or

- CDR2 is chosen from the group consisting of:
  d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
  e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
  f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

and/or

- CDR3 is chosen from the group consisting of:
  g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
  h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
  i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

In particular, such an amino acid sequence of the invention may be such that CDRI is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143; and/or CDR2 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175; and/or CDR3 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

In particular, when the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:
CDR1 is chosen from the group consisting of:
   a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
   b) amino acid sequences that have at least 80% amino acid identity with at least one of the
      amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
   c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
      amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
and
- CDR2 is chosen from the group consisting of:
   d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
   e) amino acid sequences that have at least 80% amino acid identity with at least one of the
      amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
   f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
      amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
and
- CDR3 is chosen from the group consisting of:
   g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
   h) amino acid sequences that have at least 80% amino acid identity with at least one of the
      amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
   i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
      amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207; or any
      suitable fragment of such an amino acid sequence.

In particular, such an amino acid sequence of the invention may be such that CDR1 is
chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 157,
more preferably 142 to 143; and CDR2 is chosen from the group consisting of the amino acid
sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175; and CDR3 is chosen
from the group consisting of the amino acid sequences of SEQ ID NO's: 206 to 221, more
preferably 206 to 207.

Again, preferred combinations of CDR sequences will become clear from the further
description herein.

Also, such amino acid sequences are preferably such that they can specifically bind
(as defined herein) to hCXCR4; and more in particular bind to hCXCR4 with an affinity
(suitably measured and/or expressed as a Ko-value (actual or apparent), a K_A-value (actual or
apparent), a $k_{on}$-rate and/or a $k_{off}$-rate, or alternatively as an IC50 value, as further described herein) that is as defined herein.

In one preferred, but non-limiting aspect, the invention relates to an amino acid sequence that essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 206 to 207; more preferably SEQ ID NO: 238 and SEQ ID NO: 239. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and one or more of the sequences of SEQ ID NO’s: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, in which the amino acid residues that form the framework regions are disregarded. Such amino acid sequences of the invention can be as further described herein.

In an even more specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against human CXCR4 able to block CXCL12/SDF1-dependent activation of human CXCR4 (such as a Nanobody of the invention, as further described herein), that comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO’s: 142 to 143;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 143;
d) the amino acid sequences of SEQ ID NO’s: 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 175;
g) the amino acid sequences of SEQ ID NO’s: 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the 
amino acid sequences of SEQ ID NO's: 206 to 207; 
or any suitable combination thereof.

When an amino acid sequence of the invention contains one or more amino acid 
sequences according to b) and/or c): 
i) any amino acid substitution in such an amino acid sequence according to b) and/or c) is 
preferably, and compared to the corresponding amino acid sequence according to a), a 
conservative amino acid substitution, (as defined herein); 
and/or

ii) the amino acid sequence according to b) and/or c) preferably only contains amino acid 
substitutions, and no amino acid deletions or insertions, compared to the corresponding 
amino acid sequence according to a); 
and/or

iii) the amino acid sequence according to b) and/or c) may be an amino acid sequence that 
is derived from an amino acid sequence according to a) by means of affinity maturation 
using one or more techniques of affinity maturation known per se.

Similarly, when an amino acid sequence of the invention contains one or more amino 
acid sequences according to e) and/or f): 
i) any amino acid substitution in such an amino acid sequence according to e) and/or f) is 
preferably, and compared to the corresponding amino acid sequence according to d), a 
conservative amino acid substitution, (as defined herein); 
and/or

ii) the amino acid sequence according to e) and/or f) preferably only contains amino acid 
substitutions, and no amino acid deletions or insertions, compared to the corresponding 
amino acid sequence according to d); 
and/or

iii) the amino acid sequence according to e) and/or f) may be an amino acid sequence that 
is derived from an amino acid sequence according to d) by means of affinity maturation 
using one or more techniques of affinity maturation known per se.

Also, similarly, when an amino acid sequence of the invention contains one or more 
amino acid sequences according to h) and/or i):
i) any amino acid substitution in such an amino acid sequence according to h) and/or i) is preferably, and compared to the corresponding amino acid sequence according to g), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to h) and/or i) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to g);

and/or

iii) the amino acid sequence according to h) and/or i) may be an amino acid sequence that is derived from an amino acid sequence according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last preceding paragraphs also generally apply to any amino acid sequences of the invention that comprise one or more amino acid sequences according to b), c), e), f), h) or i), respectively.

In this specific aspect, the amino acid sequence preferably comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 143;

b) the amino acid sequences of SEQ ID NO's: 174 to 175; and

c) the amino acid sequences of SEQ ID NO's: 206 to 207;

or any suitable combination thereof.

Also, preferably, in such an amino acid sequence, at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against HUMAN CXCR4.

In a more specific, but again non-limiting aspect, the invention relates to an amino acid sequence directed against human CXCR4, that comprises two or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

d) the amino acid sequences of SEQ ID NO's: 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NCTs: 174 to 175;
g) the amino acid sequences of SEQ ID NO's: 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;

such that (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e), f), g), h) or i): (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), d), e) or f),

In this specific aspect, the amino acid sequence preferably comprises two or more stretches of amino acid residues chosen from the group consisting of:
a) the amino acid sequences of SEQ ID NO's: 142 to 143;
b) the amino acid sequences of SEQ ID NO's: 174 to 175; and
c) the amino acid sequences of SEQ ID NO's: 206 to 207;

such that, (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 143, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 174 to 175 or of SEQ ID NO's: 206 to 207; (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 174 to 175, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 143 or of SEQ ID NO's: 206 to 207; or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 206 to 207, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 142 to 143 or of SEQ ID NO's: 174 to 175.
Also, in such an amino acid sequence, the at least two stretches of amino acid residues again preferably form part of the antigen binding site for binding against human CXCR4.

In an even more specific, but not limiting aspect, the invention relates to an amino acid sequence directed against human CXCR4, that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 143;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

the second stretch of amino acid residues is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and the third stretch of amino acid residues is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207.

Preferably, in this specific aspect, the first stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 143; the second stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 174 to 175; and the third stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 206 to 207.

Again, preferably, in such an amino acid sequence, the at least three stretches of amino acid residues forms part of the antigen binding site for binding against human CXCR4.

Preferred combinations of such stretches of amino acid sequences will become clear from the further disclosure herein.
Preferably, in such amino acid sequences the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 239. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and one or more of the sequences of SEQ ID NO's: 238 to 239, in which the amino acid residues that form the framework regions are disregarded. Also, such amino acid sequences of the invention can be as further described herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to human CXCR4; and more in particular bind to human CXCR4 with an affinity (suitably measured and/or expressed as a Kp-value (actual or apparent), a Kd-value (actual or apparent), a koff-rate and/or a kon-rate, or alternatively as an IC50 value, as further described herein) that is as defined herein.

When the amino acid sequence of the invention essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO's: 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;
  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

and/or

- CDR2 is chosen from the group consisting of:
  d) the amino acid sequences of SEQ ID NO's: 174 to 175;
  e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
  f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and/or

- CDR3 is chosen from the group consisting of:
  g) the amino acid sequences of SEQ ID NO's: 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207.

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 143; and/or CDR2 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 174 to 175; and/or CDR3 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 206 to 207.

In particular, when the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO's: 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;
  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

and

- CDR2 is chosen from the group consisting of:
  d) the amino acid sequences of SEQ ID NO's: 174 to 175;
  e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
  f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and

- CDR3 is chosen from the group consisting of:
  g) the amino acid sequences of SEQ ID NO's: 206 to 207;
  h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207; or any suitable fragment of such an amino acid sequence

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 142 to 143; and CDR2 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 174 to 175; and CDR3 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 206 to 207.

Again, preferred combinations of CDR sequences will become clear from the further description herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to human CXCR4; and more in particular bind to human CXCR4 with an affinity (suitably measured and/or expressed as a K_r-value (actual or apparent), a K_A-value (actual or apparent), a k_on-rate and/or a k_off-rate, or alternatively as an IC_50 value, as further described herein) that is as defined herein.

In one preferred, but non-limiting aspect, the invention relates to an amino acid sequence that essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 239. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and one or more of the sequences of SEQ ID NO's: 238 to 239, in which the amino acid residues that form the framework regions are disregarded. Such amino acid sequences of the invention can be as further described herein.

Thus, in another specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against hCD4, that comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

d) the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

g) the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303;

or any suitable combination thereof.

When an amino acid sequence of the invention contains one or more amino acid sequences according to b) and/or c):

i) any amino acid substitution in such an amino acid sequence according to b) and/or c) is preferably, and compared to the corresponding amino acid sequence according to a), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to b) and/or c) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to a);

and/or

iii) the amino acid sequence according to b) and/or c) may be an amino acid sequence that is derived from an amino acid sequence according to a) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Similarly, when an amino acid sequence of the invention contains one or more amino acid sequences according to e) and/or f):

i) any amino acid substitution in such an amino acid sequence according to e) and/or f) is preferably, and compared to the corresponding amino acid sequence according to d), a conservative amino acid substitution, (as defined herein);
and/or

ii) the amino acid sequence according to e) and/or f) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to d):

and/or

iii) the amino acid sequence according to e) and/or f) may be an amino acid sequence that is derived from an amino acid sequence according to d) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Also, similarly, when an amino acid sequence of the invention contains one or more amino acid sequences according to h) and/or i):

i) any amino acid substitution in such an amino acid sequence according to h) and/or i) is preferably, and compared to the corresponding amino acid sequence according to g), a conservative amino acid substitution, (as defined herein);

and/or

ii) the amino acid sequence according to h) and/or i) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding amino acid sequence according to g);

and/or

iii) the amino acid sequence according to h) and/or i) may be an amino acid sequence that is derived from an amino acid sequence according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last preceding paragraphs also generally apply to any amino acid sequences of the invention that comprise one or more amino acid sequences according to b), c), e), f), h) or i). respectively.

In this specific aspect, the amino acid sequence preferably comprises one or more stretches of amino acid residues chosen from the group consisting of:

i) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

ii) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295; and

iii) the amino acid sequences of SEQ ID NO's: 300 to 303. more preferably 303;

or any suitable combination thereof.

Also, preferably, in such an amino acid sequence, at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.
In a more specific, but again non-limiting aspect, the invention relates to an amino acid sequence directed against hCD4 that comprises two or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

such that (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), d), e) or f).

In this specific aspect, the amino acid sequence preferably comprises two or more stretches of amino acid residues chosen from the group consisting of:

i) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
ii) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295; and
iii) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
such that, (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295 or of SEQ ID NO’s: 300 to 303, more preferably 303; (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287 or of SEQ ID NO's: 300 to 303, more preferably 303; or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303, the second stretch of amino acid residues corresponds to one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287 or of SEQ ID NO's: 292 to 295, more preferably 295.

Also, in such an amino acid sequence, the at least two stretches of amino acid residues again preferably form part of the antigen binding site for binding against hCD4.

In an even more specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against hCD4, that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

the second stretch of amino acid residues is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303.

Preferably, in this specific aspect, the first stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287; the second stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295; and the third stretch of amino acid residues is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303.

Again, preferably, in such an amino acid sequence, the at least three stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.

Preferred combinations of such stretches of amino acid sequences will become clear from the further disclosure herein.

Preferably, in such amino acid sequences the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and one or more of the sequences of SEQ ID NO’s: 308 to 311, more preferably SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Also, such amino acid sequences of the invention can be as further described herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to hCD4; and more in particular bind to hCD4 with an affinity (suitably measured and/or expressed as a $K_{D}$-value (actual or apparent), a $K_{A}$-value (actual or apparent), a $k_{on}$-rate and/or a $k_{off}$-rate, or alternatively as an $IC_{50}$ value, as further described herein) that is as defined herein.

When the amino acid sequence of the invention essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

5 and/or

- CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;

and/or

- CDR3 is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303.

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the group consisting of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287; and/or CDR2 is chosen from the group consisting of the amino acid sequences of SEQ ID NO’s: 292 to 295. more preferably 295; and/or CDR3 is chosen from the group consisting of the amino acid sequences of SEQ ID NO’s: 300 to 303, more preferably 303.

In particular, when the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287:
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287; and

- CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

and

- CDR3 is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303; or any suitable fragment of such an amino acid sequence

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287; and CDR2 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295; and CDR3 is chosen from the group consisting of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303.

Again, preferred combinations of CDR sequences will become clear from the further description herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to hCD4; and more in particular bind to hCD4 with an affinity (suitably measured and/or expressed as a $K_D$-value (actual or apparent), a $K_A$-value (actual or apparent), a $k_{on}$-rate and/or a $k_{off}$-rate, or alternatively as an $IC_{50}$ value, as further described herein) that is as defined herein.

In one preferred, but non-limiting aspect, the invention relates to an amino acid sequence that essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which the CDR
sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and one or more of the sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Such amino acid sequences of the invention can be as further described herein.

In an even more specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against human CD4 able to block gpl20 binding to CD4 (such as a Nanobody of the invention, as further described herein), that comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO's: 287;
b) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;
d) the amino acid sequence of SEQ ID NO: 295;
e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;
g) the amino acid sequence of SEQ ID NO: 303;
h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303;

or any suitable combination thereof.

When an amino acid sequence of the invention contains one or more amino acid sequences according to b) and/or c):
i) any amino acid substitution in such an amino acid sequence according to b) and/or c) is
preferably, and compared to the corresponding amino acid sequence according to a), a
conservative amino acid substitution, (as defined herein);
and/or

ii) the amino acid sequence according to b) and/or c) preferably only contains amino acid
substitutions, and no amino acid deletions or insertions, compared to the corresponding
amino acid sequence according to a);
and/or

iii) the amino acid sequence according to b) and/or c) may be an amino acid sequence that
is derived from an amino acid sequence according to a) by means of affinity maturation
using one or more techniques of affinity maturation known per se.

Similarly, when an amino acid sequence of the invention contains one or more amino
acid sequences according to e) and/or f):

i) any amino acid substitution in such an amino acid sequence according to e) and/or f) is
preferably, and compared to the corresponding amino acid sequence according to d). a
conservative amino acid substitution, (as defined herein);
and/or

ii) the amino acid sequence according to e) and/or f) preferably only contains amino acid
substitutions, and no amino acid deletions or insertions, compared to the corresponding
amino acid sequence according to d);
and/or

iii) the amino acid sequence according to e) and/or f) may be an amino acid sequence that
is derived from an amino acid sequence according to d) by means of affinity maturation
using one or more techniques of affinity maturation known per se.

Also, similarly, when an amino acid sequence of the invention contains one or more
amino acid sequences according to h) and/or i):

i) any amino acid substitution in such an amino acid sequence according to h) and/or i) is
preferably, and compared to the corresponding amino acid sequence according to g), a
conservative amino acid substitution, (as defined herein);
and/or

ii) the amino acid sequence according to h) and/or i) preferably only contains amino acid
substitutions, and no amino acid deletions or insertions, compared to the corresponding
amino acid sequence according to g);
and/or
iii) the amino acid sequence according to h) and/or i) may be an amino acid sequence that is derived from an amino acid sequence according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last preceding paragraphs also generally apply to any amino acid sequences of the invention that comprise one or more amino acid sequences according to b), c), e), f), h) or i), respectively.

In this specific aspect, the amino acid sequence preferably comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO: 287;
b) the amino acid sequence of SEQ ID NO: 295; and
c) the amino acid sequence of SEQ ID NO: 303;

or any suitable combination thereof.

Also, preferably, in such an amino acid sequence, at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.

In a more specific, but again non-limiting aspect, the invention relates to an amino acid sequence directed against human CD4, that comprises two or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO: 287;
b) amino acid sequence that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;
d) the amino acid sequences of SEQ ID NO’s: 295;
e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;
g) the amino acid sequences of SEQ ID NO: 303;
h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;
i) amino acid sequences that have 3. 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303;
such that (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), d), e) or f).

In this specific aspect, the amino acid sequence preferably comprises two or more stretches of amino acid residues chosen from the group consisting of:

d) the amino acid sequence of SEQ ID NO: 287;

e) the amino acid sequence of SEQ ID NO: 295; and

f) the amino acid sequence of SEQ ID NO: 303:

such that, (i) when the first stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 287, the second stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 295 or of SEQ ID NO: 303; (ii) when the first stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 295, the second stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 287 or of SEQ ID NO: 303; or (iii) when the first stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 303, the second stretch of amino acid residues corresponds to the amino acid sequence of SEQ ID NO: 287 or of SEQ ID NO: 295.

Also, in such an amino acid sequence, the at least two stretches of amino acid residues again preferably form part of the antigen binding site for binding against human CD4.

In an even more specific, but non-limiting aspect, the invention relates to an amino acid sequence directed against human CD4, that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO: 287;

b) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;
the second stretch of amino acid residues is chosen from the group consisting of:

d) the amino acid sequence of SEQ ID NO: 295;

e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;

and the third stretch of amino acid residues is chosen from the group consisting of:

g) the amino acid sequence of SEQ ID NO: 303;

h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303.

Preferably, in this specific aspect, the first stretch of amino acid residues is chosen from the amino acid sequence of SEQ ID NO: 287; the second stretch of amino acid residues is chosen from the amino acid sequence of SEQ ID NO: 295; and the third stretch of amino acid residues is chosen from the amino acid sequence of SEQ ID NO: 303.

Again, preferably, in such an amino acid sequence, the at least three stretches of amino acid residues forms part of the antigen binding site for binding against human CD4.

Preferred combinations of such stretches of amino acid sequences will become clear from the further disclosure herein.

Preferably, in such amino acid sequences the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least the amino acid sequence of SEQ ID NO: 311.

This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and the sequence of SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Also, such amino acid sequences of the invention can be as further described herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to human CD4; and more in particular bind to human CD4 with an affinity (suitably measured and/or expressed as a $K_D$-value (actual or apparent), a $K_A$-value (actual or
apparent), a k\textsubscript{on} rate and/or a k\textsubscript{off} rate, or alternatively as an IC\textsubscript{50} value, as further described herein) that is as defined herein.

When the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:

- CDR1 is chosen from the group consisting of:
  - a) the amino acid sequence of SEQ ID NO: 287;
  - b) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;
  - c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;

and/or

- CDR2 is chosen from the group consisting of:
  - d) the amino acid sequence of SEQ ID NO: 295;
  - e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;
  - f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;

and/or

- CDR3 is chosen from the group consisting of:
  - g) the amino acid sequence of SEQ ID NO: 303;
  - h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;
  - i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303;

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the amino acid sequence of SEQ ID NO: 287; and/or CDR2 is chosen from the amino acid sequence of SEQ ID NO: 295; and/or CDR3 is chosen from the amino acid sequence of SEQ ID NO: 303.

In particular, when the amino acid sequence of the invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), the amino acid sequence of the invention is preferably such that:
CDR1 is chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO: 287;

b) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;

and

CDR2 is chosen from the group consisting of:

d) the amino acid sequence of SEQ ID NO: 295;

e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;

and

CDR3 is chosen from the group consisting of:

g) the amino acid sequence of SEQ ID NO: 303;

h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303; or any suitable fragment of such an amino acid sequence

In particular, such an amino acid sequence of the invention may be such that CDR1 is chosen from the amino acid sequence of SEQ ID NO: 287; and CDR2 is chosen from the amino acid sequence of SEQ ID NO: 295; and CDR3 is chosen from the amino acid sequence of SEQ ID NO: 303.

Again, preferred combinations of CDR sequences will become clear from the further description herein.

Also, such amino acid sequences are preferably such that they can specifically bind (as defined herein) to human CD4; and more in particular bind to human CD4 with an affinity (suitably measured and/or expressed as a Kp-value (actual or apparent), a $K_A$-value (actual or apparent), a $k_{on}$-rate and/or a $k_{off}$-rate, or alternatively as an IC$_{50}$ value, as further described herein) that is as defined herein.
In one preferred, but non-limiting aspect, the invention relates to an amino acid sequence that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least the amino acid sequence of SEQ ID NO: 311. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said amino acid sequence and the sequence of SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Such amino acid sequences of the invention can be as further described herein.

In such an amino acid sequence of the invention, the framework sequences may be any suitable framework sequences, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

The framework sequences are preferably (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework sequences (for example, by humanization or camelization). For example, the framework sequences may be framework sequences derived from a light chain variable domain (e.g. a Vu-sequence) and/or from a heavy chain variable domain (e.g. a \(V_{H}\)-sequence). In one particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a \(V_{H}\)-i-sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional \(V_{H}\) sequences that have been camelized (as defined herein).

The framework sequences are preferably such that the amino acid sequence of the invention is a domain antibody (or an amino acid sequence that is suitable for use as a domain antibody); is a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody); is a "dAb" (or an amino acid sequence that is suitable for use as a dAb); or is a Nanobody (including but not limited to \(V_{HH}\) sequence). Again, suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

In particular, the framework sequences present in the amino acid sequences of the invention may contain one or more of Hallmark residues (as defined herein), such that the
amino acid sequence of the invention is a Nanobody. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein.

Again, as generally described herein for the amino acid sequences of the invention, it is also possible to use suitable fragments (or combinations of fragments) of any of the foregoing, such as fragments that contain one or more CDR sequences, suitably flanked by and/or linked via one or more framework sequences (for example, in the same order as these CDR’s and framework sequences may occur in the full-sized immunoglobulin sequence from which the fragment has been derived). Such fragments may also again be such that they comprise or can form an immunoglobulin fold, or alternatively be such that they do not comprise or cannot form an immunoglobulin fold.

In one specific aspect, such a fragment comprises a single CDR sequence as described herein (and in particular a CDR3 sequence), that is flanked on each side by (part of) a framework sequence (and in particular, part of the framework sequence(s) that, in the immunoglobulin sequence from which the fragment is derived, are adjacent to said CDR sequence. For example, a CDR3 sequence may be preceded by (part of) a FR3 sequence and followed by (part of) a FR4 sequence). Such a fragment may also contain a disulphide bridge, and in particular a disulphide bridge that links the two framework regions that precede and follow the CDR sequence, respectively (for the purpose of forming such a disulphide bridge, cysteine residues that naturally occur in said framework regions may be used, or alternatively cysteine residues may be synthetically added to or introduced into said framework regions).

For a further description of these “ Expedite fragments”, reference is again made to WO 03/050531, as well as to as well as to WO 08/068280.

In another aspect, the invention relates to a compound or construct, and in particular a protein or polypeptide (also referred to herein as a "compound of the invention" or "compound of the invention", respectively) that comprises or essentially consists of one or more amino acid sequences of the invention (or suitable fragments thereof), and optionally further comprises one or more other groups, residues, moieties or binding units. As will become clear to the skilled person from the further disclosure herein, such further groups, residues, moieties, binding units or amino acid sequences may or may not provide further functionality to the amino acid sequence of the invention (and/or to the compound or construct in which it is present) and may or may not modify the properties of the amino acid sequence of the invention.
For example, such further groups, residues, moieties or binding units may be one or more additional amino acid sequences, such that the compound or construct is a (fusion) protein or (fusion) polypeptide. In a preferred but non-limiting aspect, said one or more other groups, residues, moieties or binding units are immunoglobulin sequences. Even more preferably, said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody. "dAb"'s, amino acid sequences that are suitable for use as a dAb, or Nanobodies.

Alternatively, such groups, residues, moieties or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the one or more amino acid sequences of the invention so as to provide a "derivative" of an amino acid sequence or polypeptide of the invention, as further described herein.

The polypeptides of the invention comprise or essentially consist of at least one Nanobody of the invention. Some preferred, but non-limiting examples of polypeptides of the invention are given in SEQ ID NO’s: 261 to 264, more preferably SEQ ID NO’s 263 to 264.

### Table 2: Preferred polypeptide or compound sequences (also referred herein as a sequence with a particular name or SEQ ID NO: X, wherein X is a number referring to the relevant amino acid sequence):

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Clone name</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVQLVESGGGLVQGTGSSRLSCAASGFTFSSYAMS1WVRQAPGKL</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EWSGIKSSGDSTRYAGSVKGRFTISRDNKMLQMYSKPEDE</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>TAVYCAKAKSVRSTGTYDNRQGRTQTVSSGGGSGSGSGSG</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>LVEGGLVQYTGSSRLSCAASGFTFSSYAMS1WVRQAPGKL</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>SIKSSGDSTRYAGSVKGRFTISRDNKMLQMYSKPEDEAFEY</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EVQLMESGGGLVQAGSRLSCAASGRTFNNYAMGWFRRAPGKER</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EVFAAATRSVSRGVSASIYGDYKDRFTISRDNKMLQMNSLKEPEVDITYCAASAISSGALRRFEYDYSGQQGQTQTVSSGGGG</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EVQLVESGGGLVQGTGSSRLSCAASGFTFSSYAMS1WVRQAPGKL</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EVQLVESGGGLVQAGSRLSCAASGRTFNNYAMGWFRRAPGKER</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EFVAAATRSVSRGVSASIYGDYKDRFTISRDNKMLQMNSLKEPEVDITYCAASAISSGALRRFEYDYSGQQGQTQTVSSGGGGG</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
<tr>
<td>EVQLVESGGGLVQAGSRLSCAASGFTFSSYAMS1WVRQAPGKL</td>
<td>236D2-10GS-236D2</td>
<td>261</td>
</tr>
</tbody>
</table>
Also within the scope of the present invention are compounds or constructs, that comprises or essentially consists of one or more derivatives as described herein, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers. Preferably, said one or more other groups, residues, moieties or binding units are amino acid sequences.

In the compounds or constructs described above, the one or more amino acid sequences of the invention and the one or more groups, residues, moieties or binding units may be linked directly to each other and/or via one or more suitable linkers or spacers. For example, when the one or more groups, residues, moieties or binding units are amino acid sequences, the linkers may also be amino acid sequences, so that the resulting compound or construct is a fusion (protein) or fusion (polypeptide).

The compounds or polypeptides of the invention can generally be prepared by a method which comprises at least one step of suitably linking the one or more amino acid sequences of the invention to the one or more further groups, residues, moieties or binding units, optionally via one or more suitable linkers, so as to provide the compound or polypeptide of the invention. Polypeptides of the invention can also be prepared by a method which generally comprises at least the steps of providing a nucleic acid that encodes a polypeptide of the invention, expressing said nucleic acid in a suitable manner, and recovering the expressed polypeptide of the invention. Such methods can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the methods and techniques further described herein.

The process of designing/selecting and/or preparing a compound or polypeptide of the invention, starting from an amino acid sequence of the invention, is also referred to herein as "formatting" said amino acid sequence of the invention; and an amino acid of the invention that is made part of a compound or polypeptide of the invention is said to be "formatted" or to be "in the format of" said compound or polypeptide of the invention. Examples of ways in
which an amino acid sequence of the invention can be formatted and examples of such formats will be clear to the skilled person based on the disclosure herein; and such formatted amino acid sequences form a further aspect of the invention.

In one specific aspect of the invention, a compound of the invention or a polypeptide of the invention may have an increased half-life, compared to the corresponding amino acid sequence of the invention. Some preferred, but non-limiting examples of such compounds and polypeptides will become clear to the skilled person based on the further disclosure herein, and for example comprise amino acid sequences or polypeptides of the invention that have been chemically modified to increase the half-life thereof (for example, by means of pegylation); amino acid sequences of the invention that comprise at least one additional binding site for binding to a serum protein (such as serum albumin: see for example EP 0 368 684 Bl, page 4); or polypeptides of the invention that comprise at least one amino acid sequence of the invention that is linked to at least one moiety (and in particular at least one amino acid sequence) that increases the half-life of the amino acid sequence of the invention.

Examples of polypeptides of the invention that comprise such half-life extending moieties or amino acid sequences will become clear to the skilled person based on the further disclosure herein; and for example include, without limitation, polypeptides in which the one or more amino acid sequences of the invention are suitable linked to one or more serum proteins or fragments thereof (such as (human) serum albumin or suitable fragments thereof) or to one or more binding units that can bind to serum proteins (such as, for example, domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"s, amino acid sequences that are suitable for use as a dAb, or Nanobodies that can bind to serum proteins such as serum albumin (such as human serum albumin), serum immunoglobulins such as IgG5 or transferrin; reference is made to the further description and references mentioned herein); polypeptides in which an amino acid sequence of the invention is linked to an Fc portion (such as a human Fc) or a suitable part or fragment thereof; or polypeptides in which the one or more amino acid sequences of the invention are suitable linked to one or more small proteins or peptides that can bind to serum proteins (such as, without limitation, the proteins and peptides described in WO 9 1/0 1743, WO 01/4 5746, WO 02/0 76489 and to WO 08/0 68280.

Generally, the compounds or polypeptides of the invention with increased half-life preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least
5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding amino acid sequence of the invention per se. For example, the compounds or polypeptides of the invention with increased half-life may have a half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid sequence of the invention per se.

In a preferred, but non-limiting aspect of the invention, such compounds or polypeptides of the invention have a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid sequence of the invention per se.

In another preferred, but non-limiting aspect of the invention, such compounds or polypeptides of the invention exhibit a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more. For example, compounds or polypeptides of the invention may have a half-life of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

In another aspect, the invention relates to a nucleic acid that encodes an amino acid sequence of the invention or a polypeptide of the invention (or a suitable fragment thereof). Such a nucleic acid will also be referred to herein as a "nucleic acid of the invention" and may for example be in the form of a genetic construct, as further described herein.

In another aspect, the invention relates to a host or host cell that expresses (or that under suitable circumstances is capable of expressing) an amino acid sequence of the invention and/or a polypeptide of the invention; and/or that contains a nucleic acid of the invention. Some preferred but non-limiting examples of such hosts or host cells will become clear from the further description herein.

The invention further relates to a product or composition containing or comprising at least one amino acid sequence of the invention, at least one polypeptide of the invention (or a suitable fragment thereof) and/or at least one nucleic acid of the invention, and optionally one or more further components of such compositions known per se. i.e. depending on the intended use of the composition. Such a product or composition may for example be a
pharmaceutical composition (as described herein), a veterinary composition or a product or composition for diagnostic use (as also described herein). Some preferred but non-limiting examples of such products or compositions will become clear from the further description herein.

The invention also relates to the use of an amino acid sequence. Nanobody or polypeptide of the invention, or of a composition comprising the same, in (methods or compositions for) modulating a Target of the invention, either in vitro (e.g. in an in vitro or cellular assay) or in vivo (e.g. in an a single cell or in a multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from Targets of the invention related disease or disorder).

The invention also relates to methods for modulating a Target of the invention, either in vitro (e.g. in an in vitro or cellular assay) or in vivo (e.g. in an a single cell or multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from Targets of the invention related disease or disorder), which method comprises at least the step of contacting Targets of the invention with at least one amino acid sequence, Nanobody or polypeptide of the invention, or with a composition comprising the same, in a manner and in an amount suitable to modulate Targets of the invention, with at least one amino acid sequence, Nanobody or polypeptide of the invention.

The invention also relates to the use of an one amino acid sequence, Nanobody or polypeptide of the invention in the preparation of a composition (such as, without limitation, a pharmaceutical composition or preparation as further described herein) for modulating Targets of the invention, either in vitro (e.g. in an in vitro or cellular assay) or in vivo (e.g. in an a single cell or multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from a Targets of the invention related disease or disorder).

In the context of the present invention, "modulating" or "to modulate" generally means either reducing or inhibiting the activity of, or alternatively increasing the activity of, Targets of the invention, as measured using a suitable in vitro, cellular or in vivo assay (such as those mentioned herein). In particular, "modulating" or "to modulate" may mean either reducing or inhibiting the activity of, or alternatively increasing the activity of, Targets of the invention, as measured using a suitable in vitro, cellular or in vivo assay (such as those mentioned herein), by at least 1%, preferably at least 5%. such as at least 10% or at least
25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to activity of Targets of the invention in the same assay under the same conditions but without the presence of the amino acid sequence, Nanobody or polypeptide of the invention.

As will be clear to the skilled person, "modulating" may also involve effecting a change (which may either be an increase or a decrease) in affinity, avidity, specificity and/or selectivity of Targets of the invention for one or more of its targets, ligands or substrates; and/or effecting a change (which may either be an increase or a decrease) in the sensitivity of a Target of the invention for one or more conditions in the medium or surroundings in which the Target of the invention is present (such as pH, ion strength, the presence of co-factors, etc.), compared to the same conditions but without the presence of the amino acid sequence, Nanobody or polypeptide of the invention. As will be clear to the skilled person, this may again be determined in any suitable manner and/or using any suitable assay known per se, such as the assays described herein or in the prior art cited herein.

"Modulating" may also mean effecting a change (i.e. an activity as an agonist, as an antagonist or as a reverse agonist, respectively, depending on the Target of the invention and the desired biological or physiological effect) with respect to one or more biological or physiological mechanisms, effects, responses, functions, pathways or activities in which a Target of the invention (or in which its substrate(s), ligand(s) or pathway(s) are involved, such as its signalling pathway or metabolic pathway and their associated biological or physiological effects) is involved. Again, as will be clear to the skilled person, such an action as an agonist or an antagonist may be determined in any suitable manner and/or using any suitable (in vitro and usually cellular or in assay) assay known per se, such as the assays described herein or in the prior art cited herein. In particular, an action as an agonist or antagonist may be such that an intended biological or physiological activity is increased or decreased, respectively, by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to the biological or physiological activity in the same assay under the same conditions but without the presence of the amino acid sequence, Nanobody or polypeptide of the invention.

Modulating may for example involve allosteric modulation (see for example George et al, Nat Rev Drag Discov 1:808-820 (2002); Kenakin, Trends Pharmacol Sci 25:186-192 (2002) and Rios et al., Pharmacol Ther 92:71-87 (2001)) and/or reducing or
inhibiting the binding of a Target of the invention to one of its substrates or ligands and/or competing with a natural ligand, substrate for binding to a Target of the invention. Modulating may also involve activating a Target of the invention or the mechanism or pathway in which it is involved. Modulating may be reversible or irreversible, but for pharmaceutical and pharmacological purposes will usually be in a reversible manner.

The invention further relates to methods for preparing or generating the amino acid sequences, polypeptides, nucleic acids, host cells, products and compositions described herein. Some preferred but non-limiting examples of such methods will become clear from the further description herein.

Generally, these methods may comprise the steps of:

a) providing a set, collection or library of amino acid sequences; and
b) screening said set, collection or library of amino acid sequences for amino acid sequences that can bind to and/or have affinity for Targets of the invention; and

15 c) isolating the amino acid sequence(s) that can bind to and/or have affinity for Targets of the invention.

In particular, in step b) of such a method, the set, collection or library may be screened for amino acid sequences that can bind to and/or have affinity for Targets of the invention that are expressed on the surface of a suitable cell; for amino acid sequences that can bind to and/or have affinity for an extracellular part, region, domain, loop or other extracellular epitope of a Target of the invention (as described herein); and/or for amino acid sequences that can bind to and/or have affinity for a peptide that has been derived from or based on the amino acid sequence of an extracellular part, region, domain, loop or other extracellular epitope of a Target of the invention. This can be performed using methods and techniques known per se, for example those mentioned herein.

In such a method, the set, collection or library of amino acid sequences may be any suitable set, collection or library of amino acid sequences. For example, the set, collection or library of amino acid sequences may be a set, collection or library of immunoglobulin sequences (as described herein), such as a naïve set, collection or library of immunoglobulin sequences; a synthetic or semi-synthetic set, collection or library of immunoglobulin sequences; and/or a set, collection or library of immunoglobulin sequences that have been subjected to affinity maturation.
Also, in such a method, the set, collection or library of amino acid sequences may be a
set, collection or library of heavy chain variable domains (such as \( V_\text{H} \) domains or VHH
domains) or of light chain variable domains. For example, the set, collection or library of
amino acid sequences may be a set, collection or library of domain antibodies or single
domain antibodies, or may be a set, collection or library of amino acid sequences that are
capable of functioning as a domain antibody or single domain antibody.

In a preferred aspect of this method, the set, collection or library of amino acid
sequences may be an immune set, collection or library of immunoglobulin sequences, for
example derived from a mammal that has been suitably immunized with Targets of the
invention or with a suitable antigenic determinant based thereon or derived therefrom, such as
an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular
aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other
extracellular epitope(s), or a suitable peptide derived therefrom. Alternatively, as mentioned
herein, the set, collection or library of amino acid sequences may be an immune set,
collection or library of immunoglobulin sequences, for example derived from a mammal that
has been suitably immunized with a refolded Target of the invention or with a cell, or cell
fraction or preparation derived from a cell that has a Target of the invention on its surface.

In the above methods, the set, collection or library of amino acid sequences may be
displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such
as to facilitate screening. Suitable methods, techniques and host organisms for displaying and
screening (a set, collection or library of) amino acid sequences will be clear to the person
skilled in the art, for example on the basis of the further disclosure herein. Reference is also
made to the review by Hoogenboom in Nature Biotechnology, 23, 9, 1105-1116 (2005).

In another aspect, the method for generating amino acid sequences comprises at least
the steps of:

a) providing a collection or sample of cells expressing amino acid sequences;

b) screening said collection or sample of cells for cells that express an amino acid
sequence that can bind to and/or have affinity for Targets of the invention;

and

c) either (i) isolating said amino acid sequence; or (ii) isolating from said cell a nucleic
acid sequence that encodes said amino acid sequence, followed by expressing said
amino acid sequence.
In particular, in step b) of such a method, the set, collection or library may be screened for cells that express amino acid sequences that can bind to and/or have affinity for Targets of the invention that are expressed on the surface of a suitable cell; for cells that express amino acid sequences that can bind to and/or have affinity for an extracellular part, region, domain, loop or other extracellular epitope of a Target of the invention (as described herein): and/or for cells that express amino acid sequences that can bind to and/or have affinity for a peptide that has been derived from or based on the amino acid sequence of an extracellular part, region, domain, loop or other extracellular epitope of a Target of the invention. This can be performed using methods and techniques known per se, for example those mentioned herein.

For example, when the desired amino acid sequence is an immunoglobulin sequence, the collection or sample of cells may for example be a collection or sample of B-cells. Also, in this method, the sample of cells may be derived from a mammal that has been suitably immunized with Targets of the invention or with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(ε), or a suitable peptide derived therefrom. Alternatively, as mentioned herein, the sample of cells may be derived from a mammal that has been suitably immunized with a refolded Target of the invention or with a cell, or cell fraction or preparation derived from a cell that has a Target of the invention on its surface.

The above method may be performed in any suitable manner, as will be clear to the skilled person. Reference is for example made to EP 0 542 810, WO 05/19824, WO 04/051268 and WO 04/106377. The screening of step b) is preferably performed using a flow cytometry technique such as FACS. For this, reference is for example made to Lieby et al., Blood. Vol. 97, No. 12, 3820 (2001).

In another aspect, the method for generating an amino acid sequence directed against Targets of the invention may comprise at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding amino acid sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an amino acid sequence that can bind to and/or has affinity for Targets of the invention;
and

c) isolating said nucleic acid sequence, followed by expressing said amino acid sequence.

In particular, in step b) of such a method, the set collection or library may be
screened for nucleotide sequences that encode amino acid sequences that can bind to and/or
have affinity for Targets of the invention that are expressed on the surface of a suitable cell;
for nucleotide sequences that encode amino acid sequences that can bind to and/or have
affinity for an extracellular part, region, domain, loop or other extracellular epitope of a
Target of the invention (as described herein); and/or for nucleotide sequences that encode
amino acid sequences that can bind to and/or have affinity for a peptide that has been derived
from or based on the amino acid sequence of an extracellular part, region, domain, loop or
other extracellular epitope of a Target of the invention. This can be performed using methods
and techniques known per se, for example those mentioned herein.

In such a method, the set, collection or library of nucleic acid sequences encoding
amino acid sequences may for example be a set, collection or library of nucleic acid
sequences encoding a naïve set, collection or library of immunoglobulin sequences; a set,
collection or library of nucleic acid sequences encoding a synthetic or semi-synthetic set,
collection or library of immunoglobulin sequences: and/or a set, collection or library of
nucleic acid sequences encoding a set, collection or library of immunoglobulin sequences that
have been subjected to affinity maturation.

Also, in such a method, the set, collection or library of nucleic acid sequences may
encode a set, collection or library of heavy chain variable domains (such as \( V_H \) domains or
\( V_{\text{HH}} \) domains) or of light chain variable domains. For example, the set, collection or library of
nucleic acid sequences may encode a set, collection or library of domain antibodies or single
domain antibodies, or a set, collection or library of amino acid sequences that are capable of
functioning as a domain antibody or single domain antibody.

In a preferred aspect of this method, the set, collection or library of nucleic acid
sequences may be an immune set, collection or library of nucleic acid sequences, for example
derived from a mammal that has been suitably immunized with Targets of the invention or
with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic
part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said
antigenic determinant may be an extracellular part, region, domain, loop or other extracellular
epitope(s), or a suitable peptide derived therefrom. Alternatively, as mentioned herein, the
set, collection or library of nucleic acid sequences may be an immune set, collection or
library derived from a mammal that has been suitably immunized with a Target of the invention or with a cell, or cell fraction or preparation derived from a cell that has a Target of the invention on its surface.

The set, collection or library of nucleic acid sequences may for example encode an immune set, collection or library of heavy chain variable domains or of light chain variable domains. In one specific aspect, the set, collection or library of nucleotide sequences may encode a set, collection or library of V_{\text{JH}} sequences.

In the above methods, the set, collection or library of nucleotide sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) nucleotide sequences encoding amino acid sequences will be clear to the person skilled in the art, for example on the basis of the further disclosure herein. Reference is also made to the review by Hoogenboom in Nature Biotechnology, 23, 9, 1105-1116 (2005).

In another aspect, the method for generating an amino acid sequence directed against hCXCR4 may comprise at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding amino acid sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an amino acid sequence that can bind to and/or has affinity for hCXCR4 and that is cross-blocked or is cross blocking a Nanobody of the invention, e.g. SEQ ID NO: 238 to 253 (Table 1), or a polypeptide or construct of the invention, e.g. SEQ ID NO: 261-266 (see Table 2); and

c) isolating said nucleic acid sequence, followed by expressing said amino acid sequence.

In yet another aspect, the method for generating an amino acid sequence directed against hF4 may comprise at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding amino acid sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an amino acid sequence that can bind to and/or has affinity for hCD4 and that is cross-blocked or is cross blocking a Nanobody of the invention, e.g. SEQ ID NO: 308 to 311 (Table 3); and

c) isolating said nucleic acid sequence, followed by expressing said amino acid sequence.
The invention also relates to amino acid sequences that are obtained by the above methods, or alternatively by a method that comprises the one of the above methods and in addition at least the steps of determining the nucleotide sequence or amino acid sequence of said immunoglobulin sequence; and of expressing or synthesizing said amino acid sequence in a manner known per se. such as by expression in a suitable host cell or host organism or by chemical synthesis.

Also, following the steps above, one or more amino acid sequences of the invention may be suitably humanized (or alternatively camelized); and/or the amino acid sequence(s) thus obtained may be linked to each other or to one or more other suitable amino acid sequences (optionally via one or more suitable linkers) so as to provide a polypeptide of the invention. Also, a nucleic acid sequence encoding an amino acid sequence of the invention may be suitably humanized (or alternatively camelized) and suitably expressed; and/or one or more nucleic acid sequences encoding an amino acid sequence of the invention may be linked to each other or to one or more nucleic acid sequences that encode other suitable amino acid sequences (optionally via nucleotide sequences that encode one or more suitable linkers), after which the nucleotide sequence thus obtained may be suitably expressed so as to provide a polypeptide of the invention.

The invention further relates to applications and uses of the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein, as well as to methods for the prevention and/or treatment for diseases and disorders associated with Targets of the invention. Some preferred but non-limiting applications and uses will become clear from the further description herein.

The invention also relates to the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein for use in therapy.

In particular, the invention also relates to the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein for use in therapy of a disease or disorder that can be prevented or treated by administering, to a subject in need thereof, of (a pharmaceutically effective amount of) an amino acid sequence, compound, construct or polypeptide as described herein.

More in particular, the invention relates to the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein for use in therapy of viral and bacterial induced diseases and/or disorders, such as e.g.
AIDS, Hepatitis C, Hepatitis B, adenovirus infection, hantavirus infection, herpesvirus infection, echo-virus infection, etc.

The amino acid sequences, Nanobodies and polypeptides of the invention may also find use as markers for detecting cells that express the Targets of the invention against which they are directed, for example in vitro (e.g. using Western blot, immunoprecipitation or immunofluorescence techniques) or in vivo (e.g. using suitable imaging techniques). The amino acid sequences, Nanobodies and polypeptides of the invention may also find use in affinity purification techniques for (cells expressing) the Targets of the invention against which they are directed.

Other aspects, embodiments, advantages and applications of the invention will also become clear from the further description herein, in which the invention will be described and discussed in more detail with reference to the Nanobodies of the invention and polypeptides of the invention comprising the same, which form some of the preferred aspects of the invention.

As will become clear from the further description herein, Nanobodies generally offer certain advantages (outlined herein) compared to "dAb's" or similar (single) domain antibodies or immunoglobulin sequences, which advantages are also provided by the Nanobodies of the invention. However, it will be clear to the skilled person that the more general aspects of the teaching below can also be applied (either directly or analogously) to other amino acid sequences of the invention.

Figures:
Figure 1: Schematic representation of the selection procedure. Abbreviations: CHO-CXCR4: membrane of CHO (Chinese Hamster Ovary) cells transiently transfected with human CXCR4; COS7-CXCR4: membrane of COS7 (Monkey cells) cells transiently transfected with human CXCR4; R1 is first round of selection; R2 is the second round of selection; Counterselection means that the selection was done in the presence of CHO-membrane (non expressing CXCR4); ligand is CXCL12/SDF1 (3ug in 100ul PBS), antagonist is AMD3100 (50uM), antibody is 12G5 (5ug in 100ul PBS).
Figure 2: Results from Phage ELISA (as described in example 1.5), showing that 238Cl, 238D2, 238F3 have high specificity toward CXCR4 expressing membrane compared to non-expressing membrane (-).

Figure 3: Primary and secondary screen of Nanobody clones binding to CXCR4. \[^{[25]}I\]-CXCL12 competition binding experiments were directly performed with periplasma fractions (1:10) on cell membranes from HEK293T cells transiently expressing CXCR4. All primary hits (A) were confirmed in a second screen (B). Control experiments with AMD3100 (3 \(\mu\)M) or vehicle (-) were performed to show full and no displacement, respectively.

Figure 4: Competition binding of monovalent Nanobodies and reference ligands to CXCR4. A-C) Competition binding experiments with \[^{[125]}I\]-CXCL12, \[^{[25]}I\]-238D2 or \[^{[25]}I\]-238D4 as radioligand were performed on cell membranes from HEK293T cells transiently expressing CXCR4. Control displacement experiments with AMD3100 (3 \(\mu\)M; AMD), CXCL12 (30 nM; XL12) or vehicle (-) were performed. Data are shown as means ± S.E.M (n = 2 – 6). D) Total binding (vehicle; -) and competition binding experiments (3 \(\mu\)M AMD3100; AMD) with \[^{[125]}I\]-238D2 or \[^{[12]}TJ-238D4 as radioligand were performed on cell membranes from HEK293T cells transiently expressing CXCR4 or CXCR3. Data are shown as means ± S.E.M (n = 3).

Figure 5: The monovalent antibodies 238D2 and 238D4 are potent CXCR4 antagonists. A) Inositol phosphate (IP) accumulation experiments were performed in HEK293T cells transiently expressing CXCR4 and G\(\alpha_Q\). Agonism experiments (left graph) show no intrinsic activity for 238D2 and 238D4. Antagonism experiments (right graph) were performed in the presence of CXCL12 (30 nM) following 1 h pre-incubation with 238D2 or 238D4. Control experiments with vehicle (-) or AMD3100 (3 \(\mu\)M; AMD) were performed. Data are shown as means ± S.E.M (n = 4). B) Reporter gene experiments were performed in HEK293T cells transiently transfected with pCRE/\(\beta\)-galactosidase and a plasmid encoding CXCR4. No agonist activity for 238D2 and 238D4 was observed (left graph). Data are shown as means ± S.E.M (n = 3). Experiments showing competitive antagonism of the CXCL12-induced reporter gene activation were performed by establishing concentration response curves for CXCL12 in the presence of increasing concentrations of 238D2 or 238D4 (right graphs). Schild regression analysis graphs are embedded. Data are shown as means ± S.E.M (n = 4 - 6). C) Chemotaxis experiments using ChemoTx \[^{iM}\] plates were performed with Jurkat cells endogenously expressing CXCR4. Agonism experiments (left graph) show migration of Jurkat cells from the upper compartment towards CXCL12 but not towards 238D2 and
238D4 in the lower compartment of the chemotaxis plate. Experiments showing the 
inhibition of migration towards CXCL12 (0.3 nM) were performed in the presence of 238D2 
or 238D4 in both compartments. Control experiments with AMD3100 (3 µM; AMD) were 
performed. Data are shown as means ± S.E.M (n = 4).

Figure 6: Monoclonals were sequenced and grouped based on their similarity to form 
families (more than 2 sequences). The clones on the same lane are 100% identical. U=unique 
sequences non related to each others.

Figure 7: The monovalent Nanobodies 238D2 and 238D4 do not act as agonists or 
antagonists on chemokine CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR6, β2-adrenergic 
and histamine H₁ receptors. The selectivity screen was performed with two concentrations of 
238D2 and 238D4 in the presence of a EC₅₀ - EC₅₀ of an agonist and in the absence (for the 
investigation of β₂ adrenoceptors) or the presence of forskolin (3 µM; for the investigation of 
all other receptors) on HEK293T cells transiently transfected with cDNA encoding the 
mentioned receptors (or mock for the investigation of endogenously expressed β₂ 
adrenoceptors) using the CRE/β-galactosidase reporter gene assay. Data are shown as means 
± S.E.M (n = 2 - 3).

Figure 8: Bivalent Nanobodies show increased affinity and inhibitory potency compared to 
their monovalent counterparts. A) Competition binding experiments with [¹²⁵I]-CXCL12 were 
performed on cell membranes from HEK293T cells transiently expressing CXCR4. Control 
displacement experiments with AMD3100 (3 µM; AMD), CXCL12 (30 nM; XL12) or 
vehicle (-) were performed. Data are shown as means ± S.E.M (n = 2 - 6). B) Chemotaxis 
experiments using ChemoTx™ plates were performed with Jurkat cells endogenously 
expressing CXCR4. Experiments showing the inhibition of migration towards CXCL1 2 (0.3 
nM) in the lower compartment were performed in the presence of Nanobodies in both 
compartments. Control experiments with AMD3100 (3 µM; AMD) were performed. Data are 
shown as means ± S.E.M (n = 3 - 4).

Figure 9: CD4 binding assay for a selection of clones. Negative controls are addition of 
irrelevant phage and no phage addition.

Figure 10: CD4-gpl20 blocking assay for selected Nanobodies. Monoclonal mAb A1 was 
used as positive control. Negative control was no addition of Nanobody.

Figure 11: FACS experiment showing the binding of Nanobodies to Jurkat cells expressing 
CD4. Negative controls- TOPRO, Unstained, anti mouse-PE, anti myc + anti mouse-PE. 
Positive control- Monoclonal anti CD4 antibody mAb A1.
**Detailed description of the invention:**

In some embodiments, targets of the invention are human cellular receptors for viruses and/or bacteria including hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-L, hClaudin-6 and hClaudin-9.

Viruses and bacteria target a variety of human cellular receptors. These cellular receptors include receptors that are part of the immunoglobulin superfamily, such as CD4, which is a co-receptor to the T-cell receptor, and can interact with MHC class II molecules on the surface of the antigen presenting cell. CD4 is thought to be the primary receptor used by HIV-I to gain entry into host T cells. Other receptors that are involved in entry of HIV-I are the chemokine receptors CCR5 and CXCR4.

Targets of the invention also include Toll-like receptors, such as hTLR4. Toll like receptors are a class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from viruses and bacteria.

Targets of the invention also include integrins such as human alphaV integrin, human beta3 integrin, human beta1 integrin and human alpha2 integrin. Integrin receptors are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells. Integrins can act as cellular targets for viruses, such as HIV-I. Targets of the invention also include members of the tetraspan family such as hCD81. The receptors mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility and are known to complex with integrins.

Targets of the invention also include scavenger receptors such as hSR-BI. hSR-BI facilitates high density lipoprotein cholesterol transport and correlates with protection against atherosclerosis.

Targets of the invention also include claudins, such as hClaudin-1, hClaudin-6 and hClaudin-9. Claudins are integral membrane proteins that are components of the tight junction strands and that may play a role in organ development.

Targets of the invention also include G protein coupled receptors (GPCRs) such as CXCR4 and CCR5. GPCRs are also known as seven transmembrane receptors. 7TM
receptors, heptahelical receptors, and G protein linked receptors (GPLR), which are a protein family of transmembrane receptors that transduce an extracellular signal (ligand binding) into an intracellular signal (G protein activation). GPCRs are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices. The extracellular parts of the receptor can be glycosylated. These extracellular loops also contain two highly conserved cysteine residues which build disulfide bonds to stabilize the receptor structure. An overview of GPCRs can be found in standard handbooks, such as the G Protein Coupled Receptors Handbook, L. Devi (Ed.), Humana Press, 2005; as well as from the standard databases, such as GPCR DB (see for example http://www.gPCR.org/7tm/htmls/entries.html).

In the present description, examples and aspects:


b) Unless indicated otherwise, the term "immunoglobulin sequence" - whether used herein to refer to a heavy chain antibody or to a conventional 4-chain antibody - is used as a general term 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 V_{HH} domains or V_{H}/V_{L} domains, respectively). In addition, the term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "variable domain sequence", "V_{HH} sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acids or nucleotide sequences encoding the same, unless the context requires a more limited interpretation. Also, the term "nucleotide sequence" as used herein also encompasses a nucleic acid molecule with
said nucleotide sequence, so that the terms "nucleotide sequence" and "nucleic acid" should be considered equivalent and are used interchangeably herein;

c) 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 and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews Presta, Adv. Drug Deliv. Rev. 2006, 58 (5-6): 640-56; Levin and Weiss, Mol. Biosyst. 2006, 2(1): 49-57; Irving et al., J. Immunol. Methods. 2001, 248(1-2), 31-45; Schmitz et al., Placenta. 2000, 21 Suppl. A, S106-12. Gonzales et al., Tumour Biol., 2005, 26(1), 31-43. which describe techniques for protein engineering, such as affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

d) Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as mentioned in Table A-2;
### Table A-2: One-letter and three-letter amino acid code

<table>
<thead>
<tr>
<th>Category</th>
<th>One-letter</th>
<th>Three-letter</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpolar, uncharged (at pH 6.0 - 7.0)</td>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>Val</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>Polar, uncharged (at pH 6.0-7.0)</td>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Polar, charged (at pH 6.0-7.0)</td>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>Aspartate</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>Glu</td>
<td>E</td>
</tr>
</tbody>
</table>

**Notes:**

1. Sometimes also considered to be a polar uncharged amino acid.
2. Sometimes also considered to be a nonpolar uncharged amino acid.
3. As will be clear to the skilled person, the fact that an amino acid residue is referred to in this Table as being either charged or uncharged at pH 6.0 to 7.0 does not reflect in any way on the charge said amino acid residue may have at a pH lower than 6.0 and/or at a pH higher than 7.0; the amino acid residues mentioned in the Table can be either charged and/or uncharged at such a higher or lower pH, as will be clear to the skilled person.
4. As is known in the art, the charge of a His residue is greatly dependant upon even small shifts in pH, but a His residue can generally be considered essentially uncharged at a pH of about 6.5.
e) For the purposes of comparing two or more nucleotide sequences, the percentage of "sequence identity" between a first nucleotide sequence and a second nucleotide sequence may be calculated by dividing \( \frac{\text{the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence}}{\text{the total number of nucleotides in the first nucleotide sequence}} \) and multiplying by \([100\%]\), in which each deletion, insertion, substitution or addition of a nucleotide in the second nucleotide sequence - compared to the first nucleotide sequence - is considered as a difference at a single nucleotide (position).

Alternatively, the degree of sequence identity between two or more nucleotide sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0, using standard settings.

Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0 967 284, EP 1 085 089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2 357 768-A.

Usually, for the purpose of determining the percentage of "sequence identity" between two nucleotide sequences in accordance with the calculation method outlined hereinabove, the nucleotide sequence with the greatest number of nucleotides will be taken as the "first" nucleotide sequence, and the other nucleotide sequence will be taken as the "second" nucleotide sequence;

f) For the purposes of comparing two or more amino acid sequences, the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid identity") may be calculated by dividing \( \frac{\text{the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence}}{\text{the total number of amino acid residues in the first amino acid sequence}} \) and multiplying by \([100\%]\), in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), i.e. as an "amino acid difference" as defined herein.

Alternatively, the degree of sequence identity between two amino acid sequences may be calculated using a known computer algorithm, such as those mentioned above for
determining the degree of sequence identity for nucleotide sequences, again using
standard settings.

Usually, for the purpose of determining the percentage of "sequence identity" between
two amino acid sequences in accordance with the calculation method outlined
hereinabove, the amino acid sequence with the greatest number of amino acid residues
will be taken as the "first" amino acid sequence, and the other amino acid sequence
will be taken as the "second" amino acid sequence.

Also, in determining the degree of sequence identity between two amino acid
sequences, the skilled person may take into account so-called "conservative" amino
acid substitutions, which can generally be described as amino acid substitutions in
which 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 04/037999, GB-A-3 357
768, WO 98/49185, WO 00/46383 and WO 01/09300; and (preferred) types and/or
combinations of such substitutions may be selected on the basis of the pertinent
teachings from WO 04/037999 as well as WO 98/49185 and from the further references
cited therein.

Such conservative substitutions preferably are substitutions in which one amino acid
within the following groups (a) – (e) is substituted by another amino acid residue within
the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr,
Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp,
Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large
aliphatic, nonpolar residues: Met, Leu, He, Val and Cys; and (e) aromatic residues: Phe,
Tyr and Tip.

Particularly preferred conservative 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; He into Leu or into
Val; Leu into He or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr
or into He; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr;
Tyr into Trp; and/or Phe into Val, into De or into Leu.

Any amino acid substitutions applied to the polypeptides described herein may also be
based on the analysis of the frequencies of amino acid variations between homologous

g) Amino acid sequences and nucleic acid sequences are said to be “exactly the same” if they have 100% sequence identity (as defined herein) over their entire length;
h) When comparing two amino acid sequences, the term “amino acid difference” refers to an insertion, deletion or substitution of a single amino acid residue on a position of the first sequence, compared to the second sequence: it being understood that two amino acid sequences can contain one, two or more such amino acid differences;
i) When a nucleotide sequence or amino acid sequence is said to "comprise" another nucleotide sequence or amino acid sequence, respectively, or to "essentially consist of another nucleotide sequence or amino acid sequence, this may mean that the latter nucleotide sequence or amino acid sequence has been incorporated into the firstmentioned nucleotide sequence or amino acid sequence, respectively, but more usually this generally means that the firstmentioned nucleotide sequence or amino acid sequence comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the firstmentioned sequence has actually been generated or obtained (which may for example be by any suitable method described herein). By means of a non-limiting example, when a Nanobody of the invention is said to comprise a CDR sequence, this may mean that said CDR sequence
has been incorporated into the Nanobody of the invention, but more usually this
generally means that the Nanobody of the invention contains within its sequence a
stretch of amino acid residues with the same amino acid sequence as said CDR
sequence, irrespective of how said Nanobody of the invention has been generated or
obtained. It should also be noted that when the latter amino acid sequence has a specific
biological or structural function, it preferably has essentially the same, a similar or an
equivalent biological or structural function in the firstmentioned amino acid sequence
(in other words, the firstmentioned amino acid sequence is preferably such that the
latter sequence is capable of performing essentially the same, a similar or an equivalent
biological or structural function). For example, when a Nanobody of the invention is
said to comprise a CDR sequence or framework sequence, respectively, the CDR
sequence and framework are preferably capable, in said Nanobody, of functioning as a
CDR sequence or framework sequence, respectively. Also, when a nucleotide sequence
is said to comprise another nucleotide sequence, the firstmentioned nucleotide sequence
is preferably such that, when it is expressed into an expression product (e.g. a
polypeptide), the amino acid sequence encoded by the latter nucleotide sequence forms
part of said expression product (in other words, that the latter nucleotide sequence is in
the same reading frame as the firstmentioned, larger nucleotide sequence),
j) A nucleic acid sequence or amino acid sequence is considered to be "(in) essentially
isolated (form)" - for example, 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 sequence or amino acid sequence 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 sequence or amino acid sequence 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-gel electrophoresis;
k) The term "domain" as used herein generally refers to a globular region of an amino acid
sequence (such as an antibody chain, and in particular to a globular region of a heavy
chain antibody), or to a polypeptide that essentially consists of such a globular region.
Usually, such a domain will comprise peptide loops (for example 3 or 4 peptide loops) stabilized, for example, as a sheet or by disulfide bonds. The term "binding domain" refers to such a domain that is directed against an antigenic determinant (as defined herein);

The term "antigenic determinant" refers to the epitope on the antigen recognized by the antigen-binding molecule (such as a Nanobody or a polypeptide of the invention) and more in particular by the antigen-binding site of said molecule. The terms "antigenic determinant" and "epitope" may also be used interchangeably herein,

An amino acid sequence (such as a Nanobody, an antibody, a polypeptide of the invention, or generally an antigen binding protein or polypeptide or a fragment thereof) that can (specifically) bind to, that has affinity for and/or that has specificity for a specific antigenic determinant, epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "against" or "directed against" said antigenic determinant, epitope, antigen or protein.

The term "specificity" refers to the number of different types of antigens or antigenic determinants to which a particular antigen-binding molecule or antigen-binding protein (such as a Nanobody or a polypeptide of the invention) molecule can bind. The specificity of an antigen-binding protein can be determined based on affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (K_D), is a measure for the binding strength between an antigenic determinant and an antigen-binding site on the antigen-binding protein: the lesser the value of the K_D, the stronger the binding strength between an antigenic determinant and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (K_A), which is 1/K_D). 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 a Nanobody or polypeptide of the invention) and the pertinent antigen. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule. Typically, antigen-binding proteins (such as the amino acid sequences, Nanobodies and/or polypeptides of the invention) will bind to their antigen with a dissociation constant (K_D) of 10^-5 to 10^-12 moles/liter or less, and
preferably 10^7 to 10^{-12} moles/liter or less and more preferably 10^7 to 10^{-12} moles/liter (i.e. with an association constant \( K_A \) of 10^5 to 10^{12} liter/moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles). Any \( K_D \) value greater than 10^4 mol/liter (or any \( K_A \) value lower than 10^4 M^{-1}) liters/mol is generally considered to indicate non-specific binding. Preferably, a monovalent immunoglobulin sequence of the invention will bind to the desired antigen with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Specific binding of an antigen-binding protein to an antigen or antigenic determinant can be determined in any suitable manner known per se. including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein.

The dissociation constant may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned herein. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/liter or 10^{-3} moles/liter (e.g., of 10^{-2} moles/liter).

Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant \( K_A \), by means of the relationship \[ K_D = \frac{1}{K_A}. \]

The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the \( K_D \), or dissociation constant, which has units of mol/liter (or M). The affinity can also be expressed as an association constant \( K_A \), which equals 1/\( K_D \) and has units of (mol/liter)^{-1} (or M^{-1}). In the present specification, the stability of the interaction between two molecules (such as an amino acid sequence, Nanobody or polypeptide of the invention and its intended target) will mainly be expressed in terms of the \( K_D \) value of their interaction; it being clear to the skilled person that in view of the relation \( K_A = 1/K_D \), specifying the strength of molecular interaction by its \( K_D \) value can also be used to calculate the corresponding \( K_A \) value. The Kο-value characterizes the strength of a molecular interaction also in a thermodynamic sense as it is related to the free energy (DG) of binding by the well known relation \( DG = RT \ln(K_D) \)
(equivalently \( \Delta G = -RT \ln(K_A) \)), where \( R \) equals the gas constant, \( T \) equals the absolute temperature and \( \ln \) denotes the natural logarithm.

The \( K_D \) for biological interactions which are considered meaningful (e.g. specific) are typically in the range of \( 10^{-10} \text{M} \) (0.1 nM) to \( 10^{-7} \text{M} \) (10000 nM). The stronger an interaction is, the lower is its \( K_D \).

The \( K_D \) can also be expressed as the ratio of the dissociation rate constant of a complex, denoted as \( k_{\text{off}} \), to the rate of its association, denoted \( k_{\text{on}} \) (so that \( K_D = k_{\text{off}}/k_{\text{on}} \) and \( K_A = k_{\text{on}}/k_{\text{off}} \)). The off-rate \( k_{\text{on}} \) has units \( \text{s}^{-1} \) (where \( s \) is the SI unit notation of second). The on-rate \( k_{\text{on}} \) has units \( \text{M}^{-1}\text{S}^{-1} \). The on-rate may vary between \( 10^2 \text{M}^{-1}\text{S}^{-1} \) to about \( 10^7 \text{M}^{-1}\text{V} \), approaching the diffusion-limited association rate constant for bimolecular interactions. The off-rate is related to the half-life of a given molecular interaction by the relation \( t_{1/2} = \ln(2)/k_{\text{off}} \). The off-rate may vary between \( 10^{-6} \text{S}^{-1} \) (near irreversible complex with a \( X \) of multiple days) to \( 1 \text{S}^{-1} \) \( (t_{1/2} \approx 0.69 \text{s}) \).

The affinity of a molecular interaction between two molecules can be measured via different techniques known per se, such as the well known surface plasmon resonance (SPR) biosensor technique (see for example Ober et al., Intern. Immunology, 13. 1551-1559. 2001) where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding \( k_{\text{on}} \) measurements and hence \( K_D \) (or \( K_A \)) values. This can for example be performed using the well-known BIACORE instruments.

It will also be clear to the skilled person that the measured \( K_D \) may correspond to the apparent \( K_D \) if the measuring process somehow influences the intrinsic binding affinity of the implied molecules for example by artefacts related to the coating on the biosensor of one molecule. Also, an apparent \( K_D \) may be measured if one molecule contains more than one recognition sites for the other molecule. In such situation the measured affinity may be affected by the avidity of the interaction by the two molecules.

Another approach that may be used to assess affinity is the 2-step ELISA (Enzyme-Linked Immunosorbent Assay) procedure of Friguet et al. (J. Immunol. Methods, 77, 305-19, 1985). This method establishes a solution phase binding equilibrium measurement and avoids possible artefacts relating to adsorption of one of the molecules on a support such as plastic.
However, the accurate measurement of $K_D$ may be quite labor-intensive and as consequence, often apparent $K_D$ values are determined to assess the binding strength of two molecules. It should be noted that as long all measurements are made in a consistent way (e.g. keeping the assay conditions unchanged) apparent $K_D$ measurements can be used as an approximation of the true $K_D$ and hence in the present document $K_D$ and apparent $K_D$ should be treated with equal importance or relevance.

Finally, it should be noted that in many situations the experienced scientist may judge it to be convenient to determine the binding affinity relative to some reference molecule. For example, to assess the binding strength between molecules A and B, one may e.g. use a reference molecule C that is known to bind B and that is suitably labelled with a fluorophore or chromophore group or other chemical moiety, such as biotin for easy detection in an ELISA or FACS (Fluorescent activated cell sorting) or other format (the fluorophore for fluorescence detection, the chromophore for light absorption detection, the biotin for streptavidin-mediated ELISA detection). Typically, the reference molecule C is kept at a fixed concentration and the concentration of A is varied for a given concentration or amount of B. As a result an IC$_{50}$ value is obtained corresponding to the concentration of A at which the signal measured for C in absence of A is halved. Provided $K_D^{ref}$, the $K_D$ of the reference molecule, is known, as well as the total concentration $c^{ref}$ of the reference molecule, the apparent $K_D$ for the interaction A-B can be obtained from following formula: $K_D = IC_{50}/(c^{ref}K_D^{ref})$. Note that if $c^{ref} \approx K_D$, $K_D \sim IC_{50}$ Provided the measurement of the IC$_{50}$ is performed in a consistent way (e.g. keeping $c^{ref}$ fixed) for the binders that are compared, the strength or stability of a molecular interaction can be assessed by the IC$_{50}$ and this measurement is judged as equivalent to $K_D$ or to apparent $K_D$ throughout this text.

The half-life of an amino acid sequence, compound or polypeptide of the invention can generally be defined as the time taken for the serum concentration of the amino acid sequence, compound or polypeptide to be reduced by 50%, in vivo, for example due to degradation of the sequence or compound and/or clearance or sequestration of the sequence or compound by natural mechanisms. The in vivo half-life of an amino acid sequence, compound or polypeptide of the invention can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may for example generally involve the steps of suitably administering to a warm-blooded animal (i.e. to a human or to another suitable
mammal, such as a mouse, rabbit, rat, pig, dog or a primate, for example monkeys from the genus *Macaca* (such as, and in particular, cynomolgus monkeys (*Macaca fascicularis*) and/or rhesus monkeys (*Macaca mulatto*)) and baboon (*Papio ursinus*) a suitable dose of the amino acid sequence, compound or polypeptide of the invention; collecting blood samples or other samples from said animal; determining the level or concentration of the amino acid sequence, compound or polypeptide of the invention in said blood sample; and calculating, from (a plot of) the data thus obtained, the time until the level or concentration of the amino acid sequence, compound or polypeptide of the invention has been reduced by 50% compared to the initial level upon dosing.


As will also be clear to the skilled person (see for example pages 6 and 7 of WO 04/003019 and in the further references cited therein), the half-life can be expressed using parameters such as the t1/2-alpha, t1/2-beta and the area under the curve (AUC).

In the present specification, an "increase in half-life" refers to an increase in any one of these parameters, such as any two of these parameters, or essentially all three these parameters. As used herein "increase in half-life" or "increased half-life" in particular refers to an increase in the t1/2-beta, either with or without an increase in the t1/2-alpha and/or the AUC or both.

p) In the context of the present invention, "modulating" or "to modulate" generally means either reducing or inhibiting the activity of, or alternatively increasing the activity of, a target or antigen, as measured using a suitable in vitro, cellular or in vivo assay. In particular, "modulating" or "to modulate" may mean either reducing or inhibiting the activity of, or alternatively increasing a (relevant or intended) biological activity of, a target or antigen, as measured using a suitable in vitro, cellular or in vivo assay (which will usually depend on the target or antigen involved), by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to activity of the target or antigen in the same assay under the same conditions but without the presence of the construct of the invention.
As will be clear to the skilled person, "modulating" may also involve effecting a change (which may either be an increase or a decrease) in affinity, avidity, specificity and/or selectivity of a target or antigen for one or more of its ligands, binding partners, and/or effecting a change (which may either be an increase or a decrease) in the sensitivity of the target or antigen for one or more conditions in the medium or surroundings in which the target or antigen is present (such as pH, ion strength, the presence of co-factors, etc.), compared to the same conditions but without the presence of the construct of the invention. As will be clear to the skilled person, this may again be determined in any suitable manner and/or using any suitable assay known per se, depending on the target or antigen involved.

"Modulating" may also mean effecting a change (i.e. an activity as an agonist, as an antagonist or as a reverse agonist, respectively, depending on the target or antigen and the desired biological or physiological effect) with respect to one or more biological or physiological mechanisms, effects, responses, functions, pathways or activities in which the target or antigen (or in which its substrate(s), ligand(s) or pathway(s) are involved, such as its signalling pathway or metabolic pathway and their associated biological or physiological effects) is involved. Again, as will be clear to the skilled person, such an action as an agonist or an antagonist may be determined in any suitable manner and/or using any suitable (in vitro and usually cellular or in assay) assay known per se. depending on the target or antigen involved. In particular, an action as an agonist or antagonist may be such that an intended biological or physiological activity is increased or decreased, respectively, by at least 1%, preferably at least 5%, such as at least 10% or at least 25%, for example by at least 50%. at least 60%, at least 70%, at least 80%, or 90% or more, compared to the biological or physiological activity in the same assay under the same conditions but without the presence of the construct of the invention.

Modulating may for example also involve allosteric modulation of the target or antigen; and/or reducing or inhibiting the binding of the target or antigen to one of its substrates or ligands and/or competing with a natural ligand, substrate for binding to the target or antigen. Modulating may also involve activating the target or antigen or the mechanism or pathway in which it is involved. Modulating may for example also involve effecting a change in respect of the folding or confirmation of the target or antigen, or in respect
of the ability of the target or antigen to fold, to change its confirmation (for example, upon binding of a ligand), to associate with other (sub)units, or to disassociate.

Modulating may for example also involve effecting a change in the ability of the target or antigen to transport other compounds or to serve as a channel for other compounds (such as ions).

Modulating may be reversible or irreversible, but for pharmaceutical and pharmacological purposes will usually be in a reversible manner.

q) In respect of a target or antigen, the term "interaction site" on the target or antigen means a site, epitope, antigenic determinant, part, domain or stretch of amino acid residues on the target or antigen that is a site for binding to a ligand, receptor or other binding partner, a catalytic site, a cleavage site, a site for allosteric interaction, a site involved in multimerisation (such as homomerization or heterodimerization) of the target or antigen; or any other site, epitope, antigenic determinant, part, domain or stretch of amino acid residues on the target or antigen that is involved in a biological action or mechanism of the target or antigen. More generally, an "interaction site" can be any site, epitope, antigenic determinant, part, domain or stretch of amino acid residues on the target or antigen to which an amino acid sequence or polypeptide of the invention can bind such that the target or antigen (and/or any pathway, interaction, signalling, biological mechanism or biological effect in which the target or antigen is involved) is modulated (as defined herein).

r) An amino acid sequence or polypeptide is said to be "specific for" a first target or antigen compared to a second target or antigen when it binds to the first antigen with an affinity (as described above, and suitably expressed as a $K_D$ value, $K_A$ value, $K_{on}$ rate and/or $K_{off}$ rate) that is at least 10 times, such as at least 100 times, and preferably at least 1000 times, and up to 10,000 times or more better than the affinity with which said amino acid sequence or polypeptide binds to the second target or polypeptide. For example, the first antigen may bind to the target or antigen with a $K_D$ value that is at least 10 times less, such as at least 100 times less, and preferably at least 1000 times less, such as 10,000 times less or even less than that, than the $K_D$ with which said amino acid sequence or polypeptide binds to the second target or polypeptide.

Preferably, when an amino acid sequence or polypeptide is "specific for" a first target or antigen compared to a second target or antigen, it is directed against (as defined herein) said first target or antigen, but not directed against said second target or antigen.
s) The terms "'cross-block", "cross-blocked" and "cross-blocking' are used interchangeably herein to mean the ability of an amino acid sequence or other binding agents (such as a Nanobody, polypeptide or compound or construct of the invention) to interfere with the binding of other amino acid sequences or binding agents of the invention to a given target. The extend to which an amino acid sequence or other binding agents of the invention is able to interfere with the binding of another to [target], and therefore whether it can be said to cross-block according to the invention, can be determined using competition binding assays. One particularly suitable quantitative cross-blocking assay uses a Biacore machine which can measure the extent of interactions using surface plasmon resonance technology. Another suitable quantitative cross-blocking assay uses an ELISA-based approach to measure competition between amino acid sequences or other binding agents in terms of their binding to the target.

The following generally describes a suitable Biacore assay for determining whether an amino acid sequence or other binding agent cross-blocks or is capable of cross-blocking according to the invention. It will be appreciated that the assay can be used with any of the amino acid sequences or other binding agents described herein. The Biacore machine (for example the Biacore 3000) is operated in line with the manufacturer's recommendations. Thus in one cross-blocking assay, the target protein is coupled to a CM5 Biacore chip using standard amine coupling chemistry to generate a surface that is coated with the target. Typically 200- 800 resonance units of the target would be coupled to the chip (an amount that gives easily measurable levels of binding but that is readily saturable by the concentrations of test reagent being used). Two test amino acid sequences (termed A* and B*) to be assessed for their ability to cross-block each other are mixed at a one to one molar ratio of binding sites in a suitable buffer to create the test mixture. When calculating the concentrations on a binding site basis the molecular weight of an amino acid sequence is assumed to be the total molecular weight of the amino acid sequence divided by the number of target binding sites on that amino acid sequence. The concentration of each amino acid sequence in the test mix should be high enough to readily saturate the binding sites for that amino acid sequence on the target molecules captured on the Biacore chip. The amino acid sequences in the mixture are at the same molar concentration (on a binding basis) and that concentration would typically be between 1.00 and 1.5 micromolar (on a binding site basis). Separate
solutions containing A* alone and B* alone are also prepared. A* and B* in these solutions should be in the same buffer and at the same concentration as in the test mix. The test mixture is passed over the target-coated Biacore chip and the total amount of binding recorded. The chip is then treated in such a way as to remove the bound amino acid sequences without damaging the chip-bound target. Typically this is done by treating the chip with 30 mM HCl for 60 seconds. The solution of A* alone is then passed over the target-coated surface and the amount of binding recorded. The chip is again treated to remove all of the bound amino acid sequences without damaging the chip-bound target. The solution of B* alone is then passed over the target-coated surface and the amount of binding recorded. The maximum theoretical binding of the mixture of A* and B* is next calculated, and is the sum of the binding of each amino acid sequence when passed over the target surface alone. If the actual recorded binding of the mixture is less than this theoretical maximum then the two amino acid sequences are cross-blocking each other. Thus, in general, a cross-blocking amino acid sequence or other binding agent according to the invention is one which will bind to the target in the above Biacore cross-blocking assay such that, during the assay and in the presence of a second amino acid sequence or other binding agent of the invention, the recorded binding is between 80% and 0.1% (e.g. 80% to 4%) of the maximum theoretical binding, specifically between 75% and 0.1% (e.g. 75% to 4%) of the maximum theoretical binding, and more specifically between 70% and 0.1% (e.g. 70% to 4%) of maximum theoretical binding (as just defined above) of the two amino acid sequences or binding agents in combination. The Biacore assay described above is a primary assay used to determine if amino acid sequences or other binding agents cross-block each other according to the invention. On rare occasions particular amino acid sequences or other binding agents may not bind to target coupled via amine chemistry to a CM5 Biacore chip (this usually occurs when the relevant binding site on target is masked or destroyed by the coupling to the chip). In such cases cross-blocking can be determined using a tagged version of the target, for example a N-termininal His-tagged version. In this particular format, an anti-His amino acid sequence would be coupled to the Biacore chip and then the His-tagged target would be passed over the surface of the chip and captured by the anti-His amino acid sequence. The cross blocking analysis would be carried out essentially as described above, except that after each chip regeneration cycle, new His-tagged target would be loaded back onto the anti-His amino acid
sequence coated surface. In addition to the example given using N-terminal His-tagged target, C-terminal His-tagged target could alternatively be used. Furthermore, various other tags and tag binding protein combinations that are known in the art could be used for such a cross-blocking analysis (e.g. HA tag with anti-HA antibodies; FLAG tag with anti-FLAG antibodies: biotin tag with streptavidin).

The following generally describes an ELISA assay for determining whether an amino acid sequence or other binding agent directed against a target cross-blocks or is capable of cross-blocking as defined herein. It will be appreciated that the assay can be used with any of the amino acid sequences (or other binding agents such as polypeptides of the invention) described herein. The general principal of the assay is to have an amino acid sequence or binding agent that is directed against the target coated onto the wells of an ELISA plate. An excess amount of a second, potentially cross-blocking, anti-target amino acid sequence is added in solution (i.e. not bound to the ELISA plate). A limited amount of the target is then added to the wells. The coated amino acid sequence and the amino acid sequence in solution compete for binding of the limited number of target molecules. The plate is washed to remove excess target that has not been bound by the coated amino acid sequence and to also remove the second, solution phase amino acid sequence as well as any complexes formed between the second, solution phase amino acid sequence and target. The amount of bound target is then measured using a reagent that is appropriate to detect the target. An amino acid sequence in solution that is able to cross-block the coated amino acid sequence will be able to cause a decrease in the number of target molecules that the coated amino acid sequence can bind relative to the number of target molecules that the coaled amino acid sequence can bind in the absence of the second, solution phase, amino acid sequence. In the instance where the first amino acid sequence, e.g. an Ab-X, is chosen to be the immobilized amino acid sequence, it is coated onto the wells of the ELISA plate, after which the plates are blocked with a suitable blocking solution to minimize non-specific binding of reagents that are subsequently added. An excess amount of the second amino acid sequence, i.e. Ab-Y, is then added to the ELISA plate such that the moles of Ab-Y target binding sites per well are at least 10 fold higher than the moles of Ab-X target binding sites that were used, per well, during the coating of the ELISA plate. Target is then added such that the moles of target added per well are at least 25-fold lower than the moles of Ab-X target binding sites that were used for coating each well. Following a suitable incubation
period the ELISA plate is washed and a reagent for detecting the target is added to
measure the amount of target specifically bound by the coated anti-target amino acid
sequence (in this case Ab-X). The background signal for the assay is defined as the
signal obtained in wells with the coated amino acid sequence (in this case Ab-X),
second solution phase amino acid sequence (in this case Ab-Y), target buffer only (i.e.
without target) and target detection reagents. The positive control signal for the assay is
defined as the signal obtained in wells with the coated amino acid sequence (in this case
Ab-X), second solution phase amino acid sequence buffer only (i.e. without second
solution phase amino acid sequence), target and target detection reagents. The ELISA
assay may be run in such a manner so as to have the positive control signal be at least 6
times the background signal. To avoid any artefacts (e.g. significantly different
affinities between Ab-X and Ab-Y for the target) resulting from the choice of which
amino acid sequence to use as the coating amino acid sequence and which to use as the
second (competitor) amino acid sequence, the cross-blocking assay may be run in
two formats: 1) format 1 is where Ab-X is the amino acid sequence that is coated onto
the ELISA plate and Ab-Y is the competitor amino acid sequence that is in solution and
2) format 2 is where Ab-Y is the amino acid sequence that is coated onto the ELISA
plate and Ab-X is the competitor amino acid sequence that is in solution. Ab-X and Ab-
Y are defined as cross-blocking if, either in format 1 or in format 2, the solution phase
anti-target amino acid sequence is able to cause a reduction of between 60% and 100%,
specifically between 70% and 100%, and more specifically between 80% and 100%. of
the target detection signal (i.e. the amount of target bound by the coated amino acid
sequence) as compared to the target detection signal obtained in the absence of the
solution phase anti-target amino acid sequence (i.e. the positive control wells).

t) An amino acid sequence is said to be "cross-reactive" for two different antigens or
antigenic determinants (such as serum albumin from two different species of mammal,
such as human serum albumin and cyano serum albumin) if it is specific for (as defined
herein) both these different antigens or antigenic determinants,

u) By binding that is "essentially independent of the pH" is generally meant herein that the
association constant ($K_\lambda$) of the amino acid sequence with respect to the serum protein
(such as serum albumin) at the pH value(s) that occur in a cell of an animal or human
body (as further described herein) is at least 5%, such as at least 10%, preferably at
least 25%, more preferably at least 50%, even more preferably at least 60%, such as
even more preferably at least 70%, such as at least 80% or 90% or more (or even more than 100%, such as more than 110%, more than 120% or even 130% or more, or even more than 150%, or even more than 200%) of the association constant (K_\text{a}) of the amino acid sequence with respect to the same serum protein at the pH value(s) that occur outside said cell. Alternatively, by binding that is "essentially independent of the pH" is generally meant herein that the k_{\text{on}} rate (measured by Biacore) of the amino acid sequence with respect to the serum protein (such as serum albumin) at the pH value(s) that occur in a cell of an animal or human body (as e.g. further described herein, e.g. pH around 5.5, e.g. 5.3 to 5.7) is at least 5%, such as at least 10%, preferably at least 25%. more preferably at least 50%, even more preferably at least 60%, such as even more preferably at least 70%, such as at least 80% or 90% or more (or even more than 100%, such as more than 110%. more than 120% or even 130% or more, or even more than 150%, or even more than 200%) of the k_{\text{on}} rate of the amino acid sequence with respect to the same serum protein at the pH value(s) that occur outside said cell, e.g. pH 7.2 to 7.4. By "the pH value(s) that occur in a cell of an animal or human body" is meant the pH value(s) that may occur inside a cell, and in particular inside a cell that is involved in the recycling of the serum protein. In particular, by "the pH value(s) that occur in a cell of an animal or human body" is meant the pH value(s) that may occur inside a (sub)cellular compartment or vesicle that is involved in recycling of the serum protein (e.g. as a result of pinocytosis, endocytosis, transcytosis, exocytosis and phagocytosis or a similar mechanism of uptake or internalization into said cell), such as an endosome, lysosome or pinosome.

v) As further described herein, the total number of amino acid residues in a Nanobody can be in the region of 110-120, is preferably 112-115, and is most preferably 113. It should however be noted that parts, fragments, analogs or derivatives (as further described herein) of a Nanobody are not particularly limited as to their length and/or size, as long as such parts, fragments, analogs or derivatives meet the further requirements outlined herein and are also preferably suitable for the purposes described herein;

w) The amino acid residues of a Nanobody 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 V_{HH} domains from Cameiids in the article of Riechman and Muyldermans, J. Immunol. Methods 2000 Jun 23: 240 (1-2): 185-195; or referred to
herein. According to this numbering, FR1 of a Nanobody comprises the amino acid residues at positions 1-30, CDR1 of a Nanobody comprises the amino acid residues at positions 31-35, FR2 of a Nanobody comprises the amino acids at positions 36-49, CDR2 of a Nanobody comprises the amino acid residues at positions 50-65, FR3 of a Nanobody comprises the amino acid residues at positions 66-94, CDR3 of a Nanobody comprises the amino acid residues at positions 95-102, and FR4 of a Nanobody comprises the amino acid residues at positions 103-13. [In this respect, it should be noted that - as is well known in the art for V_H domains and for V_HH domains - the total number of amino acid residues in each of the CDR's 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. Generally, however, it can be said that, according to the numbering of Kabat and irrespective of the number of amino acid residues in the CDR's, position 1 according to the Kabat numbering corresponds to the start of FR1 and vice versa, position 36 according to the Kabat numbering corresponds to the start of FR2 and vice versa, position 66 according to the Kabat numbering corresponds to the start of FR3 and vice versa, and position 103 according to the Kabat numbering corresponds to the start of FR4 and vice versa.].

Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to V_HH domains from Camelids and to Nanobodies, are the method described by Chothia et al. (Nature 342, 877-883 (1989)), the so-called "AbM definition" and the so-called "contact definition".

However, in the present description, aspects and figures, the numbering according to Kabat as applied to V_HH domains by Riechmann and Muyltermans will be followed, unless indicated otherwise; and

The Figures, Sequence Listing and the Experimental Part/Examples are only given to further illustrate the invention and should not be interpreted or construed as limiting the scope of the invention and/or of the appended aspects in any way, unless explicitly indicated otherwise herein.
For a general description of heavy chain antibodies and the variable domains thereof, reference is inter alia made to the prior art cited herein, to the review article by Muyldermans in Reviews in Molecular Biotechnology 74(2001), 277-302; as well as to the following patent applications, which are mentioned as general background art: WO 94/04678. WO 95/04079 and WO 96/34103 of the Vrije Universiteit Brussel, WO 94/25591, WO 99/37681. WO 00/40968. WO 00/43507, WO 00/65057. WO 01/40310, WO 01/44301, EP 1134231 and WO 02/48193 of Unilever; WO 97/49805, WO 01/21817. WO 03/035694. WO 03/054016 and WO 03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO 03/050531 of Algonomics N.V. and Ablynx N.V.; WO 01/90190 by the National Research Council of Canada; WO 03/025020 (= EP 1 433 793) by the Institute of Antibodies; as well as WO 04/041867, WO 04/041 862, WO 04/041865. WO 04/041863. WO 04/062551, WO 05/044858, WO 06/40153. WO 06/079372, WO 06/122786, WO 06/122787 and WO 06/122825. by Ablynx N.V. and the further published patent applications by Ablynx N.V. Reference is also made to the further prior art mentioned in these applications, and in particular to the list of references mentioned on pages 41-43 of the International application WO 06/040153. which list and references are incorporated herein by reference.

In accordance with the terminology used in the art (see the above references), the variable domains present in naturally occurring heavy chain antibodies will also be referred to as "\(V_{\text{HH}}\) domains", in order to distinguish them from the heavy chain variable domains that are present in conventional 4-chain antibodies (which will be referred to hereinbelow as "\(Y_{\text{H}}\) domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which will be referred to hereinbelow as "\(F_{\text{z}}\) domains").

As mentioned in the prior art referred to above, \(V_{\text{HH}}\) domains have a number of unique structural characteristics and functional properties which make isolated \(V_{\text{HH}}\) domains (as well as Nanobodies based thereon, which share these structural characteristics and functional properties with the naturally occurring \(V_{\text{HH}}\) domains) and proteins containing the same highly advantageous for use as functional antigen-binding domains or proteins. In particular, and without being limited thereto, \(V_{\text{HH}}\) 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) and Nanobodies can function as a single, relatively small, functional antigen-binding structural unit, domain or protein. This distinguishes the \(V_{\text{H}}\pi\) domains from the \(V_{\text{H}}\) and \(V_{\text{L}}\) domains of conventional 4-chain antibodies, which by themselves are generally not suited for practical application as single antigen-binding
proteins or 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 fragments, which consist of a \( V_H \) domain covalently linked to a \( V_L \) domain).

5 Because of these unique properties, the use of \( V_{HH} \) domains and Nanobodies as single antigen-binding proteins or as antigen-binding domains (i.e. as part of a larger protein or polypeptide) offers a number of significant advantages over the use of conventional \( V_H \) and \( V_L \) domains, scFv's or conventional antibody fragments (such as Fab- or F(ab')-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 spatial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv’s);

- \( V_{HH} \) domains and Nanobodies can be expressed from a single gene and require no post-translational folding or modifications;

10 - \( V_{HH} \) domains and Nanobodies can easily be engineered into multivalent and multispecific formats (as further discussed herein);

- \( V_{HH} \) domains and Nanobodies are highly soluble and do not have a tendency to aggregate (as with the mouse-derived "dAb's" described by Ward et al., Nature, Vol. 341. 1989, p. 544);

15 - \( V_{HH} \) domains and Nanobodies are highly stable to heat, pH, proteases and other denaturing agents or conditions (see for example Ewert et al, supra);

- \( V_{HH} \) domains and Nanobodies are easy and relatively cheap to prepare, even on a scale required for production. For example, \( V_{HH} \) domains, Nanobodies and proteins/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;

20 - \( V_{HH} \) domains and Nanobodies 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 (including but not limited to solid tumors and other dense tissues) than such conventional 4-chain antibodies and antigen-binding fragments thereof;

25 - \( V_{HH} \) domains and Nanobodies can show so-called cavity-binding properties (inter alia due to their extended CDR3 loop, compared to conventional \( V_H \) domains) and can
therefore also access targets and epitopes not accessible to conventional 4-chain antibodies and antigen-binding fragments thereof. For example, it has been shown that $V_{\text{HH}}$ domains and Nanobodies can inhibit enzymes (see for example WO 97/49805; Transue et al., Proteins 1998 Sep 1; 32(4): 515-22; Lauwereys et al., EMBO J. 1998 Jul 1; 17(13): 3512-20).

In a specific and preferred aspect, the invention provides Nanobodies against Targets of the invention, and in particular Nanobodies against Targets of the invention from a warm-blooded animal, and more in particular Nanobodies against Targets of the invention from a mammal, and especially Nanobodies against human Targets of the invention; as well as proteins and/or polypeptides comprising at least one such Nanobody.

In particular, the invention provides Nanobodies against Targets of the invention, and proteins and/or polypeptides comprising the same, that have improved therapeutic and/or pharmacological properties and/or other advantageous properties (such as, for example, improved ease of preparation and/or reduced costs of goods), compared to conventional antibodies against Targets of the invention or fragments thereof, compared to constructs that could be based on such conventional antibodies or antibody fragments (such as Fab' fragments, F(ab')$_2$ fragments, ScFv constructs, "diabodies" and other multispecific constructs (see for example the review by Holliger and Hudson, Nat Biotechnol. 2005 Sep;23(9):1 126-36)). and also compared to the so-called "dAb's" or similar (single) domain antibodies that may be derived from variable domains of conventional antibodies. These improved and advantageous properties will become clear from the further description herein, and for example include, without limitation, one or more of:

- increased affinity and/or avidity for Targets of the invention, either in a monovalent format, in a multivalent format (for example in a bivalent format) and/or in a multispecific format (for example one of the multispecific formats described hereinbelow);
- better suitability for formatting in a multivalent format (for example in a bivalent format);
- better suitability for formatting in a multispecific format (for example one of the multispecific formats described hereinbelow);
- improved suitability or susceptibility for "humanizing" substitutions (as defined herein);
- less immunogenicity, either in a monovalent format, in a multivalent format (for example in a bivalent format) and/or in a multispecific format (for example one of the multispecific formats described hereinbelow);

- increased stability, either in a monovalent format, in a multivalent format (for example in a bivalent format) and/or in a multispecific format (for example one of the multispecific formats described hereinbelow);

- increased specificity towards Targets of the invention, either in a monovalent format, in a multivalent format (for example in a bivalent format) and/or in a multispecific format (for example one of the multispecific formats described hereinbelow);

- decreased or where desired increased cross-reactivity with Targets of the invention from different species;

and/or

- one or more other improved properties desirable for pharmaceutical use (including prophylactic use and/or therapeutic use) and/or for diagnostic use (including but not limited to use for imaging purposes), either in a monovalent format, in a multivalent format (for example in a bivalent format) and/or in a multispecific format (for example one of the multispecific formats described hereinbelow).

As generally described herein for the amino acid sequences of the invention, the Nanobodies of the invention are preferably in essentially isolated form (as defined herein), or form part of a protein or polypeptide of the invention (as defined herein), which may comprise or essentially consist of one or more Nanobodies of the invention and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more amino acid sequences of the invention may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further amino acid sequences that can serve as a binding unit (i.e. against one or more other targets than Targets of the invention), so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. In particular, such a protein or polypeptide may comprise or essentially consist of one or more Nanobodies of the invention and optionally one or more (other) Nanobodies (i.e. directed against other targets than Targets of the invention), all optionally linked via one or more suitable linkers, so as to provide a monovalent, multivalent or multispecific Nanobody construct, respectively, as further
Such proteins or polypeptides may also be in essentially isolated form (as defined herein).

In a Nanobody of the invention, the binding site for binding against Targets of the invention is preferably formed by the CDR sequences. Optionally, a Nanobody of the invention may also, and in addition to the at least one binding site for binding against Targets of the invention, contain one or more further binding sites for binding against other antigens, proteins, or targets. For methods and positions for introducing such second binding sites, reference is for example made to Keck and Huston. Biophysical Journal, 71, October 1996, 2002-201 i; EP 0 640 130 and WO 06/07260.

As generally described herein for the amino acid sequences of the invention, when a Nanobody of the invention (or a polypeptide of the invention comprising the same) is intended for administration to a subject (for example for therapeutic and/or diagnostic purposes as described herein), it is preferably directed against human Targets of the invention; whereas for veterinary purposes, it is preferably directed against Targets of the invention from the species to be treated. Also, as with the amino acid sequences of the invention, a Nanobody of the invention may or may not be cross-reactive (i.e. directed against Targets of the invention from two or more species of mammal, such as against human Targets of the invention and Targets of the invention from at least one of the species of mammal mentioned herein).

Also, again as generally described herein for the amino acid sequences of the invention, the Nanobodies of the invention may generally be directed against any antigenic determinant, epitope, part, domain, subunit or confirmation (where applicable) of Targets of the invention. However, it is generally assumed and preferred that the Nanobodies of the invention (and polypeptides comprising the same) are directed against and/or have been raised against at least one extracellular region, domain, loop or other extracellular epitope of one of the Targets of the invention (or a suitable peptide derived therefrom).

As already described herein, the amino acid sequence and structure of a Nanobody can be considered - without however being limited thereto - to be comprised of four framework regions or "FR's" (or sometimes also referred to as ‘FW s’), which are referred to in the art and herein 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 complementary determining regions or "CDRV" which are referred to in the art as 'Complementarity Determining
Region 1 or "CDR1"; as "Complementarity Determining Region T" or "CDR2"; and as "Complementarity Determining Region 3" or "CDR3", respectively. Some preferred framework sequences and CDR's (and combinations thereof) that are present in the Nanobodies of the invention are as described herein. Other suitable CDR sequences can be obtained by the methods described herein.

According to a non-limiting but preferred aspect of the invention, (the CDR sequences present in) the Nanobodies of the invention are such that:

- the Nanobodies can bind to Targets of the invention with a dissociation constant ($K_D$) of $10^{-5}$ to $10^{-12}$ moles/liter or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter or less and more preferably $10^{-8}$ to $10^{-12}$ moles/liter (i.e. with an association constant ($K_A$) of $10^5$ to $10^{12}$ liter/moles or more, and preferably $10^7$ to $10^{12}$ liter/moles or more and more preferably $10^8$ to $10^{12}$ liter/moles);

and/or such that:

- the Nanobodies can bind to Targets of the invention with a $k_{on}$-rate of between $10^2$ M$^{-1}$s$^{-1}$ to about $10^7$ M$^{-1}$V$^{-1}$, preferably between $10^3$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$, more preferably between $10^4$ JVs$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$, such as between $10^5$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$;

and/or such that they:

- the Nanobodies can bind to Targets of the invention with a $k_{off}$ rate between 1 s$^{-1}$ ($t_{1/2}$=0.69 s) and $10^{-6}$ s$^{-1}$ (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between $10^2$ s$^{-1}$ and $10^{-5}$ s$^{-1}$, more preferably between $10^3$ s$^{-1}$ and $10^{-6}$ s$^{-1}$, such as between $10^4$ s$^{-1}$ and $10^{-6}$ s$^{-1}$.

Preferably, (the CDR sequences present in) the Nanobodies of the invention are such that: a monovalent Nanobody of the invention (or a polypeptide that contains only one Nanobody of the invention) is preferably such that it will bind to Targets of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

The affinity of the Nanobody of the invention against Targets of the invention can be determined in a manner known per se, for example using the general techniques for measuring $K_D$, $K_A$, $k_{on}$ or $k_{off}$ mentioned herein, as well as some of the specific assays described herein.

Some preferred IC50 values for binding of the Nanobodies of the invention (and of polypeptides comprising the same) to Targets of the invention will become clear from the further description and examples herein.
In a preferred but non-limiting aspect, the invention relates to a Nanobody (as defined herein) against hCXCR4, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

- CDR1 is chosen from the group consisting of:

  a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

and/or

- CDR2 is chosen from the group consisting of:

  d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
  e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
  f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

and/or

- CDR3 is chosen from the group consisting of:

  g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
  h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
  i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

or any suitable fragment of such an amino acid sequence.

In particular, according to this preferred but non-limiting aspect, the invention relates to a Nanobody (as defined herein) against human CXCR4, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

- CDR1 is chosen from the group consisting of:

  a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

and

- CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

and

- CDR3 is chosen from the group consisting of:

  g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

  h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

  i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

or any suitable fragment of such an amino acid sequences.

In a preferred but non-limiting aspect, the invention relates to a Nanobody (as defined herein) against hCD4, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

- CDR1 is chosen from the group consisting of:

  a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

and/or

- CDR2 is chosen from the group consisting of:

  d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

  e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

  f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
and/or
- CDR3 is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the
5 amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
or any suitable fragment of such an amino acid sequence.

In particular, according to this preferred but non-limiting aspect, the invention relates
to a Nanobody (as defined herein) against human CD4, which consists of 4 framework
regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to
CDR3 respectively), in which:
- CDR1 is chosen from the group consisting of:
a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
and
- CDR2 is chosen from the group consisting of:
d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;
and
- CDR3 is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the
amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
or any suitable fragment of such an amino acid sequences.
As generally mentioned herein for the amino acid sequences of the invention, when a Nanobody of the invention contains one or more CDR1 sequences according to b) and/or c):
  i) any amino acid substitution in such a CDR according to b) and/or c) is preferably, and compared to the corresponding CDR according to a), a conservative amino acid substitution (as defined herein);
  and/or
  ii) the CDR according to b) and/or c) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to a);

Similarly, when a Nanobody of the invention contains one or more CDR2 sequences according to e) and/or f):
  i) any amino acid substitution in such a CDR according to e) and/or f) is preferably, and compared to the corresponding CDR according to d), a conservative amino acid substitution (as defined herein);
  and/or
  ii) the CDR according to e) and/or f) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to d);
  and/or
  iii) the CDR according to e) and/or f) may be a CDR that is derived from a CDR according to d) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Also, similarly, when a Nanobody of the invention contains one or more CDR3 sequences according to h) and/or i):
  i) any amino acid substitution in such a CDR according to h) and/or i) is preferably, and compared to the corresponding CDR according to g), a conservative amino acid substitution (as defined herein);
ii) the CDR according to h) and/or i) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to g); and/or

iii) the CDR according to h) and/or i) may be a CDR that is derived from a CDR according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last three paragraphs generally apply to any Nanobody of the invention that comprises one or more CDR1 sequences, CDR2 sequences and/or CDR3 sequences according to b), c), e), f), h) or i), respectively.

Of the Nanobodies of the invention, Nanobodies comprising one or more of the CDR's explicitly listed above are particularly preferred; Nanobodies comprising two or more of the CDR's explicitly listed above are more particularly preferred; and Nanobodies comprising three of the CDR's explicitly listed above are most particularly preferred.

Some particularly preferred, but non-limiting combinations of CDR sequences, as well as preferred combinations of CDR sequences and framework sequences, are mentioned in Table A-I below, which lists the CDR sequences and framework sequences that are present in a number of preferred (but non-limiting) Nanobodies of the invention. As will be clear to the skilled person, a combination of CDR1, CDR2 and CDR3 sequences that occur in the same clone (i.e. CDR1, CDR2 and CDR3 sequences that are mentioned on the same line in Table A-I) will usually be preferred (although the invention in its broadest sense is not limited thereto, and also comprises other suitable combinations of the CDR sequences mentioned in Table A-I). Also, a combination of CDR sequences and framework sequences that occur in the same clone (i.e. CDR sequences and framework sequences that are mentioned on the same line in Table A-I) will usually be preferred (although the invention in its broadest sense is not limited thereto, and also comprises other suitable combinations of the CDR sequences and framework sequences mentioned in Table A-I, as well as combinations of such CDR sequences and other suitable framework sequences, e.g. as further described herein).

Also, in the Nanobodies of the invention that comprise the combinations of CDR's mentioned in Table A-I, each CDR can be replaced by a CDR chosen from the group consisting of amino acid sequences that have at least 80%, preferably at least 90%, more
preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with the mentioned CDR’s; in which:

i) any amino acid substitution in such a CDR is preferably, and compared to the corresponding CDR sequence mentioned in Table A-I, a conservative amino acid substitution (as defined herein);

and/or

ii) any such CDR sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR sequence mentioned in Table A-I;

and/or

iii) any such CDR sequence is a CDR that is derived by means of a technique for affinity maturation known per se, and in particular starting from the corresponding CDR sequence mentioned in Table A-I,

However, as will be clear to the skilled person, the (combinations of) CDR sequences, as well as (the combinations of) CDR sequences and framework sequences mentioned in Table A-I will generally be preferred.
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### Table A-1 (B) CDR’s and framework sequences of Nanobodies against human CD4

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Thus, in the Nanobodies of the invention, at least one of the CDR1, CDR2 and CDR3 sequences present is suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I; or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% "sequence identity" (as defined herein) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I.

In this context, by "suitably chosen" is meant that, as applicable, a CDR1 sequence is chosen from suitable CDR1 sequences (i.e. as defined herein), a CDR2 sequence is chosen from suitable CDR2 sequences (i.e. as defined herein), and a CDR3 sequence is chosen from suitable CDR3 sequence (i.e. as defined herein), respectively. More in particular, the CDR sequences are preferably chosen such that the Nanobodies of the invention bind to Targets of the invention with an affinity (suitably measured and/or expressed as a \( K_0 \)-value (actual or apparent), a \( K_A \)-value (actual or apparent), a \( k_{on} \)-rate and/or a \( k_{off} \)-rate, or alternatively as an \( IC_{50} \) value, as further described herein) that is as defined herein.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 sequences listed in Table A-I or from the group of CDR3 sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR3 sequences listed in Table A-I; and/or from the group consisting of the CDR3 sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDR3 sequences listed in Table A-I.

Preferably, in the Nanobodies of the invention, at least two of the CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I or from the group consisting of CDR1, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively,
that have 3, 2 or only 1 "amino acid difference(s)" with at least one of the CDRI, CDR2 and CDR3 sequences, respectively, listed in Table A-I.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 sequences listed in Table A-I or from the group of CDR3 sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR3 sequences listed in Table A-I, respectively; and at least one of the CDRI and CDR2 sequences present is suitably chosen from the group consisting of the CDRI and CDR2 sequences, respectively, listed in Table A-I or from the group of CDRI and CDR2 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDRI and CDR2 sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDRI and CDR2 sequences, respectively, that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDRI and CDR2 sequences, respectively, listed in Table A-I.

Most preferably, in the Nanobodies of the invention, all three CDRI, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDRI, CDR2 and CDR3 sequences, respectively, listed in Table A-I or from the group of CDRI, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDRI, CDR2 and CDR3 sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDRI, CDR2 and CDR3 sequences, respectively, that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDRI, CDR2 and CDR3 sequences, respectively, listed in Table A-I.

Even more preferably, in the Nanobodies of the invention, at least one of the CDRI, CDR2 and CDR3 sequences present is suitably chosen from the group consisting of the CDRI, CDR2 and CDR3 sequences, respectively, listed in Table A-I. Preferably, in this aspect, at least one or preferably both of the other two CDR sequences present are suitably chosen from CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDR sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the corresponding sequences, respectively, listed in Table A-I.
In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 listed in Table A-I. Preferably, in this aspect, at least one and preferably both of the CDR1 and CDR2 sequences present are suitably chosen from the groups of CDR1 and CDR2 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the CDR1 and CDR2 sequences, respectively, listed in Table A-I; and/or from the group consisting of the CDR1 and CDR2 sequences, respectively, that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDR1 and CDR2 sequences, respectively, listed in Table A-I.

Even more preferably, in the Nanobodies of the invention, at least two of the CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I. Preferably, in this aspect, the remaining CDR sequence present is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences listed in Table A-I; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the corresponding sequences listed in Table A-I.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence is suitably chosen from the group consisting of the CDR3 sequences listed in Table A-I, and either the CDR1 sequence or the CDR2 sequence is suitably chosen from the group consisting of the CDR1 and CDR2 sequences, respectively, listed in Table A-I. Preferably, in this aspect, the remaining CDR sequence present is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences listed in Table A-I; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the corresponding CDR sequences listed in Table A-I.

Even more preferably, in the Nanobodies of the invention, all three CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I.

Also, generally, the combinations of CDR’s listed in Table A-I (i.e. those mentioned on the same line in Table A-I) are preferred. Thus, it is generally preferred that, when a CDR
in a Nanobody of the invention is a CDR sequence mentioned in Table A-I or is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with a CDR sequence listed in Table A-I; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with a CDR sequence listed in Table A-I, that at least one and preferably both of the other CDR’s are suitably chosen from the CDR sequences that belong to the same combination in Table A-I (i.e. mentioned on the same line in Table A-I) or are suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the CDR sequence(s) belonging to the same combination and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the CDR sequence(s) belonging to the same combination. The other preferences indicated in the above paragraphs also apply to the combinations of CDR’s mentioned in Table A-I.

Thus, by means of non-limiting examples, a Nanobody of the invention can for example comprise a CDR1 sequence that has more than 80% sequence identity with one of the CDR1 sequences mentioned in Table A-I; a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table A-I (but belonging to a different combination); and a CDR3 sequence.

Some preferred Nanobodies of the invention may for example comprise: (1) a CDR1 sequence that has more than 80% sequence identity with one of the CDR1 sequences mentioned in Table A-I; a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table A-I (but belonging to a different combination); and a CDR3 sequence that has more than 80% sequence identity with one of the CDR3 sequences mentioned in Table A-I (but belonging to a different combination); or (2) a CDR1 sequence that has more than 80% sequence identity with one of the CDR1 sequences mentioned in Table A-I; a CDR2 sequence that has more than 80% sequence identity with one of the CDR2 sequences listed in Table A-I; and a CDR3 sequence that has 3, 2 or 1 amino acid differences with the CDR3 sequence mentioned in Table A-I that belongs to the same combination as the CDR2 sequence.

Some particularly preferred Nanobodies of the invention may for example comprise:

(1) a CDR1 sequence that has more than 80% sequence identity with one of the CDR1
sequences mentioned in Table A-I; a CDR2 sequence that has 3, 2 or 1 amino acid difference with the CDR2 sequence mentioned in Table A-I that belongs to the same combination; and a CDR3 sequence that has more than 80% sequence identity with the CDR3 sequence mentioned in Table A-I that belongs to the same combination; (2) a CDR1 sequence; a CDR 2 listed in Table A-I and a CDR3 sequence listed in Table A-I (in which the CDR2 sequence and CDR3 sequence may belong to different combinations).

Some even more preferred Nanobodies of the invention may for example comprise: (1) a CDR1 sequence that has more than 80% sequence identity with one of the CDR1 sequences mentioned in Table A-I; the CDR2 sequence listed in Table A-I that belongs to the same combination; and a CDR3 sequence mentioned in Table A-I that belongs to a different combination; or (2) a CDR1 sequence mentioned in Table A-I; a CDR2 sequence that has 3, 2 or 1 amino acid differences with the CDR2 sequence mentioned in Table A-I that belongs to the same combination; and a CDR3 sequence that has more than 80% sequence identity with the CDR3 sequence listed in Table A-I that belongs to the same or a different combination.

Particularly preferred Nanobodies of the invention may for example comprise a CDR1 sequence mentioned in Table A-I, a CDR2 sequence that has more than 80% sequence identity with the CDR2 sequence mentioned in Table A-I that belongs to the same combination; and the CDR3 sequence mentioned in Table A-I that belongs to the same combination.

In the most preferred Nanobodies of the invention, the CDR1, CDR2 and CDR3 sequences present are suitably chosen from one of the combinations of CDR1, CDR2 and CDR3 sequences, respectively, listed in Table A-I.

According to another preferred, but non-limiting aspect of the invention (a) CDR1 has a length of between 1 and 12 amino acid residues, and usually between 2 and 9 amino acid residues, such as 5, 6 or 7 amino acid residues; and/or (b) CDR2 has a length of between 13 and 24 amino acid residues, and usually between 15 and 21 amino acid residues, such as 16 and 17 amino acid residues; and/or (c) CDR3 has a length of between 2 and 35 amino acid residues, and usually between 3 and 30 amino acid residues, such as between 6 and 23 amino acid residues.

In another preferred, but non-limiting aspect, the invention relates to a Nanobody in which the CDR sequences (as defined herein) have more than 80%, preferably more than 90%, more preferably more than 95%. such as 99% or more sequence identity (as defined
herein) with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311.

Generally, Nanobodies with the above CDR sequences may be as further described herein, and preferably have framework sequences that are also as further described herein. Thus, for example and as mentioned herein, such Nanobodies may be naturally occurring Nanobodies (from any suitable species), naturally occurring \( V_{HH} \) sequences (i.e. from a suitable species of Camelid) or synthetic or semi-synthetic amino acid sequences or Nanobodies, including but not limited to partially humanized Nanobodies or \( V_{HH} \) sequences, fully humanized Nanobodies or \( V_{1\text{H}} \) sequences, camelized heavy chain variable domain sequences, as well as Nanobodies that have been obtained by the techniques mentioned herein.

Thus, in one specific, but non-limiting aspect, the invention relates to a humanized Nanobody, which consists of 4 framework regions (FRI to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which CDR1 to CDR3 are as defined herein and in which said humanized Nanobody comprises at least one humanizing substitution (as defined herein), and in particular at least one humanizing substitution in at least one of its framework sequences (as defined herein).

In another preferred, but non-limiting aspect, the invention relates to a Nanobody in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said Nanobody and one or more of the sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Such Nanobodies can be as further described herein.

In another preferred, but non-limiting aspect, the invention relates to a Nanobody with an amino acid sequence that is chosen from the group consisting of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311,
more preferably SEQ ID NO: 311 or from the group consisting of from amino acid sequences
that have more than 80%, preferably more than 90%, more preferably more than 95%, such
as 99% or more sequence identity (as defined herein) with at least one of the amino acid
sequences of SEQ ID NO's: 238 to 253. more preferably SEQ ID NO: 238 and SEQ ID NO:
239 and SEQ ID NO's: 308 to 311. more preferably SEQ ID NO: 311.

Another preferred, but non-limiting aspect of the invention relates to humanized
variants of the Nanobodies of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238
and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. that
comprise, compared to the corresponding native \( V_{\text{m}} \) sequence, at least one humanizing
substitution (as defined herein), and in particular: at least one humanizing substitution in at
least one of its framework sequences (as defined herein).

The polypeptides of the invention comprise or essentially consist of at least one
Nanobody of the invention. Thus in another preferred, but non-limiting aspect, the invention
relates to a polypeptide comprise or essentially consist of at least one Nanobody that is
chosen from the group consisting of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO:
238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311
or from the group consisting of amino acid sequences that have more than 80%, preferably
more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as
defined herein) with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253,
more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more
preferably SEQ ID NO: 311. Thus in another preferred, but non-limiting aspect, the invention
relates to a polypeptide comprise or essentially consist of amino acid sequences of SEQ ID
NO's: 261 to 264, more preferably SEQ ID NO: 263 to 264.

It will be clear to the skilled person that the Nanobodies that are mentioned herein as
"preferred" (or "more preferred", "even more preferred", etc.) are also preferred (or more
preferred, or even more preferred, etc.) for use in the polypeptides described herein. Thus,
polypeptides that comprise or essentially consist of one or more "preferred" Nanobodies of
the invention will generally be preferred, and polypeptides that comprise or essentially
consist of one or more "more preferred" Nanobodies of the invention will generally be more
preferred, etc.

Generally, proteins or polypeptides that comprise or essentially consist of a single
Nanobody (such as a single Nanobody of the invention) will be referred to herein as
"monovalent" proteins or polypeptides or as "monovalent constructs". Proteins and
polypeptides that comprise or essentially consist of two or more Nanobodies (such as at least two Nanobodies of the invention or at least one Nanobody of the invention and at least one other Nanobody) will be referred to herein as "multivalent" proteins or polypeptides or as "multivalent constructs", and these may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention. Some non-limiting examples of such multivalent constructs will become clear from the further description herein.

According to one specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least two Nanobodies of the invention, such as two or three Nanobodies of the invention. As further described herein, such multivalent constructs can provide certain advantages compared to a protein or polypeptide comprising or essentially consisting of a single Nanobody of the invention, such as a much improved avidity for Targets of the invention. Such multivalent constructs will be clear to the skilled person based on the disclosure herein, and e.g. are polypeptides that comprise or essentially consist of amino acid sequences of SEQ ID NO: 261 to 266.

According to another specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least one Nanobody of the invention and at least one other binding unit (i.e. directed against another epitope, antigen, target, protein or polypeptide), which is preferably also a Nanobody. Such proteins or polypeptides are also referred to herein as "multispecific" proteins or polypeptides or as 'multispecific constructs", and these may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention (as will become clear from the further discussion herein of some preferred, but non-limiting multispecific constructs). Such multispecific constructs will be clear to the skilled person based on the disclosure herein, and e.g. are polypeptides that comprise or essentially consist of amino acid sequences of SEQ ID NO: 263 to 264.

According to yet another specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least one Nanobody of the invention, optionally one or more further Nanobodies, and at least one other amino acid sequence (such as a protein or polypeptide) that confers at least one desired property to the Nanobody of the invention and/or to the resulting fusion protein. Again, such fusion proteins may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention. Some non-limiting examples of such amino acid sequences and of such fusion constructs will become clear from the further description herein.
It is also possible to combine two or more of the above aspects, for example to provide a trivalent bispecific construct comprising two Nanobodies of the invention and one other Nanobody, and optionally one or more other amino acid sequences. Further non-limiting examples of such constructs, as well as some constructs that are particularly preferred within the context of the present invention, will become clear from the further description herein.

In the above constructs, the one or more Nanobodies and/or other amino acid sequences may be directly linked to each other and/or suitably linked to each other via one or more linker sequences. Some suitable but non-limiting examples of such linkers will become clear from the further description herein.

In one specific aspect of the invention, a Nanobody of the invention or a compound, construct or polypeptide of the invention comprising at least one Nanobody of the invention may have an increased half-life, compared to the corresponding amino acid sequence of the invention. Some preferred, but non-limiting examples of such Nanobodies, compounds and polypeptides will become clear to the skilled person based on the further disclosure herein, and for example comprise Nanobodies sequences or polypeptides of the invention that have been chemically modified to increase the half-life thereof (for example, by means of pegylation); amino acid sequences of the invention that comprise at least one additional binding site for binding to a serum protein (such as serum albumin); or polypeptides of the invention that comprise at least one Nanobody of the invention that is linked to at least one moiety (and in particular at least one amino acid sequence) that increases the half-life of the Nanobody of the invention. Examples of polypeptides of the invention that comprise such half-life extending moieties or amino acid sequences will become clear to the skilled person based on the further disclosure herein; and for example include, without limitation, polypeptides in which the one or more Nanobodies of the invention are suitable linked to one or more serum proteins or fragments thereof (such as serum albumin or suitable fragments thereof) or to one or more binding units that can bind to serum proteins (such as, for example, Nanobodies or (single) domain antibodies that can bind to serum proteins such as serum albumin, serum immunoglobulins such as IgG, or transferrin); polypeptides in which a Nanobody of the invention is linked to an Fc portion (such as a human Fc) or a suitable part or fragment thereof; or polypeptides in which the one or more Nanobodies of the invention are suitable linked to one or more small proteins or peptides that can bind to serum proteins.
(such as, without limitation, the proteins and peptides described in WO 91/01743, WO
01/45746, WO 02/076489 and to WO 08/068280.

Again, as will be clear to the skilled person, such Nanobodies, compounds, constructs
or polypeptides may contain one or more additional groups, residues, moieties or binding
units, such as one or more further amino acid sequences and in particular one or more
additional Nanobodies (i.e. not directed against Targets of the invention), so as to provide a
tri- of multispecific Nanobody construct.

Generally, the Nanobodies of the invention (or compounds, constructs or polypeptides
comprising the same) with increased half-life preferably have a half-life that is at least 1.5
times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or
more than 20 times, greater than the half-life of the corresponding amino acid sequence of the
invention per se. For example, the Nanobodies, compounds, constructs or polypeptides of the
invention with increased half-life may have a half-life that is increased with more than 1
hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than
12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid
sequence of the invention per se.

In a preferred, but non-limiting aspect of the invention, such Nanobodies, compound,
constructs or polypeptides of the invention exhibit a serum half-life in human of at least about
12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably
at least 72 hours or more. For example, compounds or polypeptides of the invention may
have a half-life of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such
as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days),
or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days
(such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

In another one aspect of the invention, a polypeptide of the invention comprises one
or more (such as two or preferably one) Nanobodies of the invention linked (optionally via
one or more suitable linker sequences) to one or more (such as two and preferably one) amino
acid sequences that allow the resulting polypeptide of the invention to cross the blood brain
barrier. In particular, said one or more amino acid sequences that allow the resulting
polypeptides of the invention to cross the blood brain barrier may be one or more (such as
two and preferably one) Nanobodies, such as the Nanobodies described in WO 02/057445, of
which FC44 (SEQ ID NO: 189 of WO 06/040153) and FC5 (SEQ ID NO: 190 of WO
06/040154) are preferred examples.
In particular, polypeptides comprising one or more Nanobodies of the invention are preferably such that they:

- bind to Targets of the invention with a dissociation constant ($K_D$) of $1 \times 10^{-5}$ to $10^{-12}$ moles/liter or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter or less and more preferably $10^{-8}$ to $10^{-12}$ moles/liter (i.e. with an association constant ($K_A$) of $10^5$ to $10^{12}$ liter/moles or more, and preferably $10^7$ to $10^2$ liter/moles or more and more preferably $10^8$ to $10^{12}$ liter/moles);

and/or such that they:

- bind to Targets of the invention with an $k_{on}$ rate of between $10^2$ M$^{-1}$s$^{-1}$ to about $10^7$ M$^{-1}$s$^{-1}$, preferably between $10^3$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$, more preferably between $10^4$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$, such as between $10^5$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$;

and/or such that they:

- bind to Targets of the invention with a $k_{off}$ rate between $1$ s$^{-1}$ ($t_{1/2}=0.69$ s) and $10^{-6}$ s$^{-1}$ (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between $10^{-2}$ s$^{-1}$ and $10^{-6}$ s$^{-1}$, more preferably between $10^{-3}$ s$^{-1}$ and $10^{-6}$ s$^{-1}$, such as between $10^{-4}$ s$^{-1}$ and $10^{-6}$ s$^{-1}$.

Preferably, a polypeptide that contains only one amino acid sequence of the invention is preferably such that it will bind to Targets of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. In this respect, it will be clear to the skilled person that a polypeptide that contains two or more Nanobodies of the invention may bind to Targets of the invention with an increased avidity, compared to a polypeptide that contains only one amino acid sequence of the invention.

Some preferred $IC_{50}$ values for binding of the amino acid sequences or polypeptides of the invention to Targets of the invention will become clear from the further description and examples herein.

Another aspect of this invention relates to a nucleic acid that encodes an amino acid sequence of the invention (such as a Nanobody of the invention) or a polypeptide of the invention comprising the same. Again, as generally described herein for the nucleic acids of the invention, such a nucleic acid may be in the form of a genetic construct, as defined herein.

In another aspect, the invention relates to host or host cell that expresses or that is capable of expressing an amino acid sequence (such as a Nanobody) of the invention and/or a polypeptide of the invention comprising the same; and/or that contains a nucleic acid of the
invention. Some preferred but non-limiting examples of such hosts or host cells will become clear from the further description herein.

Another aspect of the invention relates to a product or composition containing or comprising at least one amino acid sequence of the invention, at least one polypeptide of the invention and/or at least one nucleic acid of the invention, and optionally one or more further components of such compositions known per se, i.e. depending on the intended use of the composition. Such a product or composition may for example be a pharmaceutical composition (as described herein), a veterinary composition or a product or composition for diagnostic use (as also described herein). Some preferred but non-limiting examples of such products or compositions will become clear from the further description herein.

The invention further relates to methods for preparing or generating the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein. Some preferred but non-limiting examples of such methods will become clear from the further description herein.

The invention further relates to applications and uses of the amino acid sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein, as well as to methods for the prevention and/or treatment for diseases and disorders associated with Targets of the invention. Some preferred but non-limiting applications and uses will become clear from the further description herein.

Other aspects, embodiments, advantages and applications of the invention will also become clear from the further description herein below.

Generally, it should be noted that the term Nanobody as used herein in its broadest sense is not limited to a specific biological source or to a specific method of preparation. For example, as will be discussed in more detail below, the Nanobodies of the invention can generally be obtained: (1) by isolating the V<sub>H</sub>H domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring V<sub>H</sub>H domain; (3) by "humanization" (as described herein) of a naturally occurring V<sub>H</sub>H domain or by expression of a nucleic acid encoding a such humanized V<sub>H</sub>H domain; (4) by "camelization" (as described herein) of a naturally occurring V<sub>H</sub> domain from any animal species, and in particular a from species of mammal, such as from a human being, or by expression of a nucleic acid encoding such a camelized V<sub>H</sub> domain; (5) by "camelisation" of a "domain antibody" or "Dab" as described by Ward et al (supra), or by expression of a nucleic acid encoding such a camelized V<sub>H</sub> domain; (6) by using synthetic or semi-synthetic
techniques for preparing proteins, polypeptides or other amino acid sequences known per se;
(7) by preparing a nucleic acid encoding a Nanobody using techniques for nucleic acid
synthesis known per se, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of one or more of the foregoing. Suitable methods and techniques for
performing the foregoing will be clear to the skilled person based on the disclosure herein
and for example include the methods and techniques described in more detail herein.

One preferred class of Nanobodies corresponds to the \( \text{V}_{\text{HH}} \) domains of naturally
occurring heavy chain antibodies directed against Targets of the invention. As further
described herein, such \( \text{V}_{\text{HH}} \) sequences can generally be generated or obtained by suitably
immunizing a species of Camelid with Targets of the invention (i.e. so as to raise an immune
response and/or heavy chain antibodies directed against Targets of the invention), by
obtaining a suitable biological sample from said Camelid (such as a blood sample, serum
sample or sample of B-cells), and by generating \( \text{V}_{\text{HH}} \) sequences directed against Targets of
the invention, stalling from said sample, using any suitable technique known per se. Such
techniques will be clear to the skilled person and/or are further described herein.

Alternatively, such naturally occurring \( \text{V}_{\text{HH}} \) domains against Targets of the invention,
can be obtained from naïve libraries of Camelid \( \text{V}_{\text{HH}} \) sequences, for example by screening
such a library using Targets of the invention, or at least one part, fragment, antigenic
determinant or epitope thereof using one or more screening techniques known per se. Such
libraries and techniques are for example described in WO 99/37681, WO 01/90190, WO
03/025020 and WO 03/035694. Alternatively, improved synthetic or semi-synthetic libraries
derived from naïve \( \text{V}_{\text{HH}} \) libraries may be used, such as \( \text{V}_{\text{HH}} \) libraries obtained from naïve \( \text{V}_{\text{ym}} \)
libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example
described in WO 00/43507.

Thus, in another aspect, the invention relates to a method for generating Nanobodies,
that are directed against Targets of the invention. In one aspect, said method at least
comprises the steps of:
a) providing a set, collection or library of Nanobody sequences; and
b) screening said set, collection or library of Nanobody sequences for Nanobody
sequences that can bind to and/or have affinity for Targets of the invention;
and
c) isolating the Nanobody or Nanobodies that can bind to and/or have affinity for Targets
of the invention.
In such a method, the set, collection or library of Nanobody sequences may be a naive set, collection or library of Nanobody sequences; a synthetic or semi-synthetic set, collection or library of Nanobody sequences; and/or a set, collection or library of Nanobody sequences that have been subjected to affinity maturation.

In a preferred aspect of this method, the set, collection or library of Nanobody sequences may be an immune set, collection or library of Nanobody sequences, and in particular an immune set, collection or library of $V_{\text{HH}}$ sequences, that have been derived from a species of Camelid that has been suitably immunized with Targets of the invention or with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

In the above methods, the set, collection or library of Nanobody or $V_{\text{HH}}$ sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) Nanobody sequences will be clear to the person skilled in the art, for example on the basis of the further disclosure herein.

Reference is also made to WO 03/054016 and to the review by Hoogenboom in Nature Biotechnology, 23, 9, 1105-1116 (2005).

In another aspect, the method for generating Nanobody sequences comprises at least the steps of:

a) providing a collection or sample of cells derived from a species of Camelid that express immunoglobulin sequences;

b) screening said collection or sample of cells for (i) cells that express an immunoglobulin sequence that can bind to and/or have affinity for Targets of the invention; and (ii) cells that express heavy chain antibodies, in which substeps (i) and (ii) can be performed essentially as a single screening step or in any suitable order as two separate screening steps, so as to provide at least one cell that expresses a heavy chain antibody that can bind to and/or has affinity for Targets of the invention;

c) either (i) isolating from said cell the $V_{\text{HH}}$ sequence present in said heavy chain antibody; or (ii) isolating from said cell a nucleic acid sequence that encodes the $V_{\text{HH}}$
sequence present in said heavy chain antibody, followed by expressing said $V_{HH}$ domain.

In another aspect, the method for generating Nanobody sequences directed against at least one of the Targets of the invention, comprises at least the steps of:

5 a. a step of suitably immunizing a Cameldid with a suitable antigen that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or with a suitable peptide derived therefrom or based thereon, such that an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s) is raised. The antigen may be any suitable antigen that is capable of raising an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s); such as, for example and without limitation, whole cells that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation, cell wall fragments thereof or any other suitable preparation derived from such cells, vesicles that have the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface, a subunit or fragment of a subunit of a Target of the invention, e.g. human CXCR4 or hCD4, that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or a synthetic or semi-synthetic peptide that comprises and/or is based on (the amino acid sequence of) the desired extracellular part, region, domain, loop or other extracellular epitope(s), more preferably, whole cells (e.g. HEK293) that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation; and

b. a step of selection for binding for the desired extracellular part, region, domain, loop or other extracellular epitope(s) using cell membranes preparation of different (than the one using in immunization) and several cell types overexpressing said Target of the invention, e.g. human CXCR4. This may for example be performed by selecting from a set, a collection or a library of cells that express heavy chain antibodies on their surface (e.g. B-cells obtained from a suitably immunized Cameldid) and using a cell membranes preparation of e.g. a first type of cells such as e.g. CHO for a first round selection and e.g. a second type of cells such as e.g. COS-7 cells for a second round selection, by selecting from a (naive or immune) library of VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and e.g. a second type of cell such as e.g. COS-7 cell for a second round
select, or by selecting from a (naïve or immune) library of nucleic acid sequences that
encode VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and e.g. a second type of cells such as e.g. COS-7 cell for a second round selection; which may all be performed in a mariner known per se; and optionally
c. washing only mildly with a buffer such as PBS without detergents; and which method may optionally further comprise one or more other suitable steps known per se, such as, for example and without limitation, a step of affinity maturation, a step of expressing the desired amino acid sequence, a step of screening for binding and/or for activity against the desired antigen (in this case, the Target of the invention, e.g. human CXCR4 or hCD4), a step of determining the desired amino acid sequence or nucleotide sequence, a step of introducing one or more humanizing substitutions (e.g. as further described herein), a step of formatting in a suitable multivalent and/or multispecific format, a step of screening for the desired biological and/or physiological properties (i.e. using a suitable assay, such as those described herein); and/or any suitable combination of one or more of such steps, in any suitable order.

In another aspect, the method for generating Nanobody sequences directed against a transmembranal protein comprises at least the steps of:

a. a step of suitably immunizing a Camelid with a suitable antigen that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or with a suitable peptide derived therefrom or based thereon, such that an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s) is raised. The antigen may be any suitable antigen that is capable of raising an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s); such as, for example and without limitation, whole cells that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation, cell wall fragments thereof or any other suitable preparation derived from such cells, vesicles that have the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface, a subunit or fragment of a subunit of a transmembrane protein, in particular multiple spanning transmembrane protein for which the native conformation cannot be reproduced in other "in vitro" system (at least at time of filing of this application), that comprises the desired extracellular part, region, domain, loop or other extracellular
epitope(s), or a synthetic or semi-synthetic peptide that comprises and/or is based on (the amino acid sequence of) the desired extracellular part, region, domain, loop or other extracellular epitope(s), more preferably, whole cells (e.g. HEK293) that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface in their native confirmation; and

b. a step of selection for binding for the desired extracellular part, region, domain, loop or other extracellular epitope(s) using cell membranes preparation of different (than the one using in immunization) and several cell types overexpressing said transmembrane protein, in particular multiple spanning transmembrane protein for which the native conformation cannot be reproduced in other "in vitro" system (at least at time of filing of this application). This may for example be performed by selecting from a set, a collection or a library of cells that express heavy chain antibodies on their surface (e.g. B-cells obtained from a suitably immunized Camelid) and using a cell membranes preparation of e.g. a first type of cells such as e.g. CHO for a first round selection and e.g. a second type of cells such as e.g. COS-7 cells for a second round selection, by selecting from a (naïve or immune) library of VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and e.g. a second type of cell such as e.g. COS-7 cell for a second round selection, or by selecting from a (naïve or immune) library of nucleic acid sequences that encode VHH sequences or Nanobody sequences by using a cell membranes preparation of e.g. a first type of cell such as e.g. CHO for a first round selection and e.g. a second type of cells such as e.g. COS-7 cell for a second round selection; which may all be performed in a manner known per se; and optionally

c. washing only mildly with a buffer such as PBS without detergents; and which method may optionally further comprise one or more other suitable steps known per se, such as, for example and without limitation, a step of affinity maturation, a step of expressing the desired amino acid sequence, a step of screening for binding and/or for activity against the desired antigen (in this case, the transmembrane protein), a step of determining the desired amino acid sequence or nucleotide sequence, a step of introducing one or more humanizing substitutions (e.g. as further described herein), a step of formatting in a suitable multivalent and/or multispecific format, a step of screening for the desired biological and/or physiological properties (i.e. using a suitable assay, such as those
described herein); and/or any suitable combination of one or more of such steps, in any suitable order.

In the method according to this aspect, the collection or sample of cells may for example be a collection or sample of B-cells.

Also, in this method, the sample of cells may be derived from a Camelid that has been suitably immunized with Targets of the invention or a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

The above method may be performed in any suitable manner, as will be clear to the skilled person. Reference is for example made to EP 0 542 810, WO 05/19824, WO 04/051268 and WO 04/106377. The screening of step b) is preferably performed using a flow cytometry technique such as FACS. For this, reference is for example made to Lieby et al., Blood, Vol. 97, No. 12, 3820. Particular reference is made to the so-called "Nanoclone" technique described in International application WO 06/079372 by Ablynx N.V.

In another aspect, the method for generating an amino acid sequence directed against Targets of the invention may comprise at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding heavy chain antibodies or Nanobody sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode a heavy chain antibody or a Nanobody sequence that can bind to and/or has affinity for Targets of the invention;

and

c) isolating said nucleic acid sequence, followed by expressing the V_H1 sequence present in said heavy chain antibody or by expressing said Nanobody sequence, respectively,

In such a method, the set, collection or library of nucleic acid sequences encoding heavy chain antibodies or Nanobody sequences may for example be a set, collection or library of nucleic acid sequences encoding a naive set, collection or library of heavy chain antibodies or Vim sequences; a set, collection or library of nucleic acid sequences encoding a synthetic or semi-synthetic set, collection or library of Nanobody sequences; and/or a set, collection or library of nucleic acid sequences encoding a set, collection or library of Nanobody sequences that have been subjected to affinity maturation.
In a preferred aspect of this method, the set, collection or library of nucleic acid sequences may be an immune set, collection or library of nucleic acid sequences encoding heavy chain antibodies or $\nu_{\text{HH}}$ sequences derived from a Camelid that has been suitably immunized with Targets of the invention or with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment- region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

In the above methods, the set, collection or library of nucleotide sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) nucleotide sequences encoding amino acid sequences will be clear to the person skilled in the art, for example on the basis of the further disclosure herein. Reference is also made to WO 03/054016 and to the review by Hoogenboom in Nature Biotechnology, 23, 9, 1105-1116 (2005).

As will be clear to the skilled person, the screening step of the methods described herein can also be performed as a selection step. Accordingly the term "screening" as used in the present description can comprise selection, screening or any suitable combination of selection and/or screening techniques. Also, when a set, collection or library of sequences is used, it may contain any suitable number of sequences, such as 1, 2, 3 or about 5, 10, 50, 100, 500, 1000, 5000, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ or more sequences.

Also, one or more or all of the sequences in the above set, collection or library of amino acid sequences may be obtained or defined by rational, or semi-empirical approaches such as computer modelling techniques or biostatics or datamining techniques.

Furthermore, such a set, collection or library can comprise one, two or more sequences that are variants from one another (e.g. with designed point mutations or with randomized positions), compromise multiple sequences derived from a diverse set of naturally diversified sequences (e.g. an immune library), or any other source of diverse sequences (as described for example in Hoogenboom et al, Nat Biotechnol 23:1105, 2005 and Binz et al. Nat Biotechnol 2005, 23:1247). Such set, collection or library of sequences can be displayed on the surface of a phage particle, a ribosome, a bacterium, a yeast cell, a mammalian cell, and linked to the nucleotide sequence encoding the amino acid sequence within these carriers. This makes such set, collection or library amenable to selection procedures to isolate the desired amino acid sequences of the invention. More generally, when a sequence is displayed
on a suitable host or host cell, it is also possible (and customary) to first isolate from said host or host cell a nucleotide sequence that encodes the desired sequence, and then to obtain the desired sequence by suitably expressing said nucleotide sequence in a suitable host organism. Again, this can be performed in any suitable manner known per se, as will be clear to the skilled person.

Yet another technique for obtaining $V_{\text{HH}}$ sequences or Nanobody sequences directed against Targets of the invention involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (i.e. so as to raise an immune response and/or heavy chain antibodies directed against Targets of the invention), obtaining a suitable biological sample from said transgenic mammal that contains (nucleic acid sequences encoding) said $V_{\text{HH}}$ sequences or Nanobody sequences (such as a blood sample, serum sample or sample of B-cells), and then generating $V_{\text{HH}}$ sequences directed against Targets of the invention, stalling from said sample, using any suitable technique known per se (such as any of the methods described herein or a hybridoma technique). For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO 02/085945, WO 04/049794 and WO 06/008548 and Janssens et al, Proc. Natl. Acad. Sci. USA. 2006 Oct 10;103(41):15130-5 can be used. For example, such heavy chain antibody expressing mice can express heavy chain antibodies with any suitable (single) variable domain, such as (single) variable domains from natural sources (e.g. human (single) variable domains, Camelid (single) variable domains or shark (single) variable domains), as well as for example synthetic or semi-synthetic (single) variable domains.

The invention also relates to the $V_{\text{HH}}$ sequences or Nanobody sequences that are obtained by the above methods, or alternatively by a method that comprises the one of the above methods and in addition at least the steps of determining the nucleotide sequence or amino acid sequence of said $V_{\text{HH}}$ sequence or Nanobody sequence; and of expressing or synthesizing said $V_{\text{HH}}$ sequence or Nanobody sequence in a manner known per se, such as by expression in a suitable host cell or host organism or by chemical synthesis.

As mentioned herein, a particularly preferred class of Nanobodies of the invention comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring $V_{\text{HH}}$ domain, but that has been "humanized", i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring $V_{\text{HH}}$ sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a $V_{H}$ domain from a
conventional 4-chain antibody from a human being (e.g. indicated above). This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art on humanization referred to herein. Again, it should be noted that such humanized Nanobodies of the invention can be obtained in any suitable manner known per se (i.e. as indicated under points (1) - (8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring $V_{HH}$ domain as a starting material.

Another particularly preferred class of Nanobodies of the invention comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring $V_{H}$ domain, but that has been "camelized", i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring $V_{H}$ domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a $V_{HH}$ domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein. Such "camelizing" substitutions are preferably inserted at amino acid positions that form and/or are present at the $V_{H}$-$V_{L}$ interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996), supra). Preferably, the $V_{H}$ sequence that is used as a starting material or starting point for generating or designing the camelized Nanobody is preferably a $V_{H}$ sequence from a mammal, more preferably the $V_{H}$ sequence of a human being, such as a $V_{H}^p$ sequence. However, it should be noted that such camelized Nanobodies of the invention can be obtained in any suitable manner known per se (i.e. as indicated under points (1) - (8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring $V_{H}$ domain as a starting material.

For example, again as further described herein, both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes a naturally occurring $V_{HH}$ domain or $V_{H}$ domain, respectively, and then changing, in a manner known per se, one or more codons in said nucleotide sequence in such a way that the new nucleotide sequence encodes a "humanized" or "camelized" Nanobody of the invention, respectively. This nucleic acid can then be expressed in a manner known per se, so as to provide the desired Nanobody of the invention. Alternatively, based on the amino acid sequence of a naturally occurring $V_{HH}$ domain or $V_{H}$ domain, respectively, the amino acid sequence of the
desired humanized or camelized Nanobody of the invention, respectively, can be designed and then synthesized *de novo* using techniques for peptide synthesis known per se. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring $V_{H H}$ domain or $V_{H}$ domain, respectively, a nucleotide sequence encoding the desired humanized or camelized Nanobody of the invention, respectively, can be designed and then synthesized *de novo* using techniques for nucleic acid synthesis known per se, after which the nucleic acid thus obtained can be expressed in a manner known per se, so as to provide the desired Nanobody of the invention.

Other suitable methods and techniques for obtaining the Nanobodies of the invention and/or nucleic acids encoding the same, starting from naturally occurring $V_{H}$ sequences or preferably $V_{H H}$ sequences, will be clear from the skilled person, and may for example comprise combining one or more parts of one or more naturally occurring $V_{H}$ sequences (such as one or more FR sequences and/or CDR sequences), one or more parts of one or more naturally occurring $V_{H H}$ sequences (such as one or more FR sequences or CDR sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a Nanobody of the invention or a nucleotide sequence or nucleic acid encoding the same (which may then be suitably expressed). Nucleotide sequences encoding framework sequences of $V_{H H}$ sequences or Nanobodies will be clear to the skilled person based on the disclosure herein and/or the further prior art cited herein (and/or may alternatively be obtained by PCR starting from the nucleotide sequences obtained using the methods described herein) and may be suitably combined with nucleotide sequences that encode the desired CDR’s (for example, by PCR assembly using overlapping primers), so as to provide a nucleic acid encoding a Nanobody of the invention.

As mentioned herein, Nanobodies may in particular be characterized by the presence of one or more "Hallmark residues" (as described herein) in one or more of the framework sequences.

Thus, according to one preferred, but non-limiting aspect of the invention, a Nanobody in its broadest sense can be generally defined as a polypeptide comprising:

a) an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 108 according to the Kabat numbering is Q; and/or:
b) an amino acid sequence that is comprised of four framework regions/sequences
interrupted by three complementarity determining regions/sequences, in which the
amino acid residue at position 45 according to the Kabat numbering is a charged amino
acid (as defined herein) or a cysteine residue, and position 44 is preferably an E;
and/or:
c) an amino acid sequence that is comprised of four framework regions/sequences
interrupted by three complementarity determining regions/sequences, in which the
amino acid residue at position 103 according to the Kabat numbering is chosen from
the group consisting of P, R and S, and is in particular chosen from the group consisting
of R and S.
Thus, in a first preferred, but non-limiting aspect, a Nanobody of the invention may
have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to
CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which
a) the amino acid residue at position 108 according to the Kabat numbering is Q;
and/or in which:

b) the amino acid residue at position 45 according to the Kabat numbering is a charged
amino acid or a cysteine and the amino acid residue at position 44 according to the
Kabat numbering is preferably an E;
and/or in which:
c) the amino acid residue at position 103 according to the Kabat numbering is chosen
from the group consisting of P, R and S, and is in particular chosen from the group
consisting of R and S;
and in which:
d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according
to one of the preferred aspects herein, and are more preferably as defined according to
one of the more preferred aspects herein.

In particular a Nanobody in its broadest sense can be generally defined as a
polypeptide comprising:
a) an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 108 according to the Kabat numbering is Q;

and/or:

b) an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;

and/or:

c) an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S.

Thus, according to a preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

\[ \text{FRI} - \text{CDR1} - \text{FR2} - \text{CDR2} - \text{FR3} - \text{CDR3} - \text{FR4} \]

in which FRI to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which

a) the amino acid residue at position 108 according to the Kabat numbering is Q;

and/or in which:

b) the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;

and/or in which:

c) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S;

and in which:

d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.
In particular, a Nanobody against Targets of the invention according to the invention may have the structure:

\[ \text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which

a) the amino acid residue at position 108 according to the Kabat numbering is Q;
and/or in which:

b) the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;
and/or in which:

c) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S;

and in which:

d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In particular, according to one preferred, but non-limiting aspect of the invention, a Nanobody can generally be defined as a polypeptide comprising an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

a-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D5G, Q, R, S, L; and is preferably chosen from the group consisting of G, E or Q; and

a-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R or C; and is preferably chosen from the group consisting of L or R; and

a-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R or S; and is preferably W or R, and is most preferably W;

a-4) the amino acid residue at position 108 according to the Kabat numbering is Q;
or in which:

b-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from
the group consisting of E and Q; and
b-2) the amino acid residue at position 45 according to the Kabat numbering is R; and
b-3) the amino acid residue at position 103 according to the Kabat numbering is chosen
from the group consisting of W, R and S; and is preferably W;

b-4) the amino acid residue at position 108 according to the Kabat numbering is chosen
from the group consisting of Q and L; and is preferably Q;

or in which:

c-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from
the group consisting of A, G, E, D, Q, R, S and L; and is preferably chosen from the
group consisting of G, E and Q; and

c-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from
the group consisting of L, R and C; and is preferably chosen from the group consisting
of L and R; and

c-3) the amino acid residue at position 103 according to the Kabat numbering is chosen
from the group consisting of P, R and S; and is in particular chosen from the group
consisting of R and S; and

c-4) the amino acid residue at position 108 according to the Kabat numbering is chosen
from the group consisting of Q and L; is preferably Q;

and in which

d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according
to one of the preferred aspects herein, and are more preferably as defined according to
one of the more preferred aspects herein.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may
have the structure

\[
\text{FR1} \quad \text{CDR1} \quad \text{FR2} \quad \text{CDR2} \quad \text{FR3} \quad \text{CDR3} \quad \text{FR4}
\]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to
CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:
a-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, G, Q, R, S, L; and is preferably chosen from the group consisting of G, E or Q;

and in which:

a-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R or C; and is preferably chosen from the group consisting of L or R;

and in which:

a-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R or S; and is preferably W or R, and is most preferably W;

and in which:

a-4) the amino acid residue at position 108 according to the Kabat numbering is Q;

and in which:

d) CDRL, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

in another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

\[
\text{FR1 - CDRI - FR2 - CDR2 - FR3 - CDR3 - FR4}
\]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDRI to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

b-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of E and Q;

and in which:

b-2) the amino acid residue at position 45 according to the Kabat numbering is R;

and in which:

b-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R and S; and is preferably W;

and in which:
b-4) the amino acid residue at position 108 according to the Kabat numbering is chosen from the group consisting of Q and L; and is preferably Q; and in which:
d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:
c-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, Q, R, S and L; and is preferably chosen from the group consisting of G, E and Q; and in which:
c-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R and C; and is preferably chosen from the group consisting of L and R; and in which:
c-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S; and is in particular chosen from the group consisting of R and S; and in which:
c-4) the amino acid residue at position 108 according to the Kabat numbering is chosen from the group consisting of Q and L; is preferably Q; and in which:
d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Two particularly preferred, but non-limiting groups of the Nanobodies of the invention are those according to a) above; according to (a-1) to (a-4) above; according to b)
above: according to (b-1) to (b-4) above; according to (c) above; and/or according to (c-1) to (c-4) above, in which either:

i) the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW (or a GLEW-like sequence as described herein) and the amino acid residue at position 108 is Q;

or in which:

ii) the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or KQRE (or a KERE-like sequence as described) and the amino acid residue at position 108 is Q or L, and is preferably Q.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

i) the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW (or a GLEW-like sequence as defined herein) and the amino acid residue at position 108 is Q;

and in which:

ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

i) the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or KQRE (or a KERE-like sequence) and the amino acid residue at position 108 is Q or L, and is preferably Q;
and in which:

ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the Nanobodies of the invention in which the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or KQRE, the amino acid residue at position 37 is most preferably F. In the Nanobodies of the invention in which the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW, the amino acid residue at position 37 is chosen from the group consisting of Y, H, I, L, V or F and is most preferably V.

Thus, without being limited hereto in any way, on the basis of the amino acid residues present on the positions mentioned above, the Nanobodies of the invention can generally be classified on the basis of the following three groups:

i) The “GLEW-group”: Nanobodies with the amino acid sequence GLEW at positions 44-47 according to the Kabat numbering and Q at position 108 according to the Kabat numbering. As further described herein, Nanobodies within this group usually have a V at position 37, and can have a W, P, R or S at position 103, and preferably have a W at position 103. The GLEW group also comprises some GLEW-like sequences such as those mentioned in Table A-3 below. More generally, and without limitation, Nanobodies belonging to the GLEW-group can be defined as Nanobodies with a G at position 44 and/or with a W at position 47, in which position 46 is usually E and in which preferably position 45 is not a charged amino acid residue and not cysteine;

ii) The “KERE-group”: Nanobodies with the amino acid sequence KERE or KQRE (or another KERE-like sequence) at positions 43-46 according to the Kabat numbering and Q or L at position 108 according to the Kabat numbering. As further described herein, Nanobodies within this group usually have a F at position 37, an L or F at position 47, and can have a W, P, R or S at position 103, and preferably have a W at position 103. More generally, and without limitation, Nanobodies belonging to the KERE-group can be defined as Nanobodies with a K, Q or R at position 44 (usually K) in which position 45 is a charged amino acid residue or cysteine, and position 47 is as further defined herein;

iii) The “103 P, R, S-group”: Nanobodies with a P, R or S at position 103. These Nanobodies can have either the amino acid sequence GLEW at positions 44-47
according to the Kabat numbering or the amino acid sequence KERE or KQRE at positions 43-46 according to the Kabat numbering, the latter most preferably in combination with an F at position 37 and an L or an F at position 47 (as defined for the KERE-group); and can have Q or L at position 108 according to the Kabat numbering, and preferably have Q.

Also, where appropriate, Nanobodies may belong to (i.e. have characteristics of) two or more of these classes. For example, one specifically preferred group of Nanobodies has GLEW or a GLEW-like sequence at positions 44-47; P, R or S (and in particular R) at position 103; and Q at position 108 (which may be humanized to L).

More generally, it should be noted that the definitions referred to above describe and apply to Nanobodies in the form of a native (i.e. non-humanized) \( V_{HH} \) sequence, and that humanized variants of these Nanobodies may contain other amino acid residues than those indicated above (i.e. one or more humanizing substitutions as defined herein). For example, and without limitation, in some humanized Nanobodies of the GLEW-group or the 103 P, R, S-group, Q at position 108 may be humanized to 108L. As already mentioned herein, other humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring \( V_{HH} \) sequence with the corresponding framework sequence of one or more closely related human \( V_{H} \) sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said \( V_{HH} \) sequence (in any manner known per se, as further described herein) and the resulting humanized \( V_{HH} \) sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) a Nanobody may be partially humanized or fully humanized.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may be a Nanobody belonging to the GLEW-group (as defined herein), and in which CDRI, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.
In another preferred, but non-limiting aspect, a Nanobody of the invention may be a Nanobody belonging to the KERE-group (as defined herein), and CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may be a Nanobody belonging to the 103 P, R, S-group (as defined herein), and in which CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Also, more generally and in addition to the 108Q, 43E/44R and 103 P,R,S residues mentioned above, the Nanobodies of the invention can contain, at one or more positions that in a conventional \( V_H \) domain would form (part of) the \( V_H/V_L \) interface, one or more amino acid residues that are more highly charged than the amino acid residues that naturally occur at the same position(s) in the corresponding naturally occurring \( V_H \) sequence, and in particular one or more charged amino acid residues (as mentioned in Table A-2). Such substitutions include, but are not limited to, the GLEW-like sequences mentioned in Table A-3 below; as well as the substitutions that are described in the International Application WO 00/29004 for so-called "microbodies", e.g. so as to obtain a Nanobody with Q at position 108 in combination with KLEW at positions 44-47. Other possible substitutions at these positions will be clear to the skilled person based upon the disclosure herein.

In one aspect of the Nanobodies of the invention, the amino acid residue at position 83 is chosen from the group consisting of L, M, S, V and W; and is preferably L.

Also, in one aspect of the Nanobodies of the invention, the amino acid residue at position 83 is chosen from the group consisting of R, K, N, E, G, I, T and Q; and is most preferably either K or E (for Nanobodies corresponding to naturally occurring \( V_{H\text{H}} \) domains) or R (for "humanized" Nanobodies, as described herein). The amino acid residue at position 84 is chosen from the group consisting of P, A, R, S, D, T, and V in one aspect, and is most preferably P (for Nanobodies corresponding to naturally occurring \( V_{H\text{H}} \) domains) or R (for "humanized" Nanobodies, as described herein).

Furthermore, in one aspect of the Nanobodies of the invention, the amino acid residue at position 104 is chosen from the group consisting of G and D; and is most preferably G.
Collectively, the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108, which in the Nanobodies are as mentioned above, will also be referred to herein as the ‘Hallmark Residues’. The Hallmark Residues and the amino acid residues at the corresponding positions of the most closely related human V\text{H} domain, V\text{H}3, are summarized in Table A-3.

Some especially preferred but non-limiting combinations of these Hallmark Residues as occur in naturally occurring V\text{H} domains are mentioned in Table A-4. For comparison, the corresponding amino acid residues of the human V\text{H}3 called DP-47 have been indicated in italics.
Table A-3: Hallmark Residues in Nanobodies

<table>
<thead>
<tr>
<th>Position</th>
<th>Human V_{H}3</th>
<th>Hallmark Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>L, V; predominantly L</td>
<td>L, M, S, V, W; preferably L</td>
</tr>
<tr>
<td>37</td>
<td>V, I, F; usually V</td>
<td>P^{(1)}, Y, H, I, L or V, preferably P^{(1)} or Y</td>
</tr>
<tr>
<td>44^{(8)}</td>
<td>G</td>
<td>G^{(2)}, E^{(3)}, A, D, Q, R, S, L; preferably G^{(2)}, E^{(3)} or Q; most preferably G^{(2)} or E^{(3)}</td>
</tr>
<tr>
<td>45^{(8)}</td>
<td>L</td>
<td>L^{(2)}, R^{(3)}, C, I, L, P, Q, V; preferably L^{(2)} or R^{(3)}</td>
</tr>
<tr>
<td>47^{(8)}</td>
<td>W, Y</td>
<td>W^{(2)}, L^{(4)} or F^{(1)}, A, G, I, M, R, S, V or Y; preferably W^{(2)}, L^{(4)}, F^{(1)} or R</td>
</tr>
<tr>
<td>83</td>
<td>R or K; usually R</td>
<td>R, K^{(5)}, N, E^{(5)}, G, I, M, Q or T; preferably K or R; most preferably K</td>
</tr>
<tr>
<td>84</td>
<td>A, T, D; predominantly A</td>
<td>P^{(5)}, A, L, R, S, T, D, V; preferably P</td>
</tr>
<tr>
<td>103</td>
<td>W</td>
<td>W^{(4)}, P^{(6)}, R^{(6)}, S; preferably W</td>
</tr>
<tr>
<td>104</td>
<td>G</td>
<td>G or D; preferably G</td>
</tr>
<tr>
<td>108</td>
<td>L, M or T; predominantly L</td>
<td>Q, L^{(7)} or R; preferably Q or L^{(7)}</td>
</tr>
</tbody>
</table>

Notes:

(1) In particular, but not exclusively, in combination with KERE or KQRE at positions 43-46.

(2) Usually as GLEW at positions 44-47.

(3) Usually as KERE or KQRE at positions 43-46, e.g. as KERE, KEREL, KQREL, KQREF or KEREG at positions 43-47. Alternatively, also sequences such as TERE (for example TEREL), KECE (for example KECEL or KECER), RERE (for example RERE), QERE (for example QEREQ), KGRE (for example KGREG), KDRE (for example KDREV) are possible. Some other possible, but less preferred sequences include for example DECKL and NVCEL.

(4) With both GLEW at positions 44-47 and KERE or KQRE at positions 43-46.

(5) Often as KP or EP at positions 83-84 of naturally occurring V_{H}H domains.

(6) In particular, but not exclusively, in combination with GLEW at positions 44-47.

(7) With the proviso that when positions 44-47 are GLEW, position 108 is always Q in (non-humanized) V_{H}H sequences that also contain a W at 103.

(8) The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW, EPEW, GLER, DQEW, DLEW, GIEW, ELEW, GPEW, EWLP, GPER, GLER and ELEW.
Table A-4: Some preferred but non-limiting combinations of Hallmark Residues in naturally occurring Nanobodies.

For humanization of these combinations, reference is made to the specification.

<table>
<thead>
<tr>
<th></th>
<th>11</th>
<th>37</th>
<th>44</th>
<th>45</th>
<th>47</th>
<th>83</th>
<th>84</th>
<th>103</th>
<th>104</th>
<th>108</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP-47 (human)</td>
<td>M</td>
<td>V</td>
<td>G</td>
<td>L</td>
<td>W</td>
<td>R</td>
<td>A</td>
<td>W</td>
<td>G</td>
<td>L</td>
</tr>
<tr>
<td>&quot;KERE&quot; group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>E</td>
<td>R</td>
<td>L</td>
<td>K</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>E</td>
<td>R</td>
<td>F</td>
<td>E</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>E</td>
<td>R</td>
<td>F</td>
<td>K</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Y</td>
<td>Q</td>
<td>R</td>
<td>L</td>
<td>K</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>L</td>
<td>R</td>
<td>V</td>
<td>K</td>
<td>P</td>
<td>Q</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>Q</td>
<td>R</td>
<td>L</td>
<td>K</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>E</td>
<td>R</td>
<td>F</td>
<td>K</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td>&quot;GLEW&quot; group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>V</td>
<td>G</td>
<td>L</td>
<td>W</td>
<td>K</td>
<td>S</td>
<td>W</td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>V</td>
<td>G</td>
<td>L</td>
<td>W</td>
<td>K</td>
<td>P</td>
<td>R</td>
<td>G</td>
<td>Q</td>
</tr>
</tbody>
</table>
In the Nanobodies, each amino acid residue at any other position than the Hallmark Residues can be any amino acid residue that naturally occurs at the corresponding position (according to the Kabat numbering) of a naturally occurring $V_{HH}$ domain.

Such amino acid residues will be clear to the skilled person. Tables A-5 to A-8 mention some non-limiting residues that can be present at each position (according to the Kabat numbering) of the FR1, FR2, FR3 and FR4 of naturally occurring $V_{HH}$ domains. For each position, the amino acid residue that most frequently occurs at each position of a naturally occurring $V_{HH}$ domain (and which is the most preferred amino acid residue for said position in a Nanobody) is indicated in bold: and other preferred amino acid residues for each position have been underlined (note: the number of amino acid residues that are found at positions 26-30 of naturally occurring $V_{HH}$ domains supports the hypothesis underlying the numbering by Chothia (supra) that the residues at these positions already form part of CDR1).

In Tables A-5 - A-8, some of the non-limiting residues that can be present at each position of a human V$_{\text{J3}}$ domain have also been mentioned. Again, for each position, the amino acid residue that most frequently occurs at each position of a naturally occurring human $V_{H}$ domain is indicated in bold; and other preferred amino acid residues have been underlined.

For reference only, Tables A-5-A-8 also contain data on the $V_{H}$ entropy (“$V_{HH}$ Ent”) and $V_{HH}$ variability (“$V_{HH}$ Var.”) at each amino acid position for a representative sample of 1118 $V_{HH}$ sequences (data kindly provided by David Lutje Hulsing and Prof. Theo Verrips of Utrecht University). The values for the $V_{HH}$ entropy and the $V_{HH}$ variability provide a measure for the variability and degree of conservation of amino acid residues between the 1118 $V_{H}$ sequences analyzed: low values (i.e. <1, such as < 0.5) indicate that an amino acid residue is highly conserved between the $V_{HH}$ sequences (i.e. little variability). For example, the G at position 8 and the G at position 9 have values for the $V_{H}$ entropy of 0.1 and 0 respectively, indicating that these residues are highly conserved and have little variability (and in case of position 9 is G in all 1118 sequences analysed), whereas for residues that form part of the CDR’s generally values of 1.5 or more are found (data not shown). Note that (1) the amino acid residues listed in the second column of Tables A-5-A-8 are based on a bigger sample than the 1118 $V_{HH}$ sequences that were analysed for determining the $V_{HH}$ entropy and $V_{HH}$ variability referred to in the last two columns; and (2) the data represented below support the hypothesis that the amino acid residues at positions 27-30 and maybe even also at positions 93 and 94 already form part of the CDR’s (although the invention is not limited to any specific hypothesis or explanation, and as mentioned above, herein the numbering
according to Kabat is used). For a general explanation of sequence entropy, sequence variability and the methodology for determining the same, see Oliveira et al. PROTEINS: Structure. Function and Genetics, 52: 544-552 (2003).

Table A-5: Non-limiting examples of amino acid residues in FRI (for the footnotes, see the footnotes to Table A-3)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Human V_{HH}$</td>
<td>$Camelid V_{HH}$'s</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>E, Q</td>
<td>Q, A, E</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>V</td>
<td>V</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>Q</td>
<td>Q, K</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>L</td>
<td>L</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>V, L</td>
<td>Q, E, L, V</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>E</td>
<td>E, D, Q, A</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>S, T</td>
<td>S, F</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>G, R</td>
<td>G</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>G</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>G, V</td>
<td>G, D, R</td>
<td>0.3</td>
</tr>
<tr>
<td>11</td>
<td><strong>Hallmark residue:</strong></td>
<td>L, M, S, V, W; preferably L</td>
<td>0.8</td>
</tr>
<tr>
<td>12</td>
<td>V, I</td>
<td>V, A</td>
<td>0.2</td>
</tr>
<tr>
<td>13</td>
<td>Q, K, R</td>
<td>Q, E, K, P, R</td>
<td>0.4</td>
</tr>
<tr>
<td>14</td>
<td>P</td>
<td>A, Q, A, G, P, S, T, V</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>G</td>
<td>G</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>G, R</td>
<td>G, A, E, D</td>
<td>0.4</td>
</tr>
<tr>
<td>17</td>
<td>S</td>
<td>S, F</td>
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</tr>
<tr>
<td>18</td>
<td>L</td>
<td>L, V</td>
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<td>19</td>
<td>R, K</td>
<td>R, K, L, N, S, T</td>
<td>0.6</td>
</tr>
<tr>
<td>20</td>
<td>L</td>
<td>L, F, I, V</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>S</td>
<td>S, A, F, T</td>
<td>0.2</td>
</tr>
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<td>22</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>A, T</td>
<td>A, D, E, P, S, T, V</td>
<td>1.3</td>
</tr>
<tr>
<td>24</td>
<td>A</td>
<td>A, I, L, S, T, V</td>
<td>1</td>
</tr>
</tbody>
</table>
Table A-5: Non-limiting examples of amino acid residues in FR1 (continued)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>S</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>26</td>
<td>G</td>
<td>0.7</td>
<td>7</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>2.3</td>
<td>13</td>
</tr>
<tr>
<td>28</td>
<td>T</td>
<td>1.7</td>
<td>11</td>
</tr>
<tr>
<td>29</td>
<td>F, V</td>
<td>1.9</td>
<td>11</td>
</tr>
<tr>
<td>30</td>
<td>S, D, G</td>
<td>1.8</td>
<td>11</td>
</tr>
</tbody>
</table>

Table A-6: Non-limiting examples of amino acid residues in FR2 (for the footnotes, see the footnotes to Table A-3)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>W</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>37</td>
<td><strong>Hallmark residue:</strong> F(1), H, L, Y or V, preferably F(1) or Y</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>38</td>
<td>R</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>39</td>
<td>Q</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>A</td>
<td>0.9</td>
<td>7</td>
</tr>
<tr>
<td>41</td>
<td>P, S, T</td>
<td>0.4</td>
<td>3</td>
</tr>
<tr>
<td>42</td>
<td>G</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>43</td>
<td>K</td>
<td>0.7</td>
<td>6</td>
</tr>
<tr>
<td>44</td>
<td><strong>Hallmark residue:</strong> G(2), E(3), A, D, Q, R, S, L; preferably G(2), E(3) or Q; most preferably G(2) or E(3)</td>
<td>1.3</td>
<td>5</td>
</tr>
<tr>
<td>45</td>
<td><strong>Hallmark residue:</strong> L(2), R(3), C, I, L, P, Q, V; preferably L(2) or R(3)</td>
<td>0.6</td>
<td>4</td>
</tr>
<tr>
<td>46</td>
<td>E, V</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>47</td>
<td><strong>Hallmark residue:</strong> W(2), L(1) or F(1), A, G, I, M, R, S, V or Y; preferably W(2), L(1), F(1) or R</td>
<td>1.9</td>
<td>9</td>
</tr>
<tr>
<td>48</td>
<td>V</td>
<td>0.4</td>
<td>3</td>
</tr>
<tr>
<td>49</td>
<td>S, A, G</td>
<td>0.8</td>
<td>3</td>
</tr>
</tbody>
</table>
Table A-7: Non-limiting examples of amino acid residues in FR3 (for the footnotes, see the footnotes to Table A-3)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>R</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>67</td>
<td>F</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>68</td>
<td>T</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>69</td>
<td>I</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>70</td>
<td>S</td>
<td>0.3</td>
<td>4</td>
</tr>
<tr>
<td>71</td>
<td>R</td>
<td>1.2</td>
<td>8</td>
</tr>
<tr>
<td>72</td>
<td>D, E</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>73</td>
<td>N, D, G</td>
<td>1.2</td>
<td>9</td>
</tr>
<tr>
<td>74</td>
<td>A, S</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>75</td>
<td>K</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>76</td>
<td>N, S</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>77</td>
<td>S, T, I</td>
<td>0.8</td>
<td>5</td>
</tr>
<tr>
<td>78</td>
<td>L, A</td>
<td>1.2</td>
<td>5</td>
</tr>
<tr>
<td>79</td>
<td>Y, H</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>80</td>
<td>L</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>81</td>
<td>Q</td>
<td>0.6</td>
<td>5</td>
</tr>
<tr>
<td>82</td>
<td>M</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>82a</td>
<td>N, G</td>
<td>0.8</td>
<td>4</td>
</tr>
<tr>
<td>82b</td>
<td>S</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>82c</td>
<td>L</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>83</td>
<td><strong>Hallmark residue:</strong> R, K$^{(5)}$, N, E$^{(5)}$, G, I, M, Q or T; preferably K or R; most preferably K</td>
<td>0.9</td>
<td>7</td>
</tr>
<tr>
<td>84</td>
<td><strong>Hallmark residue:</strong> P$^{(5)}$, A, D, L, R, S, T, V; preferably P</td>
<td>0.7</td>
<td>6</td>
</tr>
<tr>
<td>85</td>
<td>E, G</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>86</td>
<td>D</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>87</td>
<td>T, M</td>
<td>0.2</td>
<td>3</td>
</tr>
</tbody>
</table>
Table A-7: Non-limiting examples of amino acid residues in FR3 (continued)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>Camelid $V_{HH}$'s</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>A</td>
<td>A, G, S</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>89</td>
<td>V, L</td>
<td>V, A, D, I, L, M, N, R, T</td>
<td>1.4</td>
<td>6</td>
</tr>
<tr>
<td>90</td>
<td>Y</td>
<td>Y, F</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>91</td>
<td>Y, H</td>
<td>Y, D, F, H, L, S, T, V</td>
<td>0.6</td>
<td>4</td>
</tr>
<tr>
<td>92</td>
<td>C</td>
<td>C</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A-8: Non-limiting examples of amino acid residues in FR4 (for the footnotes, see the footnotes to Table A-3)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Amino acid residue(s):</th>
<th>Camelid $V_{HH}$'s</th>
<th>$V_{HH}$ Ent.</th>
<th>$V_{HH}$ Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
<td><strong>Hallmark residue:</strong> W(4), P(6), R(6), S; preferably W</td>
<td></td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>104</td>
<td><strong>Hallmark residue:</strong> G or D; preferably G</td>
<td></td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>105</td>
<td>Q, R</td>
<td>Q, E, K, P, R</td>
<td>0.6</td>
<td>4</td>
</tr>
<tr>
<td>106</td>
<td>G</td>
<td>G</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>107</td>
<td>T</td>
<td>T, A, I</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>108</td>
<td><strong>Hallmark residue:</strong> Q, L(7) or R; preferably Q or L(1)</td>
<td></td>
<td>0.4</td>
<td>3</td>
</tr>
<tr>
<td>109</td>
<td>V</td>
<td>V</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>110</td>
<td>T</td>
<td>T, I, A</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>111</td>
<td>V</td>
<td>V, A, I</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>112</td>
<td>S</td>
<td>S, F</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>113</td>
<td>S</td>
<td>S, A, L, P, T</td>
<td>0.4</td>
<td>3</td>
</tr>
</tbody>
</table>
Thus, in another preferred, but not limiting aspect, a Nanobody of the invention can be defined as an amino acid sequence with the (general) structure

\[
\text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4}
\]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

1) one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3;

and in which:

ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be \(V_{\text{H}}\) sequences or may be humanized Nanobodies. When the above Nanobody sequences are \(V_{\text{FR}}\) sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

In particular, a Nanobody of the invention can be an amino acid sequence with the (general) structure

\[
\text{FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4}
\]

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

i) (preferably) one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 (it being understood that \(V_{\text{HH}}\) sequences will contain one or more Hallmark residues: and that partially humanized Nanobodies will usually, and preferably, [still] contain one or more Hallmark residues [although it is also within the scope of the invention to provide - where suitable in accordance with the invention -
partially humanized Nanobodies in which all Hallmark residues, but not one or more of
the other amino acid residues, have been humanized]; and that in fully humanized
Nanobodies, where suitable in accordance with the invention, all amino acid residues at
the positions of the Hallmark residues will be amino acid residues that occur in a human
\( V_H^3 \) sequence. As will be clear to the skilled person based on the disclosure herein that
such \( V_H^3 \) sequences, such partially humanized Nanobodies with at least one Hallmark
residue, such partially humanized Nanobodies without Hallmark residues and such fully
humanized Nanobodies all form aspects of this invention);

and in which:

ii) said amino acid sequence has at least 80% amino acid identity with at least one of the
amino acid sequences of SEQ ID NO's: 1 to 22, in which for the purposes of
determining the degree of amino acid identity, the amino acid residues that form the
CDR sequences (indicated with X in the sequences of SEQ ID NO's: 1 to 22) are
disregarded;

and in which:

iii) CDRI, CDR2 and CDR3 are as defined herein, and are preferably as defined according
to one of the preferred aspects herein, and are more preferably as defined according to
one of the more preferred aspects herein.

The above Nanobodies may for example be \( V_H^3 \) sequences or may be humanized
Nanobodies. When the above Nanobody sequences are \( V_H^1 \) sequences, they may be suitably
humanized, as further described herein. When the Nanobodies are partially humanized
Nanobodies, they may optionally be further suitably humanized, again as described herein.
Table A-9: Representative amino acid sequences for Nanobodies of the KERE, GLEW and P,R,S 103 group.
The CDR’s are indicated with XXXXX

<table>
<thead>
<tr>
<th>Sequence No.</th>
<th>SEQ ID NO</th>
<th>Sequence 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>KERE sequence no. 1</td>
<td>SEQ ID NO-1</td>
<td>EVQLVESGGGLVQPGGLRLSRSLCAAASGIPFSXXXWFRQAPGKQRDSVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 2</td>
<td>SEQ ID NO 2</td>
<td>QVKLEESGGGLVQAGGSRLSCVSGRTFSXXXWFRLAPGKEREFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 3</td>
<td>SEQ ID NO-3</td>
<td>AVQLVDGGGGGQLVAGSDLSKCALTSAGFXXXXWFQGTRPGWGERFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 4</td>
<td>SEQ ID NO.4</td>
<td>QVQLVESGGGLVEAGGLRLSTCSTSESFRXXXWFRQTSQGERFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 5</td>
<td>SEQ ID NO.5</td>
<td>AVQLVESGGGLVQAGGLRLCAACASERIFDXXXWYRGQPGNERELVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 6</td>
<td>SEQ ID NO 6</td>
<td>DVKPFVESGGGLVQAGGLRLSCVAGFNFXXXWFRQAPGKEREVEAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 7</td>
<td>SEQ ID NO:7</td>
<td>QVRLEAESGGGLVQSGGLRLSCVAGSTYTXXXWYRGYPQKDALVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 8</td>
<td>SEQ ID NO:8</td>
<td>EVQLVESGGGLVQAGGLRLSCAASGFTSDXXXWFRQAPGKPGVXSXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 9</td>
<td>SEQ ID NO 9</td>
<td>QVQLVESGGGLVQPGGLRLSCQAGSDISTXXXWYRQYPGKLRERFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 10</td>
<td>SEQ ID NO:10</td>
<td>QVQYVEESGGGLVQAGDSRLFCAVPSFSTXXXXWFQAPGKERFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no. 11</td>
<td>SEQ ID NO 11</td>
<td>EVQLVESGGGLVQAGDSRLFCVTSSGTASXXXWFQAPGKEFVAXXXXXXRTI</td>
</tr>
<tr>
<td>KERE sequence no</td>
<td>SEQ ID NO.</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>AVQLVESGGSVQPDSQTLSCAASGRTNSXXXXXXWFROAGPKERFVLAXXXXXXRTF ISRDASKNMITYQMNKLQQTDAYYCAAXXXXXXWGQGTVQVVSS</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>AVQLVESGGGLVQAGSSLRSCLVQSLTSSXXXXXXWFRQTPWTERDFVAXXXXXXRTF ISRDNYKDTLLEMNFLKPEDTAIYCAAXXXXXXWGQGTVQVVSS</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>AVQLVESGGGLVQAGASLRLSCATSTRTLDXXXXWFROAPGRDREFVAXXXXXXRTF VSRDAAENTVALQMNSLKPEDTAYYCAAXXXXXXWGQGTVQVVSS</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>QVQLVESGGGLVQPFGSLRLSCTVSLTAAIXXXXWFROAPGKEREAVXXXXXRFTI SRYAGNTALIQMDSLKPEDTVQYCATXAXXXXXXWGQGTVQVVSS</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>EVQLVESGGGLVQAGSSLKLSCATASGRNFVXXXXXXFRRAPGKEREFEVXXXXXRTF VSRDNKNTAYLRMNLSLKPEDTYAANVXXAXLSQGTVQVVSS</td>
</tr>
<tr>
<td>GLEW sequence no</td>
<td>1</td>
<td>AVQLVESGGGSVQPGGLRSLCAASGFTFSXXXXXXVRQAPGKREWVSSXXXXXRTF ISRDNKNTLYLQMNSLKPEDTVYYCAAXXXXXXGSGQTVQVVSS</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>EVQLVESGGGSVQPGGLRSLSCVSGCTXVXXXXXXVRQAPGKAEEVWSSXXXXXRF KISRDAAKTLQYMNLSLKPEDTAMYCYQRXXXXXXRQGTVQVVSS</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>EVQLVESGGGLAPGSILTSCVFGSTFSXXXXXXVVRHTPGKEEWSXXXXXRTI SRDNKNTLYLQMNLSLKPEDTVYYCGRXXXAXRXSGQTVQVVSS</td>
</tr>
<tr>
<td>P,R,S 103 sequence no</td>
<td>20</td>
<td>AVQLVESGGGLVQAGSSLRLSCAASGRTFSXXXXXXWFROAPGKEREFVAXXXXXXRTF ISRDNKNTLYLQMNLSLKPEDTVYYCAAXXXXXXRGQTVQVVSS</td>
</tr>
<tr>
<td>P,R,S 103 sequence no</td>
<td>21</td>
<td>DVQLVESGGDLVQPGSGLRSCLASGFSDFXXXWWLRTQTPGKLEGVSXXXXXRTF ISRDNKNTLYLQMNLSLKPEDTVYYCGRXXXAXRXSGQTVQVVSS</td>
</tr>
<tr>
<td>P,R,S 103 sequence no</td>
<td>22</td>
<td>EVQLVESGGGLVQPGSGLRSCLVQSGCTXVXXXXXXVRQAPGKAEEVWSSXXXXXRF KISRDAAKTLQYMNLSLKPEDTAMYCYQRXXXXXXRQGTVQVVSS</td>
</tr>
</tbody>
</table>
In particular, a Nanobody of the invention of the KERE group can be an amino acid sequence with the (general) structure

\[
\text{FR}_1 - \text{CDR}_1 - \text{FR}_2 - \text{CDR}_2 - \text{FR}_3 - \text{CDR}_3 - \text{FR}_4
\]

in which:

i) the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid (as defined herein) or a cysteine residue, and position 44 is preferably an E;

and in which:

ii) FR1 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

| KERE FW1 sequence no | SEQ ID NO | Seq.
|----------------------|-----------|-----
| 1                    | 23        | QVQRVESGGGLVQAGGLRAASQRTS |
| 2                    | 24        | QVQLVESGGLVQAGDSRLCAATGRAFG |
| 3                    | 25        | QVKLESSEGGGLVQAGDSRLCAATGRAFG |
| 4                    | 26        | AVQLVESGGLVQPGESLGLSCASGRTS |
| 5                    | 27        | EVQLVESGGLVQAGLSCLCEVLRGRTAG |
| 6                    | 28        | QVQLVESGGWVQPGSRLCSAASSTLS |
| 7                    | 29        | QVQLVESGTTVQPGSQLCSVSGNTFN |
| 8                    | 30        | EVQLVESQUALQPGSSLQSLCSAPGFTLD |
| 9                    | 31        | AQEELVEGGGLVQAGGLRALCSAGRTFN |

iii) FR2 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:
and in which:

iv) FR3 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

<table>
<thead>
<tr>
<th>KERE FW2 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>WFRQAPGKEREFVA</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>WFRGTPGKERERFVA</td>
</tr>
<tr>
<td>3</td>
<td>43</td>
<td>WYRQAPGKQREMVA</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>WYRGPGKQRELVA</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>WIRQAPGKEREGVS</td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>WFRAPGKEREKIS</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>WYRQAPGKERDLVA</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>WFRQAPGQREEVS</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>WFRQPPGKVREFVG</td>
</tr>
</tbody>
</table>

Table A-12: Representative FW3 sequences for Nanobodies of the KERE-group.

<table>
<thead>
<tr>
<th>KERE FW3 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>RFTISRDNAKNTYLVQMNSLKPEDTAVYRCYF</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>RFAISRDNKKNTGYLVQMNSLPEPDTAVYYCAA</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>RFTVARNNAKNTVNLVENSLKPEDTAVYYCAA</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>RFTISDLIKNTVDELNMNLEPEDTAVYYCAA</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>RLTISRDNAVDMYLVQMNSLKPEDTAVYYCAA</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>RFTISRDNAKNTYLVQMDNVPEDTAIVYCAA</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>RFTISKSQKNTVYLVQMTSLKPEDTAVYCAT</td>
</tr>
<tr>
<td>8</td>
<td>57</td>
<td>RFTISRDSAKNMMLYLVQNNLKPQDTAVYYCAA</td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>RFTISREKSVTVYLQNLKPEDTAVYYCAA</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>RFTISRDYAGNTAYLVQMNSLKPEDTGVYYCAT</td>
</tr>
</tbody>
</table>

and in which:

v) FR4 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:
Table A-13: Representative FW4 sequences for Nanobodies of the KERE-group.

<table>
<thead>
<tr>
<th>KERE FW4 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>WQGTOVTVSS</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>WGGKTLTVSS</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>RGGTRVTSS</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>WGLGTOIS</td>
</tr>
</tbody>
</table>

and in which:

vi) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{\text{HH}} sequences or partially humanized Nanobodies).

Also, the above Nanobodies may for example be V_{\text{HH}} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{\text{HH}} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

With regard to framework 1, it will be clear to the skilled person that, when an amino acid sequence as outlined above is generated by expression of a nucleotide sequence, the first four amino acid sequences (i.e. amino acid residues 1-4 according to the Kabat numbering) may often be determined by the primer(s) that have been used to generate said nucleic acid. Thus, for determining the degree of amino acid identity, the first four amino acid residues are preferably disregarded.

Also, with regard to framework 1, and although amino acid positions 27 to 30 are according to the Kabat numbering considered to be part of the framework regions (and not the CDR’s), it has been found by analysis of a database of more than 1000 V_{\text{HH}} sequences that the positions 27 to 30 have a variability (expressed in terms of V_{\text{HH}} variability - see Tables A-5 to A-8) that is much greater than the variability on positions 1 to 26. Because of this, for determining the degree of amino acid identity, the amino acid residues at positions 27 to 30 are preferably also disregarded.
In view of this, a Nanobody of the KERE class may be an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

i) the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid (as defined herein) or a cysteine residue, and position 44 is preferably an E; and in which:

ii) FR1 is an amino acid sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following amino acid sequences:

<table>
<thead>
<tr>
<th>KERE FW1 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>32</td>
<td>VESGGGLVQPQGSLRLSCAAAG</td>
</tr>
<tr>
<td>11</td>
<td>33</td>
<td>VDSGGGVLQAGDSKLSCALTG</td>
</tr>
<tr>
<td>12</td>
<td>34</td>
<td>VDSGGGLVQAGDSLRSCAASG</td>
</tr>
<tr>
<td>13</td>
<td>35</td>
<td>VDSGGGLVQAGDSLRSCAASG</td>
</tr>
<tr>
<td>14</td>
<td>36</td>
<td>QDSGGGSVQAGGSLRSACSG</td>
</tr>
<tr>
<td>15</td>
<td>37</td>
<td>VQSGGRLVQAGDSLRSCAASE</td>
</tr>
<tr>
<td>16</td>
<td>38</td>
<td>VESGGTLVQSGDSLKLSCASST</td>
</tr>
<tr>
<td>17</td>
<td>39</td>
<td>MSGGDSVVQSGGSLTLSCVAG</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>QASGGGLVQAGGSLRLSCASAV</td>
</tr>
</tbody>
</table>

and in which:

iii) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the KERE-class;

and in which:

iv) CDRL, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be $V_{HH}$ sequences or may be humanized Nanobodies. When the above Nanobody sequences are $V_{HH}$ sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.
A Nanobody of the GLEW class may be an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which

i) preferably, when the Nanobody of the GLEW-class is a non-humanized Nanobody, the amino acid residue in position 108 is Q;

ii) FR1 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

| Table A-15: Representative FW1 sequences for Nanobodies of the GLEW-group. |
|---------------------------------|-----------------|---------------------------------|
| GLEW FW1 sequence no. 1        | SEQ ID NO:64    | QVQLVESGGGLVQPGGLSLCANGFTS      |
| GLEW FW1 sequence no. 2        | SEQ ID NO:65    | EVHLVESGGGLVQPGGLSLCAAFGFIK     |
| GLEW FW1 sequence no. 3        | SEQ ID NO:66    | QVKLEESGGGLAQPGGLSLCVASGFTS     |
| GLEW FW1 sequence no. 4        | SEQ ID NO:67    | EVQLVESGGGLVQPGGLSLCVSGGCT      |
| GLEW FW1 sequence no. 5        | SEQ ID NO:68    | EVQLVESGGGLALPGGLSLCVSFSGSTFS   |

and in which:

iii) FR2 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

| Table A-16: Representative FW2 sequences for Nanobodies of the GLEW-group. |
|---------------------------------|-----------------|---------------------------------|
| GLEW FW2 sequence no. 1        | SEQ ID NO:72    | WVRQAPGKVELEWVS                 |
| GLEW FW2 sequence no. 2        | SEQ ID NO:73    | WVRQAPGKGELEWVS                 |
| GLEW FW2 sequence no. 3        | SEQ ID NO:74    | WVRQAPGMELEWVS                  |
| GLEW FW2 sequence no. 4        | SEQ ID NO:75    | WVRQAPGKEPEWVS                  |
| GLEW FW2 sequence no. 5        | SEQ ID NO:76    | WVRQAPGKDPEWVS                  |
| GLEW FW2 sequence no. 6        | SEQ ID NO:77    | WVRQAPGKEWVS                    |
| GLEW FW2 sequence no. 7        | SEQ ID NO:78    | WVRQAPGKACWVS                   |
| GLEW FW2 sequence no. 8        | SEQ ID NO:79    | WVRQAPGRATEWVS                  |

and in which:

iv) FR3 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:
Table A-17: Representative FW3 sequences for Nanobodies of the GLEW-group.

<table>
<thead>
<tr>
<th>GLEW FW3 sequence no</th>
<th>SEQ ID NO:</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>RFTISRDNAKNTLYQMNLKPEDTAVYYCVK</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>RFTISRDNARNTLYQMDSLIPEDTALYYCAR</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>RFTSSRDNAKSTLYQMNLKPEDTALYYCAR</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>RFIISRDNAKNTLYQMNSLGPEDTAMYYCQR</td>
</tr>
<tr>
<td>5</td>
<td>84</td>
<td>RFTASRDNAKNTLYQMNSLKSSTARYYYCAR</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>RFTISRDNAKNTLYQMDDLQSEDSTAMYYCGR</td>
</tr>
</tbody>
</table>

and in which:

v) FR4 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

Table A-18: Representative FW4 sequences for Nanobodies of the GLEW-group.

<table>
<thead>
<tr>
<th>GLEW FW4 sequence no</th>
<th>SEQ ID NO:</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>GSQGQTQTVSS</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>LRGGTQTVSS</td>
</tr>
<tr>
<td>3</td>
<td>88</td>
<td>RGQGTQTVSS</td>
</tr>
<tr>
<td>4</td>
<td>89</td>
<td>RSRGIQTVSS</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>WGGQTQTVSS</td>
</tr>
<tr>
<td>6</td>
<td>91</td>
<td>WGGGTQTVSS</td>
</tr>
</tbody>
</table>

and in which:

vi) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

With regard to framework 1, it will again be clear to the skilled person that, for determining the degree of amino acid identity, the amino acid residues on positions 1 to 4 and 27 to 30 are preferably disregarded.

In view of this, a Nanobody of the GLEW class may be an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:
i) preferably, when the Nanobody of the GLEW-class is a non-humanized Nanobody, the amino acid residue in position 108 is Q;
and in which:

ii) FR1 is an amino acid sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following amino acid sequences:

<table>
<thead>
<tr>
<th>GLEW FW1 sequence no.</th>
<th>SEQ ID NO:</th>
<th>VESGGGLVQPGGSLRLSCASAQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLEW FW1 sequence no.</td>
<td>6</td>
<td>VESGGGLVQPGGSLRLSCASAQ</td>
</tr>
<tr>
<td>GLEW FW1 sequence no.</td>
<td>7</td>
<td>EESGGGLAQPGGLRLSCVASAQ</td>
</tr>
<tr>
<td>GLEW FW1 sequence no.</td>
<td>8</td>
<td>VESGGGLALPGGSLTLCVFGS</td>
</tr>
</tbody>
</table>

and in which:

iii) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the GLEW-class;
and in which:

iv) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be $V_{HH}$ sequences or may be humanized Nanobodies. When the above Nanobody sequences are $V_{HH}$ sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein. In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are $V_{HH}$ sequences or partially humanized Nanobodies).

A Nanobody of the P, R, S 103 class may be an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which

i) the amino acid residue at position 103 according to the Kabat numbering is different from W; and in which:
ii) preferably the amino acid residue at position 103 according to the Kabat numbering is P, R or S, and more preferably R;

and in which:

iii) FR1 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

<table>
<thead>
<tr>
<th>P,R, S 103 FW1 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>92</td>
<td>AVQLVESGGLVQAGGLSCASAGRTFS</td>
</tr>
<tr>
<td>2</td>
<td>93</td>
<td>QVQLQESGGGMVPQGGSLSCASGFDGF</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>EVHVLVESGGLVRPGGLRSLCAAAGFIFK</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>QVQLAEGGGQLVQPGGLKLSCASRTIVS</td>
</tr>
<tr>
<td>5</td>
<td>96</td>
<td>QEHLVLVESGLVDIGGLRSLCAAASERIFS</td>
</tr>
<tr>
<td>6</td>
<td>97</td>
<td>QVKLEESGGGLAQPGGLRSLCVASGFTFS</td>
</tr>
<tr>
<td>7</td>
<td>98</td>
<td>EVQLVESGGLVQPGGLRSLCVSSGCT</td>
</tr>
<tr>
<td>8</td>
<td>99</td>
<td>EVQLVESGGLALPGGLSLCFCFSGSTFS</td>
</tr>
</tbody>
</table>

and in which

iv) FR2 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:
and in which:

v) FR3 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

Table A-21: Representative FW2 sequences for Nanobodies of the P,R,S 103-group.

<table>
<thead>
<tr>
<th>P,R,S 103 FW2 sequence no.</th>
<th>SEQ ID NO:</th>
<th>WFRQAPGKEREFVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,R,S 103 FW2 sequence no. 2</td>
<td>102</td>
<td>WVRQAPGKVLEWVS</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 3</td>
<td>103</td>
<td>WVRPPGKLEWVS</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 4</td>
<td>104</td>
<td>WIRQAPGKEREGVS</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 5</td>
<td>105</td>
<td>WVRQYPKEPEWVS</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 6</td>
<td>106</td>
<td>WFRQPPGKEHEFVA</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 7</td>
<td>107</td>
<td>WYRQAPGKRTELVA</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 8</td>
<td>108</td>
<td>WLRQAPGQGLEWVS</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 9</td>
<td>109</td>
<td>WLRQTPGKLEWVG</td>
</tr>
<tr>
<td>P,R,S 103 FW2 sequence no. 10</td>
<td>110</td>
<td>WVRQAPGKAEEFVS</td>
</tr>
</tbody>
</table>

and in which:

vi) FR4 is an amino acid sequence that has at least 80% amino acid identity with at least one of the following amino acid sequences:

Table A-22: Representative FW3 sequences for Nanobodies of the P,R,S 103-group.

<table>
<thead>
<tr>
<th>P,R,S 103 FW3 sequence no.</th>
<th>SEQ ID NO:</th>
<th>RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,R,S 103 FW3 sequence no. 2</td>
<td>112</td>
<td>RFTISRDNAKNRYLQMDSLIPEDTALYYCAR</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 3</td>
<td>113</td>
<td>RFTISRDNAKEMYLQMNNLKTEDTGYYWCGA</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 4</td>
<td>114</td>
<td>RFTISSDSNRMNMYLQMNNLKPEDTAVYYCAA</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 5</td>
<td>115</td>
<td>RFTISRDNAKMLYLHNLLKSEDATVYYCRR</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 6</td>
<td>116</td>
<td>RFTISRDNAKTVYLRLNLSKPEDTAVYSCNL</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 7</td>
<td>117</td>
<td>RFTISRDNAKTVYLQMNSLKPEDTAMMYCOR</td>
</tr>
<tr>
<td>P,R,S 103 FW3 sequence no. 8</td>
<td>118</td>
<td>RFTISRDANGKTATYLMNSLKPEDTADYYCAV</td>
</tr>
</tbody>
</table>
and in which:

vii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are $V_{\text{HH}}$ sequences or partially humanized Nanobodies).

With regard to framework 1, it will again be clear to the skilled person that, for determining the degree of amino acid identity, the amino acid residues on positions 1 to 4 and 27 to 30 are preferably disregarded.

In view of this, a Nanobody of the P,R,S 103 class may be an amino acid sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

i) the amino acid residue at position 103 according to the Kabat numbering is different from W;

and in which:

ii) preferably the amino acid residue at position 103 according to the Kabat numbering is P, R or S, and more preferably R;

and in which:

iii) FR1 is an amino acid sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following amino acid sequences:

<table>
<thead>
<tr>
<th>P,R,S 103 FW4 sequence no.</th>
<th>SEQ ID NO:</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120</td>
<td>RGQGTQTVSS</td>
</tr>
<tr>
<td>2</td>
<td>121</td>
<td>LRGGTQTVSS</td>
</tr>
<tr>
<td>3</td>
<td>122</td>
<td>GNKGTLTVSS</td>
</tr>
<tr>
<td>4</td>
<td>123</td>
<td>SSPGTQTVSS</td>
</tr>
<tr>
<td>5</td>
<td>124</td>
<td>SSQGTLTVSS</td>
</tr>
<tr>
<td>6</td>
<td>125</td>
<td>RSRGTVTVSS</td>
</tr>
</tbody>
</table>
Table A-24: Representative FW1 sequences (amino acid residues 5 to 26) for Nanobodies of the P,R,S 103-group.

<table>
<thead>
<tr>
<th>P,R,S 103 FW1 sequence no.</th>
<th>SEQ ID NO</th>
<th>VESGGGLVQAGGSLRLSAAASG</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,R,S 103 FW1 sequence no. 10</td>
<td>SEQ ID NO: 101</td>
<td>AESGGGLVQPGGLKLSCAASR</td>
</tr>
</tbody>
</table>

and in which:

iv) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the P,R,S 103 class;

and in which:

v) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be $V_{HH}$ sequences or may be humanized Nanobodies. When the above Nanobody sequences are $V_{HH}$ sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are $V_{LH}$ sequences or partially humanized Nanobodies).

In another preferred, but non-limiting aspect, the invention relates to a Nanobody as described above, in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said Nanobody and one or more of the sequences of SEQ ID NO’s: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, in which the amino acid residues that form the framework regions are disregarded. Such Nanobodies can be as further described herein.

As already mentioned herein, another preferred but non-limiting aspect of the invention relates to a Nanobody with an amino acid sequence that is chosen from the group
consisting of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 or from the group consisting of from amino acid sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the amino acid sequences of SEQ ID NO's:

238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239.

Also, in the above Nanobodies:

i) any amino acid substitution (when it is not a humanizing substitution as defined herein) is preferably, and compared to the corresponding amino acid sequence of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239, a conservative amino acid substitution, (as defined herein);

and/or:

ii) its amino acid sequence preferably contains either only amino acid substitutions, or otherwise preferably no more than 5, preferably no more than 3, and more preferably only 1 or 2 amino acid deletions or insertions, compared to the corresponding amino acid sequence of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239;

and/or

iii) the CDR's may be CDR's that are derived by means of affinity maturation, for example starting from the CDR's of to the corresponding amino acid sequence of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239.

 Preferably, the CDR sequences and FR sequences in the Nanobodies of the invention are such that the Nanobodies of the invention (and polypeptides of the invention comprising the same):

- bind to hCXCR4 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^{5} to 10^{12} liter/moles or more, and preferably 10^{7} to 10^{12} liter/moles or more and more preferably 10^{8} to 10^{12} liter/moles);

and/or such that they:

- bind to hCXCR4 with a k_{off}-rate of between 10^{2} M^{-1}s^{-1} to about 10^{7} M^{-1}s^{-1}, preferably between 10^{3} M^{-1}s^{-1} and 10^{7} M^{-1}s^{-1}, more preferably between 10^{4} M^{-1}s^{-1} and 30 NT's^{-1}, such as between 10^{5} IVfV^{-1} and 10^{7} M^{-1}s^{-1};
and/or such that they:

- bind to hCXCR4 with a $k_{\text{off}}$ rate between $1 \text{s}^{-1}$ ($t_{1/2} = 0.69 \text{ s}$) and $10^6 \text{s}^{-1}$ (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between $10^{-2} \text{s}^{-1}$ and $10^{-6} \text{s}^{-1}$, more preferably between $10^{-3} \text{s}^{-1}$ and $10^{-8} \text{s}^{-1}$, such as between $10^{-4} \text{s}^{-1}$ and $10^{-5} \text{s}^{-1}$.

Preferably, CDR sequences and FR sequences present in the Nanobodies of the invention are such that the Nanobodies of the invention will bind to Targets of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

In another preferred, but non-limiting aspect, the invention relates to a Nanobody as described above, in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said Nanobody and one or more of the sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311, in which the amino acid residues that form the framework regions are disregarded. Such Nanobodies can be as further described herein.

As already mentioned herein, another preferred but non-limiting aspect of the invention relates to a Nanobody with an amino acid sequence that is chosen from the group consisting of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311 or from the group consisting of from amino acid sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311.

Also, in the above Nanobodies:

i) any amino acid substitution (when it is not a humanizing substitution as defined herein) is preferably, and compared to the corresponding amino acid sequence of SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311, a conservative amino acid substitution, (as defined herein); and/or:
ii) its amino acid sequence preferably contains either only amino acid substitutions, or otherwise preferably no more than 5, preferably no more than 3, and more preferably only 1 or 2 amino acid deletions or insertions, compared to the corresponding amino acid sequence of SEQ ID NOs: 308 to 311, more preferably SEQ ID NO: 311; and/or

iii) the CDR's may be CDR's that are derived by means of affinity maturation, for example starting from the CDR's of to the corresponding amino acid sequence of SEQ ID NOs: 308 to 311, more preferably SEQ ID NO: 311.

Preferably, the CDR sequences and FR sequences in the Nanobodies of the invention are such that the Nanobodies of the invention (and polypeptides of the invention comprising the same):

- bind to hCD4 with a dissociation constant (K_D) of 10^{-5} to 10^{-4.2} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-9} moles/liter (i.e. with an association constant (K_A) of 10^{5} to 10^{12} liter/moles or more, and

- bind to hCD4 with a k_{on}-rate of between 10^{2} M^{-1} S^{-1} to about 10^{7} M^{-1} S^{-1}, preferably between 10^{3} M^{-1} V and 10^{7} M^{-1} S^{-1}, more preferably between 10^{4} M^{-1} S^{-1} and 10^{7} M^{-1} S^{-1}, such as between 10^{5} M^{-1} S^{-1} and 10^{7} M^{-1} S^{-1}; and/or such that they:

  - bind to hCD4 with a k_{off} rate between 1 s^{-1} (t_{1/2}=0.69 s) and 10^{-6} s^{-1} (providing a near irreversible complex with a t_{1/2} of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} and more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1}, such as between 10^{-4} s^{-1} and 10^{-6} s^{-1}.

Preferably, CDR sequences and FR sequences present in the Nanobodies of the invention are such that the Nanobodies of the invention will bind to Targets of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

According to one non-limiting aspect of the invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) in at least one of the framework regions compared to the corresponding framework region of a naturally occurring human V_{H} domain, and in particular compared to the corresponding framework region of DP-47. More specifically, according to one non-limiting aspect of the
invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring human V_{H} domain, and in particular compared to the corresponding framework region of DP-47. Usually, a Nanobody will have at least one such amino acid difference with a naturally occurring V_{H} domain in at least one of FR2 and/or FR4, and in particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45).

Also, a humanized Nanobody of the invention may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) at at least one of the framework regions compared to the corresponding framework region of a naturally occurring V_{H} domain. More specifically, according to one non-limiting aspect of the invention, a humanized Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring V_{H} domain. Usually, a humanized Nanobody will have at least one such amino acid difference with a naturally occurring V_{H} domain in at least one of FR2 and/or FR4, and in particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45).

As will be clear from the disclosure herein, it is also within the scope of the invention to use natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the Nanobodies of the invention as defined herein, and in particular analogs of the Nanobodies of SEQ ID NO's: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311. Thus, according to one aspect of the invention, the term "Nanobody of the invention" in its broadest sense also covers such analogs.

Generally, in such analogs, one or more amino acid residues may have been replaced, deleted and/or added, compared to the Nanobodies of the invention as defined herein. Such substitutions, insertions or deletions may be made in one or more of the framework regions and/or in one or more of the CDR's. When such substitutions, insertions or deletions are made in one or more of the framework regions, they may be made at one or more of the Hallmark residues and/or at one or more of the other positions in the framework residues,
although substitutions, insertions or deletions at the Hallmark residues are generally less preferred (unless these are suitable humanizing substitutions as described herein).

By means of non-limiting examples, a substitution may for example be a conservative substitution (as described herein) and/or an amino acid residue may be replaced by another amino acid residue that naturally occurs at the same position in another V\textsubscript{HH} domain (see Tables A-5 to A-8 for some non-limiting examples of such substitutions), although the invention is generally not limited thereto. Thus, any one or more substitutions, deletions or insertions, or any combination thereof, that either improve the properties of the Nanobody of the invention or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of the Nanobody of the invention (i.e. to the extent that the Nanobody is no longer suited for its intended use) are included within the scope of the invention. A skilled person will generally be able to determine and select suitable substitutions, deletions or insertions, or suitable combinations of thereof, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible substitutions and determining their influence on the properties of the Nanobodies thus obtained.

For example, and depending on the host organism used to express the Nanobody or polypeptide of the invention, such deletions and/or substitutions may be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the art. Alternatively, substitutions or insertions may be designed so as to introduce one or more sites for attachment of functional groups (as described herein), for example to allow site-specific pegylation (again as described herein).

As can be seen from the data on the V\textsubscript{HH} entropy and V\textsubscript{HH} variability given in Tables A-5 to A-8 above, some amino acid residues in the framework regions are more conserved than others. Generally, although the invention in its broadest sense is not limited thereto, any substitutions, deletions or insertions are preferably made at positions that are less conserved. Also, generally, amino acid substitutions are preferred over amino acid deletions or insertions.

The analogs are preferably such that they can bind to Targets of the invention with an affinity (suitably measured and/or expressed as a K\textsuperscript{A}-value (actual or apparent), a K\textsubscript{A}-value
(actual or apparent), a \( k_{on} \) rate and/or a \( k_{irr} \) rate, or alternatively as an IC50 value, as further described herein) that is as defined herein for the Nanobodies of the invention.

The analogs are preferably also such that they retain the favourable properties the Nanobodies, as described herein.

Also, according to one preferred aspect, the analogs have a degree of sequence identity of at least 70%, preferably at least 80%, more preferably at least 90%, such as at least 95% or 99% or more; and/or preferably have at most 20, preferably at most 10, even more preferably at most 5, such as 4, 3, 2 or only 1 amino acid difference (as defined herein), with one of the Nanobodies of SEQ ID NOs: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO's: 308 to 311, more preferably SEQ ID NO: 311.

Also, the framework sequences and CDR"s of the analogs are preferably such that they are in accordance with the preferred aspects defined herein. More generally, as described herein, the analogs will have (a) a Q at position 108; and/or (b) a charged amino acid or a cysteine residue at position 45 and preferably an E at position 44, and more preferably E at position 44 and R at position 45; and/or (c) P, R or S at position 103.

One preferred class of analogs of the Nanobodies of the invention comprise Nanobodies that have been humanized (i.e. compared to the sequence of a naturally occurring Nanobody of the invention). As mentioned in the background art cited herein, such humanization generally involves replacing one or more amino acid residues in the sequence of a naturally occurring \( V_{\text{HH}} \) with the amino acid residues that occur at the same position in a human \( V_{\text{HJ}} \) domain, such as a human \( V_{\text{HJ}} \) domain. Examples of possible humanizing substitutions or combinations of humanizing substitutions will be clear to the skilled person, for example from the Tables herein, from the possible humanizing substitutions mentioned in the background art cited herein, and/or from a comparison between the sequence of a Nanobody and the sequence of a naturally occurring human \( V_{\text{H}} \) domain.

The humanizing substitutions should be chosen such that the resulting humanized Nanobodies still retain the favourable properties of Nanobodies as defined herein, and more preferably such that they are as described for analogs in the preceding paragraphs. A skilled person will generally be able to determine and select suitable humanizing substitutions or suitable combinations of humanizing substitutions, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve...
introducing a limited number of possible humanizing substitutions and determining their influence on the properties of the Nanobodies thus obtained.

Generally, as a result of humanization, the Nanobodies of the invention may become more "human-like", while still retaining the favorable properties of the Nanobodies of the invention as described herein. As a result, such humanized Nanobodies may have several advantages, such as a reduced immunogenicity, compared to the corresponding naturally occurring $V_{HH}$ domains. Again, based on the disclosure herein and optionally after a limited degree of routine experimentation, the skilled person will be able to select humanizing substitutions or suitable combinations of humanizing substitutions which optimize or achieve a desired or suitable balance between the favourable properties provided by the humanizing substitutions on the one hand and the favourable properties of naturally occurring $V_{HH}$ domains on the other hand.

The Nanobodies of the invention may be suitably humanized at any framework residue(s), such as at one or more Hallmark residues (as defined herein) or at one or more other framework residues (i.e. non-Hallmark residues) or any suitable combination thereof. One preferred humanizing substitution for Nanobodies of the "P,R,S-103 group" or the "KERE group" is Q108 into L108. Nanobodies of the "GLEW class" may also be humanized by a Q108 into L108 substitution, provided at least one of the other Hallmark residues contains a camelid (camelizing) substitution (as defined herein). For example, as mentioned above, one particularly preferred class of humanized Nanobodies has GLEW or a GLEW-like sequence at positions 44-47; P, R or S (and in particular R) at position 103. and an L at position 108.

The humanized and other analogs, and nucleic acid sequences encoding the same, can be provided in any manner known per se. For example, the analogs can be obtained by providing a nucleic acid that encodes a naturally occurring $V_{HH}$ domain, changing the codons for the one or more amino acid residues that are to be substituted into the codons for the corresponding desired amino acid residues (e.g. by site-directed mutagenesis or by PCR using suitable mismatch primers), expressing the nucleic acid/nucleotide sequence thus obtained in a suitable host or expression system; and optionally isolating and/or purifying the analog thus obtained to provide said analog in essentially isolated form (e.g. as further described herein). This can generally be performed using methods and techniques known per se, which will be clear to the skilled person, for example from the handbooks and references cited herein, the
background art cited herein and/or from the further description herein. Alternatively, a nucleic acid encoding the desired analog can be synthesized in a manner known per se (for example using an automated apparatus for synthesizing nucleic acid sequences with a predefined amino acid sequence) and can then be expressed as described herein. Yet another technique may involve combining one or more naturally occurring and/or synthetic nucleic acid sequences each encoding a part of the desired analog, and then expressing the combined nucleic acid sequence as described herein. Also, the analogs can be provided using chemical synthesis of the pertinent amino acid sequence using techniques for peptide synthesis known per se. such as those mentioned herein.

In this respect, it will be also be clear to the skilled person that the Nanobodies of the invention (including their analogs) can be designed and/or prepared starting from human V<sub>H</sub> sequences (i.e. amino acid sequences or the corresponding nucleotide sequences), such as for example from human V<sub>H</sub>3 sequences such as DP-47, DP-51 or DP-29, i.e. by introducing one or more camelizing substitutions (i.e. changing one or more amino acid residues in the amino acid sequence of said human V<sub>H</sub> domain into the amino acid residues that occur at the corresponding position in a V<sub>H</sub> domain), so as to provide the sequence of a Nanobody of the invention and/or so as to confer the favourable properties of a Nanobody to the sequence thus obtained. Again, this can generally be performed using the various methods and techniques referred to in the previous paragraph, using an amino acid sequence and/or nucleotide sequence for a human V<sub>H</sub> domain as a starting point.

Some preferred, but non-limiting camelizing substitutions can be derived from Tables A-5 – A-8. It will also be clear that camelizing substitutions at one or more of the Hallmark residues will generally have a greater influence on the desired properties than substitutions at one or more of the other amino acid positions, although both and any suitable combination thereof are included within the scope of the invention. For example, it is possible to introduce one or more camelizing substitutions that already confer at least some the desired properties, and then to introduce further camelizing substitutions that either further improve said properties and/or confer additional favourable properties. Again, the skilled person will generally be able to determine and select suitable camelizing substitutions or suitable combinations of camelizing substitutions, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a
limited number of possible camelizing substitutions and determining whether the favourable properties of Nanobodies are obtained or improved (i.e. compared to the original Vn domain). Generally, however, such camelizing substitutions are preferably such that the resulting an amino acid sequence at least contains (a) a Q at position 108; and/or (b) a charged amino acid or a cysteine residue at position 45 and preferably also an E at position 44, and more preferably E at position 44 and R at position 45; and/or (c) P, R or S at position 103; and optionally one or more further camelizing substitutions. More preferably, the camelizing substitutions are such that they result in a Nanobody of the invention and/or in an analog thereof (as defined herein), such as in a humanized analog and/or preferably in an analog that is as defined in the preceding paragraphs.

As will also be clear from the disclosure herein, it is also within the scope of the invention to use parts or fragments, or combinations of two or more parts or fragments, of the Nanobodies of the invention as defined herein, and in particular parts or fragments of the Nanobodies of SEQ ID NO:s: 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO:s: 308 to 311, more preferably SEQ ID NO: 311. Thus, according to one aspect of the invention, the term "Nanobody of the invention" in its broadest sense also covers such parts or fragments.

Generally, such parts or fragments of the Nanobodies of the invention (including analogs thereof) have amino acid sequences in which, compared to the amino acid sequence of the corresponding full length Nanobody of the invention (or analog thereof), one or more of the amino acid residues at the N-terminal end, one or more amino acid residues at the C-terminal end, one or more contiguous internal amino acid residues, or any combination thereof, have been deleted and/or removed.

The parts or fragments are preferably such that they can bind to Targets of the invention with an affinity (suitably measured and/or expressed as a Ko-value (actual or apparent), a K_A-value (actual or apparent), a k_on-rate and/or a k_off-rate, or alternatively as an IC_50 value, as further described herein) that is as defined herein for the Nanobodies of the invention.

Any part or fragment is preferably such that it comprises at least 10 contiguous amino acid residues, preferably at least 20 contiguous amino acid residues, more preferably at least 30 contiguous amino acid residues, such as at least 40 contiguous amino acid residues, of the amino acid sequence of the corresponding full length Nanobody of the invention.
Also, any part or fragment is such preferably that it comprises at least one of CDR1, CDR2 and/or CDR3 or at least part thereof (and in particular at least CDR3 or at least part thereof). More preferably, any part or fragment is such that it comprises at least one of the CDR’s (and preferably at least CDR3 or part thereof) and at least one other CDR (i.e. CDR1 or CDR2) or at least part thereof, preferably connected by suitable framework sequence(s) or at least part thereof. More preferably, any part or fragment is such that it comprises at least one of the CDR’s (and preferably at least CDR3 or part thereof) and at least part of the two remaining CDR’s, again preferably connected by suitable framework sequence(s) or at least part thereof.

According to another particularly preferred, but non-limiting aspect, such a part or fragment comprises at least CDR3. such as FR3, CDR3 and FR4 of the corresponding full length Nanobody of the invention, i.e. as for example described in the International application WO 03/050531 (Lasters et al.).

As already mentioned above, it is also possible to combine two or more of such parts or fragments (i.e. from the same or different Nanobodies of the invention), i.e. to provide an analog (as defined herein) and/or to provide further parts or fragments (as defined herein) of a Nanobody of the invention. It is for example also possible to combine one or more parts or fragments of a Nanobody of the invention with one or more parts or fragments of a human V\textsubscript{H} domain.

According to one preferred aspect, the parts or fragments have a degree of sequence identity of at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, such as at least 90%, 95% or 99% or more with one of the Nanobodies of SEQ ID NOs 238 to 253, more preferably SEQ ID NO: 238 and SEQ ID NO: 239 and SEQ ID NO’s: 308 to 311, more preferably SEQ ID NO: 311.

The parts and fragments, and nucleic acid sequences encoding the same, can be provided and optionally combined in any manner known per se. For example, such parts or fragments can be obtained by inserting a stop codon in a nucleic acid that encodes a full-sized Nanobody of the invention, and then expressing the nucleic acid thus obtained in a manner known per se (e.g. as described herein). Alternatively, nucleic acids encoding such parts or fragments can be obtained by suitably restricting a nucleic acid that encodes a full-sized Nanobody of the invention or by synthesizing such a nucleic acid in a manner known per se. Parts or fragments may also be provided using techniques for peptide synthesis known per se.
The invention in its broadest sense also comprises derivatives of the Nanobodies of the invention. Such derivatives can generally be obtained by modification, and in particular by chemical and/or biological (e.g. enzymatical) modification, of the Nanobodies of the invention and/or of one or more of the amino acid residues that form the Nanobodies of the invention.

Examples of such modifications, as well as examples of amino acid residues within the Nanobody sequence that can be modified in such a manner (i.e. either on the protein backbone but preferably on a side chain), methods and techniques that can be used to introduce such modifications and the potential uses and advantages of such modifications will be clear to the skilled person.

For example, such a modification may involve the introduction (e.g. by covalent linking or in an other suitable manner) of one or more functional groups, residues or moieties into or onto the Nanobody of the invention, and in particular of one or more functional groups, residues or moieties that confer one or more desired properties or functionalities to the Nanobody of the invention. Example of such functional groups will be clear to the skilled person.

For example, such modification may comprise the introduction (e.g. by covalent binding or in any other suitable manner) of one or more functional groups that increase the half-life, the solubility and/or the absorption of the Nanobody of the invention, that reduce the immunogenicity and/or the toxicity of the Nanobody of the invention, that eliminate or attenuate any undesirable side effects of the Nanobody of the invention, and/or that confer other advantageous properties to and/or reduce the undesired properties of the Nanobodies and/or polypeptides of the invention; or any combination of two or more of the foregoing. Examples of such functional groups and of techniques for introducing them will be clear to the skilled person, and can generally comprise all functional groups and techniques mentioned in the general background art cited hereinabove as well as the functional groups and techniques known per se for the modification of pharmaceutical proteins, and in particular for the modification of antibodies or antibody fragments (including ScFv's and single domain antibodies), for which reference is for example made to Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980). Such functional groups may for example be linked directly (for example covalently) to a Nanobody of the
invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

One of the most widely used techniques for increasing the half-life and/or reducing the immunogenicity of pharmaceutical proteins comprises attachment of a suitable pharmacologically acceptable polymer, such as 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 (single) domain antibodies and ScFv's); reference is made to for example Chapman, Nat. Biotechnol., 54, 531-545 (2002); by Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003), by Harris and Chess, Nat. Rev. Drug. Discov., 2, (2003) and in WO 04/060965, Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA.

Preferably, site-directed pegylation is used, in particular via a cysteine-residue (see for example Yang et al. Protein Engineering, 16. 10, 761-770 (2003). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a Nanobody of the invention, a Nanobody 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 of a Nanobody of the invention, all using techniques of protein engineering known per se to the skilled person.

Preferably, for the Nanobodies and proteins of the invention, a PEG is used with a molecular weight of more than 5000, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the range of 20,000-80,000.

Another, usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the Nanobody or polypeptide of the invention.

Yet another modification may comprise the introduction of one or more detectable labels or other signal-generating groups or moieties, depending on the intended use of the labelled Nanobody. Suitable labels and techniques for attaching, using and detecting them will be clear to the skilled person, and for example include, but are not limited to, fluorescent labels (such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine and fluorescent metals such as 132Eu or
others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic aciinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes (such as $^3$H, $^{125}$I, $^{32}$P, $^{35}$S, $^{14}$C, $^{51}$Cr, $^{36}$Cl, $^{57}$Co, $^{58}$Co, $^{59}$Fe, and $^{75}$Se), metals, metal chelates or metallic cations (for example metallic cations such as $^{99m}$Tc, $^{121}$I, $^{111}$In, $^{131}$I, $^{97}$Ru, $^{67}$Cu, $^{67}$Ga, and $^{68}$Ga or other metals or metallic cations that are particularly suited for use in in vivo, in vitro or in situ diagnosis and imaging, such as ($^{157}$Gd, $^{55}$Mn, $^{162}$Dy, $^{52}$Cr, and $^{56}$Fe), as well as chromophores and enzymes (such as nialate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycoporphosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels will be clear to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy.

Such labelled Nanobodies and polypeptides of the invention may for example be used for in vitro, in vivo or in situ assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays", etc.) as well as in vivo diagnostic and imaging purposes, depending on the choice of the specific label.

As will be clear to the skilled person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable chelating groups for example include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the Nanobody of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a Nanobody of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated Nanobody may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may for example also be used to bind the Nanobody of the invention to a carrier, including carriers suitable for pharmaceutical
purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh, Journal of Drug Targeting, 8, 4, 257 (2000). Such binding pairs may also be used to link a therapeutically active agent to the Nanobody of the invention.

For some applications, in particular for those applications in which it is intended to kill a cell that expresses the target against which the Nanobodies of the invention are directed (e.g. in the treatment of cancer), or to reduce or slow the growth and/or proliferation such a cell, the Nanobodies of the invention may also be linked to a toxin or to a toxic residue or moiety. Examples of toxic moieties, compounds or residues which can be linked to a Nanobody of the invention to provide - for example – a cytotoxic compound will be clear to the skilled person and can for example be found in the prior art cited above and/or in the further description herein. One example is the so-called ADEPT™ technology described in WO 03/055527.

Other potential chemical and enzymatical modifications will be clear to the skilled person. Such modifications may also be introduced for research purposes (e.g. to study function-activity relationships). Reference is for example made to Lundblad and Bradshaw, Biotechnol. Appl. Biochem., 26. 143-151 (1997).

Preferably, the derivatives are such that they bind to Targets of the invention with an affinity (suitably measured and/or expressed as a Ko-value (actual or apparent), a K_4-value (actual or apparent), a k_{on}-rate and/or a k_{cat}rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein for the Nanobodies of the invention.

As mentioned above, the invention also relates to proteins or polypeptides that essentially consist of or comprise at least one Nanobody of the invention. By "essentially consist of is meant that the amino acid sequence of the polypeptide of the invention either is exactly the same as the amino acid sequence of a Nanobody of the invention or corresponds to the amino acid sequence of a Nanobody of the invention which has a limited number of amino acid residues, such as 1-20 amino acid residues, for example 1-10 amino acid residues and preferably 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, added at the amino terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of the amino acid sequence of the Nanobody.

Said amino acid residues may or may not change, alter or otherwise influence the (biological) properties of the Nanobody and may or may not add further functionality to the Nanobody. For example, such amino acid residues:
can comprise an N-terminal Met residue, for example as result of expression in a heterologous host cell or host organism,

- may form a signal sequence or leader sequence that directs secretion of the Nanobody from a host cell upon synthesis. Suitable secretory leader peptides will be clear to the skilled person, and may be as further described herein. Usually, such a leader sequence will be linked to the N-terminus of the Nanobody, although the invention in its broadest sense is not limited thereto;

- may form a sequence or signal that allows the Nanobody to be directed towards and/or to penetrate or enter into specific organs, tissues, cells, or parts or compartments of cells, and/or that allows the Nanobody to penetrate or cross a biological barrier such as a cell membrane, a cell layer such as a layer of epithelial cells, a tumor including solid tumors, or the blood-brain-barrier. Examples of such amino acid sequences will be clear to the skilled person. Some non-limiting examples are the small peptide vectors ("Pep-trans vectors") described in WO 03/026700 and in Temsamani et al. Expert Opin. Biol. Ther., 1. 773 (2001); Temsamani and Vidal, Drug Discov. Today, 9, 1012 (004) and Rousselle. J. Pharmacol. Exp. Ther., 296, 124-131 (2001), and the membrane translocator sequence described by Zhao et al., Apoptosis, 8, 631-637 (2003). C-terminal and N-terminal amino acid sequences for intracellular targeting of antibody fragments are for example described by Cardinale et al., Methods, 34, 171 (2004).

Other suitable techniques for intracellular targeting involve the expression and/or use of so-called "intrabodies" comprising a Nanobody of the invention, as mentioned below;

- may form a "tag", for example an amino acid sequence or residue that allows or facilitates the purification of the Nanobody, for example using affinity techniques directed against said sequence or residue. Thereafter, said sequence or residue may be removed (e.g. by chemical or enzymatical cleavage) to provide the Nanobody sequence (for this purpose, the tag may optionally be linked to the Nanobody sequence via a cleavable linker sequence or contain a cleavable motif). Some preferred, but non-limiting examples of such residues are multiple histidine residues, glutathione residues and a myc-tag (see for example SEQ ID NO:3 1 of WO 06/12282).

- may be one or more amino acid residues that have been functionalized and/or that can serve as a site for attachment of functional groups. Suitable amino acid residues and functional groups will be clear to the skilled person and include, but are not limited to,
the amino acid residues and functional groups mentioned herein for the derivatives of
the Nanobodies of the invention.

According to another aspect, a polypeptide of the invention comprises a Nanobody of
the invention, which is fused at its amino terminal end. at its carboxy terminal end, or both at
its amino terminal end and at its carboxy terminal end to at least one further amino acid
sequence, i.e. so as to provide a fusion protein comprising said Nanobody of the invention
and the one or more further amino acid sequences. Such a fusion will also be referred to
herein as a "Nanobody fusion".

The one or more further amino acid sequence may be any suitable and/or desired
amino acid sequences. The further amino acid sequences may or may not change, alter or
otherwise influence the (biological) properties of the Nanobody, and may or may not add
further functionality to the Nanobody or the polypeptide of the invention. Preferably, the
further amino acid sequence is such that it confers one or more desired properties or
functionalities to the Nanobody or the polypeptide of the invention.

For example, the further amino acid sequence may also provide a second binding site,
which binding site may be directed against any desired protein, polypeptide, antigen,
antigenic determinant or epitope (including but not limited to the same protein, polypeptide,
antigen, antigenic determinant or epitope against which the Nanobody of the invention is
directed, or a different protein, polypeptide, antigen, antigenic determinant or epitope).

Example of such amino acid sequences will be clear to the skilled person, and may
generally comprise all amino acid sequences that are used in peptide fusions based on
conventional antibodies and fragments thereof (including but not limited to ScFv's and single
domain antibodies). Reference is for example made to the review by Holliger and Hudson,
Nature Biotechnology, 23.9, 1126-1136 (2005).

For example, such an amino acid sequence may be an amino acid sequence that
increases the half-life, the solubility, or the absorption, reduces the immunogenicily or the
toxicity, eliminates or attenuates undesirable side effects, and/or confers other advantageous
properties to and/or reduces the undesired properties of the polypeptides of the invention,
compared to the Nanobody of the invention per se. Some non-limiting examples of such
amino acid sequences are serum proteins, such as human serum albumin (see for example
WO 00/27435) or haptenic molecules (for example haptens that are recognized by circulating
antibodies, see for example WO 98/22141).
In particular, it has been described in the art that linking fragments of immunoglobulins (such as V_{H} domains) to serum albumin or to fragments thereof can be used to increase the half-life. Reference is for made to WO 00/27435 and WO 01/077137). According to the invention, the Nanobody of the invention is preferably either directly linked to serum albumin (or to a suitable fragment thereof) or via a suitable linker, and in particular via a suitable peptide linked so that the polypeptide of the invention can be expressed as a genetic fusion (protein). According to one specific aspect, the Nanobody of the invention may be linked to a fragment of serum albumin that at least comprises the domain III of serum albumin or part thereof. Reference is for example made to WO 07/1 12940.

Alternatively, the further amino acid sequence may provide a second binding site or binding unit that is directed against a serum protein (such as, for example, human serum albumin or another serum protein such as IgG), so as to provide increased half-life in serum. Such amino acid sequences for example include the Nanobodies described below, as well as the small peptides and binding proteins described in WO 91/01743, WO 01/45746 and WO 02/076489 and the dAb's described in WO 03/002609 and WO 04/003019. Reference is also made to Harmsen et al., Vaccine, 23 (41); 4926-42, 2005, as well as to EP 0 368 684, as well as to WO 08/028977, WO 08/043821, WO 08/043822.

Such amino acid sequences may in particular be directed against serum albumin (and more in particular human serum albumin) and/or against IgG (and more in particular human IgG). For example, such amino acid sequences may be amino acid sequences that are directed against (human) serum albumin and amino acid sequences that can bind to amino acid residues on (human) serum albumin that are not involved in binding of serum albumin to FcRn (see for example WO 06/0122787) and/or amino acid sequences that are capable of binding to amino acid residues on serum albumin that do not form part of domain III of serum albumin (see again for example WO 06/0122787); amino acid sequences that have or can provide an increased half-life (see for example WO 08/028977); amino acid sequences against human serum albumin that are cross-reactive with serum albumin from at least one species of mammal, and in particular with at least one species of primate (such as, without limitation, monkeys from the genus *Macaca* (such as, and in particular, cynomolagus monkeys (*Macaca fascicularis*) and/or rhesus monkeys (*Macaca mulatto*)) and baboon (*Papio ursinus*), reference is again made to WO 08/028977; amino acid sequences that can
bind to serum albumin in a pH independent manner (see for example WO 08/043821) and/or amino acid sequences that are conditional binders (see for example WO 08/043822).

According to another aspect, the one or more further amino acid sequences may comprise one or more parts, fragments or domains of conventional 4-chain antibodies (and in particular human antibodies) and/or of heavy chain antibodies. For example, although usually less preferred, a Nanobody of the invention may be linked to a conventional (preferably human) $V_H$ or $V_L$ domain or to a natural or synthetic analog of a $V_H$ or $V_L$ domain, again optionally via a linker sequence (including but not limited to other (single) domain antibodies, such as the dAb's described by Ward et al.).

The at least one Nanobody may also be linked to one or more (preferably human) C$n$I, C$n$2 and/or C$n$3 domains, optionally via a linker sequence. For instance, a Nanobody linked to a suitable C$n$1 domain could for example be used - together with suitable light chains - to generate antibody fragments/structures analogous to conventional Fab fragments or F(ab')$_2$ fragments, but in which one or (in case of an F(ab')$_2$ fragment) one or both of the conventional V$n$ domains have been replaced by a Nanobody of the invention. Also, two Nanobodies could be linked to a Qi3 domain (optionally via a linker) to provide a construct with increased half-life in vivo.

According to one specific aspect of a polypeptide of the invention, one or more Nanobodies of the invention may be linked (optionally via a suitable linker or hinge region) 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. For example, for this purpose, and without being limited thereto, the one or more further amino acid sequences may comprise one or more C$n$2 and/or C$n$3 domains of an antibody, such as from a heavy chain antibody (as described herein) and more preferably from a conventional human 4-chain antibody: and/or may form (part of) and Fc region, for example from IgG (e.g. from IgGl, IgG2, IgG3 or IgG4), from IgE or from another human Ig such as IgA, IgD or IgM. For example, WO 94/04678 describes heavy chain antibodies comprising a Camelid $V_{HH}$ domain or a humanized derivative thereof (i.e. a Nanobody), in which the Camelidae Qi2 and/or CH3 domain have been replaced by human C$n$2 and C$n$3 domains, so as to provide an immunoglobulin that consists of 2 heavy chains each comprising a Nanobody and human
C_H^2 and C_H^3 domains (but no C_H^1 domain), which immunoglobulin has the effector function provided by the C_H^2 and Cjp domains and which immunoglobulin can function without the presence of any light chains. Other amino acid sequences that can be suitably linked to the Nanobodies of the invention so as to provide an effector function will be clear to the skilled person, and may be chosen on the basis of the desired effector function(s). Reference is for example made to WO 04/05882O, WO 99/42077, WO 02/056910, WO 09/068628 and WO 05/017148, as well as the review by Holliger and Hudson, supra. Coupling of a Nanobody of the invention to an Fc portion may also lead to an increased half-life, compared to the corresponding Nanobody of the invention. For some applications, the use of an Fc portion and/or of constant domains (i.e. C_H^2 and/or C_H^3 domains) that confer increased half-life without any biologically significant effector function may also be suitable or even preferred.

Other suitable constructs comprising one or more Nanobodies and one or more constant domains with increased half-life in vivo will be clear to the skilled person, and may for example comprise two Nanobodies linked to a C_H^3 domain, optionally via a linker sequence. Generally, any fusion protein or derivatives with increased half-life will preferably have a molecular weight of more than 50 kD, the cut-off value for renal absorption.

In another one specific, but non-limiting, aspect, in order to form a polypeptide of the invention, one or more amino acid sequences of the invention may be linked (optionally via a suitable linker or hinge region) to naturally occurring, synthetic or semisynthetic constant domains (or analogs, variants, mutants, parts or fragments thereof) that have a reduced (or essentially no) tendency to self-associate into dimers (i.e. compared to constant domains that naturally occur in conventional 4-chain antibodies). Such monomeric (i.e. not self-associating) Fc chain variants, or fragments thereof, will be clear to the skilled person. For example, Helm et al., J Biol Chem 1996 271 7494, describe monomeric FcE chain variants that can be used in the polypeptide chains of the invention.

Also, such monomeric Fc chain variants are preferably such that they are still capable of binding to the complement or the relevant Fc receptor(s) (depending on the Fc portion from which they are derived), and/or such that they still have some or all of the effector functions of the Fc portion from which they are derived (or at a reduced level still suitable for the intended use). Alternatively, in such a polypeptide chain of the invention, the monomeric Fc chain may be used to confer increased half-life upon the polypeptide chain, in which case the monomeric Fc chain may also have no or essentially no effector functions.
Bivaleni/multivalent, bispecific/multispecific or biparatopic/multiparatopic polypeptides of the invention may also be linked to Fc portions, in order to provide polypeptide constructs of the type that is described in WO 09/068630.

The further amino acid sequences may also form a signal sequence or leader sequence that directs secretion of the Nanobody or the polypeptide of the invention from a host cell upon synthesis (for example to provide a pre-, pro- or prepro- form of the polypeptide of the invention, depending on the host cell used to express the polypeptide of the invention).

The further amino acid sequence may also form a sequence or signal that allows the Nanobody or polypeptide of the invention to be directed towards and/or to penetrate or enter into specific organs, tissues, cells, or parts or compartments of cells, and/or that allows the Nanobody or polypeptide of the invention to penetrate or cross a biological barrier such as a cell membrane, a cell layer such as a layer of epithelial cells, a tumor including solid tumors, or the blood-brain-barrier. Suitable examples of such amino acid sequences will be clear to the skilled person, and for example include, but are not limited to, the "Peptrans" vectors mentioned above, the sequences described by Cardinale et al. and the amino acid sequences and antibody fragments known per se that can be used to express or produce the Nanobodies and polypeptides of the invention as so-called "intrabodies", for example as described in WO 94/02610, WO 95/22618, US-A-7004940, WO 03/014960, WO 99/07414; WO 05/01690; EP 1 512 696; and in Cattaneo, A. & Biocca, S. (1997) Intracellular Antibodies: Development and Applications. Landes and Springer-Verlag; and in Kontermann, Methods 34, (2004), 163-170, and the further references described therein.

For some applications, in particular for those applications in which it is intended to kill a cell that expresses the target against which the Nanobodies of the invention are directed (e.g. in the treatment of cancer), or to reduce or slow the growth and/or proliferation of such a cell, the Nanobodies of the invention may also be linked to a (cyto)toxic protein or polypeptide. Examples of such toxic proteins and polypeptides which can be linked to a Nanobody of the invention to provide - for example - a cytotoxic polypeptide of the invention will be clear to the skilled person and can for example be found in the prior art cited above and/or in the further description herein. One example is the so-called ADEPT™ technology described in WO 03/055527.

According to one preferred, but non-limiting aspect, said one or more further amino acid sequences comprise at least one further Nanobody, so as to provide a polypeptide of the
invention that comprises at least two, such as three, four, five or more Nanobodies, in which said Nanobodies may optionally be linked via one or more linker sequences (as defined herein). Polypeptides of the invention that comprise two or more Nanobodies, of which at least one is a Nanobody of the invention, will also be referred to herein as "multivalent" polypeptides of the invention, and the Nanobodies present in such polypeptides will also be referred to herein as being in a "multivalent format". For example a "bivalent" polypeptide of the invention comprises two Nanobodies, optionally linked via a linker sequence, whereas a "trivalent" polypeptide of the invention comprises three Nanobodies, optionally linked via two linker sequences; etc.; in which at least one of the Nanobodies present in the polypeptide, and up to all of the Nanobodies present in the polypeptide, is/are a Nanobody of the invention.

In a multivalent polypeptide of the invention, the two or more Nanobodies may be the same or different, and may be directed against the same antigen or antigenic determinant (for example against the same part(s) or epitope(s) or against different parts or epitopes) or may alternatively be directed against different antigens or antigenic determinants; or any suitable combination thereof. For example, a bivalent polypeptide of the invention may comprise (a) two identical Nanobodies; (b) a first Nanobody directed against a first antigenic determinant of a protein or antigen and a second Nanobody directed against the same antigenic determinant of said protein or antigen which is different from the first Nanobody; (c) a first Nanobody directed against a first antigenic determinant of a protein or antigen and a second Nanobody directed against another antigenic determinant of said protein or antigen; or (d) a first Nanobody directed against a first protein or antigen and a second Nanobody directed against a second protein or antigen (i.e. different from said first antigen). Similarly, a trivalent polypeptide of the invention may, for example and without being limited thereto, comprise (a) three identical Nanobodies; (b) two identical Nanobody against a first antigenic determinant of an antigen and a third Nanobody directed against a different antigenic determinant of the same antigen; (c) two identical Nanobody against a first antigenic determinant of an antigen and a third Nanobody directed against a second antigen different from said first antigen; (d) a first Nanobody directed against a first antigenic determinant of a first antigen, a second Nanobody directed against a second antigenic determinant of said first antigen and a third Nanobody directed against a second antigen different from said first antigen; or (e) a first Nanobody directed against a first antigen, a second Nanobody directed
against a second antigen different from said first antigen, and a third Nanobody directed against a third antigen different from said first and second antigen.

**MULTIPARATOPIC**

Polypeptides of the invention that contain at least two Nanobodies, in which at least one Nanobody is directed against a first antigen (i.e. against Targets of the invention,) and at least one Nanobody is directed against a second antigen (i.e. different from Targets of the invention), will also be referred to as "multispecific" polypeptides of the invention, and the Nanobodies present in such polypeptides will also be referred to herein as being in a "multispecific format". Thus, for example, a "bispecific" polypeptide of the invention is a polypeptide that comprises at least one Nanobody directed against a first antigen (i.e. Targets of the invention,) and at least one further Nanobody directed against a second antigen (i.e. different from Targets of the invention,), whereas a "trispecific" polypeptide of the invention is a polypeptide that comprises at least one Nanobody directed against a first antigen (i.e. Targets of the invention,), at least one further Nanobody directed against a second antigen (i.e. different from Targets of the invention,) and at least one further Nanobody directed against a third antigen (i.e. different from both Targets of the invention, and the second antigen); etc.

Accordingly, in its simplest form, a bispecific polypeptide of the invention is a bivalent polypeptide of the invention (as defined herein), comprising a first Nanobody directed against Targets of the invention, and a second Nanobody directed against a second antigen, in which said first and second Nanobody may optionally be linked via a linker sequence (as defined herein); whereas a trispecific polypeptide of the invention in its simplest form is a trivalent polypeptide of the invention (as defined herein), comprising a first Nanobody directed against Targets of the invention, a second Nanobody directed against a second antigen and a third Nanobody directed against a third antigen, in which said first, second and third Nanobody may optionally be linked via one or more, and in particular one and more, in particular two, linker sequences.

However, as will be clear from the description hereinabove, the invention is not limited thereto, in the sense that a multispecific polypeptide of the invention may comprise at least one Nanobody against Targets of the invention, and any number of Nanobodies directed against one or more antigens different from Targets of the invention.
Furthermore, although it is encompassed within the scope of the invention that the specific order or arrangement of the various Nanobodies in the polypeptides of the invention may have some influence on the properties of the final polypeptide of the invention (including but not limited to the affinity, specificity or avidity for Targets of the invention, or against the one or more other antigens), said order or arrangement is usually not critical and may be suitably chosen by the skilled person, optionally after some limited routine experiments based on the disclosure herein. Thus, when reference is made to a specific multivalent or multispecific polypeptide of the invention, it should be noted that this encompasses any order or arrangements of the relevant Nanobodies, unless explicitly indicated otherwise.

Finally, it is also within the scope of the invention that the polypeptides of the invention contain two or more Nanobodies and one or more further amino acid sequences (as mentioned herein).

For multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to Conrath et al., J. Biol. Chem., Vol. 276, 10. 7346-7350, 2001; Muyldermans, Reviews in Molecular Biotechnology 74 (2001), 277-302; as well as to for example WO 96/34103 and WO 99/23221. Some other examples of some specific multispecific and/or multivalent polypeptide of the invention can be found in the applications by Ablynx K.V. referred to herein.

One preferred, but non-limiting example of a multispecific polypeptide of the invention comprises at least one Nanobody of the invention and at least one Nanobody that provides for an increased half-life. Such Nanobodies may for example be Nanobodies that are directed against a serum protein, and in particular a human serum protein, such as human serum albumin, thyroxine-binding protein, (human) transferrin, fibrinogen, an immunoglobulin such as IgG, IgE or IgM, or against one of the serum proteins listed in WO 04/003019. Of these, Nanobodies that can bind to serum albumin (and in particular human serum albumin) or to IgG (and in particular human IgG, see for example Nanobody VH-I described in the review by Muyldermans, supra) are particularly preferred (although for example, for experiments in mice or primates, Nanobodies against or cross-reactive with mouse serum albumin (MSA) or serum albumin from said primate, respectively, can be used. However, for pharmaceutical use, Nanobodies against human serum albumin or human IgG will usually be preferred). Nanobodies that provide for increased half-life and that can be
used in the polypeptides of the invention include the Nanobodies directed against serum albumin that are described in WO 04/041865, in WO 06/122787 and in the further patent applications by Ablynx N.V., such as those mentioned above.

For example, the some preferred Nanobodies that provide for increased half-life for use in the present invention include Nanobodies that can bind to amino acid residues on (human) serum albumin that are not involved in binding of serum albumin to FcRn (see for example WO 06/0122787); Nanobodies that are capable of binding to amino acid residues on serum albumin that do not form part of domain III of serum albumin (see for example WO 06/0122787); Nanobodies that have or can provide an increased half-life (see for example WO 08/028977); Nanobodies against human serum albumin that are cross-reactive with serum albumin from at least one species of mammal, and in particular with at least one species of primate (such as, without limitation, monkeys from the genus Macaca (such as, and in particular, cynomolgus monkeys (Macaca fascicularis) and/or rhesus monkeys (Macaca mulatto)) and baboon (Papio ursinus)) (see for example WO 08/028977);

Nanobodies that can bind to serum albumin in a pH independent manner (see for example WO 08/04382) and/or Nanobodies that are conditional binders (see for example WO 08/043822).

Some particularly preferred Nanobodies that provide for increased half-life and that can be used in the polypeptides of the invention include the Nanobodies ALB-I to ALB-IO disclosed in WO 06/122787 (see Tables II and III) of which ALB-8 (SEQ ID NO: 62 in WO 06/122787) is particularly preferred.

According to a specific, but non-limiting aspect of the invention, the polypeptides of the invention contain, besides the one or more Nanobodies of the invention, at least one Nanobody against human serum albumin.

Generally, any polypeptides of the invention with increased half-life that contain one or more Nanobodies of the invention, and any derivatives of Nanobodies of the invention or of such polypeptides that have an increased half-life, preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding Nanobody of the invention per se. For example, such a derivative or polypeptides with increased half-life may have a half-life that is increased with more than 1 hours, preferably more than 2 hours, more
preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding Nanobody of the invention per se.

In a preferred, but non-limiting aspect of the invention, such derivatives or polypeptides may exhibit a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more. For example, such derivatives or polypeptides may have a half-life of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

According to one aspect of the invention the polypeptides are capable of binding to one or more molecules which can increase the half-life of the polypeptide in vivo.

The polypeptides of the invention are stabilised in vivo and their half-life increased by binding to molecules which resist degradation and/or clearance or sequestration. Typically, such molecules are naturally occurring proteins which themselves have a long half-life in vivo.

Another preferred, but non-limiting example of a multispecific polypeptide of the invention comprises at least one Nanobody of the invention and at least one Nanobody that directs the polypeptide of the invention towards, and/or that allows the polypeptide of the invention to penetrate or to enter into specific organs, tissues, cells, or parts or compartments of cells, and/or that allows the Nanobody to penetrate or cross a biological barrier such as a cell membrane, a cell layer such as a layer of epithelial cells, a tumor including solid tumors, or the blood-brain-barrier. Examples of such Nanobodies include Nanobodies that are directed towards specific cell-surface proteins, markers or epitopes of the desired organ, tissue or cell (for example cell-surface markers associated with tumor cells), and the single-domain brain targeting antibody fragments described in WO 02/057445 and WO 06/040153, of which FC44 (SEQ ID NO: 189 of WO 06/040153) and FC5 (SEQ ID NO: 190 of WO 06/040154) are preferred examples.

In the polypeptides of the invention, the one or more Nanobodies and the one or more polypeptides may be directly linked to each other (as for example described in WO 99/23221) and/or may be linked to each other via one or more suitable spacers or linkers, or any combination thereof.
Suitable spacers or linkers for use in multivalent and multispecific polypeptides will be clear to the skilled person, and may generally be any linker or spacer used in the art to link amino acid sequences. Preferably, said linker or spacer is suitable for use in constructing proteins or polypeptides that are intended for pharmaceutical use.

Some particularly preferred spacers include the spacers and linkers that are used in the art to link antibody fragments or antibody domains. These include the linkers mentioned in the general background art cited above, as well as for example linkers that are used in the art to construct diabodies or ScFv fragments (in this respect, however, it should be noted that, whereas in diabodies and in ScFv fragments, the linker sequence used should have a length, a degree of flexibility and other properties that allow the pertinent V₄ and V₅ domains to come together to form the complete antigen-binding site, there is no particular limitation on the length or the flexibility of the linker used in the polypeptide of the invention, since each Nanobody by itself forms a complete antigen-binding site).

For example, a linker may be a suitable amino acid sequence, and in particular amino acid sequences of between 1 and 50, preferably between 1 and 30, such as between 1 and 10 amino acid residues. Some preferred examples of such amino acid sequences include gly-ser linkers, for example of the type \((\text{gly}_x\text{ser}_y)_z\), such as (for example \((\text{gly}_4\text{ser})_3\) or \((\text{gly}_3\text{ser}_2)_3\), as described in WO 99/42077 and the GS30, GS15, GS9 and GS7 linkers described in the applications by Ablynx mentioned herein (see for example WO 06/040153 and WO 06/122825), as well as hinge-like regions, such as the hinge regions of naturally occurring heavy chain antibodies or similar sequences (such as described in WO 94/04678).

Some other particularly preferred linkers are poly-alanine (such as AAA), as well as the linkers GS30 (SEQ ID NO: 85 in WO 06/122825) and GS9 (SEQ ID NO: 84 in WO 06/122825).

Other suitable linkers generally comprise organic compounds or polymers, in particular those suitable for use in proteins for pharmaceutical use. For instance, poly(ethyleneglycol) moieties have been used to link antibody domains, see for example WO 04/081026.

It is encompassed within the scope of the invention that the length, the degree of flexibility and/or other properties of the linker(s) used (although not critical, as it usually is for linkers used in ScFv fragments) may have some influence on the properties of the final polypeptide of the invention, including but not limited to the affinity, specificity or avidity for
Targets of the invention, or for one or more of the other antigens. Based on the disclosure herein, the skilled person will be able to determine the optimal linker(s) for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

For example, in multivalent polypeptides of the invention that comprise Nanobodies directed against a multimeric antigen (such as a multimeric receptor or other protein), the length and flexibility of the linker are preferably such that it allows each Nanobody of the invention present in the polypeptide to bind to the antigenic determinant on each of the subunits of the multimer. Similarly, in a multispecific polypeptide of the invention that comprises Nanobodies directed against two or more different antigenic determinants on the same antigen (for example against different epitopes of an antigen and/or against different subunits of a multimeric receptor, channel or protein), the length and flexibility of the linker are preferably such that it allows each Nanobody to bind to its intended antigenic determinant. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linker(s) for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

It is also within the scope of the invention that the linker(s) used confer one or more other favourable properties or functionality to the polypeptides of the invention, and/or provide one or more sites for the formation of derivatives and/or for the attachment of functional groups (e.g. as described herein for the derivatives of the Nanobodies of the invention). For example, linkers containing one or more charged amino acid residues (see Table A-2 above) can provide improved hydrophilic properties, whereas linkers that form or contain small epitopes or tags can be used for the purposes of detection, identification and/or purification. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

Finally, when two or more linkers are used in the polypeptides of the invention, these linkers may be the same or different. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

Usually, for easy of expression and production, a polypeptide of the invention will be a linear polypeptide. However, the invention in its broadest sense is not limited thereto. For example, when a polypeptide of the invention comprises three of more Nanobodies, it is
possible to link them by use of a linker with three or more "arms", which each "arm" being linked to a Nanobody, so as to provide a "star-shaped" construct. It is also possible, although usually less preferred, to use circular constructs.

The invention also comprises derivatives of the polypeptides of the invention, which may be essentially analogous to the derivatives of the Nanobodies of the invention, i.e. as described herein.

The invention also comprises proteins or polypeptides that "essentially consist" of a polypeptide of the invention (in which the wording "essentially consist of" has essentially the same meaning as indicated hereinabove).

According to one aspect of the invention, the polypeptide of the invention is in essentially isolated from, as defined herein.

The amino acid sequences, Nanobodies, polypeptides and nucleic acids of the invention can be prepared in a manner known per se, as will be clear to the skilled person from the further description herein. For example, the Nanobodies and polypeptides of the invention can be prepared in any manner known per se for the preparation of antibodies and in particular for the preparation of antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments). Some preferred, but non-limiting methods for preparing the amino acid sequences, Nanobodies, polypeptides and nucleic acids include the methods and techniques described herein.

As will be clear to the skilled person, one particularly useful method for preparing an amino acid sequence, Nanobody and/or a polypeptide of the invention generally comprises the steps of:

i) the expression, in a suitable host cell or host organism (also referred to herein as a "host of the invention") or in another suitable expression system of a nucleic acid that encodes said amino acid sequence, Nanobody or polypeptide of the invention (also referred to herein as a "nucleic acid of the invention"), optionally followed by:

ii) isolating and/or purifying the amino acid sequence, Nanobody or polypeptide of the invention thus obtained.

In particular, such a method may comprise the steps of:

i) cultivating and/or maintaining a host of the invention under conditions that are such that said host of the invention expresses and/or produces at least one amino acid sequence, Nanobody and/or polypeptide of the invention; optionally followed by:
ii) isolating and/or purifying the amino acid sequence. Nanobody or polypeptide of the invention thus obtained.

A nucleic acid of the invention can be in the form of single or double stranded DNA or RNA, and is preferably in the form of double stranded DNA. For example, the nucleotide sequences of the invention may 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 aspect of the invention, the nucleic acid of the invention is in essentially isolated from, as defined herein.

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 nucleic acids of the invention can be prepared or obtained in a manner known per se. 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. To provide analogs, nucleotide sequences encoding naturally occurring $V_{\text{HH}}$ domains can for example be subjected to site-directed mutagenesis, so at to provide a nucleic acid of the invention encoding said analog. Also, as will be clear to the skilled person, to prepare a nucleic acid of the invention, also several nucleotide sequences, such as at least one nucleotide sequence encoding a Nanobody and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

Techniques for generating the nucleic acids of the invention will be clear to the skilled person and may for instance include, but are not limited to: automated DNA synthesis; site-directed mutagenesis; combining two or more naturally occurring and/or synthetic sequences (or two or more parts thereof), introduction of mutations that lead to the expression of a truncated expression product; introduction of one or more restriction sites (e.g. to create cassettes and/or regions that may easily be digested and/or ligated using suitable restriction enzymes), and/or the introduction of mutations by means of a PCR reaction using one or more "mismatched" primers, using for example a sequence of a naturally occurring form of Targets of the invention as a template. These and other techniques will be clear to the skilled person, and reference is again made to the standard handbooks, such as Sambrook et al. and Ausubel et al., mentioned above, as well as the Examples below.
The nucleic acid of the invention may also be in the form of, be present in and/or be part of a genetic construct, as will be clear to the person skilled in the art. Such genetic constructs generally comprise at least one nucleic acid of the invention that is optionally linked to one or more elements of genetic constructs known per se, such as for example one or more suitable regulatory elements (such as a suitable promoter(s), enhancer(s), terminator(s), etc.) and the further elements of genetic constructs referred to herein. Such genetic constructs comprising at least one nucleic acid of the invention will also be referred to herein as "genetic constructs of the invention".

The genetic constructs of the invention may be DNA or RNA, and are preferably double-stranded DNA. The genetic constructs of the invention may also be in a form suitable for transformation of the intended host cell or host organism, in a form suitable for integration into the genomic DNA of the intended host cell or in a form suitable for independent replication, maintenance and/or inheritance in the intended host organism. For instance, the genetic constructs of the invention may be in the form of a vector, such as for example a plasmid, cosmid, YAC, a viral vector or transposon. In particular, the vector may be an expression vector, i.e. a vector that can provide for expression in vitro and/or in vivo (e.g. in a suitable host cell, host organism and/or expression system).

In a preferred but non-limiting aspect, a genetic construct of the invention comprises
i) at least one nucleic acid of the invention; operably connected to
ii) one or more regulatory elements, such as a promoter and optionally a suitable terminator;

and optionally also
iü) one or more further elements of genetic constructs known per se:

in which the terms "regulatory element", "promoter", "terminator" and "operably connected" have their usual meaning in the art (as further described herein); and in which said "further elements" present in the genetic constructs may for example be 3'- or 5'-UTR sequences, leader sequences, selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, and may for instance depend upon the type of construct used, the intended host cell or host organism; the manner in which the nucleotide sequences of the invention of interest are to be expressed (e.g. via constitutive, transient or inducible expression); and/or the transformation technique
to be used. For example, regulatory sequences, promoters and terminators known per se for
the expression and production of antibodies and antibody fragments (including but not
limited to (single) domain antibodies and ScFv fragments) may be used in an essentially
analogous manner.

Preferably, in the genetic constructs of the invention, said at least one nucleic acid of
the invention and said regulatory elements, and optionally said one or more further elements,
are "operably linked" to each other, by which is generally meant that they are in a functional
relationship with each other. For instance, a promoter is considered "operably linked" to a
coding sequence if said promoter is able to initiate or otherwise control/regulate the
transcription and/or the expression of a coding sequence (in which said coding sequence
should be understood as being "under the control of" said promoter). Generally, when two
nucleotide sequences are operably linked, they will be in the same orientation and usually
also in the same reading frame. They will usually also be essentially contiguous, although this
may also not be required.

Preferably, the regulatory and further elements of the genetic constructs of the
invention are such that they are capable of providing their intended biological function in the
intended host cell or host organism.

For instance, a promoter, enhancer or terminator should be "operative" in the intended
host cell or host organism, by which is meant that (for example) said promoter should be
capable of initiating or otherwise controlling/regulating the transcription and/or the
expression of a nucleotide sequence - e.g. a coding sequence - to which it is operably linked
(as defined herein).

Some particularly preferred promoters include, but are not limited to, promoters
known per se for the expression in the host cells mentioned herein; and in particular
promoters for the expression in the bacterial cells, such as those mentioned herein and/or
those used in the Examples.

A selection marker should be such that it allows - i.e. under appropriate selection
conditions - host cells and/or host organisms that have been (successfully) transformed with
the nucleotide sequence of the invention to be distinguished from host cells/organisms that
have not been (successfully) transformed. Some preferred, but non-limiting examples of such
markers are genes that provide resistance against antibiotics (such as kanamycin or
ampicillin), genes that provide for temperature resistance, or genes that allow the host cell or
host organism to be maintained in the absence of certain factors, compounds and/or (food) components in the medium that are essential for survival of the non-transformed cells or organisms.

A leader sequence should be such that - in the intended host cell or host organism - it allows for the desired post-translational modifications and/or such that it directs the transcribed mRNA to a desired part or organelle of a cell. A leader sequence may also allow for secretion of the expression product from said cell. As such, the leader sequence may be any pro-, pre-, or prepro-sequence operable in the host cell or host organism. Leader sequences may not be required for expression in a bacterial cell. For example, leader sequences known per se for the expression and production of antibodies and antibody fragments (including but not limited to single domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

An expression marker or reporter gene should be such that - in the host cell or host organism - it allows for detection of the expression of (a gene or nucleotide sequence present on) the genetic construct. An expression marker may optionally also allow for the localisation of the expressed product, e.g. in a specific part or organelle of a cell and/or in (a) specific cell(s), tissue(s), organ(s) or part(s) of a multicellular organism. Such reporter genes may also be expressed as a protein fusion with the amino acid sequence of the invention. Some preferred, but non-limiting examples include fluorescent proteins such as GFP.

Some preferred, but non-limiting examples of suitable promoters, terminator and further elements include those that can be used for the expression in the host cells mentioned herein; and in particular those that are suitable for expression in bacterial cells, such as those mentioned herein and/or those used in the Examples below. For some (further) non-limiting examples of the promoters, selection markers, leader sequences, expression markers and further elements that may be present/used in the genetic constructs of the invention - such as terminators, transcriptional and/or translational enhancers and/or integration factors - reference is made to the general handbooks such as Sambrook et al. and Ausubel et al. mentioned above, as well as to the examples that are given in WO 95/07463, WO 96/23810, WO 95/07463, WO 95/21191, WO 97/11094, WO 97/42320, WO 98/06737, WO 98/21355, US-A-7,207,410, US-A- 5,693,492 and EP 1 085 089. Other examples will be clear to the skilled person. Reference is also made to the general background art cited above and the further references cited herein.
The genetic constructs of the invention may generally be provided by suitably linking the nucleotide sequence(s) of the invention to the one or more further elements described above, for example using the techniques described in the general handbooks such as Sambrook et al. and Ausubel et al., mentioned above.

Often, the genetic constructs of the invention will be obtained by inserting a nucleotide sequence of the invention in a suitable (expression) vector known per se. Some preferred, but non-limiting examples of suitable expression vectors are those used in the Examples below, as well as those mentioned herein.

The nucleic acids of the invention and/or the genetic constructs of the invention may be used to transform a host cell or host organism, i.e. for expression and/or production of the amino acid sequence, Nanobody or polypeptide of the invention. Suitable hosts or host cells will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism, for example:

- a bacterial strain, including but not limited to gram-negative strains such as strains of *Escherichia coli*; of *Proteus*, for example of *Proteus mirabilis*; of *Pseudomonas*, for example of *Pseudomonas f uorescei*; and gram-positive strains such as strains of *Bacillus*, for example of *Bacillus subtilis* or of *Bacillus brevis*; of *Streptomyces*, for example of *Streptomyces lividans*; of *Staphylococcus*, for example of *Staphylococcus camosus*; and of *Lactococcus*, for example of *Lactococcus lactis*;

- a fungal cell, including but not limited to cells from species of *Trichoderma*, for example from *Trichoderma reesei*; of *Neurospora*, for example from *Neurospora crassa*; of *Sordaria*, for example from *Sordaria macrospora*; of *Aspergillus*, for example from *Aspergillus niger* or from *Aspergillus sojae*; or from other filamentous fungi;

- a yeast cell, including but not limited to cells from species of *Saccharomyces*, for example of *Saccharomyces cerevisiae*; of *Schizosaccharomyces*, for example of *Schizosaccharomyces pombe*; of *Pichia*, for example of *Pichia pastoris* or of *Pichia methanolica*; of *Hansenula*, for example of *Hansenula polymorpha*; of *Kluyveromyces*, for example of *Kluyveromyces lactis*; of *Arxula*, for example of *Arxula adeninivorans*; of *Yarrowia*, for example of *Yarrowia lipolytics*;

- an amphibian cell or cell line, such as *Xenopus oocytes*;
an insect-derived cell or cell line, such as cells/cell lines derived from lepidoptera, including but not limited to *Spodoptera* SF9 and Sf21 cells or cells/cell lines derived from *Drosophila*, such as Schneider and Kc cells;

- a plant or plant cell, for example in tobacco plants; and/or

- a mammalian cell or cell line, for example a cell or cell line derived from a human, a cell or a cell line from mammals including but not limited to CHO-cells, BHK-cells (for example BHK-21 cells) and human cells or cell lines such as HeLa, COS (for example COS-7) and PER.C6 cells;

as well as all other hosts or host cells known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments), which will be clear to the skilled person. Reference is also made to the general background art cited hereinafore, as well as to for example WO 94/29457; WO 96/34103; WO 99/42077; Frenken et al. (1998). supra; Riechmann and Muyldermans, (1999), supra; van der Linden, (2000), supra; Thomassen et al., (2002), supra; Joosten et al., (2003), supra; Joosten et al., (2005), supra; and the further references cited herein.

The amino acid sequences. Nanobodies and polypeptides of the invention can also be introduced and expressed in one or more cells, tissues or organs of a multicellular organism, for example for prophylactic and/or therapeutic purposes (e.g. as a gene therapy). For this purpose, the nucleotide sequences of the invention may be introduced into the cells or tissues in any suitable way, for example as such (e.g. using liposomes) or after they have been inserted into a suitable gene therapy vector (for example derived from retroviruses such as adenovirus, or paroviruses such as adeno-associated virus). As will also be clear to the skilled person, such gene therapy may be performed in vivo and/or in situ in the body of a patient by administering a nucleic acid of the invention or a suitable gene therapy vector encoding the same to the patient or to specific cells or a specific tissue or organ of the patient; or suitable cells (often taken from the body of the patient to be treated, such as explanted lymphocytes, bone marrow aspirates or tissue biopsies) may be treated in vitro with a nucleotide sequence of the invention and then be suitably (re-)introduced into the body of the patient. All this can be performed using gene therapy vectors, techniques and delivery systems which are well known to the skilled person, and for example described in Culver, K. W. "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, N.Y); Giordano, Nature F Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919;


The amino acid sequences. Nanobodies and polypeptides of the invention can for example also be produced in the milk of transgenic mammals, for example in the milk of rabbits, cows, goats or sheep (see for example US-A-6/741,957, US-A-6,304,489 and US-A-6,849,992 for general techniques for introducing transgenes into mammals), in plants or parts of plants including but not limited to their leaves, flowers, fruits, seed, roots or tubers (for example in tobacco, maize, soybean or alfalfa) or in for example pupae of the silkworm Bombix mori.

Furthermore, the amino acid sequences, Nanobodies and polypeptides of the invention can also be expressed and/or produced in cell-free expression systems, and suitable examples of such systems will be clear to the skilled person. Some preferred, but non-limiting examples include expression in the wheat germ system; in rabbit reticulocyte lysates; or in the E. coli system.

As mentioned above, one of the advantages of the use of Nanobodies is that the polypeptides based thereon can be prepared through expression in a suitable bacterial system, and suitable bacterial expression systems, vectors, host cells, regulatory elements, etc., will be clear to the skilled person, for example from the references cited above. It should however be noted that the invention in its broadest sense is not limited to expression in bacterial systems.
Preferably, in the invention, an (in vivo or in vitro) expression system, such as a bacterial expression system, is used that provides the polypeptides of the invention in a form that is suitable for pharmaceutical use, and such expression systems will again be clear to the skilled person. As also will be clear to the skilled person, polypeptides of the invention suitable for pharmaceutical use can be prepared using techniques for peptide synthesis.

For production on industrial scale, preferred heterologous hosts for the (industrial) production of Nanobodies or Nanobody-containing protein therapeutics include strains of *E. coli, Pichiapastoris, S. cerevisiae* that are suitable for large scale expression/production/fermentation, and in particular for large scale pharmaceutical (i.e. GMP grade) expression/production/fermentation. Suitable examples of such strains will be clear to the skilled person. Such strains and production/expression systems are also made available by companies such as Biovitrum (Uppsala, Sweden).

Alternatively, mammalian cell lines, in particular Chinese hamster ovary (CHO) cells, can be used for large scale expression/production/fermentation, and in particular for large scale pharmaceutical expression/production/fermentation. Again, such expression/production systems are also made available by some of the companies mentioned above.

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 Nanobody-containing recombinant protein 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. Preferably, either a human cell or cell line is used (i.e. leading to a protein that essentially has a human glycosylation pattern) or another mammalian cell line is used that can provide a glycosylation pattern that is essentially and/or functionally the same as human glycosylation or at least mimics human glycosylation. Generally, prokaryotic hosts such as *E. coli* do not have the ability to glycosylate proteins, and the use of lower eukaryotes such as yeast usually leads to a glycosylation pattern that differs from human glycosylation. Nevertheless, it should be understood that all the foregoing host cells and expression systems can be used in the invention, depending on the desired amino acid sequence, Nanobody or polypeptide to be obtained.
Thus, according to one non-limiting aspect of the invention, the amino acid sequence, Nanobody or polypeptide of the invention is glycosylated. According to another non-limiting aspect of the invention, the amino acid sequence, Nanobody or polypeptide of the invention is non-glycosylated.

According to one preferred, but non-limiting aspect of the invention, the amino acid sequence, Nanobody or polypeptide of the invention is produced in a bacterial cell, in particular a bacterial cell suitable for large scale pharmaceutical production, such as cells of the strains mentioned above.

According to another preferred, but non-limiting aspect of the invention, the amino acid sequence, Nanobody or polypeptide of the invention is produced in a yeast cell, in particular a yeast cell suitable for large scale pharmaceutical production, such as cells of the species mentioned above.

According to yet another preferred, but non-limiting aspect of the invention, the amino acid sequence, Nanobody or polypeptide of the invention is produced in a mammalian cell, in particular in a human cell or in a cell of a human cell line, and more in particular in a human cell or in a cell of a human cell line that is suitable for large scale pharmaceutical production, such as the cell lines mentioned hereinabove.

When expression in a host cell is used to produce the amino acid sequences, Nanobodies and the polypeptides of the invention, the amino acid sequences, Nanobodies and polypeptides of the invention can be produced either intracellularly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or can be produced extracellularly (e.g. in the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified. When eukaryotic host cells are used, extracellular production is usually preferred since this considerably facilitates the further isolation and downstream processing of the Nanobodies and proteins obtained. Bacterial cells such as the strains of *E. coli* mentioned above normally do not secrete proteins extracellularly, except for a few classes of proteins such as toxins and hemolysin, and secretory production in *E. coli* refers to the translocation of proteins across the inner membrane to the periplasmic space. Periplasmic production provides several advantages over cytosolic production. For example, the N-terminal amino acid sequence of the secreted product can be identical to the natural gene product after cleavage of the secretion signal sequence by a specific signal peptidase. Also, there appears to be much less
protease activity in the periplasm than in the cytoplasm. In addition, protein purification is
simpler due to fewer contaminating proteins in the periplasm. Another advantage is that
correct disulfide bonds may form because the periplasm provides a more oxidative
environment than the cytoplasm. Proteins overexpressed in *E. coli* are often found in
insoluble aggregates, so-called inclusion bodies. These inclusion bodies may be located in the
cytosol or in the periplasm; the recovery of biologically active proteins from these inclusion
bodies requires a denaturation/refolding process. Many recombinant proteins, including
therapeutic proteins, are recovered from inclusion bodies. Alternatively, as will be clear to the
skilled person, recombinant strains of bacteria that have been genetically modified so as to
secrete a desired protein, and in particular an amino acid sequence, Nanobody or a
polypeptide of the invention, can be used.

Thus, according to one non-limiting aspect of the invention, the amino acid sequence,
Nanobody or polypeptide of the invention is an amino acid sequence, Nanobody or
polypeptide that has been produced intracellularly and that has been isolated from the host
cell, and in particular from a bacterial cell or from an inclusion body in a bacterial cell.
According to another non-limiting aspect of the invention, the amino acid sequence.
Nanobody or polypeptide of the invention is an amino acid sequence, Nanobody or
polypeptide that has been produced extracellularly, and that has been isolated from the
medium in which the host cell is cultivated.

Some preferred, but non-limiting promoters for use with these host cells include,
- for expression in *E. coli*: lac promoter (and derivatives thereof such as the lacUVS
  promoter); arabinose promoter; left- (PL) and rightward (PR) promoter of phage
  lambda; promoter of the tip operon; hybrid lac/trp promoters (tac and trc); T7-promoter
  (more specifically that of T7-phage gene 10) and other T-phage promoters; promoter of
  the TnIO tetracycline resistance gene; engineered variants of the above promoters that
  include one or more copies of an extraneous regulatory operator sequence;
- for expression in *S. cerevisiae*: constitutive: ADH1 (alcohol dehydrogenase 1), ENO
  (enolase), CYC1 (cytochrome c iso-1), GAPDH (glyceraldehydes-3-phosphate
  dehydrogenase), PGK1 (phosphoglycerate kinase), PYK1 (pyruvate kinase); regulated:
  GALL1 0.7 (galactose metabolic enzymes), ADH2 (alcohol dehydrogenase 2), PHO5
  (acid phosphatase), CUP1 (copper metallothionein); heterologous: CaMV (cauliflower
  mosaic virus 35S promoter);
for expression in *Pichia pastoris*: the AOX1 promoter (alcohol oxidase I);
- for expression in mammalian cells: human cytomegalovirus (hCMV) immediate early enhancer/promoter; human cytomegalovirus (hCMV) immediate early promoter variant that contains two tetracycline operator sequences such that the promoter can be regulated by the Tet repressor; Herpes Simplex Virus thymidine kinase (TK) promoter; Rous Sarcoma Virus long terminal repeat (RSV LTR) enhancer/promoter; elongation factor 1α (hEF-1α) promoter from human, chimpanzee, mouse or rat; the SV40 early promoter; HIV-I long terminal repeat promoter; β-actin promoter;

Some preferred, but non-limiting vectors for use with these host cells include:

- vectors for expression in mammalian cells: pMAMneo (Clontech), pcDNA3 (Invitrogen), pMClneo (Stratagene), pSG5 (Stratagene), EBOpS V2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 371 10), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC37199), pRSVneo (ATCC37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460) and 1ZD35 (ATCC 37565), as well as viral-based expression systems, such as those based on adenovirus;

- vectors for expression in bacterial cells: pET vectors (Novagen) and pQE vectors (Qiagen);

- vectors for expression in yeast or other fungal cells: pYES2 (Invitrogen) and *Pichia* expression vectors (Invitrogen);

- vectors for expression in insect cells: pBlueBacII (Invitrogen) and other baculovirus vectors

- vectors for expression in plants or plant cells: for example vectors based on cauliflower mosaic virus or tobacco mosaic virus, suitable strains of *Agrobacterium*, or Ti-plasmid based vectors.

Some preferred, but non-limiting secretory sequences for use with these host cells include:

- for use in bacterial cells such as *E. coli* PeIB, Bla, OmpA, OmpC, OmpF, OmpT, StII, PhoA, PhoE, MalE, Lpp, LamB, and the like; TAT signal peptide, hemolysin C-terminal secretion signal;

- for use in yeast: α-mating factor prepro-sequence, phosphatase (phol), invertase (Sue), etc.;
for use in mammalian cells: indigenous signal in case the target protein is of eukaryotic origin; murine Ig κ-chain V-J2-C signal peptide; etc.

Suitable techniques for transforming a host or host cell of the invention will be clear to the skilled person and may depend on the intended host cell/host organism and the genetic construct to be used. Reference is again made to the handbooks and patent applications mentioned above.

After transformation, a step for detecting and selecting those host cells or host organisms that have been successfully transformed with the nucleotide sequence/genetic construct of the invention may be performed. This may for instance be a selection step based on a selectable marker present in the genetic construct of the invention or a step involving the detection of the amino acid sequence of the invention, e.g. using specific antibodies.

The transformed host cell (which may be in the form or a stable cell line) or host organisms (which may be in the form of a stable mutant line or strain) form further aspects of the present invention.

Preferably, these host cells or host organisms are such that they express, or are (at least) capable of expressing (e.g. under suitable conditions), an amino acid sequence. Nanobody or polypeptide of the invention (and in case of a host organism: in at least one cell, part, tissue or organ thereof). The invention also includes further generations, progeny and/or offspring of the host cell or host organism of the invention, that may for instance be obtained by cell division or by sexual or asexual reproduction.

To produce/obtain expression of the amino acid sequences of the invention, the transformed host cell or transformed host organism may generally be kept, maintained and/or cultured under conditions such that the (desired) amino acid sequence, Nanobody or polypeptide of the invention is expressed/produced. Suitable conditions will be clear to the skilled person and will usually depend upon the host cell/host organism used, as well as on the regulatory elements that control the expression of the (relevant) nucleotide sequence of the invention. Again, reference is made to the handbooks and patent applications mentioned above in the paragraphs on the genetic constructs of the invention.

Generally, suitable conditions may include the use of a suitable medium, the presence of a suitable source of food and/or suitable nutrients, the use of a suitable temperature, and optionally the presence of a suitable inducing factor or compound (e.g. when the nucleotide sequences of the invention are under the control of an inducible promoter); all of which may
be selected by the skilled person. Again, under such conditions, the amino acid sequences of
the invention may be expressed in a constitutive manner, in a transient manner, or only when
suitably induced.

It will also be clear to the skilled person that the amino acid sequence, Nanobody or
polypeptide of the invention may (first) be generated in an immature form (as mentioned
above), which may then be subjected to post-translational modification, depending on the
host cell/host organism used. Also, the amino acid sequence, Nanobody or polypeptide of the
invention may be glycosylated, again depending on the host cell/host organism used.

The amino acid sequence, Nanobody or polypeptide of the invention may then be
isolated from the host cell/host organism and/or from the medium in which said host cell or
host organism was cultivated, using protein isolation and/or purification techniques known
per se, such as (preparative) chromatography and/or electrophoresis techniques, differential
precipitation techniques, affinity techniques (e.g. using a specific, cleavable amino acid
sequence fused with the amino acid sequence, Nanobody or polypeptide of the invention)
and/or preparative immunological techniques (i.e. using antibodies against the amino acid
sequence to be isolated).

Generally, for pharmaceutical use, the polypeptides of the invention may be
formulated as a pharmaceutical preparation or compositions comprising at least one
polypeptide of the invention and at least one pharmaceutically acceptable carrier, diluent or
excipient and/or adjuvant, and optionally one or more further pharmaceutically active
polypeptides and/or compounds. 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 or subcutaneous injection or intravenous infusion), for topical
administration, for administration by inhalation, by a skin patch, by an implant, by a
suppository, etc. 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, and are further described herein.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that
contains at least one amino acid of the invention, at least one Nanobody of the invention or at
least one polypeptide of the invention and at least one suitable carrier, diluent or excipient
(i.e. suitable for pharmaceutical use), and optionally one or more further active substances.
Generally, the amino acid sequences, Nanobodies and polypeptides of the invention can be formulated and administered in any suitable manner known per se. for which reference is for example made to the general background art cited above (and in particular to WO 04/041862, WO 04/041863, WO 04/041865. WO 04/041 867 and WO 08/020079) as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy, 21st Edition, Lippincott Williams and Wilkins (2005) or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley. Weinheim, 2007 (see for example pages 252-255).

For example, the amino acid sequences, Nanobodies and polypeptides of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments (including ScFv's and diabodies) and other pharmaceutically active proteins. Such formulations and methods for preparing the same will be clear to the skilled person, and for example include preparations suitable for parenteral administration (for example intravenous, intraperitoneal, subcutaneous, intramuscular, intraluminal, intra-arterial or intrathecal administration) or for topical (i.e. transdermal or intradermal) administration.

Preparations for parenteral administration may for example be sterile solutions, suspensions, dispersions or emulsions that are suitable for infusion or injection. Suitable carriers or diluents for such preparations for example include, without limitation, sterile water and 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 or as well as mineral oils, animal oils and vegetable oils, for example peanut oil, soybean oil, as well as suitable mixtures thereof. Usually, aqueous solutions or suspensions will be preferred.

The amino acid sequences. Nanobodies and polypeptides of the invention can also be administered using gene therapy methods of delivery. See, e.g., U.S. Patent No. 5,399,346, which is incorporated by reference in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene encoding an amino acid sequence, Nanobody or polypeptide of the invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells and can additionally be transfected with signal and stabilization sequences for subcellularly localized expression.

Thus, the amino acid sequences. Nanobodies and polypeptides of the invention may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable
vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the amino acid sequences, Nanobodies and polypeptides of the invention may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of the amino acid sequence, Nanobody or polypeptide of the invention. Their percentage in the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of the amino acid sequence, Nanobody or polypeptide of the invention in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, com starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the amino acid sequences, Nanobodies and polypeptides of the invention, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the amino acid sequences, Nanobodies and polypeptides of the invention may be incorporated into sustained-release preparations and devices.

Preparations and formulations for oral administration may also be provided with an enteric coating that will allow the constructs of the invention to resist the gastric environment and pass into the intestines. More generally, preparations and formulations for oral administration may be suitably formulated for delivery into any desired part of the
gastrointestinal tract. In addition, suitable suppositories may be used for delivery into the gastrointestinal tract.

The amino acid sequences, Nanobodies and polypeptides of the invention may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the amino acid sequences, Nanobodies and polypeptides of the invention or their salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. 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. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the amino acid sequences, Nanobodies and polypeptides of the invention 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.
For topical administration, the amino acid sequences, Nanobodies and polypeptides of the invention may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, hydroxyalkyls or glycols or water-alcohol/glycol blends, in which the amino acid sequences, Nanobodies and polypeptides of the invention can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified cellulosics or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the amino acid sequences, Nanobodies and polypeptides of the invention to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the amino acid sequences, Nanobodies and polypeptides of the invention can be determined by comparing their in vitro activity, and in vivo activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the amino acid sequences, Nanobodies and polypeptides of the invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the amino acid sequences, Nanobodies and polypeptides of the invention required for use in treatment will vary not only with the particular amino acid sequence, Nanobody or polypeptide selected but also with the route of administration, the
nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the amino acid sequences, Nanobodies and polypeptides of the invention varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

In another aspect the invention relates to a method for the prevention and/or treatment of at least one viral and/or bacteria induced disease and/or disorders, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same.

In the context of the present invention, the term "prevention and/or treatment" not only comprises preventing and/or treating 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 may be any warm-blooded animal, but is in particular 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 of, the diseases and disorders mentioned herein.

The invention relates to a method for the prevention and/or treatment of at least one disease or disorder that is associated with Targets of the invention, with its biological or pharmacological activity, and/or with the biological pathways or signalling in which Targets of the invention is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same. In particular, the invention relates to a method for the prevention and/or treatment of at least one disease or disorder that can be treated by modulating Targets of the invention, its biological or pharmacological activity, and/or the biological pathways or signalling in which Targets of the invention is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same. In particular, said pharmaceutically effective amount may be an amount that is sufficient to modulate Targets of the invention, its biological or pharmacological activity, and/or the biological pathways or signalling in which Targets of the invention is involved; and/or an amount that provides a level of the amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention in the circulation that is sufficient to modulate Targets of the invention, its biological or pharmacological activity, and/or the biological pathways or signalling in which Targets of the invention is involved.

The invention furthermore relates to a method for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering an amino acid sequence of the invention, a Nanobody of the invention or a polypeptide of the invention to a patient, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same.

More in particular, the invention relates to a method for the prevention and/or treatment of at least one disease or disorder chosen from the group consisting of the diseases and disorders listed herein, said method comprising administering, to a subject in need
thereof, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same.

In another aspect, the invention relates to a method for immunotherapy, and in particular for passive immunotherapy, which method comprises administering, to a subject suffering from or at risk of the diseases and disorders mentioned herein, a pharmaceutically active amount of an amino acid sequence of the invention, of a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same.

In the above methods, the amino acid sequences, Nanobodies and/or polypeptides of the invention and/or the compositions comprising the same can be administered in any suitable manner, depending on the specific pharmaceutical formulation or composition to be used. Thus, the amino acid sequences, Nanobodies and/or polypeptides of the invention and/or the compositions comprising the same can for example be administered orally, intraperitoneally (e.g. intravenously, subcutaneously, intramuscularly, or via any other route of administration that circumvents the gastrointestinal tract), intranasally, transdermally, topically, by means of a suppository, by inhalation, again depending on the specific pharmaceutical formulation or composition to be used. The clinician will be able to select a suitable route of administration and a suitable pharmaceutical formulation or composition to be used in such administration, depending on the disease or disorder to be prevented or treated and other factors well known to the clinician.

The amino acid sequences, Nanobodies and/or polypeptides of the invention and/or the compositions comprising the same are administered according to a regime of treatment that is suitable for preventing and/or treating the disease or disorder to be prevented or treated. The clinician will generally be able to determine a suitable treatment regimen, depending on factors such as the disease or disorder to be prevented or treated, the severity of the disease to be treated and/or the severity of the symptoms thereof, the specific amino acid sequence, Nanobody or 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 amino acid sequences, Nanobodies and/or polypeptides of the invention, or of one or more compositions comprising the same, in one or more pharmaceutically effective amounts or
doses. The specific amount(s) or doses to administered can be determined by the clinician, again based on the factors cited above.

Generally, for the prevention and/or treatment of the diseases and disorders mentioned herein and depending on the specific disease or disorder to be treated, the potency of the specific amino acid sequence, Nanobody and polypeptide of the invention to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the amino acid sequences, Nanobodies and polypeptides of the invention will generally be administered in an amount between 1 gram and 0.01 microgram per kg body weight per day, preferably between 0.1 gram and 0.1 microgram per kg body weight per day, such as about 1, 10, 100 or 1000 microgram per kg body weight per day, either continuously (e.g. by infusion), as a single daily dose or as multiple divided doses during the day. The clinician will generally be able to determine a suitable daily dose, depending on the factors mentioned herein. It will also be clear that in specific cases, the clinician may choose to deviate from these amounts, for example on the basis of the factors cited above and his expert judgment.

Generally, some guidance on the amounts to be administered can be obtained from the amounts usually administered for comparable conventional antibodies or antibody fragments against the same target administered via essentially the same route, taking into account however differences in affinity/avidity, efficacy, biodistribution, half-life and similar factors well known to the skilled person.

Usually, in the above method, a single amino acid sequence, Nanobody or polypeptide of the invention will be used. It is however within the scope of the invention to use two or more amino acid sequences. Nanobodies and/or polypeptides of the invention in combination.

The Nanobodies, amino acid sequences and polypeptides of the invention may also be used in combination with one or more further pharmaceutically active compounds or principles, i.e. as a combined treatment regimen, which may or may not lead to a synergistic effect. Again, the clinician will be able to select such further compounds or principles, as well as a suitable combined treatment regimen, based on the factors cited above and his expert judgement.

In particular, the amino acid sequences, Nanobodies and polypeptides of the invention may be used in combination with other pharmaceutically active compounds or principles (such as e.g. a virus blocker) that are or can be used for the prevention and/or treatment of the diseases and disorders cited herein, as a result of which a synergistic effect may or may not
be obtained. Examples of such compounds and principles, as well as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician.

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, as will be clear to the skilled person.

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.

The effectiveness of the treatment regimen used according to the invention may be determined and/or followed in any manner known per se for the disease or disorder involved, as will be clear to the clinician. The clinician will also be able, where appropriate and on a case-by-case basis, to change or modify a particular treatment regimen, so as to achieve the desired therapeutic effect, to avoid, limit or reduce unwanted side-effects, and/or to achieve an appropriate balance between achieving the desired therapeutic effect on the one hand and avoiding, limiting or reducing undesired side effects on the other hand.

Generally, the treatment regimen will be followed until the desired therapeutic effect is achieved and/or for as long as the desired therapeutic effect is to be maintained. Again, this can be determined by the clinician.
In another aspect, the invention relates to the use of an amino acid sequence, Nanobody or polypeptide of the invention in the preparation of a pharmaceutical composition for prevention and/or treatment of at least one viral and/or bacteria induced disease and/or disorder; and/or for use in one or more of the methods of treatment mentioned herein.

The subject to be treated may be any warm-blooded animal, but is in particular 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 of, the diseases and disorders mentioned herein.

The invention also relates to the use of an amino acid sequence, Nanobody or polypeptide of the invention in the preparation of a pharmaceutical composition for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering an amino acid sequence. Nanobody or polypeptide of the invention to a patient.

More in particular, the invention relates to the use of an amino acid sequence, Nanobody or polypeptide of the invention in the preparation of a pharmaceutical composition for the prevention and/or treatment of viral and/or bacteria induced diseases and disorders, and in particular for the prevention and treatment of one or more of the diseases and disorders listed herein.

Again, in such a pharmaceutical composition, the one or more amino acid sequences, Nanobodies or polypeptides of the invention may also be suitably combined with one or more other active principles, such as those mentioned herein.

Finally, although the use of the Nanobodies of the invention (as defined herein) and of the polypeptides of the invention is much preferred, it will be clear that on the basis of the description herein, the skilled person will also be able to design and/or generate, in an analogous manner, other amino acid sequences and in particular (single) domain antibodies against Targets of the invention, as well as polypeptides comprising such (single) domain antibodies.

For example, it will also be clear to the skilled person that it may be possible to "graft" one or more of the CDR's mentioned above for the Nanobodies of the invention onto such (single) domain antibodies or other protein scaffolds, including but not limited to human scaffolds or non-immunoglobulin scaffolds. Suitable scaffolds and techniques for such CDR grafting will be clear to the skilled person and are well known in the art, see for example US-
example, techniques known per se for grafting mouse or rat CDR's onto human frameworks
and scaffolds can be used in an analogous manner to provide chimeric proteins comprising
one or more of the CDR's of the Nanobodies of the invention and one or more human
framework regions or sequences.

It should also be noted that, when the Nanobodies of the inventions contain one or
more other CDR sequences than the preferred CDR sequences mentioned above, these CDR
sequences can be obtained in any manner known per se, for example from Nanobodies
(preferred), V_{H} domains from conventional antibodies (and in particular from human
antibodies), heavy chain antibodies, conventional 4-chain antibodies (such as conventional
human 4-chain antibodies) or other immunoglobulin sequences directed against Targets of
the invention. Such immunoglobulin sequences directed against Targets of the invention can
be generated in any manner known per se. as will be clear to the skilled person, i.e. by
immunization with Targets of the invention or by screening a suitable library of
immunoglobulin sequences with Targets of the invention, or any suitable combination
thereof. Optionally, this may be followed by techniques such as random or site-directed
mutagenesis and/or other techniques for affinity maturation known per se. Suitable
techniques for generating such immunoglobulin sequences will be clear to the skilled person,
and for example include the screening techniques reviewed by Hoogenboom, Nature
Biotechnology. 23. 9, 1105-1 116 (2005) Other techniques for generating immunoglobulins
against a specified target include for example the Nanoclone technology (as for example
described in the published US patent application 2006-021 1088), so-called SLAM
technology (as for example described in the European patent application 0 542 810), the use
of transgenic mice expressing human immunoglobulins or the well-known hybridoma
techniques (see for example Larrick et al, Biotechnology, Vol.7, 1989, p. 934). All these
techniques can be used to generate immunoglobulins against Targets of the invention, and the
CDR's of such immunoglobulins can be used in the Nanobodies of the invention, i.e. as
outlined above. For example, the sequence of such a CDR can be determined, synthesized
and/or isolated, and inserted into the sequence of a Nanobody of the invention (e.g. so as to replace the corresponding native CDR), all using techniques known per se such as those described herein, or Nanobodies of the invention containing such CDR's (or nucleic acids encoding the same) can be synthesized de novo, again using the techniques mentioned herein.

Further uses of the amino acid sequences, Nanobodies, polypeptides, nucleic acids, genetic constructs and hosts and host cells of the invention will be clear to the skilled person based on the disclosure herein. For example, and without limitation, the amino acid sequences of the invention can be linked to a suitable carrier or solid support so as to provide a medium than can be used in a manner known per se to purify Targets of the invention from compositions and preparations comprising the same. Derivatives of the amino acid sequences of the invention that comprise a suitable detectable label can also be used as markers to determine (qualitatively or quantitatively) the presence of Targets of the invention in a composition or preparation or as a marker to selectively detect the presence of Targets of the invention on the surface of a cell or tissue (for example, in combination with suitable cell sorting techniques).

The invention will now be further described by means of the following non-limiting aspects and experimental part:
Preferred Aspects:

Aspect A-I: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention and that inhibits entry of a vims such as e.g. HIV, HCV. adenoviruses, hantavirus, herpesvirus, echo-virus 1 and others into cells.

Aspect A-2: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 1OnM, even more preferably InM.

Aspect A-3: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 10OnM, preferably 5OnM, more preferably 10nM, even more preferably InM.

Aspect A-4: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD8 1, hSR-BI, hClaudin- 1, hClaudin- 6 and hClaudin-9 and that inhibits entry of HIV into cells.

Aspect A-5: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-B1, hClaudin- 1, hClaudin-6 and hClaudm-9 and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 1OnM, even more preferably InM.

Aspect A-6: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin,
hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably InM.

5 Aspect A-7: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that inhibits entry of HIV into cells.

10 Aspect A-8: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably InM.

15 Aspect A-9: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably InM.

20 Aspect A-10: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to to hCXCR4 (SEQ ID NO: 254) and that inhibits entry of HIV into cells.

25 Aspect A-11: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to to hCXCR4 (SEQ ID NO: 254) and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably InM.

30 Aspect A-12: Amino acid sequence, e.g. single variable domain, that is directed against and/or that specifically binds to to hCXCR4 (SEQ ID NO: 254) and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably InM.
Aspect A-13: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that specifically binds to one of the Targets of the invention, e.g. 
selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, 
human alphaV integrin, human beta3 integrin, human beta1 integrin, human 
alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.

Aspect A-14: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that specifically binds to at least one of the Targets of the invention, e.g. 
CXCR4, CXCR7 or CD4.

Aspect A-15: Amino acid sequence, e.g. single variable domain according to aspect A-14, 
wherein the member of CXCR4 is human CXCR4.

Aspect A-16: Amino acid sequence, e.g. single variable domain according to aspect A-14, 
wherein the member of CD4 is human CD4.

Aspect A-17: Amino acid sequence, e.g. single variable domain according to aspect A-14, 
wherein the member of CXCR7 is human CXCR7.

Aspect A-18: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that specifically binds to one of the Targets of the invention, e.g. 
selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, 
human alphaV integrin, human beta3 integrin, human beta1 integrin, human 
alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and 
inhibits and/or blocks binding of the Target of the invention to its ligand.

Aspect A-19: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that specifically binds to hCD4 and inhibits and/or blocks binding of 
gp120 to hCD4.

Aspect A-20: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that specifically binds to hCXCR4 and inhibits and/or blocks binding of 
CXCL12 to hCXCR4.

Aspect A-21: Amino acid sequence, e.g. single variable domain, that is directed against 
and/or that a) specifically binds to one of the Targets of the invention; and b) 
fully inhibits Hgand-dependent activation of one of the Targets of the 
invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, 
hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, 
human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and
hClaudin-9, wherein the ligand is present in a concentration of 100 nM or less, more preferably 30 nM or less; and c) provides no activation of one of the Targets of the invention.

Aspect A-22: Amino acid sequence, e.g. single variable domain according to aspect A-1 to A-16, that is in essentially isolated form.

Aspect A-23: Amino acid sequence, e.g. single variable domain according to aspect A-1 or A-17, for administration to a subject, wherein said amino acid sequence does not naturally occur in said subject.

Aspect A-24: Amino acid sequence, e.g. single variable domain that can specifically bind to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4. human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin. hCD81, hSR-BI, hClaudin-1. hClaudin-6 and hClaudin-9 with a dissociation constant (K_d) of 10^5 to 10^12 moles/litre or less, and preferably 10^7 to 10^12 moles/litre or less and more preferably 10^8 to 10^12 moles/litre. Such an amino acid sequence may in particular be an amino acid sequence according to any of the preceding aspects.

Aspect A-25: Amino acid sequence, e.g. single variable domain that can specifically bind to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4. human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin. hCD81, hSR-BI, hClaudin-1. hClaudin-6 and hClaudin-9 with a rate of association (k_{on}-rate) of between 10^2 M^{-1}S^{-1} to about 10^7 M^{-1}S^{-1}, preferably between 10^3 M^{-1} and 10^7 M^{-1}S^{-1}, more preferably between 10^4 M^{-1}S^{-1} and 10^7 M^{-1}S^{-1}, such as between 10^5 M^{-1}S^{-1} and 10^7 M^{-1}S^{-1}. Such an amino acid sequence may in particular be an amino acid sequence according to any of the preceding aspects.

Aspect A-26: Amino acid sequence, e.g. single variable domain that can specifically bind to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5. hTLR4. human alphaV integrin. human beta3 integrin, human beta1 integrin, human alpha2 integrin. hCD81. hSR-BI hClaudin-1, hClaudin-6 and hClaudin-9 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^6 s^{-1}, preferably between 10^{-2} s^{-1} and 10^5 s^{-1}, more
preferably between $10^{-3}$ s$^{-1}$ and $10^{-6}$ s$^{-1}$, such as between $10^{-3}$ s$^{-1}$ and $10^{-5}$ s$^{-1}$. Such an amino acid sequence may in particular be an amino acid sequence according to any of the preceding aspects.

Aspect A-27: Amino acid sequence, e.g. single variable domain that can specifically bind to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Such an amino acid sequence may in particular be an amino acid sequence according to any of the preceding aspects.

Aspect A-28: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that is a naturally occurring amino acid sequence (from any suitable species) or a synthetic or semi-synthetic amino acid sequence.

Aspect A-29: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect A-30: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively).

Aspect A-31: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that is an immunoglobulin sequence.

Aspect A-32: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect A-33: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as affinity maturation.
Aspect A-34: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a light chain variable domain sequence (e.g. a VL-sequence); or of a heavy chain variable domain sequence (e.g. a VH-sequence).

Aspect A-35: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

Aspect A-36: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a domain antibody (or an amino acid sequence that is suitable for use as a domain antibody), of a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), of a "dAb" (or an amino acid sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a VHH sequence).

Aspect A-37: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a Nanobody.

Aspect A-38: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a Nanobody that

i) has at least 80% amino acid identity with at least one of the An amino acid sequences of SEQ ID NO:s: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3.

Aspect A-39: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a polypeptide that

i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO:s: 238 to 253, more preferably 238 to 239 and one of the amino acid sequences of SEQ ID NO:s: 308 to 311, more
preferably 311, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3.

Aspect A-40: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a Nanobody that

i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239 and one of the amino acid sequences of SEQ ID NO's: 308 to 311. more preferably 311, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

ü) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect A-41: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that essentially consists of a humanized Nanobody.

Aspect A-42: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that in addition to the at least one binding site for binding against one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-9 and hClaudin-9, contains one or more further binding sites for binding against other antigens, proteins or targets.

Aspect B-1: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4, and that comprises one or more stretches of amino acid residues chosen from the group consisting of:
a) the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 142 to 157, more preferably 142 to 143;
d) the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 174 to 189, more preferably 174 to 175;
g) the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 206 to 221, more preferably 206 to 207;

or any suitable combination thereof.

Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42.

Aspect B-2: Amino acid sequence, e.g. single variable domain according to aspect B-1, in which at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against hCXCR4.
Aspect B-3: Amino acid sequence, e.g. single variable domain that is directed against
and/or that can specifically bind hCXCR4 and that comprises two or more
stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably
   142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at
   least one of the amino acid sequences of SEQ ID NO's: 142 to 157,
   more preferably 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at
   least one of the amino acid sequences of SEQ ID NO's: 142 to 157,
   more preferably 142 to 143;

d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably
   174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at
   least one of the amino acid sequences of SEQ ID NO's: 174 to 189,
   more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at
   least one of the amino acid sequences of SEQ ID NO's: 174 to 189,
   more preferably 174 to 175;

g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably
   206 to 207;

h) amino acid sequences that have at least 80% amino acid identity with at
   least one of the amino acid sequences of SEQ ID NO's: 206 to 221,
   more preferably 206 to 207;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at
   least one of the amino acid sequences of SEQ ID NO's: 206 to 221,
   more preferably 206 to 207;

such that (i) when the first stretch of amino acid residues corresponds to one of
the amino acid sequences according to a), b) or c), the second stretch of amino
acid residues corresponds to one of the amino acid sequences according to d),
e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds
to one of the amino acid sequences according to d), e) or f), the second stretch
of amino acid residues corresponds to one of the amino acid sequences
according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid
residues corresponds to one of the amino acid sequences according to g), h) or
i), the second stretch of amino acid residues corresponds to one of the amino
acid sequences according to a), b), c), d), e) or f).

Such an amino acid sequence may in particular be an amino acid sequence according to any
of the aspects A-I to A-42, B-I or B-2.

Aspect B-4: Amino acid sequence, e.g. single variable domain according to aspect B-3, in
which the at least two stretches of amino acid residues forms part of the
antigen binding site for binding against hCXCR4.

Aspect B-5: Amino acid sequence, e.g. single variable domain that is directed against
and/or that can specifically bind hCXCR4 and that comprises three or more
stretches of amino acid residues, in which the first stretch of amino acid
residues is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably
   142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at
   least one of the amino acid sequences of SEQ ID NO's: 142 to 157,
   more preferably 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at
   least one of the amino acid sequences of SEQ ID NO's: 142 to 157,
   more preferably 142 to 143;

the second stretch of amino acid residues is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably
   174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at
   least one of the amino acid sequences of SEQ ID NO's: 174 to 189,
   more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at
   least one of the amino acid sequences of SEQ ID NO's: 174 to 189,
   more preferably 174 to 175:
and the third stretch of amino acid residues is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID N(Ts: 206 to 221, more preferably 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42 and/or B-I to B-4.

Aspect B-6: Amino acid sequence, e.g. single variable domain according to aspect B-5, in which the at least three stretches of amino acid residues forms part of the antigen binding site for binding against hCXCR4.

Aspect B-7: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4 in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity.

such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, preferably 238 to 239. Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42 and/or B-I to B-6.

Aspect B-8: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO'S: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO'S: 238 to 253, more preferably 238 to 239, wherein up to 10 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions.
Aspect B-9: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4. wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO’S: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO’S: 238 to 253, more preferably 238 to 239. wherein up to 8 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions.

Aspect B-10: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4. wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO’S: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239, wherein up to 5 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions.

Aspect C-1: Amino acid sequence, e.g. single variable domain that is directed against hCXCR4 and that cross-blocks the binding of at least one of the amino acid sequences of SEQ ID NO’s: 238 to 253, preferably 238 to 239 to hCXCR4. Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-1 to A-42 and/or according to aspects B-1 to B-11. Also, preferably, such an amino acid sequence is able to specifically bind to hCXCR4.

Aspect C-2: Amino acid sequence, e.g. single variable domain that is directed against hCXCR4 and that is cross-blocked from binding to hCXCR4 by at least one of the amino acid sequences of SEQ ID NO’s: 238 to 253, preferably 238 to 239.
Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42 and/or according to aspects B-I to B-I 1. Also, preferably, such an amino acid sequence is able to specifically bind to hCXCR4.

5 Aspect C-3: Amino acid sequence, e.g. single variable domain according to any of aspects C-I or C-2, wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect C-4: Amino acid sequence, e.g. single variable domain according to any of aspects C-I to C-3 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in an ELISA assay.

Aspect D-1: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-1 1 or C-I to C-4, that is in essentially isolated form.

Aspect D-2: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-1 1, C-I to C-4, and/or D-I for administration to a subject, wherein said amino acid sequence does not naturally occur in said subject.

Aspect D-3: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-1 1, C-I to C-4, and/or D-I to D-2 that can specifically bind to hCXCR4 with a dissociation constant (K_d) of 10^5 to 10^12 moles/litre or less, and preferably 10^-7 to 10^-12 moles/litre or less and more preferably 10^-8 to 10^-12 moles/litre.

Aspect D-4: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-1 1, C-I to C-4, and/or D-I to D-3 that can specifically bind to hCXCR4 with a rate of association (kon-rate) of between 10^2 IVT/s^-1 to about 10^7 M^-1s^-1, preferably between 10^3 M^-1S^-1 and 10^7 M^-1s^-1, more preferably between 10^4 M^-1V 1 and 10^7 IVT/S1, such as between 10^5 M^-1V 1 and 10^7 M^-1S^-1.

Aspect D-5: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-1 1, C-I to C-4, and/or D-I to D-4 that can specifically bind to hCXCR4 with a rate of dissociation (koff rate) between 1 s^-1 and 10^6 s^-1 preferably between 10^-2 s^-1 and 10^-6 s^-1, more preferably between 10^-3 s^-1 and 10^-5 s^-1, such as between 10^-2 s^-1 and 10^5 s^-1.
Aspect D-6: An amino acid sequence, e.g. a variable domain according to any of aspects B-1 to B-II, C-I to C-4, and/or D-I to D-5 that can specifically bind to hCXCR4 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Aspect D-7: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-II, C-I to C-4, and/or D-I to D-6, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with 80% sequence identity to at least one sequence selected from the group consisting of sequences having SEQ ID NO: 238 to 253, more preferably 238 to 239; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( K_D \) of \( 10^{-7} \) to \( 10^{-12} \) moles/liter or less.

Aspect D-8: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-II, C-I to C-4, and/or D-I to D-7, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239, wherein up to 10 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( K_D \) of \( 10^{-7} \) to \( 10^{-12} \) moles/liter or less.

Aspect D-9: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-II, C-I to C-4, and/or D-I to D-8, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239, wherein up to 8 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( K_D \) of \( 10^{-7} \) to \( 10^{-12} \) moles/liter or less.
Aspect D-IO: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-II, C-I to C-4, and/or D-I to D-9, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239, wherein up to 5 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant (K_D) of 10^7 to 10^12 moles/liter or less.

Aspect D-I1: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-I1, C-I to C-4, and/or D-I to D-IO, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO: 238 to 253, more preferably 238 to 239, wherein up to 3 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant (K_D) of 10^7 to 10^12 moles/liter or less.

Aspect D-12: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-I1, C-I to C-4, and/or D-I to D-I1, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO’s: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with 80% sequence identity to at least one sequences selected from the group consisting of sequences having SEQ ID NO: 238 to 253, more preferably 238 to 239; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant (K_D) of 10^8 to 10^12 moles/liter or less.

Aspect D-I3: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-I1, C-I to C-4, and/or D-I to D-12, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with sequences having SEQ ID NO: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with sequences having SEQ ID NO: 238 to 253, more
preferably 238 to 239, wherein up to 10 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( (K_D) \) of \( 10^{-8} \) to \( 10^{-12} \) moles/liter or less.

Aspect D-14: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-H, C-I to C-4, and/or D-I to D-13, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239, wherein up to 8 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( (K_D) \) of \( 10^{-8} \) to \( 10^{-12} \) moles/liter or less.

Aspect D-15: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-I1, C-I to C-4, and/or D-I to D-14, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239, wherein up to 5 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( (K_D) \) of \( 10^{-8} \) to \( 10^{-12} \) moles/liter or less.

Aspect D-16: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-I1, C-I to C-4, and/or D-I to D-15, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with SEQ ID NO's: 238 to 253, more preferably 238 to 239, wherein up to 3 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCXCR4 with a dissociation constant \( (K_D) \) of \( 10^{-8} \) to \( 10^{-12} \) moles/liter or less.
The amino acid sequences according to aspects D-I to D-16 may in particular be an amino acid sequence according to any of the aspects A-I to A-42.

Aspect B-12: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCD4, and that comprises one or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295:

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295:

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

or any suitable combination thereof.
Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42.

Aspect B-13: Amino acid sequence, e.g. single variable domain according to aspect B-12, in which at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.

Aspect B-14: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCD4 and that comprises two or more stretches of amino acid residues chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;

d) the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
such that (i) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the amino acid sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the amino acid sequences according to a), b), c), d), e) or f).

Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42, B-12 or B-13.

Aspect B-15: Amino acid sequence, e.g. single variable domain according to aspect B-14, in which the at least two stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.

Aspect B-16: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCD4 and that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 284 to 287, more preferably 2873;

d) the amino acid sequences of SEQ ID NO’s: 292 to 295, more preferably 295;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 292 to 295, more preferably 295:

and the third stretch of amino acid residues is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303.

Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42 and/or B-12 to B-15.

Aspect B-17: Amino acid sequence, e.g. single variable domain according to aspect B-16, in which the at least three stretches of amino acid residues forms part of the antigen binding site for binding against hCD4.

Aspect B-18: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCD4 in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, preferably 311. Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-I to A-42 and/or B-12 to B-17.

Aspect B-19: Amino acid sequence, e.g. single variable domain that is directed against and/or that can specifically bind hCXCR4, wherein the amino acid sequence is
selected from the group consisting of a) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311, wherein up to 10 amino acid residues are
replaced by naturally occurring amino acids and wherein said replaced amino
acids are located within the framework regions.

Aspect B-20: Amino acid sequence, e.g. single variable domain that is directed against
and/or that can specifically bind hCXCR4, wherein the amino acid sequence is
selected from the group consisting of a) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311, wherein up to 8 amino acid residues are
replaced by naturally occurring amino acids and wherein said replaced amino
acids are located within the framework regions.

Aspect B-21: Amino acid sequence, e.g. single variable domain that is directed against
and/or that can specifically bind hCXCR4, wherein the amino acid sequence is
selected from the group consisting of a) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID
NO: 308 to 311, preferably 311, wherein up to 5 amino acid residues are
replaced by naturally occurring amino acids and wherein said replaced amino
acids are located within the framework regions.

Aspect B-22: Amino acid sequence, e.g. single variable domain that is directed against
and/or that can specifically bind hCXCR4, wherein the amino acid sequence is
selected from the group consisting of a) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID
NO'S: 308 to 311, preferably 311, wherein up to 3 amino acid residues are
replaced by naturally occurring amino acids and wherein said replaced amino
acids are located within the framework regions.

Aspect C-5: Amino acid sequence, e.g. single variable domain that is directed against
hCD4 and that cross-blocks the binding of at least one of the amino acid
sequences of SEQ ID NO’S: 308 to 311, preferably 311 to hCD4. Such an
amino acid sequence may in particular be an amino acid sequence according to
any of the aspects A-1 to A-42 and/or according to aspects B-12 to B-22. Also, preferably, such an amino acid sequence is able to specifically bind to hCD4.

Aspect C-6: Amino acid sequence, e.g. single variable domain that is directed against hCD4 and that is cross-blocked from binding to hCD4 by at least one of the amino acid sequences of SEQ ID NO’s: 308 to 311, preferably 311. Such an amino acid sequence may in particular be an amino acid sequence according to any of the aspects A-1 to A-42 and/or according to aspects B-12 to B-22. Also, preferably, such an amino acid sequence is able to specifically bind to hCD4.

Aspect C-7: Amino acid sequence, e.g. single variable domain according to any of aspects C-1 or C-2, wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect C-8: Amino acid sequence, e.g. single variable domain according to any of aspects C-1 to C-3 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in an ELISA assay.

Aspect D-17: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, that is in essentially isolated form.

Aspect D-18: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8. and/or D-17 for administration to a subject, wherein said amino acid sequence does not naturally occur in said subject.

Aspect D-19: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8. and/or D-17 to D-18 that can specifically bind to hCD4 with a dissociation constant ($K_d$) of $10^5$ to $10^{-12}$ moles/litre or less, and preferably $10^{-7}$ to $10^{-12}$ moles/litre or less and more preferably $10^{-8}$ to $10^{-12}$ moles/litre.

Aspect D-20: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-19 that can specifically bind to hCD4 with a rate of association ($k_{on}$-rate) of between $10^2 \text{ M}^\cdot \text{V}$ to about $10^7 \text{ M}^\cdot \text{s}^{-1}$, preferably between $10^3 \text{ M}^\cdot \text{s}^{-1}$ and $10^7 \text{ M}^\cdot \text{s}^{-1}$, more preferably between $10^4 \text{ M}^\cdot \text{s}^{-1}$ and $10^7 \text{ M}^\cdot \text{s}^{-1}$, such as between $10^5 \text{ M}^\cdot \text{s}^{-1}$ and $10^7 \text{ M}^\cdot \text{s}^{-1}$.

Aspect D-21: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-20 that can specifically bind to
hCD4 with a rate of dissociation ($k_{\text{Off}}$ rate) between $1 \, \text{s}^{-1}$ and $10^6 \, \text{s}^{-1}$ preferably between $10^{2} \, \text{s}^{-1}$ and $10^6 \, \text{s}^{-1}$. more preferably between $10^{3} \, \text{s}^{-1}$ and $10^6 \, \text{s}^{-1}$, such as between $10^{3} \, \text{s}^{-1}$ and $10^6 \, \text{s}^{-1}$.

Aspect D-22: An amino acid sequence, e.g. a variable domain according to any of aspects B-12 to B-22 or C-5 to C-8. and/or D-17 to D-21 that can specifically bind to hCD4 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Aspect D-23: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-22, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO’s: 238 to 253, more preferably 238 to 239; and b) amino acid sequences with 80% sequence identity to at least one sequence selected from the group consisting of sequences having SEQ ID NO: 238 to 253. more preferably 238 to 239; and wherein said selected amino acid sequence from group a) and b) binds to hCD4 with a dissociation constant ($K_{D}$) of $10^{-7}$ to $10^{-12}$ moles/liter or less.

Aspect D-24: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-23, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO’s: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID NO’s: 308 to 311, preferably 311. wherein up to 10 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions: and wherein said selected amino acid sequence from group a) and b) binds to at hCD4 with a dissociation constant ($K_{D}$) of $10^{-7}$ to $10^{-12}$ moles/liter or less.

Aspect D-25: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-S, and/or D-17 to D-24. wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID NO: 308 to 311, preferably 331. wherein up to 8 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein
said selected amino acid sequence from group a) and b) binds to hCD4 with a
dissociation constant (K_D) of 10^{-7} to 10^{-12} moles/liter or less.

Aspect D-26: Amino acid sequence, e.g. single variable domain according to any of aspects
B-12 to B-22 or C-5 to C-8, and/or D-17 to D-25, wherein the amino acid
sequence is selected from the group consisting of a) amino acid sequences
with SEQ ID NO: 308 to 311, preferably 311; and b) amino acid sequences
with SEQ ID NO: 308 to 311, preferably 311, wherein up to 5 amino acid
residues are replaced by naturally occurring amino acids and wherein said
replaced amino acids are located within the framework regions; and wherein
said selected amino acid sequence from group a) and b) binds to hCD4 with a
dissociation constant (K_D) of 10^{-7} to 10^{-12} moles/liter or less.

Aspect D-27: Amino acid sequence, e.g. single variable domain according to any of aspects
B-12 to B-22 or C-5 to C-8, and/or D-17 to D-26, wherein the amino acid
sequence is selected from the group consisting of a) amino acid sequences
with SEQ ID NO: 308 to 311, preferably 311; and b) amino acid sequences
with SEQ ID NO: 308 to 311, preferably 311, wherein up to 3 amino acid
residues are replaced by naturally occurring amino acids and wherein said
replaced amino acids are located within the framework regions; and wherein
said selected amino acid sequence from group a) and b) binds to hCD4 with a
dissociation constant (K_D) of 10^{-7} to 10^{-12} moles/liter or less.

Aspect D-28: Amino acid sequence, e.g. single variable domain according to any of aspects
B-12 to B-22 or C-5 to C-8, and/or D-17 to D-27, wherein the amino acid
sequence is selected from the group consisting of a) amino acid sequences
with SEQ ID NO’s: 308 to 311, preferably 311; and b) amino acid sequences
with 80% sequence identity to at least one sequences selected from the group
consisting of sequences having SEQ ID NO: 308 to 311, preferably 311; and
wherein said selected amino acid sequence from group a) and b) binds to
hCD4 with a dissociation constant (K_D) of 10^{-8} to 10^{-12} moles/liter or less.

Aspect D-29: Amino acid sequence, e.g. single variable domain according to any of aspects
B-12 to B-22 or C-5 to C-8, and/or D-17 to D-28, wherein the amino acid
sequence is selected from the group consisting of a) amino acid sequences
with sequences having SEQ ID NO: 308 to 311, preferably 311; and b) amino
acid sequences with sequences having SEQ ID NO: 308 to 311, preferably 311, wherein up to 10 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCD4 with a dissociation constant (K_D) of 10^-8 to 10^-12 moles/liter or less.

Aspect D-30: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-29, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311, wherein up to 8 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCD4 with a dissociation constant (K_D) of 10^-8 to 10^-12 moles/liter or less.

Aspect D-31: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-30, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311, wherein up to 5 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein said selected amino acid sequence from group a) and b) binds to hCD4 with a dissociation constant (K_D) of 10^-8 to 10^-12 moles/liter or less.

Aspect D-32: Amino acid sequence, e.g. single variable domain according to any of aspects B-12 to B-22 or C-5 to C-8, and/or D-17 to D-31, wherein the amino acid sequence is selected from the group consisting of a) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311; and b) amino acid sequences with SEQ ID NO's: 308 to 311, preferably 311, wherein up to 3 amino acid residues are replaced by naturally occurring amino acids and wherein said replaced amino acids are located within the framework regions; and wherein
said selected amino acid sequence from group a) and b) binds to hCD4 with a
dissociation constant (K_D) of 10^{-8} to 10^{-12} moles/liter or less.
The amino acid sequences according to aspects D-17 to D-32 may in particular be an amino acid sequence according to any of the aspects A-I to A-42.

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Aspect E-I: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, that is a naturally occurring amino acid sequence (from any suitable species) or a synthetic or semi-

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s Synthetic amino acid sequence.

10 Aspect E-2: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect E-3: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I or E-2, that is an immunoglobulin sequence.

Aspect E-4: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-3, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect E-5: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-4 that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as

affinity maturation.

Aspect E-6: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-5 that essentially consists of a light chain variable domain sequence (e.g. a V_L-sequence); or of a heavy chain variable domain sequence (e.g. a V_H-sequence).

Aspect E-7: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-6, that essentially consists of a heavy chain variable domain sequence that is derived
from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

Aspect E-8: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, that essentially consists of a domain antibody (or an amino acid sequence that is suitable for use as a domain antibody), of a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), of a "dAb" (or an amino acid sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a \( \nu_{HH} \) sequence).

Aspect E-9: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-8 that essentially consists of a Nanobody.

Aspect E-IO: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-9 that essentially consists of a Nanobody that

i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3.

Aspect E-I1: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-IO, that essentially consists of a Nanobody that

i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239 and SEQ ID NO's: 308 to 31 L more preferably 31 L in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:
ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3.

Aspect E-12: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-I L that essentially consists of a humanized Nanobody.

Aspect E-13: Amino acid sequence, e.g. single variable domain according to any of aspects B-I to B-22 or C-I to C-8, and/or D-I to D-32, and/or E-I to E-I L, that in addition to the at least one binding site for binding formed by the CDR sequences, contains one or more further binding sites for binding against other antigens, proteins or targets.

The amino acid sequences according to aspects E-I to E-13 may in particular be an amino acid sequence according to any of the aspects A-I to A-42.

Aspect F-I: Amino acid sequence, e.g. single variable domain that essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:
- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
  c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143; and/or
- CDR2 is chosen from the group consisting of:
  d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

and/or

- CDR3 is chosen from the group consisting of:

  g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

  h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

  i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

Such an amino acid sequence is preferably directed against hCXCR4 and/or an amino acid sequence that can specifically bind to hCXCR4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-I to C-4, D1 to D-16 and/or E-I to E-13.

Aspect F-2: Amino acid sequence, e.g. single variable domain that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 is chosen from the group consisting of:

  a) the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;

  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 157, more preferably 142 to 143:

and

5 - CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 189, more preferably 174 to 175:

and

15 - CDR3 is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 221, more preferably 206 to 207.

Such an amino acid sequence is preferably directed against hCXCR4 and/or an amino acid sequence that can specifically bind to hCXCR4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-I to C-4, D-I to D-16 and/or E-I to E-13.

Aspect F-3: An amino acid sequence according to any of aspects F-1 and F-2, in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one
of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239.

Such an amino acid sequence is preferably directed against hCXCR4 and/or an amino acid sequence that can specifically bind to hCXCR4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-I to C-4, D1 to D-16 and/or E-I to E-13.

Aspect F-4: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-3 that is directed against hCXCR4 and that cross-blocks the binding to hCXCR4 of at least one of the amino acid sequences according to any of aspects the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239.

Aspect F-5: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-3 that is directed against hCXCR4 and that is cross-blocked from binding to hCXCR4 by at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, more preferably 238 to 239.

Aspect F-6: Amino acid sequence, e.g. single variable domain according to any of aspects F-4 or F-5 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect F-7: Amino acid sequence, e.g. single variable domain according to any of aspects F4 or F-5 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in an ELISA assay.

Aspect F-8: Amino acid sequence, e.g. single variable domain that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 is chosen from the group consisting of:
  a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
  b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287,
more preferably 287;

and/or

5 - CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 293 to 295, more preferably 295;

10 e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 293 to 295,
more preferably 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 293 to 295,
more preferably 295;

and/or

15 - CDR3 is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303,
more preferably 303;

20 i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO’s: 300 to 303.
more preferably 303.

Such an amino acid sequence is preferably directed against hCD4 and/or an amino acid sequence that can specifically bind to hCD4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-5 to C-8, D-17 to D-32 and/or E-I to E-13.

Aspect F-9: Amino acid sequence, e.g. single variable domain that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 is chosen from the group consisting of:
a) the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 284 to 287, more preferably 287;
and

- CDR2 is chosen from the group consisting of:
d) the amino acid sequences of SEQ ID NO's: 293 to 295, more preferably 295;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 293 to 295, more preferably 295;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 293 to 295, more preferably 295;

and

- CDR3 is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 300 to 303, more preferably 303.

Such an amino acid sequence is preferably directed against hCD4 and/or an amino acid sequence that can specifically bind to hCD4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-5 to C-8, D-17 to D-32 and/or E-I to E-13.
Aspect F-10: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 and F-2, in which the CDR sequences of said amino acid sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably 311.

Such an amino acid sequence is preferably directed against hCD4 and/or an amino acid sequence that can specifically bind to hCD4. Also, such an amino acid sequence is preferably an amino acid sequence according to any of the aspects A-I to A-42, C-5 to C-8, D-17 to D-32 and/or E-1 to E-13.

Aspect F-11: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-3 that is directed against hCD4 and that cross-blocks the binding to hCD4 of at least one of the amino acid sequences according to any of aspects the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably 311.

Aspect F-12: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-3 that is directed against hCXCR4 and that is cross-blocked from binding to hCD4 by at least one of the amino acid sequences of SEQ ID NO's: 308 to 311, more preferably 311.

Aspect F-13: Amino acid sequence, e.g. single variable domain according to any of aspects F-4 or F-5 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect F-14: Amino acid sequence, e.g. single variable domain according to any of aspects F-4 or F-5 wherein the ability of said amino acid sequence to cross-block or to be cross-blocked is detected in an ELISA assay.

Aspect F-15: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-14, that is in essentially isolated form.

Aspect F-16: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-15, for administration to a subject, wherein said amino acid sequence does not naturally occur in said subject.

Aspect F-17: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-16, that can specifically bind to Target of the invention with a
dissociation constant (K_D) of $10^{-5}$ to $10^{-12}$ moles/litre or less, and preferably $10^{-7}$ to $10^{-12}$ moles/litre or less and more preferably $10^{-8}$ to $10^{-12}$ moles/litre.

Aspect F-18: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-17, that can specifically bind to Target of the invention with a rate of association (k_{on}-rate) of between $10^2$ M^{-1}S^{-1} to about $10^7$ M^{-1}S^{-1}, preferably between $10^3$ JVF^{-1}S^{-1} and $10^7$ JVF^{-1}S^{-1}, more preferably between $10^4$ JVF^{-1}S^{-1} and $10^7$ JVF^{-1}S^{-1}, such as between $10^5$ M^{-1}S^{-1} and $10^7$ M^{-1}V^{-1}.

Aspect F-19: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-18, that can specifically bind to Target of the invention with a rate of dissociation (karate) between 1 s^{-1} and $10^{-6}$ s^{-1} preferably between $10^{-2}$ s^{-1} and $10^{-6}$ s^{-1}, more preferably between $10^{-5}$ s^{-1} and $10^{-6}$ s^{-1}, such as between $10^{-4}$ s^{-1} and $10^{-6}$ s^{-1}.

Aspect F-20: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-19, that can specifically bind to Target of the invention with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Aspect F-21: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-20, that is a naturally occurring amino acid sequence (from any suitable species) or a synthetic or semi-synthetic amino acid sequence.

Aspect F-22: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-21, that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect F-23: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-22, that is an immunoglobulin sequence.

Aspect F-24: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-23, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect F-25: Amino acid sequence, e.g. single variable domain according to any of aspects F-1 to F-24, that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as affinity maturation.
Aspect F-26: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-25, that essentially consists of a light chain variable domain sequence (e.g. a \( V_L \)-sequence); or of a heavy chain variable domain sequence (e.g. a \( V_H \)-sequence).

5 Aspect F-27: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-26, that essentially consists of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

10 Aspect F-28: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-27, that essentially consists of a domain antibody (or an amino acid sequence that is suitable for use as a domain antibody), of a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), of a "dAb^n" (or an amino acid sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a \( V_{HH} \) sequence).

15 Aspect F-29: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-28, that essentially consists of a Nanobody.

Aspect F-30: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-29, that essentially consists of a Nanobody that

20 i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; and in which:

25 ū) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect F-31: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-30, that essentially consists of a Nanobody that

30 i) has at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, preferably 238 to 239 and SEQ ID NO's: 308 to 311, preferably 311, in which for the purposes of
determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; and in which:

ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3.

Aspect F-32: Amino acid sequence, e.g. single variable domain according to any of aspects F-I to F-31, that essentially consists of a humanized Nanobody.

Aspect G-I: Amino acid sequence, e.g. single variable domain according to any of the preceding aspects, that in addition to the at least one binding site for binding formed by the CDR sequences, contains one or more further binding sites for binding against another antigen, protein or target.

Aspect K-I: Polypeptide that comprises or essentially consists of one or more amino acid sequences according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, and optionally further comprises one or more other amino acid binding units, optionally linked via one or more peptidic linkers.

Aspect K-2: Polypeptide according to aspect K-I, in which said one or more other amino acid binding units are immunoglobulin sequences.

Aspect K-3: Polypeptide according to any of aspects K-I or K-2, in which said one or more other amino acid binding units are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"'s, amino acid sequences that are suitable for use as a dAb, or Nanobodies.

Aspect K-4: Polypeptide according to any of aspects K-I to K-3, in which said one or more amino acid sequences of the invention are immunoglobulin sequences.

Aspect K-5: Polypeptide according to any of aspects K-I to K-4, in which said one or more amino acid sequences of the invention are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain
antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"s, amino acid sequences that are suitable for use as a dAb, or Nanobodies.

Aspect K-6: Polypeptide according to any of aspects K-1 to K-5, which is a multivalent construct.

Aspect K-7: Polypeptide according to any of aspects K-1 to K-6, which is a multiparatopic construct.

Aspect K-8: Polypeptide according to any of aspects K-1 to K-7, which is a multispecific construct.

Aspect K-9: Polypeptide according to any of aspects K-1 to K-9, which has an increased half-life, compared to the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect K-10: Polypeptide according to aspect K-9, in which said one or more other binding units provide the polypeptide with increased half-life, compared to the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect K-11: Polypeptide according to aspect K-9 or K-10, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Aspect K-12: Polypeptide according to any of aspects K-9 to K-11, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of human serum albumin or fragments thereof.

Aspect K-13: Polypeptide according to any of aspect K-9 to K-12, in which said one or more other binding units that provides the polypeptide with increased half-life are chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).
Aspect K-14: Polypeptide according to any of aspects K-9 to K-13, in which said one or more other binding units that provides the polypeptide with increased half-life are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"'s, amino acid sequences that are suitable for use as a dAb, or Nanobodies that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect K-15: Polypeptide according to aspect K-9 to K-14, in which said one or more other binding units that provides the polypeptide with increased half-life is a Nanobody that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect K-16: Polypeptide according to any of aspects K-9 to K-15, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-E, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect K-17: Polypeptide according to any of aspects K-9 to K-16, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect K-18: Polypeptide according to any of aspects K-I to K-17, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).
Aspect L-1: Compound or construct, that comprises or essentially consists of one or more amino acid sequences according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

Aspect L-2: Compound or construct according to aspects L-I, in which said one or more other groups, residues, moieties or binding units are amino acid sequences.

Aspect L-3: Compound or construct according to aspect L-I or L-2, in which said one or more linkers, if present, are one or more amino acid sequences.

Aspect L-4: Compound or construct according to any of aspects L-I to L-3, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.

Aspect L-5: Compound or construct according to any of aspects L-I to L-4, in which said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"'s, amino acid sequences that are suitable for use as a dAb or Nanobodies.

Aspect L-6: Compound or construct according to any of aspects L-I to L-5, in which said one or more amino acid sequences of the invention are immunoglobulin sequences.

Aspect L-7: Compound or construct according to any of aspects L-I to L-6, in which said one or more amino acid sequences of the invention are chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"'s, amino acid sequences that are suitable for use as a dAb or Nanobodies.

Aspect L-8: Compound or construct according to any of aspects L-I to L-8, which is a multivalent construct.

Aspect L-9: Compound or construct according to aspect L-8, which is a bivalent construct.
Aspect L-10: Compound or construct according to aspect L-9, which comprises two amino acid sequences that are directed against and/or capable of binding to the CXCL12 binding site on hCXCR4.

Aspect L-11: Compound or construct according to any of aspects L-9 to L-10, which comprises two amino acid sequences that are capable of competing with CXCL12 for binding to hCXCR4.

Aspect L-12: Compound or construct according to any of aspects L-10 to L-14 which can simultaneously bind both binding sites on hCXCR4.

Aspect L-13: Compound or construct according to any of aspects L-9 to L-12 which neutralizes HIV.

Aspect L-14: Compound or construct according to aspect L-9, which comprises two amino acid sequences that are directed against and/or capable of binding to the gp120 binding site on hCD4.

Aspect L-15: Compound or construct according to any of aspects L-9 or L-14, which comprises two amino acid sequences that are capable of competing with gp120 for binding to hCD4.

Aspect L-16: Compound or construct according to any of aspects L-14 to L-15 which can simultaneously bind both binding sites on hCD4.

Aspect L-17: Compound or construct according to any of aspects L-14 to L-16 which neutralizes HIV.

Aspect L-18: Compound or construct according to any of aspects L-1 to L-8, which is a multiparalopic construct.

Aspect L-19: Compound or construct according to aspect L-18, which is a biparatopic construct.

Aspect L-20: Compound or construct according to aspects L-19, which comprises at least one amino acid sequence directed against a first antigenic determinant, epitope, part or domain of a Target of the invention and at least one amino acid sequence directed against a second antigenic determinant, epitope, part or domain of the Target of the invention different from the first antigenic determinant, epitope, part or domain.

Aspect L-21: Biparatopic compound or construct according to aspect L-20, which is capable of simultaneously binding to said first antigenic determinant, epitope, part or
domain of a Target of the invention and to said second antigenic determinant, epitope, part or domain of the Target of the invention.

Aspect L-22: Compound or construct according to any of aspects L-18 to L-21, which comprises at least one amino acid sequence directed against a first antigenic determinant, epitope, part or domain of hCXCR4 and at least one amino acid sequence directed against a second antigenic determinant, epitope, part or domain of hCXCR4 different from the first antigenic determinant, epitope, part or domain.

Aspect L-23: Biparatopic compound or construct according to aspect L-22, which is capable of simultaneously binding to said first antigenic determinant, epitope, part or domain of hCXCR4 and to said second antigenic determinant, epitope, part or domain of hCXCR4.

Aspect L-24: Compound or construct according to any of aspects L-22 to L-23 wherein said compound or construct competes with CXCL12 for binding to hCXCR4.

Aspect L-25: Compound or construct according to aspects L-24, wherein said compound or construct inhibits and/or blocks binding of CXCL12 to hCXCR4.

Aspect L-26: Compound or construct according to any of aspects L-24 to L-25, wherein said compound or construct is directed against the CXCL12 binding site on hCXCR4.

Aspect L-27: Compound or construct according to any of aspects L-24 to L-26, which comprises at least one amino acid sequence that is directed against and/or capable of binding to the CXCL12 binding site on hCXCR4 and at least one further amino acid sequence that is capable of binding to at least one other antigenic determinant, epitope, part or domain on hCXCR4.

Aspect L-28: Compound or construct according to any of aspects L-24 to L-27, which comprises at least one amino acid sequence that is capable of competing with CXCL12 for binding hCXCR4 and at least one further amino acid sequence that is capable of binding to at least one other antigenic determinant, epitope, part or domain on hCXCR4.

Aspect L-29: Compound or construct according to any of aspects L-24 to L-28, which can simultaneously bind both binding sites on hCXCR4.
Aspect L-30: Compound or construct according to aspect L-18, wherein both paratopes are directed against the CXCL12 binding site on hCXCR4.

Aspect L-31: Biparatopic compound or construct according to aspect L-30, which can simultaneously bind both binding site on hCXCR4.

Aspect L-32: Compound or construct according to any of aspects L-18 to L-21, which comprises at least one amino acid sequence directed against a first antigenic determinant, epitope, part or domain of hCD4 and at least one amino acid sequence directed against a second antigenic determinant, epitope, part or domain of hCD4 different from the first antigenic determinant, epitope, part or domain.

Aspect L-33: Biparatopic compound or construct according to aspect L-32, which is capable of simultaneously binding to said first antigenic determinant, epitope, part or domain of hCD4 and to said second antigenic determinant, epitope, part or domain of hCD4.

Aspect L-34: Compound or construct according to any of aspects L-32 to L-33 wherein said compound or construct competes with gp120 for binding to hCD4.

Aspect L-35: Compound or construct according to aspects L-34, wherein said compound or construct inhibits and/or blocks binding of gp120 to hCD4.

Aspect L-36: Compound or construct according to any of aspects L-34 to L-35, wherein said compound or construct is directed against the gp120 binding site on hCD4.

Aspect L-37: Compound or construct according to any of aspects L-34 to L-36, which comprises at least one amino acid sequence that is directed against and/or capable of binding to the gp120 binding site on hCD4 and at least one further amino acid sequence that is capable of binding to at least one other antigenic determinant, epitope, part or domain on hCD4.

Aspect L-38: Compound or construct according to any of aspects L-34 to L-37, which comprises at least one amino acid sequence that is capable of competing with gp120 for binding hCD4 and at least one further amino acid sequence that is capable of binding to at least one other antigenic determinant, epitope, part or domain of hCD4.

Aspect L-39: Compound or construct according to any of aspects L-34 to L-38, which can simultaneously bind both binding sites on hCD4.
Aspect L-40: Compound or construct according to aspect L-18, wherein both paratopes are directed against the gp120 binding site on hCD4.

Aspect L-41: Biparatopic compound or construct according to aspect L-40, which can simultaneously bind both binding site on hCD4,

Aspect L-42: Compound or construct according to any of aspects L-1 to L-41, which is a multispecific construct.

Aspect 1.-43: Compound or construct according to any of aspects L-1 to L-42, which has an increased half-life, compared to the corresponding amino acid sequence according to any of aspects A-1 to A-42, B-1 to B-22, C-1 to C-8, D-1 to D-32, E-1 to E-13, F-1 to F-32 or G-1 per se.

Aspect L-44: Compound or construct according to aspect L-1 to L-43, in which said one or more other groups, residues, moiety’s or binding units provide the compound or construct with increased half-life, compared to the corresponding amino acid sequence according to any of aspects A-1 to A-42, B-1 to B-22, C-1 to C-8, D-1 to D-32, E-1 to E-13, F-1 to F-32 or G-1 per se.

Aspect L-45: Compound or construct according to aspect L-44, in which said one or more other groups, residues, moiety’s or binding units that provide the compound or construct with increased half-life is chosen from the group consisting of serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Aspect L-46: Compound or construct according to aspect L-44 or L-45, in which said one or more other groups, residues, moiety’s or binding units that provide the compound or construct with increased half-life is chosen from the group consisting of human serum albumin or fragments thereof.

Aspect L-47: Compound or construct according to any of aspects L-44 to L-46, in which said one or more other groups, residues, moiety’s or binding units that provides the compound or construct with increased half-life are chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-48: Compound or construct according to any of aspects L-44 to L-46, in which said one or more other groups, residues, moiety’s or binding units that provides the compound or construct with increased half-life are chosen from the group
consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"s, amino acid sequences that are suitable for use as a dAb, or Nanobodies that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-49: Compound or construct according to any of aspects L-44 to L-46, in which said one or more other groups, residues, moieties or binding units that provides the compound or construct with increased half-life is a Nanobody that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-50: Compound or construct according to any of aspects L-43 to L-49, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect L-51: Compound or construct according to any of aspects L-43 to L-50, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se.

Aspect L-52: Compound or construct according to any of aspects L-43 to L-51, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).
Aspect H-I: Monovalent construct, comprising or essentially consisting of one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-L

Aspect H-2: Monovalent construct according to aspect H-I, in which said amino acid sequence of the invention is chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb"s, amino acid sequences that are suitable for use as a dAb, or Nanobodies.

Aspect H-3: Monovalent construct, that is chosen from the group consisting of SEQ ID NO's: 238 to 253, preferably 238 to 239 and SEQ ID NO's: 308 to 311, preferably 311 or from the group consisting of amino acid sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the amino acid sequences of SEQ ID NO's: 238 to 253, preferably 238 to 239 and SEQ ID NO's: 308 to 311, preferably 311.

Aspect H-4: Use of a monovalent construct according to any of aspects H-I to H-3, in preparing a multivalent compound or construct according to any of aspects L-8 to L-42.

Aspect H-5: Use of a monovalent construct according to aspect H-4, in preparing a multipartatopic construct such as a bivalent, biparatopic, trivalent, triparatopic construct.

Aspect H-6: Use of a monovalent construct according to any of aspects H-4 or H-5, wherein the monovalent construct is used as a binding domain or binding unit in preparing a multivalent construct comprising two or more binding units.

Aspect H-7: Use of a monovalent construct according to any of aspects H-4 to H-6, in preparing a multivalent construct that exhibits intramolecular binding compared to intermolecular binding.

Aspect H-8: Use of a monovalent construct according to any of aspects H-4 to H-7, as a binding domain or binding unit in preparing a multivalent construct, wherein the binding domains or binding units are linked via a linker such that the
multivalent construct preferably exhibits intramolecular binding compared to intermolecular binding.

Aspect H-9: Use of a monovalent construct according to any of aspects H-4 to H-8, wherein the monovalent construct is directed against the CXCL12 binding site on hCXCR4 and/or is capable of competing with CXCL12 for binding hCXCR4.

Aspect H-10: Use of two monovalent constructs according to any of aspects H-4 to H-9 for the preparation of a bivalent compound or construct, wherein the monovalent constructs are directed against the CXCL12 binding site on hCXCR4 and/or capable of competing with CXCL12 for binding hCXCR4.

Aspect H-11: Use of a monovalent construct according to any of aspects H-4 to H-8, wherein the monovalent construct is directed against the gpl20 binding site on hCD4 and/or is capable of competing with gpl20 for binding hCD4.

Aspect H-12: Use of two monovalent constructs according to any of aspects H-4 to H-9 for the preparation of a bivalent compound or construct, wherein the monovalent constructs are directed against the gpl20 binding site on hCD4 and/or capable of competing with gpl20 for binding hCD4.

Aspect M-1: Nucleic acid or nucleotide sequence, that encodes an amino acid sequence according to any of aspects A-1 to A-42, B-1 to B-22, C-1 to C-8, D-1 to D-32, E-1 to E-13, F-1 to F-32 or G-1, a compound or construct according to any of aspects that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects H-1 to H-3.

Aspect M-2: Nucleic acid or nucleotide sequence according to aspect M-1, that is in the form of a genetic construct.

Aspect M-3: Use of a nucleic acid or nucleotide sequence according to aspect M-2, that encodes a monovalent construct according to any of aspects H-1 to H-3, for the preparation of a genetic construct that encodes a multivalent construct according to any of aspects L-8 to L-42.
Aspect M-4: Use of a nucleic acid or nucleotide sequence according to aspect M-3, wherein the genetic construct encodes a multiparatopic (such as a biparatopic) construct.

Aspect N-1: Host or host cell that expresses, or that under suitable circumstances is capable of expressing, an amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects H-I or H-3; and/or that comprises a nucleic acid or nucleotide sequence according to aspect M-I or a genetic construct according to aspect M-2.

Aspect O-I: Composition comprising at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3, or nucleic acid or nucleotide sequence according to aspects M-I or M-2.

Aspect O-2: Composition according to aspect O-I, which is a pharmaceutical composition.

Aspect O-3: Composition according to aspect O-2, which is a pharmaceutical composition, that further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and that optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

Aspect P-I: Method for producing an amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the
same, or a monovalent construct according to any of aspects H-I or H-3, said method at least comprising the steps of:

a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid or nucleotide sequence according to aspect M-I, or a genetic construct according to aspect M-2;

optionally followed by:

b) isolating and/or purifying the amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52, or a monovalent construct according to any of aspects H-I or H-3 thus obtained.

Aspect P-2: Method for producing an amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects H-I or H-3, said method at least comprising the steps of:

a) cultivating and/or maintaining a host or host cell according to aspect N-I under conditions that are such that said host or host cell expresses and/or produces at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52, or a monovalent construct according to any of aspects H-I or H-3;

optionally followed by:

b) isolating and/or purifying the amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, a polypeptide according to any of aspects K-I to K-18, a compound or construct according to any of aspects L-I to L-52, or
monovalent construct according to any of aspects H-I or H-3 thus obtained.

Aspect P-3: Method for preparing and/or generating a bivalent or multivalent construct according to any of aspects L-8 to L-42, said method comprising at least the steps of linking two or more monovalent amino acid sequences or monovalent construct according to any of aspects H-I to H-3 and for example one or more linkers.

Aspect P-4: Method according to aspect P-3, comprising the steps of:

a. linking two or more nucleic acid sequences according to aspect M-1, encoding a monovalent construct according to any of aspects H-I to H-3 (and also for example nucleic acids encoding one or more linkers and further one or more further elements of genetic constructs known per se) to obtain a genetic construct according to aspect M-2;

b. expressing, in a suitable host cell or host organism or in another suitable expression system, the genetic construct obtained in a) optionally followed by:

c. isolating and/or purifying the biparatopic or triparatopic construct according to any of aspects L-8 to L-42 thus obtained.

Aspect Q-1: Method for screening amino acid sequences directed against a Target of the invention that comprises at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding amino acid sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an amino acid sequence that can bind to and/or has affinity for the Target of the invention and that is cross-blocked or is cross blocking a Nanobody of the invention, e.g. SEQ ID NO's: 238 to 253 (Table-1) or SEQ ID NO's: 308 to 311, or a polypeptide or construct of the invention, e.g. SEQ ID NO: 261 to 266 (see Table 2); and

c) isolating said nucleic acid sequence, followed by expressing said amino acid sequence.
Aspect R-1: Method for the prevention and/or treatment of at least one viral and/or bacterial induced/related disease or disorder, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect O-2 or 0-3.

Aspect R-2: Method for the prevention and/or treatment of at least one disease or disorder that is associated with a Target of the invention, with its biological or pharmacological activity, and/or with the biological pathways or signalling in which the Target of the invention is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect 0-2 or 0-3.

Aspect R-3: Method for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering, to a subject in need thereof, at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect 0-2 or 0-3. said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-Ho F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to
L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect 0-2 or O-3.

Aspect R-4: Method for immunotherapy, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-1 to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect 0-2 or 0-3.

Aspect R-5: Use of an amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3 in the preparation of a pharmaceutical composition for prevention and/or treatment of at least one viral or bacterial induced/related disease or disorder; and/or for use in one or more of the methods according to aspects R-1 to R-4.

Aspect R-6: Amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-1 to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, polypeptide according to any of aspects K-I to K-18, compound or construct according to any of aspects L-I to L-52, monovalent construct according to any of aspects H-I or H-3; or composition according to aspect 0-2 or 0-3 for the prevention and/or treatment of at least one viral or bacterial induced/related disease or disorder.

Aspect R-7: Method of generating an amino acid sequence or single variable domain according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, at least one building block of compound or construct according to any of aspects L-8 to L-42, at least one building block of monovalent construct according to any of aspects H-I to H-3, comprising at least the steps of:

   a. immunizing a Camelid, preferably Llama, with whole cells that are alive and overexpress the desired extracellular part, region, domain, loop or other extracellular epitope(s) of one of the Targets of the invention, e.g.
selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, on their surface in their native confirmation; and

b. selecting for binding for the desired extracellular part, region, domain, loop or other extracellular epitope(s) using cell membranes preparation of different (other than the one used in immunization) cell types overexpressing at least one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9; and optionally

c. washing only mildly with a buffer such as PBS without detergents.

Aspect R-8: Method of screening to identify an amino acid sequence, e.g. a single variable domain, that is directed against one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, comprising the step of contacting any of the amino acid sequences or single variable domains according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I, any of the compounds or constructs according to any of aspects L-I to L-52, any of the monovalent constructs according to any of aspects H-1 to H-3 with one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.

Aspect S-1: Part or fragment of an amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I.

Aspect S-2: Part or fragment according to aspect S-I, that can specifically bind to a Target of the invention.
Aspect S-3: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention and that inhibits entry of a virus such as e.g. HIV, HCV, adenoviruses, hantavirus, herpesvirus, echo-vims 1 and others into cells.

5 Aspect S-4: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 1OnM, even more preferably InM.

Aspect S-5: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 10OnM, preferably 5OnM, more preferably 1OnM, even more preferably InM.

Aspect S-6: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and that inhibits entry of HIV into cells.

10 Aspect S-7: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 1OnM, even more preferably InM.

Aspect S-8: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay)
equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect S-9: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that inhibits entry of HIV into cells.

Aspect S-10: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect S-11: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect S-12: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and that inhibits entry of HIV into cells.

Aspect S-13: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect S-14: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect S-15: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4,

Aspect S-16: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to at least one of the Targets of the invention, e.g. CXCR4, CXCR7 or CD4.

Aspect S-17: Part of fragment according to aspect S-16, wherein the member of CXCR4 is human CXCR4.

Aspect S-18: Part of fragment according to aspect S-16, wherein the member of CD4 is human CD4.

Aspect S-19: Part of fragment according to aspect S-16, wherein the member of CXCR7 is human CXCR7.

Aspect S-20: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and inhibits and/or blocks binding of the Target of the invention to its ligand.

Aspect S-21: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to hCD4 and inhibits and/or blocks binding of gp120 to hCD4.

Aspect S-22: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that specifically binds to hCXCR4 and inhibits and/or blocks binding of CXCL12 to hCXCR4.

Aspect S-23: Part of fragment according to any of aspects S-1 or S-2, that is directed against and/or that a) specifically binds to one of the Targets of the invention; and b) fully inhibits ligand-dependent activation of one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, wherein the ligand is present in a concentration of 100 nM or less, more preferably 30 nM or less; and c) provides no activation of one of the Targets of the invention.
Aspect S-24: Part of fragment according to any of aspects S-I or S-23, that can specifically bind to a Target of the invention with a dissociation constant ($K_D$) of $10^{-5}$ to $10^{12}$ moles/litre or less, and preferably $10^{-7}$ to $10^{-12}$ moles/litre or less and more preferably $10^{-5}$ to $10^{-12}$ moles/litre.

Aspect S-25: Part or fragment according to any of aspects S-I to S-24, that can specifically bind to a Target of the invention with a rate of association ($k^a$-rate) of between $10^2$ M$^{-1}$V$^1$ to about $10^7$ M$^{-1}$V$^1$, preferably between $10^3$ M$^{-1}$V$^1$ and $10^7$ M$^{-1}$V$^1$, more preferably between $10^4$ M$^{-1}$s$^{-1}$ and $10^7$ M$^{-1}$s$^{-1}$, such as between $10^5$ M$^{-1}$s$^{-1}$ and 10$^7$ M$^{-1}$V.

Aspect S-26: Part or fragment according to any of aspects S-I to S-25, that can specifically bind to a Target of the invention with a rate of dissociation ($k_d$-rate) between 1 s$^{-1}$ and $10^6$ s$^{-1}$ preferably between $10^2$ s$^{-1}$ and $10^6$ s$^{-1}$, more preferably between $10^3$ s$^{-1}$ and $10^6$ s$^{-1}$, such as between $10^4$ s$^{-1}$ and $10^6$ s$^{-1}$.

Aspect S-27: Compound or construct, that comprises or essentially consists of one or more parts or fragments according to any of aspects S-I to S-26, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

Aspect S-28: Compound or construct according to aspect S-27, in which said one or more other groups, residues, moieties or binding units are amino acid sequences.

Aspect S-29: Compound or construct according to aspect S-27 or S-28, in which said one or more linkers, if present, are one or more amino acid sequences.

Aspect S-30: Nucleic acid or nucleotide sequence, that encodes a part or fragment according to any of aspects S-1 to S-26 or a compound or construct according to any of aspects S-8 to S-29.

Aspect S-31: Composition, comprising at least one part or fragment according to any of aspects S-1 to S-26, compound or construct according to any of aspects S-27 to S-29, or nucleic acid or nucleotide sequence according to aspect S-30.

Aspect T-1: Derivative of an amino acid sequence according to any of aspects A-1 to A-42, B-1 to B-22, C-1 to C-8, D-1 to D-32, E-1 to E-13, F-1 to F-32 or G-1.

Aspect T-2: Derivative according to aspect T-1, that can specifically bind to a Target of the invention.
Aspect T-3: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention and that inhibits entry of a virus such as e.g. HIV, HCV, adenoviruses, hantavirus, herpesvirus, echo-virus 1 and others into cells.

5 Aspect T-4: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 10nM, even more preferably InM.

Aspect T-5: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 10OnM, preferably 5OnM, more preferably 10nM, even more preferably InM.

Aspect T-6: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta3 integrin, human beta3 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and that inhibits entry of HIV into cells.

Aspect T-7: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta3 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and wherein the IC50 in the HIV-I infection assays is equal or below 10OnM, preferably 5OnM, more preferably 10nM, even more preferably 3nM.

Aspect T-8: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human beta3 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay)
equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect T-9: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that inhibits entry of HIV into cells.

Aspect T-10: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect T-11: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279 and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect T-12: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and that inhibits entry of HIV into cells.

Aspect T-13: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect T-14: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to hCXCR4 (SEQ ID NO: 254) and that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

Aspect T-15: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4,
human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.

Aspect T-16: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to at least one of the Targets of the invention, e.g. CXCR4, CXCR7 or CD4.

Aspect T-17: Derivative according to aspect T-16, wherein the member of CXCR4 is human CXCR4.

Aspect T-18: Derivative according to aspect T-16, wherein the member of CD4 is human CD4.

Aspect T-19: Derivative according to aspect T-16, wherein the member of CXCR7 is human CXCR7.

Aspect T-20: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4,

human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI hClaudin-1, hClaudin-6 and hClaudin-9 and inhibits and/or blocks binding of the Target of the invention to its ligand.

Aspect T-21: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to hCD4 and inhibits and/or blocks binding of gpl20 to hCD4.

Aspect T-22: Derivative according to any of aspect T-1 or T-2, that is directed against and/or that specifically binds to hCXCR4 and inhibits and/or blocks binding of CXCL12 to hCXCR4.

Aspect T-23: Derivative according to any of aspect T-1 or T-2, that is directed against

and/or that a) specifically binds to one of the Targets of the invention: and b)

fully inhibits ligand-dependent activation of one of the Targets of the invention, e.g. selected from the group consisting of hCD4, hCXCR4, JbCCRS, hTLR4, human alphaV integrin, human beta3 integrin, human beta1 integrin, human alpha2 integrin, hCD81, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9, wherein the ligand is present in a concentration of 100 nM or less, more preferably 30 nM or less; and c) provides no activation of one of the Targets of the invention.
Aspect T-24: Derivative according to any of aspects T-I or T-23, that can specifically bind to the Target of the invention with a dissociation constant ($K_D$) of $10^{-5}$ to $10^{12}$ moles/litre or less, and preferably $10^{-7}$ to $10^{-12}$ moles/litre or less and more preferably $10^{-8}$ to $10^{-12}$ moles/litre.

Aspect T-25: Derivative according to any of aspects T-I to T-24, that can specifically bind to the Target of the invention with a rate of association ($k_{on}$-rate) of between $10^2 M^{-1} s^{-1}$ to about $10^7 M^{-1} s^{-1}$, preferably between $10^3 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$, more preferably between $10^4 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$, such as between $10^5 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$.

Aspect T-26: Derivative according to any of aspects T-I to T-25, that can specifically bind to the Target of the invention with a rate of dissociation ($k_{off}$-rate) between $1 s^{-1}$ and $10^6 s^{-1}$ preferably between $10^2 s^{-1}$ and $10^6 s^{-1}$, more preferably between $10^3 s^{-1}$ and $10^6 s^{-1}$, such as between $10^4 s^{-1}$ and $10^6 s^{-1}$.

Aspect T-27: Derivative of a polypeptide according to any of aspects K-I to K-18 or compound or construct according to any of aspects L-I to L-52.

Aspect T-28: Derivative according to aspect T-27, that can specifically bind to a Target of the invention.

Aspect T-29: Derivative according to any of aspects T-27 or T-28, that can specifically bind to the Target of the invention with a dissociation constant ($K_D$) of $10^{-5}$ to $10^{12}$ moles/liter or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter or less and more preferably $10^{-8}$ to $10^{-12}$ moles/liter.

Aspect T-30: Derivative according to any of aspects T-27 to T-29, that can specifically bind to the Target of the invention with a rate of association ($k_{on}$-rate) of between $10^2 M^{-1} s^{-1}$ to about $10^7 M^{-1} s^{-1}$, preferably between $10^3 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$, more preferably between $10^4 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$, such as between $10^5 M^{-1} s^{-1}$ and $10^7 M^{-1} s^{-1}$.

Aspect T-31: Derivative according to any of aspects T-27 to T-30, that can specifically bind to the Target of the invention with a rate of dissociation ($k_{off}$-rate) between $1 s^{-1}$ and $10^6 s^{-1}$ preferably between $10^2 s^{-1}$ and $10^6 s^{-1}$, more preferably between $10^3 s^{-1}$ and $10^6 s^{-1}$, such as between $10^4 s^{-1}$ and $10^6 s^{-1}$.

Aspect T-32: Derivative according to any of aspects T-1 to T-31, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for
example at least 10 times or more than 20 times, greater than the half-life of the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se, polypeptide according to any of aspects K-I to K-18 or compound or construct according to any of aspects L-I to L-52 per se.

Aspect T-33: Derivative according to any of aspects T-I to T-132, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding amino acid sequence according to any of aspects A-I to A-42, B-I to B-22, C-I to C-8, D-I to D-32, E-I to E-13, F-I to F-32 or G-I per se, polypeptide according to any of aspects K-I to K-18 or compound or construct according to any of aspects L-I to L-52 per se.

Aspect T-34: Derivative according to any of aspects T-I to T-33, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

Aspect T-35: Derivative according to any of aspects T-I to T-34, that is a pegylated derivative.

Aspect T-36: Compound or construct, that comprises or essentially consists of one or more derivatives according to any of aspects T-I to T-34, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

Aspect T-37: Compound or construct according to aspect T-36, in which said one or more other groups, residues, moieties or binding units are amino acid sequences.

Aspect T-38: Compound or construct according to aspect T-36, in which said one or more linkers, if present, are one or more amino acid sequences.
Aspect T-39: Nucleic acid encoding a compound or construct according to aspect T-36 or T-37.

Aspect T-40: Composition, comprising at least one derivative to any of aspects T-1 to T-35, compound or construct according to any of aspects T-36 to T-38, or nucleic acid or nucleotide sequence according to aspect T-39.
EXAMPLES

Example 1: Generation of CXCR4 Nanobodies

**Methods:**

**Cell culture and transfection** ~ HEK293T cells were maintained at 37 °C in a humidified 5% CO₂, 95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM l-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 10% fetal calf serum. Jurkat cells were cultured in a humidified 5% CO₂, 95% air atmosphere in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 2 mM l-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 10% fetal calf serum. HEK293T cells were transiently transfected with a constant amount of total DNA using linear 25 kDa polyethyleneimine (Polysciences, Warrington, PA) as carrier as previously described (Verzijl et al.), Noncompetitive Antagonism and Inverse Agonism as Mechanism of Action of Nonpeptidergic Antagonists at Primate and Rodent CXCR3

Chemokine Receptors. Journal of Pharmacology and Experimental Therapeutics (2008) 325(2):544-55). cDNA encoding chemokine receptors CCR5, CCR7, CXCR1, CXCR2, CXCR3 and CXCR7 obtained from cdna.org (Missouri S&T cDNA Resource Center, Rolla, MO) were amplified by PCR and cloned into an expression vector.

\(^{[125]}I\)-labelling - Radiolabeling of Nanobodies with \(^{125}I\) was performed using the Iodo-gen method (Pierce, Rockford, IL) according to the manufacturer's protocol. \(^{125}I\)-labeled Nanobody was separated from free iodine (>99%) using a Sephadex G-25 gel filtration column (Amersham Biosciences, Piscataway, NJ). Iodine incorporation and specific activity were controlled via precipitation of the protein with trichloroacetic acid. myo-[\(^2\text{H}\)]-inositol (10-20 Ci/mmol) and \([^{125}\text{I}]\)-labeled CXCL12 (2,200 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA).

**Competition binding assays** - Membranes from HEK293T cells transiently expressing CXCR3 or CXCR4 were prepared 48 h after transfection as follows. Cells were washed and scraped from the cell culture dishes with ice-cold PBS containing 1 mM EDTA. The scraped cells were pelleted at 1500 x g for 10 min at 4°C. The pellet was washed and then resuspended in ice-cold membrane buffer (15 mM Tris, pH 7.5, 1 mM EGTA, 0.3 mM EDTA, and 2 mM MgCl₂). The cell suspension was homogenized by 10 strokes at 1200 rpm using a Teflon-glass homogenizer and rotor and further subjected to three freeze-thaw cycles.
using liquid nitrogen. Membranes were separated by centrifugation at 40,000 g for 25 min at 4°C. The membrane pellet was washed and resuspended in ice-cold Tris-sucrose buffer (20 nM Tris, pH 7.4, and 250 mM sucrose) and frozen in liquid nitrogen. The total protein was determined using a Bradford assay (Bio-Rad).

Periplasmas (1:10) or ligands were pre-incubated with membranes in binding buffer (50 mM HEPES (pH 7.4), 1 mM CaCl₂, 5 mM MgCl₂, 100 mM NaCl 0.5% bovine serum albumin) supplemented with 0.5% BSA for 1 h at 22°C before the addition of [¹²⁵I]-CXCL12 (40 pM) or [¹³¹I]-238D2 (3 nM) or [¹²⁵I]-238D4 (3 nM) for additional 2 h at 22°C. The non-specific binding was determined in the presence of AMD3100 (3 μM). Membranes were then harvested over polyethylenimine (0.5%)-treated Whatman GF/C filter plates and washed three times with ice cold binding buffer containing 500 mM NaCl. Plates were counted by liquid scintillation.

**Inositol phosphate accumulation assay** - 24 hours post transfection with pcDNA3.1-CXCR4 and pcDNA1-HA-mG (alpha) qi5 (see Verzijl et al, 2008 supra), 250,000 cells were seeded into 24-wells plates and labelled overnight using inositol-free minimal essential medium supplemented with 1 μCi myo-[2-³H]-inositol. The next day, the cells were washed once to remove non-incorporated myo-[2-³H]-inositol. In antagonist experiments, the cells were pre-incubated with test compounds at 37°C in assay medium (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose and 0.05% (w/v) bovine serum albumin, pH 7.4) for 1 h before stimulation with LiCl (10 mM) and CXCL12 (30 nM) for further 2 h at 37°C. In agonist experiments, the cells were directly stimulated with test compounds and LiCl (10 mM) in assay buffer for 2 h at 37°C. The stimulation was stopped by aspirating the stimulation medium and adding ice-cold 10 mM formic acid. The accumulated inositol phosphates were isolated by anion exchange chromatography and counted by liquid scintillation.

**CRE reporter gene assay** - HEK239T cells were transfected with pCRE/β-galactosidase (Chen W, Shields TS, Stork PJS, Cone RD (1995) Anal Biochem 226:349-354) and plasmids (pcDEFa or pcDNA3.1) encoding the indicated receptors. 40,000 transfected cells per well were seeded into 96 well plates and grown in DMEM supplemented with 10% fetal calf serum. The medium was replaced 32 h after transfection by serum-free DMEM supplemented with 0.5% bovine serum albumin and ligands as indicated. Following 16 h of ligand incubation, the medium was removed, the cells were lysed in 100 μl of assay buffer
(100 HiM sodium phosphate buffer at pH 8.0, 4 nM 2-nitrophenol-β-D-pyranoside, 0.5%
Triton X-100, 2 mM MgSO$_4$, 0.1 mM MnCl$_2$, and 40 nM β-mercaptoethanol) and incubated
at room temperature. The β-galactosidase activity was determined by the measurement of
absorption at 420 nm with a PowerwaveX340 plate reader (Bio-Tek Instruments Inc.,
Winooski, VT) after incubation with the assay buffer when the OD$_{420}$ value for forskolin (3
µM) controls reached 0.4 - 0.6.

Chemotaxis assay - The chemotactic responsiveness of Jurkat 3D cells was assessed
using ChemoTx™ plates (Receptor Technologies Ltd., Oxon, UK) in which a upper cell-
containing compartment is separated from a lower chemoattractant-containing compartment
by a polystyrene-free polycarbonate filter with 5-µm pores. Cells were harvested, washed
and resuspended in RPMI containing 0.5% bovine serum albumin and then loaded to
150,000 cells per well in a volume of 25 µl into the upper compartment of the chemotaxis
chamber. To stimulate cells migrating through the membrane, CXCL12 and/or test
compounds were loaded in a final volume of 31 µl at indicated concentrations into the lower
compartment (agonist experiments). For the characterization of antagonistic properties,
AMD3100 or test compounds were loaded to the lower CXCL12 (300 pM) containing
compartment and additionally pre-incubated with the cells in the upper compartment. The
chemotaxis chambers were incubated at 37°C, 100% humidity, and 5% CO$_2$ for 4 h. The
number of cells migrating into each lower compartment was determined by fluorescence
measurement at 535 nm following incubation with calcein AM and calibration with 0 to
50,000 Jurkat 3D cells per well.

HW-I infection assays - The CXCR4-using (X4) HIV-1 molecular clone NL4.3 was
obtained from the National Institutes of Health NIAID AIDS Reagent program (Bethesda,
MD), the CCR5-using (R5) HIV-1 strain BaL was obtained from the Medical Research
Council AIDS reagent project (Herts, UK). The dual-tropic (R5/X4) HIV-1 HE strain was
initially isolated from a patient at the University Hospital in Leuven, and had been routinely
cultured in MT-4 cells (Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ,
343:470-474). The MT-4 cells were seeded out in 96-well plate and the U87 cells in 24-well
plates. The test compounds were added at different concentrations together with HIV-1 and
the plates were maintained at 37 °C in 10% CO2. Cytopathic effect induced by the virus was
monitored by daily microscopic evaluation of the virus-infected cell cultures. At clay 4-5 after infection, when strong cytopathic effect was observed in the positive control (i.e., untreated HIV-infected cells), the cell viability was assessed via the in situ reduction of the tetrazolium compound MTS, using the CellTiter 96® AQ®-One Solution Cell Proliferation Assay (Promega, Madison, WI). The absorbance was then measured spectrophotometrically at 490 nm with a 96-well plate reader (Molecular Devices, Sunnyvale, CA) and compared with four cell control replicates (cells without virus and drugs) and four virus control wells (virus-infected cells without drugs). The 50% inhibitory concentration (IC50, i.e. the drug concentration that inhibits HIV-induced cell death by 50%), was calculated for each compound from the dose-response curve. The CC50 or 50% cytotoxic concentration of each of the compounds was determined from the reduction of viability of uninfected cells exposed to the agents, as measured by the MTS method described above.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density centri (ligation (Lymphoprep; Nycomed Pharma, AS Diagnostics, Oslo, Norway) and stimulated with phytohemagglutinin (PHA) (Sigma Chemical Co., Bornem Belgium) for 3 days. The activated cells (PHA-stimulated blasts) were washed with PBS and viral infections were performed as described previously (Schols D, Struyf S, Van Damme J, Este JA, Henson G, De Clercq E. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J Exp Med 1997; 186:1383-1388). At 8-10 days after the start of the infection, viral p24 Ag was detected in the culture supernatant by an enzyme-linked immunosorbent assay (Perkin Elmer. Brussels, Belgium).

Data analysis and presentation - Data are presented as mean ± S.E.M. from n independent experiments. Concentration response curves (E/[A] curves) were fitted to the Hill equation using an iterative, least-squares method (GraphPad Prism 4.0, GraphPad Software, San Diego, CA) to provide maximal inhibitory effects (I50max), half maximal effective (EC50) or inhibitory concentrations (IC50). Competition binding affinities and functional antagonist affinities (pKᵢ) were calculated using the Cheng and Prusoff equation

\[ pKᵢ = \frac{IC50}{IC50 + [agonist]/EC50} \]

(Cheng & Prusoff, 1973). Antagonist affinities were optionally expressed as pKβ values using the method of Arunlakshana and Schild (1959) based on the equation

\[ pKβ = -\log [antagonist] + \log (CR-I) \]

where CR represents the ratio of the agonist EC50 in the presence and the absence of an antagonist.
Results were compared using Student's t-test or oneway analysis of variance followed by Bonferroni corrected t-test for stepwise comparison, when multiple comparison was made. P values <0.05 were considered to be significant.

Sequence targets - Synonyms: CXCR-4/ Stromal cell-derived factor 1 receptor (SDF-1 receptor)/ Fusin/ Leukocyte-derived seven transmembrane domain receptor (LESTR)/ LCRI/ FB22 / NPYRL/ HM89/ CD84 antigen. Human CXCR4 was used for selection:

Table B-1: HOMOLOGY AGAINST HUMAN SEQUENCE:

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<td>238E3</td>
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</tr>
<tr>
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<td>238A6</td>
<td>248</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>237D1</td>
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</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>237E1</td>
<td>250</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
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<td>251</td>
</tr>
<tr>
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<td>238C4</td>
<td>252</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>237C1</td>
<td>253</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>238C5, 238G2, 238H5, 238C3, 238D6, 238E6</td>
<td>254</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
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<td>308</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>01E2</td>
<td>309</td>
</tr>
<tr>
<td>EVQLVESGGGLVQPGSSRSLCCASAAGSFSSISNLGSGMQVYQAAPGKEEFVAYRHWSGDAGLYADSVKGRFTISRGNKNTVQLSQMNLSKEDTAYCAYAAGMITSNQXLYLWYGQGQTQTVSS</td>
<td>01N12</td>
<td>310</td>
</tr>
<tr>
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<td>03F1</td>
<td>311</td>
</tr>
</tbody>
</table>
Example 1.1: Immunizations

For immunization, HEK293 cells (human embryonic kidney) transiently expressing human CXCR4 were used as "antigen". Two llamas were immunized according to standard protocols with 6 boosts of a cells (1*10E7 cells) at day 0, 7, 21, 32, 43 and 56. Blood were collected from these animals at 4 and 8 days after the 6th boost.

Example 1.2: Library construction

Peripheral blood mononuclear cells were prepared from blood samples using Ficoll-Hypaque according to the manufacturer’s instructions. Next, total RNA extracted was extracted from these cells as well as from the lymph node bow cells and used as starting material for RT-PCR to amplify Nanobody encoding gene fragments. These fragments were cloned into a phagemid vector. Phage was prepared according to standard methods (see for example the prior art and applications filed by applicant cited herein) and stored at 4°C for further use, making phage library 217 and 218.

Example 1.3: Selections using 2 rounds of trypsin elution

To identify Nanobodies recognizing CXCR4, phage libraries (217, 218) were used in a phage display selection.

Because hCXCR4 is an integral transmembrane protein, it is essential to conserve the native conformation of hCXCR4. Therefore, the phage display selection was done on cell membranes preparation of CHO and COS7 cells overexpressing hCXCR4. Membranes were coated onto Maxisorp plate overnight at 4°C (10ug in 100ul PBS). The next day, after blocking in 4% milk-PBS 1 hour, phages from the libraries were incubated with the coated membrane in the presence (and in parallel without) of 1% milk-PBS and CHO-membrane preparation expressing a non-relevant membrane protein. After 2 hours incubation, the plates were washed extensively with PBS. After washing, bound phages were eluted using trypsin (lug/ml) for 15min at RT. Phages were rescued and reamplified in TGl as usual giving R1 polyclonal phages.

Those R1 phages were used for a second round of selection like the first round with the only difference that phages selected on CHO-CXCR4 membrane in the first round were also used on COS7-CXCR4 membrane and reverse. This unique strategy allows the depletion
on non-CXCR4 specific phages. After 2 hours incubation, the plates were washed extensively with PBS and bound phages were eluted using trypsin (lug/ml) for 15min at RT.

Output of R2 selections were analyzed for enrichment factor (phage present in eluate relative to controls). Based on these parameters the best selections were chosen for further analysis. The polyclonal output was rescued in TG1, individual TG1 colonies were picked and grown in 96 deep well plates (1 ml volume) to produce monoclonal phages (addition of helper phage) or monoclonal periplasmic Nanobody fractions (addition of IPTG). Periplasmic extracts (volume: ~90 ul) were prepared according to standard methods (see for example the prior art and applications filed by applicant cited herein).

A schematic representation of the selection can be found in Figure 1.

**Example 1.4: Selections using 2 rounds of specific (competitive) elution**

An alternative to the non-specific trypsin elution is to use specific CXCR4 binding compound to elute (compete out) the phages binding to the site of the compound binding. In this case, 2.5ug of membrane preparation was coated overnight at 4C in 100ul PBS and elution was done for 30min at RT with an excess of:
- CXCL12/SDF1 (3ug in 100ul PBS), the natural Hgand for CXCR4,
- AMD3 100 (50uM), a know chemical antagonist (from Sigma Aldrich)
- 12G5 (5ug in 100ul PBS), a known neutralizing antibody (from R&D System).

Eluted phages were rescued and reamplified in TG1 as usual giving R1 polyclonal phages.

Those R1 phages were used for a second round of selection like the first round with the only difference that phages selected on CHO-CXCR4 membrane in the first round were also used on COS7-CXCR4 membrane and reverse. This unique strategy allows the depletion on non-CXCR4 specific phages (membrane specific). After 2 hours incubation, the plates were washed extensively with PBS and bound phages were eluted as the first round. This way, 2 rounds of CXCL1 2/SDF1 was done as well as 2 rounds of AMD3100.

Output of R2 selections were analyzed for enrichment factor (phage present in eluate relative to controls). Based on these parameters the best selections were chosen for further analysis. The polyclonal output was rescued in TG1, individual TG1 colonies were picked and grown in 96 deep well plates (1 ml volume) to produce monoclonal phages (addition of helper phage) or monoclonal periplasmic Nanobody fractions (addition of IPTG). Periplasmic
extracts (volume: ~90 ul) were prepared according to standard methods (see for example the prior art and applications filed by applicant cited herein).

A schematic representation of the selection can be found in Figure 1.

5 Example 1.5: Screening for binding

In order to determine binding specificity of the Nanobodies, 15ul of the produced phage were tested in an Phage ELISA binding assay,

In short. 2ug in 100ul PBS of membrane expressing either CXCR4 (CHO-CXCR4) or a non relevant GPCR (CHO) were coated directly on maxisorop microtiter plates (Nunc) overnight at 4C. Free binding sites were blocked using 4% Marvel in PBS for 1h. Next, 15 ul of monoclonal phages was added in 100 ul 1% Marvel PBS for 2hours. After incubation and an extensive PBS washing step, phage binding was revealed using an anti-M13-HRPO antibody. Binding specificity (binding to CHO-CXCR4) was determined based on OD values compared to controls (binding to CHO).

An example is shown in Figure 2.

5 Example 1.6: Screening of CXCR4-binding nanobodies by displacement of [125I]-CXCL12

180 clones were analyzed and their periplasma fractions were screened using a CXCR4 competition binding assay. In a primary screen with membranes from HEK293T cells transiently expressing CXCR4, approximately 13% of the clones were found to compete with the radiolabeled endogenous CXCR4 ligand [125I]-CXCL12 for binding to CXCR4 and produce an inhibition of specific [125I]-CXCL12 binding of at least 30% (Figure 3). A total amount of five clones (approximately 3%) strongly inhibit specific [125I]-CXCL12 binding by more than 70%. No inhibition was observed for control phages expressing Nanobodies directed against membrane proteins different from CXCR4.

All primary hits were confirmed in a second screen (Figure 3b) and therefore the V\textsubscript{H}-encoding DNA of CXCL12-displacing Nanobody-producing clones were sequenced. Sequencing analysis results in seven pools of identical or highly similar clones strongly (2 pools) or partially (5 pools) displacing [125I]-CXCL12 (Table B-2). Nanobodies representing these pools, namely 237A6, 237D1, 237D2, 237G7, 238C5, 238D2 and 238D4 were purified and further pharmacologically analyzed.
Characterization of Nanobody binding to CXCR4 - Following purification, receptor binding characteristics for 237A6, 237D1, 237D2, 237G7, 238C5, 238D2 and 238D4 were investigated on cell membranes from transiently CXCR4 expressing HEK293T cells. The Nanobodies 238D2 and 238D4 fully displace all specifically bound \([^{125}\text{I}]\)-CXCL12 and show affinities to CXCR4 in the low nanomolar range (Table B2). All other Nanobodies were unable to displace \([^{125}\text{I}]\)-CXCL12 even at the highest test concentration of 0.5 µM (237A6, 237D1, 237D2, 237G7 and 238C5) (Figure 4A: Table B-3).

In order to further investigate the binding properties of the two potently \([^{125}\text{I}]\)-CXCL12-displacing Nanobodies 238D2 and 238D4 to CXCR4, we generated \(^{125}\text{I}\)-labeled Nanobodies for competition binding studies. Both, \([^{125}\text{I}]\)-238D2 and \([^{125}\text{I}]\)-238D4 selectively bound to membranes from HEK293T cells transiently expressing CXCR4 compared to those expressing CXCR3 (Figure 4D). Both Nanobodies compete for binding to CXCR4 as shown by the full displacement of \([^{125}\text{I}]\)-238D2 by 238D4 and by the full inhibition of \([^{125}\text{I}]\)-238D4 binding by 238D2 (Figures 4B, 4C). Furthermore, the small molecule ligand AMD3100 displaced \([^{125}\text{I}]\)-238D2 and \([^{125}\text{I}]\)-238D4 with affinities comparable to those obtained against \([^{125}\text{I}]\)-CXCL12 (Table B-3) indicating that also AMD3100 compete with the Nanobodies 238D2 and 238D4 for the same receptor. The monoclonal antibody 12G5 that has previously been reported to label a certain subpopulation of CXCR4 (J. Virol. Baribaud et al. 75 (19): 8957) potently but incompletely displace specifically bound \([^{85}\text{I}]\)-CXCL12, \([^{125}\text{I}]\)-238D2 and \([^{125}\text{I}]\)-238D4 from CXCR4. 237A6, 237D1, 237D2 and 237G7 were unable to inhibit binding of \([^{125}\text{I}]\)-238D2 or \([^{125}\text{I}]\)-238D4 to CXCR4. 238C5 displaced \([^{125}\text{I}]\)-238D2 and \([^{125}\text{I}]\)-238D4 but not \([^{125}\text{I}]\)-CXCL12 at high concentrations (> 100 nM) indicating that this Nanobody binds to the receptor as a low affinity allosteric CXCR4 ligand (Figures 4B, 4C).

Table B-2: Screening and sequencing of Nanobody clones displacing \([^{125}\text{I}]\)-CXCL12. Binding efficiency was determined by competition binding with \([^{125}\text{I}]\)-CXCL12 on membranes from HEK293T cells transiently expressing CXCR4.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Clones</th>
<th>Binding&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>237A6</td>
<td>237A6</td>
<td>–</td>
</tr>
<tr>
<td>237D1</td>
<td>237D1</td>
<td>+</td>
</tr>
<tr>
<td>237D2</td>
<td>237B6, 237C1, 237C5, 237D2, 237D4</td>
<td>–/+</td>
</tr>
</tbody>
</table>
Table B-3: Receptor affinity (pKᵢ) and maximal displacement of [¹²⁵I]-CXCL12, [¹²⁵I]-238D2 and [¹²⁵I]-238D4 for monovalent Nanobodies and CXCR4 reference ligands. The experiments were performed on membranes from HEK293T cells transiently expressing CXCR4. Data were shown as means ± S.E.M. The number of experiments is given as n.

<table>
<thead>
<tr>
<th></th>
<th>[¹²⁵I]-CXCL12</th>
<th>[¹²⁵I]-238D2</th>
<th>[¹²⁵I]-238D4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Displacem. (%)</td>
<td>pKᵢ</td>
<td>n</td>
</tr>
<tr>
<td>238D2</td>
<td>93 ± 5</td>
<td>8.01 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>238D4</td>
<td>99 ± 5</td>
<td>8.22 ± 0.16</td>
<td>6</td>
</tr>
<tr>
<td>237A6</td>
<td>0ᵃ</td>
<td>&lt; 6.3</td>
<td>4</td>
</tr>
<tr>
<td>237D1</td>
<td>0ᵃ</td>
<td>&lt; 6.3</td>
<td>3</td>
</tr>
<tr>
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<td>4</td>
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<td>238C5</td>
<td>0ᵃ</td>
<td>&lt; 6.3</td>
<td>4</td>
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<td>116B2</td>
<td>0ᵃ</td>
<td>&lt; 6.3</td>
<td>2</td>
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<tr>
<td>CXCL12</td>
<td>105 ± 2</td>
<td>9.84 ± 0.13</td>
<td>3</td>
</tr>
<tr>
<td>AMD3100</td>
<td>94 ± 2</td>
<td>7.41 ± 0.28</td>
<td>3</td>
</tr>
<tr>
<td>12G5</td>
<td>54 ± 5ᵃ</td>
<td>9.19 ± 0.19</td>
<td>3</td>
</tr>
</tbody>
</table>

a No significant displacement at 0.5 µM.
b Maximum not reached at the highest test concentration of 0.5 µM, displacement at 0.5 µM.
c Significantly different from 100%.
Example 1.7: Inhibition of CXCR4-mediated signal transduction

In an effort to functionally characterize the Nanobodies 238D2 and 238D4, we measured their ability to activate G-protein signaling or to inhibit the CXCL12-induced G-protein signaling in HEK293T cells transiently co-transfected with cDNAs encoding CXCR4 and G\(\alpha_{q,5}\). This assay is based upon the use of the chimeric G\(\alpha_{q,5}\)-protein that contains a G\(\alpha_q\) backbone with the 5 C-terminal amino acids replaced by those from Ga,. The chimeric G-protein is activated by CXCR4 like a Ga, subunit but transduces signals like G\(\alpha_q\) proteins (Coward, P., et al, Chimeric G Proteins Allow a High-Throughput Signaling Assay of Gl-Coupled Receptors, Analytical Biochemistry (1999) 270: 242-248). Thus, the activation of Ga\(q,5\) can be quantified by the measurement of accumulated inositol phosphates. CXCL12 stimulates inositol phosphate accumulation with a pEC\(_{50}\) of 7.89 ± 0.21 (n = 4). No agonist activity was observed for the Nanobodies 238D2 or 238D4 up to a concentration of 100 nM. However, 238D2 and 238D4 fully inhibited the CXCL12-induced accumulation of inositol phosphates in a concentration-dependent manner (Figure 5A).

In addition, we investigated the ability of the Nanobodies 238D2 and 238D4 to inhibit the CXCL12-induced activation at a later step of signal transduction in HEK293T cells transiently transfected with pcDNA3.1-CXCR4 and a β-galactosidase reporter gene under the control of a cAMP response element (CRE). Stimulation of G\(_f\) protein-coupled receptors like CXCR4 may result in an inhibition the forskolin-induced activation of CRE. Indeed, CXCL12 potently inhibited the forskolin (3 μM)-induced activation of the CRE-dependent transcription of β-galactosidase with a pEC\(_{50}\) of 9.78 ± 0.09 (n = 11), whereas the Nanobodies 238D2 and 238D4 did not show any agonist activity in the absence of CXCL12 (Figure 5B). However, both nanobodies inhibited the CXCL12 response by parallel rightward-shifts of the concentration response curves of CXCL12 without affecting its maximal effect upon increasing Nanobody concentrations. Schild analysis showed linearity between log (CR - 1) and -log [Nanobody] (M) with slopes of 0.91 ± 0.20 and 0.71 ± 0.17 (not significantly different from unity) for both 238D2 and 238D4, respectively (Figure 5B). These results indicate competitive antagonism of the CXCL12-induced activation of signal transduction for both Nanobodies. Based on the Schild plot data, pK\(_B\) values of 7.64 ± 0.16 and 7.70 ± 0.16 were calculated for 238D2 and 238D4, respectively.

In order to demonstrate the specificity of the Nanobodies for CXCR4, we also investigated the effects of 238D2 and 238D4 on other chemokine and non-chemokine
receptor signaling by using the CRE/β-galactosidase reporter gene. Sub-maximally effective agonist concentrations (50 – 80% $E_{\text{max}}$) in the absence or the presence of 3 μM forskolin were used to stimulate the cells. The Nanobodies 238D2 and 238D4 even in concentrations up to 2.5 μM did not alter the agonist-induced inhibition of the forskolin (3 μM)-induced activation of CRE in HEK293T cells transiently transfected with cDNA encoding CXCR1, CXCR2, CXCR3, CXCR6, CCR5, CCR7 or histamine H4 receptors, respectively (Figure 7). Furthermore, 238D2 and 238D4 (2.5 μM) did not inhibit the activation of endogenously expressed β2-adrenoceptors by the β2-adrenoceptor agonist salbutamol (100 nM). These results demonstrate an over 100-fold selectivity of 238D2 and 238D4 for CXCR4 over all other receptors tested.

The chemoattractant or anti-chemoattractant effects of CXCL12 and the Nanobodies were investigated in Jurkat leukaemia T cells endogenously expressing CXCR4. CXCL12 induced migration of Jurkat cells with a typical bell-shaped profile with a pECso of 9.41 ± 0.26 (n = 5) for the first phase of the concentration response curve. 238D2 and 238D4 were unable to induce any significant migration of Jurkat cells by themselves (Figure 5C). In contrast, both 238D2 and 238D4 concentration-dependently inhibited the migration of Jurkat cells towards 300 pM CXCL12 (Figure 5C).

Table B-4: Maximal inhibition ($I_{\text{max}}$) and functional inhibitory potency ($pK_i$ or $pIC_{50}$) of the Nanobodies 238D2 and 238D4. Data were shown as means ± S.E.M. The number of experiments is given as $n$.

<table>
<thead>
<tr>
<th></th>
<th>238D2</th>
<th>238D4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{\text{max}}$ (%)</td>
<td>$pK_i$ or $pIC_{50}$</td>
</tr>
<tr>
<td><strong>IP accumulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293T-CXCR4-Gαq5/30 nM CXCL12</td>
<td>86 ± 12</td>
<td>8.51 ± 0.11</td>
</tr>
<tr>
<td><strong>CRE activation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293T-CXCR4-CREβgal / 0.01 – 100 nM CXCL12</td>
<td>104 ± 5a</td>
<td>7.64 ± 0.16b</td>
</tr>
</tbody>
</table>

*Chemotaxis*
**Example 1.8: HIV-I activity**

The ability of 238D2 and 238D4 to block the entry of the X4 HIV-I strain NL4.3, the R5 HIV-I strain BaL and the dual-tropic (R5/X4) HIV-I strain HE was investigated on endogenously CXCR4 expressing MT-4 cells, endogenous^ CXCR4 and CCR5 expressing PBMCs or U87.CD4 cells stably transfected with CCR5 or CXCR4, respectively. Control experiments with the small molecule antagonists for CXCR4 (AMD3100) and CCR5 (maraviroc) were simultaneously performed. Additional control experiments in the absence of any virus confirm that the Nanobodies show no toxicity at the highest test concentrations (500 nM in MT-4 and U87.CD4 cells; 100 nM in PBMCs). 238D2 and 238D4 were equipotently active against the CXCR4-using strains NL4.3 and HE in both MT-4 cells and PBMCs in nanomolar concentrations (Table B-4). Furthermore, both Nanobodies were active against NL4.3 in U87 cells expressing recombinant CD4 and CXCR4. However, 238D2 and 238D4 failed to inhibit the entry and infectivity of the CCR5-using HIV-I particles into PBMCs and U87.CD4.CCR5 cells. For comparison, maraviroc was potently active against BaL on PBMCs (IC<sub>50</sub> = 3.13 nM) and U87.CD4.CCR5 cells (IC<sub>50</sub> = 0.72 nM) in control experiments. The activity of the Nanobodies against X4-HIV-1 and failure to inhibit R5 viruses show that their anti-retroviral activity is directly linked to their interaction with CXCR4.

**Example 2: Generation of Nanobodies directed against human CXCR7**

The same approach as for Nanobodies directed against human CXCR4 is used. In particular, the method uses at least the following steps:
a) immunization with whole living cell (e.g. HEK293) overexpressing human CXCR7;  
b) Immunization and selection using different cell types (e.g. HEK293 for immunization,  
CHO-membrane enriched for human CXCR7 for 1st round selection, C0S7 membranes  
enriched for human CXCR7 for 2nd round selection);  
c) Optionally, washing with mild buffer, e.g. PBS (without detergents).  

Reference is also made to the human protein sequence of CXCR7 that can e.g. be  
found in the SwissProt database under "P25106".

Example 3: Examples of HIV in vitro neutralization assays

Example 3.1: Single-round pseudovirus neutralization assay

Reference is made to James M. Binley, Terri Wrin, Bette Korber, Michael B. Zwick,  
Meng Wang, Colombe Chappey, Gabriela Stiegler. Renate Kunert, Susan Zolla-Pazner,  
Hermann Katinger, Christos J, Petropoulos, and Dennis R. Burton (Comprehensive Cross-  
Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1  
23).

A recombinant-virus assay involving a single round of virus infection is used to  
measure neutralization. Recombinant luciferase pseudovimses are incubated for 1 h at 37°C  
with 10 serial fourfold dilutions of MAbs or heat-inactivated plasma, usually starting from 50  
µg/ml (MAbs) or a 1:20 dilution (plasma). In a variant protocol, virus is incubated with  
antibody for 18 h before the mixture is added to target cells, U87 cells expressing CD4 plus  
the CCR5 and CXCR4 coreceptors are inoculated with virus-antibody (Ab) dilutions in the  
absence of added cations. Virus stocks are screened to ensure that they were functional and  
yielded a high luciferase reporter light signal in target cell lysates. Input virus used in each  
experiment is not standardized. Virus infectivity is determined 72 h postinoculation by  
measuring the amount of luciferase activity expressed in infected cells. Neutralizing activity  
is reported as the concentration or dilution of each MAb or plasma required to confer 50%  
(IC$_{50}$) or 90% (IC$_{90}$) inhibition of infection (percent inhibition = [1 - [luciferase +  
Ab/luciferase - Ab] ] X 100). To eliminate nonspecific neutralization, the criterion for genuine  
neutralization is that the titer must be at least 2.5-fold higher against HIV-1 than it is against  
the amphotropic control MuLV. Due to the large size of this study, each individual virus-Ab  
combination is in general tested only once. To ensure that the results are reproducible, the
control viruses JR-CSF (R5-tropic) and NL4-3 (X4-tropic) are run at least six times in all assays. The reproducibility of the assay within and between runs is assessed by looking at these controls.

Example 3.2: GHOST assay


These cells are derived from human osteosarcoma cells and are transfected with the gene coding for human CD4, one of the HIV co-receptors (CCR5 or CXCR4) and green fluorescent protein under the control of the HIV-2 LTR promoter. The number of infected cells is measured by FACS. Plasma samples are diluted 1/20 and purified IgGs to a concentration of 500 ug/ml. The format of the neutralization assay is 24/24/48 where 24/x/x is the time in hours during which antibody and virus are pre-incubated, x/24/x is the time in hours during which cells are exposed to these mixtures and x/x/48 is the time in hours between the start of viral inoculation and the FACS analysis. The percentage neutralization is calculated as 100- [% infected cells of tested sample/× infected cells of seronegative control]

Example 3.4: PBMC assay

Reference is made to Beirnaert et al., 2000.

Virus neutralization assays are carried out as described previously with some minor modifications. Briefly, culture supernatant of virus infected PBMCs (50 TCID50/well) and twofold serial dilutions (1/10-1/1,280) of heat-inactivated serum (30 min at 56°C) are mixed in a 96-well tray and incubated for 1 hr at 37°C in a 5% CO2 atmosphere. In each experiment, an HIV (-) serum is assayed in the same conditions as the sample sera, to serve as a negative control. Subsequently, 7.5 × 104/well PHAstimulated, IL-2 maintained PBMCs are added. After 2 hr incubation, the cells are washed three times and incubated in RPMI 1640 medium supplemented with 20 U/ml IL-2, 15% FCS, 0.03% L-Glutamine, 2 mg/ml polybrene, 5 mg/ml hydrocortisone and antibiotics. For every neutralization experiment, the...
virus is titrated again to compare the infectivity of the virus stock in different donor PBMCs. If the virus titer differed from the input virus titer by more than a factor 3, the neutralization experiment is considered invalid. Viral replication is assessed after 7 days using a non-commercial antigen capture ELISA that captures antigen of HIV-I belonging to Group M as well as to Group O. [Beirnaert et al., 1998, Identification and characterization of sera from HIV-infected individuals with broad cross-neutralizing activity against group M (env clade A-H) and group O primary HIV-I isolates. J Med Virol. 2000 Sep;62(1):14-24], Fifty-percent inhibitory doses (ID50) are defined as the reciprocal of the highest serum dilution that produced 50% reduction in absorbance value in the antigen capture assay compared to the negative serum control. Serum neutralizing titers of <1/10 are considered negative. Sera are assayed in duplicate and tests are carried out at least three times.

**Example 3.5: HIV in vivo neutralization models**

**Example 3.5.1: Hu-PBL (NOD/SCID)**


To assess virus-inhibiting activity in vivo, human polyclonal immunoglobulins are administered to huPBL-NOD/Scid mice 6 days after reconstitution and 1 day before viral challenge. All injections are given intraperitoneally (Lp.). Each experimental group may consist of four mice. The minimal viral inoculum needed to infect all mice is determined in preliminary titrations. The chimeric mice that survived the graft-versus-host reaction (82%) are sacrificed 14 days after challenge and viral load is measured in their plasma using COBAS Amplicor HIV-I Monitor™ version 1.5 (Roche) according manufacturer's instructions. Due to the limited availability of mouse plasma, these may be diluted 1/100 and therefore the lower limit of detection of this assay is about 3.70 log equiv/ml.

**Example 3.5.2: SHIV macaque model**

For antibody infusions, vaginal challenge, and blood and mucosal collections, macaques are lightly anesthetized with ketamine HCl. A SHIV89.9PD challenge stock is grown and titrated in rhesus PBMC. Antibodies are infused intravenously 24 h prior to virus
challenge. Vaginal SHIV challenge is done by gently introducing 1 ml of a 1:5 dilution of virus stock (600 TCID50) into the vaginal canal of macaques using a 1-ml syringe. Macaques are kept in a prone position for at least 15 min postchallenge. Thirty days prior to vaginal challenge, macaques may have received 30 mg of medroxyprogesterone acetate (Depo-Provera, Upjohn, Kalamazoo, Michigan) by intramuscular injection. Recent titration experiments in progesterone treated macaques demonstrated that the monkeys are exposed to 10-50 animal infectious doses of SHIV89.6PD. After vaginal challenge, monkeys are followed clinically and by routine hematology, lymphocyte subset, and blood chemistry measurements. Inguinal lymph node biopsies for viral co-culture and PCR for viral DNA are done on all monkeys at 3 weeks post-virus challenge.

**Example 4: Optimization of the functional Nanobody profile directed against hCXCR4**

In order to improve the functional inhibitory profile by engineering, a series of bivalent Nanobodies on the basis of 238D2 and 238D4 were generated (Table B-5).

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Clone name</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVQLVESGGGLVQTVGSLRCLAGASGVTSSYAMSVVRQAPGKL</td>
<td>238D2-10GS-238D2</td>
<td>261</td>
</tr>
<tr>
<td>EVQLVESGGGLVQTVGSLRCLAGASGVTSSYAMSVVRQAPGKL</td>
<td>238D2-20GS-238D4</td>
<td>262</td>
</tr>
<tr>
<td>EVQLVESGGGLVQTVGSLRCLAGASGVTSSYAMSVVRQAPGKL</td>
<td>238D2-15GS-238D4</td>
<td>263</td>
</tr>
<tr>
<td>EVQLVESGGGLVQTVGSLRCLAGASGVTSSYAMSVVRQAPGKL</td>
<td>238D2-20GS-238D4</td>
<td>264</td>
</tr>
</tbody>
</table>
Recombinant linking of 238D2 to 238D2 and 238D4 to 238D4 using amino acid linkers with repetitive GGGGS sequences of different sizes resulted in a 14 and 4.4-fold increase in affinity to CXCR4, respectively (Table B-6, Table B-7). A significant increase in apparent affinity was also observed when 238D2 was linked to 238D4. In case of the obtained heterobivalent Nanobody 238D2-20GS-238D4, the affinity to CXCR4 was increased by 27 and 17-fold over the respective monovalent counterparts 238D2 and 238D4. Alteration in the linker size between 15 amino acids and 20 amino acids did not show any influence in respect to the receptor affinity. However, linking of 238D2 to the inactive Nanobody 238B10 or to the low affinity Nanobody 238C5 did not result in an increase but even lowered the receptor affinity. These results exclude the possibility that the linker increases the receptor affinity by its own. Furthermore, competition in binding between 238D2 and 238D4 (Figures 4B,4C) and lacking increase of [125I]-CXCL12 displacement potency by equi-molar mixing of 238D2 and 238D4 argues against a positive cooperative effect due to allosteric binding on the same receptor molecule.

<table>
<thead>
<tr>
<th>Displacement (%)</th>
<th>pK\textsubscript{j}</th>
<th>Rel. Potency</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>238D2-20GS-238C5</td>
<td>265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>238D2-20GS-238B10</td>
<td>266</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table B-6: Receptor affinity (pK\textsubscript{j}), relative potency and maximal displacement of [125I]-CXCL12 by bivalent Nanobodies in comparison to their monovalent counterparts. The experiments were performed on membranes from HEK293T cells transiently expressing CXCR4. Data are shown as means ± S.E.M. The number of experiments is given as n.
The most potent bivalent Nanobodies 238D2-15GS-238D4 and 238D2-20GS-238D4 were further functionally characterized. Both, 238D2-15GS-238D4 and 238D2-20GS-238D4 fully antagonized the chemoattractant effects of CXCL12 in subnanomolar concentrations.

### Table B-7: Chemotaxis - Maximal inhibition ($I_{\text{max}}$) and functional inhibitory potency ($pK_i$ or $pIC_{50}$) of the compound 238D2-15GS-238D4 and compound 238D2-20GS-238D4.

Data are shown as means ± S.E.M. The number of experiments is given as $n$.

<table>
<thead>
<tr>
<th></th>
<th>238D2-15GS-238D4</th>
<th></th>
<th>238D2-20GS-238D4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{\text{max}}$ (%)</td>
<td>$pK_i$ or $pIC_{50}$</td>
<td>$I_{\text{max}}$ (%)</td>
<td>$pK_i$ or $pIC_{50}$</td>
</tr>
<tr>
<td><strong>Chemotaxis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jurkat / 0.3 nM CXCL12</td>
<td>107 ± 5</td>
<td>9.86 ± 0.04</td>
<td>3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td><strong>Anti-HIV-1 activity (in nM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT-4 / NL4.3</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT-4 / HE</td>
<td>0.16</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM C / NL4.3</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM C / HE</td>
<td>1.5</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM C / BaL</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87.CD4.CXCR4 / NL4.3</td>
<td>0.36</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87.CD4.CCR5 / BaL</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Potency relative to those of monovalent 238D2.

$^b$ Potency relative to those of monovalent 238D4.

$^c$ Maximum not reached at the highest test concentration of 0.5 µM, displacement at 0.5 µM.
(pK_L = 9.86 ± 0.04 and 10.19 ± 0.27, respectively; n = 3). Furthermore, 238D2-15GS-238D4
and 238D2-20GS-238D4 inhibited the entry of the X4 HIV-I strain NL4.3 into MT-4,
PBMCs and stably CD4 and CXCR4 expressing U87 cells in subnanomolar concentrations
(IC50: 0.2-0.5 nM). Both Nanobodies also potently inhibit the entry of the dual-tropic HIV-I
strain HE into MT-4 cells, endogenously expressing CXCR4 but not CCR5. The bivalent
Nanobodies were somewhat less potent in the inhibition of HIV-I HE into PBMCs which
endogenously express CCR5 in addition to CXCR4 and thus can partially circumvent a
blocking of CXCR4. Indeed, the lack of anti-retroviral efficiency in control experiments with
the CCR5-using HIV-I strain BaL confirms that the anti-retroviral activity of the bivalent
Nanobodies 238D2-15GS-238D4 and 238D2-20GS-238D4 is dependent on CXCR4 as co-
receptor for virus entry (Table B-7). In conclusion, linking of the Nanobodies 238D2 and
238D4 to a single chain molecule result in a significant increase of anti-chemotactic and anti-
retroviral potency via blocking of CXCR4 by even one to two orders of magnitude (see Table
B-7 but also Table B-4; Figure 8).

**Example 5: HIV-I infection assays**

The anti-HIV-1 potencies of Nanobodies on the X4 HIV-I clone NL4.3 or primary
virus strains UG270 (X4, Clade D), CI#17 (X4, clade B), the R5 HIV-I strain BaL or the
R5/X4 HIV-I HE strain in MT-4, U87.CD4.CXCR4, U87.CD4.CCR5 and/or
phytohemagglutinin-stimulated PBMCs from different donors were determined by measuring
the cytopathic effect of HIV-1 (in MT-4 and U87 cell lines) microscopically and by
tetrazolium-based colorimetric evaluation or by quantification of the viral p24 antigen
production in the culture supernatant (PBMCs) as described in detail. Primary virus strains
were grown 1 or 2 times in PBMC only.

The CXCR4-using (X4) HIV-I clone NL4.3 was obtained from the National Institutes
of Health NIAID AIDS Reagent program (Bethesda, MD). The CCR5-using (R5) HIV-I
strain BaL was obtained from the Medical Research Council AIDS reagent project (Herts,
UK). The dual-tropic (R5/X4) HIV-I HE strain was initially isolated from a patient at the
University Hospital in Leuven.

The MT-4 cells were seeded out in 96-well plate and the U87 cells in 24-well plates.
The test compounds were added at different concentrations together with HIV-I and the
plates were maintained at 37 °C in 10% CO2. Cytopathic effect induced by the virus was
monitored by daily microscopic evaluation of the virus-infected cell cultures. At day 4-5 after infection, when strong cytopathic effect was observed in the positive control (i.e., untreated HIV-infected cells), the cell viability was assessed via the in situ reduction of the tetrazolium compound MTS, using the CellTiter 96® AQUous One Solution Cell Proliferation Assay (Promega, Madison, WI). The absorbance was then measured spectrophotometrically at 490 nm with a 96-well plate reader (Molecular Devices, Sunnyvale, CA) and compared with four cell control replicates (cells without virus and drugs) and four virus control wells (virus-infected cells without drugs). The 50% inhibitory concentration (IC50, i.e. the drug concentration that inhibits HIV-induced cell death by 50%). was calculated for each compound from the dose-response curve. The CC50 or 50% cytotoxic concentration of each of the compounds was determined from the reduction of viability of uninfected cells exposed to the agents, as measured by the MTS method described above.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density centrifugation (Lymphoprep; Nycomed Pharma, AS Diagnostics, Oslo, Norway) and stimulated with phytohemagglutinin for 3 days. The activated cells were washed with PBS and viral infections were performed as described previously (Schols D, et al. (1997) Inhibition of T-tropic FIIV strains by selective antagonization of the chemokine receptor CXCR4, J Exp Med 386:1383-1388). At 8-10 days after the start of the infection, viral p24 Ag was detected in the culture supernatant by an enzyme-linked immunosorbent assay (Perkin Elmer, Brussels, Belgium).
Table B-7: Inhibition by Nanobodies 238D2, 238D4 and biparatopes of infection of PBMC by primary virus isolates

<table>
<thead>
<tr>
<th></th>
<th>MT-4/NL4.3</th>
<th>PBMC+NL4.3</th>
<th>PBMC+NL4.3</th>
<th>PBMC+BaL</th>
<th>PBMC+BaL</th>
<th>PBMC+UG270</th>
<th>PBMC+UG270</th>
<th>PBMC+CI17</th>
<th>PBMC+CI17</th>
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<tbody>
<tr>
<td></td>
<td>exp. 1</td>
<td>donor 1</td>
<td>donor 2</td>
<td>donor 1</td>
<td>donor 2</td>
<td>donor 2</td>
<td>donor 1</td>
<td>donor 2</td>
<td>donor 2</td>
</tr>
<tr>
<td>238D2 IC50 (ng/ml)</td>
<td>701.4</td>
<td>468.4</td>
<td>307.4</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>492.5</td>
<td>447.2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>238D4 IC90 (ng/ml)</td>
<td>849</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<td>&gt;1000</td>
<td>&gt;1000</td>
<td>851.3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>238D2-20GS IC50 (ng/ml)</td>
<td>832.9</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<td>&gt;1000</td>
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<tr>
<td>AMD3100 IC50 (ng/ml)</td>
<td>6.9</td>
<td>18.2</td>
<td>9.2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>24.2</td>
<td>83.1</td>
<td>100.9</td>
<td>120</td>
</tr>
<tr>
<td>238D4 IC90 (ng/ml)</td>
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<td>97.2</td>
<td>73.1</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>139.1</td>
<td>167.8</td>
<td>468.1</td>
<td>654.0</td>
</tr>
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<td>238D2-15GS IC50 (ng/ml)</td>
<td>16.6</td>
<td>10</td>
<td>17.9</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>21.2</td>
<td>89.4</td>
<td>28.5</td>
<td>35.5</td>
</tr>
<tr>
<td>238D4 IC90 (ng/ml)</td>
<td>71.1</td>
<td>109.5</td>
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<td>&gt;1000</td>
<td>78.9</td>
<td>170.3</td>
<td>196.5</td>
<td>607.6</td>
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<td>maraviroc IC50 (ng/ml)</td>
<td>6.4</td>
<td>1.1</td>
<td>&gt;40</td>
<td>nd</td>
<td>nd</td>
<td>3.6</td>
<td>17.9</td>
<td>12.1</td>
<td>14.9</td>
</tr>
<tr>
<td>maraviroc IC90 (ng/ml)</td>
<td>&gt;40</td>
<td>9.9</td>
<td>&gt;40</td>
<td>nd</td>
<td>nd</td>
<td>15.4</td>
<td>34.1</td>
<td>&gt;40</td>
<td>37.9</td>
</tr>
<tr>
<td>maraviroc IC50 (ng/ml)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1.5</td>
<td>0.8</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>maraviroc IC90 (ng/ml)</td>
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<td>27.3</td>
<td>13.1</td>
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</tr>
</tbody>
</table>
Example 6: Nanobodies against CD4

Example 6.1: Animal Immunizations

Llama 58, 59 and 60 were immunized according to standard protocols with 6 boosts, each of them with approximately $10^8$ of human peripheral blood lymphocytes (hPBLs). Blood was collected 39 and 43 days after boost 1. In addition, approximately 1 g of lymph node was collected from both animals 39 days after boost 1.

Exarçppie 6.2: Library construction

Peripheral blood mononuclear cells were prepared from blood samples using Ficoll-Hypaque according to the manufacturer’s instructions. Next, total RNA was extracted from
these cells and lymph node tissue, if available, and used as starting material for RT-PCR to
amplify Nanobody encoding gene fragments. These fragments were cloned into a phagemid
vector. Phage was prepared according to standard methods (see for example the prior art and
applications filed by applicant cited herein).

Example 6.3: Selections of phage displaying CD4 binding Nanobodies

Phage libraries 58, 59 and 60 were used for selections on recombinant human CD4
(ImmunoDiagnostics. Inc., cat#7001, lot # 5S30/1.5). rhCD4 was immobilized directly on
Maxisorp 96 well microliter plates (Nunc) at 10 ug/ml, 0.1 ug/ml and 0 ug/ml (control).
Following incubation with the phage libraries and extensive washing, bound phage was
eluted with 100 mM triethylamine (TEA). The eluted phage were amplified and applied in a
similar second round of selection. After elution with TEA the second round obtained phage
were again amplified and applied into a similar third round selection in which following
incubation with the phage and extensive washing, bound phage was aspecifically eluted with
TEA, or specifically with 250 nM of gpl 20 HIV-1 IIIB (Immunodiagnostic).

Individual colonies obtained from the eluted phage pools were grown and i) induced
for new phage production and ii) induced with IPTG for Nanobody expression and extraction
(periplasmic extracts) according to standard methods (see for example the prior art and
applications filed by applicant cited herein).

Example 6.4: Screening for CD4 binding Nanobodies

In order to determine binding specificity to CD4, the selected clones were tested in an
ELISA binding assay setup, using the monoclonal phage pools. Shortly, 1 ug/ml receptor
recombinant human CD4 (ImmunoDiagnostics, Inc., cat#700i, lot # 5S30/1.5) was
immobilized on Maxisorp ELISA plates (Nunc) and free binding sites were blocked using 4%
Marvel skimmed milk in PBS. Next, 15 ul of supernatant from the monoclonal phage
inductions of the different clones in 100 ul 1% Marvel PBS were allowed to bind to the
immobilized antigen. After incubation and a wash step, phage binding was revealed using a
HRP-conjugated monoclonal-anti-M13 antibody (Gentaur Cat# 27942101). Binding
specificity was determined based on OD values compared to controls having received no
phage or an irrelevant phage.

Figure 9 shows a selection of clones binding to CD4.
Example 6.5: Screening for Nanobodies blocking CD4-gpl20 interaction

Clones tested positive in the CD4 binding assay were screened for their ability to block gpl20 binding to CD4. The monovalent his-tagged Nanobodies were purified from periplasmic extracts by affinity and desalting chromatography and used in an ELISA-based competition setup. In short, 1 μg/m3 of gpl20 HIV-I IHB (Immunodiagnostic) was captured by 20 μg/ml of sheep anti-gpl20 antibody D7324 (Aalto Bio Reagents) previously coated in 96 well Maxisorp microtiter plates (Nunc) and blocked with 1% casein in PBS. In parallel, 0.5 μg/ml of biotinylated CD4 was incubated with 500 nM of the different purified Nanobodies in 100 μl 0.1% Casein/PBS. After 1 hour, the biotinylated CD4-Nanobody pre-mixes were incubated 1 hour with the captured gpl20. Bound biotinylated CD4 was detected using HRP-conjugated Extravidin (Sigma E2886).

A blocking mouse anti-CD4 monoclonal antibody (Diaclone, clone B-Al cat#854.030.000) was used as positive control. Blocking activity was determined as loss of OD signal, as compared to wells where no Nanobody was added.

Figure 10 shows results of this blocking assay using a selection of clones binding to rh CD4. These results indicates that Nanobodies 03Fl1 and 01B6 are potential blockers for the in vitro interaction of CD4 with gpl20.

Example 6.6: Binding of selected Nanobodies to Jurkat-CD4 expressing cells

To check whether the selected Nanobodies recognize cell-surface-native form of CD4, a Flow cytometry experiment was performed where the Nanobodies were tested for specific binding to Jurkat cell expressing CD4.

Periplasmic fractions diluted 2.5 or 5 fold were allowed to bind to 10^5 cells of Jurkat cells for 30 minutes at 4°C in a final volume of 100 μl of 10% FBS (Invitrogen, Cat 10270-106, Lot#41G41 170K) in PBS (Invitrogen # 14190). Bound Nanobodies were detected with mouse anti-myc antibody (Serotec, Cat#MCA2200, Lot#0407) followed by goat anti-mouse-PE antibody (Jackson #115-1 15-164. Lot# 79725). Dead cells percentage population was determined by staining the cells with TOPRO3 (Molecular probes T3605; Lot #413969).

Using a BD FACSArray Bioanalyzer system, a PE filter 585/42 and a Topro filter 661/16, twenty thousand events were acquired. First a P1 population which represents more than 80% of the total population was selected. From this population the TOPRO3+ cells
(dead cells) were excluded. For the P1/TPORO3- population the MCF (mean channel fluorescence) was calculated. Expression of CD4 in Jurkat cells was confirmed by using 10 ng/ml of an anti-CD4 monoclonal antibody (Diaclone, clone B-A1 cat#854.030.000). Staining of the cells with an irrelevant Nanobody 03G6, anti myc and/or the goat anti mouse-PE antibodies antibody was performed as negative controls.

The results obtained for the different Nanobodies and control antibodies are shown in Figure 11. These data showed only clear binding of 03Fl1 to CD4 positive Jurkat.

**Example 6.7: Inhibition by the CD4 binding Nanobody 03Fl11 of infection of MT4 cells by NL4.3 virus**

In a first experiment the 03Fl1, 01E2, 01H12 and 01B6 were tested for anti-HIV activity using the NL4.3 strain on MT4 cells. To demonstrated replication p24 levels were determined. Of the four Nanobodies tested only 03Fl1 showed strong inhibitory activity (ICSO= 15 nM) while the other 3 showed no anti-HIV activity even at 500 nM. In a second experiment again using MT4 cell and the NL4.3 virus strain but now using the MTS method to detect HIV replication, again only 03Fl1 inhibited infection of cells by NL4.3 was observed (ICSO= 85 nM). Again no inhibition was observed with the other 3 Nanobodies even when used at 500 nM.
<table>
<thead>
<tr>
<th>Target of invention</th>
<th>Amino Acid Sequence</th>
<th>SEQ ID NO.</th>
</tr>
</thead>
<tbody>
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<td>hCD4</td>
<td>MNRGVFRRHLVLQLALLPATAQCKVVLGKOTVTLCTATCSQKSTQFHKNQSNQQIKLGNQGSQLTFLKGFSLHNLQDSRRLMLQDQNNPNFLIKNKLIESTDYYCEVQKEQFGVQVAVGFTLVSNQSLTSNLIILLTVIETDQQKSSQPGCPSVYSCRSFGRNCGKNTSVLQGQSTCNQSGKTVTQLVQCKKVIITFDSLQNKEVSKVDTLPQMQKKFLHHTLQFALPQYAGSNNLTLAELAQTGKLQQVEVNLQMLLQKSLKLNKENAFLVSRKFWAVNNCPAGMQQCLLDSDLGQALSSNDVFLVXWTPVQMPALTVLGCVAGLFLGIFCVCRCRRHRQRQERMSTQKRLSSSECTFCQPHSF</td>
<td></td>
</tr>
<tr>
<td>hCXCR4</td>
<td>MEGSISIPLELFIQTVSDNYETEMGSGYDYCSDKMCFRCEENAANFKNIFLPCTISIFLTGIVGCVSLVPMGSKKLMRSTDKRYKHLSSVACDLLFVTLIMFMAVDAWNYPPFVNCVKAVAVHVITYNLYSASLIALFLTRVTAIHVNSQPRKLAKVEEYGGYVWIPALLLEIDFIFANVEADDDYRCDYFPDNLYMVQQFPQHTMVLIGPVTIYDSCSIIKLSHSGKQKRLAKKTVTILLAPAFCWLPYTYGQDSTFLLIELKQGCQFENCVQHKWSEITALEFHCCINIPLYAPLGAFTQTSagogueTSVRGSLIKSLKRGHRGHSVSESSSFSHS</td>
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<td>hCCR5</td>
<td>MDYQVSSTVHDNYYSTSPCQKINVKQIAARLPPLYSLVFIFGVCVMNLVLVILNCRLKSLSDTLYLNNLALISEDFLFLTVPFWAAYAAMQPCNTMKCQLLQLYFLFGFSGFPIIIITLDVYLVHAVAFLDKARTVTFGVSITMTYWEVAASFLGPITAROSKEGLHVTYCSHSHFYSQHOFKNQFTILKVILGLVLPLMVQHYCLILESQALWELGHRNFLRNERAVLFLTIVLYFLMPAVPNYVLLMIQFPQFSGMCNCSNSNRLDAQAKHYTQLLMGHTCINIPILYAVFLGKFRNYYYLVQVQKHIARKRRCKCSCISIPQFQAEFAASSVYTRSTGEQIESVGL</td>
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<td>MELNFYKFIDNLFLSTKNLDSFNLRLHSYSGYFSFSFPFLQVLDLSRCSEITQTEDQAGYQSLHLSTTLTGTFSQPQSLAFLGSSLQLKLYAVETNALSLENFPIFLHGHTLKLVMAHLQIQISFKLFPYFSLNLTENLHDLSNHIQSIYCTDLRLVLQHEPMELNLSDSLINMNPIQGAFKEIRLKLTLRNIFSDLNVMMKTCIQGCALVHRLVLGQRHGFNGERNLKEFQSALEGONLITEFRRALYLYLDLDIDILPNCTNVSSFLVSLSTVIEKDFSYNFGWHOLELVNCFPQQGFTKLQKLRLTFTSNNKGNASEFVDELPSLEFDLRSNRLSFSGCCSQDFSCTSTKLYDLQSPGVIYMTSNSNLGCEQLEQHELDFQHPSNLKQMSEFVSTFLSNLNLQLYINHMTSIIKIQELQFHPFLALNFNTQDPCCTCHEQSFILQWINLVQVQOLQLEQLSTPFNASLSSQLVLNMNSNNFSSFSLTDTDYPKCNLSILVSINLYIMHMTSKQLEOQLFHPSLALNFNTQDPCCTCHEQSFILQWINLVQVQOLQLEQLSTPFNASLSSQLVLNMNSNNFSSFSLTDTDYPPCLQWLVYYPHIFMLLMALICIKYGRGEPYDFAVYIYSQDQDNNVINNLVKNLEEGYPPCLQRITFPIVAANIIHEHFKRSKRVJSVSSQHHQFQSRKICFYEIAQQTWQLSSRAGIFTIFEKRLQETVQQLLRRLLRNTRYQEEDEGCRHWHRLKALDLGKSNFECVTGCGNWQAE</td>
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</tr>
</tbody>
</table>
| human alphaV integrin | MAPPPRRRLGLPGRLPLSLGSLPLPCRAFNLVDPSPAYESGPGGSGYFSGFAVDFVFPVAQSMRFLVQAPKANTQPGIVEGWWWLKDWSSTRRCQPIBDFATDQKNYACDQPEFQSHQWFGAVSKQDKITACAQLYHFRTMEXQEREQVFCTQQLQTFKTQAYACFQRSDQIDAGQCGFCQGSGFIDTKQADVLQEGPSQFYQQLQISOVAEVSKYDNYSIKYNQNQATRAIGFDSVLGYSVAVGDNNGGTIDDFGSVPVARAATKLGMVYIDYGNMSSLYNFTGEQMAAYGFVSVAATDINGDDAYFVIGAPLMDFREGSDKQECQVSQVSLSQARSDGQFTQKLQGEVFAFQIRGSLATAPRLGIDDOFNDIAIAIAAYGPEDDKQIVYIFNGRSTRGLANSPTLQLEGQWAARSMSPFSFGYSMKATDICKNGYP | 268 254 269 270 271
Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following aspects.

All references disclosed herein are incorporated by reference in their entirety for the purpose and information indicated in the specification.
Claims:

1. Polypeptide comprising one or more Nanobodies that specifically bind one of the Targets of the invention and that inhibit viral entry.

2. Polypeptide according to claim 1, wherein the IC50 in the HIV-I infection assays is equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

3. Polypeptide according to any of claim 1 or 2, that has an antiviral activity (expressed as IC50 measured in the HIV-I infection assay) equal or below 100nM, preferably 50nM, more preferably 10nM, even more preferably 1nM.

4. Polypeptide according to any of claim 1 or 3, wherein the one or more Nanobodies specifically binds one of the Targets of the invention selected from the group consisting of hCD4, hCXCR4, hCCR5, hTLR4, human alphaV integrin, human beta3 integrin, human betal integrin, human alpha2 integrin, hCDS1, hSR-BI, hClaudin-1, hClaudin-6 and hClaudin-9.

5. Polypeptide according to any of claims 1 to 4, that specifically binds to one of the Targets of the invention selected from the group consisting of amino acid sequences with SEQ IDs: 254, 268 to 279.

6. Polypeptide according to any of claims 1 to 5, that specifically binds to hCXCR4 (SEQ ID NO: 254).

7. Polypeptide according to any of claims 1 to 5, that specifically binds to hCD4 (SEQ ID NO: 268).

8. Polypeptide of claim 1 to 7, that comprises two or more Nanobodies.

9. Polypeptide of claim 8, that comprises two Nanobodies.
10. Polypeptide of claim 1 to 9, wherein at least one Nanobody essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

5  
a) the amino acid sequences of SEQ ID NO's: 142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

and/or

- CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 175;

e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and/or

- CDR3 is chosen from the group consisting of:

g) the amino acid sequences of SEQ ID NO's: 206 to 207:

h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207.

11. Polypeptide of claim 1 to 10, wherein two Nanobodies essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDRI is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142 to 143;

b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143; and/or
- CDR2 is chosen from the group consisting of:

d) the amino acid sequences of SEQ ID NO's: 174 to 175;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and/or
- CDR3 is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 206 to 207;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207.

12. Polypeptide of claim 1 to 9, wherein one Nanobody essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

a) the amino acid sequences of SEQ ID NO's: 142;
b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142; and
- CDR2 is chosen from the group consisting of:
d) the amino acid sequences of SEQ ID NO's: 174;
e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174;
and
- CDR3 is chosen from the group consisting of:
g) the amino acid sequences of SEQ ID NO's: 206;
h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206;

wherein another Nanobody essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which - CDR] is chosen from the group consisting of:

j) the amino acid sequences of SEQ ID NO's: 143;
k) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 143;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 143;

and
- CDR2 is chosen from the group consisting of:
m) the amino acid sequences of SEQ ID NO's: 175;
n) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 175;
o) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 175;

and
- CDR3 is chosen from the group consisting of:
p) the amino acid sequences of SEQ ID NO's: 207;
q) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 207;
r) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 207.
13. Polypeptide of claim 1 to 12, wherein the Nanobodies are selected from the group consisting of Nanobodies having at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's 238 to 239.

14. Polypeptide of claim 13, wherein the polypeptide is selected from the group consisting of polypeptides having at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's 261 to 264.

15. Nanobody that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

   a) the amino acid sequences of SEQ ID NO's: 142 to 143;
   b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;
   c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 142 to 143;

and

   d) CDR2 is chosen from the group consisting of:
   e) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;
   f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 174 to 175;

and

   g) CDR3 is chosen from the group consisting of:
   h) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207;
   i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO's: 206 to 207.
16. Polypeptide of claim 1 to 9, wherein at least one Nanobody essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

- the amino acid sequence of SEQ ID NO: 287;
- amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;
- amino acid sequences that have 3, 2, or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 287;
- CDR2 is chosen from the group consisting of:
  - the amino acid sequence of SEQ ID NO: 295;
  - amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;
- amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;
- CDR3 is chosen from the group consisting of:
  - the amino acid sequence of SEQ ID NO: 303;
  - amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;
  - amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303.

17. Polypeptide of claim 1 to 10, wherein two Nanobodies essentially consists of 4 framework regions (FRI to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

- the amino acid sequence of SEQ ID NO: 287;
- amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;
c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;

and/or

- CDR2 is chosen from the group consisting of:

d) the amino acid sequence of SEQ ID NO: 295;

e) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 295;

f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 295;

and/or

- CDR3 is chosen from the group consisting of:

g) the amino acid sequence of SEQ ID NO: 303;

h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;

i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303.

18. Polypeptide of claim 1 to 17, wherein the Nanobodies are selected from the group consisting of Nanobodies having at least 80% amino acid identity with SEQ ID NO: 311.

19. Nanobody that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

a) the amino acid sequence of SEQ ID NO: 287;

b) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 287;

c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 287;

and

- CDR2 is chosen from the group consisting of:

d) the amino acid sequence of SEQ ID NO: 291;
337
c) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 291;
f) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 291;

and

- CDR3 is chosen from the group consisting of:

g) the amino acid sequence of SEQ ID NO: 303;
h) amino acid sequences that have at least 80% amino acid identity with at least the amino acid sequence of SEQ ID NO: 303;
i) amino acid sequences that have 3, 2, or 1 amino acid difference with at least the amino acid sequence of SEQ ID NO: 303.

20. Nucleic acid or nucleotide sequence, that encodes a polypeptide or Nanobody according to any of claims 1 to 19.

21. Host cell that under suitable circumstances is capable of expressing, a polypeptide or Nanobody according to any of claims 1 to 19.

22. Pharmaceutical composition comprising a polypeptide or Nanobody according to any of claims 1 to 19.

23. Pharmaceutical composition according to claim 22 that further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and that optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

24. Method for screening amino acid sequences directed against human CXCR4 (SEQ ID NO: 254) that comprises at least the steps of:

a) providing a set, collection or library of nucleic acid sequences encoding amino acid sequences;

b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an amino acid sequence that can bind to and/or has affinity
for a Target of the invention such as e.g. human CXCR4 or hCD4 and that is cross-blocked or is cross blocking a Nanobody or polypeptide according to claims 1 to 19; and

c) isolating said nucleic acid sequence, followed by expressing said amino acid sequence.

25. Method/use for treatment of viral and/or bacterial induced disease or disorder, e.g. AIDS, said method/use comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide or Nanobody according to any of claims 1 to 19.
Figure 2: Phage binding to coated membrane (Phage ELISA)

- CXCR4
- -

Monoclonal phages

238H3
238G3
238F3
238E3
238D3
238C3
238B3
238A3
238G2
238F2
238E2
238D2
238C2
238B2
238A2
238H1
238G1
238F1
238E1
238D1
238C1
238B1
238A1

0.0000
0.0200
0.0400
0.0600
0.0800
0.1000
0.1200
0.1400

SUBSTITUTE SHEET (RULE 26)
Figure 4a:

[Diagram of a plot showing binding efficiency of different antibodies against CXCL12. The x-axis is labeled 'Specific [125I]-CXCL12 Binding (%)' and the y-axis is labeled 'log [Nanobody] (M).']
Figure 4b:
Figure 5b:

Inhibition of CRE (% 20 nM CXCL12) vs. log [Ligand] (M)

- CXCL12
- 238D2
- 238D4

Inhibition of CRE (% 20 nM CXCL12) vs. log [CXCL12] (M)

- 10 nM
- 30 nM
- 100 nM
- 300 nM

Inhibition of CRE (% 20 nM CXCL12) vs. log [238D2] (M)
**Figure 6:**

### CXCR4 FAMILY GROUPING

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>237B6, A5, D2, D3, E4, F4, G2, G4, xh5, F1, C5, G1</td>
<td>FAM 1</td>
</tr>
<tr>
<td>2</td>
<td>237D4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>238B10</td>
<td>FAM 2</td>
</tr>
<tr>
<td>4</td>
<td>238F7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>238C5, G2, xH5, C3, D6, E6</td>
<td>u (4)</td>
</tr>
<tr>
<td>6</td>
<td>238D4, G3</td>
<td>FAM 3</td>
</tr>
<tr>
<td></td>
<td>238C4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>238C1, D2</td>
<td>u (5)</td>
</tr>
<tr>
<td>8</td>
<td>237B5</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>238H2</td>
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<td>10</td>
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</tr>
<tr>
<td>11</td>
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<td>?</td>
</tr>
<tr>
<td>12</td>
<td>237F11</td>
<td>?</td>
</tr>
<tr>
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<td>u</td>
</tr>
<tr>
<td>14</td>
<td>xx237C1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>237D1</td>
<td></td>
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<tr>
<td>16</td>
<td>237E1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>237G7</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8:

A

\[ ^{125}\text{I}-\text{CXCL12} \text{ binding (\%)} \]

\[ \text{log [Nanobody]} \text{ (M)} \]

\[ \text{XL12AMD} \]

△ 238D2
▼ 238D4
♦ 238D2 + 238D4
△ 238D2-15GS-238D4
▼ 238D2-20GS-238D4
○ 238D2-10GS-238D2
♦ 238D4-20GS-238D4
□ 238D2-20GS-238C5
★ 238D2-20GS-238B10

B

\[ \text{Migrated cells (% 0.3 nM CXCL12)} \]

\[ \text{log [Nanobody]} \text{ (M)} \]

\[ \text{AMD} \]

△ 238D2
▼ 238D4
▼ 238D2-15GS-238D4
△ 238D2-20GS-238D4
Figure 9:

Phage binding CD4 ELISA

<table>
<thead>
<tr>
<th>Sample</th>
<th>O.D. (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01B6</td>
<td>0.2</td>
</tr>
<tr>
<td>01E2</td>
<td>0.5</td>
</tr>
<tr>
<td>01H12</td>
<td>0.2</td>
</tr>
<tr>
<td>03F11</td>
<td>0.3</td>
</tr>
<tr>
<td>Irrelevant</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 10:

CD4-gp120 Blocking ELISA

O.D. (450 nm)

0.0  0.2  0.4  0.6

03F11  01H12  01E2  01B6  No Nanobody  mAb A1
Figure 11:

CD4 Jurkat cells controls

MCF

TOPRO
Unstained
anti mouse-PE
anti myc + anti mouse-PE
mAb A1
IgG2a

0
5000
10000
15000
20000

0
1000
2000
3000
4000

01B6
01E2
01H12
03F11
Irrelevant

P.E.

1/5
1/2.5