# Improved Nanobodies™ against Tumor Necrosis Factor-alpha

The present invention relates to improved Nanobodies™ against Tumor Necrosis Factor-alpha (TNF-alpha), as well as to polypeptides comprising or essentially consisting of one or more of such Nanobodies. *[Note: Nanobody™, Nanobodies™ and Nanoclone™ are trademarks of Ablynx N.V.]*

The invention also relates to nucleic acids encoding such Nanobodies and polypeptides; to methods for preparing such Nanobodies and polypeptides; to host cells expressing or capable of expressing such Nanobodies or polypeptides; to compositions comprising such Nanobodies, polypeptides, nucleic acids or host cells; and to uses of such Nanobodies, such polypeptides, such nucleic acids, such host cells or such compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned below.

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

WO 04/041862 by applicant relates to Nanobodies against TNF-alpha and to the preparation and use thereof, in particular for the prevention and/or treatment of diseases and disorders associated with and/or mediated by TNF-alpha, such as inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, multiple sclerosis, addison's disease, autoimmune hepatitis, autoimmune parotitis, diabetes type 1, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, male infertility, multiple sclerosis, myasthenia gravis, pemphigus, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, and vasculitis.

The anti-TNF Nanobodies according to WO 04/041862 may be humanized and may be monovalent or multivalent, the latter of which leads to increased affinity for TNF. The anti-TNF Nanobodies™ according to WO 04/041862 may also be multispecific, and may in particular be in the form of a multispecific construct comprising two or more Nanobodies against TNF and a further Nanobody directed against a serum protein such as human serum albumin, which leads to an increased half-life in vivo.

WO 04/041862 also relates to methods for the preparation of the anti-TNF Nanobodies, to nucleic acids or constructs encoding the anti-TNF Nanobodies, as well as

to pharmaceutical compositions comprising the anti-TNF Nanobodies, which may be suitable for intravenous, subcutaneous, oral, sublingual, topical, nasal, vaginal or rectal administration, or for administration by inhalation. The anti-TNF Nanobodies according to WO 04/041862 may also be used for diagnostic purposes, optionally in the form of a kit-of-parts.

EP 0 486 526 describes TNF-alpha binding ligands against a specific epitope of TNF. Among the binding ligands, single domain antibodies ("dAbs") are mentioned.

Reiter et al., *J. Mol. Biol.* (1999), 290, 685-698 describe single domain antibodies against TNF-alpha obtained from a randomized phage display library that was generated starting from a VH domain scaffold from a mouse hybridoma.

WO 00/29004 describes murine single domain antibodies ("microbodies") against TNF-alpha.

WO 04/003019 *inter alia* describes ligands comprising a first binding domain against TNF-alpha and a second binding domain against a serum protein such as serum albumin.

It is a general object of the present invention to provide Nanobodies against TNF-alpha, in particular against human TNF-alpha.

In particular, it is an object of the present invention to provide Nanobodies against TNF-alpha, in particular against human TNF-alpha, and to provide proteins or polypeptides comprising the same, that are suitable for therapeutic and/or diagnostic use, and in particular for the prevention, treatment and/or diagnosis of one or more diseases and disorders associated with and/or mediated by TNF-alpha such as those mentioned above, and/or that can be used in the preparation of a pharmaceutical composition for the prevention and/or treatment of one or more diseases associated with and/or mediated by TNF-alpha, such as those mentioned above.

More in particular, it is an object of the invention to provide Nanobodies against TNF-alpha, and to provide proteins and polypeptides comprising the same, that are either an alternative to the Nanobodies and polypeptides against TNF-alpha described in WO 04/041862 and/or that have one or more improved properties or characteristics, compared to the Nanobodies and polypeptides against TNF-alpha described in WO 04/041862.

More in particular, it is an object of the invention to provide Nanobodies against TNF-alpha, and to provide proteins or polypeptides comprising the same, that are

improved compared to the Nanobodies and polypeptides against TNF-alpha described in WO 04/041862 with respect to one or more of the following properties or characteristics:

- increased affinity for TNF-alpha, 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 in WO 04/041862 or 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 in WO 04/041862 or hereinbelow);
- improved suitability or susceptibility for "humanizing" substitutions (as defined herein); and/or
- 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 in WO 04/041862 or hereinbelow) in a monovalent format;
- 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 in WO 04/041862 or hereinbelow) in a monovalent format;
- increased specificity towards TNF-alpha, 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 in WO 04/041862 or hereinbelow) in a monovalent format;
- decreased or where desired increased cross-reactivity with TNF-alpha 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 in WO 04/041862 or hereinbelow).

These objects are achieved by the Nanobodies, proteins and polypeptides described herein. These Nanobodies are also referred to herein as "*Nanobodies of the invention*"; and these proteins and polypeptides are also collectively referred to herein "*polypeptides of the invention*".

Since the Nanobodies and polypeptides described herein are mainly intended for therapeutic and/or diagnostic use, they are directed against (as defined herein) human TNF-alpha. It is however not excluded (but also not required) that Nanobodies and polypeptides described herein show cross-reactivity with TNF-alpha from one or more other species of warm-blooded animals, for example with TNF-alpha from one or more other species of primates and/or with TNF-alpha from one or more species of animals that are often used in animal models for diseases (for example mouse, rat, rabbit, pig or dog), and in particular in animal models for diseases and disorders associated with TNF-alpha (such as the species and animal models mentioned herein). In this respect, it will be clear to the skilled person that such cross-reactivity, when present, may have advantages from a drug development point of view, since it allows the Nanobodies and polypeptides against human TNF-alpha to be tested in such disease models.

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 TNF-alpha against which the Nanobodies and polypeptides of the invention are directed.

However, in a preferred embodiment, the Nanobodies, proteins or polypeptides described herein are directed against and/or can bind to an epitope of TNF-alpha that lies in and/or forms part of the TNF receptor binding site(s) (e.g. the binding sites for the TNF-RI, THF-RII, also known as p55 or p75). As is well known in the art, a TNF trimer comprises three receptor binding sites, which are essentially equivalent and which are formed by/at the interface of two TNF monomers within the TNF trimer. For example, the Nanobodies, proteins or polypeptides described herein are preferably directed against and/or can bind to an epitope of TNF-alpha that comprises the following amino acid residues of TNF-alpha: Gln at position 88, Lys at position 90, and/or Glu at position 146).

In particular, the Nanobodies, proteins or polypeptides described herein are directed against and/or can bind to an epitope of the TNF-alpha trimer, which lies in and/or forms part of the TNF receptor binding site(s). For example, the Nanobodies, proteins or polypeptides described herein may be directed against and/or can bind to an

epitope of the TNF-alpha trimer that comprises the following amino acid residues: Gln at position 88 and Lys at position 90 on a first TNF monomer (referred to herein as "monomer A"), and Glu at position 146 on a second TNF monomer (referred to herein as "monomer B") (in which Monomer A and Monomer B together, in the TNF trimer, form the TNF receptor binding site(s)).

More particularly, the Nanobodies, proteins or polypeptides described herein may be directed against and/or can bind to an epitope of the TNF-alpha trimer that comprises the aforementioned amino acids (Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B), and in addition at least one, preferably two or more, more preferably 5 or more, and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A: Gly at position 24, Gln at position 25, Thr at position 72, His at position 73, Val at position 74, Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 83, Thr at position 89, Val at position 91. Asn at position 92, Ile at position 97, Arg at position 131, Glu at position 135, Ile at position 136, Asn at position 137, Arg at position 138, Pro at position 139, Asp at position 140 and the following residues in monomer B: Pro at position 20, Arg at position 32, Lys at position 65, Lys at position 112, Tyr at position 115, Ala at position 145, Ser at position 147.

Alternatively, the Nanobodies, proteins or polypeptides described herein may be directed against and/or can bind to an epitope of TNF-alpha that comprises the aforementioned amino acids (Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B), and in addition at least one, preferably two or more, more preferably 5 or more and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A: Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 80, Ser at position 81, Tyr at position 87, Thr at position 89, Val at position 91, Asn at position 92, Ser at position 95, Ile at position 97, Glu at position 135, Ile at position 136, Asn at position 137 and the following residues in monomer B: Ala at position 33, Ala at position 145, Ser at position 147.

Such epitope can be delineated from structural analysis of the nanobody crystallized in complex with the TNF molecule, or from other approaches such as epitope mapping via pepscan analysis.

By comparison, from crystallographic data (not shown), it can be seen that the Nanobody 3E from WO 04/041862 binds to a different epitope (i.e. an epitope comprising

Tyr at position 141, Asp at position 140, Gln at position 67, Gly at position 24 and Glu at position 23) than the preferred epitope of the invention.

Thus, in another aspect, the present invention relates to an immunoglobulin variable domain (or a suitable fragment thereof) that can bind to an epitope of TNF-alpha that lies in and/or forms part of the TNF receptor binding site, and preferably to an epitope that comprises at least one, preferably two or more, and preferably all, of the following amino acid residues of TNF-alpha: Gln at position 88; Lys at position 90 and Glu at position 146. Such an immunoglobulin variable domain is preferably a heavy chain variable domain or a light chain variable domain, and in particular a heavy chain variable domain, which may be any mammalian heavy chain variable domain, including but not limited to human heavy chain variable domains, mouse heavy chain variable domains and Camelid heavy chain variable domains (such as the heavy chain variable domains from Camelid 4-chain immunoglobulins or the heavy chain variable domains (VHH domains) from so-called heavy chain antibodies). The immunoglobulin variable domain is preferably a domain antibody or single domain antibody or suitable for use as a (single) domain antibody. Most preferably, the immunoglobulin variable domain is a Nanobody (as defined herein), and some preferred, but non-limiting examples of Nanobodies that are suitable for use in this aspect of the invention are PMP1C2 (TNF1, SEQ ID NO:52) and PMP5F10 (TNF3, SEQ ID NO: 60), as well as humanized and other variants thereof (as further described herein).

The aforementioned immunoglobulin variable domain may also be humanized (as for example, and without limitation) described herein with respect to Nanobodies. The invention also relates to proteins and polypeptides that comprise or essentially consist of such immunoglobulin variable domains, which may for example be as defined herein. Alternatively, such variable domains may form part of ScFv constructs, dual-specific constructs, chimeric antibody or antibody structures and other immunoglobulin constructs, as for example reviewed by Hoogenboom (Nature Biotechnology (1997), 15:125-126). Preferably, however, the immunoglobulin variable domains directed against the above epitope are Nanobodies, in which case the proteins and polypeptides comprising such Nanobodies may be as further described herein.

Thus, some preferred aspects of the invention relate to:

I) A Nanobody which is directed against the same epitope on the trimer of TNF-alpha as Nanobody TNF1 (SEQ ID NO: 52).

II) A Nanobody which is directed against the same epitope on the trimer of TNF-alpha as Nanobody TNF3 (SEQ ID NO: 60).

III) A Nanobody which is directed against an epitope of the trimer of TNF-alpha that at least comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B.

IV) A Nanobody which is directed against an epitope of the trimer of TNF-alpha that comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B; and that further comprises at least comprises at least one, preferably two or more, more preferably 5 or more, and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A: Gly at position 24, Gln at position 25, Thr at position 72, His at position 73, Val at position 74, Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 83, Thr at position 89, Val at position 91. Asn at position 92, Ile at position 97, Arg at position 131, Glu at position 135, Ile at position 136, Asn at position 137, Arg at position 138, Pro at position 139, Asp at position 140 and the following residues in monomer B: Pro at position 20, Arg at position 32, Lys at position 65, Lys at position 112, Tyr at position 115, Ala at position 145, Ser at position 147.

V) A Nanobody which is directed against an epitope of the trimer of TNF-alpha that comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B; and that further comprises at least one, preferably two or more, more preferably 5 or more, and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 80, Ser at position 81, Tyr at position 87, Thr at position 89, Val at position 91, Asn at position 92, Ser at position 95, Ile at position 97, Glu at position 135, Ile at position 136, Asn at position 137 and the following residues in monomer B: Ala at position 33, Ala at position 145, Ser at position 147.

- A Nanobody in accordance with any of of I) to V) above, which has a $K_{off}$ rate for TNF of better than $2.10^{-3}$ (1/s), preferably better than $1.10^{-3}$.
- A Nanobody in accordance with any one of I) to V) above , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID

NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody.

- A Nanobody in accordance with any one of I) to V) above , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 12nM; or a humanized variant of such a Nanobody..
- A Nanobody in accordance with any one of I) to V) above , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody..
- A Nanobody in accordance with any one of I) to V) above , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody..
- A Nanobody in accordance with any one of I) to V) above , which is a humanized Nanobody.

and some preferred aspects of this embodiment relate to:

- A Nanobody in accordance with any one of I) to V) above , which is a GLEW-class Nanobody.
- A Nanobody in accordance with any one of I) to V) above , which is a humanized GLEW-class Nanobody.
- A Nanobody in accordance with any one of I) to V) above , which contains an arginine residue (R) at position 103.
- A Nanobody in accordance with any one of I) to V) above ,which contains an arginine residue (R) at position 103, and which is humanized
- A Nanobody in accordance with any one of I) to V) above , which is a GLEW-class Nanobody, and which contains an arginine residue (R) at position 103, and which is humanized.
- A Nanobody in accordance with any one of I) to V) above , which contains a leucine residue (L) at position 108.
- A Nanobody in accordance with any one of I) to V) above , which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 52 (TNF1), 76 (TNF13), 77 (TNF14), 95 (TNF29) or 96 (TNF30)
- A Nanobody in accordance with any one of I) to V) above , in which

a) CDR1 comprises:

- the amino acid sequence DYWMY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence DYWMY;

and

b) CDR2 comprises:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of I) to V) above , in which CDR1 comprises the amino acid sequence DYWMY.
- A Nanobody in accordance with any one of I) to V) above , in which CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG.
- A Nanobody in accordance with any one of I) to V) above , in which CDR3 comprises the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of I) to V) above , in which:
  - CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN; or

- CDR1 comprises the amino acid sequence DYWMY; and CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; or
- CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 comprises the amino acid sequence SPSGFN

- A Nanobody in accordance with any one of I) to V) above , in which CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of I) to V) above , in which CDR1 comprises the amino acid sequence DYWMY; CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG and CDR3 comprises the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of I) to V) above, in which

a) CDR1 is:

- the amino acid sequence DYWMY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence DYWMY;

and in which:

b) CDR2 is:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and in which

c) CDR3 is:

- the amino acid sequence SPSGFN; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of I) to V) above , in which CDR1 is the amino acid sequence DYWMY.
- A Nanobody in accordance with any one of I) to V) above , in which CDR2 is the amino acid sequence EINTNGLITKYPDSVKG.
- A Nanobody in accordance with any one of I) to V) above , in which CDR3 is the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of I) to V) above , in which:
  - CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN; or
  - CDR1 is the amino acid sequence DYWMY; and CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; or
  - CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 is the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of I) to V) above , in which CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of I) to V) above , in which CDR1 is the amino acid sequence DYWMY; CDR2 is the amino acid sequence EINTNGLITKYPDSVKG and CDR3 is the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of I) to V) above , in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

and some other preferred aspects of this embodiment relate to:

- A Nanobody in accordance with any one of I) to V) above , which is a KERE-class Nanobody

- A Nanobody in accordance with any one of I) to V) above , which is a humanized KERE-class Nanobody
- A Nanobody in accordance with any one of I) to V) above , which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 50 (TNF3), 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 98 (TNF33).
- A Nanobody in accordance with any one of I) to V) above , in which

a) CDR1 is:

- the amino acid sequence NYYMG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

and

b) CDR2 is:

- the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 is:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with any one of I) to V) above , in which CDR1 is the amino acid sequence NYYMG.
- A Nanobody in accordance with any one of I) to V) above , in which CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG.
- A Nanobody in accordance with any one of I) to V) above , in which CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with any one of I) to V) above , in which:
  - CDR1 is the amino acid sequence NYYMG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 is the amino acid sequence NYYMG; and CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with any one of I) to V) above , in which CDR1 is the amino acid sequence NYYMG; CDR2 is the amino acid sequence SILPLSDDPGWNTY and CDR3 is the amino acid sequence ILPLSDDPGWNTY.
- A Nanobody in accordance with any one of I) to V) above , in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

and with yet some other particularly preferred aspects being:

VI) A protein or polypeptide, which comprises a or essentially consists a Nanobody in accordance with any one of I) to V) above .

VII) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody in accordance with any one of I) to V) above .

VIII) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of I) to V) above .

IX) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of I) to V) above , and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor

crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

X) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of I) to V) above , and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

XI) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of I) to V) above , linked via a suitable linker.

XII) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of I) to V) above , linked via a suitable linker, and which is pegylated.

XIII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above , and which further comprises at least one Nanobody directed against human serum albumin.

XIV) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above , and which further comprises at least one Nanobody directed against human serum albumin, and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XV) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above , and which further comprises at least one Nanobody directed against human serum albumin and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

XVI) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above , and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of I) to V) above is linked, optionally via a suitable linker, to the one Nanobody directed against human serum albumin.

XVII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above , and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of I) to V) above is linked, optionally via a suitable linker, to the one Nanobody directed against human serum albumin, and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the

TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XVIII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of I) to V) above, and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of I) to V) above is linked, optionally via a suitable linker, to the one Nanobody directed against human serum albumin, and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of VI) to XVIII) above , in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of VI) to XVIII) above , in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of VI) to XVIII) above , in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of VI) to XVIII) above , in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of VI) to XVIII) above , which comprises or essentially consists of two humanized Nanobodies in accordance with any one of I) to V) above , and one humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).

It should be noted that when a Nanobody is mentioned above as being "*in accordance with any one of I) to V) above*", it is at least according to one of I) to V), may be according to two or more of I) to V), and may also include any one or more of the other aspects that are indicated as being "in accordance with any one of I) to V) above. Similarly, when a protein or polypeptide is mentioned above as being "*in accordance with any one of VI) to XVIII) above*", it is at least according to one of VI) to XVIII), may be according to two or more of VI) to XVIII), and may also include any one or more of the

other aspects that are indicated as being "in accordance with any one of VI) to XVIII) above.

It is also within the scope of the invention that, where applicable, a Nanobody or polypeptide of the invention can bind to two or more antigenic determinants, epitopes, parts, domains, subunits or confirmations of TNF-alpha. In such a case, the antigenic determinants, epitopes, parts, domains or subunits of TNF-alpha to which the Nanobodies and/or polypeptides of the invention bind may be the essentially same (for example, if TNF-alpha contains repeated structural motifs or is present as a multimer) or may be different (and in the latter case, the Nanobodies and polypeptides of the invention may bind to such different antigenic determinants, epitopes, parts, domains, subunits of TNF-alpha with an affinity and/or specificity which may be the same or different). Also, for example, when TNF-alpha exists in an activated conformation and in an inactive conformation, the Nanobodies and polypeptides of the invention may bind to either one of these conformation, or may bind to both these conformations (i.e. with an affinity and/or specificity which may be the same or different). Also, for example, the Nanobodies and polypeptides of the invention may bind to a conformation of TNF-alpha in which it is bound to a pertinent ligand, may bind to a conformation of TNF-alpha 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).

It is also expected that the Nanobodies and polypeptides of the invention will generally bind to all naturally occurring or synthetic analogs, variants, mutants, alleles, parts and fragments of TNF-alpha, or at least to those analogs, variants, mutants, alleles, parts and fragments of TNF-alpha 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 Nanobodies and polypeptides of the invention bind in TNF-alpha (e.g. in wild-type TNF-alpha). Again, in such a case, the Nanobodies 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 different from (i.e. higher than or lower than), the affinity and specificity with which the Nanobodies of the invention bind to (wild-type) TNF-alpha. It is also included within the scope of the invention that the Nanobodies and polypeptides of the invention bind to some analogs, variants, mutants, alleles, parts and fragments of TNF-alpha, but not to others.

Generally, the Nanobodies and polypeptides of the invention will at least bind to those forms (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.

Also, as TNF-alpha exists in a monomeric form and in multimeric forms, and in particular in trimeric form, it is within the scope of the invention that the Nanobodies and polypeptides of the invention only bind to TNF-alpha in monomeric form, or that the Nanobodies and polypeptides of the invention in addition also bind to one or more of such multimeric forms, such as the trimeric form of TNF; or may only bind to such a multimeric (e.g. trimeric) form. Thus, generally, when in this description reference is made to a Nanobody, protein or polypeptide that is directed to TNF-alpha, it should be understood that this also comprises Nanobodies directed against TNF-alpha in its trimeric form (including but not limited to Nanobodies against the receptor binding sites (e.g. the binding sites for the TNF-RI, THF-RII, also known as p55 or p75) of such a trimer). In all these cases, the Nanobodies 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 Nanobodies and polypeptides of the invention bind to TNF-alpha in its monomeric and non-associated state.

Also, generally, polypeptides of the invention that contain two or more Nanobodies directed against TNF-alpha may bind with higher avidity than the corresponding monomeric Nanobody or Nanobodies.

For example, and without limitation, a multivalent (as defined herein) protein or polypeptide that contains two or more Nanobodies that are directed against different epitopes of TNF-alpha multivalent (as defined herein) protein or polypeptide that contains two or more Nanobodies that are directed against different epitopes of TNF-alpha may bind to TNF-alpha with higher avidity than the corresponding monomers.

More importantly, a multivalent (as defined herein) protein or polypeptide that contains two or more Nanobodies that are directed against TNF-alpha may (and usually will) bind with higher avidity to a multimer of TNF-alpha than to a monomer of TNF-alpha, and will usually also bind with higher avidity than the corresponding monomeric Nanobodies. In such a multivalent protein or polypeptide, the two or more Nanobodies may for example be directed against the same epitopes, substantially equivalent epitopes,

or different epitopes. In one embodiment of such a multivalent protein or polypeptide, the two or more Nanobodies may be the same (and therefore be directed against the same epitope).

The latter is of particular importance, as it is known that the primary mode of signal transduction by TNF involves crosslinking by TNF receptors by a trimer of TNF molecules, which contains three receptor binding sites (see for example Peppel et al., *J. Exp. Med.*, 174 (1991), 1483-1489; Engelmann et al., *J. Biol. Chem.*, 265 (1990), 14497; Smith and Baglioni, *J. Biol. Chem.*, 264 (1989), 14646). For example, as described by Peppel et al., an engineered monovalent extracellular domain of the TNF receptor - which was only capable of blocking a single receptor binding site on aTNF trimer - was unable to prevent crosslinking of the TNF receptors by the remaining two receptor binding sites; whereas an engineered protein that comprises two such extracellular domains – thus being capable of blocking two receptor binding sites – provided a striking efficacy compared to the monovalent extracellular domain.

In the present invention, it has been found that monovalent Nanobodies are capable of binding to TNF alpha in such a way that the activity of TNF is reduced, both in in vitro models, in cellular models and in ex vivo models (see the Experimental Section below). Although the invention is not limited to any specific mechanism, explanation or hypothesis, it is assumed that because of their small size and high affinity for TNF-alpha, two or three monovalent Nanobodies of the invention are capable of simultaneously occupying two or three different receptor binding sites on the TNF trimer, thus preventing the trimer to initiate receptor crosslinking and thereby to initiate signal transduction (however, other mechanisms of action are not excluded: for example, depending on the epitope against which it is directed, a Nanobody of the invention may also inhibit the association of TNF into the trimeric state).

It should also be noted that, in addition or as an alternative to binding to two or more receptor binding sites on a single TNF-trimer, the proteins or polypeptides of the present invention that comprises or essentially consists of two or more immunoglobulin variable domains (or suitable fragments thereof) that are directed against epitopes of TNF-alpha may bind (e.g. intermolecularly) epitopes on two separate TNF-alpha molecules (e.g. two separate trimers).

However, according to one particularly preferred embodiment, the invention relates to a protein or polypeptide that comprises or essentially consists of two or more

immunoglobulin variable domains (or suitable fragments thereof) that are each directed against epitopes on TNF-alpha (and in particular of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site(s) of the TNF trimer, such that said polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking.

In particular, according to this preferred embodiment, the invention relates to a protein polypeptide that comprises or essentially consist of two or more immunoglobulin variable domains (or suitable fragments thereof) that are each directed against epitopes on TNF-alpha (and in particular of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site(s) of the TNF-trimer, wherein said immunoglobulin variable domains are linked to each other in such a way that the protein or polypeptide is capable of simultaneously binding to two or more receptor binding sites on a single TNF trimer (in other words, is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer). In this embodiment, the two or more immunoglobulin variable domains are preferably as defined above and are most preferably Nanobodies (so that the protein or polypeptide is a multivalent Nanobody construct, as further described herein). Also, in this embodiment, the two or more immunoglobulin variable domains may be the same or different; and may directed against different epitopes within the TNF receptor binding site(s), but are preferably directed against the same epitope.

In one preferred aspect of this embodiment, the two or more immunoglobulin variable domains are directed against epitopes of the TNF-alpha trimer, which epitopes lie in and/or form part of the TNF receptor binding site(s). For example, the two or more immunoglobulin variable domains are preferably directed against and/or can bind to an epitope of the TNF-alpha trimer that comprises the following amino acid residues: Gln at position 88 and Lys at position 90 on a first TNF monomer (referred to herein as "monomer A"), and Glu at position 146 on a second TNF monomer (referred to herein as "monomer B") (in which Monomer A and Monomer B together, in the TNF trimer, form the TNF receptor binding site(s)).

As further described below in more details with respect to Nanobodies, in such a protein or polypeptide, the at least two immunoglobulin variable domains are preferably linked in such a way that the distance between the N-terminus and the C-terminus of the two immunoglobulin variable domains present in such a protein or polypeptide is

preferably at least 50 Angstroms, and more preferably in the region of 55-200 Angstroms, and more preferably in the region of Angstroms, and in particular in the region of 65-150 Angstroms.

In a particularly preferred aspect of this embodiment, these two or more immunoglobulin sequences are Nanobodies, and are preferably chosen from the Nanobodies described herein. Some particularly preferred Nanobodies for use in this embodiment of the invention are PMP1C2 (TNF1, SEQ ID NO:52) and/or PMP5F10 (TNF3, SEQ ID NO: 60), as well as humanized and other variants thereof (as described herein); with PMP1C2 (TNF1, SEQ ID NO:52) and its humanized variants being particularly preferred.

Accordingly, the present embodiment will now be described in more detail with reference to Nanobodies. However, it will be clear to the skilled person that the teaching herein may be applied analogously to immunoglobulin variable domains.

In this embodiment of the invention, the two or more immunoglobulin sequences will usually be linked via one or more suitable linkers, which linkers are such that each immunoglobulin sequence can bind to a different receptor binding site on the same TNF trimer. Suitable linkers will inter alia depend on (the distance between) the epitopes on the TNF trimer to which the immunoglobulin sequences bind, and will be clear to the skilled person based on the disclosure herein, optionally after some limited degree of routine experimentation. For example, when the two or more immunoglobulin sequences are (single) domain antibodies or Nanobodies, suitable linkers may be chosen from the linkers described herein, but with a linker length that is such that the two or more (single) domain antibodies or Nanobodies can each bind to a different receptor binding site on the same TNF trimer.

Also, when the two or more immunoglobulin sequences that bind to the receptor binding sites of TNF-alpha are (single) domain antibodies or Nanobodies, they may also be linked to each other via a third (single) domain antibody or Nanobody (in which the two or more immunoglobulin sequences may be linked directly to the third (single) domain antibody/Nanobody or via suitable linkers). Such a third (single) domain antibody or Nanobody may for example be a (single) domain antibody or Nanobody that provides for an increased half-life, as further described herein. For example, the latter (single) domain antibody or Nanobody may be a (single) domain antibody or Nanobody that is

capable of binding to a (human) serum protein such as (human) serum albumin, as further described herein.

Alternatively, the two or more immunoglobulin sequences that bind to the receptor binding site(s) of TNF-alpha may be linked in series (either directly or via a suitable linker) and the third (single) domain antibody or Nanobody (which may provide for increased half-life, as decribed above) may be connected directly or via a linker to one of these two or more aforementioned immunoglobulin sequences. Some non-limiting examples of such constructs are the constructs of SEQ ID NOS: 93 or 94.

In particular, it has been found in the invention (see the crystallography data referred to herein) that, when the Nanobodies present in a multivalent or multispecific protein or polypeptide of the invention bind to the particular epitope described above (which is the epitope of TNF1 and its humanized variants, as well as of TNF3 and its humanized variants) then preferably, the two (or more) anti-TNF Nanobodies present in such a protein or polypeptide should be linked in such a way that the distance between the N-terminus and the C-terminus of two anti-TNF Nanobodies present in such a protein or polypeptide should preferably be at least 50 Angstroms, and more preferably in the region of 55-200 Angstroms, and in particular in the region of 65-150 Angstroms (with the upper limit being less critical, and being chosen for reasons of convenience, e.g. with a view to expression/production of the protein); or more generally that said distance should be such that it allows the protein or polypeptide to undergo intramolecular binding to the TNF-trimer (i.e. instead of intermolecular binding). The distance between the N-terminus and the C-terminus of two anti-TNF Nanobodies can be determined by any suitable means, such as by crystallography or molecular modelling (as described herein). These techniques generally also make it possible to determine whether a specific multivalent or multispecific protein or polypeptide is capable of providing intramolecular modelling. Alternatively, the present invention also provides a simple experiment using size-exclusion chromatography (as described by Santora et al., Anal. Biochem., 299: 119-129) that can be used to determine whether a given protein or polypeptide of the invention will (predominantly) provide intramolecular binding to a TNF-trimer or (predominantly) intermolecular binding between two or more TNF-trimers. Thus, in one particular embodiment of the invention, a protein or polypeptide of the invention is preferably such that in this experiment, it predominantly or essentially exclusively leads to intramolecular binding However, as emphasized above, it should be noted that proteins or polypeptides of

the invention that operate via intermolecular binding of separate TNF-alpha molecules (e.g. trimers) are also within the scope of the present invention.

Thus, in another preferred aspect, the invention provides for a multivalent or multispecific protein or polypeptide that comprises at least two Nanobodies against TNF-alpha (and in particular of the TNF-alpha trimer), in which said Nanobodies are preferably directed to essentially the same epitope as Nanobody PMP1C2 (as mentioned herein), and in which said at least two Nanobodies are linked in such a way that the distance the distance between the N-terminus and the C-terminus of the at least two anti-TNF Nanobodies is such that the protein or polypeptide is capable of undergoing intramolecular binding (as described herein) with a TNF-trimer. Preferably, in such a protein or polypeptide, the distance between the N-terminus and the C-terminus of two anti-TNF Nanobodies is at least 50 Angstroms, and more preferably in the region of 55-200 Angstroms, and in particular in the region of 65-150 Angstroms.

In such a preferred protein or polypeptide, the two or more Nanobodies may be linked in any suitable fashion, as long as the preferred distance between the N-terminus and the C-terminus of the at least two anti-TNF Nanobodies can be achieved, and/or as long as the protein or polypeptide is capable of undergoing intramolecular binding (as described herein) with a TNF-trimer.

For example, in its simplest form, the at least two Nanobodies are directly linked via a suitable linker or spacer that provides for the preferred distance between the N-terminus and the C-terminus of the at least two anti-TNF Nanobodies and which may allow the protein or polypeptide to undergo intramolecular binding (as described herein) with a TNF-trimer. Suitable linkers are described herein, and may - for example and without limitation - comprise an amino acid sequence, which amino acid sequence preferably has a length of 14 amino acids, more preferably at least 17 amino acids, such as about 20-40 amino acid sequence (which, using an average distance of 3.5 Angstrom for one amino acid, corresponds to linker lengths of 49 Angstroms, 59.5 Angstroms and about 70 Angstroms, respectively; with the maximum amount of amino acids being calculated in the same way based on the distances mentioned above). Preferably, such an amino acid sequence should also be such that it allows the protein or polypeptide to undergo intramolecular binding (as described herein) with a TNF-trimer.

Thus, in another preferred aspect, the invention provides for a multivalent or multispecific protein or polypeptide that comprises at least two Nanobodies against TNF-

alpha (and in particular of the TNF-alpha trimer), in which said Nanobodies are preferably directed to essentially the same epitope as Nanobody PMP1C2 (as mentioned herein), and in which said at least two Nanobodies are directly linked to each other using a suitable linker or spacer such that the distance the distance between the N-terminus and the C-terminus of the at least two anti-TNF Nanobodies is such that the protein or polypeptide is capable of undergoing intramolecular binding (as described herein) with a TNF-trimer. Preferably, in such a protein or polypeptide, the distance between the N-terminus and the C-terminus of two anti-TNF Nanobodies (and thereby the preferred length of the linker or spacer) is at least 50 Angstroms, and more preferably in the region of 55-200 Angstroms, and in particular in the region of 65-150 Angstroms.

More preferably, in this preferred aspect, the linker or spacer is an amino acid sequence that comprises at least 14, preferably at least 17, more preferably at least 20 amino acids (with a non-critical upper limit chosen for reasons of convenience being abut 50, and preferably about 40 amino acids). In one preferred, but non-limiting embodiment, the linker essentially consists of glycine and serine residues (as further described below). For example, one suitable linker is the GS30 linker described herein, which comprises 30 amino acid residues.

In another embodiment, the at least two Nanobodies against TNF-alpha are linked to each other via another moiety (optionally via one or two linkers), such as another protein or polypeptide. In this embodiment, it may be desirable to have the preferred distance (i.e. as mentioned above) between the N-terminus and the C-terminus of the at least two anti-TNF Nanobodies, for example such that the protein or polypeptide can still undergo intramolecular binding (as described herein) with a TNF-trimer. In this embodiment, the at least two Nanobodies may be linked directly to the other moiety, or using a suitable linker or spacer, again as long as the preferred distance and/or desired intramolecular binding can still be achieved. The moiety may be any suitable moiety which does not detract (too much) from the binding of the protein or polypeptide to TNF and/or from the further desired biological or pharmacological properties of the protein or polypeptide. As such, the moiety may be essentially inactive or may be biologically active, and as such may or may not improve the desired properties of the protein or polypeptide and/or may confer one or more additional desired properties to the protein or polypeptide. For example, and without limitation, the moiety may improve the half-life of the protein or polypeptide, and/or may reduce its immunogenicity or improve any other desired property.

In one preferred embodiment, the moiety may be another Nanobody (including but not limited to a third Nanobody against TNF-alpha, although this is not necessary and usually less preferred), and in particular another Nanobody that improves the half-life of the protein or polypeptide, such as a Nanobody that is directed against a serum protein, for example against human serum albumin. Examples of such proteins and polypeptides are described herein.

Thus, in one embodiment, the invention relates to a multivalent multispecific construct comprising two or more immunoglobulin sequences (or suitable fragments thereof) that are each directed against epitopes on TNF-alpha (e.g. of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site, and that are linked to each other via at least one immunoglobulin sequence that provides for increased half-life (and optionally via one or more suitable linkers), such that said polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking and/or the signal transduction that is mediated by said TNF trimer. Such a polypeptide may be such such that said firstmentioned two or more immunoglobulin sequences can each bind to a different receptor binding site on a TNF trimer.

In particular, in this embodiment, the polypeptide may comprise a trivalent bispecific Nanobody, that comprises two Nanobodies that are each directed against epitopes on TNF-alpha (and in particular of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site, in which said Nanobodies are linked to each other via a third Nanobody that provides for an increased half-life (e.g. a Nanobody that is directed to a serum protein such as human serum albumin), in which each of the firstmentioned two Nanobodies may be directly linked to said third Nanobody or via one or more suitable linkers, such that said polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking and/or the signal transduction that is mediated by said TNF trimer. Such a polypeptide may be such that said firstmentioned two Nanobodies can each bind to a different receptor binding site on a TNF trimer. Again, some particularly preferred Nanobodies for use in this embodiment of the invention are PMP1C2 (TNF1, SEQ ID NO:52) and/or PMP5F10 (TNF3, SEQ ID NO: 60), as well as humanized and other variants thereof (as described herein); with PMP1C2 (TNF1, SEQ ID NO:52) and its humanized variants being particularly preferred; and the Nanobodies directed against human serum albumin described herein. Some preferred, but non-limiting constructs of this embodiment of the invention are TNF 24 (SEQ ID NO: 90), TNF 26

(SEQ ID NO: 92), TNF 27 (SEQ ID NO: 93), TNF 28 (SEQ ID NO: 94), TNF 60 (SEQ ID NO: 417) and TNF 62 (SEQ ID NO:418), of which TNF 60 is particularly preferred.

Thus, some preferred aspects of this embodiment of the invention relate to:

XIX) A protein or polypeptide that comprises or essentially consists of two or more immunoglobulin variable domains (or suitable fragments thereof) that are each directed against epitopes on TNF-alpha (and in particular of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site(s) of the TNF trimer, such that said polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XX) A protein or polypeptide that comprises or essentially consists of two or more immunoglobulin variable domains (or suitable fragments thereof) that are each directed against epitopes on TNF-alpha (and in particular of the TNF-alpha trimer) that lie in and/or form part of the receptor binding site(s) of the TNF trimer, such that said polypeptide is capable intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said immunoglobulin variable domains are linked to each other in such a way that the protein or polypeptide is capable of simultaneously binding to two or more receptor binding sites on a single TNF trimer.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said immunoglobulin variable domains are capable of binding to the same epitope as Nanobody TNF1 (SEQ ID NO: 52).
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said immunoglobulin variable domains are capable of binding against the epitope within the TNF receptor binding site of the TNF trimer that at least comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said immunoglobulin variable domains are capable of binding against the epitope within the TNF receptor binding site of the TNF trimer that at least comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B; and that further

comprises at least one, preferably two or more, more preferably 5 or more, and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A: Gly at position 24, Gln at position 25, Thr at position 72, His at position 73, Val at position 74, Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 83, Thr at position 89, Val at position 91. Asn at position 92, Ile at position 97, Arg at position 131, Glu at position 135, Ile at position 136, Asn at position 137, Arg at position 138, Pro at position 139, Asp at position 140 and the following residues in monomer B: Pro at position 20, Arg at position 32, Lys at position 65, Lys at position 112, Tyr at position 115, Ala at position 145, Ser at position 147.

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said immunoglobulin variable domains are capable of binding against the epitope within the TNF receptor binding site of the TNF trimer that at least comprises the following amino acid residues: Gln at position 88 in monomer A; Lys at position 90 in monomer A and Glu at position 146 in monomer B; and that further comprises at least one, preferably two or more, more preferably 5 or more, and preferably all or essentially all, of the following amino acid residues of TNF-alpha monomer A Leu at position 75, Thr at position 77, Thr at position 79, Ile at position 80, Ser at position 81, Tyr at position 87, Thr at position 89, Val at position 91, Asn at position 92, Ser at position 95, Ile at position 97, Glu at position 135, Ile at position 136, Asn at position 137 and the following residues in monomer B: Ala at position 33, Ala at position 145, Ser at position 147.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the at least two immunoglobulin variable domains are linked in such a way that the distance between the N-terminus of the first immunoglobulin variable domain and the C-terminus of the second immunoglobulin variable domain present in such a protein or polypeptide is at least 50 Angstroms.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the distance between the N-terminus of the first immunoglobulin variable domain and the C-terminus of the second immunoglobulin variable domain is between 55-200 Angstroms
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the distance between the N-terminus of the first immunoglobulin variable

domain and the C-terminus of the second immunoglobulin variable domain is between 65-150 Angstroms

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domain are linked to each other via a linker or spacer.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer is an amino acid sequence.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer is comprises at least 14 amino acid residues.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer is comprises at least 17 - 50 amino acid residues.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer essentially consists of glycine and serine residues.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer is GS30 (SEQ ID NO: 69).
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domain are linked to each other via another moiety.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said other moiety is a protein or polypeptide moiety.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said other moiety confers at least one desired property to the protein or polypeptide, or improves at least one desired property of the protein or polypeptide.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which said other moiety improves the half-life of the protein or polypeptide and/or reduces the immunogenicity of the protein or polypeptide.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which each of the first and second immunoglobulin variable domain are linked to said other moiety via a linker or spacer.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer is an amino acid sequence.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the linker or spacer essentially consists of glycine and serine residues.

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the said other moiety is a Nanobody.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the other moiety is a Nanobody directed against human serum albumin.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with a $K_{off}$ rate for TNF of better than $2.10^{-3}$ (1/s), preferably better than $1.10^{-3}$ (1/s); or a humanized variant of such a Nanobody.,
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody..
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with an EC50 value in the cell-based assay using KYM cells described in Example 1,

under 3), of WO 04/041862 that is better than 12nM; or a humanized variant of such a Nanobody..

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody..
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody..
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies in accordance with any one of XIX) to XX) above , which are humanized;

with some particularly preferred aspects of this embodiment being:

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are GLEW-class Nanobodies.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with an arginine residue (R) at position 103.
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are GLEW-class Nanobodies with an arginine residue (R) at position 103..
- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies with at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 52 (TNF1), 76 (TNF13), 77 (TNF14), 95 (TNF29) or 96 (TNF30)
- A protein or polypeptide in accordance with any one of XIX) to XX) above, in which the first and second immunoglobulin variable domains are Nanobodies as

described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which:

a) CDR1 comprises:

- the amino acid sequence DYWMY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence DYWMY;

and

b) CDR2 comprises:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence SPSGFN.

- A protein or polypeptide in accordance with any one of XIX) to XX) above , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 comprises the amino acid sequence DYWMY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for

the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG.

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR3 comprises the amino acid sequence SPSGFN
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which:
  - CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN; or
  - CDR1 comprises the amino acid sequence DYWMY; and CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; or
  - CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 comprises the amino acid sequence SPSGFN
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 comprises the amino acid sequence DYWMY; CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG and CDR3 comprises the amino acid sequence SPSGFN.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which
  a) CDR1 is:
     - the amino acid sequence DYWMY; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence DYWMY;

and in which:

b) CDR2 is:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and in which

c) CDR3 is:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SPSGFN.

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 is the amino acid sequence DYWMY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR2 is the amino acid sequence EINTNGLITKYPDSVKG.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for

the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR3 is the amino acid sequence SPSGFN

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which:
  - CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN; or
  - CDR1 is the amino acid sequence DYWMY; and CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; or
  - CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 is the amino acid sequence SPSGFN
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 is the amino acid sequence DYWMY; CDR2 is the amino acid sequence EINTNGLITKYPDSVKG and CDR3 is the amino acid sequence SPSGFN.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are chosen from the group consisting of the Nanobody TNF 1 (SEQ ID NO: 52) and humanized variants of the Nanobody TNF 1 (SEQ ID NO: 52).

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are chosen from the group consisting of TNF 13 (SEQ ID NO: 76), TNF 14 (SEQ ID NO: 77), TNF 29 (SEQ ID NO: 95) and TNF 30 (SEQ ID NO:96).
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are TNF 30 (SEQ ID NO:96);

and with some particularly preferred aspects of this embodiment being:

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are KERE-class Nanobodies.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies with at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 50 (TNF3), 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 98 (TNF33).
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which:

  a) CDR1 comprises:
     - the amino acid sequence NYYMG; or
     - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
     - an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

  and

  b) CDR2 comprises:
     - the amino acid sequence NISWRGYNIYYKDSVKG; or
     - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or

- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR1 comprises the amino acid sequence NYYMG.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) in which CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which:
  - CDR1 comprises the amino acid sequence NYYMG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 comprises the amino acid sequence NYYMG; and CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which CDR1 comprises the amino acid sequence NYYMG; CDR2 comprises the amino acid sequence SILPLSDDPGWNTY and CDR3 comprises the amino acid sequence ILPLSDDPGWNTY (SEQ ID NO: 436).
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which

a) CDR1 is:
   - the amino acid sequence NYYMG; or
   - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
   - an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

and

b) CDR2 is:
   - the amino acid sequence NISWRGYNIYYKDSVKG; or
   - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
   - an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 is:
   - the amino acid sequence SILPLSDDPGWNTY; or
   - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or

- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which CDR1 is the amino acid sequence NYYMG.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which:
  - CDR1 is the amino acid sequence NYYMG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 is the amino acid sequence NYYMG; and CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which CDR1 is the amino acid sequence NYYMG; CDR2 is the amino acid sequence SILPLSDDPGWNTY and CDR3 is the amino acid sequence ILPLSDDPGWNTY.
- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are Nanobodies as described for the proteins or polypeptides in accordance with any one of XIX) to XX) , in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or

- said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are chosen from the group consisting of the Nanobody TNF 3 (SEQ ID NO: 60) and humanized variants of the Nanobody TNF 3 (SEQ ID NO: 60).

- A protein or polypeptide in accordance with any one of XIX) to XX) , in which the first and second immunoglobulin variable domains are chosen from the group consisting of TNF20 (SEQ ID NO:83), TNF21 (SEQ ID NO: 84), TNF 22 (SEQ ID NO: 85), TNF23 (SEQ ID NO: 86) or TNF33 (SEQ ID NO: 99).

It should be noted that when a protein or polypeptide is mentioned above as being "*in accordance with any one of XIX) to XX) above*", it is at least according to one of XIX) to XX), and may be according to both XIX) and XX), and may also include any one or more of the other aspects that are indicated as being "in accordance with any one of XIX) to XX) above".

However, it should be noted that the invention is not limited to any specific mechanism of action or hypothesis. In particular, it has been found that the monovalent Nanobodies of the invention may be also active in the assays and models described herein, which confirms that intramolecular binding of he TNF trimer, although preferred in one specific embodiment of the invention, is not required to obtain the desired action and effect of the Nanobodies, proteins and polypeptides described herein. Similarly, it is also encompassed within the scope of the invention that the proteins and polypeptides described herein achieve their desired action via any appropriate mechanism (i.e. by intramolecular binding, intermolecular binding or even by binding to monomeric TNF, thus inhibiting the formation of TNF trimers).

It is also within the scope of the invention to use parts, fragments, analogs, mutants, variants, alleles and/or derivatives of the Nanobodies and polypeptides of the invention, and/or to use proteins or polypeptides comprising or essentially consisting of the same, as long as these are suitable for the uses envisaged herein. Such parts, fragments, analogs, mutants, variants, alleles, derivatives, proteins and/or polypeptides will be described in the further description herein.

In another aspect, the invention relates to a Nanobody (as defined herein), against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

(i) CDR1 is an amino acid sequence chosen from the group consisting of the CDR1 sequences of SEQ ID NOS: 15 to 21 or from the group consisting of the CDR1 sequences of SEQ ID NOS: 164 to 197;

or 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 at least one of the CDR1 sequences from the group consisting of SEQ ID NOS: 15 to 21 or with at least one of the CDR1 sequences from the group consisting of SEQ ID NOS: 164 to 197, in which

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 2 or only 1 "amino acid difference(s)" (as defined herein) with at least one of the CDR1 sequences from the group consisting of SEQ ID NOS: 15 to 21 or with at least one of the CDR1 sequences from the group consisting of SEQ ID NOS: 164 to 197, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

(ii) CDR2 is an amino acid sequence chosen from the group consisting of the CDR2 sequences of SEQ ID NOS: 22 to 28 or from the group consisting of the CDR2 sequences of SEQ ID NOS: 232 to 265;

or 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 at least one of the CDR2 sequences

from the group consisting of SEQ ID NOS: 22 to 28 or with at least one of the CDR2 sequences from the group consisting of SEQ ID NOS: 232 to 265, in which

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with at least one of the CDR2 sequences from the group consisting of SEQ ID NOS: 22 to 28 or with at least one of the CDR2 sequences from the group consisting of SEQ ID NOS: 232 to 265, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

(iii) CDR3 is an amino acid sequence chosen from the group consisting of the CDR3 sequences of SEQ ID NOS: 29 to 33 or from the group consisting of the CDR3 sequences of SEQ ID NOS: 300-333;

or 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 at least one of the CDR3 sequences from the group consisting of SEQ ID NOS: 29 to 33 or with at least one of the CDR3 sequences from the group consisting of SEQ ID NOS: 300-333, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) or with at least one of the CDR3 sequences from the group consisting of SEQ ID NOS: 300-333, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

or from the group consisting of the CDR3 sequences of SEQ ID NOS: 34 and 35 or 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 at least one of the CDR3 sequences of SEQ ID NOS: 34 and 35, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with at least one of the CDR3 sequences of SEQ ID NOS: 34 and 35, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

The Nanobodies against TNF-alpha as described above and as further described hereinbelow are also referred to herein as Nanobodies of the invention.

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 can be seen in Table I below, which lists the CDR's 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 which are mentioned on the same line in Table 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 I).

Also, in the Nanobodies of the invention that comprise the combinations of CDR's mentioned in Table 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

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or chosen from the group consisting of amino acid sequences that have 3, 2 or only 1 (as indicated in the preceding paragraph) "amino acid difference(s)" (as defined herein) with the mentioned CDR(s) one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

However, as will be clear to the skilled person, the (combinations of) CDR sequences mentioned in Table I will generally be preferred.

Table I: Preferred combinations of framework and CDR sequences

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PMP1C2 (TNF1) | 13.0 | QVQLVESGGGLVQPG GSLRLSCAASGFTFS | 16.4 | DYWMY | 19.8 | WVRQAPGKG LEWVS | 23.2 | EINTNGLITKYP DSVKG | 26.6 | RFTISRDNAKNTLYLQMNSL KPEDTALYYCAR | 30.0 | SPSGFN | 33.4 | RGQGT QVTVSS |
| PMP1G11 | 13.1 | QVQLQESGGGMVQPG GSLRLSCAASGFDFG | 16.5 | VSWMY | 19.9 | WVRQAPGKG LEWVS | 23.3 | EINTNGLITKYP DSVKG | 26.7 | RFTISRDNAKTTLYLQMNSL KPEDTALYYCAR | 30.1 | SPSGSF | 33.5 | RGQGT QVTVSS |
| PMP1H6 | 13.2 | EVQLVESGGGLVQPG *GSLRLSCATSGFDFS* | 16.6 | *VSWMY* | 20.0 | WVRQAPGKG *LEWVS* | 23.4 | EINTNGLITKYV DSVKG | 26.8 | RFTISRDNAKNTLYLQMDSL *IPEDTALYYCAR* | 30.2 | *SPSGSF* | 33.6 | RGQGT QVTVSS |
| PMP1G5 (TNF2) | 13.3 | QVQLVESGGGLVQAG GSLRLSCAASGRTFS | 16.7 | EPSGYT YTIG | 20.1 | WFRQAPGKE REFVA | 23.5 | RIYWSSGLTYY ADSVKG | 26.9 | RFTISRDIAKNTVDLLMNSL KPEDTAVYYCAA | 30.3 | RDGIPTSRSV GSYNY | 33.7 | WGQGT QVTVSS |
| PMP1H2 | 13.4 | QVKLEESGGGLVQPG DSLRLSCAASGRTFS | 16.8 | DYSGYT YTVG | 20.2 | WFRQAPGKE REFVA | 23.6 | RIYWSSGNTYY ADSVKG | 27.0 | RFTISRDIAKNTVDLLMNNL EPEDTAVYYCAA | 30.4 | RDGIPTSRSV ESYNY | 33.8 | WGQGT QVTVSS |
| *PMP3G2* | 13.5 | AVQLVESGGGLVQPG *DSLRLSCAASGRTFS* | 16.9 | DYSGYT *YTVG* | 20.3 | WFRQAPGKE *REFVA* | 23.7 | RIYWSSGNTYY ADSVKG | 27.1 | RFTISRDIAKNTVDLLMNNL EPEDTAVYYCAA | 30.5 | RDGIPTSRSV *ESYNY* | 33.9 | WGQGT QVTVSS |
| PMP1D2 | 13.6 | AVQLVDSGGGLVQAG GSLRLSCAASGRTFS | 17.0 | AHSVYT MG | 20.4 | WFRQAPGKE REFVA | 23.8 | RIYWSSANTYY ADSVKG | 27.2 | RFTISRDNAKNTVDLLMNCL KPEDTAVYYCAA | 30.6 | RDGIPTSRSV EAYNY | 34.0 | WGQGT QVTVSS |

Table I (continued):

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PMP3D10 | 13 7 | QVQLVESGGGLVQAGG SLSLSCAASGRSFT | 17 1 | GYYMG | 20 5 | WFRQAPG KERQLLA | 23 9 | SISWRGDNTYYKE SVKG | 27 3 | RFTISRDDAKNTIYLQMN SLKPEDTAVYYCAA | 30 7 | SILPLSDDPG WNTN | 34 1 | WGQG TQVTV SS |
| PMP5F10 (TNF3) | 13 8 | EVQLVESGGGLVQAGG SLSLSCSASGRSLS | 17 2 | NYYMG | 20 6 | WFRQAPG KERELLG | 24 0 | NISWRGYNIYYKDS VKG | 27 4 | RFTISRDDAKNTIYLQMN RLKPEDTAVYYCAA | 30 8 | SILPLSDDPG WNTY | 34 2 | WGQG TQVTV SS |
| NC55TNF_S1C4 | 13 9 | EVQLVESGGGLVQAGD SLRLSCAASQIIFG | 17 3 | SHVAA | 20 7 | WFRQAPG REREFVA | 24 1 | EIRPSGDFGPEGE FEHVTASLKG | 27 5 | RFTIAKNSVDNTVYLQM NSLKPEDTAVYYCAA | 30 9 | APYRGGRDYR WEYEYEY | 34 3 | WGQG TQVTV SS |
| NC55TNF_S1C3 | 14 0 | EVQLVESGGGLVQPGG SLRLSCKNAGSTSN | 17 4 | AYATG | 20 8 | WFRRAPG KEREFVA | 24 2 | GIQWSGGDAFYRN SVKG | 27 6 | RFRITRDPDNTVYLQMN DLKPEDTAIYYCAQ | 31 0 | KLSPYYNDFD SSNYEY | 34 4 | WGQG TQVTV SS |
| NC55TNF_S2C1 | 14 1 | EVQLVESGGDLVQPGG SLRLSCAVSGQLFS | 17 5 | TNDVG | 20 9 | WYRRAPG KQRELVA | 24 3 | TITDDGTTDYGDD VKG | 27 7 | RFVISREGEMVYLEMNS LKPEDTAVYYCNI | 31 1 | NRLRSTWGIR YDV | 34 5 | WGQG TQVTV SS |
| NC55TNF_S2C5 | 14 2 | EVQLVESGGGLVQPGG SLRLSCVVSGFTFS | 17 6 | TTSMT | 21 0 | WVRQAPG KFEEWVS | 24 4 | FINSDGSSTTYADS VKG | 27 8 | RFTISRDNAKNTLYLQMN SLKPEDTAMYYCGR | 31 2 | RGYGRD | 34 6 | RSKGI QVTVA S |
| NC55TNF_S3C7 | 14 3 | EVQLVESGGGTVQAGD SLRLSCAASGRSFS | 17 7 | SVAMG | 21 1 | WFRQAPG KQREFLA | 24 5 | GVGYDGSSIRYAE SVKG | 27 9 | RFTIARGNRESTVFLQM ENLKPEDTAVYFCTA | 31 3 | EPIGAYEGLW TY | 34 7 | WGQG TQVTV SS |

Table I (continued):

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14 | TNF- | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | WGQG |
| NC55TNF | 44 | ALPHAVESGGGLMQPG | 78 | | 11 | WFRQAPGK | 46 | TISWNGGSS | 80 | RFTISRDNAKNTVYLQMNG | 14 | SYSNGNPHRFS | 48 | TQVTV |
| _S3C1 | 45 | GSLKLSCAASGFMFS | 89 | DSAMG | 23 | EREFVA | 67 | SYADFVKG | 01 | LTPQDTAIYYCAG | 45 | QYQY | 89 | SS |
| | 14 | | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | WGQG |
| NC55TNF | 45 | EVQLVESGGGLVQAGG | 79 | | 12 | WFRQAPGK | 47 | AISWGGGSIV | 81 | RFTISRDNAKXTMYLQMDS | 15 | | 49 | TQVTV |
| _BMP1B2 | 56 | SLRLSCAASGRTFG | 90 | TYAMG | 34 | EREFVA | 78 | YAESAKG | 12 | LKPEDTAVYYCAA | 56 | ANNIATLRQGS | 90 | SS |
| | 14 | | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | LGSGT |
| NC55TNF | 48 | EVQLVESGGELVQAGGS | 81 | | 12 | WFRRAPGKE | 48 | SISWSGDTT | 82 | RFTVSRDNGKNTAYLRMN | 16 | VQVIDPSWSGV | 56 | QVTVS |
| _BMP1D2 | 60 | LKLSCTASGRNFV | 00 | TYAMS | 45 | REFVA | 89 | YYSNSVKG | 23 | SLKPEDTADYYCAV | 67 | NLDDYDY | 01 | S |
| | 14 | | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | WGQG |
| NC55TNF | 48 | EVQLVESGGRLVQPGG | 81 | | 12 | WFRRAPGKE | 48 | GIQWSGGDA | 82 | RFRITRDPDNTVYLQMNDL | 16 | KLSPYYNDFDS | 56 | TQVTV |
| _BMP1E2 | 71 | SLRLSCKNAGSTSN | 10 | AYATG | 56 | REFVA | 98 | FYRNSVKG | 34 | KPEDTAIYYCAQ | 78 | SNYEY | 12 | SS |
| | 14 | | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | WGQG |
| NC55TNF | 48 | EVQLVESGGGLVQPGG | 81 | | 12 | WYRQAPGK | 57 | SITIGSRTNY | 82 | RFTISRDNAKNTVYLQMNS | 16 | | 56 | TQVTV |
| _BMP1G2 | 82 | SLRLSCAASATISS | 21 | IVMLG | 67 | QREWVA | 01 | ADSVKG | 45 | LKPEDTAVYFCNA | 89 | VPPRDDY | 23 | SS |
| | 14 | | 17 | | 21 | | 24 | | 28 | | 31 | | 35 | WGQG |
| NC55TNF | 49 | EVQLVESGGGLVQAGG | 81 | | 12 | WFRQAPGE | 59 | RISGSDGSTY | 82 | RFTISRDNTKNMVYLQMD | 16 | PRYENQWSSY | 56 | TQVTV |
| _BMP2A2 | 93 | SLRLSCAASGQTSS | 32 | SYDMG | 78 | GREFVA | 12 | YSDRAKD | 56 | RLKPDDTAVYYCRV | 90 | DY | 34 | SS |
| | 15 | | 18 | | 22 | | 25 | | 29 | | 32 | | 36 | WGKGT |
| NC55TNF | 58 | EVQLVESGGGLVQPGG | 80 | | 11 | WVRQAPGK | 58 | GIDSGGGSP | 82 | RFTVSRDNAKNTLYLQMN | 26 | FSTGADGGSW | 58 | QVTVS |
| _BMP2C2 | 06 | SLRLSCAASGSTFS | 43 | TYDMS | 89 | GLEWVS | 26 | MYVDSVKG | 67 | SLKPEDTAVYYCAK | 01 | YWSYGMDS | 45 | S |

Table I (continued):

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NC55TNF_BMP2F2 | 15 1 | EVQLVESGGGLVQAG DSLRLSCEASERSSN | 18 5 | RYNMA | 21 9 | WFRQAPGK EREFLA | 25 3 | RVDVSGGNT LYGDSVKD | 28 7 | RFTVSRINGKNAMYLQMNNL KPEDTAIYYCAA | 32 1 | GGWGTTQYDY DY | 35 5 | WGQG TQVTV SS |
| NC55TNF_NC10 | 15 2 | EVQLVESGGGLVQPG GSLRLSCVCVSSGCT | 18 6 | FSAYSMT | 22 0 | WVRQAPGK AEEWVS | 25 4 | FINSDGSSTT YADSVNG | 28 8 | RFKISRDNAKKTLYLQMNSL GPEDTAMYYCQR | 32 2 | RGYALD | 35 6 | RGQGT QVTVS S |
| NC55TNF_NC11 | 15 3 | EVQLVESGGGLVQAG DSLTLSCASSGRGFY | 18 7 | KNAMG | 22 1 | WFRQPPGK EREFVA | 25 5 | SIKWNGNNT YYADSVRG | 28 9 | RFTISRGNAKNTENTVSLQM NSLKPEDTADYYCAA | 32 3 | DSSHYSYVYSK AYEYDY | 35 7 | WGQG TQVTV SS |
| NC55TNF_NC1 | 15 4 | EVQLVESGGGLVQPG GSLRLSCVFSGFAFS | 18 8 | ASSMA | 22 2 | WVRQAPGK YEEWVS | 25 6 | FINSDGSSTT YADSVQG | 29 0 | RFTISRDNAKNTLYLQMNSL KSEDTAMYYCGR | 32 4 | RGYGRD | 35 8 | RSQGI QVTVS S |
| NC55TNF_NC2 | 15 5 | EVQLVESGGGLVQAG GSLRLSCAASGRTFS | 18 9 | SYAMG | 22 3 | WFRQAPGK EREFVA | 25 7 | AISWSGTITN YADSVKG | 29 1 | RFTISRDNGKNTVHLQMNSL KPEDTAVYHCAV | 32 5 | VQPYSGGDYY TGVEEYDY | 35 9 | WGXG TQVTV SS |
| NC55TNF_NC3 | 15 6 | EVQLVESGGGLVQPG GSLRLSCVVSGFTFS | 19 0 | ATSMT | 22 4 | WVRQAPGK AEEWVS | 25 8 | FINSDGSSTT YADSVKG | 29 2 | RFTISRDNAKNTLYLQMDDL QSEDTAMYYCGR | 32 6 | RGYGRD | 36 0 | RSRGI QVTVS S |
| NC55TNF_NC5 | 15 7 | EVQLVESGGGLVQAG GSLRLSCAASGGAFS | 19 1 | NYDVG | 22 5 | WFRQAPGE GREIVA | 25 9 | RISGSGDST YSSNRAKG | 29 3 | RFTISRDNAKNTVYLQMNSL KREDTAVYYCRA | 32 7 | ARYNGTWSSN DY | 36 1 | WGQG TQVTV SS |

Table I (continued):

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NC55TN F_NC6 | 15 28 | EVQLVESGGGLVQPGGSL RLSCECVSSGCT | 19 2 | FSAYSM T | 22 6 | WVRQAPGKA EEFVS | 26 0 | FINSDGSSTT YANSVNG | 29 4 | RFKISRDNAKKTLYLQMNSL GPEDTAMYYCQR | 32 8 | RGYALD | 36 2 | RGQGTQ VTVSS |
| NC55TN F_NC7 | 15 29 | QVQLVESGGGLVQAGGS LRLSCTASGQTSS | 19 3 | TADMG | 22 7 | WFRQPPGKG REFVA | 26 1 | RISGIDGTTY YDEPVKG | 29 5 | RFTISRDKAQNTVYLQMDSL KPEDTAVYYCRS | 32 9 | PRYADQW SAYDY | 36 3 | WGQGTQ VTVSS |
| NC55TN F_NC8 | 16 00 | EVQLVESGGGLVQPGGSL RLSCVVSGFTFS | 19 4 | TTSMT | 22 8 | WVRQAPGKF EEWVS | 26 2 | FINSDGSSTT YADSVKG | 29 6 | RFTISRDNAKNTLYLQMNSL KPEDTAMYYCGR | 33 0 | RGYGRD | 36 4 | RSKGIQV TVSS |
| NC55TN F_S2C2 | 16 01 | EVQLVESGGGLVQPGGSL RLSCVASASGVK | 19 5 | VNDMG | 22 9 | WYRQAPGKE RELVA | 26 3 | TITDDGRTNY EDFAKG | 29 7 | RFTISRDNAKNTVYLQMNSL LPEDTAVYYCNA | 33 1 | RTYWAHL PTY | 36 5 | WGQGTQ VTVSS |
| NC55TN F_S1C6 | 16 02 | EVQLVESGGGLVQAGGSL RLSCAASGRSFG | 19 6 | SVAMG | 23 0 | WFRQAPGKE REFVA | 26 4 | AIGYDGNSIR YGDSVKG | 29 8 | RFTISRDNIKNTMYLEMENL NADDTARYLCAA | 33 2 | EPLARYE GLWTY | 36 6 | WGQGTQ VTVSS |
| NC55TN F_S3C2 | 16 03 | EVQLVESGGGLVQAGASL RLSCTTSTRTN | 19 7 | DRFNMA | 23 1 | WFHQAPGKD REFVS | 26 5 | RIDVAGYNTA YGDFVKG | 29 9 | RFTVSRDSAENTVVLQMNSL RPEDTGVYYCAA | 33 3 | GGWGISQ SDYDL | 36 7 | WGQGTQ VTVSS |

Notes to Table I:

- ID refers to the SEQ ID NO's in the attached sequence listing

- For CDR1: SEQ ID NO: 164 corresponds to SEQ ID NO: 15, SEQ ID NO: 167 corresponds to SEQ ID NO: 16; SEQ ID NO: 172 corresponds to SEQ ID NO: 17, SEQ ID NOS: 165 and 166 correspond to SEQ ID NO: 18, SEQ ID NO: 170 corresponds to SEQ ID NO: 19, SEQ ID NO: 171 corresponds to SEQ ID NO: 20, and SEQ ID NOS: 168 and 169 correspond to SEQ ID NO: 21.

- For CDR2: SEQ ID NOS: 232 and 233 correspond to SEQ ID NO: 22, SEQ ID NO: 235 corresponds to SEQ ID NO: 23, SEQ ID NO: 240 corresponds to SEQ ID NO: 24, SEQ ID NO: 234 corresponds to SEQ ID NO: 25, SEQ ID NOS: 236 and 237 correspond to SEQ ID NO: 26, SEQ ID NO: 238 corresponds to SEQ ID NO: 27, and SEQ ID NO: 239 corresponds to SEQ ID NO: 28.

- For CDR3: SEQ ID NO: 303 corresponds to SEQ ID NO: 29, SEQ ID NO: 308 corresponds to SEQ ID NO: 30, SEQ ID NO: 306 corresponds to SEQ ID NO: 31, SEQ ID NO: 307 corresponds to SEQ ID NO: 32, SEQ ID NOS: 304 and 305 corresponds to SEQ ID NO: 33, SEQ ID NO: 300 corresponds to SEQ ID NO: 34, and SEQ ID NOS: 301 and 302 correspond to SEQ ID NO: 35.

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 I; or from the group 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" (as defined herein) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table 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 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 sequences (i.e. as defined herein), respectively.

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 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 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 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 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 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 CDR1, CDR2 and CDR3 sequences, respectively, listed in Table 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 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 I, respectively; and at least one of the CDR1 and CDR2 sequences present is suitably chosen from the group consisting of the CDR1 and CDR2 sequences, respectively, listed in Table I or from the group 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 at least one of the CDR1 and CDR2 sequences, respectively, listed in Table 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 I.

Most 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 I or from the group 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 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 CDR1, CDR2 and CDR3 sequences, respectively, listed in Table I.

Even more preferably, 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 I. Preferably, in this embodiment, at least one or preferably both of the other two CDR sequences present are suitably chosen from CDR sequences that 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 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 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 I. Preferably, in this embodiment, 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 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 listed in Table 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 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 I. Preferably, in this embodiment, the remaining CDR sequence present are suitably chosen from the group of CDR sequences that 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 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 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 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 I. Preferably, in this embodiment, the remaining CDR sequence present are suitably chosen from the group of CDR sequences that 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 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 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 I.

Also, generally, the combinations of CDR's listed in Table I (i.e. those mentioned on the same line in Table 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 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 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 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 I (i.e. mentioned on the same line in Table 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 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 I, a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table 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 I; a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table 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 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 I; a CDR2 sequence, and one of the CDR3 sequences listed in Table I; or (3) a CDR1 sequence; a CDR2 sequence that has more than 80% sequence identity with one of the CDR2 sequence listed in Table I; and a CDR3 sequence that has 3, 2 or 1 amino acid differences with the CDR3 sequence mentioned in Table 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 I; a CDR2 sequence that has 3, 2 or 1 amino acid difference with the CDR2 sequence mentioned in Table 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 I that belongs to the same combination; (2) a CDR1

sequence; a CDR 2 listed in Table I and a CDR3 sequence listed in Table 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 I; the CDR2 sequence listed in Table I that belongs to the same combination; and a CDR3 sequence mentioned in Table I that belongs to a different combination; or (2) a CDR1 sequence mentioned in Table I; a CDR2 sequence that has 3, 2 or 1 amino acid differences with the CDR2 sequence mentioned in Table I that belongs to the same combination; and more than 80% sequence identity with the CDR3 sequence listed in Table I that belongs to same different combination.

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

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

Preferably, when a CDR sequence 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 (as defined herein) with one of the CDR sequences listed in Table I; and/or when a CDR sequence is suitably chosen from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with one of the CDR sequences listed in Table I:

i) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

ii) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the CDR sequence listed in Table I.

According to a non-limiting but preferred embodiment of the invention, the CDR sequences in the the Nanobodies of the invention are as defined above and are also such that the Nanobody of the invention binds to TNF-alpha with an dissociation constant ($K_D$) of $10^{-5}$ to $10^{-12}$ moles/liter (M) or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter (M) or less and more preferably $10^{-8}$ to $10^{-12}$ moles/liter (M), and/or with an association constant ($K_A$)

of at least $10^7$ $M^{-1}$, preferably at least $10^8$ $M^{-1}$, more preferably at least $10^9$ $M^{-1}$, such as at least $10^{12}$ $M^{-1}$;and in particular with a $K_D$ less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. The $K_D$ and $K_A$ values of the Nanobody of the invention against TNF-alpha can be determined in a manner known per se, for example using the assay described herein.

According to another preferred, but non-limiting embodiment 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 one aspect, the invention provides Nanobodies against TNF-alpha that are better performing than Nanobody 3E, the best performing Nanobody according to WO 04/041862.

More specifically, some preferred aspects of this embodiment of the invention are:

XXI) A Nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), which has a Koff rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s); or a humanized variant of such a Nanobody.

XXII) A Nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody.

XXIII) A Nanobody against TNF-alpha, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 12nM; or a humanized variant of such a Nanobody.

XXIV) A Nanobody against TNF-alpha, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody.

XXV) A Nanobody against TNF-alpha, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody;

with some particularly preferred aspects being:

- A Nanobody in accordance with any one of XXI) to XXV), which is a GLEW-class Nanobody.
- A Nanobody in accordance with any one of XXI) to XXV), which contains an arginine residue (R) at position 103.
- A Nanobody in accordance with any one of XXI) to XXV), which is a humanized Nanobody.
- A Nanobody in accordance with any one of XXI) to XXV), which contains a leucine residue (L) at position 108.
- A Nanobody in accordance with any one of XXI) to XXV), which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 52 (TNF1), 76 (TNF13), 77 (TNF14), 95 (TNF29) or 96 (TNF30).
- A Nanobody in accordance with any one of XXI) to XXV), in which
  a) CDR1 comprises:
     - the amino acid sequence DYWMY; or
     - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
     - an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence DYWMY;

  and

  b) CDR2 comprises:
     - the amino acid sequence EINTNGLITKYPDSVKG; or
     - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
     - an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 comprises the amino acid sequence DYWMY.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR3 comprises the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of XXI) to XXV), in which:
  - CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN; or
  - CDR1 comprises the amino acid sequence DYWMY; and CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; or
  - CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 comprises the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 comprises the amino acid sequence DYWMY; CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG and CDR3 comprises the amino acid sequence SPSGFN.
- A Nanobody in accordance with any one of XXI) to XXV), in which

a) CDR1 is:

- the amino acid sequence DYWMY; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence DYWMY;

and in which:

b) CDR2 is:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and in which

c) CDR3 is:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 is the amino acid sequence DYWMY.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR2 is the amino acid sequence EINTNGLITKYPDSVKG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR3 is the amino acid sequence SPSGFN
- A Nanobody in accordance with any one of XXI) to XXV), in which:
  - CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN; or
  - CDR1 is the amino acid sequence DYWMY; and CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; or

- CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 is the amino acid sequence SPSGFN

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 is the amino acid sequence DYWMY; CDR2 is the amino acid sequence EINTNGLITKYPDSVKG and CDR3 is the amino acid sequence SPSGFN.

- A Nanobody in accordance with any one of XXI) to XXV), in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

and with some other particularly preferred aspects being:

- A Nanobody in accordance with any one of XXI) to XXV), which is a KERE-class Nanobody.

- A Nanobody in accordance with any one of XXI) to XXV), which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 50 (TNF3), 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 98 (TNF33).

- A Nanobody in accordance with any one of XXI) to XXV), in which

  a) CDR1 comprises:
     - the amino acid sequence NYYMG; or
     - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
     - an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

  and

  b) CDR2 comprises:
     - the amino acid sequence NISWRGYNIYYKDSVKG; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 comprises the amino acid sequence NYYMG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with any one of XXI) to XXV), in which:
  - CDR1 comprises the amino acid sequence NYYMG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 comprises the amino acid sequence NYYMG; and CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 comprises the amino acid sequence NYYMG; CDR2 comprises the amino acid sequence SILPLSDDPGWNTY and CDR3 comprises the amino acid sequence ILPLSDDPGWNTY.
- A Nanobody in accordance with any one of XXI) to XXV), in which

a) CDR1 is:

- the amino acid sequence NYYMG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

and

b) CDR2 is:

- the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 is:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 is the amino acid sequence NYYMG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG.
- A Nanobody in accordance with any one of XXI) to XXV), in which CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with any one of XXI) to XXV), in which:

- CDR1 is the amino acid sequence NYYMG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY; or
- CDR1 is the amino acid sequence NYYMG; and CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; or
- CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with any one of XXI) to XXV), in which CDR1 is the amino acid sequence NYYMG; CDR2 is the amino acid sequence SILPLSDDPGWNTY and CDR3 is the amino acid sequence ILPLSDDPGWNTY.
- A Nanobody in accordance with any one of XXI) to XXV), in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A Nanobody in accordance with any one of XXI) to XXV), which is a humanized Nanobody.

and with yet some other particularly preferred aspects being:

XXVI) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody in accordance with any one of XXI) to XXV).

XXVII) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of XXI) to XXV).

XXVIII) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XXIX) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

XXX) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of XXI) to XXV), linked via a suitable linker.

XXXI) A protein or polypeptide, which comprises two Nanobodies in accordance with any one of XXI) to XXV), linked via a suitable linker, and which is pegylated.

XXXII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises at least one Nanobody directed against human serum albumin.

XXXIII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises at least one Nanobody directed against human serum albumin, and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XXXIV) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises at least one Nanobody directed against human serum albumin and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

XXXV) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of XXI) to XXV) is linked, optionally via a suitable linker, to the one Nanobody directed against human serum albumin.

XXXVI) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of XXI) to XXV) is linked, optionally via a suitable linker, to the one Nanobody directed against human serum albumin, and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XXXVII) A protein or polypeptide which comprises two Nanobodies in accordance with any one of XXI) to XXV), and which further comprises one Nanobody directed against human serum albumin, in which each of the two Nanobodies in accordance with any one of XXI) to XXV) is linked, optionally via a suitable linker, to the one

Nanobody directed against human serum albumin, and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of XXVI) to XXXVII), in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of XXVI) to XXXVII), in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of XXVI) to XXXVII), in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of XXVI) to XXXVII), in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of XXVI) to XXXVII), which comprises or essentially consists of two humanized Nanobodies Nanobodies in accordance with any one of XXI) to XXV), and one humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).

It should be noted that when a Nanobody is mentioned above as being "*in accordance with any one of XXI) to XXV) above*", it is at least according to one of XXI) to XXV), may be according to two or more of XXI) to XXV), and may also include any one or more of the other aspects that are indicated as being "in accordance with any one of XXI) to XXV) above. Similarly, when a protein or polypeptide is mentioned above as being "*in accordance with any one of XXVI) to XXXVII) above*", it is at least according to one of XXVI) to XXXVII), may be according to two or more of XXVI) to XXXVII), and may also include any one or more of the other aspects that are indicated as being "in accordance with any one of XXVI) to XXXVII) above.

A clone that has been found to be particularly useful as an anti-TNF Nanobody is the clone PMP1C2 (TNF1). As can be seen from the comparative data from the KYM-assay in Table 39, TNF1 has an $EC_{50}$ value that is more then 4 times better than the best monovalent Nanobody described in WO 04/41862 (Nanobody 3E), i.e. 2,466 nM for

PMP1C2 vs. 12 nM for 3E (As can be seen from Table 39, all Nanobodies TNF1 to TNF 9 of the invention gave a better EC50 value in this assay than 3E). In this respect, it should also be noted that Nanobody 3E from WO 04/41862 belongs to the "KERE class" (as described herein), and can therefore be humanized to a lesser degree than Nanobody PMP1C2 (which belongs to the "GLEW class"). When Nanobody PMP1C2 is compared to the Nanobody 1A from WO 04/41862, a GLEW-class Nanobody with the highest degree in sequence homology with PMP1C2 (in both the CDR's and the frameworks), the $EC_{50}$ value obtained for PMP1C2 in the KYM assay is more than 50 times better, i.e. 2.466 nM for PMP1C2 compared to 100 nM for 1A.

Accordingly, Nanobodies that comprise one or more, preferably any two and more preferably all three of the CDR's present in the clone PMP1C2 (or CDR sequences that are derived therefrom or correspond thereto) are particularly preferred in the invention. Also, these Nanobodies preferably belong to the "103 P,R,S group" (as defined herein), and most preferably have an R at position 103, and preferably also have GLEW or a GLEW-like sequence at positions 44-47. Also, when these Nanobodies belong to the "103 P, R, S group" (and in particular when they have an R at position 103), one preferred, but non-limiting humanizing substitution is 108Q to 108L. Other preferred, but non-limiting humanizing substitutions in these preferred Nanobodies are one or more of those present in the humanized variants of TNF1 described herein, such as TNF13, TNF14, TNF 29 or TNF30, as will immediately be clear from a comparison between the sequence of TNF1 and these humanized sequences.

Thus, in a particularly preferred Nanobody of the invention, at least one of the CDR1, CDR2 and CDR3 sequences present is suitably chosen from the group consisting of the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1), or from the group 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" (as defined herein) with the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively; 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 the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively.

Preferably, in these preferred 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 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1), 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 the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively; 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 the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively.

Most preferably, in these preferred Nanobodies of the invention, all three CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1), or from the group 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 the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively; 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 the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively.

Even more preferably, in these preferred 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 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1). Preferably, in this embodiment, at least one or preferably both of the other two CDR sequences present are suitably suitably chosen from CDR sequences that 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 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of

SEQ ID NO: 300, respectively; and/or suitably chosen from the group consisting of the CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively.

Even more preferably, in these preferred 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 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1). Preferably, in this embodiment, the remaining CDR sequence present are suitably chosen from the group of CDR sequences that 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 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively.

Particularly preferred Nanobodies of the invention comprise the CDR1 sequence of SEQ ID NO: 164, the CDR2 sequence of SEQ ID NO: 232, and the CDR3 sequence of SEQ ID NO: 300, respectively (i.e. the CDR sequences present in clone TNF1).

Nanobodies with the above CDR sequences preferably have framework sequences that are as further defined herein. Some particularly preferred, but non-limiting combinations of framework sequences can be seen in the above Table I. As will be clear to the skilled person, a combination of FR1, FR2, FR3 and FR4 sequences that occur in the same clone (i.e. FR1, FR2, FR3 and FR4 sequences which are mentioned on the same line in Table I) will usually be preferred (although the invention in its broadest sense is not limited thereto, and also comprises other suitable combinations of the framework sequences mentioned in Table I).

More specifically, some preferred aspects of this embodiment of the invention are:

XXXVIII) A nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

a) CDR1 comprises:

- the amino acid sequence DYWMY; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence DYWMY;

and

b) CDR2 comprises:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence SPSGFN.

- A nanobody in accordance with XXXVIII) , in which CDR1 comprises the amino acid sequence DYWMY.
- A nanobody in accordance with XXXVIII) , in which CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG.
- A nanobody in accordance with XXXVIII) , in which CDR3 comprises the amino acid sequence SPSGFN.
- A nanobody in accordance with XXXVIII) , in which:
  - CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN; or
  - CDR1 comprises the amino acid sequence DYWMY; and CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; or

- CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 comprises the amino acid sequence SPSGFN

- A nanobody in accordance with XXXVIII) , in which CDR1 comprises the amino acid sequence DYWMY; and CDR3 comprises the amino acid sequence SPSGFN.
- A nanobody in accordance with XXXVIII) in which CDR1 comprises the amino acid sequence DYWMY; CDR2 comprises the amino acid sequence EINTNGLITKYPDSVKG and CDR3 comprises the amino acid sequence SPSGFN.
- A Nanobody in accordance with XXXVIII) , in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A Nanobody in accordance with XXXVIII) , which is a GLEW-class Nanobody.
- A Nanobody in accordance with XXXVIII) , which contains an arginine residue (R) at position 103.
- A Nanobody in accordance with XXXVIII) , which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 52 (TNF1), 76 (TNF13), 77 (TNF14), 95 (TNF29) or 96 (TNF30).
- A Nanobody in accordance with XXXVIII) , which is a humanized Nanobody.
- A Nanobody in accordance with XXXVIII) , which contains a leucine residue (L) at position 108.
- A Nanobody in accordance with XXXVIII) , which has a $K_{off}$ rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s); or a humanized variant of such a Nanobody;
- A Nanobody in accordance with XXXVIII) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XXXVIII) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody.

- A Nanobody in accordance with XXXVIII) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody.

XXXIX) A Nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which

a) CDR1 is:

- the amino acid sequence DYWMY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence DYWMY; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence DYWMY;

and in which:

b) CDR2 is:

- the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence EINTNGLITKYPDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence EINTNGLITKYPDSVKG;

and in which

c) CDR3 is:

- the amino acid sequence SPSGFN; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SPSGFN; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence SPSGFN.

- A Nanobody in accordance with XXXIX) , in which CDR1 is the amino acid sequence DYWMY.

- A Nanobody in accordance with XXXIX) , in which CDR2 is the amino acid sequence EINTNGLITKYPDSVKG.
- A Nanobody in accordance with XXXIX) , in which CDR3 is the amino acid sequence SPSGFN
- A Nanobody in accordance with XXXIX) , in which:
  - CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN; or
  - CDR1 is the amino acid sequence DYWMY; and CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; or
  - CDR2 is the amino acid sequence EINTNGLITKYPDSVKG; and CDR3 is the amino acid sequence SPSGFN
- A Nanobody in accordance with XXXIX) , in which CDR1 is the amino acid sequence DYWMY; and CDR3 is the amino acid sequence SPSGFN.
- A Nanobody in accordance with XXXIX) , in which CDR1 is the amino acid sequence DYWMY; CDR2 is the amino acid sequence EINTNGLITKYPDSVKG and CDR3 is the amino acid sequence SPSGFN.
- A Nanobody in accordance with XXXIX) , in which:
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A Nanobody in accordance with XXXIX) , which is a GLEW-class Nanobody.
- A Nanobody in accordance with XXXIX) , which contains an arginine residue (R) at position 103.
- A Nanobody in accordance with XXXIX) , which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 52 (TNF1), 76 (TNF13), 77 (TNF14), 95 (TNF29) or 96 (TNF30).
- A Nanobody in accordance with XXXIX) , which is a humanized Nanobody.
- A Nanobody in accordance with XXXIX) , which contains a leucine residue (L) at position 108.

- A Nanobody in accordance with XXXIX) , which has a $K_{off}$ rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s); or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XXXIX) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XXXIX) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XXXIX) , which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XXXIX) , which is chosen from the group consisting of TNF 13 (SEQ ID NO: 76), TNF 14 (SEQ ID NO: 77), TNF 29 (SEQ ID NO: 95) and TNF 30 (SEQ ID NO:96).
- A Nanobody in accordance with XXXIX) , which is TNF 30 (SEQ ID NO: 96);

with some other preferred aspects being:

XL) A protein or polypeptide, which comprises or essentially consists of a Nanobody in accordance with XXXVIII) or XXXIX).

XLI) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody in accordance with XXXVIII) or XXXIX) .

- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises two Nanobodies in accordance with XXXVIII) or XXXIX) .
- A protein or polypeptide in accordance with any one of XL) or XLI),, which comprises two Nanobodies in accordance with XXXVIII) or XXXIX) , and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..
- A protein or polypeptide in accordance with any one of XL) or XLI),, which comprises two Nanobodies in accordance with XXXVIII) or XXXIX) , and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises two Nanobodies in accordance with XXXVIII) or XXXIX) , which are directly linked to each other or linked to each other via a linker.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises two Nanobodies in accordance with XXXVIII) or XXXIX), which are linked to each other via an amino acid sequence.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises two Nanobodies in accordance with XXXVIII) or XXXIX), which are linked to each other via an amino acid sequence (such as, without limitation, an amino acid sequence that comprises glycine and serine residues) that comprises at least 14 amino acids, more preferably at least 17 amino acids, such as about 20-40 amino acids (such as the linker GS30).
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists of the polypeptide TNF 7 (SEQ ID NO: 73), in which both Nanobodies TNF 1 have been humanized.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists the polypeptide TNF 55 (SEQ ID NO: 419) or TNF 56 (SEQ ID NO: 420).
- A protein or polypeptide in accordance with any one of XL) or XLI), which is pegylated.
- A protein or polypeptide in accordance with any one of XL) or XLI),which comprises two Nanobodies in accordance with XXXVIII) or XXXIX) , and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking; and/or which is such that said protein or polypeptide is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer, and which protein or polypeptide further comprises at least one Nanobody directed against human serum albumin.
- A protein or polypeptide in accordance with any one of XL) or XLI),, which comprises two Nanobodies in accordance with XXXVIII) or XXXIX), and which protein or polypeptide further comprises at least one Nanobody directed against human serum albumin, in which the two Nanobodies in accordance with XXXVIII)

or XXXIX) are linked to each other via the at least one Nanobody directed against human serum albumin, and in which the two Nanobodies in accordance with XXXVIII) or XXXIX) are either linked directly to the at least one Nanobody directed against human serum albumin, or are linked to the at least one Nanobody directed against human serum albumin via a linker.

- A protein or polypeptide in accordance with any one of XL) or XLI),, which comprises two Nanobodies in accordance with XXXVIII) or XXXIX), and which protein or polypeptide further comprises at least one Nanobody directed against human serum albumin, in which the two Nanobodies in accordance with XXXVIII) or XXXIX) are linked to each other via the at least one Nanobody directed against human serum albumin, and in which the two Nanobodies in accordance with XXXVIII) or XXXIX) are either linked directly to the at least one Nanobody directed against human serum albumin, or are linked to the at least one Nanobody directed against human serum albumin via a linker, in which the linker is an amino acid sequence (such as, without limitation, a linker that comprises glycine and serine residues), and in particular an amino acid sequence that comprises between 3 and 40 amino acid residues, such as between 5 and 15 amino acid residues (such as the linker GS9).
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises two Nanobodies in accordance with XXXVIII) or XXXIX), and which protein or polypeptide further comprises at least one Nanobody directed against human serum albumin, in which the two Nanobodies in accordance with XXXVIII) or XXXIX) are linked to each other via the at least one Nanobody directed against human serum albumin, and in which the two Nanobodies in accordance with XXXVIII) or XXXIX) are either linked directly to the at least one Nanobody directed against human serum albumin, or are linked to the at least one Nanobody directed against human serum albumin via a linker, and which protein or polypeptide is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking; and/or which protein or polypeptide is such that said protein or polypeptide is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of XL) or XLI), in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of XL) or XLI), in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of XL) or XLI), in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of XL) or XLI), in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists of two humanized Nanobodies in accordance with any one of XL) or XLI) and one humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists of the polypeptide TNF24 (SEQ ID NO: 90), in which both the Nanobody TNF 1 as well as the Nanobody ALB 1 has been humanized.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists of two Nanobodies TNF 30 and one Nanobody ALB 8.
- A protein or polypeptide in accordance with any one of XL) or XLI), which comprises or essentially consists the polypeptide TNF 60 (SEQ ID NO: 417).

It should be noted that when a Nanobody is mentioned above as being "*in accordance with XXXVIII" or "in accordance with XXXIX*", it is at least according to one of XXXVIII) and/or XXXIX), and may also include any one or more of the other aspects that are indicated as being "in accordance with XXXVIII" or "in accordance with XXXIX" above. Similarly, when a protein or polypeptide is mentioned above as being "*in accordance with any one of XL) or XLI)*", it is at least according to one of XL) to XLI), may be according to two or more of VI) to XVIII), and may also include any one or more

of the other aspects that are indicated as being "in accordance with any one of XL) or XLI) above.

For the Nanobodies based on Nanobody TNF1 above (including but not limited to the humanized Nanobodies), the framework sequences may generally be as described herein, and preferably are as follows:

a) FR1 comprises or is:

- the amino acid sequence of SEQ ID NO: 130;or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 130; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 130;

and

b) FR2 comprises or is:

- the amino acid sequence of SEQ ID NO: 198; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 198; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence of SEQ ID NO: 198;

and

c) FR3 comprises or is:

- the amino acid sequence of SEQ ID NO: 266; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 266; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 266.

and

d) FR4 comprises or is:

- the amino acid sequence of SEQ ID NO: 334; or

- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 334; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 334;

in which the amino acid differences present in the framework sequences are more preferably as described herein.

Nanobodies against TNF-alpha, which have framework regions as described above (i.e. similar to TNF1), and in which at least one of the framework regions (such as any two, any three or all four framework regions) have been humanized, form a further aspect of the invention. Such Nanobodies may in particular have CDR's that are such that the Nanobody has a Koff rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s); and/or have CDR's that are such that the Nanobody has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; and in particular better than 12nM, more in particular better than 5 nM, even more in particular better than 3nM. Also, or alternatively, such Nanobodies are preferably directed against the same epitope of TNF (i.e. the TNF trimer) as TNF1.

In particular, the invention relates to a Nanoobody against TNF-alpha, which is a humanized variant of a Nanobody against TNF-alpha, which Nanobody against TNF-alpha has the following framework sequences: FR1: SEQ ID NO: 130; FR2: SEQ ID NO: 198; FR3: SEQ ID NO: 266; and FR4: SEQ ID NO: 334. Such a Nanobody may in particular have CDR's that are such that the Nanobody has a Koff rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s); and/or have CDR's that are such that the Nanobody has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; and in particular better than 12nM, more in particular better than 5 nM, even more in particular better than 3nM. Also, or alternatively, such Nanobodies are preferably directed against the same epitope of TNF (i.e. the TNF trimer) as TNF1.

Another clone that has been found to be particularly useful as an anti-TNF Nanobody is the clone PMP5F10 (TNF3, SEQ ID NO: 60). As can be seen from the

comparative data from the KYM-assay in Table 39, TNF3 has an $EC_{50}$ value that is more than 15 times better than the best monovalent Nanobody described in WO 04/41862.

Accordingly, Nanobodies that comprise one or more, preferably any two and more preferably all three of the CDR's present in the clone PMP5F10 (or CDR sequences that are derived therefrom or correspond thereto) are particularly preferred in the invention. Also, these Nanobodies preferably belong to the KERE class.

More specifically, some preferred aspects of this embodiment of the invention are:

XLII) A Nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which

a) CDR1 comprises:

- the amino acid sequence NYYMG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

and

b) CDR2 comprises:

- the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 comprises:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or

- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with XLII), in which CDR1 comprises the amino acid sequence NYYMG.
- A Nanobody in accordance with XLII), in which CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG.
- A Nanobody in accordance with XLII), in which CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with XLII), in which:
  - CDR1 comprises the amino acid sequence NYYMG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 comprises the amino acid sequence NYYMG; and CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 comprises the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 comprises the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with XLII), in which CDR1 comprises the amino acid sequence NYYMG; CDR2 comprises the amino acid sequence SILPLSDDPGWNTY and CDR3 comprises the amino acid sequence ILPLSDDPGWNTY.
- A Nanobody in accordance with XLII), in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A Nanobody in accordance with XLII), which is a KERE-class Nanobody.
- A Nanobody in accordance with XLII), which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 50 (TNF3), 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 99 (TNF33).
- A Nanobody in accordance with XLII), which is a humanized Nanobody.

- A Nanobody in accordance with XLII), which has a $K_{off}$ rate for TNF of better than $2.10^{-3}$ (1/s), preferably better than $1.10^{-3}$ (1/s) ; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody.

XLIII) A Nanobody against TNF-alpha, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which

a) CDR1 is:
   - the amino acid sequence NYYMG; or
   - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NYYMG; or
   - an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence NYYMG;

and

b) CDR2 is:
   - the amino acid sequence NISWRGYNIYYKDSVKG; or
   - an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence NISWRGYNIYYKDSVKG; or
   - an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence NISWRGYNIYYKDSVKG;

and

c) CDR3 is:

- the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence SILPLSDDPGWNTY; or
- an amino acid sequences that has 2 or only 1 amino acid difference with the amino acid sequence SILPLSDDPGWNTY.

- A Nanobody in accordance with XLIII), in which CDR1 is the amino acid sequence NYYMG.
- A Nanobody in accordance with XLIII), in which CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG.
- A Nanobody in accordance with XLIII), in which CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with XLIII), in which:
  - CDR1 is the amino acid sequence NYYMG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY; or
  - CDR1 is the amino acid sequence NYYMG; and CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; or
  - CDR2 is the amino acid sequence NISWRGYNIYYKDSVKG; and CDR3 is the amino acid sequence SILPLSDDPGWNTY.
- A Nanobody in accordance with XLIII), in which CDR1 is the amino acid sequence NYYMG; CDR2 is the amino acid sequence SILPLSDDPGWNTY and CDR3 is the amino acid sequence ILPLSDDPGWNTY.
- A Nanobody in accordance with XLIII), in which
  - any amino acid substitution is preferably a conservative amino acid substitution; and/or
  - said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).
- A Nanobody in accordance with XLIII), which is a KERE-class Nanobody.
- A Nanobody in accordance with XLIII), which has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence

identity (as defined herein) with one of the amino acid sequences of SEQ ID NO's 50 (TNF3), 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 99 (TNF33).

- A Nanobody in accordance with XLIII), which is a humanized Nanobody.
- A Nanobody in accordance with XLIII), which has a $K_{off}$ rate for TNF of better than $2.10^{-3}$ (1/s), preferably better than $2.10^{-3}$ (1/s) ; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLIII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLIII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLIII), which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM; or a humanized variant of such a Nanobody.
- A Nanobody in accordance with XLIII), which is chosen from the group consisting of SEQ ID NO's, 83 (TNF20), 85 (TNF21), 85 (TNF22), 96 (TNF23) or 98 (TNF33)TNF 13 (SEQ ID NO: 76), TNF 14 (SEQ ID NO: 77), TNF 29 (SEQ ID NO: 95) and TNF 30 (SEQ ID NO:96)

with some other preferred aspects being:

XLIV) A protein or polypeptide, which comprises or essentially consists of a Nanobody in accordance with XLII) or XLIII).

XLV) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody in accordance with XLII) or XLIII).

XLVI) A protein or polypeptide, which comprises two Nanobodies in accordance with XLII) or XLIII).

XLVII) A protein or polypeptide, which comprises two Nanobodies in accordance with XLII) or XLIII), and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

XLVIII) A protein or polypeptide, which comprises two Nanobodies in accordance with XLII) or XLIII), and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

- A protein or polypeptide in accordance with any one of XLIV) or XLVIII),which comprises or essentially consists of the polypeptide TNF 6 (SEQ ID NO: 72) or TNF 9 (SEQ ID NO: 75, in which both Nanobodies TNF 3 have been humanized
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), which is pegylated.
- A protein or polypeptide, which comprises two Nanobodies in accordance with XLII) or XLIII), and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking; and/or which is such that said protein or polypeptide is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer, and which protein or polypeptide further comprises at least one Nanobody directed against human serum albumin.
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), which comprises or essentially consists of two humanized Nanobodies in accordance with any one of XLIV) or XLVIII) and one humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).

- A protein or polypeptide in accordance with any one of XLIV) or XLVIII), which comprises or essentially connsists of the polypeptide TNF26 (SEQ ID NO: 92), in which both the Nanobodies TNF 3 as well as the Nanobody ALB 1 has been humanized.

It should be noted that when a Nanobody is mentioned above as being "*in accordance with XLII" or "in accordance with XLIII"*, it is at least according to one of XLII) and/or XLIII), and may also include any one or more of the other aspects that are indicated as being "in accordance with XLII)" or "in accordance with XLIII)" above. Similarly, when a protein or polypeptide is mentioned above as being "*in accordance with any one of XLIV) or XLVIII)*", it is at least according to one of XL) to XLI), may be according to two or more of XLIV) to XLVIII), and may also include any one or more of the other aspects that are indicated as being "in accordance with any one of XLIV) or XLVIII) above.

For the Nanobodies based on Nanobody TNF3 above (including but not limited to the humanized Nanobodies), the framework sequences may generally be as described herein, and preferably are as follows:

a) FR1 comprises or is:

- the amino acid sequence of SEQ ID NO: 138;or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 138; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 138;

and

b) FR2 comprises or is:

- the amino acid sequence of SEQ ID NO: 206; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 206; or
- an amino acid sequences that has 2 or only 1 amino acid difference(s) with the amino acid sequence of SEQ ID NO: 206;

and

c) FR3 comprises or is:

- the amino acid sequence of SEQ ID NO: 274; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 274; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 274.

and

d) FR4 comprises or is:

- the amino acid sequence of SEQ ID NO: 342; or
- an amino acid sequence that has at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 342; or
- an amino acid sequences that has only 1 amino acid difference with the amino acid sequence of SEQ ID NO: 342;

in which the amino acid differences present in the framework sequences are more preferably as described herein.

In another aspect, the invention relates to a Nanobody with an amino acid sequence that is chosen from the group consisting of SEQ ID NO's: 52 to 60, from the group consisting of SEQ ID NO's: 76 to 86, from the group consisting of SEQ ID NO's: 95 to 99, from the group consisting of SEQ ID NO's 105 to 129 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 one or more of the amino acid sequences of SEQ ID NO's: 52 to 60, SEQ ID NO's: 76 to 86, SEQ ID NO's: 95 to 99 or SEQ ID NO's 105 to 129, in which the latter amino acid sequences most preferably have framework sequences that are as further defined below under the general description of the framework sequences of Nanobodies.

According to a specific, but non-limiting embodiment, the latter amino acid sequences are "humanized", as further described herein.

Most preferably, the Nanobodies of the invention are chosen from the group consisting of SEQ ID NO's: 52 to 60, from the group consisting of SEQ ID NO's: 76 to 86, from the group consisting of SEQ ID NO's: 95 to 99, or from the group consisting of SEQ ID NO's 105 to 129, of which the "humanized" Nanobodies of SEQ ID NO's 76 to 86 and SEQ ID NO's: 95 to 99 may be particularly preferred.

As mentioned above, a particularly preferred Nanobody of the invention is the clone PMP1C2 (TNF1; SEQ ID NO: 52). Thus, in a preferred aspect, the invention relates to a Nanobody with an amino acid sequence that is chosen from the group consisting of SEQ ID NO: 52 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 the amino acid sequence of SEQ ID NO:52, in which the latter amino acid sequences most preferably have framework sequences that are as further defined below under the general description of the framework sequences of Nanobodies.

Particularly preferred are humanized variants of the clone PMP1C2 (TNF1; SEQ ID NO: 52). Some preferred, but non-limiting examples of such humanized variants are the clones TNF13 (SEQ ID NO: 76 ), TNF14 (SEQ ID NO:77), TNF29 (SEQ ID NO: 95) and TNF 30 (SEQ ID NO: 96). Thus, in a preferred aspect, the invention relates to a humanized Nanobody with an amino acid sequence that is chosen from the group consisting of SEQ ID NO's: 76, 77, 95 or 96, 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 one of the amino acid sequences of SEQ ID NO's: 76, 77, 95 or 96, in which the latter amino acid sequences most preferably have framework sequences that are as further defined below under the general description of the framework sequences of Nanobodies.

According to one preferred embodiment, the Nanobody of the invention is a humanized variant of the Nanobody TNF 1 (SEQ ID NO: 52).

Some preferred aspects of this embodiment of the invention are:

XLIX) A humanized variant of the Nanobody TNF 1, which has a Koff rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s).

L) A humanized variant of the Nanobody TNF 1, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay.

LI) A humanized variant of the Nanobody TNF 1, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM.

LII) A humanized variant of the Nanobody TNF 1, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM.

LIII) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody which is a humanized variant of the Nanobody TNF 1 in accordance with any one of XLIX) to LII)

LIV) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 1 in accordance with any one of XLIX) to LII) (optionally linked via a linker).

LV) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 1 in accordance with any one of XLIX) to LII) (optionally linked via a linker), and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

LVI) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 1 in accordance with any one of XLIX) to LII) and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

LVII) A protein or polypeptide which comprises or essentially consists of the polypeptide TNF 7 (SEQ ID NO: 73), in which both Nanobodies TNF 1 have been humanized.

LVIII) A protein or polypeptide which comprises or essentially consists of the polypeptide TNF 7 (SEQ ID NO: 73), in which both Nanobodies TNF 1 have been humanized, and which is pegylated.

LIX) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 1 in accordance with any one of XLIX) to LII), and which further comprises at least one Nanobody directed against human serum albumin.

LX) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 1 in accordance with any one of XLIX) to LII), which are each linked (optionally linked via a linker) to one Nanobody directed against human serum albumin.

- A protein or polypeptide in accordance with any one of LIII) to LX), in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of LIII) to LX), in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of LIII) to LX), in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of LIII) to LX), in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of LIII) to LX), which comprises or essentially consists of the polypeptide TNF24 (SEQ ID NO: 90), in which both the Nanobody TNF 1 as well as the Nanobody ALB TNF 1 has been humanized.
- A protein or polypeptide in accordance with any one of LIII) to LX), which comprises or essentially consists of two Nanobodies TNF 30 and one Nanobody ALB 8.

According to one preferred embodiment, the Nanobody of the invention is a humanized variant of the Nanobody TNF 3 (SEQ ID NO: 60).

Some preferred aspects of this embodiment of the invention are:

LXI) A humanized variant of the Nanobody TNF 3, which has a Koff rate for TNF of better than 2.10-3 (1/s), preferably better than 1.10-3 (1/s).

LXII) A humanized variant of the Nanobody TNF 3, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Nanobody VHH 3E (SEQ ID NO:4) of WO 04/041862 in the same assay.

LXIII) A humanized variant of the Nanobody TNF 3, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 5nM.

LXIV) A humanized variant of the Nanobody TNF 3, which has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 3nM.

LXV) A protein or polypeptide, which comprises or essentially consists of at least one Nanobody which is a humanized variant of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV)

LXVI) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV) (optionally linked via a linker).

LXVII) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV) (optionally linked via a linker), and which is such that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking..

LXVIII) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV) and which is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer.

LXIX) A protein or polypeptide which comprises or essentially consists of the polypeptide TNF 6 (SEQ ID NO: 72) or TNF 9 (SEQ ID NO: 75), in which both Nanobodies TNF 3 have been humanized.

LXX) A protein or polypeptide which comprises or essentially consists of the polypeptide TNF 6 (SEQ ID NO: 72) or TNF 9 (SEQ ID NO: 75), in which both Nanobodies TNF 3 have been humanized, and which is pegylated.

LXXI) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV), and which further comprises at least one Nanobody directed against human serum albumin.

LXXII) A protein or polypeptide, which comprises or essentially consists of two Nanobodies which are humanized variants of the Nanobody TNF 3 in accordance with any one of LXI) to LXIV), which are each linked (optionally linked via a linker) to one Nanobody directed against human serum albumin.

- A protein or polypeptide in accordance with any one of LXV) to LXXII), in which the at least one Nanobody directed against human serum albumin is a humanized Nanobody.
- A protein or polypeptide in accordance with any one of LXV) to LXXII), in which the at least one Nanobody directed against human serum albumin is a humanized variant of the Nanobody ALB 1 (SEQ ID NO: 63).
- A protein or polypeptide in accordance with any one of LXV) to LXXII), in which the at least one Nanobody directed against human serum albumin is a chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQ ID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102) ALB 9 (SEQ ID NO: 103) and ALB 10 (SEQ ID NO: 104).
- A protein or polypeptide in accordance with any one of LXV) to LXXII), in which the at least one Nanobody directed against human serum albumin is ALB 8.
- A protein or polypeptide in accordance with any one of LXV) to LXXII), which comprises or essentially consists of the polypeptide TNF26 (SEQ ID NO: 92), in which both the Nanobodies TNF 3 as well as the Nanobody ALB 1 has been humanized.

In another aspect, the invention relates to a polypeptide that comprises or essentially consists of at least one Nanobody against TNF-alpha as defined herein. Such polypeptides are also referred to herein as "polypeptides of the invention" and may be as further described hereinbelow and/or as generally described in WO 04/041862 for the Nanobodies disclosed therein, and may for example be multivalent polypeptides or multispecific polypeptides, again as further described hereinbelow.

Preferably, a polypeptide of the invention is either bivalent or trivalent (i.e. comprising two or three Nanobodies of the invention, respectively, optionally linked via one or two linkers as defined below, respectively) or a multispecific polypeptide, comprising one or two, and preferably two, Nanobodies of the invention and at least one Nanobody directed against a serum protein, and in particular against a human serum protein, such as against human serum albumin.

In one preferred, but non-limiting embodiments, the Nanobodies of the invention present in the polypeptides of the invention are chosen from the group consisting of SEQ ID NO's: 52 to 60 and SEQ ID NO's 105-129 or from humanized variants thereof, and in particular from the "humanized" Nanobodies of SEQ ID NO's 76 to 86 and SEQ ID NO's:

95 to 99. The Nanobodies against human serum albumin present in the polypeptides of the invention are preferably as defined below, and are more preferably chosen from the group consisting of SEQ ID NO's: 61 to 67, SEQ ID NO's: 87 to 89 and SEQ ID NO's: 100-104, and in particular from the "humanized" Nanobodies against human serum albumin of SEQ ID NO's 76 to 86 and SEQ ID NO's 100-104.

With respect to the Nanobodies that are present in the polypeptides of the invention, it will be clear to the skilled person that the Nanobodies that are mentioned herein as "preferred" (or as "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 of the invention will generally be more preferred, etc..

Thus, in the invention, polypeptides that comprise one or more Nanobodies that essentially consist of one of the preferred variants of clone PMP1C2 (TNF1; SEQ ID NO: 52), in which said preferred variants are as defined herein, are particularly preferred. Even more preferred are polypeptides that comprise one or more Nanobodies that essentially consist of one of the humanized variants of clone PMP1C2 (TNF1; SEQ ID NO: 52), in which said humanized variants are as defined herein (examples being, without limitation, TNF13, TNF14, TNF29 and TNF30). TNF30 is a particularly preferred humanized "building block" for use in the polypeptides of the invention.

Some preferred, but non-limiting examples of such proteins and polypeptides are PMP1C2 itself, the humanized variants TNF13, TNF14, TNF29 and TNF30; the constructs of SEQ ID NO: 70 (TNF4), SEQ ID NO: 73 (TNF7), SEQ ID NO: 90 (TNF24), SEQ ID NO: 93 (TNF27); and the constructs of SEQ ID NO: 417 (TNF60), SEQ ID NO: 419 (TNF55) and SEQ ID NO: 420 (TNF56), in which the latter three constructs contain the humanized variant TNF 30 as a building block.

As mentioned herein, the Nanobodies and constructs described herein may be pegylated, or contain one or more (additional) amino acid residues that allow for pegylation and/or facilitate pegylation. Two preferred, but non-limiting examples of such polypeptides are TNF55 and TNF56, which both contain an additional cysteine residue for easy attachment of a PEG-group.

Some preferred, but non-limiting examples of polypeptides of the invention are the bivalent polypeptides of the invention of SEQ ID NO's: 70 to 75 and the multispecific polypeptides of the invention of SEQ ID NO's: 90 to 94 and SEQ ID NO's 417 to 420.

As can be seen from the data represented below, and in particular from the data given in the Comparative Example, the Nanobodies and/or polypeptides of the invention have improved properties. In particular, the proteins and polypeptides of the invention may havean improved affinity for human TNF-alpha (expressed as the $EC_{50}$-value in the KYM assay described herein), compared to the commercially available anti-TNF biologicals Enbrel™, Humira™ and Remicade™. Also, the Nanobodies described herein may have an improved affinity for TNF-alpha compared to best performing Nanobody described in the International application WO 04/041862. It can thus be expected that polypeptides of the invention comprising at least one of the Nanobodies of the invention will also have improved properties compared to polypeptides that comprise only the Nanobodies against TNF-alpha described in WO 04/041862.

More in particular, a polypeptide as described herein that comprises two or more (and preferably two) Nanobodies as herein (and optionally for example a Nanobody against human serum albumin), has an an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than the EC50 value of Humira® en Remicade ®, and preferably also better than Enbrel® in the same assay.

For example, such a protein or polypeptide preferably has an EC50 value in the cell-based assay using KYM cells described in Example 1, under 3), of WO 04/041862 that is better than 0.2 nM, preferably better than 0.1 nM, such as betet than 0.7 Nm and in particular better than 0.4 nM.

It has also been shown by applicants that Nanobodies against mouse TNF-alpha and polypeptides comprising Nanobodies against mouse TNF-alpha show a beneficial biological activity in the following disease models (data not shown):

- The dextran sulfate sodium ("DSS") model of colitis, using both regular mice as well as IL-10 knock out mice, as described by Okayasu *et al.* (*Gastroenterol* 1990, 98(3): 694)
- The collagen induced arthritis ("CIA") model of arthritis ("RA"), as described by Courtenay *et al.* (*Nature* 1980, 283(5748): 666), using both regular mice as well as IL-10 knock out mice;

- The IL-10 knockout mice model of IBD, as for example described by Rennick *et al.* (*Clin Immunol Immunopathol* 1995, 76(3 Pt 2): S174)
- The Kollias model of RA as for example described by Keffer *et al.* (*EMBO J* 1991, 10(13): 4025)
- The 2,4,6-trinitrobenzenesulphonic acid ("TNBS") model of IBD, as described by Elson *et al.* (*J Immunol* 1996, 157(5): 2174)
- The CIA model of RA, described by Koppieters et al (manuscript in preparation);
- The synovial-derived fibroblast model (described below); and
- The murine air pouch model.

Preferably, the Nanobodies described herein are better than Nanobody 1A from WO 04/041862 in at least one of these models, and preferably in all of these models; and more preferably are better than Nanobody 3E from WO 04/041862 in at least one of these models, and preferably in all of these models. Also, the polypeptides described herein are preferably equivalent to or better than Humira® or Remicade ® in at least one of these models, and preferably in all of these models; and more preferably also equivalent to or better than Enbrel® in at least one of these models, and preferably in all of these models.

These data confirm that Nanobodies against TNF-alpha and polypeptides containing the same, such as the Nanobodies and polypeptides described in WO 04/041862 and in particular the Nanobodies and polypeptides described herein, should have therapeutic efficacy against TNF mediated diseases and disorders, such as the diseases and disorders mentioned above.

In another aspect, the invention relates to a nucleic acid that encodes a Nanobody of the invention and/or a polypeptide of the invention. Such a nucleic acid will also be referred to below as a "nucleic acid of the invention" and may for example be in the form of a genetic construct, as defined below.

In another aspect, the invention relates to host or host cell that expresses or is capable of expressing a Nanobody of the invention and/or a polypeptide of the invention; and/or that contains a nucleic acid encoding a Nanobody of the invention and/or a polypeptide of the invention. Such a host or a host cell may also be analogous to the hosts and host cells described in WO 04/041862, but expressing or capable of expressing a Nanobody of the invention and/or a polypeptide of the invention and/or containing a nucleic acid as described herein.

The invention further relates to a product or composition containing or comprising a Nanobody of the invention, a polypeptide of the invention; and/or a nucleic acid of the invention. Such a product or composition may for example be a pharmaceutical composition (as described below) or a product or composition for diagnostic use (as also described below). Such a product or composition may also be analogous to the products and compositions described in WO 04/041862, but containing or comprising a Nanobody of the invention, a polypeptide of the invention or a nucleic acid of the invention.

The invention further relates to methods for preparing or generating the Nanobodies, polypeptides, nucleic acids, host cells, products and compositions as described herein, which methods are as further described below. Also, generally, the Nanobodies, polypeptides, nucleic acids, host cells, products and compositions described herein may also be prepared and used in a manner analogous to the manner described in WO 04/041862.

The invention further relates to applications and uses of the above Nanobodies, polypeptides, nucleic acids, host cells, products and compositions described herein, which applications and uses include, but are not limited to, the applications and uses described hereinbelow and/or the further uses and applications for Nanobodies against TNF-alpha and/or for polypeptides containing the same in WO 04/041862.

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

*Detailed description of the invention*

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

a) Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" ( 2nd.Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987); Lewin, "Genes II", John Wiley & Sons, New York, N.Y., (1985); Old et al., "Principles of Gene Manipulation: An Introduction to Genetic Engineering", 2nd edition, University of California Press, Berkeley, CA (1981); Roitt et al., "Immunology" (6th. Ed.), Mosby/Elsevier, Edinburgh (2001); Roitt et al., Roitt's Essential Immunology, 10th

Ed. Blackwell Publishing, UK (2001); and Janeway et al., "Immunobiology" (6th Ed.), Garland Science Publishing/Churchill Livingstone, New York (2005), as well as to the general background art cited herein;

b) Unless indicated otherwise, the term "*immunoglobulin sequence*" - whether it 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 acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation;

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, to the general background art referred to above and to the further references cited therein;

d) Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as mentioned in Table 1;

**Table 1: one-letter and three-letter amino acid code**

| | | | |
|---|---|---|---|
| Nonpolar, uncharged (at pH 6.0 – 7.0)[3] | Alanine | Ala | A |
| | Valine | Val | V |
| | Leucine | Leu | L |
| | Isoleucine | Ile | I |
| | Phenylalanine | Phe | F |
| | Methionine[1] | Met | M |
| | Tryptophan | Trp | W |
| | Proline | Pro | P |
| Polar, uncharged (at pH 6.0-7.0) | Glycine[2] | Gly | G |
| | Serine | Ser | S |
| | Threonine | Thr | T |
| | Cysteine | Cys | C |
| | Asparagine | Asn | N |
| | Glutamine | Gln | Q |
| | Tyrosine | Tyr | Y |
| Polar, charged (at pH 6.0-7.0) | Lysine | Lys | K |
| | Arginine | Arg | R |
| | Histidine[4] | His | H |
| | Aspartate | Asp | D |
| | Glutamate | Glu | E |

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 residu 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 [*the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence*] by [*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 may be calculated by dividing [*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*] by [*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 below.

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-2 357 768, 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, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp.

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; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile 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 proteins of different species developed by Schulz et al., Principles of Protein Structure, Springer-Verlag, 1978, on the analyses of structure forming potentials developed by Chou and Fasman, Biochemistry 13: 211, 1974 and Adv. Enzymol., 47: 45-149, 1978, and on

the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al., Proc. Nad. Acad Sci. USA 81: 140-144, 1984; Kyte & Doolittle; J Molec. Biol. 157: 105-132, 198 1, and Goldman et al., Ann. Rev. Biophys. Chem. 15: 321-353, 1986, all incorporated herein in their entirety by reference. Information on the primary, secondary and tertiary structure of Nanobodies given in the description herein and in the general background art cited above. Also, for this purpose, the crystal structure of a VHH domain from a llama is for example given by Desmyter et al., Nature Structural Biology, Vol. 3, 9, 803 (1996); Spinelli et al., Natural Structural Biology (1996); 3, 752-757; and Decanniere et al., Structure, Vol. 7, 4, 361 (1999). Further information about some of the amino acid residues that in conventional VH domains form the VH/VL interface and potential camelizing substitutions on these positions can be found in the prior art on Nanobodies cited herein;

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) 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-gelelectrophoresis;

j) The term "domain" as used herein generally refers to a globular region of 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.

k) 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.

l) 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 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.

m) 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 (KD), 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 KD, 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 (KA), which is 1/KD). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known per se, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as 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 Nanobodies and/or polypeptides of the invention) will bind with a dissociation constant (KD) of $10^{-5}$ to $10^{-12}$ moles/liter (M) or less, and preferably $10^{-7}$ to $10^{-12}$ moles/liter (M) or less and more preferably $10^{-8}$ to $10^{-12}$ moles/liter, and/or with an association constant($K_A$) of at least $10^7$ $M^{-1}$, preferably at least $10^8$ $M^{-1}$, more preferably at least $10^9$ M-1, such as at least $10^{12}$ $M^{-1}$.

Any $K_D$ value greater than $10^{-4}$ M is generally considered to indicate non-specific binding. Preferably, a Nanobody or polypeptide of the invention will bind to the desired antigen with an $K_D$ 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.

n) as further described hereinbelow, 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", which are referred to in the art and hereinbelow as "*Framework region 1*" or "*FR1*"; as "*Framework region 2*" or"*FR2*"; as "*Framework region 3*" or "*FR3*"; and as "*Framework region 4*" or "*FR4*", respectively; which framework regions are interrupted by three complementary determining regions or "CDR's", which are referred to in the art as "*Complementarity Determining Region 1*"or "*CDR1*"; as "*Complementarity Determining Region 2*" or "*CDR2*"; and as "*Complementarity Determining Region 3*" or "*CDR3*", respectively;

o) as also further describe hereinbelow, 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 or analogs (as further described hereinbelow) of a Nanobody are not particularly limited as to their length and/or size, as long as such parts, fragments or analogs meet the further requirements outlined hereinbelow and are also preferably suitable for the purposes described herein;

p) 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 Camelids in the article of Riechmann and Muyldermans, referred to above (see for example Figure 2 of said reference). 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 31-36, 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 103-113. [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, claims and figures, the numbering according to Kabat as applied to $V_{HH}$ domains by Riechmann and Muyldermans will be followed, unless indicated otherwise; and

q) 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 claims 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 following references, 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

applicant; 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 04/041862, WO 04/041865, WO 04/041863, WO 04/062551 by applicant and the further published patent applications by applicant;

Hamers-Casterman et al., Nature 1993 June 3; 363 (6428): 446-8; Davies and Riechmann, FEBS Lett. 1994 Feb 21; 339(3): 285-90; Muyldermans et al., Protein Eng. 1994 Sep; 7(9): 1129-3; Davies and Riechmann, Biotechnology (NY) 1995 May; 13(5): 475-9; Gharoudi et al., 9th Forum of Applied Biotechnology, Med. Fac. Landbouw Univ. Gent. 1995; 60/4a part I: 2097-2100; Davies and Riechmann, Protein Eng. 1996 Jun; 9(6): 531-7; Desmyter et al., Nat Struct Biol. 1996 Sep; 3(9): 803-11; Sheriff et al., Nat Struct Biol. 1996 Sep; 3(9): 733-6; Spinelli et al., Nat Struct Biol. 1996 Sep; 3(9): 752-7; Arbabi Ghahroudi et al., FEBS Lett. 1997 Sep 15; 414(3): 521-6; Vu et al., Mol Immunol. 1997 Nov-Dec; 34(16-17): 1121-31; Atarhouch et al., Journal of Camel Practice and Research 1997; 4: 177-182; Nguyen et al., J. Mol. Biol. 1998 Jan 23; 275(3): 413-8; Lauwereys et al., EMBO J. 1998 Jul 1; 17(13): 3512-20; Frenken et al., Res Immunol. 1998 Jul-Aug;149(6):589-99; Transue et al., Proteins 1998 Sep 1; 32(4): 515-22; Muyldermans and Lauwereys, J. Mol. Recognit. 1999 Mar-Apr; 12 (2): 131-40; van der Linden et al., Biochim. Biophys. Acta 1999 Apr 12; 1431(1): 37-46.; Decanniere et al., Structure Fold. Des. 1999 Apr 15; 7(4): 361-70; Ngyuen et al., Mol. Immunol. 1999 Jun; 36(8): 515-24; Woolven et al., Immunogenetics 1999 Oct; 50 (1-2): 98-101; Riechmann and Muyldermans, J. Immunol. Methods 1999 Dec 10; 231 (1-2): 25-38; Spinelli et al., Biochemistry 2000 Feb 15; 39(6): 1217-22; Frenken et al., J. Biotechnol. 2000 Feb 28; 78(1): 11-21; Nguyen et al., EMBO J. 2000 Mar 1; 19(5): 921-30; van der Linden et al., J. Immunol. Methods 2000 Jun 23; 240 (1-2): 185-95; Decanniere et al., J. Mol. Biol. 2000 Jun 30; 300 (1): 83-91; van der Linden et al., J. Biotechnol. 2000 Jul 14; 80(3): 261-70; Harmsen et al., Mol. Immunol. 2000 Aug; 37(10): 579-90; Perez et al., Biochemistry 2001 Jan 9; 40(1): 74-83; Conrath et al., J. Biol. Chem. 2001 Mar 9; 276 (10): 7346-50; Muyldermans et al., Trends Biochem Sci. 2001 Apr;26(4):230-5; Muyldermans S., J. Biotechnol. 2001 Jun; 74 (4): 277-302; Desmyter et al., J. Biol. Chem. 2001 Jul 13 ;276 (28): 26285-90; Spinelli et al., J. Mol. Biol. 2001 Aug 3; 311 (1): 123-9; Conrath et al., Antimicrob Agents Chemother. 2001 Oct; 45 (10): 2807-12; Decanniere et al., J. Mol. Biol. 2001 Oct 26; 313(3): 473-8; Nguyen et al., Adv Immunol. 2001; 79: 261-96; Muruganandam et al., FASEB J. 2002 Feb; 16 (2): 240-2; Ewert et al., Biochemistry 2002

Mar 19; 41 (11): 3628-36; Dumoulin et al., Protein Sci. 2002 Mar; 11 (3): 500-15; Cortez-Retamozo et al., Int. J. Cancer. 2002 Mar 20; 98 (3): 456-62; Su et al., Mol. Biol. Evol. 2002 Mar; 19 (3): 205-15; van der Vaart JM., Methods Mol Biol. 2002; 178: 359-66; Vranken et al., Biochemistry 2002 Jul 9; 41 (27): 8570-9; Nguyen et al., Immunogenetics 2002 Apr; 54 (1): 39-47; Renisio et al., Proteins 2002 Jun 1; 47 (4): 546-55; Desmyter et al., J. Biol. Chem. 2002 Jun 28; 277 (26): 23645-50; Ledeboer et al., J. Dairy Sci. 2002 Jun; 85 (6): 1376-82; De Genst et al., J. Biol. Chem. 2002 Aug 16; 277 (33): 29897-907; Ferrat et al., Biochem. J. 2002 Sep 1; 366 (Pt 2): 415-22; Thomassen et al., Enzyme and Microbial Technol. 2002; 30: 273-8; Harmsen et al., Appl. Microbiol. Biotechnol. 2002 Dec; 60 (4): 449-54; Jobling et al., Nat Biotechnol. 2003 Jan; 21 (1): 77-80; Conrath et al., Dev. Comp. Immunol. 2003 Feb; 27 (2): 87-103; Pleschberger et al., Bioconjug. Chem. 2003 Mar-Apr; 14 (2): 440-8; Lah et al., J. Biol. Chem. 2003 Apr 18; 278 (16): 14101-11; Nguyen et al., Immunology. 2003 May; 109 (1): 93-101; Joosten et al., Microb. Cell Fact. 2003 Jan 30; 2 (1): 1; Li et al., Proteins 2003 Jul 1; 52 (1): 47-50; Loris et al., Biol Chem. 2003 Jul 25; 278 (30): 28252-7; van Koningsbruggen et al., J. Immunol. Methods. 2003 Aug; 279 (1-2): 149-61; Dumoulin et al., Nature. 2003 Aug 14; 424 (6950): 783-8; Bond et al., J. Mol. Biol. 2003 Sep 19; 332 (3): 643-55; Yau et al., J. Immunol. Methods. 2003 Oct 1; 281 (1-2): 161-75; Dekker et al., J. Virol. 2003 Nov; 77 (22): 12132-9; Meddeb-Mouelhi et al., Toxicon. 2003 Dec; 42 (7): 785-91; Verheesen et al., Biochim. Biophys. Acta 2003 Dec 5; 1624 (1-3): 21-8; Zhang et al., J Mol Biol. 2004 Jan 2; 335 (1): 49-56; Stijlemans et al., J Biol Chem. 2004 Jan 9; 279 (2): 1256-61; Cortez-Retamozo et al., Cancer Res. 2004 Apr 15; 64 (8): 2853-7; Spinelli et al., FEBS Lett. 2004 Apr 23; 564 (1-2): 35-40; Pleschberger et al., Bioconjug. Chem. 2004 May-Jun; 15 (3): 664-71; Nicaise et al., Protein Sci. 2004 Jul; 13 (7): 1882-91; Omidfar et al., Tumour Biol. 2004 Jul-Aug; 25 (4): 179-87; Omidfar et al., Tumour Biol. 2004 Sep-Dec; 25(5-6): 296-305; Szynol et al., Antimicrob Agents Chemother. 2004 Sep;48(9):3390-5; Saerens et al., J. Biol. Chem. 2004 Dec 10; 279 (50): 51965-72; De Genst et al., J. Biol. Chem. 2004 Dec 17; 279 (51): 53593-601; Dolk et al., Appl. Environ. Microbiol. 2005 Jan; 71(1): 442-50; Joosten et al., Appl Microbiol Biotechnol. 2005 Jan; 66(4): 384-92; Dumoulin et al., J. Mol. Biol. 2005 Feb 25; 346 (3): 773-88; Yau et al., J Immunol Methods. 2005 Feb; 297 (1-2): 213-24; De Genst et al., J. Biol. Chem. 2005 Apr 8; 280 (14): 14114-21; Huang et al., Eur. J. Hum. Genet. 2005 Apr 13; Dolk et al., Proteins. 2005 May 15; 59 (3): 555-64; Bond et al., J.

Mol. Biol. 2005 May 6;348(3):699-709; Zarebski et al., J. Mol. Biol. 2005 Apr 21; [E-publication ahead of print].

In accordance with the terminology used in the above references, the variable domains present in naturally occurring heavy chain antibodies will also be referred to as "*$V_{HH}$ 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 "*$V_H$ domains*") and from the light chain variable domains that are present in conventional 4-chain antibodies (which will be referred to hereinbelow as "*$V_L$ domains*").

As mentioned in the prior art referred to above, $V_{HH}$ domains have a number of unique structural characteristics and functional properties which make isolated $V_{HH}$ domains (as well as Nanobodies based thereon, which share these structural characteristics and functional properties with the naturally occurring $V_{HH}$ domains) and proteins containing the same highly advantageous for use as functional antigen-binding domains or proteins. In particular, and without being limited thereto, $V_{HH}$ 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 $V_{HH}$ domains from the $V_H$ and $V_L$ 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).

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')$_2$-fragments):

- only a single domain is required to bind an antigen with high affinity and with high selectivity, so that there is no need to have two separate domains present, nor to assure that these two domains are present in the right spacial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv's);

- $V_{HH}$ domains and Nanobodies can be expressed from a single gene and require no post-translational folding or modifications;
- $V_{HH}$ 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 antigen-binding domains described by Ward et al., Nature, Vol. 341, 1989, p. 544);
- $V_{HH}$ 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;
- $V_{HH}$ 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;
- $V_{HH}$ 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 accessable to conventional 4-chain antibodies and antigen-binding fragments thereof. For example, it has been shown that $V_{HH}$ domains and Nanobodies can inhibit enzymes (see for example WO 97/49805; Transue et al., (1998), supra; Lauwereys et al., (1998), supra.

As mentioned above, the invention generally relates to Nanobodies directed against TNF-alpha, as well as to polypeptides comprising or essentially consisting of one or more of such Nanobodies, that can be used for the prophylactic, therapeutic and/or diagnostic purposes described below and in WO 04/041862.

As also mentioned above and further described below, the invention further relates to nucleic acids encoding such Nanobodies and polypeptides, to methods for preparing such Nanobodies and polypeptides, to host cells expressing or capable of expressing such

Nanobodies or polypeptides, to uses of such Nanobodies, polypeptides, nucleic acids or host cells, and to compositions comprising such Nanobodies, polypeptides, nucleic acids or host cells.

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 be obtained (1) by isolating the $V_{HH}$ domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring $V_{HH}$ domain; (3) by "humanization" (as described below) of a naturally occurring $V_{HH}$ domain or by expression of a nucleic acid encoding a such humanized $V_{HH}$ domain; (4) by "camelization" (as described below) of a naturally occurring $V_H$ domain from any animal species, in particular a species of mammal, such as from a human being, or by expression of a nucleic acid encoding such a camelized $V_H$ 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 $V_H$ domain; (6) using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences; (7) by preparing a nucleic acid encoding a Nanobody using techniques for nucleic acid synthesis, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination 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 hereinbelow.

However, according to a specific embodiment, the Nanobodies of the invention do not have an amino acid sequence that is exactly the same as (i.e. as a degree of sequence identity of 100% with) the amino acid sequence of a naturally occurring $V_H$ domain, such as the amino acid sequence of a naturally occurring $V_H$ domain from a mammal, and in particular from a human being.

One 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 $V_{HH}$ 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 $V_{HH}$ sequence 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 below 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.

A preferred, but non-limiting humanzing substitution for Nanobodies belonging to the 103 P,R,S-group and/or the GLEW-group (as defined herein) is 108Q to 108L.

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 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 below. Reference is also made to WO 94/04678. Such camelization may preferentially occur at amino acid positions which are present at the $V_H$-$V_L$ interface and at the so-called Camelidae hallmark residues (see for example also WO 94/04678), as also mentioned below. Preferably, the $V_H$ domain or 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. 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 below, both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes such 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 such that the new nucleotide sequence encodes a humanized or camelized Nanobody of the invention, respectively, and then expressing the nucleotide sequence thus obtained 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 $V_{HH}$ 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 nucleotide sequence thus obtained can be expressed in a manner known per se so as to provide the desired Nanobody of the invention.

Other suitable ways and techniques for obtaining Nanobodies of the invention and/or nucleotide sequences and/or nucleic acids encoding the same, starting from (the amino acid sequence of) naturally occurring $V_H$ domains or preferably $V_{HH}$ domains and/or from nucleotide sequences and/or nucleic acid sequences encoding the same will be clear from the skilled person, and may for example comprising combining one or more amino acid sequences and/or nucleotide sequences from naturally occurring $V_H$ domains (such as one or more FR's and/or CDR's) with one or more amino acid sequences and/or nucleotide sequences from naturally occurring $V_{HH}$ domains (such an one or more FR's or CDR's), in a suitable manner so as to provide (a nucleotide sequence or nucleic acid encoding) a Nanobody of the invention.

According to one preferred, but non-limiting aspect of the 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 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, 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

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

and/or in which:

ii) 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:

iii) 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

iv) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

In particular, a Nanobody against TNF-alpha according to 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 residue at position 108 according to the Kabat numbering is Q;

and/or in which:

ii) 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:

iii) 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

iv) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

In particular, according to one preferred, but non-limiting aspect of the 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 G, E, D, G, 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 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.

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 residue at position 44 according to the Kabat numbering is chosen from the group consisting of G, E, D, G, Q, R, S, L; and is preferably chosen from the group consisting of G, E or Q;

and in which:

ii) 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:

iii) 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

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

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

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 residue at position 44 according to the Kabat numbering is chosen from the group consisting of E and Q;

and in which:

ii) the amino acid residue at position 45 according to the Kabat numbering is R;

and in which:

iii) 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:

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

and in which:

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

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 residue at position 44 according to the Kabat numbering is chosen from the group consisting of G, E, D, Q, R, S and L; and is preferably chosen from the group consisting of G, E and Q;

and in which:

ii) 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:

iii) 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:

iv) 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:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

Two particularly preferred, but non-limiting groups of the Nanobodies of the invention are those according to a) above; according to I) 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;

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

or in which:

b) 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.

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 below) and the amino acid residue at position 108 is Q;

and in which:

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

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 above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

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, V or F, and is most preferably F.

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 is on the basis of the following three groups:

a) 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 2 below;

b) The "*KERE-group*": Nanobodies with the amino acid sequence KERE or KQRE or 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;

c) 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 of 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.

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 CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

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 in which CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

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 above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

Also, more generally and in addition to the 108Q, 43E/44R and 103P,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, contain 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 $V_H$ or $V_{HH}$ domain, and in particular one or more charged amino acid residues (as mentioned in Table 1).

Such substitutions include, but are not limited to the GLEW-like sequences mentioned in Table 2 below; as well as the substitutions that are described in the International Application WO 00/29004 for so-called "*microbodies*", e.g. a Q at position 108 and KLEW at positions 44-47.

In some embodiments 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 some embodiments of the Nanobodies of the invention, the amino acid residue at position 83 is chosen from the group consisting of R, K, N, E, I and Q; and is most preferably either K or E (for Nanobodies corresponding to naturally occurring $V_{HH}$

domains) or R (for "humanized" Nanobodies, as described below). The amino acid residue at position 84 in some embodiments is chosen from the group consisting of P, A, R, S, D and V, and is most preferably P (for Nanobodies corresponding to naturally occurring $V_{HH}$ domains) or R (for "humanized" Nanobodies, as described below).

Furthermore, in some embodiments 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 VH domain, VH3, are summarized in Table 2.

Some especially preferred combinations of these Hallmark Residues as occur in naturally occurring $V_{HH}$ domains are mentioned in Table 3. For comparison, the corresponding amino acid residues of the human $V_H3$ called DP-47 have been indicated in italics.

**Table 2: Hallmark Residues in Nanobodies**

| Position | Human $V_H3$ | Hallmark Residues |
|---|---|---|
| 11 | L, V; predominantly L | L, M, S, V,W; preferably L |
| 37 | V, I, F; usually V | $F^{(1)}$, Y, H, I or V, preferably $F^{(1)}$ or Y |
| $44^{(8)}$ | G | $G^{(2)}$, $E^{(3)}$, D, Q, R, S, L;<br>preferably $G^{(2)}$, $E^{(3)}$ or Q;<br>most preferably $G^{(2)}$ or $E^{(3)}$. |
| $45^{(8)}$ | L | $L^{(2)}$, $R^{(3)}$, C, I, L, P, Q, V; preferably $L^{(2)}$ or $R^{(3)}$ |
| $47^{(8)}$ | W, Y | $W^{(2)}$, $L^{(1)}$ or $F^{(1)}$, A, G, I, M, R, S or Y;<br>preferably $W^{(2)}$, $L^{(1)}$, $F^{(1)}$ or R |
| 83 | R or K; usually R | R, $K^{(3)}$, N, $E^{(3)}$, I, M or Q; preferably K or R; most preferably K |
| 84 | A, T, D; predominantly A | $P^{(3)}$, A, L, R, S, D, V; preferably P |

| | | |
|---|---|---|
| 103 | W | $W^{(4)}$, $P^{(6)}$, $R^{(6)}$, S; preferably W |
| 104 | G | G or D; preferably G |
| 108 | L, M or T; predominantly L | Q, $L^{(7)}$ or R; preferably Q or $L^{(7)}$ |

Notes:

(1): In particular, but not exclusively, in combination with KERE (SEQ ID NO: 437) or KQRE (SEQ ID NO: 438) at positions 43-46.

(2): Usually as GLEW (SEQ ID NO: 439) at positions 44-47.

(3): Usually as KERE or KQRE at positions 43-46, e.g. as KEREL (SEQ ID NO: 440), KEREF (SEQ ID NO: 441), KQREL (SEQ ID NO: 442), KQREF (SEQ ID NO: 443) or KEREG (SEQ ID NO: 444) at positions 43-47. Alternatively, also sequences such as TERE (SEQ ID NO: 445) (for example TEREL (SEQ ID NO: 446)), KECE (SEQ ID NO: 447) (for example KECEL (SEQ ID NO: 448) or KECER (SEQ ID NO: 449)), RERE (SEQ ID NO: 450) (for example REREG (SEQ ID NO: 451)), QERE (SEQ ID NO: 452) (for example QEREG (SEQ ID NO: 453)), KGRE (SEQ ID NO: 454) (for example KGREG (SEQ ID NO: 455)), KDRE (SEQ ID NO: 456) (for example KDREV (SEQ ID NO: 457)) are possible. Some other possible, but less preferred sequences include for example DECKL (SEQ ID NO: 458) and NVCEL (SEQ ID NO: 459).

(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 $V_{HH}$ 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.

(8): The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW (SEQ ID NO: 460), EPEW (SEQ ID NO: 461), GLER (SEQ ID NO: 462), DQEW (SEQ ID NO: 463), DLEW (SEQ ID NO: 464), GIEW (SEQ ID NO: 465), ELEW (SEQ ID NO: 466), GPEW (SEQ ID NO: 467), EWLP (SEQ ID NO: 468), GPER (SEQ ID NO: 469), GLER (SEQ ID NO: 470) and ELEW.

**Table 3: Some preferred combinations of Hallmark Residues in naturally occurring Nanobodies.**

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

| | 11 | 37 | 44 | 45 | 47 | 83 | 84 | 103 | 104 | 108 |
|---|---|---|---|---|---|---|---|---|---|---|
| *DP-47 (human)* | *M* | *V* | *G* | *L* | *W* | *R* | *A* | *W* | *G* | *L* |
| | | | | | | | | | | |
| "KERE" group | L | F | E | R | L | K | P | W | G | Q |
| | L | F | E | R | F | E | P | W | G | Q |
| | L | F | E | R | F | K | P | W | G | Q |
| | L | Y | Q | R | L | K | P | W | G | Q |
| | L | F | L | R | V | K | P | Q | G | Q |
| | L | F | Q | R | L | K | P | W | G | Q |
| | L | F | E | R | F | K | P | W | G | Q |
| | | | | | | | | | | |
| "GLEW" group | L | V | G | L | W | K | S | W | G | Q |
| | M | V | G | L | W | K | P | R | G | Q |

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 4 - 7 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 Chothia (supra) that the residues at these positions already form part of CDR1.)

In Tables 4 - 7, some of the non-limiting residues that can be present at each position of a human $V_H3$ 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 $V_H3$ domain is indicated in bold; and other preferred amino acid residues have been underlined.

**Table 4: Non-limiting examples of amino acid residues in FR1 (for the footnotes, see the footnotes to Table 2)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 1 | E, Q | Q, A, E, D, H, R |
| 2 | V | V, A, E, G, L, M, Q |
| 3 | Q | Q, K, E, H, P, R, Y |
| 4 | L | L, F, P, R, V |
| 5 | V, L | Q, E, L, V, M, P, A, I |
| 6 | E | E, D, Q, A, H |
| 7 | S, T | S, F, H |
| 8 | G, R | G, A, R |
| 9 | G | G, E |
| 10 | G, V | G, D, R, A, E, N, T, V |
| 11 | **Hallmark residue:** L, M, S, V,W, F, N, P, T, Y; preferably L | |
| 12 | V, I | V, A, G, M |
| 13 | Q, K, R | Q, E, K, D, G, A, H, L, N, P, R, T |
| 14 | P | A, Q, A, G, P, T, V, E, F, I, N, S |
| 15 | G | G |
| 16 | G, R | G, A, E, D, N, P, R, S, V, W |
| 17 | S | S, F, T, N, P, A, C |
| 18 | L | L, V, M, Q, R |
| 19 | R, K | R, K, L, N, S, T, A, F, G, I, M, Q |
| 20 | L | L, F, I, V, M, S |
| 21 | S | S, F, T, G, H, P, A |
| 22 | C | C |
| 23 | A, T | A, D, P, S, T, V, E, G, I, L, Q, R |
| 24 | A | A, I, S, T, V, C, E, F, G, L, N, P, Q, Y |

**Table 4: Non-limiting examples of amino acid residues in FR1 (continued)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 25 | S | S, A, F, P, T, L, V |
| 26 | G | G, D, E, R, S, V, A, I, M, P, T |
| 27 | F | S, F, R, L, P, G, N, A, D, E, H, I, K, M, Q, T, V, Y |
| 28 | T | N, T, E, D, S, I, R, A, G, R, F, Y, L, M, P, V |
| 29 | F, V | F,L, D, S, I, G, V, A, E, P, T, Y |
| 30 | S, D, G | N, S, E, G, A, D, M, T, H, I, P, R, V, W |

**Table 5: Non-limiting examples of amino acid residues in FR2 (for the footnotes, see the footnotes to Table 2)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 36 | W | W |
| 37 | Hallmark residue: $F^{(1)}$, Y, H, I, A, L, P, S or V preferably $F^{(1)}$ or Y | |
| 38 | R | R |
| 39 | Q | Q, H, P, R, A, D, G, L, E |
| 40 | A | A, F, G, P, T, V, I, L, N, R, S, Y |
| 41 | P, S, T | P, A, L, S, I, Q, T |
| 42 | G | G, E, D, R, T, V |
| 43 | K | K, D, E, N, Q, R, T, V, A, L, M, S |
| 44 | Hallmark residue: $G^{(2)}$, $E^{(3)}$, D, Q, R, S, L, A, F, K, M, N, P, V, W, Y; preferably $G^{(2)}$, $E^{(3)}$ or Q; most preferably $G^{(2)}$ or $E^{(3)}$. | |
| 45 | Hallmark residue: $L^{(2)}$, $R^{(3)}$, C, I, L, P, Q, V, D, E, G, H, K, T; preferably $L^{(2)}$ or $R^{(3)}$ | |

| | | |
|---|---|---|
| 46 | E, V | E, D, K, Q, V, A, G, N |
| 47 | Hallmark residue: $W^{(2)}$, $L^{(1)}$ or $F^{(1)}$, A, G, I, M, R, S, D, E, H, K, Q, T, V or Y; preferably $W^{(2)}$, $L^{(1)}$, $F^{(1)}$ or R | |
| 48 | V | V, I, L, A, C, E, F, G, H, M, P, Q, R, S, T, V, W, Y |
| 49 | S, A, G | A, S, A, G, T, V, D, E, I, L, Q, R, Y |

**Table 6: Non-limiting examples of amino acid residues in FR3 (for the footnotes, see the footnotes to Table 2)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 66 | R | R |
| 67 | F | F, L, V, A, D, I, S, Y |
| 68 | T | T, A, S, D, F, G, I, K, N |
| 69 | I | I, M, V, A, F, L, R, S, T |
| 70 | S | S, A, F, E, G, K, P, T, V |
| 71 | R | R, G, I, K, Q, S, T, W, A, F, L, M, N |
| 72 | D, E | D, E, G, N, V, A, H, I, L, Q, S, T |
| 73 | N, D, G | N, D, F, I, K, S, T, Y, A, G, H, L, M, R, V |
| 74 | A, S | A, D, G, N, P, S, T, F, H, I, L, R, V, Y |
| 75 | K | K, A, E, K, L, N, Q, R, D, G, I, M, S, T, V, W |
| 76 | N, S | N, D, K, R, S, T, Y, E, G, H, I, Q |
| 77 | S, T, I | T, A, E, I, M, S , K, L, N, R, V |
| 78 | L, A | V, L, A, F, G, I, M, E, N, Q, R, S, T, W |
| 79 | Y, H | Y, A, D, F, H, S, T, C, E, I, L, N, V, W |

| | | |
|---|---|---|
| 80 | **L** | **L**, F, V, M |
| 81 | **Q** | **Q**, E, R, T, G, H, I, K, L, M, N |
| 82 | **M** | **M**, I, L, V, G, P, T |
| 82a | N, G | **N**, D, G, H, S, T, A, E, I, K, R, V |
| 82b | **S** | **S**, N, D, G, R, A, C, E, F, I, K, M, P, T, V |
| 82c | **L** | **L**, P, M, T, V |
| 83 | **Hallmark residue:** R, K[3], N, E[3], I, M, A, D, G, L, Q, S, T or Q; preferably K or R; most preferably K | |
| 84 | **Hallmark residue:** P[3], A, L, R, S, D, V, F, G, H, N, T, Y; preferably P | |
| 85 | **E**, G | **E**, D, G, Q, A, N, R, V, Y |
| 86 | **D** | **D**, E, F, Y |
| 87 | **T**, M | **T**, S, A, C, M |

**Table 6: Non-limiting examples of amino acid residues in FR3 (continued)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 88 | A | A, G, S, D, L, N, P |
| 89 | V, L | V, A, D, I, L, M, N, R, T, E, F, S |
| 90 | Y | Y, F, E, H, N |
| 91 | Y, H | Y, D, F, H, L, S, T, V, C, I, N, R, W |
| 92 | C | C |
| 93 | A, K, T | A, N, G, H, K, R, S, T, V, Y, E, F, I, L, M, Q |
| 94 | K, R, T | A, V, C, F, G, I, L, R, S, D, E, K, M, N, P, Q, T, W, Y T or K; |

**Table 7: Non-limiting examples of amino acid residues in FR4 (for the footnotes, see the footnotes to Table 2)**

| Pos. | Amino acid residue(s): | |
|---|---|---|
| | *Human $V_H3$* | *Camelid $V_{HH}$'s* |
| 103 | **Hallmark residue:** W[4], P[6], R[6], S, F, G, K, L, N, Q, V, Y; preferably W | |
| 104 | **Hallmark residue:** G, A, R, S, T or D; preferably G | |
| 105 | Q, R | Q, E, K, P, R, G, H, L, S, V |
| 106 | G | G |
| 107 | T | T, A, I, N, P |
| 108 | **Hallmark residue:** Q, L[7], E, H, N, P, T or R; preferably Q or L[7] | |
| 109 | V | V |
| 110 | T | T, I, A |
| 111 | V | V, A, I, G |
| 112 | S | S, F, A, L, P, T, Y |
| 113 | S | S, A, L, P, F, T |

Thus, in another preferred, but not limiting aspect, a Nanobody of the invention can 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 Hallmark residues are as defined above;

and in which:

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

In another preferred, but not limiting aspect, a Nanobody of the invention can 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:

and in which

i) FR1 is chosen from the group consisting of the amino acid sequence:

[1] QVQLQESGGGXVQAGGSLRLSCAASG [26] [SEQ ID NO: 1]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

ii) FR2 is chosen from the group consisting of the amino acid sequence:

[36] W<u>X</u>RQAPGK<u>XX</u>E<u>X</u>VA [49] [SEQ ID NO: 2]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

iii) FR3 is chosen from the group consisting of the amino acid sequence:

[66] RFTISRDNAKNTVYLQMNSLXXEDTAVYYCAA [94] [SEQ ID NO: 3]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

iv) FR4 is chosen from the group consisting of the amino acid sequence:

[103] XXQGTXVTVSS [113] [SEQ ID NO: 4]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 7; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 7; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above;

in which the Hallmark Residues are indicated by "X" and are as defined hereinabove and in which the numbers between brackets refer to the amino acid positions according to the Kabat numbering.

In another preferred, but not limiting aspect, a Nanobody of the invention can 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:

and in which

i) FR1 is chosen from the group consisting of the amino acid sequence:

[1] QVQLQESGGGLVQAGGSLRLSCAASG [26] [SEQ ID NO: 5]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residue at position is as indicated in the sequence above;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residue at position is as indicated in the sequence above;

and in which:

ii) FR2 is chosen from the group consisting of the amino acid sequences:

[36] WFRQAPGKERELVA [49] [SEQ ID NO: 6]

[36] WFRQAPGKEREFVA [49] [SEQ ID NO: 7]

[36] WFRQAPGKEREGA [49] [SEQ ID NO: 8]

[36] WFRQAPGKQRELVA [49] [SEQ ID NO: 9]

[36] WFRQAPGKQREFVA [49] [SEQ ID NO: 10]
[36] WYRQAPGKGLEWA [49] [SEQ ID NO: 11]

or 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 one of the above amino acid sequences; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in each of the sequences above;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in each of the sequences above;

and in which:

iii) FR3 is chosen from the group consisting of the amino acid sequence:

[66] RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA [94] [SEQ ID NO: 12]

or 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 above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 83 and 84 are as indicated in each of the sequences above;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 83 and 84 are as indicated in each of the sequences above;

and in which:

iv) FR4 is chosen from the group consisting of the amino acid sequences:

| | |
|---|---|
| [103] WGQGTQVTVSS [113] | [SEQ ID NO: 13] |
| [103] WGQGTLVTVSS [113] | [SEQ ID NO: 14] |

or 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 one of the above amino acid sequence; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in each of the sequences above;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in each of the sequences above;

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

In another preferred, but not limiting aspect, a Nanobody of the invention can 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: and in which

i) FR1 is chosen from the group consisting of the amino acid sequence:

[1] QVQLQESGGGLVQAGGSLRLSCAASG [26] [SEQ ID NO: 5]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residue at position is as indicated in the sequence above;

and in which:

ii) FR2 is chosen from the group consisting of the amino acid sequences:

| | |
|---|---|
| [36] WFRQAPGKERELVA [49] | [SEQ ID NO: 6] |
| [36] WFRQAPGKEREFVA [49] | [SEQ ID NO: 7] |
| [36] WFRQAPGKEREGA [49] | [SEQ ID NO: 8] |
| [36] WFRQAPGKQRELVA [49] | [SEQ ID NO: 9] |
| [36] WFRQAPGKQREFVA [49] | [SEQ ID NO: 10] |

and/or from the group consisting of amino acid sequences that have 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in each of the sequences above;

and in which:

iii) FR3 is chosen from the group consisting of the amino acid sequence:

[66] RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA [94] [SEQ ID NO: 12]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 83 and 84 are as indicated in each of the sequences above;

and in which:

iv) FR4 is chosen from the group consisting of the amino acid sequences:

[103] WGQGTQVTVSS [113] [SEQ ID NO: 13]

[103] WGQGTLVTVSS [113] [SEQ ID NO: 14]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 7; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in each of the sequences above;

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

In another preferred, but not limiting aspect, a Nanobody of the invention can 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: and in which

i) FR1 is chosen from the group consisting of the amino acid sequence:

[1] QVQLQESGGGLVQAGGSLRLSCAASG [26] [SEQ ID NO: 5]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residue at position is as indicated in the sequence above;

and in which:

ii) FR2 is chosen from the group consisting of the amino acid sequence:

[36] WYRQAPGKGLEWA [49] [SEQ ID NO: 11]

and/or from the group consisting of amino acid sequences that have 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in each of the sequences above;

and in which:

iii) FR3 is chosen from the group consisting of the amino acid sequence:

[66] RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA [94] [SEQ ID NO: 12]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 83 and 84 are as indicated in each of the sequences above;

and in which:

iv) FR4 is chosen from the group consisting of the amino acid sequence:

[103] WGQGTQVTVSS [113] [SEQ ID NO: 13]

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 7; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s); and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in each of the sequences above;

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

Some other framework sequences that can be present in the Nanobodies of the invention can be found in the European patent EP 656 946 mentioned above (see for example also the granted US equivalent 5,759,808).

In another preferred, but not limiting aspect, a Nanobody of the invention can 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:
and in which

i) FR1 is chosen from the group consisting of the FR1 sequences present in the Nanobodies of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99,

or 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 one of said FR1 sequences; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR1 sequence; and

(3) the Hallmark residue at position is as indicated in said FR1 sequence;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of said FR1 sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 4; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR1 sequence; and

(3) the Hallmark residue at position is as indicated in said FR1 sequence;

and in which:

ii) FR2 is chosen from the group consisting of the FR2 sequences present in the Nanobodies of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99,

or 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 one of said FR2 sequences; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR2 sequence; and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in said FR2 sequence;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of said FR2 sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 5; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR2 sequence; and

(3) the Hallmark residues at positions 37, 44, 45 and 47 are as indicated in said FR2 sequence;

and in which:

iii) FR3 is chosen from the group consisting of the FR3 sequences present in the Nanobodies of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99,

or 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 one of said FR3 sequences; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR3 sequence; and

(3) the Hallmark residues at positions 83 and 84 are as indicated in said FR3 sequence;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of said FR3 sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR3 sequence; and

(3) the Hallmark residues at positions 83 and 84 are as indicated in said FR3 sequence;

and in which:

iv) FR4 is chosen from the group consisting of the FR4 sequences present in the Nanobodies of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to

99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99,

or 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 one of said FR4 sequences; in which

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR4 sequence; and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in said FR4 sequence;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of said FR4 sequences, in which:

(1) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Table 6; and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to said FR4 sequence; and

(3) the Hallmark residues at positions 103, 104 and 108 are as indicated in said FR4 sequence;

and in which:

v) CDR1, CDR2 and CDR3 are as defined above, and are preferably as defined according to one of the preferred definitions above, and are more preferably as defined according to one of the more preferred definitions above.

Some particularly preferred Nanobodies of the invention can be chosen from the group consisting of the amino acid sequences of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99 or 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 one of the amino acid

sequences of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99; in which

(1) the Hallmark residues can be as indicated in Table 2 above;

(2) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Tables 4-7; and/or

(3) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

Some even more particularly preferred Nanobodies of the invention can be chosen from the group consisting of the amino acid sequences of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular in the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99 or 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 one of the amino acid sequences of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99; in which

(1) the Hallmark residues are as indicated in the pertinent sequence chosen from SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99;

(2) any amino acid substitution at any position other than a Hallmark position is preferably either a conservative amino acid substitution (as defined herein) and/or an amino acid substitution as defined in Tables 4-7; and/or

(3) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the pertinent sequence chosen from SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99.

Some of the most preferred Nanobodies of the invention can be chosen from the group consisting of the amino acid sequences of SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, and in particular from the humanized Nanobodies of SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99.

As will be clear from the above, the term Nanobodies of the invention as used herein in its broadest sense also comprises natural or synthetic mutants, variants, alleles, analogs and

orthologs (hereinbelow collectively referred to as "*analogs*") of the Nanobodies mentioned in the SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99.

Generally, such analogs can for example comprise homologous sequences, functional portions, or a functional portion of a homologous sequence (as further defined below) of a Nanobody. Generally, in such analogs, each amino acid residue (other than the Hallmark Residue) in each of the framework regions can be replaced by any other amino acid residue, provided that the total degree of sequence identity of the framework regions remains as defined above. Preferably, however, in such analogs:

- one or more amino acid residues in the above framework sequences are replaced by one or more amino acid residues that naturally occur at the same position in a naturally occurring $V_{HH}$ domain. Some examples of such substitutions are mentioned in Tables 4-7 above;

and/or:

- one or amino acid residues in the above framework sequences are replaced by one or more amino acid residues that can be considered a "conservative" amino acid substitution, as described hereinabove;

and/or:

- one or amino acid residues in the above framework sequences are replaced by one or more amino acid residues that naturally occur at the same position in a naturally occurring $V_H$ domain of a human being. This is generally referred to as "humanization" of the naturally occurring $V_{HH}$/Nanobody in general and of said position in particular, and will be discussed in more detail hereinbelow;

and:

- positions for which only one amino acid residue is mentioned for both the $V_H$ domain and the $V_{HH}$ domain in Tables 4 – 7 above are preferably not replaced.

Also, although generally less preferred, in such analogs, one or more amino acid residues may be deleted from the framework regions and/or inserted into the framework regions (optionally in addition to one or more amino acid substitutions as mentioned above), provided that the total degree of sequence identity of the framework regions remains as defined above. The Hallmark residues should not be deleted. Also, most preferably, amino acid residues for which only one amino acid residue is mentioned for both the $V_H$ domain and the $V_{HH}$ domain in Tables 4 – 7 above are preferably not deleted.

Preferably, such analogs should be such that they still can bind to, have affinity for and/or have specificity for TNF-alpha, i.e. with an affinity and/or a specificity which is at least 10%, preferably at least 50%, more preferably at least 70%, even more preferably at least 80%, such as at least 90%, at least 95%, at least 99% or more, of the affinity and/or specificity of at least one of the Nanobodies of SEQ ID No's SEQ ID NO's 52 to 60, SEQ ID NO's 76 to 86 or SEQ ID NO's 95 to 99, as determined using a suitable assay, for example an assay to determine binding of the analog to TNF, and in particular one of the assays as used in the Examples below.

Generally, such analogs can for example 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 humanized into the codons for the corresponding human amino acid residue(s), 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 (as defined hereinabove). 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 and/or from the further description hereinbelow. Alternatively, and for example, a nucleic acid encoding an 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 be expressed in a suitable host or expression system, upon which the analog thus obtained can optionally be isolated and/or purified so as to provide said analog in essentially isolated form (as defined hereinabove). Another way to provide the analogs involves chemical synthesis of the pertinent amino acid sequence using techniques for peptide synthesis known per se, such as those mentioned hereinbelow.

It will be also generally be clear to the skilled person that Nanobodies (including analogs thereof) can also be prepared starting from human $V_H$ sequences (i.e. amino acid sequences or the corresponding nucleotide sequences), such as for example human $V_H3$ sequences such as DP-47, DP-51, DP-54 or DP-29, by changing one or more amino acid residues in the amino acid sequence of said human $V_H$ domain, so as to provide an amino acid sequence that has (a) a Q at position 108; and/or (b) E at position 44 and/or R at position 45, and preferably E at position 44 and R at position 45; and/or (c) P, R or S at position 103, as described above. 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 $V_H$ domain as a starting point.

The term Nanobodies as used herein in its broadest sense also comprises parts or fragments of the Nanobodies (including analogs) of the invention as defined above, which can again be as further described below.

Generally, parts or fragments of the Nanobodies and/or analogs have amino acid sequences in which, compared to the amino acid sequence of the corresponding full length Nanobody or analog, 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. It is also possible to combine one or more of such parts or fragments to provide a Nanobody of the invention.

Preferably, the amino acid sequence of a Nanobody that comprises one or more parts or fragments of a full length Nanobody and/or analog should have a degree of sequence identity of at least 50%, preferably at least 60%, more preferably at least 70%, such as at least 80%, at least 90% or at least 95%, with the amino acid sequence of the corresponding full length Nanobody.

Also, the amino acid sequence of a Nanobody that comprises one or more parts or fragments of a full length Nanobody and/or analog is preferably such that is 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.

Generally, such parts or fragments of the Nanobodies of the invention will have amino acid sequences in which, compared to the amino acid sequence of the corresponding full length Nanobody of the invention, 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. It is also possible to combine one or more of such parts or fragments to provide a Nanobody of the invention.

According to one preferred embodiment, a fragment as used herein comprises at least one of the CDR's present in a full-sized Nanobody of the invention, preferably at least two of the CDR's present in a full-sized Nanobody of the invention, more preferably at least CDR2

and CDR3 present in a full-sized Nanobody of the invention, such as for example all three CDR's present in a full-sized Nanobody of the invention.

According to another particularly preferred, but non-limiting embodiment, such a part or fragment comprises at least 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.).

Preferably, such parts or fragments should be such that they still can bind to, have affinity for and/or have specificity for TNF-alpha, i.e. with an affinity and/or a specificity which is at least 10%, preferably at least 50%, more preferably at least 70%, even more preferably at least 80%, such as at least 90%, at least 95%, at least 99% or more, of the affinity and/or specificity of the corresponding full-sized Nanobody of the invention, for example an assay to determine binding of the analog to TNF, and in particular one of the assays as used in the Examples below.

From the description hereinabove, it will be clear that the amino acid sequences of the Nanobodies used herein differ at at least one amino acid position in at least one of the framework regions from the amino acid sequences of naturally occurring $V_H$ domains, such as the amino acid sequences of naturally occurring $V_H$ domains of antibodies from human beings. In particular, it will be clear that the amino acid sequences of the Nanobodies used herein differ at at least one of the Hallmark Residues from amino acid sequences of naturally occurring $V_H$ domains, such as the amino acid sequences of naturally occurring $V_H$ domains from antibodies from Camelids and/or human beings.

Thus, according to one specific embodiment, a Nanobody of the invention has an amino acid sequence that differs at at least one amino acid position in one of the framework regions from the amino acid sequence of a naturally occurring $V_H$ domain. According to a more specific, but non-limiting embodiment of the invention, a Nanobody of the invention has an amino acid sequence that differs at at least one of the Hallmark residues from the amino acid sequence of a naturally occurring $V_H$ domain.

From the description hereinabove, it will also be clear that the amino acid sequences of the some of the Nanobodies of the invention, such as the humanized Nanobodies of the invention, will differ at at least one amino acid position in at least one of the framework regions (i.e. either at the position of a Hallmark residue or at another position) from the amino acid sequences of naturally occurring $V_{HH}$ domains. Thus, according to one specific, but non-limiting embodiment, a Nanobody of the invention has an amino acid sequence that

differs at at least one amino acid position in one of the framework regions from the amino acid sequence of a naturally occurring $V_{HH}$ domain. According to a more specific, but non-limiting embodiment of the invention, a Nanobody of the invention has an amino acid sequence that differs at at least one of the Hallmark residues from the amino acid sequence of a naturally occurring $V_{HH}$ domain.

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 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 the reducing immunogenicity of pharmaceutical proteins comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) 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.

With regard to pegylation, its should be noted that generally, the invention also encompasses any Nanobody of the invention and/or polypeptide of the invention that has been pegylated at one or more amino acid positions, preferably in such a way that said pegylation either (1) increases the half-life in vivo; (2) reduces immunogenicity; (3) provides one or more further beneficial properties known per se for pegylation; (4) does not essentially affect the affinity of the Nanobody and/or polypeptide for TNF-alpha (e.g. does not reduce said affinity by more than 90%, preferably not by more than 50 %, and more preferably not

by more than 10%, as determined by a suitable assay, such as those described in the Examples below); and/or (4) does not affect any of the other desired properties of the Nanobodies and/or polypeptides of the invention. Suitable PEG-groups and methods for attaching them, either specifically or non-specifically, will be clear to the skilled person. Suitable kits and reagents for such pegylation can for example be obtained from Nektar (CA, USA).

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 $^{152}Eu$ or others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic acridinium 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, metals chelates or metallic cations (for example metallic cations such as $^{99m}Tc$, $^{123}I$, $^{111}In$, $^{131}I$, $^{97}Ru$, $^{67}Cu$, $^{67}Ga$, 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 malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-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 Targetting, 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 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).

As mentioned above, the invention also relates to proteins or polypeptides comprising at least one $V_{HH}$ domain (i.e. as identified using the methods of the invention) or at least one Nanobody based thereon.

According to one non-limiting embodiment of the invention, such a polypeptide of the invention essentially consists of a Nanobody. 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 (as mentioned above) or corresponds to the amino acid sequence of a Nanobody in which a limited number of amino acid residues, such as 1-10 amino acid residues and preferably 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, have been added to the amino terminal end, to the carboxy terminal end, or both to the amino terminal end and to 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:

a) can comprise an N-terminal Met residue, for example as result of expression in a heterologous host cell or host organism.

b) 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;

c) 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;

d) 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, glutatione residues and a myc-tag such as AAAEQKLISEEDLNGAA [SEQ ID NO: 476];

e) 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 embodiment, 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.

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 immunogenicity 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).

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). For example, the further amino acid sequence may provide a second binding site 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. Reference is for example made to EP 0 368 684, WO 91/01743, WO 01/45746 and WO 04/003019 (in which various serum proteins are mentioned), the International application by applicant entitled "Nanobodies™ against amyloid-beta and polypeptides comprising the same for the treatment of degenerative neural diseases such as Alzheimer's disease" (in which various other proteins are mentioned), as well as to Harmsen et al., Vaccine, 23 (41); 4926-42.

According to another embodiment, 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 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) $CH_1$, $CH_2$ and/or $CH_3$ domains, optionally via a linker sequence. For instance, a Nanobody linked to a suitable $CH_1$ 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 $V_H$ domains have been replaced by a Nanobody of the invention. Also, two Nanobodies could be linked to a CH3 domain (optionally via a linker) to provide a construct with increased half-life in vivo.

According to one specific embodiment of a polypeptide of the invention, one or more Nanobodies of the invention may linked to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the invention and/or may confer the ability to bind to one or more Fc receptors. For example, for this purpose, and without being limited thereto, the one or more further amino acid sequences may comprise one or more $CH_2$ and/or $CH_3$ 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, from IgE or from another human Ig. 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 $CH_2$ and/or $CH_3$ domain have been replaced by human $CH_2$ and $CH_3$ domains, so as to provide an immunoglobulin that consists of 2 heavy chains each comprising a Nanobody and human CH2 and CH3 domains (but no CH1 domain), which immunoglobulin has the effector function provided by the CH2 and CH3 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 04/058820, WO 99/42077 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. $CH_2$ and/or $CH_3$ 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 CH3 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.

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 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 WO 03/055527.

According to one non-limiting embodiment, one or more amino acid residues can be added to, inserted in and/or substituted in the amino acid sequence of a Nanobody or polypeptide of the invention, so as to provide one or more specific amino acid residues for attachment of a PEG-group.

The efficacy of protein pharmaceuticals depends on its potency to neutralize the target but also on the intrinsic pharmacokinetics of the potential drug. Because the kidney generally filters out molecules below 60,000 Daltons (Da), efforts to reduce clearance have focussed on increasing the molecular weight of the biopharmaceutical through protein fusions (Syed et al., 1997), glycosylations or modification with polyethylene glycol polymers, i.e., PEGylation (Lee et al., 1999; Abuchowski et al., 1977; Nucci et al., 1991; Lecolley, et al. Chem

Commun, 2004; Tao et al., J Am Chem Soc, 2004; Mantovani et al., 2005). These methods successfully extend the in vivo exposure of the biopharmaceutical.

Alternatively, the half-life can be extended using another pegylating agent, POLY PEG for conjugation to the bivalent Nanobodies,TNF56 or TNF55. POLY PEG are comb shape polymers with PEG teeth on a methacrylic backbone. POLY PEGs can vary on the length of the PEG chain, on the methacrylic backbone and on the active end-group which determines the method of conjugation of the POLY PEG to the Nanobody. Site-specific conjugation to the C-terminal cysteine present in the Nanobodies can be achieved through the active maleimide end-group in the POLY PEG.

The invention also encompasses any Nanobody of the invention and/or polypeptide of the invention that has been glycosylated at one or more amino acid positions, usually depending upon the host used to express the Nanobody or polypeptide of the invention (as further described below).

According to one non-limiting embodiment, one or more amino acid residues can be added to, inserted in and/or substituted in the amino acid sequence of a Nanobody or polypeptide of the invention, so as to provide one or more specific amino acid residues and/or a site that can be glycosylated by the host organism used. By means of a preferred, but non-limiting example, the N-residue on position 50 within CDR2 of a Nanobody of the invention can for example be replaced by a Q, D or S residue so as to provide a glycosylation site, e.g. for glycosylation by *Pichia*.

According to another embodiment, a polypeptide of the invention can comprise the amino acid sequence of a Nanobody, 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 with at least one further amino acid sequence.

Again, said further amino acid sequence(s) 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, according to one preferred, but non-limiting embodiment, said further amino acid sequence may comprise at least one further Nanobody, so as to provide a polypeptide of the invention that comprises at least two, such as three, four or five, Nanobodies, in which said Nanobodies may optionally be linked via one or more linker sequences (as defined herein).

Polypeptides of the invention comprising two or more Nanobodies will also referred to herein as "multivalent" polypeptides. 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 a multivalent polypeptide of the invention, the two or more Nanobodies may be the same or different. For example, the two or more Nanobodies in a multivalent polypeptide of the invention:

- may be directed against the same antigen, i.e. against the same parts or epitopes of said antigen or against two or more different parts or epitopes of said antigen; and/or:
- may be directed against the different antigens;

or a combination thereof.

Thus, a bivalent polypeptide of the invention for example:

- may comprise two identical Nanobodies;
- may comprise a first Nanobody directed against a first part or epitope of an antigen and a second Nanobody directed against the same part or epitope of said antigen or against another part or epitope of said antigen;
- or may comprise a first Nanobody directed against a first antigen and a second Nanobody directed against a second antigen different from said first antigen;

whereas a trivalent Polypeptide of the Invention for example:

- may comprises three identical or different Nanobodies directed against the same or different parts or epitopes of the same antigen;
- may comprise two identical or different Nanobodies directed against the same or different parts or epitopes on a first antigen and a third Nanobody directed against a second antigen different from said first antigen; or
- may comprise 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,

Polypeptides of the invention that contain at least two Nanobodies, in which at least one Nanobody is directed against a first antigen and at least one Nanobody is directed against a second antigen different from the first antigen, will also be referred to as "multispecific" Nanobodies. Thus, a "bispecific" Nanobody is a Nanobody that comprises at least one Nanobody directed against a first antigen and at least one further Nanobody directed against a

second antigen, whereas a "trispecific" Nanobody is a Nanobody that comprises at least one Nanobody directed against a first antigen, at least one further Nanobody directed against a second antigen, and at least one further Nanobody directed against a third antigen; etc.

Accordingly, in their simplest form, a bispecific polypeptide of the invention is a bivalent polypeptide of the invention (as defined herein), comprising a first Nanobody directed against a first antigen 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 a first antigen, 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 any number of Nanobodies directed against two or more different antigens.

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, as well as to EP 0 822 985.

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, its 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 $V_H$ and $V_L$ 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).

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 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-3 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 on 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 on some limited routine experiments.

Linkers for use in multivalent and multispecific polypeptides will be clear to the skilled person, and for example include gly-ser linkers, for example of the type $(gly_xser_y)_z$, such as (for example $(gly_4ser)_3$ or $(gly_3ser_2)_3$, as described in WO 99/42077, hinge like regions such as the hinge regions of naturally occurring heavy chain antibodies or similar sequences. For other suitable linkers, reference is also made to the general background art cited above. Some particularly preferred linkers are given in SEQ ID NO's 68 and 69.

Linkers can also provide some functionality for the multivalent or multispecific polypeptide. For example, linkers containing one or more charged amino acid residues (see Table 1 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.

As mentioned herein, in a protein or polypeptide of the invention, the anti-TNF Nanobodies mentioned herein are preferably linked in such a way that said protein or polypeptide, upon binding to a TNF trimer, is capable inhibiting or reducing the TNF receptor crosslinking that is mediated by said TNF trimer and/or the signal transduction that is mediated by such receptor crosslinking; and/or in such a way that the protein or polypeptide is capable of intramolecular binding to at least two TNF receptor binding sites on a TNF trimer. Suitable linkers are as described herein.

As also mentioned herein, whether a protein or polypeptide provides intermolecular binding or extramolecular binding can be assessed (at least initially) by a size exclusion chromatography. By Size Exclusion Chromatography the complexes of TNF-alpha and antibodies can be analyzed for determining the number and the ratio of antibody and TNF-alpha molecules in the complex. From these data it can be deduced if inter- or intramolecular binding occurs, as was done by Santora and colleagues (Santora, L.C., et al, Anal Biochem. 2001) for establishing the stoichiometry of binding of monoclonal antibody D2E7 (Humira) to TNF-alpha at different ratios of antibody and target. From the molecular weight of the complex it was concluded that three antibody molecules complexed with three TNF trimers, thereby indicating that the antibody binds in an intermolecular mode. Similar experiments were performed with bivalent Nanobodies, in which a very short linker induced the formation of large molecular complexes, which were obtained by intermolecular bonds. However, the same bivalent Nanobodies constructs with longer linkers eluted from the gel filtration column as discrete small complexes, thereby demonstrating that intramolecular bonds were formed. Combined with the bioassay data, in which the longer linker containing Nanobody TNF1 had an optimal potency (complete neutralization of amount of TNF used in the assay, i.e. 10 pM), it can be concluded that intramolecular binding of the bivalent Nanobody efficiently prevents cross-linking of two cell bound receptors and the associated receptor activation. Known monoclonal antibodies such as Humira or Remicade can not form such intramolecular bonds, leaving always two receptor bindingsites on the trimeric TNF molecule to a certain degree available for interaction with cell bound receptor, which translates into less potent neutralization as measured in the bioassay.

Alternatively, whether a protein or polypeptide provides intermolecular binding or extramolecular binding can be assessed by crystallography and/or molecular modelling (or other suitable *in silico* techniques). A model of a trimeric TNF30/TNF-alpha complex was generated based on the crystal structure of the monomeric wild type TNF1/TNF-alpha

complex. From this structure the final TNF30-linker-ALB8-linker-TNF30 construct was modeled. The TNF30-linker-ALB8-linker-TNF30 construct was modelled starting from the trimer of TNFα with two TNF30 molecules bound. As the structure of the ALB8 is not known, a third TNF30 molecule was used instead, which was placed in between the other two Nanobodies along the line between the N- and C-termini. The 9 amino acid linkers were then added manually.

The model is shown in Figure 62. Clearly, the 9 amino acid linkers together with the ALB8 provide ample room to span the about 66 Å between the two TNF30 domains bound to TNFα. ALB8 by itself already spans 40 Å, and each linker can span another ~27 Å in completely extended conformation. As a result, the ALB8 has quite some flexibility of movement, and it is not expected that its binding to albumin would interfere much with the binding to TNFα.

Moreover, it is likely that the linkers can be shortened without affecting avidity, especially in the case of the linker that is C-terminal of ALB8. This may have the beneficial effect of increased binding to the same TNFα trimer versus crosslinking trimers, because the probability that the second TNF30 associates with a different TNFα increases with the length of the linker.

As also further described herein, a multispecific polypeptide of the invention directed against a desired antigen and against at least one serum protein, such as the serum proteins mentioned hereinbelow, and in particular against human serum albumin, may show increased half-life in serum, compared to the corresponding monovalent Nanobody.

As mentioned hereinabove, the methods described herein are particularly suited for generating such multivalent of multispecific polypeptides of the invention.

In a polypeptide of the invention, the at least one Nanobody may also be linked to a conventional $V_H$ domain or to a natural or synthetic analog of a $V_H$ domain, optionally via a linker sequence.

In a polypeptide of the invention, the at least one Nanobody may also be linked to a $V_L$ domain or to a natural or synthetic analog of a $V_L$ domain, optionally via a linker sequence, so as to provide a polypeptide of the invention that is in the form analogous to a conventional scFv fragment, but containing a Nanobody instead of a $V_H$ domain.

In a polypeptide of the invention, the at least one Nanobody may also be linked to one or more of a CH1, CH2 and/or CH3 domain, optionally via a linker sequence. For instance, a Nanobody linked to a suitable CH1 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 $V_H$ domains have been replaced by a Nanobody. Such fragments may also be heterospecific or bispecific, i.e. directed against two or more antigens. A Nanobody linked to suitable CH2 and CH3 domains, for example derived from Camelids, could be used to form a monospecific or bispecific heavy chain antibody. Finally, a Nanobody linked to suitable CH1, CH2 and CH3 domains, for example derived from a human being, could be used – together with suitable light chains - to form an antibody that is analogous to a conventional 4-chain antibody, but in which one or both of the conventional $V_H$ domains have been replaced by a Nanobody.

Also, in addition to the one or more Nanobodies, Polypeptides of the Invention can also contain functional groups, moieties or residues, for example therapeutically active substances, such as those mentioned below, and/or markers or labels, such as fluorescent markers, isotopes, etc., as further described hereinbelow.

The Nanobodies of the invention, the polypeptides of the invention, and nucleic acids encoding the same, can be prepared in a manner known per se, as will be clear to the skilled person from the further description herein. Some preferred, but non-limiting methods for preparing the Nanobodies, polypeptides and nucleic acids include the methods and techniques mentioned above and/or further described hereinbelow .

As will be clear to the skilled person, one particularly useful method for preparing a Nanobody and/or a polypeptide of the invention generally comprises the steps of:

- 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 Nanobody or polypeptide of the invention (also referred to herein as a "*nucleic acid of the invention*"), optionally followed by:
- isolating and/or purifying the Nanobody or polypeptide of the invention thus obtained.

In particular, such a method may comprise the steps of:

- 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 Nanobody and/or polypeptide of the invention; optionally followed by:
- isolating and/or purifying the 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 embodiment of the invention, the nucleic acid of the invention is in essentially isolated from, as defined hereinabove.

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 $V_{HH}$ domains can for example be subjected to site-directed mutagenesis, so as 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 casettes 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 GPCR 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 hereinbelow. 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 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 embodiment, a genetic construct of the invention comprises

a) at least one nucleic acid of the invention; operably connected to

b) one or more regulatory elements, such as a promoter and optionally a suitable terminator;

c) and optionally also

d) 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 below); 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.

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 promotor). 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 "*operable*" 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 bacterial cells, such as those mentioned hereinbelow 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 (succesfully) transformed with the nucleotide sequence of the invention to be distinguished from host cells/organisms that have not been (succesfully) transformed. Some preferred, but non-limiting examples of such markers are genes that provide resistance against antibiotics (such as kanamycine or ampicilline), 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.

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 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-6,207,410, 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 hereinbelow.

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 below.

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. The host cell or host organism may 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 fluorescens*; 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 carnosus*; 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 lipolytica*;
- 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 derived a cell or cell line derived from a human, from the 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 hereinabove, 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 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 parvoviruses 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, for example 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; Anderson, Science 256 (1992),808-813; Verma, Nature 389 (1994),239; Isner, Lancet 348 (1996),370-374; Muhlhauser, Circ. Res. 77 (1995),1077-1086; Onodera, Blood 91; (1998),30- 36; Verma, Gene Ther. 5 (1998),692-699; Nabel, Ann. N.Y. Acad. Sci. : 811 (1997), 289-292; Verzeletti, Hum. Gene Ther. 9 (1998), 2243-51; Wang, Nature Medicine 2 (1996),714-716; WO 94/29469; WO 97/00957, US 5,580,859; US 5,589,466; or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640. For example, in situ expression of ScFv fragments (Afanasieva et al., Gene Ther., 10, 1850-1859 (2003)) and of diabodies (Blanco et al., J. Immunol, 171, 1070-1077 (2003)) has been described in the art.

For expression of the Nanobodies in a cell, they may also be expressed as so-called "intrabodies", as for example described in WO 94/02610, WO 95/22618 and US-A-7004940; WO 03/014960; in Cattaneo, A. & Biocca, S. (1997) Intracellular Antibodies: Development and Applications. Landes and Springer-Verlag; and in Kontermann, Methods 34, (2004), 163-170.

For production, the 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 Bombyx mori.

Furthermore, the 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* Zubay 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*, *Pichia pastoris*, *S. cerevisiae* that are suitable for large scale expression/production/fermentation, and in particular for large scale pharmaceutical 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 are 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 Nanobody or protein to be obtained.

Thus, according to one non-limiting embodiment of the invention, the Nanobody or polypeptide of the invention is glycosylated. According to another non-limiting embodiment of the invention, the Nanobody or polypeptide of the invention is non-glycosylated.

According to one preferred, but non-limiting embodiment of the invention, the 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 embodiment of the invention, the 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 embodiment of the invention, the 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 Nanobodies and the proteins of the invention, the Nanobodies and proteins of the invention can be produced either intracellullarly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or 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 hosts 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 a Nanobody or a polypeptide of the invention, can be used.

Thus, according to one non-limiting embodiment of the invention, the Nanobody or polypeptide of the invention is a 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 embodiment of the invention, the Nanobody or polypeptide of the invention is a 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 lacUV5 promoter); arabinose promoter; left- (PL) and rightward (PR) promoter of phage lambda; promoter of the trp 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 Tn10 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: GAL1,10,7 (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-1 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), pMC1neo (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 37110), 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 bacterials 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: PelB, 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 (pho1), invertase (Suc), 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 succesfully 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 of 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 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 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 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 of the invention may be glycosylated, again depending on the host cell/host organism used.

The amino acid sequence 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 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 of the inventions may be formulated as a pharmaceutical preparation 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 hereinbelow.

Generally, the 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 and WO 04/041867) as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990) or Remington, the Science and Practice of Pharmacy, 21th Edition, Lippincott Williams and Wilkins (2005).

For example, the 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 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 a 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 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 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 Nanobody or polypeptide of the invention. The percentage of 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 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, corn 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 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 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 Nanobodies and polypeptides of the invention may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the 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 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 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 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 celluloses 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 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 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 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 Nanobodies and polypeptides of the invention required for use in treatment will vary not only with the particular 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 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 TNF-relates disease or disorder as mentioned herein, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount 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 from, the diseases and disorders mentioned herein.

The invention also 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 a Nanobody or polypeptide of the invention to a patient, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount 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 a Nanobody of the invention, of a polypeptide of the invention, and/or of a pharmaceutical composition comprising the same.

In another embodiment, 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 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 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 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 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 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 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 Nanobody and polypeptide of the invention to be used, the specific route of

administration and the specific pharmaceutical formulation or composition used, the 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 Nanobody or polypeptide of the invention will be used. It is however within the scope of the invention to use two or more Nanobodies and/or polypeptides of the invention in combination.

The Nanobodies 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 Nanobodies and polypeptides of the invention may be used in combination with other pharmaceutically active compounds or principles 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 administered to be 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.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that contains at least one Nanobody of the invention or at least one polypeptide of the invention and at least one suitable carrier (i.e. a carrier suitable for veterinary use), and optionally one or more further active substances.

The invention also relates to the use of a Nanobody of the invention and/or of a polypeptide of the invention in the preparation of a pharmaceutical composition, in particular in the preparation of a pharmaceutical composition for the prevention and/or treatment (including but not limiting to the alleviation of at least one symptom) of a disease or disorder mediated by TNF-alpha and/or associated with TNF-alpha (for example associated with an

abnormal activity of TNF-alpha, abnormal levels of TNF-alpha, abnormal expression of TNF-alpha and/or abnormal sensitivity or response to TNF-alpha), or of one of the biological phenomena associated with TNF-alpha, such as one of the diseases or disorders mentioned above.

The invention also relates to a method for preventing and/or treating (including but not limiting to the alleviation of at least one symptom) of a disease or disorder mediated by TNF-alpha and/or associated with TNF-alpha (for example associated with an abnormal activity of TNF-alpha, abnormal levels of TNF-alpha, abnormal expression of TNF-alpha and/or abnormal sensitivity or response to TNF-alpha, or of one of the biological phenomena associated with TNF-alpha), such as one of the diseases or disorders mentioned above, which method comprises administering to a subject in need thereof a therapeutically active amount of a Nanobody of the invention, of polypeptide of the invention, and/or of a pharmaceutical composition as described above.

The present invention provides polypeptides comprising one or more nanobodies directed towards tumor necrosis factor alpha (TNF-alpha). The present invention further relates to their use in diagnosis and therapy. Such antibodies may have a framework sequence with high homology to the human framework sequences. Compositions comprising antibodies to tumor necrosis factor alpha (TNF-alpha) alone or in combination with other drugs are described.

Tumor necrosis factor alpha (TNF-alpha) is believed to play an important role in various disorders, for example in inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis. Both TNF-alpha and the receptors (CD120a, CD120b) have been studied in great detail. TNF-alpha in its bioactive form is a trimer and the groove formed by neighboring subunits is important for the cytokine-receptor interaction. Several strategies to antagonize the action of the cytokine have been developed and are currently used to treat various disease states.

A TNF-alpha inhibitor which has sufficient specificity and selectivity to TNF-alpha may be an efficient prophylactic or therapeutic pharmaceutical compound for preventing or treating disorders where TNF-alpha has been implicated as causative agent. Methods of treating toxic shock (EP 486526), tumor regression, inhibition of cytotoxicity (US 6448380, US 6451983, US 6498237), autoimmune disease such as RA and Crohn's disease (EP 663836, US 5672347, US 5656272), graft versus host reaction (US 5672347), bacterial meningitis (EP 585705) by means of an antibody to TNF-alpha have been described.

Yet none of the presently available drugs are completely effective for the treatment of autoimmune disease, and most are limited by severe toxicity. In addition, it is extremely difficult and a lengthy process to develop a new chemical entitiy (NCE) with sufficient potency and selectivity to such target sequence. Antibody-based therapeutics on the other hand have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. In addition, the development time can be reduced considerably when compared to the development of new chemical entities (NCE's). However, conventional antibodies are difficult to raise against multimeric proteins where the receptor-binding domain of the ligand is embedded in a groove, as is the case with TNF-alpha. Heavy chain antibodies described in the invention which are derived from Camelidae, are known to have cavity-binding propensity (WO97/49805; Lauwereys et al, EMBO J. 17, 5312, 1998)). Therefore, such heavy chain antibodies are inherently suited to bind to receptor binding domains of such ligands as TNF. In addition, such antibodies are known to be stable over long periods of time, therefore increasing their shelf-life (Perez et al, Biochemistry, 40, 74, 2001). Furthermore, such heavy chain antibody fragments can be produced 'en-masse' in fermentors using cheap expression systems compared to mammalian cell culture fermentation, such as yeast or other microorganisms (EP 0 698 097).

The use of antibodies derived from sources such as mouse, sheep, goat, rabbit etc., and humanised derivatives thereof as a treatment for conditions which require a modulation of inflammation is problematic for several reasons. Traditional antibodies are not stable at room temperature, and have to be refrigerated for preparation and storage, requiring necessary refrigerated laboratory equipment, storage and transport, which contribute towards time and expense. Refrigeration is sometimes not feasible in developing countries. Furthermore, the manufacture or small-scale production of said antibodies is expensive because the mammalian cellular systems necessary for the expression of intact and active antibodies require high levels of support in terms of time and equipment, and yields are very low. Furthermore the large size of conventional antibodies, would restrict tissue penetration, for example, at the site of inflamed tissue. Furthermore, traditional antibodies have a binding activity which depends upon pH, and hence are unsuitable for use in environments outside the usual physiological pH range such as, for example, in treating gastric bleeding, gastric surgery. Furthermore, traditional antibodies are unstable at low or high pH and hence are not suitable for oral administration. However, it has been demonstrated that camelidae antibodies resist harsh conditions, such as extreme pH, denaturing reagents and high temperatures

(Dumoulin et al, Protein Science 11, 500, 2002), so making them suitable for delivery by oral administration. Furthermore, traditional antibodies have a binding activity, which depends upon temperature, and hence are unsuitable for use in assays or kits performed at temperatures outside biologically active-temperature ranges (e.g. 37 ± 20°C).

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, it is known by the skilled addressee that an antibody which has been obtained for a therapeutically useful target requires additional modification in order to prepare it for human therapy, so as to avoid an unwanted immunological reaction in a human individual upon administration thereto. The modification process is commonly termed "humanisation". It is known by the skilled artisan that antibodies raised in species, other than in humans, require humanisation to render the antibody therapeutically useful in humans ( (1) CDR grafting : Protein Design Labs: US 6180370, US 5693761; Genentech US 6054297; Celltech: 460167, EP 626390, US 5859205; (2) Veneering: Xoma: US 5869619, US 5766886, US 5821123). There is a need for a method for producing antibodies which avoids the requirement for substantial humanisation, or which completely obviates the need for humanisation. There is a need for a new class of antibodies which have defined framework regions or amino acid residues and which can be administered to a human subject without the requirement for substantial humanisation, or the need for humanisation at all.

Another important drawback of conventional antibodies is that they are complex, large molecules and therefore relatively unstable, and they are sensitive to breakdown by proteases. This means that conventional antibody drugs cannot be administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation because they are not resistant to the low pH at these sites, the action of proteases at these sites and in the blood and/or because of their large size. They have to be administered by injection (intravenously, subcutaneously, etc.) to overcome some of these problems. Administration by injection requires specialist training in order to use a hypodermic syringe or needle correctly and safely. It further requires sterile equipment, a liquid formulation of the therapeutic polypeptide, vial packing of said polypeptide in a sterile and stable form and, of the subject, a suitable site for entry of the needle. Furthermore, subjects commonly experience physical and psychological stress prior to and upon receiving an injection. Therefore, there is need for a method for the delivery of therapeutic polypeptides which avoids the need for injection which

is not only cost/time saving, but which would also be more convenient and more comfortable for the subject.

Nanobody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, improving further their intrinsic and functional affinity can lead to many benefits for a patient such as reduced dose of therapeutic, faster therapy, and reduced side effects.

One embodiment of the present invention is an anti-TNF-alpha nanobody, which nanobody is preferably as further defined above.

One embodiment of the present invention is an anti-TNF-alpha polypeptide comprising at least one anti-TNF-alpha nanobody, which polypeptide is preferably as further defined above.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above further comprising at least one nanobody directed against a serum protein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above wherein said serum protein is any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, or fibrinogen.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above further comprising at least one nanobody selected from the group consisting of anti-IFN-gamma nanobody, anti-TNF-alpha receptor nanobody and anti-IFN-gamma receptor nanobody.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, wherein the number of nanobodies directed against TNF-alpha is at least two.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, wherein at least one nanobody is a humanized Camelidae $V_{HH}$s.

Another embodiment of the present invention is a composition comprising an anti-TNF-alpha polypeptide as described above and at least one nanobody from the group consisting of anti-IFN-gamma nanobody, anti-TNF-alpha receptor nanobody and anti-IFN-gamma receptor nanobody, for simultaneous, separate or sequential administration to a subject.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above, wherein said nanobody is an

homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length nanobody.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above, wherein the anti-TNF-alpha polypeptide is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length anti-TNF-alpha polypeptide.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above wherein at least one nanobody is a Camelidae $V_{HH}$.

Another embodiment of the present invention is a nucleic acid encoding an anti-TNF-alpha polypeptide as described above.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of an anti-TNF-alpha polypeptide as described above, to Tumor Necrosis Factor-alpha comprising the steps of:

(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of an anti-TNF-alpha polypeptide as described above and Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a method of identifying an agent that modulates Tumor Necrosis Factor-alpha-mediated disorders through the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified, said candidate modulator as an agent that modulates Tumor Necrosis Factor alpha-mediated disorders.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of Tumor Necrosis Factor alpha to its receptor through the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor-alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of Tumor Necrosis Factor-alpha to its receptor.

Another embodiment of the present invention is a kit for screening for agents that modulate Tumor Necrosis Factor-alpha-mediated disorders comprising an anti-TNF-alpha polypeptide as described above and Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is an unknown agent that modulates the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha, identified according to the method as described above.

Another embodiment of the present invention is an unknown agent that modulates Tumor Necrosis Factor-alpha-mediated disorders, identified according to the methods as described above.

Another embodiment of the present invention is an unknown agent as described above wherein said disorders are one or more of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a nucleic acid as described above, or a composition as described above, or an agent as described above for treating and/or preventing and/or alleviating disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a nucleic acid as described above, or a composition as described above, or an agent as described above for the preparation of a medicament for treating and/or preventing and/or alleviating disorders relating to inflammatory reactions.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or

alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the gastric environment without the substance being inactivated.

Another embodiment of the present invention is an use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the gastric environment without the substance being inactivated.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a

medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the tissues beneath the tongue effectively.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the tissues beneath the tongue effectively.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the skin effectively.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the skin effectively.

Another embodiment of the present invention is a method as described above, a kit as described above, a nucleic acid or agent as described above, use of a nucleic acid or agent as described above, a composition as described above, use of a composition as described above, an anti-TNF-alpha polypeptide as described above, use of an anti-TNF-alpha polypeptide as described above wherein said disorders are any of inflammation, rheumatoid arthritis, COPD, asthma, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, multiple sclerosis, Addison's disease, Autoimmune hepatitis, Autoimmune parotitis, Diabetes Type I, Epididymitis, Glomerulonephritis, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, Hemolytic anemia, Systemic lupus erythematosus, Male infertility, Multiple sclerosis, Myasthenia Gravis, Pemphigus, Psoriasis, Rheumatic fever, Rheumatoid arthritis, Sarcoidosis, Scleroderma, Sjogren's syndrome, Spondyloarthropathies, Thyroiditis, and Vasculitis.

Another embodiment of the present invention is a composition comprising a nucleic acid or agent as described above, an anti-TNF-alpha polypeptide as described above, or a composition as described above, and a suitable pharmaceutical vehicle.

Another embodiment of the present invention is a method of diagnosing a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha comprising:

(a) contacting a sample with an anti-TNF-alpha polypeptide as described above,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a kit for screening for a disorder as cited above, using a method as described above.

Another embodiment of the present invention is a kit for screening for a disorder as cited above comprising an isolated anti-TNF-alpha polypeptide as described above.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above for the purification of said Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above for inhibiting the interaction between Tumor Necrosis Factor-alpha and one or more Tumor Necrosis Factor-alpha receptors.

Another embodiment of the present invention is a method for producing an anti-TNF-alpha polypeptide as described above comprising the steps of:

(a) obtaining double stranded DNA encoding a Camelidae $V_{HH}$ directed to Tumor Necrosis Factor alpha,

(b) cloning and expressing the DNA selected in step (b).

Another embodiment of the present invention is a method of producing an anti-TNF-alpha polypeptide as described above comprising:

(a) culturing host cells comprising nucleic acid capable of encoding an anti-TNF-alpha polypeptide as described above, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

Another embodiment of the present invention is a method as described above, wherein said host cells are bacterial or yeast.

Another embodiment of the present invention is a kit for screening for any of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome or multiple sclerosis comprising an anti-TNF-alpha polypeptide as described above.

$V_{HH}$s, according to the present invention, and as known to the skilled addressee are heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from Camelidae as described in WO 94/04678 (and referred to hereinafter as $V_{HH}$ domains or nanobodies). $V_{HH}$ molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies. Furthermore, in vitro expression of $V_{HH}$s produces high yield, properly folded functional $V_{HH}$s. In addition, antibodies generated in Camelids will recognize epitopes other than those recognised by antibodies generated in vitro through the use of antibody libraries or via immunisation of mammals other than Camelids (WO 9749805). As such, anti-TNF-alpha $V_{HH}$'s may interact more efficiently with TNF-alpha than conventional antibodies, thereby blocking its interaction with the TNF-alpha receptor more efficiently.

TNF-alpha is also a fragment of TNF-alpha, capable of eliciting an immune response. TNF-alpha is also a fragment of TNF-alpha, capable of binding to a nanobody raised against the full length TNF-alpha.

A nanobody directed against TNF-alpha means nanobody that it is capable of binding to TNF-alpha with an affinity of better than $10^{-6}$ M.

One embodiment of the present invention is an anti-TNF polypeptide, wherein the nanobodies comprise Camelidae $V_{HH}$ directed against TNF-alpha.

The one or more nanobodies of the anti-TNF polypeptide which are directed against a TNF-alpha may be of the same sequence. Alternatively they may not all have the same sequence. It is within the scope of the invention that an anti-TNF polypeptide comprises anti-TNF-alpha nanobodies which do not all share the same sequence, but which are directed against the same target, one or more antigens thereof.

The present invention further relates to an anti-TNF-alpha polypeptide, wherein said nanobody is a $V_{HH}$ directed against TNF-alpha, wherein the $V_{HH}$ belongs to a class having human-like sequences. The class is characterised in that the $V_{HH}$s carry an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine,

tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 and a tryptophan at position 103, according to the Kabat numbering. Another human-like class of Camelidae nanobodies have been described in WO03035694 and contain the hydrophobic FR2 residues typically found in conventional antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in VH from double-chain antibodies. As such, peptides belonging to these two classes show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanisation. The invention also relates to nucleic acids capable of encoding said polypeptides.

Any of the $V_{HH}$s as used by the invention may be of the traditional class or of the classes of human-like Camelidae antibodies. Said antibodies may be directed against whole TNF-alpha or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include the full length Camelidae antibodies, namely Fc and $V_{HH}$ domains, chimeric versions of heavy chain Camelidae antibodies with a human Fc domain or $V_{HH}$'s by themselves or derived fragments.

Anti-serum albumin $V_{HH}$'s may interact in a more efficient way with serum albumin than conventional antibodies which is known to be a carrier protein. As a carrier protein some of the epitopes of serum albumin may be inaccessible by bound proteins, peptides and small chemical compounds. Since $V_{HH}$'s are known to bind into 'unusual' or non-conventional epitopes such as cavities (WO 97/49805), the affinity of such $V_{HH}$'s to circulating albumin may be increased.

The present invention also relates to the finding that an anti-TNF polypeptide as described herein further comprising one or more nanobodies directed against one or more serum proteins of a subject, surprisingly has significantly prolonged half-life in the circulation of said subject compared with the half-life of the anti-TNF-alpha nanobody when not part of said construct. Furthermore, the said polypeptides were found to exhibit the same favourable properties of nanobodies such as high stability remaining intact in mice, extreme pH resistance, high temperature stability and high target affinity.

The serum protein may be any suitable protein found in the serum of subject. In one aspect of the invention, the serum protein is serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, or fibrinogen. Depending on the intended use such as

the required half-life for effective treatment and/or compartimentalisation of the target antigen, the $V_{HH}$-partner can be directed to one of the above serum proteins.

According to a specific, but non-limiting aspect of the invention, the Nanobody against human serum albumin consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

(iv) CDR1 is an amino acid sequence chosen from the group consisting of:

| | |
|---|---|
| SFGMS | [SEQ ID NO: 36] |
| LNLMG | [SEQ ID NO: 37] |
| INLLG | [SEQ ID NO: 38] |
| NYWMY; | [SEQ ID NO: 39] |

and/or from the group consisting of amino acid sequences that have 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

and in which:

(v) CDR2 is an amino acid sequence chosen from the group consisting of:

| | |
|---|---|
| SISGSGSDTLYADSVKG | [SEQ ID NO: 40] |
| TITVGDSTNYADSVKG | [SEQ ID NO: 41] |
| TITVGDSTSYADSVKG | [SEQ ID NO: 42] |
| SINGRGDDTRYADSVKG | [SEQ ID NO: 43] |
| AISADSSTKNYADSVKG | [SEQ ID NO: 44] |
| AISADSSDKRYADSVKG | [SEQ ID NO: 45] |
| RISTGGGYSYYADSVKG | [SEQ ID NO: 46] |

or 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 one of the above amino acid sequences; in which

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

and in which:

(vi) CDR3 is an amino acid sequence chosen from the group consisting of:

DREAQVDTLDFDY [SEQ ID NO: 47]

or 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 one of the above amino acid sequences; in which

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

or from the group consisting of:

GGSLSR [SEQ ID NO: 48]

| | |
|---|---|
| RRTWHSEL | [SEQ ID NO: 49] |
| GRSVSRS | [SEQ ID NO: 50] |
| GRGSP | [SEQ ID NO: 51] |

and/or from the group consisting of amino acid sequences that have 3, 2 or only 1 "amino acid difference(s)" (as defined herein) with one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences.

In another aspect, the invention relates to a Nanobody against human serum albumin, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), which is chosen from the group consisting of domain antibodies and/or single domain antibodies with the one of the following combinations of CDR1, CDR2 and CDR3, respectively:

- **CDR1:** SFGMS; **CDR2:** SISGSGSDTLYADSVKG; **CDR3:** GGSLSR;
- **CDR1:** LNLMG; **CDR2:** TITVGDSTNYADSVKG; **CDR3:** RRTWHSEL;
- **CDR1:** INLLG; **CDR2:** TITVGDSTSYADSVKG; **CDR3:** RRTWHSEL;
- **CDR1:** SFGMS; **CDR2:** SINGRGDDTRYADSVKG; **CDR3:** GRSVSRS;
- **CDR1:** SFGMS; **CDR2:** AISADSSDKRYADSVKG; **CDR3:** GRGSP;
- **CDR1:** SFGMS; **CDR2:** AISADSSDKRYADSVKG; **CDR3:** GRGSP;
- **CDR1:** NYWMY; **CDR2:** RISTGGGYSYYADSVKG; **CDR3:** DREAQVDTLDFDY.

In the Nanobodies of the invention that comprise the combinations of CDR's mentioned above, 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

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences;

and/or chosen from the group consisting of amino acid sequences that have 3, 2 or only 1 (as indicated in the preceding paragraph) "amino acid difference(s)" (as defined herein) with the mentioned CDR(s) one of the above amino acid sequences, in which:

(1) any amino acid substitution is preferably a conservative amino acid substitution (as defined herein); and/or

(2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequences.

However, of the Nanobodies of the invention that comprise the combinations of CDR's mentioned above, Nanobodies comprising one or more of the CDR's listed above are particularly preferred; Nanobodies comprising two or more of the CDR's listed above are more particularly preferred; and Nanobodies comprising three of the CDR's listed above are most particularly preferred.

In these Nanobodies against human serum albumin, the Framework regions FR1 to FR4 are preferably as defined hereinabove for the Nanobodies of the invention.

Particularly preferred Nanobodies against human serum albumin are chosen from the group consisting of SEQ ID NO's: 61 to 67, SEQ ID NO's 87 to 89 and SEQ ID NO's 100-104. The preferred combinations of CDR's and framework regions present in these Nanobodies are also listed in Table II

Table II: Preferred combination of Framework sequences and CDR's in Nanobodies against human serum albumin.

| Clone | ID | FR1 | ID | CDR1 | ID | FR2 | ID | CDR2 | ID | FR3 | ID | CDR3 | ID | FR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PMP6A8 (ALB2) | 36-38 | AVQLVESGGGLVQGGG SLRLACAASERIFD | 37-45 | LNLMG | 38-42 | WYRQGPGN ERELVA | 38-39 | TCITVGDSTNYA DSVKG | 39-46 | RFTISMDYTKQTVYLHMN SLRPEDTGLYYCKI | 40-43 | RRTWHSEL | 41-40 | WGQGTQV TVSS |
| PMP6B4 | 36-39 | EVQLVESGGGLVQEGG SLRLACAASERIWD | 37-46 | INLLG | 38-43 | WYRQGPGN ERELVA | 39-40 | TITVGDSTSYAD SVKG | 39-47 | RFTISRDYDKNTLYLQMN SLRPEDTGLYYCKI | 40-44 | RRTWHSEL | 41-41 | WGQGTQV TVSS |
| PMP6A6 (ALB1) | 37-40 | AVQLVESGGGLVQPGN SLRLSCAASGFTFR | 37-47 | SFGMS | 38-44 | WVRQAPGK EPEWVS | 39-41 | SISGSGSDTLYA DSVKG | 39-48 | RFTISRDNAKTTLYLQMN SLKPEDTAVYYCTI | 40-45 | GGSLSR | 41-42 | SSQGTQV TVSS |
| PMP6C1 | 37-41 | AVQLVDSGGGLVQPGG SLRLSCAASGFSFG | 37-48 | SFGMS | 38-45 | WVRQYPGK EPEWVS | 39-42 | SINGRGDDTRYA DSVKG | 39-49 | RFSISRDNAKNTLYLQMN SLKPEDTAEYYCTI | 40-46 | GRSVSRS | 41-43 | RTQGTQV TVSS |
| PMP6G8 | 37-42 | AVQLVESGGGLVQPGG SLRLTCTASGFTFR | 37-49 | SFGMS | 38-46 | WVRQAPGK DQEWVS | 39-43 | AISADSSTKNYA DSVKG | 40-00 | RFTISRDNAKKMLYLEMN SLKPEDTAVYYCVI | 40-47 | GRGSP | 41-44 | SSPGTQV TVSS |
| PMP6A5 | 37-43 | QVQLAESGGGLVQPGG SLRLTCTASGFTFG | 38-00 | SFGMS | 38-47 | WVRQAPGE GLEWVS | 39-44 | AISADSSDKRYA DSVKG | 40-01 | RFTISRDNAKKMLYLEMN SLKSEDTAVYYCVI | 40-48 | GRGSP | 41-45 | ASQGTQV TVSS |
| PMP6G7 | 37-44 | QVQLVESGGGLVQPGG SLRLSCAASGFTFS | 38-01 | NYWMY | 38-48 | 'WVRVAPGK GLERIS | 39-45 | RDISTGGGYSYY ADSVKG | 40-02 | RFTISRDNAKNTLYLQMN SLKPEDTALYYCAK | 40-09 | DREAQVDTLD FDY | 41-46 | RGQGTQV TVSS |

Another aspect of the invention is an anti-TNF-alpha polypeptide as disclosed herein further comprising at least one polypeptide selected from the group consisting of an anti-IFN-gamma polypeptide, an anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide.

According to one aspect of the invention, a nanobody is directed against TNF-alpha receptor. Said nanobody may be a Camelidae $V_{HH}$.

According to one aspect of the invention, a nanobody is directed against IFN-gamma receptor. Said nanobody may be a Camelidae $V_{HH}$.

Another aspect of the invention is a method of treating an autoimmune disease or condition as cited herein, comprising administering to a patient an effective amount of an anti-TNF-alpha polypeptide further comprising a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, such polypeptides joined to each other as described below.

Such multi-specific constructs may have improved potency as inflammatory therapeutic compound over mono-specific constructs.

One aspect of the invention is a composition comprising an anti-TNF-alpha polypeptide as disclosed herein and at least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, for simultaneous, separate or sequential administration to a subject.

One aspect of the invention is a method for treating autoimmune disease comprising administering to an individual an effective amount of an anti-TNF-alpha polypeptide and a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, simultaneously, separately or sequentially.

Another aspect of the invention is a kit containing an anti-TNF-alpha polypeptide and a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide for simultaneous, separate or sequential administration to a subject. It is an aspect of the invention that the kit may be used according to the invention. It is an aspect of the invention that the kit may be used to treat the diseases as cited herein.

By simultaneous administration means the polypeptides are administered to a subject at the same time. For example, as a mixture of the polypeptides or a composition comprising said polypeptides. Examples include, but are not limited to a solution administered intraveneously, a tablet, liquid, topical cream, etc., wherein each preparation comprises the polypeptides of interest.

By separate administration means the polypeptides are administered to a subject at the same time or substantially the same time. The polypeptides are present in the kit as separate, unmixed preparations. For example, the different polypeptides may be present in the kit as individual tablets. The tablets may be administered to the subject by swallowing both tablets at the same time, or one tablet directly following the other.

By sequential administration means the polypeptides are administered to a subject sequentially. The polypeptides are present in the kit as separate, unmixed preparations. There is a time interval between doses. For example, one polypeptide might be administered up to 336, 312, 288, 264, 240, 216, 192, 168, 144, 120, 96, 72, 48, 24, 20, 16, 12, 8, 4, 2, 1, or 0.5 hours after the other component.

In sequential administration, one polypeptide may be administered once, or any number of times and in various doses before and/or after administration of another polypeptide. Sequential administration may be combined with simultaneous or sequential administration.

The medical uses of the anti-TNF-alpha polypeptide described below, also apply to the composition comprising an anti-TNF-alpha polypeptide as disclosed herein and at least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, for simultaneous, separate or sequential administration to a subject as disclosed here above.

According to one aspect of the invention, an anti-IFN-gamma polypeptide anti-TNF-alpha a nanobody directed against IFN-gamma. Said nanobody may be a Camelidae $V_{HH}$.

According to one aspect of the invention, anti-TNF-alpha a nanobody directed against TNF-alpha receptor. Said nanobody may be a Camelidae $V_{HH}$.

According to one aspect of the invention, an anti-IFN-gamma receptor polypeptide anti-TNF-alpha a nanobody directed against IFN-gamma receptor. Said nanobody may be a Camelidae $V_{HH}$.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein, wherein the number of nanobodies directed against TNF-alpha is two or

more. Such multivalent anti-TNF-alpha polypeptides have the advantage of unusually high functional affinity for the target, displaying much higher than expected inhibitory properties compared to their monovalent counterparts.

The multivalent anti-TNF-alpha polypeptides have functional affinities that are several orders of magnitude higher than the monovalent parent anti-TNF-alpha polypeptides. The inventors have found that the functional affinities of these multivalent polypeptides are much higher than those reported in the prior art for bivalent and multivalent antibodies. Surprisingly, anti-TNF-alpha polypeptides of the present invention linked to each other directly or via a short linker sequence show the high functional affinities expected theoretically with multivalent conventional four-chain antibodies.

The inventors have found that such large increased functional activities can be detected preferably with antigens composed of multidomain and multimeric proteins, either in straight binding assays or in functional assays, e.g. cytotoxicity assays.

The nanobodies may be joined to form any of the polypeptides disclosed herein comprising more than one nanobody using methods known in the art or any future method. For example, they may be fused by chemical cross-linking by reacting amino acid residues with an organic derivatising agent such as described by Blattler et al, Biochemistry 24,1517-1524; EP294703. Alternatively, the nanobody may be fused genetically at the DNA level i.e. a polynucleotide construct formed which encodes the complete polypeptide construct comprising one or more anti-target nanobodies and one or more anti-serum protein nanobodies. A method for producing bivalent or multivalent $V_{HH}$ polypeptide constructs is disclosed in PCT patent application WO 96/34103. One way of joining multiple nanobodies is via the genetic route by linking nanobody coding sequences either directly or via a peptide linker. For example, the C-terminal end of the first nanobody may be linked to the N-terminal end of the next nanobody. This linking mode can be extended in order to link additional nanobodies for the construction and production of tri-, tetra-, etc. functional constructs.

According to one aspect of the present invention, the nanobodies are linked to each other directly, without use of a linker. Contrary to joining bulky conventional antibodies where a linker sequence is needed to retain binding activity in the two subunits, polypeptides of the invention can be linked directly thereby avoiding potential problems of the linker sequence, such as antigenicity when administered to a human subject, instability of the linker sequence leading to dissociation of the subunits.

According to another aspect of the present invention, the nanobodies are linked to each other via a peptide linker sequence. Such linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. The linker sequence is expected to be non-immunogenic in the subject to which the anti-TNF-alpha polypeptide is administered. The linker sequence may provide sufficient flexibility to the multivalent anti-TNF-alpha polypeptide, at the same time being resistant to proteolytic degradation. A non-limiting example of a linker sequences is one that can be derived from the hinge region of $V_{HH}$s described in WO 96/34103.

According to another aspect of the invention, multivalent nanobodies comprising more than two nanobodies can be linked to each other either directly or via a linker sequence. Such constructs are difficult to produce with conventional antibodies and due to steric hindrance of the bulky subunits, functionality will be lost or greatly diminished rather than increased considerably as seen with $V_{HH}$'s of the invention compared to the monovalent construct.

The polypeptide constructs disclosed herein may be made by the skilled artisan according to methods known in the art or any future method. For example, $V_{HH}$s may be obtained using methods known in the art such as by immunising a camel and obtaining hybridomas therefrom, or by cloning a library of nanobodies using molecular biology techniques known in the art and subsequent selection by using phage display.

According to an aspect of the invention an anti-TNF-alpha polypeptide may be a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a functional portion of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a functional portion of a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to an aspect of the invention an anti-TNF-alpha polypeptide may comprise a sequence of an anti-TNF-alpha polypeptide.

According to an aspect of the invention a nanobody used to form an anti-TNF-alpha polypeptide may be a complete nanobody (e.g. a $V_{HH}$) or a homologous sequence thereof. According to another aspect of the invention, a nanobody used to form the polypeptide construct may be a functional portion of a complete nanobody. According to another aspect of the invention, a nanobody used to form the polypeptide construct may be a homologous

sequence of a complete nanobody. According to another aspect of the invention, a nanobody used to form the polypeptide construct may be a functional portion of a homologous sequence of a complete nanobody.

As used herein, an homologous sequence of the present invention may comprise additions, deletions or substitutions of one or more amino acids, which do not substantially alter the functional characteristics of the polypeptides of the invention. The number of amino acid deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence according to the present invention may a polypeptide modified by the addition, deletion or substitution of amino acids, said modification not substantially altering the functional characteristics compared with the unmodified polypeptide.

A homologous sequence according to the present invention may be a polypeptide modified by the addition, deletion or substitution of amino acids, said modification not substantially altering the functional characteristics compared with the unmodified polypeptide.

A homologous sequence according to the present invention may be a sequence which exists in other Camelidae species such as, for example, camel, dromedary, llama, alpaca, guanaco etc.

Where homologous sequence indicates sequence identity, it means a sequence which presents a high sequence identity (more than 70%, 75%, 80%, 85%, 90%, 95% or 98% sequence identity) with the parent sequence and is preferably characterised by similar properties of the parent sequence, namely affinity, said identity calculated using known methods.

Alternatively, an homologous sequence may also be any amino acid sequence resulting from allowed substitutions at any number of positions of the parent sequence according to the formula below:

Ser substituted by Ser, Thr, Gly, and Asn;

Arg substituted by one of Arg, His, Gln, Lys, and Glu;

Leu substituted by one of Leu, Ile, Phe, Tyr, Met, and Val;

Pro substituted by one of Pro, Gly, Ala, and Thr;

Thr substituted by one of Thr, Pro, Ser, Ala, Gly, His, and Gln;
Ala substituted by one of Ala, Gly, Thr, and Pro;
Val substituted by one of Val, Met, Tyr, Phe, Ile, and Leu;
Gly substituted by one of Gly, Ala, Thr, Pro, and Ser;
Ile substituted by one of Ile, Met, Tyr, Phe, Val, and Leu;
Phe substituted by one of Phe, Trp, Met, Tyr, Ile, Val, and Leu;
Tyr substituted by one of Tyr, Trp, Met, Phe, Ile, Val, and Leu;
His substituted by one of His, Glu, Lys, Gln, Thr, and Arg;
Gln substituted by one of Gln, Glu, Lys, Asn, His, Thr, and Arg;
Asn substituted by one of Asn, Glu, Asp, Gln, and Ser;
Lys substituted by one of Lys, Glu, Gln, His, and Arg;
Asp substituted by one of Asp, Glu, and Asn;
Glu substituted by one of Glu, Asp, Lys, Asn, Gln, His, and Arg;
Met substituted by one of Met, Phe, Ile, Val, Leu, and Tyr.

A homologous nucleotide sequence according to the present invention may refer to nucleotide sequences of more than 50, 100, 200, 300, 400, 500, 600, 800 or 1000 nucleotides able to hybridize to the reverse-complement of the nucleotide sequence capable of encoding the patent sequence, under stringent hybridisation conditions (such as the ones described by Sambrook et al., Molecular Cloning, Laboratory Manuel, Cold Spring, Harbor Laboratory press, New York).

As used herein, a functional portion refers to a sequence of a nanobody that is of sufficient size such that the interaction of interest is maintained with affinity of $1 \times 10^{-6}$ M or better.

Alternatively, a functional portion comprises a partial deletion of the complete amino acid sequence and still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the target.

As used herein, a functional portion refers to less than 100% of the complete sequence (e.g., 99%, 90%, 80%, 70%, 60% 50%, 40%, 30%, 20%, 10%, 5%, 1% etc.), but comprises 5 or more amino acids or 15 or more nucleotides.

Targets as mentioned herein such as TNF-alpha, TNF-alpha receptor, serum proteins (e.g. serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, fibrinogen) and IFN-gamma, IFN-gamma receptor may be fragments of said targets. Thus a target is also a fragment of said target, capable of eliciting an immune response. A target is

also a fragment of said target, capable of binding to a nanobody raised against the full length target.

A fragment as used herein refers to less than 100% of the sequence (e.g., 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% etc.), but comprising 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids. A fragment is of sufficient length such that the interaction of interest is maintained with affinity of $1 \times 10^{-6}$ M or better.

A fragment as used herein also refers to optional insertions, deletions and substitutions of one or more amino acids which do not substantially alter the ability of the target to bind to a nanobody raised against the wild-type target. The number of amino acid insertions deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence of the present invention may include an anti-TNF-alpha polypeptide which has been humanised. The humanisation of antibodies of the new class of $V_{HH}$s would further reduce the possibility of unwanted immunological reaction in a human individual upon administration.

One embodiment of the present invention relates to a method for preparing modified polypeptides based upon llama antibodies by determining the amino acid residues of the antibody variable domain ($V_{HH}$) which may be modified without diminishing the native affinity of the domain for antigen and while reducing its immunogenicity with respect to a heterologous species; the use of $V_{HH}$s having modifications at the identified residues which are useful for administration to heterologous species; and to the $V_{HH}$ so modified.

More specifically, the invention relates to the preparation of modified $V_{HH}$s, which are modified for administration to humans, the resulting $V_{HH}$ themselves, and the use of such "humanized" $V_{HH}$s in the treatment of diseases in humans. By humanised is meant mutated so that immunogenicity upon administration in human patients is minor or nonexistent. Humanising a polypeptide, according to the present invention, comprises a step of replacing one or more of the Camelidae amino acids by their human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, i.e. the humanisation does not significantly affect the antigen binding capacity of the resulting polypeptide. Such methods are known by the skilled addressee.

Humanization of Camelidae nanobodies requires the introduction and mutagenesis of a limited amount of amino acids in a single polypeptide chain. This is in contrast to humanization of scFv, Fab, (Fab)2 and IgG, which requires the introduction of amino acid changes in two chains, the light and the heavy chain and the preservation of the assembly of both chains.

As described in WO 04/041862, an anti-TNF nanobody can be humanized. Humanization may for example involve mutagenesis of residues in FR1 at position 1 and 5 which were introduced by the primer used for repertoire cloning and do not occur naturally in the llama sequence. Mutagenesis of those residues did not result in loss of binding and/or inhibition activity. Humanization may also involve mutagenesis of residues in FR3 at position 74, 76, 83, 84, 93. Mutagenesis of those residues did not result in a dramatic loss of binding and/or inhibition activity. Combining the mutations of FR1 and FR3 therefore did not affect the binding and/or inhibition activity. Humanization may also involve mutagenesis of residues in FR4 at position 108. Mutagenesis of Q108L resulted in lower production level in Escherichia coli. Position 108 is solvent exposed in camelid $V_{HH}$, while in human antibodies this position is buried at the VH-VL interface (Spinelli, 1996; Nieba, 1997). In isolated VHs position 108 is solvent exposed. The introduction of a non-polar hydrophobic Leu instead of polar uncharged Gln can have a drastic effect on the intrinsic folding/stability of the molecule. Also, replacement of the hydrophilic residues by human hydrophobic residues at positions 44 and 45 (E44G and R45L), did not have an effect on binding and/or inhibition. However, loss of binding and/or inhibition activity was observed when F37V and F47W were introduced. Modeling data confirmed the critical residue 37 to preserve the integrity of the CDR3 loop conformation and hence on activity (all numbering according to the Kabat ).

According to one embodiment of the present invention, humanization involves replacing of any of the following residues either alone or in combination:

- FR1 position 1, 5, 28 and 30,
- the hallmark amino acid at position 44 and 45 in FR2,
- FR3 residues 74, 75, 76, 83, 84, 93 and 94 ,
- and positions 103, 104, 108 and 111 in FR4 ;
- numbering according to the Kabat numbering.

One embodiment of the present invention is an anti-TNF-alpha polypeptide, or a nucleic acid capable of encoding said polypeptide for use in treating, preventing and/or alleviating the symptoms of disorders relating to inflammatory processes. TNF-alpha is

involved in inflammatory processes, and the blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disease states such as, for example, Crohn's disease. The Examples demonstrate $V_{HH}$s according to the invention which bind TNF-alpha and moreover, block its binding to the TNF-alpha receptor.

The anti-TNF-alpha polypeptides of the present invention are applicable to autoimmune diseases, such as Addison's disease (adrenal), Autoimmune diseases of the ear (ear), Autoimmune diseases of the eye (eye), Autoimmune hepatitis (liver), Autoimmune parotitis (parotid glands), Crohn's disease (intestine), Diabetes Type I (pancreas), Epididymitis (epididymis), Glomerulonephritis (kidneys), Graves' disease (thyroid), Guillain-Barre syndrome (nerve cells), Hashimoto's disease (thyroid), Hemolytic anemia (red blood cells), Systemic lupus erythematosus (multiple tissues), Male infertility (sperm), Multiple sclerosis (nerve cells), Myasthenia Gravis (neuromuscular junction), Pemphigus (primarily skin), Psoriasis (skin), Rheumatic fever (heart and joints), Rheumatoid arthritis (joint lining), Sarcoidosis (multiple tissues and organs), Scleroderma (skin and connective tissues), Sjogren's syndrome (exocrine glands, and other tissues), Spondyloarthropathies (axial skeleton, and other tissues), Thyroiditis (thyroid), Vasculitis (blood vessels).
Within parenthesis is the tissue affected by the disease. This listing of autoimmune diseases is intended to be exemplary rather than inclusive.

Autoimmune conditions for which the anti-TNF-alpha polypeptides of the present invention is applicable include, for example, AIDS, atopic allergy, bronchial asthma, eczema, leprosy, schizophrenia, inherited depression, transplantation of tissues and organs, chronic fatigue syndrome, Alzheimer's disease, Parkinson's disease, myocardial infarction, stroke, autism, epilepsy, Arthus's phenomenon, anaphylaxis, and alcohol and drug addiction. In the above-identified autoimmune conditions, the tissue affected is the primary target, in other cases it is the secondary target. These conditions are partly or mostly autoimmune syndromes. Therefore, in treating them, it is possible to use the same methods, or aspects of the same methods that are herein disclosed, sometimes in combination with other methods.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide according to the invention, or a nucleic acid capable of encoding said polypeptide for the preparation of a medicament for treating a disorder relating to inflammatory processes. Examples of disorders include rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

Polypeptides and nucleic acids according to the present invention may be administered to a subject by conventional routes, such as intravenously. However, a special property of the anti-TNF-alpha polypeptides of the invention is that they penetrate barriers such as tissue membranes and/or tumours and act locally thereon, and they are sufficiently stable to withstand extreme environments such as in the stomach. Therefore, another aspect of the present invention relates to the delivery of anti-TNF-alpha polypeptides.

When the Nanobodies and/or polypeptides of the invention are used for, or are intended for use in, the prevention or treatment of diseases and disorders of the gastro-intestinal tract, in particular by means of oral administration or other administration into the gastrointestinal tract, it will usually not be necessary to use polypeptides of the invention that have increased half-life in serum (i.e. that have been pegylated or that contain a Nanobody directed against a serum protein). Thus, for such indications, polypeptides of the invention can be used that only contain Nanobodies of the invention. In particular, it has been found that for oral administration for the prevention and treatment of diseases or disorders of the gastro-intestinal tract associated with and/or mediated by TNF-alpha (such as IBD and the other diseases and disorders of the gastro-intestinal tract mentioned above), the use of a monovalent Nanobody of the invention or of a polypeptide of the invention that essentially consists of a monovalent Nanobody of the invention may be preferred. For other indications, such as the treatment of rheumatoid arthritis (RA), the use of a bivalent Nanobody of the invention may be preferred. When such a Nanobody has to reach its intended site of action via the blood stream, the use of a polypeptide of the invention that has increased half-life in serum may be preferred.

A subject according to the invention can be any mammal susceptible to treatment by therapeutic polypeptides.

Oral delivery of anti-TNF-alpha polypeptides of the invention results in the provision of such molecules in an active form in the colon at local sites that are affected by the disorder. These sites may be highly inflamed and contain TNF-alpha-producing cells. The anti-TNF-alpha polypeptides of the invention which bind to TNF-alpha can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as Micrococcus lactis are able to secrete antibody or functional portions thereof. Such modified microorganisms can be used as vehicles for local production and delivery of antibodies or functional portions thereof

in the intestine. By using a strain which produces an anti-TNF-alpha polypeptide, inflammatory bowel syndrome could be treated.

Another aspect of the invention involves delivering anti-TNF polypeptides by using surface expression on or secretion from non-invasive bacteria, such as Gram-positive host organisms like Lactococcus spec. using a vector such as described in WO00/23471.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the gastric environment without the substance being inactivated.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, and multiple sclerosis. As known by persons skilled in the art, once in possession of said polypeptide construct, formulation technology may be applied to release a maximum amount of polypeptide in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disorders whose targets are located in the gut system.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the gastric environment without being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the gastric environment without being inactivated.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the gut system without said substance being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject without the substance being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms or disorders

susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the vaginal and/or rectal tract.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, and multiple sclerosis. In a non-limiting example, a formulation according to the invention comprises an anti-TNF-alpha polypeptide as disclosed herein, in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time (such formulations are described in EP 707473, EP 684814, US 5629001).

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the vaginal and/or rectal tract.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the vaginal and/or rectal tract without being said substance being inactivated, by administering to the vaginal and/or rectal tract of a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject without said substance being inactivated, by administering to the vaginal and/or rectal tract of a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein, for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the nose, upper respiratory tract and/or lung.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, and multiple sclerosis. In a non-limiting example, a formulation according to the invention, comprises an anti-TNF-alpha polypeptide as disclosed herein in the form of a nasal spray

(e.g. an aerosol) or inhaler. Since the polypeptide construct is small, it can reach its target much more effectively than therapeutic IgG molecules.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the upper respiratory tract and lung, by administering to a subject an anti-TNF-alpha polypeptide as disclosed herein, by inhalation through the mouth or nose.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the nose, upper respiratory tract and/or lung, without said polypeptide being inactivated.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the nose, upper respiratory tract and lung without inactivation, by administering to the nose, upper respiratory tract and/or lung of a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject without inactivation by administering to the nose, upper respiratory tract and/or lung of a subject an anti-TNF-alpha polypeptide as disclosed herein.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa. Because of their small size, an anti-TNF-alpha polypeptide as disclosed herein can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, $V_{HH}$ is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a second $V_{HH}$ which is fused to the therapeutic $V_{HH}$. Such fusion constructs are made using methods known in the art. The "carrier" $V_{HH}$ binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the intestinal mucosa without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide of the invention.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide of the invention.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, an anti-TNF-alpha polypeptide as described herein is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a $V_{HH}$ which is fused to said polypeptide. Such fusion constructs made using methods known in the art. The "carrier" $V_{HH}$ binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the tissues beneath the tongue effectively.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, and multiple sclerosis. A formulation of said polypeptide construct as disclosed herein, for

example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able to pass through the tissues beneath the tongue.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the tissues beneath the tongue without being inactivated, by administering sublingually to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide as disclosed herein.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the skin effectively.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, and multiple sclerosis. A formulation of said polypeptide construct, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the skin effectively, by topically administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a Nanobody or polypeptide of the invention which is able pass through the skin effectively.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the skin without being inactivated, by administering topically to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a Nanobody or polypeptide of the invention to the bloodstream of a subject, by administering topically to a subject an anti-TNF-alpha polypeptide as disclosed herein.

In another embodiment of the present invention, an anti-TNF-alpha polypeptide further comprises a carrier nanobody (e.g. $V_{HH}$) which acts as an active transport carrier for transport said anti-TNF-alpha polypeptide, from the lung lumen to the blood.

An anti-TNF-alpha polypeptide further comprising a carrier binds specifically to a receptor present on the mucosal surface (bronchial epithelial cells) resulting in the active transport of the polypeptide from the lung lumen to the blood. The carrier nanobody may be fused to the polypeptide construct. Such fusion constructs may be made using methods known in the art and are describe herein. The "carrier" nanobody binds specifically to a receptor on the mucosal surface which induces an active transfer through the surface.

Another aspect of the present invention is a method to determine which nanobodies (e.g. $V_{HH}$s) are actively transported into the bloodstream upon nasal administration. Similarly, a naïve or immune $V_{HH}$ phage library can be administered nasally, and after different time points after administration, blood or organs can be isolated to rescue phages that have been actively transported to the bloodstream. A non-limiting example of a receptor for active transport from the lung lumen to the bloodstream is the Fc receptor N (FcRn). One aspect of the invention includes the $V_{HH}$ molecules identified by the method. Such $V_{HH}$ can then be used as a carrier $V_{HH}$ for the delivery of a therapeutic $V_{HH}$ to the corresponding target in the bloodstream upon nasal administration.

In one aspect of the invention, one can use an anti-TNF-alpha polypeptide as disclosed herein, in order to screen for agents that modulate the binding of the polypeptide to TNF-alpha. When identified in an assay that measures binding or said polypeptide displacement alone, agents will have to be subjected to functional testing to determine whether they would modulate the action of the antigen in vivo.

In an example of a displacement experiment, phage or cells expressing TNF-alpha or a fragment thereof are incubated in binding buffer with polypeptide of the invention which has been labeled, in the presence or absence of increasing concentrations of a candidate modulator. To validate and calibrate the assay, control competition reactions using

increasing concentrations of said polypeptide and which is unlabeled, can be performed. After incubation, cells are washed extensively, and bound, labeled polypeptide is measured as appropriate for the given label (e.g., scintillation counting, fluorescence, etc.). A decrease of at least 10% in the amount of labeled polypeptide bound in the presence of candidate modulator indicates displacement of binding by the candidate modulator. Candidate modulators are considered to bind specifically in this or other assays described herein if they displace 50% of labeled polypeptide (sub-saturating polypeptide dose) at a concentration of 1 μM or less.

Alternatively, binding or displacement of binding can be monitored by surface plasmon resonance (SPR). Surface plasmon resonance assays can be used as a quantitative method to measure binding between two molecules by the change in mass near an immobilized sensor caused by the binding or loss of binding of the polypeptide of the invention from the aqueous phase to TNF-alpha immobilized in a membrane on the sensor. This change in mass is measured as resonance units versus time after injection or removal of the said polypeptide or candidate modulator and is measured using a Biacore Biosensor (Biacore AB). TNF-alpha can be for example immobilized on a sensor chip (for example, research grade CM5 chip; Biacore AB) in a thin film lipid membrane according to methods described by Salamon et al. (Salamon et al., 1996, Biophys J. 71: 283-294; Salamon et al., 2001, Biophys. J. 80: 1557-1567; Salamon et al., 1999, Trends Biochem. Sci. 24: 213-219, each of which is incorporated herein by reference.). Sarrio et al. demonstrated that SPR can be used to detect ligand binding to the GPCR A(1) adenosine receptor immobilized in a lipid layer on the chip (Sarrio et al., 2000, Mol. Cell. Biol. 20: 5164-5174, incorporated herein by reference). Conditions for the binding of a polypeptide of the invention to TNF-alpha in an SPR assay can be fine-tuned by one of skill in the art using the conditions reported by Sarrio et al. as a starting point.

SPR can assay for modulators of binding in at least two ways. First, a polypeptide of the invention can be pre-bound to immobilized TNF-alpha followed by injection of candidate modulator at a concentration ranging from 0.1 nM to 1 μM. Displacement of the bound polypeptide can be quantitated, permitting detection of modulator binding. Alternatively, the membrane-bound TNF-alpha can be pre-incubated with a candidate modulator and challenged with the polypeptide of the invention. A difference in binding affinity between said polypeptide and TNF-alpha pre-incubated with the modulator, compared with that between said polypeptide and TNF-alpha in absence of the modulator

will demonstrate binding or displacement of said polypeptide in the presence of modulator. In either assay, a decrease of 10% or more in the amount of said polypeptide bound in the presence of candidate modulator, relative to the amount of said polypeptide bound in the absence of candidate modulator indicates that the candidate modulator inhibits the interaction of TNF-alpha and said polypeptide.

Another method of detecting inhibition of binding of, for example, a polypeptide of the invention, to TNF-alpha uses fluorescence resonance energy transfer (FRET). FRET is a quantum mechanical phenomenon that occurs between a fluorescence donor (D) and a fluorescence acceptor (A) in close proximity to each other (usually < 100 Å of separation) if the emission spectrum of D overlaps with the excitation spectrum of A. The molecules to be tested, e.g. a polypeptide polypeptide of the invention and a TNF-alpha are labelled with a complementary pair of donor and acceptor fluorophores. While bound closely together by the TNF-alpha: polypeptide interaction, the fluorescence emitted upon excitation of the donor fluorophore will have a different wavelength from that emitted in response to that excitation wavelength when the said polypeptide and TNF-alpha are not bound, providing for quantitation of bound versus unbound molecules by measurement of emission intensity at each wavelength. Donor fluorophores with which to label the TNF-alpha are well known in the art. Of particular interest are variants of the A. Victoria GFP known as Cyan FP (CFP, Donor (D)) and Yellow FP (YFP, Acceptor (A)). As an example, the YFP variant can be made as a fusion protein with TNF-alpha. Vectors for the expression of GFP variants as fusions (Clontech) as well as fluorophore-labeled reagents (Molecular Probes) are known in the art. The addition of a candidate modulator to the mixture of fluorescently-labelled polypeptide and YFP-TNF-alpha will result in an inhibition of energy transfer evidenced by, for example, a decrease in YFP fluorescence relative to a sample without the candidate modulator. In an assay using FRET for the detection of TNF-alpha: polypeptide interaction, a 10% or greater decrease in the intensity of fluorescent emission at the acceptor wavelength in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the candidate modulator inhibits the TNF-alpha:polypeptide interaction.

A sample as used herein may be any biological sample containing TNF-alpha such as clinical (e.g. cell fractions, whole blood, plasma, serum, tissue, cells, etc.), derived from clinical, agricultural, forensic, research, or other possible samples. The clinical samples may

be from human or animal origin. The sample analysed can be both solid or liquid in nature. It is evident when solid materials are used, these are first dissolved in a suitable solution.

A variation on FRET uses fluorescence quenching to monitor molecular interactions. One molecule in the interacting pair can be labelled with a fluorophore, and the other with a molecule that quenches the fluorescence of the fluorophore when brought into close apposition with it. A change in fluorescence upon excitation is indicative of a change in the association of the molecules tagged with the fluorophore:quencher pair. Generally, an increase in fluorescence of the labelled TNF-alpha is indicative that anti-TNF-alpha polypeptide bearing the quencher has been displaced. For quenching assays, a 10% or greater increase in the intensity of fluorescent emission in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the candidate modulator inhibits TNF-alpha: anti-TNF-alpha polypeptide interaction.

In addition to the surface plasmon resonance and FRET methods, fluorescence polarization measurement is useful to quantitate binding. The fluorescence polarization value for a fluorescently-tagged molecule depends on the rotational correlation time or tumbling rate. Complexes, such as those formed by TNF-alpha associating with a fluorescently labelled anti-TNF-alpha polypeptide, have higher polarization values than uncomplexed, labelled polypeptide. The inclusion of a candidate inhibitor of the TNF-alpha:anti-TNF-alpha polypeptide interaction results in a decrease in fluorescence polarization, relative to a mixture without the candidate inhibitor, if the candidate inhibitor disrupts or inhibits the interaction of TNF-alpha with said polypeptide. Fluorescence polarization is well suited for the identification of small molecules that disrupt the formation of TNF-alpha:anti-TNF-alpha polypeptide complexes. A decrease of 10% or more in fluorescence polarization in samples containing a candidate modulator, relative to fluorescence polarization in a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the TNF-alpha : anti-TNF-alpha polypeptide interaction.

Another alternative for monitoring TNF-alpha : anti-TNF-alpha polypeptide interactions uses a biosensor assay. ICS biosensors have been described in the art (Australian Membrane Biotechnology Research Institute; Cornell B, Braach-Maksvytis V, King L, Osman P, Raguse B, Wieczorek L, and Pace R. "A biosensor that uses ion-channel switches" Nature 1997, 387, 580). In this technology, the association of TNF-alpha and a anti-TNF-alpha polypeptide is coupled to the closing of gramicidine-facilitated ion channels in suspended membrane bilayers and thus to a measurable change in the admittance (similar to

impedance) of the biosensor. This approach is linear over six orders of magnitude of admittance change and is ideally suited for large scale, high throughput screening of small molecule combinatorial libraries. A 10% or greater change (increase or decrease) in admittance in a sample containing a candidate modulator, relative to the admittance of a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the interaction of TNF-alpha and said polypeptide. It is important to note that in assays testing the interaction of TNF-alpha with an anti-TNF-alpha polypeptide, it is possible that a modulator of the interaction need not necessarily interact directly with the domain(s) of the proteins that physically interact with said polypeptide. It is also possible that a modulator will interact at a location removed from the site of interaction and cause, for example, a conformational change in the TNF-alpha. Modulators (inhibitors or agonists) that act in this manner are nonetheless of interest as agents to modulate the binding of TNF-alpha to its receptor.

Any of the binding assays described can be used to determine the presence of an agent in a sample, e.g., a tissue sample, that binds to TNF-alpha , or that affects the binding of, for example, a polypeptide polypeptide of the invention to the TNF-alpha. To do so a TNF-alpha is reacted with said polypeptide in the presence or absence of the sample, and polypeptide binding is measured as appropriate for the binding assay being used. A decrease of 10% or more in the binding of said polypeptide indicates that the sample contains an agent that modulates the binding of said polypeptide to the TNF-alpha. Of course, the above-generalized method might easily be applied to screening for candidate modulators which alter the binding between any anti-TNF-alpha polypeptide of the invention, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof, and TNF-alpha or a fragment thereof.

One embodiment of the present invention is an unknown agent identified by the method disclosed herein.

One embodiment of the present invention is an unknown agent identified by the method disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders relating to inflammatory processes.

Examples of disorders include rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

A cell that is useful according to the invention is preferably selected from the group consisting of bacterial cells such as, for example, E. coli, yeast cells such as, for example, S. cerevisiae, P. pastoris, insect cells or mammal cells.

A cell that is useful according to the invention can be any cell into which a nucleic acid sequence encoding a polypeptide comprising an anti-TNF-alpha of the invention, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof according to the invention can be introduced such that the polypeptide is expressed at natural levels or above natural levels, as defined herein. Preferably a polypeptide of the invention that is expressed in a cell exhibits normal or near normal pharmacology, as defined herein.

According to a preferred embodiment of the present invention, a cell is selected from the group consisting of COS7-cells, a CHO cell, a LM (TK-) cell, a NIH-3T3 cell, HEK-293 cell, K-562 cell or a 1321N1 astrocytoma cell but also other transfectable cell lines.

In general, "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve the desired result or results (modulating TNF-alpha binding; treating or preventing inflammation). One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the various compounds that modulate TNF-alpha binding used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to an anti-TNF-alpha polypeptide of the present invention, a composition, or a nucleic acid capable of encoding said polypeptide or an agent identified according to the screening method described herein or said polypeptide comprising one or more derivatised amino acids.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Anti-TNF-alpha polypeptides as disclosed herein is useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound or composition.

Anti-TNF polypeptides of the present invention are useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound or composition that binds TNF-alpha.

Anti-TNF-alpha polypeptides as disclosed here in are useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The anti-TNF-alpha polypeptides as disclosed here in are useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

Conditions mediated by TNF-alpha include, but are not limited rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

A compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or a domestic animal in a variety of forms adapted to the chosen route of administration, i.e., orally or parenterally, by intranassally by inhalation, intravenous, intramuscular, topical or subcutaneous routes.

A compound of the present 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 for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound 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 active compound 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 active compound. The percentage of 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 active compound 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, corn 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 active compound, 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 active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its 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 active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound 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 present compound 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 celluloses 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 compound 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 compound 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 compound(s) 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 compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt 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 compound 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.

The invention provides for an agent that is a modulator of TNF-alpha / TNF-alpha-receptor interactions.

The candidate agent may be a synthetic agent, or a mixture of agents, or may be a natural product (e.g. a plant extract or culture supernatant). A candidate agent according to

the invention includes a small molecule that can be synthesized, a natural extract, peptides, proteins, carbohydrates, lipids etc.

Candidate modulator agents from large libraries of synthetic or natural agents can be screened. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based agents. Synthetic agent libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Combinatorial libraries are available and can be prepared. Alternatively, libraries of natural agents in the form of bacterial, fungal, plant and animal extracts are available from e.g., Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible by methods well known in the art. Additionally, natural and synthetically produced libraries and agents are readily modified through conventional chemical, physical, and biochemical means.

Useful agents may be found within numerous chemical classes. Useful agents may be organic agents, or small organic agents. Small organic agents have a molecular weight of more than 50 yet less than about 2,500 daltons, preferably less than about 750, more preferably less than about 350 daltons. Exemplary classes include heterocycles, peptides, saccharides, steroids, and the like. The agents may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an agent may be used to identify, generate, or screen additional agents. For example, where peptide agents are identified, they may be modified in a variety of ways to enhance their stability, such as using an unnatural amino acid, such as a D-amino acid, particularly D-alanine, by functionalizing the amino or carboxylic terminus, e.g. for the amino group, acylation or alkylation, and for the carboxyl group, esterification or amidification, or the like.

For primary screening, a useful concentration of a candidate agent according to the invention is from about 10 mM to about 100 μM or more (i.e. 1 mM, 10 mM, 100 mM, 1 M etc.). The primary screening concentration will be used as an upper limit, along with nine additional concentrations, wherein the additional concentrations are determined by reducing the primary screening concentration at half-log intervals (e.g. for 9 more concentrations) for secondary screens or for generating concentration curves.

A high throughput screening kit according to the invention comprises all the necessary means and media for performing the detection of an agent that modulates TNF-

alpha/TNF-alpha receptor interactions by interacting with TNF-alpha in the presence of a polypeptide, preferably at a concentration in the range of 1μM to 1 mM.

The kit comprises the following. Recombinant cells of the invention, comprising and expressing the nucleotide sequence encoding TNF-alpha , which are grown according to the kit on a solid support, such as a microtiter plate, more preferably a 96 well microtiter plate, according to methods well known to the person skilled in the art especially as described in WO 00/02045. Alternatively TNF-alpha is supplied in a purified form to be immobilized on, for example, a 96 well microtiter plate by the person skilled in the art. Alternatively TNF-alpha is supplied in the kit pre-immobilized on, for example, a 96 well microtiter plate. The TNF-alpha may be whole TNF-alpha or a fragment thereof.

Modulator agents according to the invention, at concentrations from about 1 μM to 1 mM or more, are added to defined wells in the presence of an appropriate concentration of anti-TNF-alpha polypeptide, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof, said concentration of said polypeptide preferably in the range of 1 μM to 1 mM. Kits may contain one or more anti-TNF-alpha polypeptides of the invention.

Binding assays are performed as according to the methods already disclosed herein and the results are compared to the baseline level of, for example TNF-alpha binding to an anti-TNF-alpha polypeptide, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof , but in the absence of added modulator agent. Wells showing at least 2 fold, preferably 5 fold, more preferably 10 fold and most preferably a 100 fold or more increase or decrease in TNF-alpha-polypeptide binding (for example) as compared to the level of activity in the absence of modulator, are selected for further analysis.

The invention provides for other kits useful for screening for modulators of TNF-alpha/TNF-alpha receptor binding, as well as kits useful for diagnosis of disorders characterised by dysfunction of TNF-alpha. The invention also provides for kits useful for screening for modulators of disorders as well as kits for their diagnosis, said disorders characterised by one or more process involving TNF-alpha. Kits useful according to the invention can include an isolated TNF-alpha . Alternatively, or in addition, a kit can comprise cells transformed to express TNF-alpha. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding TNF-alpha. In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of

TNF-alpha. Kits useful according to the invention can comprise an isolated TNF-alpha polypeptide, a homologue thereof, or a functional portion thereof. A kit according to the invention can comprise cells transformed to express said polypeptide. Kits may contain more than one polypeptide. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding TNF-alpha . In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of a macromolecule such as, for example, TNF-alpha. All kits according to the invention will comprise the stated items or combinations of items and packaging materials therefore. Kits will also include instructions for use.

Furthermore, 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 other "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-A-7,180,370, WO 01/27160, EP 0 605 522, EP 0 460 167, US-A-7,054,297, Nicaise et al., Protein Science (2004), 13:1882-1891; Ewert et al., Methods, 2004 Oct; 34(2):184-199; Kettleborough et al., Protein Eng. 1991 Oct; 4(7): 773-783; O'Brien and Jones, Methods Mol. Biol. 2003: 207: 81-100; and Skerra, J. Mol. Recognit. 2000: 13: 167-187, and Saerens et al., J. Mol. Biol. 2005 Sep 23;352(3):597-607, and the further references cited therein. For 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 human framework regions or sequences.

Thus, in another embodiment, the invention comprises a chimeric polypeptide comprising at least one CDR sequence chosen from the group consisting of CDR1 sequences, CDR2 sequences and CDR3 sequences mentioned herein for the Nanobodies of the invention. Preferably, such a chimeric polypeptide comprises at least one CDR sequence chosen from the group consisting of the CDR3 sequences mentioned herein for the Nanobodies of the invention, and optionally also at least one CDR sequence chosen from the group consisting of the CDR1 sequences and CDR2 sequences mentioned herein for the Nanobodies of the invention. For example, such a chimeric polypeptide may comprise one CDR sequence chosen from the group consisting of the CDR3 sequences mentioned herein for the Nanobodies of the invention, one CDR sequence chosen from the group consisting of the CDR1 sequences mentioned herein for the Nanobodies of the invention and one CDR

sequence chosen from the group consisting of the CDR1 sequences and CDR2 sequences mentioned herein for the Nanobodies of the invention. The combinations of CDR's that are mentioned herein as being preferred for the Nanobodies of the invention will usually also be preferred for these chimeric polypeptides.

In said chimeric polypeptides, the CDR's may be linked to further amino acid sequences and/or may be linked to each other via amino acid sequences, in which said amino acid sequences are preferably framework sequences or are amino acid sequences that act as framework sequences, or together form a scaffold for presenting the CDR's. Reference is again made to the prior art mentioned in the last paragraph. According to one preferred embodiment, the amino acid sequences are human framework sequences, for example $V_H3$ framework sequences. However, non-human, synthetic, semi-synthetic or non-immunoglobulin framework sequences may also be used. Preferably, the framework sequences used are such that (1) the chimeric polypeptide is capable of binding xxxx, i.e. with an affinity that is at least 1%, preferably at least 5%, more preferably at least 10%, such as at least 25% and up to 50% or 90% or more of the affinity of the corresponding Nanobody of the invention; (2) the chimeric polypeptide is suitable for pharmaceutical use; and (3) the chimeric polypeptide is preferably essentially non-immunogenic under the intended conditions for pharmaceutical use (i.e. indication, mode of administration, dosis and treatment regimen) thereof (which may be essentially analogous to the conditions described herein for the use of the Nanobodies of the invention).

According to one non-limiting embodiment, the chimeric polypeptide comprises at least two CDR sequences (as mentioned above) linked via at least one framework sequence, in which preferably at least one of the two CDR sequences is a CDR3 sequence, with the other CDR sequence being a CDR1 or CDR2 sequence. According to a preferred, but non-limiting embodiment, the chimeric polypeptide comprises at least two CDR sequences (as mentioned above) linked at least two framework sequences, in which preferably at least one of the three CDR sequences is a CDR3 sequence, with the other two CDR sequences being CDR1 or CDR2 sequences, and preferably being one CDR1 sequence and one CDR2 sequence. According to one specifically preferred, but non-limiting embodiment, the chimeric polypeptides have the structure FR1' - CDR1 - FR2' - CDR2 - FR3' - CDR3 - FR4', in which CDR1, CDR2 and CDR3 are as defined herein for the CDR's of the Nanobodies of the invention, and FR1', FR2', FR3' and FR4' are framework sequences. FR1', FR2', FR3' and FR4' may in particular be Framework 1, Framework 2, Framework 3 and Framework 4

sequences, respectively, of a human antibody (such as $V_H3$ sequences) and/or parts or fragments of such Framework sequences. It is also possible to use parts or fragments of a chimeric polypeptide with the structure FR1' - CDR1 - FR2' - CDR2 - FR3' - CDR3 - FR4. Preferably, such parts or fragments are such that they meet the criteria set out in the preceding paragraph.

The invention also relates to proteins and polypeptides comprising and/or essentially consisting of such chimeric polypeptides, to nucleic acids encoding such proteins or polypeptides; to methods for preparing such proteins and polypeptides; to host cells expressing or capable of expressing such proteins or polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such proteins or polypeptides, nucleic acids or host cells; and to uses of such proteins or polypeptides, such nucleic acids, such host cells and/or such compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein. For example, such proteins, polypeptides, nucleic acids, methods, host cells, compositions and uses may be analogous to the proteins, polypeptides, nucleic acids, methods, host cells, compositions and use described herein for the Nanobodies of the invention.

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 any suitable (i.e. suitable for the purposes described herein) CDR sequences and/or 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 TNF. Such immunoglobulin sequences directed against xxxx can be generated in any manner known per se, as will be clear to the skilled person, i.e. by immunization with TNF or by screening a suitable library of immunoglobulin sequences with TNF, 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, 1105-1116 (2005) . Other techniques for generating immunoglobulins against a specified target include for example the Nanoclone technology (as for example

described in the non-prepublished US provisional patent application 60/648,922), 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 TNF, 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.

The invention will now be further described by means of the following non-limiting examples and figures, in which the Figures show:

Monovalent TNFα nanobodies

Figure 1: Sequence alignment of human TNFα nanobodies

Figure 2: Sequence alignment of serum albumin specific TNFα nanobodies

Figure 3: Binding of albumin specific TNFα nanobodies to human serum albumin

Figure 4: Binding of albumin specific TNFα nanobodies to rhesus serum albumin

Figure 5: Binding of albumin specific TNFα nanobodies to mouse serum albumin

Figure 6: Purity of TNFα and serum albumin nanobodies (SDS-PAGE)

Figure 7: Western Blot analysis of TNFα and serum albumin nanobodies

Figure 8: Binding of TNFα nanobodies to human TNFα (ELISA)

Figure 9: Binding of TNFα nanobodies to rhesus TNFα (ELISA)

Figure 10: Receptor-inhibition assay on Enbrel for human TNFα

Figure 11: Receptor-inhibition assay on Enbrel for rhesus TNFα

Figure 12: Binding of TNFα nanobodies to human TNFα (Biacore)

Figure 13: Binding of TNFα nanobodies to rhesus TNFα (Biacore)

Figure 14: Binding of TNFα nanobodies to Protein A (Biacore)

Figure 15: Temperature treatment of TNFα and serum albumin nanobodies (Western Blot)

Figure 16: Stability: temperature treatment of TNFα nanobodies (ELISA)

Figure 17: Temperature treatment of serum albumin nanobodies (Biacore)

Bivalent TNFα nanobodies

Figure 18: Purity of bivalent TNFα nanobodies (SDS-PAGE)

Figure 19: Western Blot analysis of bivalent TNFα nanobodies

Figure 20: Receptor-inhibition assay on Enbrel for bivalent TNFα nanobodies

Figure 21: Stability: temperature treatment of bivalent TNFα nanobodies (ELISA)

Humanised monovalent TNFα nanobodies

Figure 22: Multiple sequence alignment of TNF1 humanised nanobodies

Figure 23: Multiple sequence alignment of TNF2 humanised nanobodies

Figure 24: Multiple sequence alignment of TNF3 humanised nanobodies

Figure 25: Multiple sequence alignment of ALB1 humanised nanobodies

Figure 26: Purity of humanised TNFα and serum albumin nanobodies (SDS-PAGE)

Figure 27: Western Blot analysis of humanised TNFα and serum albumin nanobodies

Figure 28: Binding of humanised TNFα nanobodies to human TNFα

Figure 29: Binding of humanised serum albumin nanobodies to human serum albumin

Figure 30: Stability: temperature treatment of humanised TNFα nanobodies (ELISA)

Trivalent TNFα nanobodies

Figure 31: Purity of trivalent TNFα nanobodies (SDS-PAGE)

Figure 32: Western Blot analysis of trivalent TNFα nanobodies

Figure 33: Stability: temperature treatment of trivalent TNFα nanobodies (ELISA)

Humanised monovalent TNFα nanobodies (second round)

Figure 34: Multiple sequence alignment of TNF1 humanised nanobodies

Figure 35: Multiple sequence alignment of TNF2 humanised nanobodies

Figure 36: Multiple sequence alignment of TNF3 humanised nanobodies

Figure 37: Multiple sequence alignment of ALB1 humanised nanobodies

Figure 38: Purity of humanised TNFα nanobodies (SDS-PAGE)

Figure 39: Western Blot analysis of humanised TNFα nanobodies

Figure 40: Binding of humanised TNFα nanobodies to human TNFα

Figure 41: Stability: temperature treatment of humanised TNFα nanobodies (ELISA)

Figure 42: Analysis of purified TNF60 on Silver stained SDS-PAGE gel (A) Coomassie stained SDS-PAGE gel (B) and in Western blot analysis using anti-NB (C) for detection

Figure 43: Chromatogram of analytical size exclusion of TNF60 on Superdex HR75

Figure 44: Binding of TNF60 to human TNF-alpha

Figure 45:Dose response curve obtained in cytotoxicity assay with human TNF-alpha using Nanobody™ TNF60 in comparison with Enbrel (Etanercept), Humira (Adalimumab) and Remicade (Infliximab)

Figure 46: Dose response curve obtained in cytotoxicity assay with rhesus TNFα using Nanobody™ TNF60 in comparison with Enbrel (Etanercept), Humira (Adalimumab) and Remicade (Infliximab)

Figure 47: Pharmacokinetic profile of TNF60 in mice

Figure 48: Immunogenicity profile of TNF60 in mice

Figure 49: Analysis of purified TNF56-PEG40, TNF56-PEG60, TNF56-biotine, TNF55-PEG40, TNF55-PEG60 and TNF55-biotine on Coomassie stained SDS-PAGE gel

Figure 50: Analysis of purified TNF56-PEG40 on SDS-PAGE gel using Silver stain (A) Coomassie stain (B) and in Western blot analysis using anti-NB (C) for detection

Figure 51: Chromatogram of analytical size exclusion of TNF56-PEG40 on Superdex HR 75

Figure 52: Chromatogram of analytical size exclusion of TNF56-PEG40 on Superdex HR 200

Figure 53: Dose response curve obtained in cytotoxicity assay with human TNFα using Nanobody™ TNF56-PEG40 and the monovalent wild-type Nanobody™ TNF1 in comparison with Enbrel (Etanercept), Humira (Adalimumab) and Remicade (Infliximab)

Figure 54: Dose response curve obtained in cytotoxicity assay with rhesus TNFα using Nanobody™ TNF56-PEG40 in comparison with Enbrel (Etanercept), Humira (Adalimumab) and Remicade (Infliximab)

Figure 55: Pharmacokinetic analysis of pegylated bivalent Nanobody™ TNF56-PEG40 and TNF56-PEG60 after intravenous administration in mice

Figure 56: Pharmacokinetic analysis of pegylated bivalent Nanobody™ 3E-3E-PEG20, pegylated bivalent Nanobody™ 3E-3E-PEG40 and bispecific Nanobody™ 3E-3E-AR1 after intravenous administration in mice

Figure 57: Immunogenicity profile of TNF56-PEG40 and TNF56-PEG60 in mice

Figure 58: Efficacy of TNF60 in the prevention of chronic polyarthritis in mice

Figure 59: Efficacy of TNF60 in therapeutic treatment of chronic polyarthritis in mice

Figure 60: Effect of TNF60 Nanobody™ formatting on efficacy in the prevention of chronic polyarthritis in mice

Figure 61: Sequence alignment of Nanobodies™ PMP1C2, 3E, 1A and 3G

Figure 62: Molecular model of TNF-60

The appended Tables form an integral part of the present specification and are as follows:

Monovalent TNFα nanobodies

Table 8: Sequence listing of TNFα nanobodies

Table 9: Koff values of human TNFα nanobodies

Table 10: Homology of TNFα and serum albumin nanobodies to human germline sequences

Table 11: Expression levels of TNFα and serum albumin nanobodies

Table 12: ELISA binding to human and rhesus TNFα

Table 13: Receptor-inhibition assay of TNFα nanobodies

Table14: Biacore analysis of TNFα nanobodies

Table 15: Binding of TNFα nanobodies to TNFα ($K_D$-values)

Table 16: Potency of TNFα nanobodies to neutralize human (a) and rhesus (b) TNFα

Table 17: OD280nm of TNFα and serum albumin nanobodies after temperature treatment

Table 18: Potency of TNFα nanobodies after temperature treatment

Bivalent TNFα nanobodies

Table 19: Sequence listing of bivalent TNFα nanobodies and linker sequences

Table 20: Bivalent TNFα nanobody constructs

Table 21: Expression levels of bivalent TNFα nanobodies

Table 22: Receptor-inhibition assay of bivalent TNFα nanobodies

Table 23: Potency of TNFα nanobodies to neutralize human (a) and rhesus (b) TNFα

Table 24: OD280nm of bivalent TNFα nanobodies

Humanised monovalent TNFα nanobodies

Table 25: Sequence listing of humanised monovalent TNFα and serum albumin nanobodies

Table 26: Expression levels of humanised TNFα and serum albumin nanobodies

Table 27: Potency of TNFα nanobodies to neutralize human TNFα

Table 28: OD280nm of humanised TNFα and serum albumin nanobodies

Trivalent TNFα nanobodies

Table 29: Sequence listing of trivalent TNFα nanobodies

Table 30: Trivalent TNFα nanobody constructs

Table 31: Expression levels of trivalent TNFα nanobodies

Table 32: Potency of trivalent TNFα nanobodies to neutralize human TNFα

Table 33: Binding of trivalent nanobodies to serum albumin ($K_D$-values)

Table 34: OD280nm of trivalent TNFα nanobodies

Humanised monovalent TNFα nanobodies (second round)

Table 35: Sequence listing of second round humanised monovalent TNFα nanobodies

Table 36: Expression levels of humanised TNFα nanobodies

Table 37: Potency of TNFα nanobodies to neutralize human TNFα

Table 38: OD280nm of humanised TNFα nanobodies

Table 39: Comparing bio-activity of nanobodies

Further tables

Table 40: Overview of oligonucleotides used in formatting of trivalent Nanobodies™

Table 41: Overview of oligonucleotides used in cloning of trivalent Nanobodies™

Table 42: EC50 values obtained in cytotoxicity assay using trivalent Nanobody™ TNF60 in comparison with commercial controls (Enbrel, Remicade, Humira)

Table 43: Affinity determination of TNF60 and TNF24 on human serum albumin in Biacore. Nd, not determined.

Table 44: Overview of oligonucleotides used in formatting of bivalent Nanobodies™

Table 45: EC50 values obtained in cytotoxicity assay using bivalent Nanobodies™ in comparison with commercial controls (Enbrel, Remicade, Humira)

Table 46: Results of synovium derived fibroblast studies

Table 47: Results of murine air pouch studies

## EXAMPLES

### Example 1: Identification of TNFα and serum albumin specific nanobodies

Antagonistic nanobodies were identified using two llamas (*Llama glama*) immunized with human TNFα by 6 injections of 100 µg of the cytokine at weekly intervals. Screening was performed using a competition based assay, in which individual nanobodies were analyzed for their capability to inhibit binding of labeled TNFα to its receptor. The albumin specific nanobodies were identified from a llama immunized with human serum albumin. Screening of individual nanobodies was performed by ELISA using human, rhesus and mouse albumin, yielding a panel of nanobodies cross-reacting with the serum albumin of various species.

### Example 2: Sequence analysis of isolated nanobodies

Different classes of nanobodies were identified based on sequence analysis (Figure 1) using a BLOSUM62 scoring matrix and a similarity significance value cut-off of ≥ 60%: Class I (PMP1 C2, PMP1 G11, PMP1 H6), Class II (PMP1 G5, PMP1 H2, PMP3 G2), Class IIb (PMP1 D2), Class III (PMP3 D10, PMP5 F10). Table 8 lists the sequences of these TNFα nanobodies (SEQ ID NOs: 52 to 60).

Based on sequence analysis (Figure 2) different classes of serum albumin nanobodies were identified using the BLOSUM62 scoring matrix and a similarity significance value cut-off of ≥ 60%.Table 8 lists the sequences of these serum albumin nanobodies (SEQ ID NOs: 61 to 67).

**Example 3: Biacore analysis**

TNFα

Binding of nanobodies to TNFα was characterised by surface plasmon resonance in a Biacore 3000 instrument. TNF from different species was covalently bound to CM5 sensor chips surface via amine coupling until an increase of 250 response units was reached. Remaining reactive groups were inactivated. Nanobody binding was assessed at one concentration (1 in 1,000 diluted). Each nanobody was injected for 4 minutes at a flow rate of 45 µl/min to allow for binding to chip-bound antigen. Binding buffer without nanobody was sent over the chip at the same flow rate to allow spontaneous dissociation of bound nanobody for 4 hours. $K_{off}$-values were calculated from the sensorgrams obtained for the different nanobodies.

Of each class of nanobodies unpurified proteins were analyzed in Biacore. $K_{off}$ data is listed in Table 9.

Representative nanobodies from each class were retained for further analysis based on $k_{off}$ value. For Class I PMP1C2 (TNF1) was selected; PMP1G5 (TNF2) was selected as representative of Class II; PMP5F10 (TNF3) was selected as representative of Class III.

Serum albumin

Binding was assayed as described above except that 1 in 20 dilutions were used. Figures 3, 4 and 5 illustrate screening of albumin specific TNFα nanobodies versus human, rhesus and mouse serum albumin using unpurified protein.

The nanobodies are ranked according to $k_{off}$-values, see Table III below:

| Class | Human | Rhesus | Mouse |
|---|---|---|---|
| C | PMP6A8 | PMP6A8 | PMP6B4 |
| C | PMP6B4 | PMP6B4 | PMP6A8 |
| B | PMP6A6 | PMP6A6 | PMP6A6 |
| B | PMP6C1 | PMP6C1 | PMP6C1 |
| A | PMP6G8 | PMP6G8 | PMP6G8 |
| A | PMP6A5 | PMP6A5 | PMP6A5 |
| D | PMP6G7 | PMP6G7 | PMP6G7 |

Table III

The best $k_{off}$ were obtained for members of family C and family B. Cross-reactivity between mouse, human and rhesus serum albumin was also observed for members of those families. A representative nanobody from class B and C was defined for further analysis: PMP6A6 (ALB1) was selected as representative of Class B and PMP6A8 (ALB2) was selected as representative of Class C.

**Example 4: Cloning of monovalent nanobodies in pAX051**

Description of Escherichia coli expression vector

pAX051 is a derivative of pUC19. It contains the LacZ promoter which enables a controlled induction of expression using IPTG. The vector has a resistance gene for Ampicillin or Carbenicillin. The multicloning sites harbours several restriction sites of which *SfiI* and *BstEII* are frequently used for cloning of Nanobodies™. In frame with the NB coding sequence the vector codes for a C-terminal c-myc tag and a (His)6 tag. The signal peptide is the gen3 leader sequence which translocates the expressed Nanobody™ to the periplasm.

The DNA coding for the selected nanobodies TNF1 (PMP1C2), TNF2 (PMP1G5), TNF3 (PMP5F10), ALB1 (PMP6A6) and ALB2 (PMP6A8) was cloned in pAX051 and the construct was transformed to TG1 electrocompetent cells. Clones were analyzed for PCR insert and the nucleotide sequence was determined from 4 positive clones. Glycerol stocks were prepared from clones containing the correct sequence and stored at -80°C.

**Example 5: Expression of monovalent nanobodies**

A preculture was started by inoculating a single colony of the clone expressing the respective nanobodies at 37°C in Luria Broth, Ampicillin/Carbenicillin (100µg/ml) and 2% glucose overnight. This preculture was used to inoculate. Inoculum is 1% percent (v/v) of the production culture (TB medium + Ampicillin/Carbenicillin + 0.1% Glucose). The production culture is grown at 37°C until an OD600nm of 5-10 is reached and nanobody expression is induced by adding IPTG (1mM final concentration). Protein expression is allowed to continue either for 4h at 37°C or overnight at 28°C, at which point cells are collected by centrifugation and stored as wet cell paste at -20°C.

Preparative periplasmic extracts of the -20°C stored wet cell paste are made by resuspending the pellet in Peri-buffer (50mM $NaH_2PO_4$, 300mM NaCl, adjusted pH to 8.0), rotating the mixture for 30min at 4°C and centrifuging the mixture using a preparative centrifuge (Sorvall RC-3C *Plus* with H-6000A rotor) to pellet the cells. Supernatant, representing a rough extract of the periplasmic space, is collected for further purification.

The His(6)-tagged nanobodies are purified on Immobilized Metal Affinity Chromatography (IMAC). The TALON resin (Clontech) is processed according to the manufacturer's instructions. The extracts are incubated with the resin for 30 min at RT on a rotator. The resin is washed with PBS and transferred to a column. The packed resin is washed with 15 mM Imidazole. The nanobodies are eluted from the column using 150 mM Imidazole. The eluated fractions are analyzed by spotting on Hybond Membrane and visualization with Ponceau. Fractions containing protein are pooled and dialysed against PBS. Dialysed proteins are collected, filter sterilized, concentration determined and stored in aliquots at -20°C.

**Characterisation of monovalent TNFα nanobodies**

**Example 6: Homology to human germline sequences**

The nanobody amino acid sequences were compared to the human germline sequences as represented in Table 10. In order of homology to human sequences the nanobodies rank as follows: TNF1 > TNF2 > TNF3 for the TNFα nanobodies; ALB1 > ALB2 for the serum albumin nanobodies.

**Example 7: Expression level**

Expression levels were calculated and represented in Table 11. In order of yield the nanobodies rank as follows: TNF1>TNF2>TNF3 for the TNFα nanobodies; ALB1 > ALB2 for the serum albumin nanobodies.

**Example 8: SDS-Page analysis**

To determine the purity, protein samples were analyzed on a 15% SDS-PAGE gel. 10µl Laemmli sample buffer was added to 10µl (1ug) purified protein, the sample was heated for 10 minutes at 95°C, cooled and loaded on a 15% SDS-PAGE gel. The gel was processed according to general procedures and stained with Coomassie Brilliant Blue (CBB). Figure 6 represents the SDS-PAGE for the TNFα-specific and serum albumin-specific nanobodies.

**Example 9: Western Blot analysis**

100 ng of purified protein was loaded on the gel. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane using the Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad). The membrane was blocked overnight in PBS, 1% casein at 4°C. As all constructs were fused to c-myc tag, mouse monoclonal anti-myc antibody was used as a detection tool. In addition, rabbit polyclonal anti-Nanobody (R23) was used as a detection tool. The blot was incubated for 1h at room temperature with agitation in 1/2000 diluted anti-myc antibody in PBS or 1/2000 anti-Nanobody antibody in PBS, 1% casein. The membrane was washed 5 times in PBS before the secondary antibody was applied (rabbit-anti-mouse IgG alkaline phosphatase conjugate, Sigma, A1902, diluted 1/1000 in PBS or goat anti-rabbit IgG alkaline phosphatase conjugate, Sigma, A8025, 1% casein). After incubation with gentle agitation for 1h at room temperature, the membrane was washed 5 times in PBS. Blots were developed using BCIP/NBT solutions and the reaction was stopped by washing the blot with milliQ water when bands were clearly visible. Figure 7 represents the Western Blot analysis.

**Example 10: ELISA binding to human and rhesus TNFα**

An ELISA was performed to examine binding to human and rhesus TNFα. A 96-well Maxisorp plate was coated with 2 µg/ml Neutravidin in PBS ON at 4°C. Plates were blocked with 1 % caseine for 2hrs at RT. Biotinylated TNFα (400 ng/ml) was added to the wells and incubated for 1hr at RT. Nanobody samples were diluted starting at 2 µg/ml and using 1 in 3 dilutions. Nanobodies were detected using mouse anti-myc (1/2000 diluted) and rabbit anti-

mouse alkaline phosphatase (1/2000 diluted, Sigma, A1902) and pNPP (2mg/ml) as substrate. Figures 9 and 10 respresent the binding in ELISA to human and rhesus TNFα.

Results are summarized in Table12. TNF1 and TNF3 show binding to both human and rhesus TNFα. TNF2 is binding to human TNFα but is only weakly reactive to rhesus TNFα.

**Example 11: Receptor-Inhibition assay**

The ability to inhibit receptor-ligand interaction was analyzed for rhesus and human TNFα. A 96-well Maxisorp plate was coated with 2 µg/ml Enbrel in PBS ON at 4°C. Plates were blocked with 1 % Caseine for 2hrs at RT. Nanobody samples were pre-incubated for 30 min at RT with biotinylated TNFα (10ng/ml) starting at a concentration of 5 µg/ml and using 1 in 2 dilutions. Samples were added to the plates and incubated for 1 hr at RT. Biotinylated TNFα was detected using Extravidin alkaline phosphatase (1/2000 diluted) and pNPP (2mg/ml) as substrate. Figures 11 and 12 represent an inhibition ELISA for human and rhesus TNFα. Results are summarized in Table 13. Inhibition of ligand/receptor binding is observed for TNF1 and TNF3 for both human and rhesus TNF, while TNF2 is only inhibiting human TNFα.

**Example 12: Biacore analysis**

TNFα binding

The analysis was performed as described in Example 3. Figures 13 and 14 illustrate the binding to human and rhesus TNFα via Biacore analysis. Results are summarized in Table 14. Binding experiments in Biacore confirm the ELISA results: cross-reactive binding for TNF1 and TNF3, while TNF2 only significantly binds human TNFα.

Serum albumin

Binding was assayed as described above except that series of different concentrations were used. Each concentration was injected for 4 minutes at a flow rate of 45 µl/min to allow for binding to chip-bound antigen. Binding buffer without analyte was sent over the chip at

the same flow rate to allow for dissociation of bound nanobody. After 15 minutes, remaining bound analyte was removed by injection of the regeneration solution (25 mM NaOH).

From the sensorgrams obtained for the different concentrations of each analyte $K_D$-values were calculated via steady state affinity when equilibrium was reached.

Results are summarized in Table 15. Cross-reactivity is observed for both ALB1 and ALB2. The highest affinity is observed for ALB2 on human and rhesus TNFα. However, the difference in affinity for human/rhesus versus mouse serum albumin is more pronounced for ALB2 (factor 400), while for ALB1 a difference of a factor 12 is observed.

**Example 13: Bio-assay**

The TNFα sensitive mouse fibroblast cell line L929s was used for measuring the anti-TNFα activity of the selected nanobodies. At a sufficiently high concentration of TNFα in the medium, i.e. cytotoxic dose, L929s cells undergo necrosis. The inhibition of TNFα interaction with its receptor was determined by pre-incubating a series of antibody dilutions with a cytotoxic concentration of TNFα before adding the mixture to the cells. The presence of actinomycin D in the medium sensitises the cells further to TNFα, resulting in increased sensitivity of the bioassay for free TNFα.

The L929 cells were grown to nearly confluency, plated out in 96-well microtiter plates at 5000 cells per well and incubated overnight. Actinomycin D was added to the cells at a final concentration of 1 µg/ml. Serial dilutions of the nanobodies to be tested were mixed with a cytotoxic concentration of TNFα (final assay concentration is 0.5 ng/ml or 15 IU/ml). After at least 30 minutes of incubation at 37°C, this mixture was added to the plated cells. Plates were incubated for 24 hours at 37°C and 5% $CO_2$. Cell viability was determined by use of the tetrazolium salt WST-1. Dose-response curves and $EC_{50}$ values were calculated with Graphpad Prism.

The results are summarized in Table 16 for human and rhesus TNFα. Based on their potency to neutralize cytotoxic activity, the molecules are ranked as follows: TNF3>TNF1>TNF2 for human TNFα, and TNF1 = TNF3 > TNF2 for rhesus TNFα.

**Example 14: Protein A binding**

Figure 14 represents Protein A binding analyzed in Biacore as described in Example 12. Positive binding was obtained for TNF1, TNF2, ALB1. No or weak binding was observed for TNF3 and ALB2.

**Example 15: Temperature stability**

Samples were diluted at 200 µg/ml and divided in 8 aliquots containing 500 µl. The different vials were incubated each at a given temperature ranging from RT to 90°C. After treatment the samples were cooled down for 2hrs at RT, they were kept at 4°C. Precipitates were removed by centrifugation for 30 min at 14,000 rpm. SN was carefully removed and further analysed.

OD280nm

OD at 280 nm was measured and the concentration was calculated. Results are summarized in Table 17. A decrease in protein content was observed for TNF2 and TNF3 starting at 80°C, while for ALB2 a decrease is observed starting from 70°C.

Western Blot

2 µg of treated protein was separated on a 15% SDS-PAGE and transferred to a nitrocellulose membrane and treated as described above. Detection was performed using polyclonal anti-Nanobody (R23, 1/2000 diluted) and anti-rabbit horse radish peroxidase (DAKO, P0448, 1/2000 diluted). Figure 15 represents the Western Blot analysis. A clear drop in protein concentration was observed for ALB2 treated at 70, 80 and 90°C. Aggregation was still observed for TNF1 treated at 70, 80 and 90°C; for TNF3 treated at 90°C; for ALB1 treated at 90°C, meaning that the SN still contains traces of precipitates which result in a higher OD280nm read-out. This explains why the protein concentration as measured at OD280nm does not decrease for TNF1, TNF3 and ALB1 treated at these higher T.

ELISA

The ELISA to detect binding to human TNFα was essentially performed as described in Example 10. Results are presented in Figure 16. Human TNFα binding is decreased for TNF1, TNF2, TNF3 starting at 80°C.

Bio-assay

The bio-assay was performed as described in Example 13. The results are summarized in Table 18. Potency of the nanobodies is decreased for TNF1 starting at 70°C; for TNF2 and TNF3 starting at 80°C.

Biacore

Binding to human serum albumin was determined as described in Example 12. A fixed concentration was used (1 in 50 diluted). Results are presented in Figure 17. Temperature treatment is not influencing binding to serum albumin for ALB1. The treatment has an effect on the $k_{on}$ for ALB2 starting from T=70°C.

**Bivalent nanobodies**

**Example 16: Formatting of bivalent TNFα specific nanobodies**

TNF1, TNF2 and TNF3 were formatted to bivalent nanobodies. As spacer between the two building blocks either a 9AA GlySer linker (Table 19 SEQ ID No: 68) or a 30 AA GlySer linker (Table 19 SEQ ID No: 69) was used. This generated the constructs represented by Table 20. Table 19 lists the sequences of these bivalent TNFα nanobodies (SEQ ID NOs: 70 to 75).

**Example 17: Expression of bivalent TNFα specific nanobodies**

Expression was performed as described in Example 5. The His(6)-tagged nanobodies were purified on Immobilized Metal Affinity Chromatography (IMAC). The Ni-NTA resin (Qiagen) was processed according to the manufacturer's instructions. The extracts were incubated with the resin and incubated for 30 min at RT on a rotator. The resin was washed with PBS and transferred to a column. The packed resin was washed with PBS (1 in 10 diluted). The column was pre-eluted with 15 mM Imidazole. The nanobodies were eluted from the column using 25 mM Citric Acid pH=4. The eluated fractions were analyzed by spotting on Hybond Membrane and by visualization with Ponceau. Fractions containing protein were pooled and further purified on Cation exchange followed by size exclusion. Purified proteins were collected, filter sterilized, concentration determined and stored in aliquots at -20°C.

**Characterisation of bivalent TNFα specific nanobodies**

**Example 18: Expression level**

Expression levels of the bivalent TNFα nanobodies were calculated and represented in Table 21. The linker has no significant effect on the expression level of the nanobodies.

**Example 19: SDS-PAGE**

SDS-Page was performed as described in Example 8. Figure 18 shows the result of the SDS-Page.

**Example 20: Western Blot**

Western Blot analysis was performed as described in Example 9. Figure 19 represents the Western Blot results.

**Example 21: Receptor-Inhibition assay**

The assay was performed as described in Example 11. Figure 20 and Table 22 represent the results. Enhancement of inhibition of ligand/receptor binding was observed for all bivalent nanobodies compared to the monovalent format.

**Example 22: Bio-assay**

The assay was performed as described in Example 13. Results are summarized in Table 23. Based on their potency to neutralize cytotoxic activity TNF8, TNF7, TNF9 and TNF5 have a potency in the range of Enbrel.

**Example 23: Temperature stability**

Samples were analysed as described in Example 15.

OD280 nm

OD at 280 nm was measured and the concentration was calculated. Results are summarized in Table 24. A decrease in protein content was observed for TNF4 and TNF7 starting at 70°C, while for TNF5, TNF6, TNF8 and TNF9 a decrease was observed starting from 80°C.

Western Blot

Samples were analyzed for the presence of aggregates as described in Example 15.

ELISA

The ELISA to detect binding to human TNFα was essentially performed as described above. Results are presented in Figure 21. Human TNFα binding was decreased for TNF5, TNF6, TNF8 and TNF9 starting at 80°C, for TNF4 and TNF7 starting from 70°C.

Humanised monovalent nanobodies

**Example 24: Identification of non-human amino acid positions in TNFα and serum albumin specific nanobodies**

Figure 22 (TNF1), Figure 23 (TNF2), Figure 24 (TNF3) and Figure 25 (ALB1) represent multiple sequence alignments (Clustal W 1.7) with DP51, DP53, DP54 and DP29 sequences.

In addition to the amino acid mutations, codon optimization was performed yielding the sequences of Table 25 SEQ ID NOs: 76 to 89 (Nanobodies against TNF-alpha and human serum albumin, respectively).

**Example 25: Generation of codon optimised mutants**

Oligonucleotides were synthesised spanning the entire sequence of the nanobodies.

**Example 26: Expression of bivalent TNFα specific nanobodies**

Expression was performed as described in Example 5.

Characterisation of humanised nanobody

**Example 27: Expression level**

Table 26 represents calculated expression levels. Expression was achieved with yields in the range of 3.5-11.7 mg/ml. Induction time did not influence the yield.

**Example 28: SDS-PAGE**

SDS-PAGE was performed as described in Example 8. Figure 26 represents the SDS-PAGE gel.

**Example 29: Western Blot**

Western Blot analysis was performed as described in Example 9. Figure 27 represents the Western Blot results.

**Example 30: Bio-assay**

The assay was performed as described in Example 13.

The results of the humanised nanobodies are summarized in Table 27. The wildtype nanobodies are included as reference.

**Example 31: Biacore**

The analysis was performed as described in Example 12. Figure 28 and 31 shows Biacore results.

**Example 32: Temperature stability**

Samples were analysed as described in Example 15.

OD280 nm

OD at 280 nm was measured and the concentration was calculated. Results are summarized in Table 28.

No significant decrease in protein concentration is observed for the humanised TNF1 nanobodies (TNF13-14). A decrease in protein concentration is observed for humanised TNF2 (TNF15-19) and TNF3 (TNF20-23) starting at 80°C. A decrease in protein concentration is observed for humanized ALB1 (ALB4-5) starting at 70°C and for ALB3 starting at 60°C.

Western Blot

Samples were analyzed for the presence of aggregates as described in Example 15.

ELISA

The ELISA to detect binding to human TNFα was essentially performed as described in Example 15. Results are presented in Figure 30.

Human TNFα binding is comparable for temperature treated WT TNF1 and the humanized TNF13 and 14; for temperature treated WT TNF2 and the humanized TNF15-19; human TNFα binding is decreased for TNF21 and 22, and to a less extent for TNF23, while no effect is observed for TNF20 compared to the temperature treated WT TNF3.

**Trivalent TNFα nanobodies**

**Example 33: Formatting of trivalent TNFα specific nanobodies**

TNF1, TNF2, TNF3 and ALB1 were formatted to trivalent nanobodies. As spacer between 2 building blocks either a 9AA GlySer linker (Table 19 SEQ ID No 68) or a 30 AA GlySer linker (Table 19 SEQ ID No 69) was used. This generated the constructs of Table 30. Table 29 lists the sequences of trivalent TNFα nanobodies (SEQ ID NOs: 91 to 94).

**Example 34: Expression of trivalent TNFα specific nanobody**

Expression was performed as described in Example 5. The His(6)-tagged nanobodies are purified on Immobilized Metal Affinity Chromatography (IMAC). The Ni-NTA resin (Qiagen) is processed according to the manufacturer's instructions. The extracts are incubated with the resin and incubated for 30 min at RT on a rotator. The resin is washed with PBS and transferred to a column. The packed resin is washed with PBS (1 in 10 diluted). Pre-elute with 15 mM Imidazole. The nanobodies are eluted from the column using 25 mM Citirc Acid pH=4. The eluated fractions are analyzed by spotting on Hybond Membrane and visualization with Ponceau. Fractions containing protein are pooled and further purified on Cation exchange followed by size exclusion. Purified proteins are collected, filter sterilized, concentration determined and stored in aliquots at -20°C.

**Characterization of trivalent TNFα/SA specific nanobodies**

**Example 35: Expression level**

Expression levels were calculated and represented in Table 31.

**Example 36: SDS-PAGE analysis**

SDS-PAGE was performed as described in Example 8. Figure 31 represents the SDS-PAGE gel.

**Example 37: Western Blot analysis**

Western Blot analysis was performed as described in Example 9. Figure 32 represents the Western Blot analysis.

**Example 38: Bio-assay**

The assay was performed as described in Example 13.

The results of the bivalent nanobodies are summarized in Table 32. Based on their potency to neutralize cytotoxic activity, the molecules are equally potent and comparable to their potency as bivalent molecules.

**Example 39: Binding to Human Serum Albumin**

Binding was assayed as described above except that series of different concentrations were used. Each concentration was injected for 4 minutes at a flow rate of 45 µl/min to allow for binding to chip-bound antigen. Next, binding buffer without analyte was sent over the chip at the same flow rate to allow for dissociation of bound nanobody. After 15 minutes, remaining bound analyte was removed by injection of the regeneration solution (25 mM NaOH).

From the sensorgrams obtained for the different concentrations of each analyte $K_D$-values were calculated via steady state affinity when equilibrium was reached.

Results are summarized in Table 33. A decrease in affinity was observed for the formatted ALB1 binder compared to the wild type ALB1. The affinity however is still in the range of 7.2-14 nM.

**Example 40: Temperature stability**

Samples were analysed as described in Example 15.

OD280 nm

OD at 280 nm was measured and the concentration was calculated. Results are summarized in Table 34. A decrease in protein content is observed for TNF24, TNF27 and TNF28 starting at 60°C, while for TNF25 and TNF26 starting from 70°C.

Western Blot

Samples were analyzed for the presence of aggregates as described in Example 15.

ELISA

The ELISA to detect binding to human TNFα was essentially performed as described above. Results are presented in Figure 33. Human TNFα binding is decreased for TNF24 and TNF27, starting from 60°C and for TNF25, TNF26 and TNF28 starting at 70°C.

Humanised monovalent nanobodies (second round)

**Example 41: Identification of non-human amino acid positions in TNFα and serum albumin specific nanobodies**

Figure 34 (TNF1), Figure 35 (TNF2), Figure 36 (TNF3) and Figure 37 (ALB1) represent multiple sequence alignments (Clustal W 1.7) with DP51, DP53, DP54 and DP29 sequences. The mutated molecules were expressed and purified as described above, yielding the sequences of Table 35 SEQ ID NOs: 95 to 104 (against TNF-alpha and human serum albumin, respectively).

Characterisation of humanised nanobody

**Example 42: Expression level**

Table 36 represents calculated expression levels. Expression was achieved with yields in the range of 0.5-2.7 mg/ml.

**Example 43: SDS-PAGE**

SDS-Page was performed as described in Example 8. Figure 38 represents the SDS-Page gel.

**Example 44: Western Blot**

Western Blot analysis was performed as described in Example 9. Figure 39 represents the Western Blot results.

**Example 45: Bio-assay**

The assay was performed as described in Example 13.

The results of the humanised nanobodies are summarized in Table 37. The wildtype nanobodies and first round of humanised nanobodies are included as reference.

**Example 46: Biacore**

The analysis was performed as described in Example 12. Figure 40 shows Biacore results.

**Example 47: Temperature stability**

Samples were analysed as described in Example 15.

OD280 nm

OD at 280 nm was measured and the concentration was calculated. Results are summarized in Table 38.

No significant decrease in protein concentration is observed for the humanised TNF1 nanobodies (TNF29-30). A decrease in protein concentration is observed for humanised TNF2 (TNF31-32) and TNF3 (TNF33) starting at 80°C.

Western Blot

Samples were analyzed for the presence of aggregates as described in Example 15.

ELISA

The ELISA to detect binding to human TNFα was essentially performed as described in Example 15. Results are presented in Figure 41.

Human TNFα binding is comparable for WT TNF1 and the humanised TNF29 and TNF30; comparable for WT TNF2 and the humanised TNF31 and TNF32; and also for WT TNF3 and humanised TNF33.

**Comparative Example**

In this Comparative Example, nine Nanobodies of the invention were compared with three Nanobodies from WO 04/041862, called "$V_{HH}$#1A" or "1A", "$V_{HH}$3E" or "3E" and "$V_{HH}$#3G" or "3G" respectively (SEQ ID NOS:1, 4 and 5 in WO 04/041862). The assay used was the cell based assay using KYM-cells referred to in WO 04/41862 (see for example Example 1, under 3)). The results are mentioned in Table 39 below. As can be seen, the Nanobodies of the invention have an EC50 value in this assay that is 18-fold better than the EC50 value of 3E, the best performing Nanobody according to WO 04/041862.

**Example 48: Generation of trivalent bispecific humanized Nanobodies™**

Trivalent bispecific Nanobodies were formatted and cloned in the E. coli expression vector pAX054 first and then rescued through PCR and cloned in the pPICZαA expression vector.

***Description of Escherichia coli expression vector***

pAX54 is a derivative of pUC19. It contains the LacZ promoter which enables a controlled induction of expression using IPTG. The vector has a resistance gene for Ampicillin or Carbenicillin. The multicloning sites harbours several restriction sites of which *Sfi*I and *BstE*II are frequently used for cloning of Nanobodies™. The signal peptide is the gen3 leader sequence which translocates the expressed Nanobody™ to the periplasm.

***Description of Pichia pastoris expression vector***

pPICZαA contains a pUC-derived origin of replication allowing propagation in *E coli*. It contains the promoter of the *Pichia pastoris AOX1* (alcohol oxidase 1) gene. This 942 bp promoter region (i) allows methanol-inducible, high-level expression of the gene of interest, and (ii) targets plasmid integration to the *AOX1* locus following transformation of Pichia with vector DNA that is linearized within the 5′ *AOX1* promoter region. Note that pPICZα vectors do not contain a yeast origin of replication and that, consequently, transformants can only be isolated if recombination occurs between the plasmid and the Pichia genome. The vector specifies resistance to the antibiotic Zeocin in both *E. coli* and *Pichia pastoris* host cells. The vector incorporates the secretion signal of the *Saccharomyces cerevisiae* α-mating factor allowing for efficient secretion of most proteins to the culture medium. The initiation ATG in the α-factor signal sequence corresponds to the native initiation ATG of the *AOX1* gene. The multicloning site harbours several restriction sites of which *Xho*1/*EcoR1* or *Xho*1/*Not*1 are typically used for fusion of the Nanobody™ coding sequences to the secretion signal. The multicloning site is followed by the *AOX1* transcription termination region. More details on this expression vector can be found on the website of Invitrogen (http://www.invitrogen.com/content/sfs/manuals/ppiczalpha_man.pdf).

***Formatting trivalent Nanobodies***

Three separate PCR reactions were set up to amplify the N-terminal, the middle and the C-terminal Nanobody™ subunit using the oligo combinations indicated in the WPA-0012. The N-terminal Nanobody™ was amplified using M13_rev/ Rev_9GlySer_L108; the middle Nanobody™ was amplified using For_GlySer/Short and Rev_15BspEI_L108; the C-terminal Nanobody™ was amplified using For_BspEI/M13_for. A PCR reaction of 1 µl plasmid DNA (50-100 ng), 1.5 µl forward primer (10 µM → 300 nM), 1.5 µl reverse primer

(10 µM → 300 nM), 1 µl dNTPs (10 mM → 0.2 mM), 5 µl buffer (10x → 1x), 0.75 µl enzyme (3.5 U/µl → 2.6 U/µl) and 39.25 µl $H_2O$ with a total volume of 50 µl was prepared. Primer sequences are given in Table 40. A PCR program was started with 2 minutes at 94°C. A cycle of 30 seconds at 94°C, 30 seconds at 50°C and 1 minute at 72°C was repeated 30 times and followed by 10 minutes at 72°C. Amplification was checked by separating 5 µl of the PCR reaction on a 2% agarose gel. The PCR product was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. One column was used and eluted with 50 µl EB buffer. The N-terminal VHH fragment was prepared by incubating 50 µl DNA and 2 µl *BamHI* (10 U/µl) in the appropriate buffer recommended by the manufacturer at 37°C for 2 hours. Subsequently, 2 µl *SfiI* (10U/µl) was added and the mixture was incubated at 55°C for 2 hours. The middle VHH fragment was prepared by incubating 50 µl DNA and 2 µl *BamHI* (10 U/µl) and 2 µl *BspEI* (10 U/µl) in the appropriate buffer recommended by the manufacturer at 37°C for 2 hours. The C-terminal VHH fragment was prepared by incubating 50 µl DNA with 2 µl *BspEI* (10 U/µl) in the appropriate buffer recommended by the manufacturer at 37°C for 2 hours. Subsequently, 2 µl *BstEII* (10U/µl) was added and the mixture was incubated at 60°C for 1 hours. The previous digestion reactions were separated on a 2% agarose gel. The VHH bands (350-450 bp) were cut out of the gel and the DNA was purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. One column (with a maximum of 400 mg agarose gel per column) was used and the bound DNA was eluted with 50 µl EB buffer. DNA concentration was determined by measuring $OD_{260}$ (1 OD unit = 50 µg/ml). A ligation mixture with a final volume of 10 µl containing 100 ng vector pAX54, 12 ng N-terminal VHH, 12 ng middle VHH fragment, 12 ng C-terminal VHH fragment, 1 µl ligation buffer and 1 µl ligase (3U) was prepared and incubated for 2 hours at room temperature. Transformation of *E. coli*, TG1 was performed by using 2 µl of ligation mixture. Colonies are analysed using PCR as described in WPA-0010. Sequence analysis is performed on positive clones. Plasmid preparation was performed using the Qiaprep spin Miniprep kit (Qiagen) according to the manufacturer's instructions and described above. Sequencing was performed at the VIB sequence facility, Antwerp, Belgium.

***Amplification of coding DNA***

The Nanobody™ coding region cloned in the pAX054 *E. coli* expression vector is rescued through PCR using an appropriate primer pair. To ensure that the Nanobody™ is

expressed with a native N-terminus, the coding region is cloned in frame with the Kex2 cleavage site of the secretion signal. The forward primer fuses the C-terminal part of the secretion signal, up to the *Xho*1 recognition site, to the Nanobody™ coding region. A PCR reaction of 1 µl plasmid DNA (50-100 ng), 1.5 µl forward primer (10 µM → 300 nM), 1.5 µl reverse primer (10 µM → 300 nM), 1 µl dNTPs (10 mM → 0.2 mM), 5 µl buffer (10x → 1x), 0.75 µl enzyme (3.5 U/µl → 2.6 U) and 39.25 µl $H_2O$ with a total volume of 50 µl was prepared. Primer sequences are given in Table 41. A PCR program was started with 2 minutes at 94°C. A cycle of 30 seconds at 94°C, 30 seconds at 50°C and 2 minutes at 72°C was repeated 20 times and followed by 10 minutes at 72°C. Amplification was checked by separating 5 µl of the PCR reaction on a 2% agarose gel. The PCR product was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. One column was used and the bound DNA was eluted with 50 µl EB buffer.

***Cloning strategy***

The DNA fragment coding for the NB as well as the pPICZαA expression vector is digested with the appropriate restriction enzymes (*Xho*I + *Not*I). The insert is obtained by incubating 50 µl PCR product with 2 µl *Xho*I (10 U/µl) and 2 µl *Not*I (10 U/µl) in the appropriate buffer recommended by the manufacturer for 3 hrs at 37°C. Vector is obtained similarly, adapting the amount of restriction enzymes to the amount of plasmid. Both the vector and the NB coding fragment are purified and the DNA concentration is quantified using the BioPhotometer (Eppendorf). The fragment and the acceptor vector are ligated in equimolar ratio's using 1 Unit T4 ligase (Promega) for 30 minutes at room temperature or overnight at 16°C. The DNA (20-30 ng) is transformed to TG1 cells. Colonies are analysed through PCR using the 3'AOX1 R and 5'AOX1 F primers. Sequence analysis is performed on positive clones. TNF30, TNF33 and ALB8 were formatted to trivalent bispecific Nanobodies™. As spacer between the building blocks a 9AA GlySer linker was used.

***Transformation P. pastoris***

To isolate plasmid DNA, a preculture is started by inoculating a single colony of the clone in 50 ml Luria Broth + Ampicillin or Carbenicillin (100µg/ml) + 2% glucose and incubation at 37°C overnight. Plasmid DNA is prepared using the Plasmid Midi kit (Qiagen) according to the manufacturer's instructions. The DNA is linearized by incubating 30 µg plasmid DNA with 6 µl *BstX*1 (10 U/µl) in the appropriate buffer according to the

manufacturer's instructions for 3 hrs at 45°C. Digested DNA is purified using the PCR Purification kit (Qiagen) according to the manufacturer's instructions. The DNA is concentrated using EtOH precipitation according to standard procedures. X-33 electrocompetent cells are transformed with 10 µg linearized DNA and cells are allowed to grow for 48 hrs on a selective YPD agar plate containing Zeocin (100/250/500 µg/ml). X-33 is a wild type *Pichia pastoris* strain; the strain itself as well as the derived recombinant strains contain the native *AOX1* gene and are able to metabolize methanol ($Mut^+$).

Clones are screened for expression level by inoculating single colonies in 1 ml BGCM in a 24-well plate and growing them for 48 hrs at 30°C at 120 rpm. Cells are centrifuged and fresh BGCM is added to the cells for growth at 30°C at 120 rpm during 48 hrs. Next, MeOH is added to a final concentration of 0.5 % and cells are grown at 30°C at 120 rpm during 8 hrs, after which MeOH is added again to a final concentration of 0.5 %. Cells are grown overnight at 30°C at 120 rpm. Cells are centrifuged and the supernatant is harvested and analysed in ELISA as described in example 10.

**Example 49: Expression and purification of trivalent bispecific humanized Nanobodies™**

***Production in Pichia pastoris***

Composition of buffers, solutions and others can be found on the website of Invitrogen (http://www.invitrogen.com/content/sfs/manuals/ppiczalpha_man.pdf).

A preculture was started by inoculating a single colony from plate in 5 ml YPD. The culture was grown overnight at 180 rpm and 30°C. The next day, the pre-culture was diluted to 50 ml of YPD and grown overnight at 180 rpm and 30°C. Production cultures were started by inoculating the pre-culture to a final OD600nm = 0.04-0.08. Cultures were grown in BGCM for 24 hrs at 30 °C at 180 rpm and centrifuged at 4,500 rpm for 30 minutes. Cells were resuspended in 1/3 of the original volume in BGCM medium with a final OD600 nm = 15-20. Cells were induced with MeOH at regular time points, typically 3 times/day, never exceeding the 1 % MeOH content. After 50 hours of induction the supernatant is harvested.

***Purification of Nanobody expressed in Pichia pastoris***

Culture supernatant is filtered over a .22 µm filtration membrane Micro filtration (Hydrosart, Sartorius). Sample is concentrated using diafiltration on 10kDa ultra filtration membrane (HydroSart, Sartorius) and concentrated to 0.5-1L.

Nanobodies™ are purified using Protein A affinity chromatography (MabSelect Xtra, GE Healthcare) using PBS as running buffer and Glycine [100mM pH=2.5] for elution. Samples are neutralized using 1.5 M Tris pH=8.8. Nanobodies™ are further processed in Anion Exchange Chromatography (Source 30Q, GE Healthcare). Samples are diluted 10-fold with 10mM Piperazine pH=10.2 and adjusted to pH=10.2 with 1M NaOH and a conductivity of <2mS/cm with MilliQ water.

Nanobodies are processed in Size Exclusion chromatography (Superdex 75pg, Hiload XK26/60, GE Healthcare) and LPS is removed via Anion Exchange Chromatography (Source 30Q, GE Healthcare) by passage through 5ml column, which is sanitized with 1M NaOH and equilibrated in Dulbecco PBS.

To determine the purity, protein samples were analyzed on a 15% SDS-PAGE gel as described in example 8. The gel is processed using the SilverQuest™ according to general procedures described by the manufacturer (Invitrogen). Alternatively, gel is processed using coomassie brilliant blue or in western blot as described in example 8 and 9. Results are given in Figure 42.

**Example 50: Characterization of trivalent bispecific humanized Nanobodies™**

TNF60 consists of 363 amino acids. The protein has a molecular weight of 38,441 Da. The pI is 8.71. The extinction coefficient at 280 nm is 1.736.

*Mass spectrophotometry*

The mass of the protein was determined in ESI-MS according to standard procedures. The theoretical mass of TNF60 is 38,441 Da. The protein has 2 S-S bridges which should result in a mass of 38,435 Da in ESI-MS. The mass that was experimentally determined for TNF60 derived from 3 different batches ranges from 38,433 Da to 38,435 Da, differing maximally 0.005% with the theoretical mass.

*N-terminal sequencing*

N-terminal sequencing was performed by Edman degradation according to standard procedures. N-terminal sequencing showed that the protein sequence for the first 7 amino acids is as follows: EVQLVES. This is consistent with the theoretical protein sequence, which indicates proper N-terminal processing.

*Analytical sizing*

Samples (100 ug) were analysed on the high resolution Superdex75 column, to characterize the different batches of Nanobody™. Size exclusion chromatography of the Nanobody™ typically yields a symmetrical peak, with a retention time of 11.5 min on Superdex75. The absorbance is typically recorded at 280, 254 and 214 nm. The 214 nm measurement permits higher detection sensitivity. Analytical sizing in PBS provides a symmetrical peak. No contaminants were observed. The retention time observed for 3 different batches is 11,5-11,55 min. A representative profile is shown in Figure 43.

**Example 51: Binding of TNF60 to human TNFα in ELISA**

The functionality of TNF60, i.e. binding to human TNFα was analyzed in ELISA as described in example 10. The results are summarized in Figure 44 and clearly demonstrate a dose-dependent and saturable binding of 2 batches of TNF60 to human TNFα.

**Example 52: Functionality in Cell-based assay**

The potency to neutralize the cytotoxic activity of TNFα was analyzed in a cell-based assay as described in example 13. The results are summarized in Table 42 and in Figures 45 and 46.

The data show that TNF60 has potency in the range of Enbrel/Etanercept and a 10-fold better potency than Humira/Adalimumab and Remicade/Infliximab.

**Example 53: Binding of TNF60 to serum albumin**

Binding to human and rhesus serum albumin was analyzed in Biacore as described in example 12. KD, kon and koff values are represented in Table 43. TNF60 is compared to TNF24, which is the trivalent bispecific parent Nanobody™ with wild-type building blocks.

Affinity of TNF60 for human and rhesus serum albumin is similar. Affinity is 2-fold lower as compared to the affinity observed for TNF24 which is the wild type analogue of TNF60. $K_{on}$ is identical for both molecules, but the $k_{off}$ is 2-fold higher for TNF60.

**Example 54: Pharmacokinetic and immunogenicity analysis of trivalent bispecific humanized Nanobodies in mice**

*Animals*

DBA1 or BALBc mice were warmed up under an infrared lamp and 200 µl Nanobody™ (100 µg per mouse) was injected intravenously in the tail. Blood samples were obtained at different time points by making a small incision in the tail and collecting the blood in a microtube. Typically, blood was sampled at t=15 min, 2hrs, 4hrs, 6 hrs, 1 day, 2 days, 3 days, 4 days, 7 days, 14 days. Serum was prepared according to standard procedures.

*Determination of Nanobody™ concentration in mouse serum*

A microtiterplate (NUNC, Maxisorb) was coated with 2 µg/ml neutravidin overnight at 4°C. The plate was washed 5 times with PBS/0.05% Tween-20 and blocked for 2 hours at RT with PBS/1% casein. Biotinylated human TNFα (1/2000 in PBS/0.2% casein; 400 ng/ml) was applied to the wells and incubated for 1hr at RT. The standard reference Nanobody™ was applied starting at a concentration of 5 µg/ml and using 5-fold dilutions in PBS containing 1% mouse plasma. The Nanobodies™ were allowed to bind for 2 hours at RT. The plate was washed 5 times and rabbit polyclonal anti-Nanobody (R23) was applied at a 2000-fold dilution for one hour at RT. After washing of the plate, binding was detected with goat-polyclonal-anti-rabbit-HRP (DAKO) at a 3000-fold dilution for one hour at RT, and stained with ABTS/$H_2O_2$. The OD405nm was measured.

This first ELISA was used to determine the linear range of the standard reference. In a second ELISA, the standard reference was used at concentrations in this linear range and typically using 2-fold dilutions. In this second ELISA, serum test samples were diluted 100-fold and further 5-fold dilutions were made in 1% mouse plasma, to determine the dilution at which the serum samples provide a read-out in the linear range of the standard curve. In a third ELISA, serum samples are diluted at an appropriate concentration determined in the second ELISA and using 2-fold dilutions for accurate determination of the Nanobody™ concentration in the serum samples.

Experiments were performed to determine the pharmacokinetic profile of TNF60 in mice (n=3). A Cmax value of 103,84 ± 31 µg/ml was reached 15 minutes after administration. The half-life (t1/2β) was determined to be 1.9 days, similar to the half-life of mouse serum albumin, indicating that TNF60 adopts the half-life of serum albumin. Data are presented in Figure 47.

*Determination of anti-Nanobody antibodies in mice*

Nanobody™ was coated at 5 µg/ml in PBS at 4°C overnight. The plate was washed 5 times with PBS/0.05% Tween-20 and blocked for 2 hours at RT with PBS/1% casein. Serum samples were diluted 100-fold and applied to the wells for incubation during 1 hr at room temperature. Detection was performed using 1000-fold diluted polyclonal rabbit anti-mouse-HRP (DAKO, P0260) and using ABTS as substrate.

Serum samples were diluted 50-fold and analyzed for the presence of mouse anti-TNF60 antibodies. Lack of immunogenicity was demonstrated for TNF60. Data are presented in Figure 48.

**Example 55: Generation of bivalent long half-lived humanized Nanobodies™**

*Description of Pichia pastoris expression vector*

See example 48

*Formatting bivalent Nanobodies™*

Two separate PCR reactions were set up to amplify the N-terminal and the C-terminal Nanobody™ subunit using the procedures as indicated in the WPA-0011. For the amplification of the N-terminal Nanobody™ PiForLong and Rev_30GlySer_L108 were used as primer combination; for the amplification of the C-terminal Nanobody™ For_GlySer and PiRevCys1hum were used or alternatively For_GlySer and PiRevCys2hum, introducing the restriction sites required for formatting and free cysteine residues required for C-terminal modifications.

PCR reaction of 1 µl plasmid DNA (50-100 ng), 1.5 µl forward primer (10 µM → 300 nM), 1.5 µl reverse primer (10 µM → 300 nM), 1 µl dNTPs (10 mM → 0.2 mM), 5 µl buffer (10x → 1x), 0,75 µl enzyme (3.5 U/µl → 2.6 U/µl) and 39.25 µl $H_2O$ with a total volume of 50 µl was prepared. Primer sequences are given in Table 44. A PCR program was started with 2 minutes at 94°C. A cycle of 30 seconds at 94°C, 30 seconds at 50°C and 1 minute at 72°C was repeated 30 times and followed by 10 minutes at 72°C. Amplification was checked by separating 5 µl of the PCR reaction on a 2% agarose gel. The PCR product was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. One column was used and eluted with 50 µl EB buffer. The N-terminal VHH fragment was prepared by incubating 50 µl DNA and 2 µl *BamHI* (10 U/µl) and 2 µl *XhoI* (10 U/µl)in the appropriate buffer recommended by the manufacturer at 37°C for 1.5 hours. The C-terminal

VHH fragment was prepared by incubating 50 µl DNA and 2 µl *BamHI* (10 U/µl) and 2 µl *EcoRI* (10 U/µl) in the appropriate buffer recommended by the manufacturer at 37°C for 1 hour. The previous digestion reactions were separated on a 2% agarose gel. The VHH bands (350-450 bp) were cut out of the gel and the DNA was purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. One column (with a maximum of 400 mg agarose gel per column) was used and the bound DNA was eluted with 50 µl EB buffer. DNA concentration was determined by measuring $OD_{260}$ (1 OD unit = 50 µg/ml). A ligation mixture with a final volume of 10 µl containing 100 ng vector pPICZαA, linearized with *XhoI*/*EcoRI*, 30 ng N-terminal VHH, 30 ng C-terminal VHH fragment, 1 µl ligation buffer and 1 µl ligase (3U) was prepared and incubated for 1 hour at RT. Transformation of *E. coli*, TG1 was performed by using 2 µl of ligation mixture. Colonies are analysed using PCR as decribed in WPA-0010 but using the AOXIFor/AOXIRev primer combination. Sequence analysis is performed on positive clones. Plasmid preparation was performed using the Qiaprep spin Miniprep kit (Qiagen) according to the manufacturer's instructions and described above. Sequencing was performed at the VIB sequence facility, Antwerp, Belgium.

***Transformation of P. pastoris***

See example 48.

TNF30 was formatted to bivalent Nanobodies™. As spacer between the 2 building blocks a 30 AA GlySer linker was used. To allow for C-terminal site-specific modifications a free cysteine was introduced, either as the last AA of the Nanobody™ or with an extra spacer consisting of GlyGlyGlyCys (SEQ ID NO: 471).

**Example 56: Expression and purification of bivalent long half-lived humanized Nanobodies™**

***Production in Pichia pastoris***

See example 49

***Purification of bivalent Nanobodies***

The culture medium was made cell-free via centrifugation and 0.22µm filtration. The sterile medium was stored at 4°C until further processed. Low molecular weight contaminants were reduced via ultra filtration on a 10kDa ultra filtration (UF) membrane

contaminants were reduced via ultra filtration on a 10kDa ultra filtration (UF) membrane (HydroSart Sartocon Slice Cassette, Sartorius) as follows: four liter medium was concentrated to 0.5-1 lit, then diluted with 5lit PBS and again concentrated to 0.5lit. This action was carried out twice.

The retentate of the UF was filtered through nylon 47mm membranes 0,45µm (Alltech #2024).

In a next step bivalent Nanobody™ was captured from the concentrated medium via Protein A affinity purification (using MabSelectXtraTM, GE Healthcare). The column [35X100mm] was equilibrated in PBS and after sample application washed extensively with PBS. TNF56 was eluted with Glycine [100mM, pH=2.5].

The eluted fractions of MabSelectXtraTM were neutralized with Tris [1,5M, pH 8,8] and stored at 4°C. TNF56 was concentrated and purified via AEX (A= 10mM piperazin, pH 10,8 and B=1M NaCl in 50mM Tris, pH 7,5) using Source 30Q (GE Healthcare). To this end the Nanobody™ fractions were diluted with A buffer (10mM piperazin, pH 10,8) to a conductivity of 5mS/cm and the pH was adjusted to 10,8. The column [25X100mm] was equilibrated in A buffer before loading the sample onto the column. TNF56 was eluted with a 5 Column Volume (CV) gradient. The pH of the collected fractions was adjusted to 7.8 using 1M Tris pH=7.8.

**Pegylation of bivalent Nanobodies™ expressed in *Pichia pastoris***

***Reduction of C-terminal cysteines***

Dithiotreitol (DTT, Aldrich Cat 15,046-0) was added to the neutralized fractions to reduce potential disulfide bridges that formed between the carboxy terminal cysteines of the Nanobodies™ (usually around 20%). A final concentration of 10mM DTT and incubation overnight at 4°C was found to be optimal. The reduction was evaluated by analytical size exclusion chromatography (SEC). Therefore 25µl of the reduced Nanobody™ was added to 75µl D-PBS and injected on a Sup75 10/300 GL column equilibrated in Dulbecco's PBS (D-PBS, GibcoTM REF 14190-094).

Non reduced Nanobody™ and DTT was removed by preparative SEC on a Hiload 26/60 Superdex75 prep grade column equilibrated in D-PBS.

The concentration of the reduced Nanobody™ was measured by measuring the Absorbance at 280nm. A Uvikon 943 Double Beam UV/VIS Spectrophotometer (method: see

SOP ABL-0038) was used. The absorption was measured in a wavelength scan 245-330nm. Two Precision cells made of Quartz Suprasil® cells were used (Hellma type No.: 104-QS; light path: 10mm). First the absorption of the blank was measured at 280nm by placing two cells filled by 900µl D-PBS. The sample was diluted (1/10) by adding 100µl of the sample to the first cell. The absorption of the sample was measured at 280nm.

The concentration was calculated with following formula: $\frac{OD_{280}\text{Sample} - OD_{280}\text{blank}}{\varepsilon \times l} \times 10$

For TNF55: ε=1,85

For TNF56: ε=1,83.

***PEGylation***

To PEGylate Nanobody™ a 5X molar excess of freshly made 1mM PEG40 solution was added to the reduced Nanobody™ solution. (MPEG2-MAL-40K of NEKTAR™ Transforming Therapeutics (2D3YOTO1) Mw = 40,000 g/mol; MPEG2-MAL-60K of NEKTAR™ Transforming Therapeutics (2D3YOVO1) Mw = 60,000 g/mol).

The Nanobody™-PEG mixture was incubated for 1h at room temperature (RT) with gentle agitation and then transferred to 4°C. The PEGylation was evaluated via analytical SEC. Therefore 25µl of the Nanobody™ was added to 75µl D-PBS and injected on a Sup75HR 10/300 column equilibrated in D-PBS. Pegylated Nanobody™ eluted in the range of the exclusion volume of the column (>75kDa).

The PEGylated and non PEGylated Nanobody™ were separated via cation exchange chromatography (CEX, using Source30S, GE Healthcare; A buffer =25mM citric acid pH=4 and B=1M NaCl in PBS). The sample was diluted to a conductivity of <5mS/cm and the pH was adjusted to 4,0. The column [25X100mm] was equilibrated and after sample application washed extensively with A-buffer. Pegylated Nanobody™ was eluted with a 3 CV gradient. The collected Nanobody™ was buffer exchanged to D-PBS by SEC on a Hiload 26/60 Superdex 75 prep grade column equilibrated in D-PBS.

Finally the Nanobody™ was made LPS-free via passage over an anion exchange column (Source30Q). The column (10x100mm) was sanitized overnight in NaOH [1M] and afterwards equilibrated in endotoxin free D-PBS.

***Biotinylation***

To biotinylate Nanobody™ a 5X Molar excess of biotin (EZ-Link® Maleimide-PO2-Biotin, Pierce #21901) from a 10mM stock solution was added to the reduced Nanobody™

(see 5.5.1). The biotin-Nanobody™ mixture was incubated for 1h at RT with gentle agitation and then stored at 4°C.

The purity of biotinylated Nanobody™ was controlled via analytical SEC. Therefore 25µl of biotinylated Nanobody™ was added to 75µl D-PBS and injected on a Sup75HR 10/300 column equilibrated in D-PBS. From the obtained chromatogram could be concluded that the Nanobody™-biotin needs no further purification: no dimerization of Nanobody™ via an oxidation of free sulfidrils could be detected. A buffer change to D-PBS was done by a passage over a desalting column Sephadex G25 fine (90ml) column.

Finally the Nanobody™-biotin was made LPS-free by passage over an anion exchange column (Source30Q, GE Healthcare). The column (1x10cm) was sanitized overnight in 1M NaOH and then equilibrated in D-PBS.

To determine the purity, protein samples were analyzed on a 15% SDS-PAGE gel as described in example 8 and 49. Results are presented in Figure 49 and 50.

**Example 57: Characterization of bivalent long half-lived humanized Nanobodies™**

***Biochemical characterization***

TNF55 consists of 260 amino acids. The protein has a molecular weight of 27,106 Da. The pI is 8.67. The extinction coefficient at 280 nm is 1.850.

TNF56 consists of 264 amino acids. The protein has a molecular weight of 27,365 Da. The pI is 8.67. The extinction coefficient at 280 nm is 1.830.

***Mass spectrophotometry***

The theoretical mass of TNF55 is 27,106 Da. The TNF55-Biotine protein has 2 S-S bridges and a biotine modification which should result in a mass of 27,627 Da in ESI-MS. The mass that was experimentally determined for TNF55-biotine is 27,627 Da.

The theoretical mass of TNF56 is 27,365 Da. The TNF55-Biotine protein has 2 S-S bridges and a biotine modification which should result in a mass of 27,886 Da in ESI-MS. The mass that was experimentally determined for TNF55-biotine is 27,886 Da.

*N-terminal sequencing*

N-terminal sequencing of TNF56-PEG40 showed that the protein sequence for the first 7 amino acids is as follows: EVQLVES. This is consistent with the theoretical protein sequence, which indicates proper N-terminal processing.

*Analytical sizing*

Analytical sizing of TNF56-PEG40 in PBS provides a symmetrical peak. No contaminants were observed. The retention time observed is 8.5 ml on Superdex HR 75 and 10.32 ml on Superdex HR 200. A representative profile is shown in Figures 51 and 52.

**Example 58: functionality in cell-based assay**

The potency to neutralize the cytotoxic activity of TNFα was analyzed in a cell-based assay. Potency was examined at different concentrations of Nanobody™ as well as of the commercially available Enbrel, Humira and Remicade on a molar base. The higher the EC50 observed the lower the activity of the compound to neutralize TNFα.

The results are summarized in Table 45 and Figures 53 and 54.

The data show an increase in potency for the bivalent Nanobodies™ when compared to the monovalent Nanobody™ TNF1. Potency of TNF55 derivatives is similar to TNF56 derivatives, which is in the range of Enbrel and 10-fold better than Humira and Remicade.

**Example 59: Pharmacokinetic and immunogenicity analysis of bivalent long half-lived humanized Nanobodies in mice**

See example 54

Experiments were performed in order to examine the half-life of pegylated Nanobodies™ in mice. The half-life of bivalent TNF56-PEG40 was compared to the half-life of TNF56-PEG60. Both Nanobodies™ have comparable half-life of ~ 2days. The results are presented in Figure 55.

In addition, the half-life of pegylated bivalent 3E-3E was explored. The half-life of 3E-3E-PEG20 was compared to the half-life of 3E-3E-PEG40 after intravenous administration of 100 μg of the Nanobodies™. 3E-3E-PEG20 has a half-life of 17 hrs, while 3E-3E-PEG40 has a half-life of 2.1 days, comparable to the half-life of 3E-3E-MSA21. The results are presented in Figure 56.

Serum samples were diluted 100-fold and analyzed for the presence of mouse anti-TNF56-PEG40 or anti-TNF56-PEG60 antibodies. Lack of immunogenicity was demonstrated for both molecules. Data are presented in Figure 57.

**Example 60: Efficacy of anti-TNF-α Nanobody TNF60 (TNF60) in prevention of chronic polyarthritis**

Transgenic mouse lines carrying and expressing a 3'-modified human tumour necrosis factor (hTNF-alpha, cachectin) transgene were used as a model to study the efficacy of TNF60 (TNF60) in preventing the development of arthritis (EMBO J. 10, 4025-4031). These mice have been shown to develop chronic polyarthritis with 100% incidence at four to seven weeks of age.

From the third week of age, litters of transgenic mice were divided into groups of eight animals. Before initializing the study, the average body weight was calculated for each group. From then on, during the whole study animal weights were recorded once a week for each group.

To test the efficacy of TNF60 in the prevention of chronic polyarthritis, intraperitoneal injections were given twice a week to each animal of a particular group according to the following scheme:

-Group 1 (negative control): phosphate buffered saline (PBS) (formulation buffer)
-Group 2 (Nanobody treatment): TNF60 at a final dose of 30 mg/kg
-Group 3 (Nanobody treatment): TNF60 at a final dose of 10 mg/kg
-Group 4 (Nanobody treatment): TNF60 at a final dose of 3 mg/kg
-Group 5 ($1^{st}$ positive control): Enbrel at a final dose of 30 mg/kg
-Group 6 ($1^{st}$ positive control): Enbrel at a final dose of 10 mg/kg
-Group 7 ($2^{nd}$ positive control): Remicade at a final dose of 30 mg/kg
-Group 8 ($2^{nd}$ positive control): Remicade at a final dose of 10 mg/kg
-Group 9 ($2^{nd}$ positive control): Remicade at a final dose of 3 mg/kg

For each group, dates of injection and injection volumes were noted.

Injections continued for seven weeks. During this period, clinical scores were recorded by observing macroscopic changes in joint morphology for each animal.

At 10 weeks of age, all mice were sacrificed and sera and joints were collected. Sera were stored at -70°C and ankle joints were conserved in formalin.

For selected groups, ankle joints were embedded in paraffin and sectioned. Ankle joint sections were subsequently used for histopathological evaluation of disease progression.

Results are depicted in figure 58.

**Example 61: Efficacy of anti-TNF-α Nanobody TNF60 (TNF60) in therapeutic *treatment of chronic polyarthritis***

Transgenic mouse lines carrying and expressing a 3'-modified human tumour necrosis factor (hTNF-alpha, cachectin) transgene were used as a model to study the efficacy of TNF60 (TNF60) in therapeutic treatment of arthritis (EMBO J. 10, 4025-4031). These mice have been shown to develop chronic polyarthritis with 100% incidence at four to seven weeks of age.

From the sixth week of age, litters of transgenic mice were divided into groups of eight animals. Before initializing the study, the average body weight was calculated for each group. From then on, during the whole study animal weights were recorded once a week for each group.

To test the efficacy of TNF60 in the therapeutic treatment of chronic polyarthritis, intraperitoneal injections were given twice a week to each animal of a particular group according to the following scheme:

-Group 1 (negative control): phosphate buffered saline (PBS) (formulation buffer)

-Group 2 (Nanobody treatment): TNF60 at a final dose of 30 mg/kg

-Group 3 (Nanobody treatment): TNF60 at a final dose of 10 mg/kg

-Group 4 ($1^{st}$ positive control): Enbrel at a final dose of 30 mg/kg

-Group 5 ($2^{nd}$ positive control): Remicade at a final dose of 30 mg/kg

For each group, dates of injection and injection volumes were noted.

Injections continued for seven weeks. During this period, clinical scores were recorded by observing macroscopic changes in joint morphology for each animal.

At 13 weeks of age, all mice were sacrificed and sera and joints were collected. Sera were stored at -70°C and ankle joints were conserved in formalin.

For selected groups, ankle joints were embedded in paraffin and sectioned. Ankle joint sections were subsequently used for histopathological evaluation of disease progression.

Results are depicted in figure 59.

**Example 62: Effect of formatting on efficacy of an anti-TNF-α Nanobody in prevention of chronic polyarthritis**

Transgenic mouse lines carrying and expressing a 3'-modified human tumour necrosis factor (hTNF-alpha, cachectin) transgene were used as a model to study the efficacy of an anti-TNF-α Nanobody formatted in different ways in the prevention of chronic polyarthritis (EMBO J. 10, 4025-4031). These mice have been shown to develop chronic polyarthritis with 100% incidence at four to seven weeks of age.

From the third week of age, litters of transgenic mice were divided into groups of eight animals. Before initializing the study, the average body weight was calculated for each group. From then on, during the whole study animal weights were recorded once a week for each group.

To study the efficacy of an anti-TNF-α Nanobody in different formats for prevention of chronic polyarthritis, intraperitoneal injections were given twice a week to each animal of a particular group according to the following scheme:

-Group 1 (negative control): phosphate buffered saline (PBS) (formulation buffer)
-Group 2 (Nanobody format 1): TNF60 at a final dose of 10 mg/kg
-Group 3 (Nanobody format 1): TNF60 at a final dose of 2.5 mg/kg
-Group 4 (Nanobody format 1): TNF60 at a final dose of 1 mg/kg
-Group 5 (Nanobody format 2): TNF56-PEG40 at a final dose of 10 mg/kg
-Group 6 (Nanobody format 2): TNF56-PEG40 at a final dose of 1.8 mg/kg
-Group 7 (Nanobody format 2): TNF56-PEG40 at a final dose of 0.7 mg/kg
-Group 8 (Nanobody format 3): TNF56-biot at a final dose of 1.8 mg/kg
-Group 9 (Nanobody format 4): TNF30 at a final dose of 1 mg/kg
-Group 10 (Nanobody format 5): TNF1 at a final dose of 1 mg/kg
-Group 11 ($1^{st}$ positive control): Enbrel at a final dose of 10 mg/kg
-Group 12 ($2^{nd}$ positive control): Remicade at a final dose of 10 mg/kg

For each group, dates of injection and injection volumes were noted.

Injections continued for seven weeks. During this period, clinical scores were recorded by observing macroscopic changes in joint morphology for each animal.

At 10 weeks of age, all mice were sacrificed and sera and joints were collected. Sera were stored at -70°C and ankle joints were conserved in formalin.

For selected groups, ankle joints were embedded in paraffin and sectioned. Ankle joint sections were subsequently used for histopathological evaluation of disease progression.

Results are depicted in figure 60.

**Example 63: Pharmacokinetic study of anti-TNF-α Nanobodies TNF60 (TNF60) and TNF56-PEG40 in rhesus monkey**

Captive-bred rhesus monkeys (*Macaca mulatta*) are used to determine the pharmacokinetic profile of TNF60 and TNF56-PEG40.

Sixteen animals are used in this study (eight males and eight females) and are divided into four groups (two males and two females per group). All animals weighed approximately 5 kg and are disease-free for at least six weeks prior to use. Sniff® Pri vegetarisch V3994 serves as food. Sixty g/kg b.w. are offered to each monkey. The residue is removed. At regular intervals (at least twice a year) the food is analyzed based on EPA/USA for contaminants by LUFA-ITL. Tap water is offered ad libitum. The animals in each treatment group are housed in a block of several adjacent cages within the monkey unit. The monkeys are kept singly in $V_2A$ steel cages with a size of 90 cm x 82 cm x 96 cm. The room temperature is maintened at 23°C ± 3°C (maximum range) and the relative humidity at 60% ± 20% (maximum range). Deviations from the maximum range caused for example during cleaning procedure are dealt with in SOPs. The rooms are lit and darkened for periods of 12 hours each.

Two groups are infused with TNF60 and two groups are infused with TNF56-PEG40. Intravenous infusions of TNF60 and TNF56-PEG40 (dissolved in PBS) into the vena cephalica of the right or the left arm using indwelling catheters and a TSE infusion pump (see below) are given at a fixed dose of 2 mg/kg.

Four single administrations are performed, separated by a wash-out period of at least fourteen days. After the last administration the follow-up period is at least eight weeks. Two out of the four groups are treated with TNF60 or TNF56-PEG40 in combination with methotrexate (MTX) (dissolved in PBS). Group 2 is treated with TNF60 and MTX; group 4 is treated with TNF56-PEG40 and MTX. MTX is dosed weekly intramuscularly at 0.2 mg/kg. On the administration days, MTX is given approximately 30 minutes prior to administration. Dosing starts at the first Nanobody administration and will continue throughout the eight week wash-out after the fourth dose. There are fourteen single MTX administrations, separated by a wash-out period of at least one week starting at the first test item administration.

**Example 64: Synovium-derived fibroblast studies**

In this study the ability of the anti-TNF biologicals, ALX0071 and Etanercept, to attenuate TNFα-induced IL-6 production by RA-synovium derived fibroblasts was assessed.

*Isolation of synovial fibroblasts*

Synovial joint tissue from consenting RA patients was stored in DMEM-based medium with antibiotics at 4°C for up to 96 hours after joint replacement surgery. Synovial cells were isolated from dissected synovium by collagenase digestion at 37 °C for 2 hours. The resultant cell suspension was then washed by a series of centrifugation and resuspension steps and the resultant cells then cultured at 37 °C in DMEM-based culture medium supplemented with 10% FCS (v/v). The resultant fibroblasts were used for the following experiment at either the second or third passage. Cells from four donors were used in individual experiments. Fibroblasts were seeded into 96-well flat bottom polystyrene plates at 1.5 $x10^4$ cells in a final volume of 250 µL of DMEM-based culture medium supplemented with 10% FCS (v/v) per well and cultured overnight.

*Stimulation of synovial fibroblasts*

Cells were then incubated for 72 hours in DMEM-based culture medium supplemented with TNFα at 50 ng per mL (3 nM (R&D Systems 210-TA/CF) alone or in the presence of increasing doses of ALX0071 (0.575 to 1920 ng per mL; 0.015 to 50 nM) or Etanercept (Wyeth Labs; 3.75 to 11250 ng per mL; 0.025 to 75 nM). The final volume in each well was 250 µL and each assessment was performed in triplicate. After 72 hours, the supernatant media was removed and stored at –40 °C prior to analysis by IL-6 ELISA (R&D Systems). The inhibition of TNFα-induced IL-6 production was determined and $IC_{50}$ values were calculated for both ALX0071 and Etanercept.

*Summary of results*

Both ALX10071 and Etanercept dose-dependently reduced TNFα-induced IL-6 production by RA synovium derived fibroblasts from all four donors. There was a similar potency between the two reagents under these assay conditions.

**Example 65: Murine Air pouch studies**

In this study the ability of the anti-TNF biologicals, ALX0071 and Etanercept, to attenuate TNFα-induced cell infiltration in to a murine air pouch was assessed.

*Creation of Air Pouch*

Air pouches were formed by the sub-cutaneous (s.c.) injection of 2.5mL of sterile air in to the dorsal surface of anaesthetised male C57Bl/6/J mice (25-30g, Harlan). The pouch was re-inflated by injecting 2.5 ml of sterile air 3 days later.

*TNFα stimulation*

Six days after the initial creation of the air pouch, the animals were anaesthetised and the pouch injected with 1 ml of 0.5% CarboxyMethylCellulose (CMC) vehicle containing 0.1 μg recombinant human TNFα (R & D Systems, 210-TA-050/CF). In three other groups of animals, ALX0071 (0.0625, 0.125 and 0.25 mg/kg) was injected s.c. 19 hours prior to the injection of TNFα. A second three groups of animals were injected (s.c.) with Etanercept (Wyeth Labs, 0.125, 0.25 and 0.5 mg/kg) immediately prior to the injection of TNFα.

24 hours following TNFα injection, mice were culled with a rising concentration of $CO_2$. Pouches were lavaged with 2 ml of ice cold endotoxin free sterile PBS containing 5 IU/ml heparin. Volumes were recorded and 0.5 ml aliquots were separated for counting of the total white blood cell (WBC) population on a Sysmex XT-Vet cell counter. The mean and standard error of the mean (SEM) total WBC counts for each group were calculated per ml of lavage fluid withdrawn. Statistical analysis was by ANOVA with Kruskal-Wallis post-test on untransformed data.

*Summary of results*

Both ALX0071 and Etanercept attenuated the TNFα-induced WBC infiltration in to the air pouches (Table). While this attenuation reached statistical significance at both the 0.125 ($P<0.01$) and 0.25 mg/kg ($P<0.05$) ALX0071 dose groups, statistical significance was not observed with any Etanercept dose group.

**Table 8**

| Name | SEQ ID NO | Sequence |
|---|---|---|
| PMP1C2(TNF1) | 52 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA LYYCARSPSGFNRGQGTQVTVSS |
| PMP1G11 | 53 | QVQLQESGGGMVQPGGSLRLSCAASGFDFGVSWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKTTLYLQMNSLKPEDTA LYYCARSPSGSFRGQGTQVTVSS |
| PMP1H6 | 54 | EVQLVESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLE WVSEINTNGLITKYVDSVKGRFTISRDNAKNTLYLQMDSLIPEDTA LYYCARSPSGSFRGQGTQVTVSS |
| PMP1G5(TNF2) | 55 | QVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP GKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLK PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| PMP1H2 | 56 | QVKLEESGGGLVQPGDSLRLSCAASGRTFSDYSGYTYTVGWFRQAP GKEREFVARIYWSSGNTYYADSVKGRFTISRDIAKNTVDLLMNNLE PEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS |
| PMP3G2 | 57 | AVQLVESGGGLVQPGDSLRLSCAASGRTFSDYSGYTYTVGWFRQAP GKEREFVARIYWSSGNTYYADSVKGRFTISRDIAKNTVDLLMNNLE PEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS |
| PMP1D2 | 58 | AVQLVDSGGGLVQAGGSLRLSCAASGRTFSAHSVYTMGWFRQAPGK EREFVARIYWSSANTYYADSVKGRFTISRDNAKNTVDLLMNCLKPE DTAVYYCAARDGIPTSRSVEAYNYWGQGTQVTVSS |
| PMP3D10 | 59 | QVQLVESGGGLVQAGGSLSLSCAASGRSFTGYYMGWFRQAPGKERQ LLASISWRGDNTYYKESVKGRFTISRDDAKNTIYLQMNSLKPEDTA VYYCAASILPLSDDPGWNTNWGQGTQVTVSS |
| PMP5F10(TNF3) | 60 | EVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERE LLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTA VYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| PMP6A8(ALB2) | 61 | AVQLVESGGGLVQGGGSLRLACAASERIFDLNLMGWYRQGPGNERE LVATCITVG.DSTNYADSVKGRFTISMDYTKQTVYLHMNSLRPEDT GLYYCKIRRTWHSELWGQGTQVTVSS |
| PMP6B4 | 62 | EVQLVESGGGLVQEGGSLRLACAASERIWDINLLGWYRQGPGNERE LVATITVG.DSTSYADSVKGRFTISRDYDKNTLYLQMNSLRPEDTG LYYCKIRRTWHSELWGQGTQVTVSS |
| PMP6A6(ALB1) | 63 | AVQLVESGGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKEPE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA VYYCTIGGSLSRSSQGTQVTVSS |
| PMP6C1 | 64 | AVQLVDSGGGLVQPGGSLRLSCAASGFSFGSFGMSWVRQYPGKEPE WVSSINGRGDDTRYADSVKGRFSISRDNAKNTLYLQMNSLKPEDTA EYYCTIGRSVSRSRTQGTQVTVSS |
| PMP6G8 | 65 | AVQLVESGGGLVQPGGSLRLTCTASGFTFRSFGMSWVRQAPGKDQE WVSAISADSSTKNYADSVKGRFTISRDNAKKMLYLEMNSLKPEDTA VYYCVIGRGSPSSPGTQVTVSS |

| | | |
|---|---|---|
| PMP6A5 | 66 | QVQLAESGGGLVQPGGSLRLTCTASGFTFGSFGMSWVRQAPGEGLE<br>WVSAISADSSDKRYADSVKGRFTISRDNAKKMLYLEMNSLKSEDTA<br>VYYCVIGRGSPASQGTQVTVSS |
| PMP6G7 | 67 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSNYWMYWVRVAPGKGLE<br>RISRDISTGGGYSYYADSVKGRFTISRDNAKNTLYLQMNSLKPEDT<br>ALYYCAKDREAQVDTLDFDYRGQGTQVTVSS |
| NC55TNF_S1C4 | 105 | EVQLVESGGGLVQAGDSLRLSCAASQIIFGSHVAAWFRQAPGRERE<br>FVAEIRPSGDFGPEGEFEHVTASLKGRFTIAKNSVDNTVYLQMNSL<br>KPEDTAVYYCAAAPYRGGRDYRWEYEYEYWGQGTQVTV |
| NC55TNF_S1C3 | 106 | EVQLVESGGGLVQPGGSLRLSCKNAGSTSNAYATGWFRRAPGKERE<br>FVAGIQWSGGDAFYRNSVKGRFRITRDPDNTVYLQMNDLKPEDTAI<br>YYCAQKLSPYYNDFDSSNYEYWGQGTQVTV |
| NC55TNF_S2C1 | 107 | EVQLVESGGDLVQPGGSLRLSCAVSGQLFSTNDVGWYRRAPGKQRE<br>LVATITDDGTTDYGDDVKGRFVISREGEMVYLEMNSLKPEDTAVYY<br>CNINRLRSTWGIRYDVWGQGTQVTVSS |
| NC55TNF_S2C5 | 108 | EVQLVESGGGLVQPGGSLRLSCVVSGFTFSTTSMTWVRQAPGKFEE<br>WVSFINSDGSSTTYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTA<br>MYYCGRRGYGRDRSKGIQVTVAS |
| NC55TNF_S3C7 | 109 | EVQLVESGGGTVQAGDSLRLSCAASGRSFSSVAMGWFRQAPGKQRE<br>FLAGVGYDGSSIRYAESVKGRFTIARGNRESTVFLQMENLKPEDTA<br>VYFCTAEPIGAYEGLWTYWGQGTQVTVSS |
| NC55TNF_S3C1 | 110 | XXXXVESGGGLMQPGGSLKLSCAASGFMFSDSAMGWFRQAPGKERE<br>FVATISWNGGSSSYADFVKGRFTISRDNAKNTVYLQMNGLTPQDTA<br>IYYCAGSYSNGNPHRFSQYQYWGQGTQVTVSS |
| NC55TNF_BMP1B2 | 111 | EVQLVESGGGLVQAGGSLRLSCAASGRTFGTYAMGWFRQAPGKERE<br>FVAAISWGGGSIVYAESAKGRFTISRDNAKXTMYLQMDSLKPEDTA<br>VYYCAAANNIATLRQGSWGQGTQVTVSS |
| NC55TNF_BMP1D2 | 112 | EVQLVESGGELVQAGGSLKLSCTASGRNFVTYAMSWFRRAPGKERE<br>FVASISWSGDTTYYSNSVKGRFTVSRDNGKNTAYLRMNSLKPEDTA<br>DYYCAVVQVIDPSWSGVNLDDYDYLGSGTQVTVSS |
| NC55TNF_BMP1E2 | 113 | EVQLVESGGRLVQPGGSLRLSCKNAGSTSNAYATGWFRRAPGKERE<br>FVAGIQWSGGDAFYRNSVKGRFRITRDPDNTVYLQMNDLKPEDTAI<br>YYCAQKLSPYYNDFDSSNYEYWGQGTQVTVSS |
| NC55TNF_BMP1G2 | 114 | EVQLVESGGGLVQPGGSLRLSCAASATISSIVMLGWYRQAPGKQRE<br>WVASITIGSRTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAV<br>YFCNAVPPRDDYWGQGTQVTVSS |
| NC55TNF_BMP2A2 | 115 | EVQLVESGGGLVQAGGSLRLSCAASGQTSSSYDMGWFRQAPGEGRE<br>FVARISGSDGSTYYSDRAKDRFTISRDNTKNMVYLQMDRLKPDDTA<br>VYYCRVPRYENQWSSYDYWGQGTQVTVSS |
| NC55TNF_BMP2C2 | 116 | EVQLVESGGGLVQPGGSLRLSCAASGSTFSTYDMSWVRQAPGKGLE<br>WVSGIDSGGGSPMYVDSVKGRFTVSRDNAKNTLYLQMNSLKPEDTA<br>VYYCAKFSTGADGGSWYWSYGMDSWGKGTQVTVSS |
| NC55TNF_BMP2F2 | 117 | EVQLVESGGGLVQAGDSLRLSCEASERSSNRYNMAWFRQAPGKERE<br>FLARVDVSGGNTLYGDSVKDRFTVSRINGKNAMYLQMNNLKPEDTA<br>IYYCAAGGWGTTQYDYDYWGQGTQVTVSS |
| NC55TNF_NC10 | 118 | EVQLVESGGGLVQPGGSLRLSCVCVSSGCTFSAYSMTWVRQAPGKA<br>EEWVSFINSDGSSTTYADSVNGRFKISRDNAKKTLYLQMNSLGPED<br>TAMYYCQRRGYALDRGQGTQVTVSS |
| NC55TNF_NC11 | 119 | EVQLVESGGGLVQAGDSLTLSCASSGRGFYKNAMGWFRQPPGKERE<br>FVASIKWNGNNTYYADSVRGRFTISRGNAKNTENTVSLQMNSLKPE<br>DTADYYCAADSSHYSYVYSKAYEYDYWGQGTQVTVSS |
| NC55TNF_NC1 | 120 | EVQLVESGGGLVQPGGSLRLSCVFSGFAFSASSMAWVRQAPGKYEE<br>WVSFINSDGSSTTYADSVQGRFTISRDNAKNTLYLQMNSLKSEDTA<br>MYYCGRRGYGRDRSQGIQVTVSS |
| NC55TNF_NC2 | 121 | EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKERE<br>FVAAISWSGTITNYADSVKGRFTISRDNGKNTVHLQMNSLKPEDTA<br>VYHCAVVQPYSGGDYYTGVEEYDYWGXGTQVTVSS |

| | | |
|---|---|---|
| NC55TNF_NC3 | 122 | EVQLVESGGGLVQPGGSLRLSCVVSGFTFSATSMTWVRQAPGKAEE WVSFINSDGSSTTYADSVKGRFTISRDNAKNTLYLQMDDLQSEDTA MYYCGRRGYGRDRSRGIQVTVSS |
| NC55TNF_NC5 | 123 | EVQLVESGGGLVQAGGSLRLSCAASGGAFSNYDVGWFRQAPGEGRE IVARISGSGDSTYSSNRAKGRFTISRDNAKNTVYLQMNSLKREDTA VYYCRAARYNGTWSSNDYWGQGTQVTVSS |
| NC55TNF_NC6 | 124 | EVQLVESGGGLVQPGGSLRLSCECVSSGCTFSAYSMTWVRQAPGKA EEFVSFINSDGSSTTYANSVNGRFKISRDNAKKTLYLQMNSLGPED TAMYYCQRRGYALDRGQGTQVTVSS |
| NC55TNF_NC7 | 125 | QVQLVESGGGLVQAGGSLRLSCTASGQTSSTADMGWFRQPPGKGRE FVARISGIDGTTYYDEPVKGRFTISRDKAQNTVYLQMDSLKPEDTA VYYCRSPRYADQWSAYDYWGQGTQVTVSS |
| NC55TNF_NC8 | 126 | EVQLVESGGGLVQPGGSLRLSCVVSGFTFSTTSMTWVRQAPGKFEE WVSFINSDGSSTTYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTA MYYCGRRGYGRDRSKGIQVTVSS |
| NC55TNF_S2C2 | 127 | EVQLVESGGGLVQPGGSLRLSCVASASGVKVNDMGWYRQAPGKERE LVATITDDGRTNYEDFAKGRFTISRDNAKNTVYLQMNSLLPEDTAV YYCNARTYWAHLPTYWGQGTQVTVSS |
| NC55TNF_S1C6 | 128 | EVQLVESGGGLVQAGGSLRLSCAASGRSFGSVAMGWFRQAPGKERE FVAAIGYDGNSIRYGDSVKGRFTISRDNIKNTMYLEMENLNADDTA RYLCAAEPLARYEGLWTYWGQGTQVTVSS |
| NC55TNF_S3C2 | 129 | EVQLVESGGGLVQAGASLRLSCTTSTRTNDRFNMAWFHQAPGKDRE FVSRIDVAGYNTAYGDFVKGRFTVSRDSAENTVVLQMNSLRPEDTG VYYCAAGGWGISQSDYDLWGQGTQVTVSS |

**Table 9**

| nanobody | Class | Estimated koff (1/s) |
|---|---|---|
| PMP5 F10 | III | 2,63E-04 |
| PMP1 G5 | II | 3,59E-04 |
| PMP1 C2 | I | 4,39E-04 |
| PMP1 G11 | I | 1,15E-03 |
| PMP1 H6 | I | 2,14E-03 |
| PMP1 H2 | II | 3,65E-03 |
| PMP3 G2 | II | 1,09E-02 |

**Table 10**

| **Nanobody** | **Germline sequence** | **#AA differences / total #AA** | **% AA identity** |
|---|---|---|---|
| ALB1 | DP51/DP53 | 13/87 | 85.1 |
| ALB2 | DP54 | 26/87 | 70.2 |
| TNF1 | DP51/DP53 | 6/87 | 93.2 |
| TNF2 | DP54 | 16/87 | 81.7 |
| TNF3 | DP29 | 18/87 | 79.4 |

**Table 11**

| **Nanobody** | **Induction time** | **Yield (mg/L)** |
|---|---|---|
| ALB1 | short/37°C | 18 |
| ALB2 | short/37°C | 4 |
| TNF1 | short/37°C | 8.3 |
| TNF2 | short/37°C | 5 |
| TNF3 | short/37°C | 0.8 |

**Table 12**

| | **50% binding (ng/ml)** | |
|---|---|---|
| ***ID*** | ***Human TNFα*** | ***Rhesus TNFα*** |
| TNF1 | 12 | 12 |
| TNF2 | 20 | >3000 |
| TNF3 | 18 | 16 |

**Table 13**

| | 50% inhibition (ng/ml) | |
|---|---|---|
| ID | Human TNFα | Rhesus TNFα |
| TNF1 | 530 | 220 |
| TNF2 | 3500 | >5000 |
| TNF3 | 100 | 100 |

**Table 14**

| Human TNFα | Kd (1/s) |
|---|---|
| TNF1 | 1,05E-03 |
| TNF2 | 1,33E-03 |
| TNF3 | 3,02E-04 |

**Table 15**

| | | Human albumin | Rhesus albumin | Mouse albumin |
|---|---|---|---|---|
| ALB1 | KD (nM) | 0,57 | 0,52 | 6,5 |
| | ka (1/Ms) | 1,11E+06 | 1,05E+06 | 1,11E+06 |
| | kd (1/s) | 6,30E-04 | 5,46E-04 | 7,25E-03 |
| ALB2 | KD (nM) | 0,092 | 0,036 | 15,7 |
| | ka (1/Ms) | 8,15E+05 | 1,94E+06 | 1,95E+05 |
| | kd (1/s) | 7,52E-05 | 7,12E-05 | 3,07E-03 |

**Table 16**

***assay :*** ***L929s + Act D (5000c/w)***

***TNF :*** ***human* TNFα @ *0.5ng/ml***

| | | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|---|
| **Nanobody** | | **mean** | **stdev** | **#** | **mean** | **stdev** |
| TNF1 | 1C2 | 0.707 | 0.265 | 14 | 0.015 | 0.007 |
| TNF2 | 1G5 | 1.412 | 0.622 | 14 | 0.007 | 0.002 |
| TNF3 | 5F10 | 0.224 | 0.133 | 14 | 0.048 | 0.019 |
| Enbrel | | 0.009 | 0.005 | 45 | 1.002 | 0.011 |
| Humira | | 0.079 | 0.043 | 39 | 0.097 | 0.069 |
| Remicade | | 0.083 | 0.037 | 45 | 0.103 | 0.058 |

***assay :*** ***L929s + Act D (5000c/w)***

***TNF :*** ***rhesus* TNFα @ *0.5ng/ml***

| | | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|---|
| **Nanobody** | | **mean** | **stdev** | **#** | **mean** | **stdev** |
| TNF1 | 1C2 | 0.693 | 0.305 | 9 | 0.015 | 0.009 |
| TNF2 | 1G5 | >50 | | 9 | | |
| TNF3 | 5F10 | 0.602 | 0.283 | 9 | 0.017 | 0.010 |
| Enbrel | | 0.009 | 0.003 | 7 | 1 | 0.000 |
| Humira | | 0.071 | 0.025 | 8 | 0.103 | 0.059 |
| Remicade | | >6.7 | | 7 | | |

**Table 17**

| % | Untreated | RT | 37°C | 50°C | 60°C | 70°C | 80°C | 90°C |
|---|---|---|---|---|---|---|---|---|
| TNF1 | 100 | 98 | 98 | 98 | 98 | 95 | 92 | 90 |
| TNF2 | 100 | 99 | 100 | 99 | 97 | 96 | 63 | 50 |
| TNF3 | 100 | 96 | 97 | 98 | 96 | 94 | 75 | 70 |
| ALB1 | 100 | 101 | 102 | 101 | 100 | 64 | 94 | 90 |
| ALB2 | 100 | 100 | 102 | 100 | 100 | 28 | 8 | 17 |

**Table 18**

| nanobody | temp in °C | $EC_{50}$ in nM | # | relative potency |
|---|---|---|---|---|
| TNF1 | control | 0,916 | 1 | 0,013 |
| 92#2302nr1.TNF1 | RT | 0,873 | 1 | 0,014 |
| | 37 | 0,901 | 1 | 0,013 |
| | 50 | 0,908 | 1 | 0,013 |
| | 60 | 0,891 | 1 | 0,013 |
| | 70 | 1,218 | 1 | 0,010 |
| | 80 | 2,655 | 1 | 0,004 |
| | 90 | 5,797 | 1 | 0,002 |
| TNF2 | control | 2,500 | 1 | 0,005 |
| 92#2302nr2.TNF2 | RT | 2,165 | 1 | 0,005 |
| | 37 | 2,212 | 1 | 0,005 |
| | 50 | 2,241 | 1 | 0,005 |
| | 60 | 1,782 | 1 | 0,007 |
| | 70 | 2,487 | 1 | 0,005 |
| | 80 | 2,818 | 1 | 0,004 |
| | 90 | 6,135 | 1 | 0,002 |
| TNF3 | control | 0,278 | 1 | 0,043 |
| 92#2302nr3.TNF3 | RT | 0,289 | 1 | 0,041 |
| | 37 | 0,295 | 1 | 0,040 |
| | 50 | 0,290 | 1 | 0,041 |
| | 60 | 0,281 | 1 | 0,042 |
| | 70 | 0,293 | 1 | 0,040 |
| | 80 | 0,576 | 1 | 0,021 |
| | 90 | 0,861 | 1 | 0,014 |

**Table 19**

| Name | SEQ ID NO | Sequence |
|---|---|---|
| GS9 | 68 | GGGGSGGGS |
| GS30 | 69 | GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS |
| TNF1-GS9-TNF1(TNF4) | 70 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTALYYCARSPSGFNRGQGTQVTVSSGGGGSGGGSQVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTALYYCARSPSGFNRGQGTQVTVSS |
| TNF2-GS9-TNF2(TNF5) | 71 | QVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAPGKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSSGGGGSGGGSQVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAPGKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF3-GS9-TNF3(TNF6) | 72 | EVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| TNF1-GS30-TNF1(TNF7) | 73 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTALYYCARSPSGFNRGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSQVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTALYYCARSPSGFNRGQGTQVTVSS |
| TNF2-GS30-TNF2(TNF8) | 74 | QVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAPGKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSQVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAPGKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF3-GS30-TNF3(TNF9) | 75 | EVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| TNF30-30GS-TNF30-C (TNF 55) | 419 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSSggggsggggsggggsggggsgggggsggggsEVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSC |
| TNF30-30GS-TNF30-gggC (TNF56) | 420 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSSggggsggggsggggsggggsgggggsggggsEVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSSgggC |

**Table 20**

| ID | Format | Linker |
|---|---|---|
| TNF4 | TNF1-TNF1 | 9 AA GlySer |
| TNF5 | TNF2-TNF2 | 9 AA GlySer |
| TNF6 | TNF3-TNF3 | 9 AA GlySer |
| TNF7 | TNF1-TNF1 | 30 AA GlySer |
| TNF8 | TNF2-TNF2 | 30 AA GlySer |
| TNF9 | TNF3-TNF3 | 30 AA GlySer |

**Table 21**

| Nanobody™ | Induction time | Yield (mg/L) |
|---|---|---|
| TNF4 | ON/28°C | 3.2 |
| TNF5 | short/37°C | 5.5 |
| TNF6 | short/37°C | 1.19 |
| TNF7 | ON/28°C | 2.7 |
| TNF8 | short/37°C | 6.6 |
| TNF9 | ON/28°C | 1.3 |

**Table 22**

| | 50% inhibition (ng/ml) |
|---|---|
| ID | Human TNFα |
| TNF4 | 13 |
| TNF5 | 6.3 |
| TNF6 | 30 |
| TNF7 | 16 |
| TNF8 | 23 |
| TNF9 | 18 |

Table 23

*assay :* *L929s + Act D (5000c/w)*

*TNF :* *human TNFa @ 0.5ng/ml*

| | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|
| Nanobody | mean | stdev | # | mean | stdev |
| TNF4 | 0.236 | 0.049 | 4 | 0.033 | 0.012 |
| TNF5 | 0.020 | 0.010 | 9 | 0.566 | 0.275 |
| TNF6 | 0.078 | 0.047 | 8 | 0.179 | 0.168 |
| TNF7 | 0.013 | 0.005 | 8 | 0.673 | 0.211 |
| TNF8 | 0.007 | 0.002 | 2 | 1.240 | 0.137 |
| TNF9 | 0.012 | 0.005 | 6 | 0.729 | 0.242 |
| Enbrel | 0.009 | 0.005 | 45 | 1.002 | 0.011 |
| Humira | 0.079 | 0.043 | 39 | 0.097 | 0.069 |
| Remicade | 0.083 | 0.037 | 45 | 0.103 | 0.058 |

*assay :* *L929s + Act D (5000c/w)*

*TNF :* *rhesus TNFa @ 0.5ng/ml*

| | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|
| Nanobody | mean | stdev | # | mean | stdev |
| TNF4 | 0.141 | 0.025 | 4 | 0.065 | 0.015 |
| TNF5 | 35.000 | 16.000 | 5 | 0.000 | 0.000 |
| TNF6 | 0.398 | 0.074 | 6 | 0.024 | 0.003 |
| TNF7 | 0.011 | 0.005 | 4 | 0.860 | 0.142 |
| TNF8 | 1.026 | 0.444 | 2 | 0.010 | 0.001 |
| TNF9 | 0.038 | 0.012 | 4 | 0.249 | 0.032 |
| Enbrel | 0.009 | 0.003 | 7 | 1.000 | 0.000 |
| Humira | 0.071 | 0.025 | 8 | 0.103 | 0.059 |
| Remicade | >6.7 | | 7 | | |

Table 24

| % | untreated | RT | 37°C | 50°C | 60°C | 70°C | 80°C | 90°C |
|---|---|---|---|---|---|---|---|---|
| TNF4 | 100 | 99 | 99 | 99 | 98 | 55 | 34 | 17 |
| TNF5 | 100 | 99 | 101 | 99 | 98 | 92 | 26 | 22 |
| TNF6 | 100 | 103 | 104 | 103 | 105 | 99 | 7 | 7 |
| TNF7 | 100 | 100 | 100 | 98 | 96 | 66 | 33 | 40 |
| TNF8 | 100 | 99 | 100 | 99 | 100 | 89 | 11 | 8 |
| TNF9 | 100 | 101 | 101 | 101 | 101 | 99 | 17 | 18 |

**Table 25**

| Name | SEQ ID NO | Sequence |
|---|---|---|
| TNF13(TNF1 HUM1) | 76 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA VYYCARSPSGFNRGQGTQVTVSS |
| TNF14(TNF1 HUM2) | 77 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA LYYCARSPSGFNRGQGTLVTVSS |
| TNF15(TNF2 HUM1) | 78 | EVQLVESGGGLVQPGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLK PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF16(TNF2 HUM2) | 79 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLK PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF17(TNF2 HUM3) | 80 | EVQLVESGGGLVQPGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDNAKNTVDLQMNSLK PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF18(TNF2 HUM4) | 81 | EVQLVESGGGLVQPGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLR PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF19(TNF2 HUM5) | 82 | EVQLVESGGGLVQPGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLK PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTLVTVSS |
| TNF20(TNF3 HUM1) | 83 | EVQLVESGGGLVQPGGSLRLSCAASGRSLSNYYMGWFRQAPGKGRE LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLKPEDTA VYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| TNF21(TNF3 HUM2) | 84 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMGWFRQAPGKGRE LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLKPEDTA VYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| TNF22(TNF3 HUM3) | 85 | EVQLVESGGGLVQPGGSLRLSCAASGRSLSNYYMGWFRQAPGKGRE LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLKTEDTA VYYCAASILPLSDDPGWNTYWGQGTQVTVSS |
| TNF23(TNF3 HUM4) | 86 | EVQLVESGGGLVQPGGSLRLSCAASGRSLSNYYMGWFRQAPGKGRE LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLKPEDTA VYYCAASILPLSDDPGWNTYWGQGTLVTVSS |
| ALB3(ALB1 HUM1) | 87 | EVQLVESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKEPE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA VYYCTIGGSLSRSSQGTQVTVSS |
| ALB4(ALB1 HUM2) | 88 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMSWVRQAPGKEPE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA VYYCTIGGSLSRSSQGTQVTVSS |
| ALB5(ALB1 HUM3) | 89 | EVQLVESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA VYYCTIGGSLSRSSQGTQVTVSS |

Table 26

| Nanobody | Induction time | Yield (mg/L) |
|---|---|---|
| TNF13 | ON/28°C | 8.8 |
| TNF14 | ON/28°C | 7 |
| TNF15 | Short/37°C | 7.6 |
| TNF16 | Short/37°C | 8.7 |
| TNF17 | Short/37°C | 7.2 |
| TNF18 | Short/37°C | 4.8 |
| TNF19 | Short/37°C | 8 |
| TNF20 | ON/28°C | 3.5 |
| TNF21 | ON/28°C | 7.5 |
| TNF22 | ON/28°C | 6 |
| TNF23 | ON/28°C | 2.8 |
| ALB3 | ON/28°C | 11.8 |
| ALB4 | ON/28°C | 9 |
| ALB5 | ON/28°C | 11.7 |

Table 27

*assay :* ***L929s + Act D (5000c/w)***

*TNF :* ***human TNFa @ 0.5ng/ml***

| | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|
| **Nanobody** | **mean** | **stdev** | **#** | **mean** | **stdev** |
| **TNF1** | **0.707** | **0.265** | **14** | **0.015** | **0.007** |
| TNF13 | 0.988 | 0.014 | 3 | 0.014 | 0.003 |
| TNF14 | 0.981 | 0.007 | 3 | 0.014 | 0.003 |
| **TNF2** | **1.412** | **0.622** | **14** | **0.007** | **0.002** |
| TNF15 | 1.669 | 1.253 | 4 | 0.002 | 0.000 |
| TNF16 | 1.898 | 0.192 | 4 | 0.005 | 0.001 |
| TNF17 | 3.023 | 0.562 | 4 | 0.001 | 0.001 |
| TNF18 | 1.508 | 0.481 | 4 | 0.004 | 0.001 |
| TNF19 | 2.191 | 0.941 | 4 | 0.001 | 0.001 |
| **TNF3** | **0.224** | **0.133** | **14** | **0.048** | **0.019** |
| TNF20 | 0.380 | 0.080 | 3 | 0.035 | 0.005 |
| TNF21 | 0.889 | 0.019 | 3 | 0.015 | 0.003 |
| TNF22 | 0.303 | 0.005 | 3 | 0.044 | 0.011 |
| TNF23 | 0.3 | 0.011 | 3 | 0.044 | 0.011 |
| Enbrel | 0.009 | 0.005 | 45 | 1.002 | 0.011 |
| Humira | 0.079 | 0.043 | 39 | 0.097 | 0.069 |
| Remicade | 0.083 | 0.037 | 45 | 0.103 | 0.058 |

Table 28

| % | Untreated | RT | 37C | 50C | 60C | 70C | 80C | 90C |
|---|---|---|---|---|---|---|---|---|
| TNF13 | 100 | 104 | 99 | 98 | 99 | 84 | 93 | 93 |
| TNF14 | 100 | 98 | 101 | 95 | 99 | 96 | 99 | 90 |
| TNF15 | 100 | 100 | 91 | 99 | 95 | 90 | 59 | 46 |
| TNF16 | 100 | 97 | 102 | 101 | 94 | 101 | 58 | 48 |
| TNF17 | 100 | 102 | 98 | 100 | 90 | 90 | 69 | 59 |
| TNF18 | 100 | 100 | 101 | 97 | 91 | 93 | 63 | 50 |
| TNF19 | 100 | 102 | 111 | 98 | 92 | 91 | 60 | 49 |
| TNF20 | 100 | 94 | 93 | 93 | 93 | 92 | 85 | 67 |
| TNF21 | 100 | 98 | 99 | 101 | 98 | 96 | 36 | 40 |
| TNF22 | 100 | 102 | 101 | 105 | 99 | 93 | 25 | 31 |
| TNF23 | 100 | 98 | 97 | 99 | 97 | 98 | 87 | 55 |
| ALB3 | 100 | 100 | 99 | 98 | 25 | 18 | 60 | 62 |
| ALB4 | 100 | 100 | 100 | 100 | 99 | 29 | 61 | 55 |
| ALB5 | 100 | 100 | 100 | 99 | 94 | 32 | 61 | 48 |

**Table 29**

| Name | SEQ ID NO | Sequence |
|---|---|---|
| TNF1-9GS-ALB1-9GS-TNF1(TNF24) | 90 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE<br>WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA<br>LYYCARSPSGFNRGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQP<br>GNSLRLSCAASGFTFRSFGMSWVRQAPGKEPEWVSSISGSGSDTLY<br>ADSVKGRFTISRDNAKTTLYLQMNSLKPEDTAVYYCTIGGSLSRSS<br>QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFT<br>FSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDN<br>AKNTLYLQMNSLKPEDTALYYCARSPSGFNRGQGTQVTVSS |
| TNF2-9GS-TNF2-9GS-ALB1(TNF25) | 91 | QVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP<br>GKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLK<br>PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSSGGGGSGGGS<br>EVQLVESGGGLVQAGGSLRLSCAASGRTFSEPSGYTYTIGWFRQAP<br>GKEREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLLMNSLK<br>PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSSGGGGSGGGS<br>EVQLVESGGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKEPE<br>WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA<br>VYYCTIGGSLSRSSQGTQVTVSS |
| TNF3-9GS-ALB1-9GS-TNF3(TNF26) | 92 | EVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERE<br>LLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTA<br>VYYCAASILPLSDDPGWNTYWGQGTQVTVSSGGGGSGGGSEVQLVE<br>SGGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKEPEWVSSIS<br>GSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTAVYYCTI<br>GGSLSRSSQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLSL<br>SCSASGRSLSNYYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKG<br>RFTISRDDAKNTIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTY<br>WGQGTQVTVSS |
| TNF1-30GS-TNF1-9GS-ALB1(TNF27) | 93 | QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE<br>WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA<br>LYYCARSPSGFNRGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGG<br>GSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQ<br>APGKGLEWVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNS<br>LKPEDTALYYCARSPSGFNRGQGTQVTVSSGGGGSGGGSEVQLVES<br>GGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKEPEWVSSISG<br>SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTAVYYCTIG<br>GSLSRSSQGTQVTVSS |
| TNF3-30GS-TNF3-9GS-ALB1(TNF28) | 94 | EVQLVESGGGLVQAGGSLSLSCSASGRSLSNYYMGWFRQAPGKERE<br>LLGNISWRGYNIYYKDSVKGRFTISRDDAKNTIYLQMNRLKPEDTA<br>VYYCAASILPLSDDPGWNTYWGQGTQVTVSSGGGGSGGGGSGGGGS<br>GGGGSGGGSSGGGGSEVQVVESGGGLVQAGGSLSLSCSASGRSLSN<br>YYMGWFRQAPGKERELLGNISWRGYNIYYKDSVKGRFTISRDDAKN<br>TIYLQMNRLKPEDTAVYYCAASILPLSDDPGWNTYWGQGTQVTVSS<br>GGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFRSFGMSWV<br>RQAPGKEPEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQM<br>NSLKPEDTAVYYCTIGGSLSRSSQGTQVTVSS |
| TNF30-9GS-ALB8-9GS-TNF30 (TNF60) | 417 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE<br>WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTA<br>VYYCARSPSGFNRGQGTLVTVSSggggsgggsEVQLVESGGGLVQP<br>GNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLY<br>ADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSS<br>QGTLVTVSSggggsgggsEVQLVESGGGLVQPGGSLRLSCAASGFT<br>FSDYWMYWVRQAPGKGLEWVSEINTNGLITKYPDSVKGRFTISRDN<br>AKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSS |

| | | |
|---|---|---|
| TNF33-9GS-ALB8-9GS-TNF33 (TNF62) | 418 | EVQLVESGGGLVQPGGSLRLSCAASGRSLSNYYMGWFRQAPGKGRE<br>LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLRPEDTA<br>VYYCAASILPLSDDPGWNTYWGQGTLVTVSSggggsgggsEVQLVE<br>SGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSIS<br>GSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTI<br>GGSLSRSSQGTLVTVSSggggsgggsEVQLVESGGGLVQPGGSLRL<br>SCAASGRSLSNYYMGWFRQAPGKGRELLGNISWRGYNIYYKDSVKG<br>RFTISRDDSKNTIYLQMNSLRPEDTAVYYCAASILPLSDDPGWNTY<br>WGQGTLVTVSS |

**Table 30**

| ID | Format |
|---|---|
| TNF24 | TNF1-9GS-ALB1-9GS-TNF1 |
| TNF25 | TNF2-9GS-TNF2-9GS-ALB1 |
| TNF26 | TNF3-9GS-ALB1-9GS-TNF3 |
| TNF27 | TNF1-30GS-TNF1-9GS-ALB1 |
| TNF28 | TNF3-30GS-TNF3-9GS-ALB1 |

**Table 31**

| Nanobody | Induction time | Yield (mg/L) |
|---|---|---|
| TNF24 | ON/28°C | 1.7 |
| TNF25 | short/37°C | 0.445 |
| TNF26 | short/37°C | 0.167 |
| TNF27 | ON/28°C | 2.2 |
| TNF28 | short/37°C | 1 |

**Table 32**

***assay :*** ***L929s + Act D (5000c/w)***

***TNF :*** ***human TNFa @ 0.5ng/ml***

| | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|
| Nanobody | mean | stdev | # | mean | stdev |
| TNF24 | 0.011 | 0.003 | 11 | 0.878 | 0.248 |
| TNF25 | 0.018 | 0.008 | 14 | 0.603 | 0.243 |
| TNF26 | 0.020 | 0.009 | 14 | 0.583 | 0.210 |
| TNF27 | 0.012 | 0.003 | 3 | 0.810 | 0.037 |
| TNF28 | 0.021 | 0.008 | 6 | 0.548 | 0.360 |
| Enbrel | 0.009 | 0.005 | 45 | 1.002 | 0.011 |
| Humira | 0.079 | 0.043 | 39 | 0.097 | 0.069 |
| Remicade | 0.083 | 0.037 | 45 | 0.103 | 0.058 |

**Table 33**

| Human albumin | KD (nM) | ka (1/Ms) | kd (1/s) |
|---|---|---|---|
| 6A6 (ALB1) | 0,57 | 1,11E+6 | 6,30E-4 |
| 1C2-GS-6A6-GS-1C2 (TNF24) | 11 | 2,26E+05 | 2,48E-03 |
| 1G5-GS-1G5-GS-6A6 (TNF25) | 7,2 | 2,91E+05 | 2,10E-03 |
| 5F10-GS-6A6-GS-5F10 (TNF26) | 7,3 | 2,81E+05 | 2,05E-03 |
| 1C2-GS6-1C2-GS-6A6 (TNF27) | 8,9 | 3,19E+05 | 2,84E-03 |
| 5F10-GS6-5F10-GS-6A6 (TNF28) | 14 | 1,55E+05 | 2,13E-03 |

**Table 34**

| % | untreated | RT | 37C | 50C | 60C | 70C | 80C | 90C |
|---|---|---|---|---|---|---|---|---|
| TNF24 | 100 | 100 | 99 | 98 | 5 | 3 | 8 | 18 |
| TNF25 | 100 | nd | 103 | 102 | 95 | 5 | 4 | 6 |
| TNF26 | 100 | 109 | 115 | 112 | 107 | 10 | 8 | 10 |
| TNF27 | 100 | 102 | 103 | 102 | 22 | 9 | 26 | 34 |
| TNF28 | 100 | 97 | 99 | 99 | 66 | 3 | 6 | 10 |

**Table 35**

| Name | SEQ ID NO | Sequence |
|---|---|---|
| TNF29(TNF1 HUM1) | 95 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLKPEDTA VYYCARSPSGFNRGQGTLVTVSS |
| TNF30(TNF1 HUM2) | 96 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYWVRQAPGKGLE WVSEINTNGLITKYPDSVKGRFTISRDNAKNTLYLQMNSLRPEDTA VYYCARSPSGFNRGQGTLVTVSS |
| TNF31(TNF2 HUM1) | 97 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLR PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTQVTVSS |
| TNF32(TNF2 HUM2) | 98 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSEPSGYTYTIGWFRQAP GKGREFVARIYWSSGLTYYADSVKGRFTISRDIAKNTVDLQMNSLR PEDTAVYYCAARDGIPTSRSVGSYNYWGQGTLVTVSS |
| TNF33(TNF3 HUM1) | 99 | EVQLVESGGGLVQPGGSLRLSCAASGRSLSNYYMGWFRQAPGKGRE LLGNISWRGYNIYYKDSVKGRFTISRDDSKNTIYLQMNSLRPEDTA VYYCAASILPLSDDPGWNTYWGQGTLVTVSS |
| ALB6(ALB1 HUM1) | 100 | EVQLVESGGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLKPEDTA VYYCTIGGSLSRSSQGTLVTVSS |
| ALB7(ALB1 HUM2) | 101 | EVQLVESGGGLVQPGNSLRLSCAASGFTFRSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTA VYYCTIGGSLSRSSQGTLVTVSS |
| ALB8(ALB1 HUM3) | 102 | EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTA VYYCTIGGSLSRSSQGTLVTVSS |
| ALB9(ALB1 HUM4) | 103 | EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKNTLYLQMNSLRPEDTA VYYCTIGGSLSRSSQGTLVTVSS |
| ALB10(ALB1 HUM5) | 104 | EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLE WVSSISGSGSDTLYADSVKGRFTISRDNAKNTLYLQMNSLRPEDTA VYYCTIGGSLSRSGQGTLVTVSS |

**Table 36**

| Nanobody™ | Induction time | yield |
|---|---|---|
| TNF29 | ON/28C | 2.1 mg/L |
| TNF30 | ON/28C | 2.7 mg/L |
| TNF31 | ON/28C | 2 mg/L |
| TNF32 | ON/28C | 1.5 mg/L |
| TNF33 | ON/28C | 0.5 mg/L |

Table 37

*assay : L929s + Act D (5000c/w)*
*TNF : human TNFa @ 0.5ng/ml*

| | $EC_{50}$ in nM | | | relative potency | |
|---|---|---|---|---|---|
| $V_{HH}$ | mean | stdev | # | mean | stdev |
| **TNF1** | **0.707** | **0.265** | **14** | **0.015** | **0.007** |
| TNF13 | 0.988 | 0.014 | 3 | 0.014 | 0.003 |
| TNF14 | 0.981 | 0.007 | 3 | 0.014 | 0.003 |
| TNF29 | 1.336 | | 1 | 0.013 | |
| TNF30 | 0.985 | | 1 | 0.017 | |
| **TNF2** | **1.412** | **0.622** | **14** | **0.007** | **0.002** |
| TNF15 | 5.896 | 1.253 | 4 | 0.002 | 0.000 |
| TNF16 | 2.422 | 0.192 | 4 | 0.005 | 0.001 |
| TNF17 | 7.555 | 0.562 | 4 | 0.001 | 0.001 |
| TNF18 | 3.134 | 0.481 | 4 | 0.004 | 0.001 |
| TNF19 | 7.372 | 0.941 | 4 | 0.001 | 0.001 |
| TNF31 | 2.195 | | 1 | 0.008 | |
| TNF32 | 2.506 | | 1 | 0.007 | |
| **TNF3** | **0.224** | **0.133** | **14** | **0.048** | **0.019** |
| TNF20 | 0.380 | 0.080 | 3 | 0.035 | 0.005 |
| TNF21 | 0.889 | 0.019 | 3 | 0.015 | 0.003 |
| TNF22 | 0.303 | 0.005 | 3 | 0.004 | 0.011 |
| TNF23 | 0.3 | 0.011 | 3 | 0.04 | 0.011 |
| TNF33 | 0.3 | | 1 | 0.057 | |
| Enbrel | 0.009 | 0.005 | 45 | 1.002 | 0.011 |
| Humira | 0.079 | 0.043 | 39 | 0.097 | 0.069 |
| Remicade | 0.083 | 0.037 | 45 | 0.103 | 0.058 |

Table 38

| | untreated | RT | 37C | 50C | 60C | 70C | 80C | 90C |
|---|---|---|---|---|---|---|---|---|
| **TNF29** | 100 | 100 | 100 | 100 | 100 | 96 | 91 | 89 |
| **TNF30** | 100 | 100 | 100 | 99 | 100 | 96 | 92 | 89 |
| **TNF31** | 100 | 100 | 100 | 98 | 91 | 84 | 56 | 43 |
| **TNF32** | 100 | 99 | 98 | 97 | 87 | 78 | 45 | 39 |
| **TNF33** | 100 | 98 | 97 | 97 | 94 | 91 | 79 | 49 |

Table 39

*assay : alphaKYM (10000c/w)*
*TNF : human TNFa @ 1ng/ml*

| Nanobody | $EC_{50}$ in nM |
|---|---|
| TNF1 | 2.466 |
| TNF2 | 4.236 |
| TNF3 | **0.655** |
| TNF4 | 0.069 |
| TNF5 | 0.008 |
| TNF6 | 0.121 |
| TNF7 | 0.009 |
| TNF8 | 0.013 |
| TNF9 | 0.020 |
| Enbrel | 0.040 |
| Humira | 0.103 |
| **Remicade** | **0.100** |

***Results from WO 04/41862***

| Nanobody | SEQ ID No | $EC_{50}$ in nM |
|---|---|---|
| 1A | 1 | 100 |
| 3E | 4 | **12** |
| 3G | 5 | 20 |
| **Remicade** | | **0,080** |

Table 40

| | | |
|---|---|---|
| M13 rev | SEQ ID NO: 421 | GGATAACAATTTCACACAGG |
| Rev 9GlySer L108 | SEQ ID NO: 422 | TCAGTAACCTGGATCCGCCACCGCTGCCTCCACCGCCTGAGGAGACGGTGACCAG |
| For GS/Short | SEQ ID NO: 423 | AGGTTACTGAGGATCCGAGGTGCAGCTGGTGGAGTCTGG |
| Rev 15BspEI L108 | SEQ ID NO: 424 | TCAGTAACCTTCCGGAACCGCCACCGCCTGAGGAGACGGTGACAAG |
| For BspEI | SEQ ID NO: 425 | AGGTTACTGATCCGGAGGCGGTAGCGAGGTGCAGCTGGTGGAGTCTGG |
| M13 for | SEQ ID NO: 426 | CACGACGTTGTAAAACGAC |

Table 41

| **Reverse primer** | | **Sequence** |
|---|---|---|
| PiRevhumNot/a40c (Not1) | SEQ ID NO: 427 | ATGGTGGTGTGCGGCCGCCTATTATGAGGAGACGGTGACCAGG |
| **Forward primer** | | |
| Pi2for (Xho1) | SEQ ID NO: 428 | AGGGGTATCTCTCGAGAAAAGAGAGGTGCAGCTGGTGGAGTCTGG |

Table 42

| *Human TNFa* | $EC_{50}$ in nM | | |
|---|---|---|---|
| **VHH** | **mean** | **stdev** | **Number of assays** |
| TNF60 | 0.010 | 0.002 | 6 |
| Enbrel | 0.014 | 0.009 | 33 |
| Humira | 0.141 | 0.074 | 33 |
| Remicade | 0.120 | 0.037 | 33 |

Table 43

| | | human albumin | rhesus albumin |
|---|---|---|---|
| **TNF60** | $K_D$ (nM) | **24,4** | **24,1** |
| | $k_{on}$ (1/Ms) | 2,05E+05 | 2,09E+05 |
| | $k_{off}$ (1/s) | 5,02E-03 | 5,04E-03 |
| **TNF24** | $K_D$ (nM) | **11** | Nd |
| | $k_{on}$ (1/Ms) | 2.26E+05 | Nd |
| | $k_{off}$ (1/s) | 2.48E-03 | Nd |

**Table 44**

| | | |
|---|---|---|
| PiForLong | SEQ ID NO: 429 | GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGTGCAGCTGGTGGAGTCTGG |
| Rev_30GlySer_L108 | SEQ ID NO: 430 | TCAGTAACCTGGATCCCCCGCCACCGCTGCCTCCACCGCCGCTACCCCCGCCACCGC TGCCTCCACCGCCTGAGGAGACGGTGACAAG |
| For_GlySer | SEQ ID NO: 431 | AGGTTACTGAGGATCCGGCGGTGGAGGCAGCGGTGGCGGGGGTAGCGAGGTGCAGCTGGTGGAGTCTGG |
| PiRevCys1hum | SEQ ID NO: 432 | ATGGTGGTGTGAATTCTTATTAGCAGGAGACGGTGACAAGG |
| PiRevCys2hum | SEQ ID NO: 433 | ATGGTGGTGTGAATTCTTATTAGCAACCTCCACCTGAGGAGACGGTGACAAGG |
| AOXIFor | SEQ ID NO: 434 | GACTGGTTCCAATTGACAAGC |
| AOXIRev | SEQ ID NO: 435 | GCAAATGGCATTCTGACATCC |

**Table 45**

| *Human TNFα* | $EC_{50}$ in nM | | |
|---|---|---|---|
| VHH | mean | stdev | Number of assays |
| TNF1 | 0.748 | 0.153 | 27 |
| TNF55-PEG40 | 0.004 | 0.001 | 8 |
| TNF55-PEG60 | 0.004 | 0.002 | 6 |
| TNF55-Biotine | 0.012 | 0.003 | 5 |
| TNF56-PEG40 | 0.006 | 0.003 | 57 |
| TNF56-PEG60 | 0.005 | 0.003 | 7 |
| TNF56-Biotine | 0.017 | 0.009 | 13 |
| Enbrel | 0.013 | 0.006 | 71 |
| Humira | 0.127 | 0.058 | 67 |
| Remicade | 0.144 | 0.061 | 67 |

**Table 46**

| | DS534 P4 | DS592 P4 | DS605 P3 | KM05-179 P4 |
|---|---|---|---|---|
| p[IC]50 | | | | |
| Etanercept | 9.55 | 9.49 | 9.51 | 9.51 |
| Accipiter | 9.88 | 9.44 | 9.37 | 9.27 |
| | | | | |
| pM | | | | |
| Etanercept | 282 | 324 | 309 | 309 |
| Accipiter | 132 | 363 | 427 | 537 |

**Table 47**

| | Total WBC count ($x10^6$/mL) | |
|---|---|---|
| Dose Group | ALX0071 | Etanercept |
| CMC vehicle | 0.86 ± 0.09 | |
| 0.1 μg human TNFα | 3.72 ± 0.21 | |
| 0.0625 mg/kg | 3.03 ± 0.6 | - |
| 0.125 mg/kg | 1.23 ± 0.3** | 2.40 ± 0.39 |
| 0.25 mg/kg | 1.37 ± 0.17* | 2.47 ± 0.54 |
| 0.5 mg/kg | - | 2.19 ± 0.10 |

*P<0.05, **P<0.01 vs 0.1 μg human TNFα

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 claims.

All references disclosed herein are incorporated by reference in their entirety.

Claims

1. Nanobody against human serum albumin, which consists of 4 framework regions (FRl to FR4 respectively) and 3 complementarity determining regions (CDRl to CDR3 respectively), in which:
CDRl comprises or is the amino acid sequence SFGMS (SEQ ID NO:36),
CDR2 comprises or is the amino acid sequence SISGSGSDTLYADSVKG (SEQ ID NO: 40), and
CDR3 comprises or is the amino acid sequence GGSLSR (SEQ ID NO: 48).

2. Nanobody against human serum albumin according to claim 1, which is chosen from the group consisting of ALB 3 (SEQ ID NO: 87), ALB 4 (SEQ ID NO: 88), ALB 5 (SEQID NO: 89), ALB 6 (SEQ ID NO: 100), ALB 7 (SEQ ID NO: 101), ALB 8 (SEQ ID NO: 102), ALB 9 (SEQ IDNO: 103) and ALB 10 (SEQ ID NO: 104).

3. The Nanobody against human serum albumin according to claim 1, in which:
(i) FR1 is an amino acid sequence chosen from the group consisting of: SEQ ID NO: 370, 368, 369, 371, 372, 373 and 374;
(ii) FR2 is an amino acid sequence chosen from the group consisting of: SEQ ID NO: 384, 383 and 385 – 388;
(iii) FR3 is an amino acid sequence chosen from the group consisting of: SEQ ID NO: 398, 396, 397, 399, 400 and 402; and
(iv) FR4 is an amino acid sequence chosen from the group consisting of: SEQ ID NO: 412, 410, 411 and 413 – 416.

4. Protein or polypeptide comprising or essentially consisting of the Nanobody according to any one of claims 1 – 3.

5. Protein or polypeptide comprising two or more Nanobodies, optionally linked via (a) linker sequence(s), said protein or polypeptide comprising at least one Nanobody according to any of claims 1 – 3.

6. Protein or polypeptide comprising three Nanobodies, optionally linked via two linker sequences, said protein or polypeptide comprising at least one Nanobody according to any of claims 1 – 3.

7. Protein or polypeptide according to claims 5 or 6, in which the linker consists of an amino acid(s).

8. Protein or polypeptide according to claim 7, in which the linker comprises at least 14 amino acids, at least 17 amino acids, or 20-40 amino acids.

9. Protein or polypeptide according to claim 7 or 8, in which the linker comprises glycine and serine residues.