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(54) **Title:** IMPROVED POLYPEPTIDES DIRECTED AGAINST IgE

(57) **Abstract:** Polypeptides are provided that bind IgE, as well as constructs and compositions comprising the same. The poly-
peptides, constructs and compositions provided by the invention show improved stability during production and storage, as well as
improved potency for IgE. The invention also relates to the uses of such polypeptides, constructs and compositions for prophylactic
and/or therapeutic purposes.



Improved polypeptides directed against IgE

FIELD OF THE INVENTION

The present disclosure relates to polypeptides, constructs and compositions that are directed
5 against IgE, and uses of such polypeptides, constructs and compositions.

BACKGROUND ART

WO 04/041867 describes VHH's that are directed against IgE (see for example SEQ ID NO's: 1 to
11 of WO 04/041867). WO 04/041867 further mentions that these VHH's may be humanized and
10 may be suitably linked to one or more VHH's that are directed against a serum protein such as serum
albumin, to provide a protein construct that has increased half-life as well as the favorable properties
that are associated with VHH's and Nanobodies. [*Nanobody® and Nanobodies® are trademarks of
Ablynx N.V.*].

WO 04/041865 describes specific protein constructs that are directed against IgE that comprise
15 at least one VHH or humanized VHH against IgE and at least one Nanobody that is directed against a
serum protein such as (human) serum albumin, which because of the presence of the serum albumin-
binding Nanobody, have increased half-life *in vivo* compared to the corresponding VHH's against IgE
alone. Examples of such bispecific anti-IgE/anti-serum albumin constructs are given in SEQ ID NO's:
22 to 24 of WO 04/041865.

WO 06/122787 describes a number of Nanobodies against (human) serum albumin. These
20 Nanobodies include the Nanobody called Alb-1 (SEQ ID NO: 52 in WO 06/122787; SEQ ID NO: 37
herein) and humanized variants thereof, such as Alb-8 (SEQ ID NO: 62 in WO 06/122787; SEQ ID NO:
38 herein).

The use of Nanobodies against (human) serum albumin for extending the half-life of therapeutic
25 moieties such as Nanobodies has been validated by means of clinical trials. For example, the safety,
tolerability, immunogenicity and pharmacokinetics (PK) of ALX-0141, a protein construct that
comprises two Nanobodies against RANK-L and the Nanobody Alb-8, has been confirmed in phase I
clinical trials (data presented by Ablynx N.V. on May 27, 2011 at the Annual European Congress of
Rheumatology (EULAR) in London). Also, numerous published patent applications of Ablynx N.V. give
30 examples of constructs with increased half-life that comprise one or more Nanobodies against a
therapeutic target and one or more Nanobodies against serum albumin (such as Alb-8). Reference is
for example made to WO 04/041862, WO 06/122786, WO 08/020079, WO 08/142164, WO
09/068627 and WO 09/147248.

SUMMARY OF THE INVENTION

The present disclosure relates to polypeptides and constructs, such as protein constructs, that are directed against IgE; to nucleic acids that encode such polypeptides and protein constructs; to methods for preparing such polypeptides and constructs; to host cells expressing or capable of
5 expressing such polypeptides and protein constructs; to compositions, and in particular pharmaceutical compositions, that comprise such polypeptides and constructs; and to uses of such polypeptides, constructs and compositions, in particular for prophylactic and/or therapeutic purposes, such as the prophylactic and/or therapeutic purposes mentioned herein.

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

Although it has been established that the use of Nanobodies against (human) serum albumin is a good and broadly applicable way of extending the half-life of Nanobodies and other therapeutic entities, when representative Nanobodies according to WO 06/122787 are applied to extending the half-life of Nanobodies that are directed to IgE (in the manner generally described in WO 04/041865
15 and WO 04/041867), the constructs thus obtained, even though they are sufficiently biologically active against IgE and have a half-life that is suitable for therapeutic applications, have some properties that would benefit from further improvement.

In particular, it has been found that polypeptides that comprise a Nanobody against IgE and Alb-8 have limited storage stability (see for example Example 14 herein).

20 The present invention provides improved polypeptides and (other) constructs, such as protein constructs, that comprise at least one immunoglobulin single variable domain (such as a Nanobody) against IgE and that have an increased half-life *in vivo* (compared to the immunoglobulin single variable domain against IgE per se) and that are not associated with the same problem(s) as observed when using a serum albumin-binding Nanobody described in WO 06/122787.

25 More specifically, the present invention provides polypeptides (also referred to as "*polypeptide(s) of the invention*") and constructs, such as protein constructs, that are directed against IgE and that have improved prophylactic, therapeutic and/or pharmacological properties, in addition to other advantageous properties (such as, for example, improved stability, improved ease of preparation and/or reduced costs of goods), compared to the prior art polypeptides and antibodies.

30 WO 2012/175740 describes a Nanobody against IgE (IgE026; SEQ ID NO: 27 in the present application) that is cross-reactive for human IgE and cynomolgus IgE and that shows improved binding characteristics and potency.

WO 2012/175400 describes Nanobodies against human serum albumin (based on Nanobody Alb-23; SEQ ID NO: 39 in the present application) with improved storage stability and a reduced tendency

to form dimers under certain formulation conditions (for example, at high concentrations in certain aqueous formulation buffers).

The polypeptides of the present invention combine the improved properties of this unique immunoglobulin single variable domain building block directed against IgE with the improved properties of these unique immunoglobulin single variable domain building blocks directed against human serum albumin (HSA). The polypeptides of the invention comprise or essentially consist of one or more immunoglobulin single variable domain(s) directed against IgE selected from Nanobody IGE026 (SEQ ID NO: 27) and variants thereof and an immunoglobulin single variable domain directed against HSA selected from Nanobody Alb-23 (SEQ ID NO: 39) and variants thereof. More specifically, the present invention provides polypeptides that comprise or essentially consist of one or more immunoglobulin single variable domain(s) directed against IgE and an immunoglobulin single variable domain directed against HSA wherein:

A. the one or more immunoglobulin single variable domain(s) directed against IgE is selected from:

a) SEQ ID NO: 32;

b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:

i) the amino acid has an Aspartic acid (Asp, D) at position 1 (said position determined according to Kabat numbering); and

ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32;

B. the immunoglobulin single variable domain directed against HSA is selected from:

a) SEQ ID NO: 39;

b) amino acid variants that have no more than 6, preferably no more than 5, no more than 4, more preferably no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 39, provided that:

i) the amino acid variant binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to SEQ ID NO: 39.

Without being limiting, the advantages that the improved polypeptides may provide over previously described polypeptides and antibodies, such as *e.g.* the Nanobodies described in WO 04/041865 and WO 06/122787 may include:

- improved binding characteristics (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein);
 - improved potency (see for example the data presented in the Experimental Part)
 - 5 - cross-reactivity between human IgE and cynomolgus monkey IgE (as further described herein);
 - they may block interaction of human and cyno IgE both with the high affinity IgE receptor (Fc(epsilon)RI) and the low affinity receptor (Fc(epsilon)RII) (see Examples 6, 7, 8 and 10); and generally have a desirable "balance" between affinity for the human receptor(s) and the cyno receptor(s);
 - 10 - they neutralize soluble IgE and also displace Fc(epsilon)RI-bound IgE (e.g. in an in vitro cell based degranulation assay as described herein);
 - they have a desirable "balance" between affinity for the human high affinity IgE receptor and the low affinity IgE receptor (see again the data presented in the Experimental Part);
 - improved stability (such as improved thermal stability as determined by measuring the T_m);
 - 15 - improved storage stability (as for example measured in the SE-HPLC experiment described in Example 14);
 - reduced pyroglutamate post-translational modification of the N-terminus and hence increased product stability;
 - a reduced tendency to form dimers under certain formulation conditions (for example, at high
 - 20 concentrations in certain aqueous formulation buffers); and/or
 - increased expression yields (as for example determined by SDS-Page analysis),
- or any combination of any or all of the foregoing.

The variants of Nanobody IGE026 (SEQ ID NO: 27) that bind IgE are selected from:

- a) SEQ ID NO: 32;
- 25 b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:
 - i) the amino acid variant has an Aspartic acid (Asp, D) at position 1 (said position determined according to Kabat numbering); and
 - 30 ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32;

In a preferred aspect, the variants of Nanobody IGE026 are selected from:

- 35 a) SEQ ID NO: 32;

b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:

- i) the amino acid difference is a substitution in the framework 1 and/or CDR1 region, preferably at a position selected from positions 6, 29, 31 and 35, such as *e.g.* selected from Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering); and
- ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the amino acid has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32.

Non-limiting examples of some variants of the Nanobody IGE026 are SEQ ID NO's: 28-36. An alignment of Nanobody IGE026 with the variant sequences of SEQ ID NO's: 28-36 is given in Figure 1. In a preferred aspect, the polypeptide of the invention comprises an immunoglobulin single variable domain directed against IgE selected from any of SEQ ID NO: 28-36.

In a preferred aspect, the one or more immunoglobulin single variable domain against IgE binds human IgE (full length) and constructs made from human IgE such as *e.g.*, the a human IgE c(epsilon)2-c(epsilon)3-c(epsilon)4 fragment of IgE. In another preferred aspect, the one or more immunoglobulin single variable domain against IgE binds cyno IgE (full length) and constructs made from cyno IgE such as *e.g.* the a cyno IgE c(epsilon)2-c(epsilon)3-c(epsilon)4 fragment of IgE.

The one or more immunoglobulin single variable domains against IgE present in the anti-IgE polypeptides of the invention may in particular be immunoglobulin single variable domains that are cross-reactive between the human IgE Fc sequence of SEQ ID NO: 1 and the and IgE Fc sequence from cynomolgus monkey given in SEQ ID NO: 2.

The variants of Nanobody Alb-23 (SEQ ID NO: 39) that binds HSA may be selected from amino acid variants that have no more than 6, preferably no more than 5, or no more than 4, more preferably no more than 3, or no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 39, provided that the amino acid variant binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to SEQ ID NO: 39.

In a preferred aspect, the amino acid difference is a substitution in one or more of the framework regions, preferably at a position selected from positions 1, 14, 30, 87 and 108, such as *e.g.*, selected from Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering). Apart from this or in addition, in another preferred aspect the amino acid difference is an addition of 1 to 3 amino acid residues at the C-terminal part of the

immunoglobulin single variable domain against HSA, such as the addition of one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly at the C-terminal end of the immunoglobulin single variable domain against HSA.

Non-limiting examples of some Alb-23 variants are given in SEQ ID NO's: 40 to 48. An alignment of Alb-23 with the variant sequences of SEQ ID NO's: 40-48 is given in Figure 2. In a preferred aspect, the polypeptide of the invention comprises an immunoglobulin single variable domain directed against HSA selected from any of SEQ ID NO: 39-48. The variants of SEQ ID NO's: 43-48 (or other Alb-23 variants with 1 to 3 amino acid residues at the C-terminus, which may each be independently chosen from naturally occurring amino acid residues and may for example be independently chosen from A, G, V, L and I) may in particular be used when the albumin-binding Nanobody is provided at the C-terminal end of the polypeptide or construct.

Accordingly, in another preferred aspect, the immunoglobulin single variable domain directed against HSA is located at the C-terminal part of the polypeptide of the invention. In this case, the one or more immunoglobulin single variable domain(s) directed against IgE is preferably located at the N-terminal side of the polypeptide of the invention.

The one or more immunoglobulin single variable domain(s) directed against IgE and the immunoglobulin single variable domain against HSA may be linked directly or via one or more linkers or spacers. Accordingly, in one aspect the invention provides a polypeptide, wherein the one or more immunoglobulin single variable domain(s) against IgE and the immunoglobulin single variable domain against HSA are directly linked to each other. In another aspect, the invention provides a polypeptide wherein the one or more immunoglobulin single variable domain(s) against IgE and the immunoglobulin single variable domain directed against HSA are linked to each other via one or more linkers or spacers (such as *e.g.*, SEQ ID NO's: 49-63). In a preferred aspect, the linker is SEQ ID NO: 52.

In a further aspect, the present invention relates to a polypeptide that is directed against IgE, selected from the following polypeptides:

- a) SEQ ID NO: 82;
- b) polypeptides that have no more than 10, preferably no more than 9, no more than 8, no more than 7, no more than 6, more preferably no more than 5, no more than 4, no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 82, provided that:
 - i) the polypeptide has an Aspartic acid (Asp, D) at position 1; and
 - ii) the polypeptide binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the polypeptide has the same, about the same, or a higher potency (as determined in a degranulation assay as

defined in Example 11) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference; and

- iii) the polypeptide binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference.

In a specific aspect, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be a substitution in the immunoglobulin single variable domain directed against IgE at a position selected from positions 6, 29, 31 and 35, preferably selected from Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering).

Apart from this or in addition, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be a substitution in the immunoglobulin single variable domain directed against HSA at a position selected from positions 1, 14, 30, 87 and 108, preferably selected from Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering).

Apart from this or in addition, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be an addition of 1 to 3 amino acid residues at the C-terminal end of the immunoglobulin single variable domain against HSA, such as *e.g.* the addition of one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly at the C-terminal end of the immunoglobulin single variable domain against HSA.

In a preferred aspect, the polypeptide of the invention is selected from any of SEQ ID NO's: 82-91.

The invention further relates to protein constructs (also referred to herein as a "*protein construct(s) of the invention*" or "*construct(s) of the invention*") that comprise or essentially consist of one or more polypeptides of the invention (or suitable fragments thereof), and optionally further comprise one or more amino acids. As will become clear to the skilled person from the further disclosure herein, such further amino acid sequences may or may not provide further functionality to the polypeptide of the invention (and/or to the protein construct in which it is present) and may or may not modify the properties of the polypeptide of the invention.

The invention also relates to nucleic acids or nucleotide sequences that encode a polypeptide of the invention (or a suitable fragment thereof). Such a nucleic acid will also be referred to herein as "*nucleic acid(s) of the invention*" and may for example be in the form of a genetic construct, as further described herein. Accordingly, the present invention also relates to a nucleic acid or nucleotide sequence that is in the form of a genetic construct.

The invention further relates to a host or host cell that expresses (or that under suitable circumstances is capable of expressing) a polypeptide of the invention and/or a protein construct of the invention; and/or that contains a nucleic acid of the invention. Some preferred but non-limiting

examples of such hosts or host cells will become clear from the further description herein. The host is preferably a non-human host.

The invention further relates to constructs (also referred to herein as a “*construct(s) of the invention*”) that comprise or essentially consist of one or more polypeptides of the invention, and optionally further comprise one or more other groups, residues, moieties or binding units. As will become clear to the skilled person from the further disclosure herein, such further groups, residues, moieties, binding units or amino acid sequences may or may not provide further functionality to the polypeptide of the invention (and/or to the construct in which it is present) and may or may not modify the properties of the polypeptide of the invention.

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

The invention further relates to methods for preparing the polypeptides, constructs, nucleic acids, host cells, products and compositions described herein.

The invention further relates to applications and uses of the polypeptides, constructs, nucleic acids, host cells, products and compositions described herein, such as the use of the polypeptides, constructs, nucleic acids, host cells, products and compositions in prophylaxis and/or therapy.

Other applications and uses of the polypeptides, constructs and compositions of the invention will become clear to the skilled person from the further disclosure herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Sequence alignment of the various humanized and sequence-optimized variants of 39D11 (SEQ ID NOs: 19-36) compared to 39D11 (SEQ ID NO: 13). The sequence alignments are shown on two pages, and individual sequences are identified by SEQ ID NO on the second page of Figure 1.

Figure 2: Sequence alignment of Alb-23 (SEQ ID NO: 39) with some of the Alb-23 variants (SEQ ID NOs: 40-48). The sequence alignments are shown on two pages, and individual sequences are identified by SEQ ID NO on the second page of Figure 2.

Figure 3: Inhibition of IgE-mediated degranulation of basophils by IGE122 (▲) and Omalizumab (●). A serial dilution of both compounds was pre-incubated with anti-NP IgE prior to incubation on

the basophils for 4h. Subsequently the basophils were incubated with NIP-BSA and the degranulation was monitored in real time using the xCELLigence System. Crosslinking was done using the allergen.

Figure 4: Binding of IGE122 to HSA in the HSA binding potency assay. A dose range concentrations of IGE122 was incubated on a HSA coated plate. Bound IGE122 was detected sequentially via a flag-tagged anti-Nb-Nb and an HRP labeled anti-flag mAb and visualized using a colorimetric substrate (TMB). The resulting dose range curve is analysed using a 5 parameter logistic fit.

Figure 5: Species cross-reactivity of IGE047 for species serum albumin (SA). Titration series of IGE047 were incubated on microtiter plates coated with human, cynomolgus monkey, mouse, rat, guinea pig, dog, pig or rabbit SA and subsequently detected with biotinylated R345 antibody and HRP-labeled streptavidin.

Figures 6A and 6B: Species cross-reactivity of IGE122 in competition ELISA. Titration series of IGE123 (= Flag-tagged IGE122) were pre-incubated with IgE or IgE Fc fragment from human, cynomolgus monkey, mouse or guinea pig, incubated on human IgE coated plates and subsequently detected with a HRP-labeled monoclonal anti-FLAG antibody.

Figure 7: Species cross-reactivity of IGE122 for binding IgE in the degranulation assay. A titration series of IGE122 was pre-incubated with human or cynomolgus monkey IgE Fc-fragment and subsequently incubated on tissue culture E-plates seeded with RBL2H3 basophil cells expressing either human Fc(epsilon)RIalpha or cynomolgus monkey Fc(epsilon)RIalpha.

Figure 8: Displacement of Fc(epsilon)RI-bound IgE by IGE122 in an *in vitro* cell based degranulation assay. RBL2H3 cells were incubated with a saturating concentration of chimaeric IgE anti-NIP. After washing, a dose range concentrations of IGE122 or Omalizumab was applied in presence or absence of HSA. Degranulation was triggered by addition of NIP-BSA and monitored in realtime using the xCELLigence system.

Figure 9: Binding of Nanobody IGE047 to complexes of human Fc(epsilon) RI and human IgE. Report points on the graphs:

- Baseline_1: binding level of directly immobilized Fc(epsilon)RIA;
- Binding_1 / stability_1: binding level of human IgE (resp. before and after the stop of injection of human IgE);
- Baseline_2: binding level of human IgE just before injection of control Nanobody;
- Binding_2 / stability_2: binding level of control Nanobody (resp. before and after the stop of injection of the control Nanobody);
- Baseline_3: binding level of human IgE just before injection of IGE047;

- Binding_3 / stability_3: binding level of IGE047 (resp. before and after the stop of injection of IGE047).

DETAILED DESCRIPTION OF THE INVENTION

5 Immunoglobulin single variable domain of the invention

Unless indicated otherwise, the term "immunoglobulin" - whether used herein to refer to a heavy chain antibody or to a conventional 4-chain antibody - is used as a general term to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as V_{HH} domains or
10 V_H/V_L domains, respectively).

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

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

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

The term "immunoglobulin single variable domain", interchangeably used with "single variable domain", defines molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from
35 "conventional" immunoglobulins or their fragments, wherein two immunoglobulin domains, in

particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (VH) and a light chain variable domain (VL) interact to form an antigen binding site. In conventional immunoglobulins, the complementarity determining regions (CDRs) of both VH and VL will contribute to the antigen binding site, *i.e.*, a total of 6 CDRs will be involved in antigen binding site formation.

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

In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single VH/VHH or VL domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs.

As such, the single variable domain may be a light chain variable domain sequence (*e.g.* a VL-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (*e.g.* a VH-sequence or VHH sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (*i.e.*, a functional antigen binding unit that essentially consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

The unique immunoglobulin single variable domains used in the polypeptides of the present invention are derived from heavy chain variable domain sequences from a heavy chain antibody.

"VHH domains", also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (*i.e.* of "antibodies devoid of light chains"; Hamers-Casterman et al. 1993, Nature 363: 446-448). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains" or "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_L domains" or "VL domains"). For a further description of VHH's and Nanobodies, reference is made to the review article by Muyldermans 2001 (Reviews in Molecular Biotechnology 74: 277-302), as well

as to the following patent applications, which are mentioned as general background art: WO 94/04678, WO 95/04079 and WO 96/34103 of the Vrije Universiteit Brussel; WO 94/25591, WO 99/37681, WO 00/40968, WO 00/43507, WO 00/65057, WO 01/40310, WO 01/44301, EP 1134231 and WO 02/48193 of Unilever; WO 97/49805, WO 01/21817, WO 03/035694, WO 03/054016 and
5 WO 03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO 03/050531 of Algonomics N.V. and Ablynx N.V.; WO 01/90190 by the National Research Council of Canada; WO 03/025020 (= EP 1433793) by the Institute of Antibodies; as well as WO 04/041867, WO 04/041862, WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858, WO 06/40153, WO 06/079372, WO 06/122786, WO 06/122787 and WO 06/122825, by Ablynx N.V. and the further published patent applications by
10 Ablynx N.V. Reference is also made to the further prior art mentioned in these applications, and in particular to the list of references mentioned on pages 41-43 of the International application WO 06/040153, which list and references are incorporated herein by reference. As described in these references, Nanobodies (in particular VHH sequences and partially humanized Nanobodies) can in particular be characterized by the presence of one or more "Hallmark residues" in one or more of the
15 framework sequences. A further description of the Nanobodies, including humanization and/or camelization of Nanobodies, as well as other modifications, parts or fragments, derivatives or "Nanobody fusions", multivalent constructs (including some non-limiting examples of linker sequences) and different modifications to increase the half-life of the Nanobodies and their preparations can be found *e.g.* in WO 08/101985 and WO 08/142164. For a further general
20 description of Nanobodies, reference is made to the prior art cited herein, such as *e.g.* described in WO 08/020079 (page 16).

The amino acid residues of a VHH domain are numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown
25 *e.g.* in Figure 2 of Riechmann and Muyldermans 1999 (J. Immunol. Methods 231: 25-38). Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat applied to VHH domains as described above will be followed, unless indicated otherwise.

30 It should be noted that - as is well known in the art for V_H domains and for VHH domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat
35 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. The total number of amino acid residues in a VH domain and a VHH domain will usually be in the range of from 110 to 120, often between 112 and 115.

In the CDR determination according to Kabat, FR1 of a VHH comprises the amino acid residues at positions 1-30, CDR1 of a VHH comprises the amino acid residues at positions 31-35, FR2 of a VHH comprises the amino acids at positions 36-49, CDR2 of a VHH comprises the amino acid residues at positions 50-65, FR3 of a VHH comprises the amino acid residues at positions 66-94, CDR3 of a VHH comprises the amino acid residues at positions 95-102, and FR4 of a VHH comprises the amino acid residues at positions 103-113.

The present invention provides unique immunoglobulin single variable domains against IgE and unique immunoglobulin single variable domains against human serum albumin (HSA) (also referred to as "*immunoglobulin single variable domain(s) of the invention*") derived from a camelid heavy chain antibody. As further described in the Experimental part, the unique immunoglobulin single variable domains are VHH that have been further sequence optimized (*e.g.* by techniques such as humanization and affinity maturation). These unique immunoglobulin single variable domains are used in the preparation of the polypeptides of the invention.

The unique immunoglobulin single variable domains of the invention against IgE and the unique immunoglobulin single variable domains of the invention against HSA are based respectively on Nanobody IgE026 (SEQ ID NO: 27) described in WO 2012/175740 and variant amino acids thereof and on Nanobody Alb-23 (SEQ ID NO: 39) and variant amino acids thereof described in WO 2012/175400. More specifically, the unique immunoglobulin single variable domains of the invention encompass variant amino acids (also referred to herein as "*(amino acid) variant(s) of the invention*") of SEQ ID NO: 27 and variant amino acids of SEQ ID NO: 39 that have no more than 6, preferably no more than 5, no more than 4, more preferably no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 27 or with SEQ ID NO: 39 respectively.

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. With respect to the amino acid sequences described in the present invention, the term "amino acid difference" may also refer to the addition of one to three amino acid residue(s) at the C-terminal part of the second sequence compared to the first sequence.

More particularly, in the polypeptides of the present invention, the term "amino acid difference" refers to:

- an insertion, deletion or substitution of a single amino acid residue on a position of a first immunoglobulin single variable domain against IgE compared to a second immunoglobulin single variable domain against IgE; it being understood that the second immunoglobulin single variable

domain against IgE can contain one, two, three, four or maximal five such amino acid differences compared to the first immunoglobulin single variable domain against IgE;

- an insertion, deletion or substitution of a single amino acid residue on a position of a first immunoglobulin single variable domain against HSA, compared to a second immunoglobulin single variable domain against HSA; it being understood that the second immunoglobulin single variable domain against HSA can contain one, two, three, four, or maximal five such amino acid differences compared to the first immunoglobulin single variable domain against HSA;
- an addition of one to three amino acid residue(s) at the C-terminal part of the immunoglobulin single variable domain against HSA.

The “amino acid difference” can be any substitution, deletion or insertion, or any combination thereof, that either improves the properties of the polypeptide of the invention or that at least does not detract too much from the desired properties or from the balance or combination of desired properties of the polypeptide of the invention. In this respect, the resulting polypeptide of the invention should at least bind IgE with the same, about the same, or a higher affinity compared to the polypeptide without the substitution, deletion or insertion (said affinity as measured by surface plasmon resonance or KinExA) and/or should at least have a potency that is the same, about the same or higher compared to the polypeptide without the substitution, deletion or insertion (said potency as measured by any suitable *in vivo* or *in vitro* assay as further described herein). The resulting polypeptide of the invention should furthermore at least bind HSA with the same, about the same, or a higher affinity compared to the polypeptide without the substitution, deletion or insertion (said affinity as measured by surface plasmon resonance or KinExA).

In a specific aspect, the amino acid difference is an amino acid substitution. The amino acid substitution in the immunoglobulin single variable domain may be a conservative amino acid substitution. “Conservative” amino acid substitutions are generally 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 resulting immunoglobulin single variable domain. Such conservative amino acid substitutions are well known in the art, for example from WO 04/037999, GB 3357768, 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 residue 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.

When such substitutions, insertions or deletions are made in one or more of the framework regions, they may be made at one or more of the Hallmark residues (as *e.g.* defined in WO 08/020079; Tables A-3 to A-8). By means of non-limiting examples, an amino acid residue may be replaced by another amino acid residue that naturally occurs at the same position in another V_{HH} domain (see WO 08/020079, Tables A-5 to A-8). As can be seen from the data on the V_{HH} entropy and V_{HH} variability given in Tables A-5 – A-8 of WO 08/020079, some amino acid residues in the framework regions are more conserved than others. Generally, although the invention in its broadest sense is not limited thereto, any substitutions, deletions or insertions are preferably made at positions that are less conserved.

Substitutions, insertions or deletions made (preferably) in one or more of the framework regions may be humanizing substitutions (*i.e.*, replacing one or more amino acid residues in the amino acid sequence of a naturally occurring V_{HH} sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a V_H domain from a conventional 4-chain antibody from a human being). Potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring V_{HH} sequence with the corresponding framework sequence of one or more closely related human V_H sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said V_{HH} sequence (in any manner known per se, as further described herein) and the resulting humanized V_{HH} sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) an immunoglobulin single variable domain, such as a Nanobody (including VHH domains) may be partially humanized or fully humanized.

In another specific aspect, the amino acid substitution in the immunoglobulin single variable domain may provide the immunoglobulin single variable domain with increased affinity for binding to IgE or for binding HSA respectively. Accordingly, the immunoglobulin single variable domain of the

invention can also be subjected to affinity maturation by introducing one or more alterations in the amino acid sequence of one or more CDRs, which alterations result in an improved affinity of the resulting (second) immunoglobulin single variable domain for IgE or HSA respectively, as compared to the first immunoglobulin single variable domain. Affinity-matured immunoglobulin single variable domain molecules of the invention may be prepared by methods known in the art, for example, as described by Marks et al. 1992 (Biotechnology 10:779-783), Barbas et al. 1994 (Proc. Nat. Acad. Sci, USA 91: 3809-3813), Shier et al. 1995 (Gene 169: 147-155), Yelton et al. 1995 (Immunol. 155: 1994-2004), Jackson et al. 1995 (J. Immunol. 154: 3310-9), Hawkins et al. 1992 (J. Mol. Biol. 226: 889 896), Johnson and Hawkins 1996 (Affinity maturation of antibodies using phage display, Oxford University Press), or techniques described in WO 09/004065, WO 05/003345, WO 06/023144, EP527809, EP397834. Without being limiting, affinity maturation of immunoglobulin single variable domains has been described in WO 09/004065.

Depending on the host organism used to express the polypeptide of the invention, such deletions and/or substitutions may also be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the art. Alternatively, substitutions or insertions may be designed so as to introduce one or more sites for attachment of functional groups (as described herein), for example to allow site-specific pegylation.

During the production of certain immunoglobulin single variable domains and polypeptides comprising the same, a high level of pyro glutamate (pGlu) on the amino terminus of the immunoglobulin single variable domain and/or polypeptide has been detected (by RP-HPLC). Levels of more than 15% pGlu have been detected following fermentation, which were steadily increasing upon storage during stability studies. Such a modification therefore leads to heterogeneity of the final product and needs to be avoided. The control/prevention of pGlu formation is therefore critical to keep therapeutic proteins within their set specifications.

The possibility of pGlu post-translational modification of the N-terminus can be eliminated by changing the N-terminal Glutamic acid (E) [HOOC-(CH₂)₂ -protein] into an Aspartic acid (D) [HOOC-CH₂ -protein]. This will lead to increased product stability. Accordingly, the present invention also relates to amino acid variants wherein the amino acid difference is the change of Glutamic acid at position 1 (said position determined according to Kabat numbering) into an Aspartic acid.

The unique immunoglobulin single variable domains of the invention directed against IgE are selected from SEQ ID NO: 27 and from variants of SEQ ID NO: 27 that have at least one amino acid difference with SEQ ID NO: 27. In a preferred aspect, the variants of the invention have one to five, such as *e.g.* one, two, three, four or five amino acid differences compared to SEQ ID NO: 27.

Accordingly, the one or more immunoglobulin single variable domain(s) of the invention against IgE encompassed in the polypeptides of the invention is selected from:

- a) SEQ ID NO: 27
- b) amino acid variants that have no more than 5, preferably no more than 4, no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 27, provided that:
 - i) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as *e.g.* described in Example 11) compared to SEQ ID NO: 27.

In a preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 27 which is a substitution in the framework 1 region and/or in the CDR1 region, preferably at one or more of positions 1, 6, 29, 31 and 35. In a preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 27 selected from one or more of: Glu1Asp, Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly, such as *e.g.* Glu6Gln (SEQ ID NO: 28), Glu6Gln and Ala35Gly (SEQ ID NO: 28), Glu6Gln and Asn31Ser (SEQ ID NO: 30), Glu6Gln and Phe29Tyr (SEQ ID NO: 31), Glu1Asp (SEQ ID NO: 32); Glu1Asp and Glu6Gln (SEQ ID NO: 33), Glu1Asp, Glu6Gln and Ala35Gly (SEQ ID NO: 34), Glu1Asp, Glu6Gln and Asn31Ser (SEQ ID NO: 35), Glu1Asp, Glu6Gln and Phe29Tyr (SEQ ID NO: 36).

Accordingly, in a preferred aspect, the one or more immunoglobulin single variable domain(s) against IgE encompassed in the polypeptides of the invention comprises or essentially consists of any of SEQ ID NO's: 27-36.

The present invention provides a number of sequence optimized immunoglobulin single variable domains against IgE that show increased stability during fermentation and upon storage. The invention therefore also provides variants as described above, wherein the first amino acid (Glutamic acid) has been changed into Aspartic acid. Accordingly, the unique immunoglobulin single variable domain of the invention against IgE encompassed in the polypeptides of the invention may be selected from the following:

- a) SEQ ID NO: 32;
- b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:
 - i) the amino acid variant has an Aspartic acid (Asp, D) at position 1 (said position determined according to Kabat numbering); and

- ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32.

5 In a preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 32 which is a substitution in the framework 1 and/or in the CDR1 region, preferably at one or more of positions 6, 29, 31 and 35. In another preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 32 selected from one or more of: Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly, such as *e.g.* Glu6Gln (SEQ ID NO: 33), Glu6Gln and Ala35Gly (SEQ
10 ID NO: 34), Glu6Gln and Asn31Ser (SEQ ID NO: 35), Glu6Gln and Phe29Tyr (SEQ ID NO: 36).

Preferably, the one or more immunoglobulin single variable domain(s) against IgE encompassed in the polypeptides of the invention comprises or essentially consists of any of SEQ ID NO's: 32-36.

Immunoglobulin single variable domains as described above have shown advantageous properties for use as prophylactic, therapeutic and/or pharmacologically active agents such as *e.g.*
15 improved binding characteristics and/or potency.

More in particular, these immunoglobulin single variable domains of the invention can bind IgE with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), or alternatively as an IC_{50} value, as further described herein) preferably such that they bind to IgE with a dissociation constant (K_D) of 10 nM to 0.01 nM or less, preferably 1 nM to 0.01 nM or less, more
20 preferably 0.1 nM to 0.01 nM or less, such as 0.05 nM or less or 0.02 nM or less (*e.g.* as measured by KinExA).

The immunoglobulin single variable domains of the invention against IgE are capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RI (the high affinity IgE receptor), for example as measured
25 in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 7; in the Alphascreen assay described in Example 6; in the FACS assay described in Example 10). They are capable of inhibiting the HuIgE/HuFc(epsilon)RI interaction (for example, in the Alphascreen assay described in Example 6) with an IC_{50} value of $5 \cdot 10^{-10}$ M or lower, preferably $2 \cdot 10^{-10}$ M or lower, such as 10^{-10} M or lower, $5 \cdot 10^{-11}$ M or lower, $2 \cdot 10^{-11}$ M or lower, or
30 even 10^{-11} M or lower.

The immunoglobulin single variable domains of the invention against IgE are also capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RII (the low affinity IgE receptor), for example as measured
35 in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 8; in the FACS assay described in Example 10). They may be capable

of inhibiting the H α lgE/HuFc(epsilon)RII interaction (for example, in the ELISA assay described in Example 8) with an IC₅₀ value of 5.10⁻⁸M or lower, preferably 2.10⁻⁸M or lower, such as 10⁻⁸M or lower, 5.10⁻⁹M or lower, 2.10⁻⁹M or lower, 10⁻⁹M or lower, 5.10⁻¹⁰M or lower, or 2.10⁻¹⁰ M or lower.

Also, the immunoglobulin single variable domain of the invention against IgE has an IC₅₀ value in the degranulation assay described in Example 11 which is 100 nM or less, preferably 50 nM or less, more preferably 20nM or less, such as 5nM or less, 1 nM or less, or even 0.5 nM or less.

In a further aspect, the unique immunoglobulin single variable domains of the invention directed against HSA are selected from SEQ ID NO: 39 and from variants of SEQ ID NO: 39 that have at least one amino acid difference with SEQ ID NO: 39. In a preferred aspect, the variants of the invention have one to six, such as *e.g.* one, two, three, four, five or six amino acid differences compared to SEQ ID NO: 39.

Accordingly, the immunoglobulin single variable domain of the invention against HSA encompassed in the polypeptides of the invention is selected from:

a) SEQ ID NO: 39;

b) amino acid variants that have no more than 6, preferably no more than 5, no more than 4, more preferably no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 39, provided that:

i) the amino acid variant binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to SEQ ID NO:

39.

In a preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 39 which is a substitution in one or more of the framework regions, preferably at one or more of positions 1, 14, 30, 87 and 108. In a preferred aspect, the variant of the invention has an amino acid difference with SEQ ID NO: 39 selected from one or more of: Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln, such as *e.g.* Glu1Ala (SEQ ID NO: 40), Glu1Ala and Leu108Gln (SEQ ID NO: 41), Leu108Gln (SEQ ID NO: 42). Preferably, the immunoglobulin single variable domain against HSA encompassed in the polypeptides of the invention comprises or essentially consists of any of SEQ ID NO's: 39-42.

Apart from this and/or in addition, the variant of the invention has an amino acid difference with SEQ ID NO: 39 which is an addition of 1 to 3 amino acid residues at the C-terminal part of the SEQ ID NO: 39. The 1 to 3 amino acid residues may each be independently chosen from naturally occurring amino acid residues and may for example be independently chosen from Ala, Gly, Val, Leu and Ile. In a preferred aspect, the variant of the invention has an addition at the C-terminal end of SEQ ID NO: 39 selected from one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly, such as *e.g.* Ala (SEQ ID NO: 43), Ala-Ala (SEQ ID NO: 44), Ala-Ala-Ala (SEQ ID NO: 45), Gly (SEQ ID NO: 46), Gly-Gly (SEQ ID

NO: 47) and Gly-Gly-Gly (SEQ ID NO: 48). Preferably, the immunoglobulin single variable domain against HSA encompassed in the polypeptides of the invention comprises or essentially consists of any of SEQ ID NO's: 43-48.

The immunoglobulin single variable domains against HSA as described above have shown advantageous properties for use as prophylactic, therapeutic and/or pharmacologically active agents such as *e.g.* improved stability, and improved binding characteristics and/or potency.

These immunoglobulin single variable domains of the invention can bind to HSA with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent)) of 100 nM to 0.1 nM or less, preferably 10 nM to 0.1 nM or less, more preferably 1 nM to 0.1 nM or less (*e.g.* as measured by Surface Plasmon Resonance or by KinExA).

In a storage stability assay (such as *e.g.* described in Example 14), the immunoglobulin single variable domains of the invention against HSA (when used as part of a polypeptide of the invention that further comprises an immunoglobulin single variable domain of the invention against IgE such as *e.g.* IGE045 and optionally a linker such as *e.g.* a 9GS linker), after 1 month storage at 25°C (under the further conditions given in Example 5), show a pre-peak on SE-HPLC which is less than 10%, preferably less than 5%. Furthermore, the immunoglobulin single variable domains of the invention against HSA (when used as part of a construct that further comprises an immunoglobulin single variable domain of the invention against IgE such as *e.g.* IGE045 and a linker such as *e.g.* a 9GS linker), after 1 month storage at 40°C (under the further conditions given in Example 5), show a pre-peak on SE-HPLC which is less than 20%, preferably less than 15%. Reference is for example made to the comparative results in Table 16.

In addition, the immunoglobulin single variable domains of the invention against HSA, when encompassed in the polypeptides of the invention, show improved T_m values and improved expression yields compared to the albumin binding Nanobodies (such *e.g.* as Alb-1 (SEQ ID NO: 37) and Alb-8 (SEQ ID NO: 38)) previously described.

Polypeptides of the invention

The immunoglobulin single variable domains described herein form part of a polypeptide (also referred to as "*polypeptide of the invention*"), which may comprise or essentially consist of one or more immunoglobulin single variable domain(s) against IgE and an immunoglobulin single variable domain against human serum albumin (HSA). Accordingly, the invention relates to a polypeptide (also referred to herein as a "*polypeptide of the invention*") that comprises or essentially consists of one or more immunoglobulin single variable domain(s) against IgE and an immunoglobulin single variable domain against HSA.

"One or more immunoglobulin single variable domain(s) against IgE" means that the number of immunoglobulin single variable domain(s) against IgE present in the polypeptide can be one, but it can also be more than one such as *e.g.* two, three or even more. In a preferred aspect, the number of immunoglobulin single variable domain(s) against IgE in the polypeptide of the invention is one.

5 Accordingly, the present invention relates to a polypeptide that comprises or essentially consists of an immunoglobulin single variable domain of the invention against IgE and an immunoglobulin single variable domain of the invention against HSA.

More specifically, the present invention provides a polypeptide that comprises or essentially consists of one or more immunoglobulin single variable domain(s) against IgE selected from SEQ ID
10 NO: 27 and variants thereof (as described herein) and an immunoglobulin single variable domain against serum albumin selected from SEQ ID NO: 39 and variants thereof (as described herein). In a specific aspect, the present invention provides a polypeptide that comprises or essentially consists of one or more immunoglobulin single variable domain(s) against IgE selected from variants of SEQ ID
15 NO: 27 and an immunoglobulin single variable domain against HSA selected from SEQ ID NO: 39 and variants thereof. In another specific aspect, the present invention provides a polypeptide that comprises or essentially consists of one or more immunoglobulin single variable domain(s) against IgE selected from SEQ ID NO: 27 and variants thereof and an immunoglobulin single variable domain against HSA selected from variants of SEQ ID NO: 39. In another specific aspect, the present invention provides a polypeptide that comprises or essentially consists of one or more immunoglobulin single
20 variable domain(s) against IgE selected from variants of SEQ ID NO: 27 and an immunoglobulin single variable domain against HSA selected from variants of SEQ ID NO: 39.

The process of designing/selecting and/or preparing a polypeptide of the invention, starting from an immunoglobulin single variable domain of the invention, is also referred to herein as "*formatting*" said immunoglobulin single variable domain; and an immunoglobulin single variable
25 domain that is made part of a polypeptide of the invention is said to be "*formatted*" or to be "*in the format of*" said polypeptide of the invention. Examples of ways in which an immunoglobulin single variable domain can be formatted and examples of such formats will be clear to the skilled person based on the disclosure herein. In a preferred aspect, the one or more immunoglobulin single variable domain(s) directed against IgE is located at the N-terminal side of the polypeptide of the
30 invention. In another preferred aspect, the immunoglobulin single variable domain directed against HSA is located at the C-terminal part of the polypeptide. In another preferred aspect, the one or more immunoglobulin single variable domain(s) directed against IgE is located at the N-terminal side of the polypeptide of the invention and the immunoglobulin single variable domain directed against HSA is located at the C-terminal part of the polypeptide.

The polypeptides of the invention can generally be prepared by a method which comprises at least the step of suitably linking the immunoglobulin single variable domains of the invention, optionally via the one or more suitable linkers, so as to provide the polypeptide of the invention.

In one aspect of the invention, the one or more immunoglobulin single variable domain(s) against IgE and the immunoglobulin single variable domain against HSA are directly linked to each other (without the addition of one or more linkers or spacers).

In another aspect of the invention, the one or more immunoglobulin single variable domain(s) against IgE and the immunoglobulin single variable domain against HSA are linked to each other via one or more linkers or spacers. Suitable spacers or linkers for use in the polypeptides of the invention will be clear to the skilled person, and may generally be any linker or spacer used in the art to link amino acids. Preferably, said linker or spacer is suitable for use in constructing proteins or polypeptides that are intended for pharmaceutical use.

Some particularly preferred spacers include the spacers and linkers that are used in the art to link antibody fragments or antibody domains. These include the linkers mentioned in the general background art cited above, as well as for example linkers that are used in the art to construct diabodies or ScFv fragments (in this respect, however, it should be noted that, whereas in diabodies and in ScFv fragments, the linker sequence used should have a length, a degree of flexibility and other properties that allow the pertinent 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 immunoglobulin single variable domain by itself forms a complete antigen-binding site).

For example, a linker may be a suitable amino acid, and in particular amino acids of between 1 and 50, preferably between 1 and 30, such as between 1 and 10 amino acid residues. Some preferred examples of such amino acids 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 (such as described in WO 94/04678).

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 polypeptides of the invention). For example, linkers containing one or more charged amino acid residues can provide improved hydrophilic properties,

whereas linkers that form or contain small epitopes or tags can be used for the purposes of detection, identification and/or purification. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

5 Finally, when two or more linkers are used in the polypeptides of the invention, these linkers may be the same or different. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

10 Some other particularly preferred linkers are poly-alanine (such as Ala-Ala-Ala; SEQ ID NO: 63), as well as other linkers mentioned in Table A-6, of which 9GS (SEQ ID NO: 52) is particularly preferred.

Accordingly, in a preferred aspect, the polypeptide of the invention comprises or essentially consists of one or more variant(s) of SEQ ID NO: 27 (as described herein), a 9GS linker (SEQ ID NO: 52) and SEQ ID NO: 39 or variants thereof (as described herein).

15 As such, the invention also relates to variants of IGE047 (SEQ ID NO: 72) that have no more than 11, no more than 10, preferably no more than 9, no more than 8, no more than 7, no more than 6, more preferably no more than 5, no more than 4, no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 72, provided that:

- 20 i) the polypeptide binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the polypeptide has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to the polypeptide without the 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference; and
- 25 ii) the polypeptide binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to the polypeptide without the 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference.

In a preferred aspect, the polypeptide of the invention comprises or essentially consists of SEQ ID NO: 72, wherein:

- 30 – the first Glutamic acid has been changed into Aspartic acid;
and/or
- in the immunoglobulin single variable domain directed against IgE one or more (such as two, three or four) amino acid residues have been mutated, preferably at positions 6, 29, 31 and/or 35, such as *e.g.* selected from the following: Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering);
- 35 and/or

- in the immunoglobulin single variable domain directed against HSA one or more (such as two, three, four or five) amino acid residues have been mutated, preferably at positions 1, 14, 30, 87 and/or 108, such as *e.g.* selected from the following: Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering);

and/or

- at the C-terminal end of the immunoglobulin single variable domain against HSA one or more (such as two or three) amino acid residues have been added, which may each be independently chosen from naturally occurring amino acid residues and may for example be independently chosen from A, G, V, L and I, such as *e.g.* selected from the following: Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly.

More specifically, the polypeptide of the invention may comprise or essentially consist of SEQ ID NO: 72, wherein:

- the first Glutamic acid has been changed into Aspartic acid;

and/or

- in the immunoglobulin single variable domain directed against IgE following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):
 - Glu6Gln;
 - Glu6Gln and Ala35Gly;
 - Glu6Gln and Asn31Ser; or
 - Glu6Gln and Asn31Pro;

and/or

- in the immunoglobulin single variable domain directed against HSA following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):
 - Glu1Ala;
 - Glu1Ala and Leu108Gln; or
 - Leu108Gln;

and/or

- at the C-terminal end of the immunoglobulin single variable domain against HSA following amino acid residue(s) have been added:
 - Ala;
 - Ala-Ala;
 - Ala-Ala-Ala;
 - Gly;

- Gly-Gly; or
- Gly-Gly-Gly.

In a preferred aspect, the polypeptide of the invention comprises or essentially consists of any of SEQ ID NO's: 73-91.

5 Polypeptides as described above have shown advantageous properties for use as prophylactic, therapeutic and/or pharmacologically active agents such as *e.g.* improved stability, less immunogenicity, and improved binding characteristics and/or potency.

More in particular, these polypeptides of the invention can bind IgE with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), or alternatively as an IC_{50} value, as
10 further described herein) preferably such that they bind to IgE with a dissociation constant (K_D) of 10 nM to 0.01 nM or less, preferably 1 nM to 0.01 nM or less, more preferably 0.1 nM to 0.01 nM or less, such as 0.05 or less or 0.02 or less (*e.g.* as measured by KinExA).

These polypeptides of the invention can bind to HSA with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent)) of 100 nM to 0.1 nM or less, preferably 10 nM to 0.1
15 nM or less, more preferably 1 nM to 0.1 nM or less (said affinity as measured by surface plasmon resonance or KinExA).

The polypeptides of the invention against IgE are capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RI (the high affinity IgE receptor), for example as measured in a suitable assay, such as one
20 of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 7; in the Alphascreen assay described in Example 6; in the FACS assay described in Example 10). They are capable of inhibiting the HuIgE/HuFc(epsilon)RI interaction (for example, in the Alphascreen assay described in Example 6) with an IC_{50} value of $5 \cdot 10^{-10}$ M or lower, preferably $2 \cdot 10^{-10}$ M or lower, such as 10^{-10} M or lower, $5 \cdot 10^{-11}$ M or lower, $2 \cdot 10^{-11}$ M or lower, or even 10^{-11} M or lower.

25 The polypeptides of the invention against IgE are also capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RII (the low affinity IgE receptor), for example as measured in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 8; in the FACS assay described in Example 10). They may be capable of inhibiting the
30 HuIgE/HuFc(epsilon)RII interaction (for example, in the ELISA assay described in Example 8) with an IC_{50} value of $5 \cdot 10^{-8}$ M or lower, preferably $2 \cdot 10^{-8}$ M or lower, such as 10^{-8} M or lower, $5 \cdot 10^{-9}$ M or lower, $2 \cdot 10^{-9}$ M or lower, 10^{-9} M or lower, $5 \cdot 10^{-10}$ M or lower, or $2 \cdot 10^{-10}$ M or lower.

More in particular, these polypeptides of the invention have an IC_{50} value in a degranulation assay (such as *e.g.* described in Example 15) which is 100 nM or less, preferably 50 nM or less, more
35 preferably 20 nM or less, such as 5 nM or less, or even 1 nM or less.

The possibility of pGlu post-translational modification at the N-terminus of the polypeptide of the invention can be eliminated by changing the N-terminal Glutamic acid (E) into an Aspartic acid (D). This will lead to increased product stability. Accordingly, the present invention also relates to polypeptides wherein the Glutamic acid at position 1 (said position determined according to Kabat numbering) is changed into an Aspartic acid. More specifically, the polypeptide of the invention may comprise or essentially consist of SEQ ID NO: 72, wherein the first Glutamic acid has been changed into Aspartic acid, and in which optionally:

- in the immunoglobulin single variable domain directed against IgE one or more (such as two, three or four) amino acid residues have been mutated, preferably at positions 6, 29, 31 and/or 35, such as *e.g.* selected from the following: Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering);
- in the immunoglobulin single variable domain directed against HSA one or more (such as two, three, four or five) amino acid residues have been mutated, preferably at positions 6, 29, 31 and/or 35, such as *e.g.* selected from the following: Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering); and/or
- at the C-terminal end of the immunoglobulin single variable domain against HSA one or more (such as two or three) amino acid residues have been added, which may each be independently chosen from naturally occurring amino acid residues and may for example be independently chosen from A, G, V, L and I, such as *e.g.* selected from the following: one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly.

In a preferred aspect, the polypeptide of the invention comprises or essentially consists of SEQ ID NO: 72, in which:

- in the immunoglobulin single variable domain directed against IgE, following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):
 - Glu1Asp;
 - Glu1Asp and Glu6Gln;
 - Glu1Asp Glu6Gln and Ala35Gly;
 - Glu1Asp Glu6Gln and Asn31Ser; or
 - Glu1Asp Glu6Gln and Phe29Tyr;
 and/or
- in the immunoglobulin single variable domain directed against HSA following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):
 - Glu1Ala;

- Glu1Ala and Leu108Gln; or
- Leu108Gln; and/or

and/or

- at the C-terminal end of the immunoglobulin single variable domain against HSA following amino acid residue(s) have been added:

- Ala;
- Ala-Ala;
- Ala-Ala-Ala;
- Gly;
- Gly-Gly; or
- Gly-Gly-Gly.

In a further aspect, the present invention relates to a polypeptide that is directed against IgE, selected from the following polypeptides:

a) SEQ ID NO's: 82-91;

b) Polypeptides that have no more than 10, preferably no more than 9, no more than 8, no more than 7, no more than 6, more preferably no more than 5, no more than 4, no more than 3, no more than 2, most preferably no more than one amino acid difference with one of SEQ ID NO's: 82-91, provided that:

i) the polypeptide has an Aspartic acid (Asp, D) at position 1; and

ii) the polypeptide binds IgE with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) and/or the polypeptide has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference; and

iii) the polypeptide binds HSA with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference.

In a specific aspect, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be a substitution in the one or more immunoglobulin single variable domain(s) directed against IgE at a position selected from positions 6, 29, 31 and 35, preferably selected from Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering).

Apart from this or in addition, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be a substitution in the immunoglobulin single variable domain directed against HSA at a position selected

from positions 1, 14, 30, 87 and 108, preferably selected from Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering).

Apart from this or in addition, the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference may be an addition of 1 to 3 amino acid residues at the C-terminal end of the immunoglobulin single variable domain against HSA, which may be independently chosen from naturally occurring amino acid residues and may for example be independently chosen from A, G, V, L and I, such as *e.g.* the addition of one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly at the C-terminal end of the immunoglobulin single variable domain against HSA.

In a preferred aspect, the polypeptide of the invention is selected from any of SEQ ID NO's: 82-91.

Polypeptides of the invention can be prepared by a method which generally comprises at least the steps of providing a nucleic acid that encodes a polypeptide of the invention, expressing said nucleic acid in a suitable manner, and recovering the expressed polypeptide of the invention. Such methods can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the methods and techniques further described herein.

The polypeptides provided by the invention are preferably in essentially isolated form (as defined herein), or form part of a protein construct (also referred to as "*protein construct of the invention*"), which may comprise or essentially consist of one or more polypeptides of the invention and which may optionally further comprise one or more further amino acids (all optionally linked via one or more suitable linkers).

Constructs of the invention

The polypeptides provided by the invention are preferably in essentially isolated form (as defined herein), or form part of a protein construct (also referred to as "*protein construct of the invention*"), which may comprise or essentially consist of one or more polypeptides of the invention and which may optionally further comprise one or more further amino acids (all optionally linked via one or more suitable linkers). The polypeptides provided by the invention may also form part of a construct (also referred to as "*construct of the invention*"), which may comprise or essentially consist of one or more polypeptides of the invention and which may optionally further comprise one or more further groups, residues, moieties, binding units or amino acid sequences.

Accordingly, in a further aspect, the invention relates to a construct, and in particular a protein construct that comprises or essentially consists of one or more polypeptides of the invention, and optionally further comprises one or more other groups, residues, moieties, binding units and or amino acids. As will become clear to the skilled person from the further disclosure herein, such further groups, residues, moieties, binding units and/or amino acids may or may not provide further

functionality to the polypeptide of the invention (and/or to the construct in which it is present) and may or may not modify the properties of polypeptide of the invention.

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

Also within the scope of the present invention are constructs that comprise or essentially consist of one or more derivatives as described herein, and optionally further comprise one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers. Preferably, said one or more other groups, residues, moieties or binding units are amino acid sequences.

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

A construct of the invention may comprise a polypeptide 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, i.e. so as to provide a fusion protein comprising said polypeptide of the invention and the one or more further amino acid.

The one or more further amino acid may be any suitable and/or desired amino acid. The further amino acid may or may not change, alter or otherwise influence the (biological) properties of the polypeptide of the invention, and may or may not add further functionality to polypeptide of the invention. Preferably, the further amino acid is such that it confers one or more desired properties or functionalities to the polypeptide of the invention.

Example of such amino acids will be clear to the skilled person, and may generally comprise all amino acids 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 2005 (Nature Biotechnol. 23: 1126-1136).

For example, such an amino acid may be an amino acid that increases 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 construct of the invention, compared to the polypeptide of the invention per se. Some non-limiting examples of such amino acids are haptenic molecules (for example haptens that are recognized by circulating antibodies, see for example WO 98/22141).

The further amino acid may also provide a second binding site, which binding site may be directed against any desired protein, polypeptide, antigen, antigenic determinant or epitope.

According to another embodiment, the one or more further amino acid 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 polypeptide of the invention may be linked to a conventional (preferably human) V_H or V_L domain or to a natural or synthetic analog of a V_H or V_L domain, again optionally via a linker sequence (including but not limited to other (single) domain antibodies, such as the dAb's described by Ward et al. 1989 (Nature 341: 544)).

Accordingly, in the construct of the invention, said one or more other amino acids may be chosen from the group consisting of domain antibodies, amino acid sequences that are suitable for use as a domain antibody, single domain antibodies, amino acid sequences that are suitable for use as a single domain antibody, "dAb's", amino acid sequences that are suitable for use as a dAb, or Nanobodies.

The further amino acids may also form a signal sequence or leader sequence that directs secretion of 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 may also form a sequence or signal that allows the 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 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. Suitable examples of such amino acids will be clear to the skilled person, and for example include, but are not limited to, the "Peptrans" vectors mentioned above, and the amino acids and antibody fragments known per se that can be used to express or produce the polypeptides of the invention as so-called "intrabodies", for example as described in WO 94/02610, WO 95/22618, US 7,004,940, WO 03/014960, WO 99/07414; WO 05/01690; EP 1512696; and in Cattaneo and Biocca 1997 (Intracellular Antibodies: Development and Applications. Landes and Springer-Verlag); and in Kontermann 2004 (Methods 34: 163-170), and the further references described therein.

Constructs as described above should have the same advantageous properties for use as prophylactic, therapeutic and/or pharmacologically active agents as described for the polypeptides of the invention.

Preferably, these constructs of the invention are such that they bind IgE with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), or alternatively as an IC_{50} value, as further described herein) preferably such that they bind to IgE with a dissociation constant (K_D) of 10

nM to 0.01 nM or less, preferably 1 nM to 0.01 nM or less, more preferably 0.1 nM to 0.01 nM or less, such as 0.05 or less or 0.02 or less (said affinity as measured by surface plasmon resonance or KinExA).

Preferably, these constructs of the invention are such that they bind to HSA with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent)) of 100 nM to 0.1 nM or less, preferably 10 nM to 0.1 nM or less, more preferably 1 nM to 0.1 nM or less (said affinity as measured by surface plasmon resonance or surface plasmon resonance or KinExA).

Preferably, these constructs of the invention are such that they are capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RI (the high affinity IgE receptor), for example as measured in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 7; in the Alphascreen assay described in Example 6; in the FACS assay described in Example 10). They are capable of inhibiting the HulgE/HuFc(epsilon)RI interaction (for example, in the Alphascreen assay described in Example 6) with an IC50 value of $5 \cdot 10^{-10}$ M or lower, preferably $2 \cdot 10^{-10}$ M or lower, such as 10^{-10} M or lower, $5 \cdot 10^{-11}$ M or lower, $2 \cdot 10^{-11}$ M or lower, or even 10^{-11} M or lower.

Preferably, these constructs of the invention are such that they are also capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RII (the low affinity IgE receptor), for example as measured in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 8; in the FACS assay described in Example 10). They may be capable of inhibiting the HulgE/HuFc(epsilon)RII interaction (for example, in the ELISA assay described in Example 8) with an IC50 value of $5 \cdot 10^{-8}$ M or lower, preferably $2 \cdot 10^{-8}$ M or lower, such as 10^{-8} M or lower, $5 \cdot 10^{-9}$ M or lower, $2 \cdot 10^{-9}$ M or lower, 10^{-9} M or lower, $5 \cdot 10^{-10}$ M or lower, or $2 \cdot 10^{-10}$ M or lower.

More in particular, these constructs of the invention are such that they have an IC50 value in a degranulation assay (such as *e.g.* described in Example 15) which is 100 nM or less, preferably 50 nM or less, more preferably 20nM or less, such as 5nM or less, or even 1 nM or less.

The invention in its broadest sense also comprises derivatives of the polypeptides and constructs 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 polypeptides or constructs of the invention and/or of one or more of the amino acid residues that form the polypeptides or constructs of the invention.

Examples of such modifications, as well as examples of amino acid residues within the polypeptide or construct sequences 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 polypeptide or construct 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 polypeptide or construct 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 absorption of the polypeptide or construct of the invention, that reduce the immunogenicity and/or the toxicity of the polypeptide or construct of the invention, that eliminate or attenuate any undesirable side effects of the polypeptide or construct of the invention, and/or that confer other advantageous properties to and/or reduce the undesired properties of polypeptide or construct 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 1980 (16th Ed., Mack Publishing Co., Easton, PA). Such functional groups may for example be linked directly (for example covalently) to a polypeptide or construct of the invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

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 polypeptide or construct 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 polypeptide or construct of the invention. 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 ¹⁵²Eu 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 ³H, ¹²⁵I, ³²P, ³⁵S, ¹⁴C, ⁵¹Cr, ³⁶Cl, ⁵⁷Co, ⁵⁸Co, ⁵⁹Fe, and ⁷⁵Se), 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 polypeptides or constructs 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 polypeptide or construct 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 polypeptide or construct 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 polypeptide or construct of the invention 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 polypeptide or construct of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example is the liposomal formulations described by Cao and Suresh 2000 (J. Drug Targeting 8: 257). Such binding pairs may also be used to link a therapeutically active agent to the polypeptide or construct of the invention.

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 1997 (Biotechnol. Appl. Biochem. 26: 143-151).

Preferably the derivatives as described above should have the same advantageous properties for use as prophylactic, therapeutic and/or pharmacologically active agents as described for the polypeptides of the invention.

Preferably, these derivatives are such that they bind IgE with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), or alternatively as an IC_{50} value, as further described herein) preferably such that they bind to IgE with a dissociation constant (K_D) of 10 nM to 0.01 nM or less, preferably 1 nM to 0.01 nM or less, more preferably 0.1 nM to 0.01 nM or less, such as 0.05 or less or 0.02 or less (said affinity as measured by surface plasmon resonance or KinExA).

Preferably, these derivatives are such that they bind to HSA with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent)) of 100 nM to 0.1 nM or less, preferably 10 nM to 0.1 nM or less, more preferably 1 nM to 0.1 nM or less (said affinity as measured by surface plasmon resonance or KinExA).

Preferably, these derivatives are such that they are capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RI (the high affinity IgE receptor), for example as measured in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 7; in the Alphascreen assay described in Example 6; in the FACS assay described in Example 10). They are capable of inhibiting the HuIgE/HuFc(epsilon)RI interaction (for example, in the Alphascreen assay described in Example 6) with an IC_{50} value of $5 \cdot 10^{-10}$ M or lower, preferably $2 \cdot 10^{-10}$ M or lower, such as 10^{-10} M or lower, $5 \cdot 10^{-11}$ M or lower, $2 \cdot 10^{-11}$ M or lower, or even 10^{-11} M or lower.

Preferably, these derivatives are such that they are also capable of modulating, and in particular inhibiting or blocking (fully or partially) the interaction between (human) IgE and the (human) Fc(epsilon)RII (the low affinity IgE receptor), for example as measured in a suitable assay, such as one of the assays used in the Experimental Part below (for example, in the ELISA assay described in Example 8; in the FACS assay described in Example 10). They may be capable of inhibiting the HuIgE/HuFc(epsilon)RII interaction (for example, in the ELISA assay described in Example 8) with an IC_{50} value of $5 \cdot 10^{-8}$ M or lower, preferably $2 \cdot 10^{-8}$ M or lower, such as 10^{-8} M or lower, $5 \cdot 10^{-9}$ M or lower, $2 \cdot 10^{-9}$ M or lower, 10^{-9} M or lower, $5 \cdot 10^{-10}$ M or lower, or $2 \cdot 10^{-10}$ M or lower.

More in particular, these derivatives should have an IC_{50} value in a degranulation assay (such as *e.g.* described in Example 15) which is 100 nM or less, preferably 50 nM or less, more preferably 20 nM or less, such as 5 nM or less, or even 1 nM or less.

Such constructs of the invention and derivatives may also be in essentially isolated form (as defined herein).

Preparation of the polypeptides, constructs and derivatives of the invention

The invention also relates to methods for preparing the polypeptides and constructs described herein. The polypeptides and constructs of the invention can be prepared in a manner known per se, as will be clear to the skilled person from the further description herein. For example, the polypeptides and constructs of the invention can be prepared in any manner known per se for the preparation of antibodies and in particular for the preparation of antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments). Some preferred, but non-limiting methods for preparing the polypeptides and constructs include the methods and techniques described herein.

The method for producing a polypeptide or protein construct of the invention may comprise the following steps:

- 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 polypeptide or protein construct of the invention,

optionally followed by:

- isolating and/or purifying the polypeptide or protein construct 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 polypeptide or protein construct of the invention;

optionally followed by:

- isolating and/or purifying the polypeptide or protein construct of the invention thus obtained.

Accordingly, the present invention also relates to a nucleic acid or nucleotide sequence that encodes a polypeptide or protein construct of the invention (also referred to as "*nucleic acid of the invention*" or "*nucleotide sequence of the invention*"). 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 form, as defined herein. The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form.

The nucleic acids of the invention can be prepared or obtained in a manner known per se, based on the information on the polypeptides or protein constructs of the invention given herein, and/or can be isolated from a suitable natural source. 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 an immunoglobulin single variable domain of the invention and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

Techniques for generating the nucleic acids of the invention will be clear to the skilled person and may for instance include, but are not limited to, automated DNA synthesis; site-directed mutagenesis; combining two or more naturally occurring and/or synthetic sequences (or two or more parts thereof), introduction of mutations that lead to the expression of a truncated expression product; introduction of one or more restriction sites (*e.g.* to create cassettes and/or regions that may easily be digested and/or ligated using suitable restriction enzymes), and/or the introduction of mutations by means of a PCR reaction using one or more "mismatched" primers. 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 herein, as well as the Examples below.

The nucleic acid of the invention may also be in the form of, be present in and/or be part of a genetic construct, as will be clear to the person skilled in the art. Such genetic constructs generally comprise at least one nucleic acid of the invention that is optionally linked to one or more elements of genetic constructs known per se, such as for example one or more suitable regulatory elements (such as a suitable promoter(s), enhancer(s), terminator(s), etc.) and the further elements of genetic constructs referred to herein. Such genetic constructs comprising at least one nucleic acid of the invention will also be referred to herein as "*genetic constructs of the invention*".

The genetic constructs of the invention may be DNA or RNA, and are preferably double-stranded DNA. The genetic constructs of the invention may also be in a form suitable for transformation of the intended host cell or host organism, in a form suitable for integration into the genomic DNA of the intended host cell or in a form suitable for independent replication, maintenance and/or inheritance in the intended host organism. For instance, the genetic constructs of the invention may be in the form of a vector, such as for example a plasmid, cosmid, YAC, a viral vector or transposon. In particular, the vector may be an expression vector, *i.e.* a vector that can provide for expression *in vitro* and/or *in vivo* (*e.g.* in a suitable host cell, host organism and/or expression system).

In a preferred but non-limiting 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; and optionally also
- c) one or more further elements of genetic constructs known per se;

in which the terms "regulatory element", "promoter", "terminator" and "operably connected" have their usual meaning in the art (as further described herein); and in which said "further elements"

present in the genetic constructs may for example be 3'- or 5'-UTR sequences, leader sequences, selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, and may for instance depend upon the type of construct used; the intended host cell or host organism; the manner in which the nucleotide sequences of the invention of interest are to be expressed (*e.g.* via constitutive, transient or inducible expression); and/or the transformation technique to be used. For example, regulatory sequences, promoters and terminators known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

Preferably, in the genetic constructs of the invention, said at least one nucleic acid of the invention and said regulatory elements, and optionally said one or more further elements, are "operably linked" to each other, by which is generally meant that they are in a functional relationship with each other. For instance, a promoter is considered "operably linked" to a coding sequence if said promoter is able to initiate or otherwise control/regulate the transcription and/or the expression of a coding sequence (in which said coding sequence should be understood as being "under the control of" said promoter). Generally, when two nucleotide sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

The nucleic acids of the invention and/or the genetic constructs of the invention may be used to transform a host cell or host organism, i.e. for expression and/or production of the polypeptide or protein construct of the invention. The host is preferably a non-human host. Suitable hosts or host cells will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism, for example:

- a bacterial strain, including but not limited to gram-negative strains such as strains of *Escherichia coli*; of *Proteus*, for example of *Proteus mirabilis*; of *Pseudomonas*, for example of *Pseudomonas 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 adenivorans*; of *Yarrowia*, for example of *Yarrowia lipolytica*;

- an amphibian cell or cell line, such as *Xenopus oocytes*;
- 5 - an insect-derived cell or cell line, such as cells/cell lines derived from lepidoptera, including but not limited to *Spodoptera* SF9 and Sf21 cells or cells/cell lines derived from *Drosophila*, such as Schneider and Kc cells;
- a plant or plant cell, for example in tobacco plants; and/or
- a mammalian cell or cell line, for example a cell or cell line derived from a human, a cell or a cell
- 10 line from mammals including but not limited to CHO-cells, BHK-cells (for example BHK-21 cells) and human cells or cell lines such as HeLa, COS (for example COS-7) and PER.C6 cells;

as well as all other hosts or host cells known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments), which will be clear to the skilled person. Reference is also made to the general background art cited

15 hereinabove, as well as to for example WO 94/29457; WO 96/34103; WO 99/42077; Frenken et al. 1998 (Res. Immunol. 149: 589-99); Riechmann and Muyldermans 1999 (J. Immunol. Met. 231: 25-38); van der Linden 2000 (J. Biotechnol. 80: 261-70); Joosten et al. 2003 (Microb. Cell Fact. 2: 1); Joosten et al. 2005 (Appl. Microbiol. Biotechnol. 66: 384-92); and the further references cited herein.

For expression of the polypeptides or constructs 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 7004940; WO

20 03/014960; in Cattaneo and Biocca 1997 (Intracellular Antibodies: Development and Applications. Landes and Springer-Verlag); and in Kontermann 2004 (Methods 34: 163-170).

According to one preferred, but non-limiting embodiment of the invention, the polypeptide or protein construct of the invention is produced in a bacterial cell, in particular a bacterial cell suitable

25 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 polypeptide or protein construct 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

30 polypeptide or construct 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.

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
5 have been successfully transformed with the nucleotide sequence/genetic construct of the invention may be performed. This may for instance be a selection step based on a selectable marker present in the genetic construct of the invention or a step involving the detection of the polypeptide 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
10 (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), a polypeptide or protein construct of the invention (and in case of a host organism: in at least one cell, part, tissue or organ thereof). The
15 invention also includes further generations, progeny and/or offspring of the host cell or host organism of the invention, for instance obtained by cell division or by sexual or asexual reproduction.

Accordingly, in another aspect, the invention relates to a host or host cell that expresses (or that under suitable circumstances is capable of expressing) a polypeptide or protein construct of the invention; and/or that contains a nucleic acid encoding the same. Some preferred but non-limiting
20 examples of such hosts or host cells can be as generally described in WO 04/041867, WO 04/041865 or WO 09/068627. For example, polypeptides and protein constructs of the invention may with advantage be expressed, produced or manufactured in a yeast strain, such as a strain of *Pichia pastoris*. Reference is also made to WO 04/25591, WO 2010/125187, WO 2011/003622, and WO2012/056000 which also describes the expression/production in *Pichia* and other hosts/host cells
25 of immunoglobulin single variable domains and polypeptides comprising the same.

To produce/obtain expression of the polypeptides or protein constructs 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) polypeptide or protein construct of the invention is expressed/produced. Suitable conditions will be clear to the skilled person and will usually depend
30 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
35 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 polypeptides or protein constructs of the invention may be expressed in a constitutive manner, in a transient manner, or only when suitably induced.

5 It will also be clear to the skilled person that the polypeptide or protein construct 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 polypeptide or protein construct of the invention may be glycosylated, again depending on the host cell/host organism used.

10 The polypeptide or protein construct 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 polypeptide or
15 construct of the invention) and/or preparative immunological techniques (*i.e.* using antibodies against the amino acid sequence to be isolated).

The constructs of the invention can generally be prepared by a method which comprises at least the step of suitably linking polypeptides of the invention to the one or more further groups, residues, moieties or binding units, optionally via the one or more suitable linkers, so as to provide the
20 constructs of the invention. The polypeptides and constructs of the invention can then further be modified, and in particular by chemical and/or biological (*e.g.* enzymatical) modification, of one or more of the amino acid residues that form the polypeptides or constructs of the invention, to obtain derivatives of the polypeptides or constructs of the invention.

25 Products and compositions

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

Generally, for pharmaceutical use, the polypeptides and constructs as described herein may be formulated as a pharmaceutical preparation or compositions comprising at least one polypeptide or
35 construct as described herein and at least one pharmaceutically acceptable carrier, diluent or

excipient and/or adjuvant, and optionally one or more further pharmaceutically active polypeptides and/or compounds. By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (such as by intravenous, intramuscular or subcutaneous injection or intravenous infusion), for topical administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, etc. Such suitable administration forms - which may be solid, semi-solid or liquid, depending on the manner of administration - as well as methods and carriers for use in the preparation thereof, will be clear to the skilled person, and are further described herein.

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

Generally, the polypeptides and constructs as described herein can be formulated and administered in any suitable manner known per se, for which reference is for example made to the general background art cited above (and in particular to WO 04/041862, WO 04/041863, WO 04/041865, WO 04/041867 and WO 08/020079) as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy, 21th Edition, Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

For example, the polypeptides and constructs as described herein 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, those mentioned on page 143 of WO 08/020079. Usually, aqueous solutions or suspensions will be preferred.

Thus, the polypeptides and constructs as described herein 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 polypeptides and constructs 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 polypeptide or construct as described herein. Their percentage in the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of the polypeptide or construct as described herein 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 binders, excipients, disintegrating agents, lubricants and sweetening or flavouring agents, for example those mentioned on pages 143-144 of WO 08/020079. 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 polypeptides or constructs as described herein, 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 polypeptides or constructs as described 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 polypeptides or 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 polypeptides or constructs as described herein may also be administered intravenously or intraperitoneally by infusion or injection, as further described on pages 144 and 145 of WO 08/020079.

For topical administration, the polypeptides and constructs as described herein 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, as further described on page 145 of WO 08/020079.

Generally, the concentration of the polypeptides and constructs as described herein 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 polypeptides and constructs as described herein required for use in treatment will vary not only with the particular polypeptide or construct 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.

5 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.

10 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., 4th Ed., Mack Publishing Co., Easton, PA). The dosage can also be adjusted by the
15 individual physician in the event of any complication.

Uses of the polypeptides and constructs of the invention

The invention also relates to the use of a polypeptide or a construct as described herein, or of a composition comprising the same, in (methods or compositions for) modulating (as generally defined
20 in WO 09/068627) IgE and/or one or more biological actions, mechanisms or responses associated with IgE, either *in vitro* (*e.g.*, in an *in vitro* or cellular assay) or *in vivo* (*e.g.*, in a single cell or in a multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from a disease or disorder associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity)
25 for IgE).

The invention also relates to methods for modulating IgE and/or one or more biological actions, mechanisms or responses associated with IgE, either *in vitro* (*e.g.*, in an *in vitro* or cellular assay) or *in vivo* (*e.g.*, in a single cell or multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from a disease
30 associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE), which method comprises at least the step of contacting IgE with a polypeptide or construct as described herein, or with a composition comprising the same, in a manner and in an amount suitable to modulate IgE (or the intended or desired action(s), mechanism(s) or response(s) associated with IgE).

The invention also relates to the use of a polypeptide or construct as described herein in the preparation of a composition (such as, without limitation, a pharmaceutical composition or preparation as further described herein) for modulating IgE or one or more biological actions, mechanisms or responses associated with IgE, either *in vitro* (e.g., in an *in vitro* or cellular assay) or *in vivo* (e.g., in a single cell or multicellular organism, and in particular in a mammal, and more in particular in a human being, such as in a human being that is at risk of or suffers from a disease or disorder associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE).

The invention also relates to a polypeptide or construct as described herein for use in therapy.

In particular, the invention relates to a polypeptide or construct as described herein for use in the prevention and/or treatment of a disease or disorder that can be prevented and/or treated by administering, to a subject in need thereof, of (a pharmaceutically effective amount of) a polypeptide or construct as described herein (or a suitable composition comprising the same).

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

The subject to be treated may be any warm-blooded animal, but is in particular a mammal, and more in particular a human being. As will be clear to the skilled person, the subject to be treated will in particular be a person suffering from, or at risk of, the diseases and disorders mentioned herein.

The invention furthermore relates to a method for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering a polypeptide or construct as described herein to a patient, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of polypeptide or construct as described herein, and/or of a pharmaceutical composition comprising the same.

The invention also relates to the use of a polypeptide or construct as described herein in the preparation of a pharmaceutical composition for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering a polypeptide or construct of the invention to a patient.

More in particular, the invention relates to a polypeptide or construct as described herein for use in prevention and/or treatment of a disease or disorder associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE, with its biological or pharmacological activity, and/or with the biological pathways or signalling in which IgE is involved, or more generally any disease or disorder mediated by IgE (also referred to herein as "*IgE mediated disease and disorders*"). Such "*IgE mediated diseases and disorders*" will be clear to the skilled person and may for example include, without limitation: conditions such as asthma, allergic rhinitis, hay fever, conjunctivitis, eczema, urticaria, food allergies and other allergies, including serious and/or life-threatening allergic reactions such as those to insect bites or stings, snake bites etc., as well as to allergic reaction to medication; and more generally any disease or disorder associated with anaphylactic hypersensitivity and/or (atopic) allergy.

For example, a monoclonal against IgE called omalizumab (Xolair®) is currently marketed by Genentech for IgE mediated disorders. It is envisaged that the polypeptides and constructs of the invention, as well as compositions comprising the same, can be used in the prevention or treatment of the same diseases and disorders that omalizumab has been approved for prior to the priority date of the present invention and/or will be approved for after the priority date of the present application. From the further description and data presented herein, it will also be clear to the skilled person that the polypeptides and constructs of the invention, as well as compositions comprising the same may have substantial advantages over omalizumab (see for example the Experimental Part, in which omalizumab (obtained from a commercial source) and a Fab obtained through papain digest of commercial omalizumab were used as reference compounds).

The invention relates to a method for the prevention and/or treatment of at least one disease or disorder that is associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE, with its biological or pharmacological activity, and/or with the biological pathways or signalling in which IgE is involved, or more generally any IgE mediated disease or disorder, and in particular for the prevention and treatment of one or more of such diseases and disorders listed herein, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of polypeptide or construct as described herein, and/or of a pharmaceutical composition comprising the same.

In another aspect, the invention relates to the use of a polypeptide or construct as described herein in the preparation of a pharmaceutical composition for prevention and/or treatment of at least one disease or disorder that is associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE, with its biological or pharmacological activity, and/or with the biological pathways or signalling in which IgE is involved, or more generally any IgE mediated disease or disorder, and in particular for the prevention and

treatment of one or more of such diseases and disorders listed herein; and/or for use in one or more of the methods of treatment mentioned herein.

In particular, the invention 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 modulating IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of polypeptide or construct as described herein, and/or of a pharmaceutical composition comprising the same. In particular, said pharmaceutically effective amount may be an amount that is sufficient to modulate IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved; and/or an amount that provides a level polypeptide or construct as described herein that is sufficient to modulate IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved.

In another aspect, the invention relates to a method for immunotherapy, and in particular for passive immunotherapy, which method comprises administering, to a subject suffering from or at risk of the diseases and disorders mentioned herein, a pharmaceutically active amount of polypeptide or construct described herein, and/or of a pharmaceutical composition comprising the same.

In the above methods, the polypeptides or constructs as described herein 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 polypeptides or constructs as described herein 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 polypeptides or constructs as described herein 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 polypeptide or construct to be used, the specific route of administration and pharmaceutical formulation or composition to be used, the age, gender, weight, IgE level in serum or plasma, diet, general condition of the patient, and similar factors well known to the clinician.

Generally, the treatment regimen will comprise the administration of one or more polypeptides or constructs as described herein, 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 administer can be determined by the clinician, again based on the factors cited above.

5 Depending on the specific disease or disorder to be treated, the potency of the specific polypeptide or construct to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the clinician will generally be able to determine a suitable daily dose. Generally, some guidance on the amounts to be administered can be obtained from the amounts usually administered for comparable conventional antibodies or antibody
10 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 methods, a single polypeptide or construct as described herein is used. It is however within the scope of the invention to use two or more polypeptides or constructs as
15 described herein.

The polypeptides or constructs as described herein 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
20 the factors cited above and his expert judgment.

In particular, the polypeptides or constructs as described herein 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
25 as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician.

When two or more substances or principles are to be used as part of a combined treatment regimen, they can be administered via the same route of administration or via different routes of administration, at essentially the same time or at different times (*e.g.*, essentially simultaneously, consecutively, or according to an alternating regime). When the substances or principles are to be
30 administered simultaneously via the same route of administration, they may be administered as different pharmaceutical formulations or compositions or part of a combined pharmaceutical formulation or composition, as will be clear to the skilled person.

Also, when two or more active substances or principles are to be used as part of a combined
35 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.

Further uses of the polypeptides, constructs, genetic constructs, hosts and host cells of the invention will be clear to the skilled person based on the disclosure herein. For example, and without limitation, the polypeptides of the invention can be linked to a suitable carrier or solid support so as to provide a medium that can be used in a manner known per se to purify IgE from compositions and preparations comprising the same. Derivatives of the polypeptides of the invention that comprise a suitable detectable label can also be used as markers to determine (qualitatively or quantitatively) the presence of IgE in a composition or preparation or as a marker to selectively detect the presence of IgE on the surface of a cell or tissue (for example, in combination with suitable cell sorting techniques).

Definitions

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. 1989 (Molecular Cloning: A Laboratory Manual (2nd Ed.) Vols. 1-3, Cold Spring Harbor Laboratory Press), Ausubel et al. 1987 (Current protocols in molecular biology, Green Publishing and Wiley Interscience, New York), Lewin 1985 (Genes II, John Wiley & Sons, New York, N.Y.), Old et al. 1981 (Principles of Gene Manipulation: An Introduction to Genetic Engineering (2nd Ed.) University

of California Press, Berkeley, CA); Roitt et al. 2001 (Immunology (6th Ed.) Mosby/Elsevier, Edinburgh), Roitt et al. 2001 (Roitt's Essential Immunology (10th Ed.) Blackwell Publishing, UK), and Janeway et al. 2005 (Immunobiology (6th Ed.) Garland Science Publishing/Churchill Livingstone, New York), as well as to the general background art cited herein.

Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known per se, as will be clear to the skilled person. Reference is for example again made to the standard handbooks and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews Presta 2006 (Adv. Drug Deliv. Rev. 58: 640-56), Levin and Weiss 2006 (Mol. Biosyst. 2: 49-57), Irving et al. 2001 (J. Immunol. Methods 248: 31-45), Schmitz et al. 2000 (Placenta 21 Suppl. A: S106-12), Gonzales et al. 2005 (Tumour Biol. 26: 31-43), which describe techniques for protein engineering, such as affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

The term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "variable domain sequence", "V_{HH} sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acids or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code. Reference is made to Table A-2 on page 48 of WO 08/020079.

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

When a nucleotide sequence or amino acid sequence is said to "comprise" another nucleotide sequence or amino acid sequence, respectively, or to "essentially consist of" another nucleotide sequence or amino acid sequence, this may mean that the latter nucleotide sequence or amino acid sequence has been incorporated into the first mentioned nucleotide sequence or amino acid sequence, respectively, but more usually this generally means that the first mentioned nucleotide

sequence or amino acid sequence comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the first mentioned sequence has actually been generated or obtained. By means of a non-limiting example, when a polypeptide of the

5 invention is said to comprise an immunoglobulin single variable domain, this may mean that said immunoglobulin single variable domain sequence has been incorporated into the sequence of the polypeptide of the invention, but more usually this generally means that the polypeptide of the invention contains within its sequence the sequence of the immunoglobulin single variable domains irrespective of how said polypeptide of the invention has been generated or obtained. Also, when a
10 nucleic acid or nucleotide sequence is said to comprise another nucleotide sequence, the first mentioned nucleic acid or nucleotide sequence is preferably such that, when it is expressed into an expression product (e.g. a polypeptide), the amino acid sequence encoded by the latter nucleotide sequence forms part of said expression product (in other words, that the latter nucleotide sequence is in the same reading frame as the first mentioned, larger nucleic acid or nucleotide sequence).

15 By "essentially consist of" is meant that the combination of unique immunoglobulin single variable domains described in the present invention either is exactly the same as the polypeptide of the invention or corresponds to the polypeptide of the invention which has a limited number of amino acid residues, such as 1-20 amino acid residues, for example 1-10 amino acid residues and preferably 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, added at the amino
20 terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of the combination of specific immunoglobulin single variable domains.

Said amino acid residues may or may not change, alter or otherwise influence the (biological) properties of the polypeptide of the invention and may or may not add further functionality to the polypeptide of the invention. For example, such amino acid residues:

- 25 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 polypeptide 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-
30 terminus of the polypeptide, although the invention in its broadest sense is not limited thereto;
- c) may form a sequence or signal that allows the polypeptide 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 polypeptide 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-
35 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. 2001 (Expert Opin. Biol. Ther. 1: 773); Temsamani and Vidal 2004 (Drug Discov. Today 9: 1012) and Rousselle 2001 (J. Pharmacol. Exp. Ther. 296: 124-131), and the membrane translocator sequence described by Zhao et al. 2003 (Apoptosis 8: 631-637).

5 C-terminal and N-terminal amino acid sequences for intracellular targeting of antibody fragments are for example described by Cardinale et al. 2004 (Methods 34: 171). Other suitable techniques for intracellular targeting involve the expression and/or use of so-called "intrabodies" comprising a polypeptide of the invention;

d) may form a "tag", for example an amino acid sequence or residue that allows or facilitates the
10 purification of the polypeptide, 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 polypeptide (for this purpose, the tag may optionally be linked to the polypeptide sequence via a cleavable linker sequence or contain a cleavable motif). Some preferred, but non-limiting examples of such residues are multiple histidine residues,
15 glutathione residues and a myc-tag such as AAAEQKLISEEDLNGAA (SEQ ID NO: 92);

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 polypeptides of the invention.

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

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

The term "specificity" has the meaning given to it in paragraph n) on pages 53-56 of WO 08/020079; and as mentioned therein refers to the number of different types of antigens or antigenic
35 determinants to which a particular antigen-binding molecule or antigen-binding protein (such as an

immunoglobulin single variable domain and/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, as described on pages 53-56 of WO 08/020079 (incorporated herein by reference), which also describes some preferred techniques for measuring binding between an antigen-binding molecule (such as an immunoglobulin single variable domain and/or polypeptide of the invention) and the pertinent antigen. Typically, antigen-binding proteins (such as the immunoglobulin single variable domains and/or polypeptides of the invention) will bind to their antigen with a dissociation constant (K_D) of 10^5 to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles). Any K_D value greater than 10^4 mol/liter (or any K_A value lower than 10^4 M⁻¹) liters/mol is generally considered to indicate non-specific binding. Specific binding of an antigen-binding protein to an antigen or antigenic determinant can be determined in any suitable manner known per se, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein. As will be clear to the skilled person, and as described on pages 53-56 of WO 08/020079, the dissociation constant may be the actual or apparent dissociation constant. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned on pages 53-56 of WO 08/020079.

The affinity of a molecular interaction between two molecules can be measured via different techniques known per se, such as the well the known surface plasmon resonance (SPR) biosensor technique (see for example Ober et al. 2001, Intern. Immunology, 13: 1551-1559) where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding k_{on} , k_{off} measurements and hence K_D (or K_A) values. This can for example be performed using the well-known BIACORE instruments. Another technique to measure the affinity is KinExA, e.g. as described by Blake et al. 1999 (Analytical Biochemistry 272: 123-134), Darling et al. 2004 (Assay and Drug Development Technology 2 (6): 647-657) or Rathanaswami et al. 2005 (Biochemical and Biophysical Research Communications 334: 1004-1013); and/or as described in the Experimental Part.

An immunoglobulin single variable domain and/or polypeptide is said to be "specific for" a first target or antigen compared to a second target or antigen when it binds to the first antigen with an affinity (as described above, and suitably expressed as a K_D value, K_A value, K_{off} rate and/or K_{on} rate) that is at least 10 times, such as at least 100 times, and preferably at least 1000 times, and up to 10000 times or more better than the affinity with which immunoglobulin single variable domain

and/or polypeptide binds to the second target or antigen. For example, the immunoglobulin single variable domain and/or polypeptide may bind to the first target or antigen with a K_D value that is at least 10 times less, such as at least 100 times less, and preferably at least 1000 times less, such as 10.000 times less or even less than that, than the K_D with which said immunoglobulin single variable domain and/or polypeptide binds to the second target or antigen. Preferably, when an immunoglobulin single variable domain and/or polypeptide is "specific for" a first target or antigen compared to a second target or antigen, it is directed against (as defined herein) said first target or antigen, but not directed against said second target or antigen.

A polypeptide is said to be "cross-reactive" for two different antigens or antigenic determinants (such as *e.g.* serum albumin from two different species of mammal, such as *e.g.* human serum albumin and cyno serum albumin; such as *e.g.* IgE from two different species of mammal, such as *e.g.* human serum albumin and cyno serum albumin) if it is specific for (as defined herein) both these different antigens or antigenic determinants.

The term "potency" of a polypeptide of the invention, as used herein, is a function of the amount of polypeptide of the invention required for its specific effect to occur. It is measured simply as the inverse of the IC_{50} for that polypeptide. It refers to the capacity of said polypeptide of the invention to modulating IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved. The potency may be measured by any suitable assay known in the art or described herein, such as *e.g.* an *in vitro* degranulation assays (*e.g.* as described by Gibbs et al. 2006, J. Allergy Clin. Immunol. 118-5: 1060-1067); and/or the degranulation assay as described in the Example section (see *e.g.* Examples 11, 13B, 13E and 15B) and/or *in vivo* assays (*e.g.* the cynomolgus model described in WO 2012/175740 or as described by Foc et al. 1996, J. Pharmacol. And Exp. Therapeutics 279: 10001008) or the *in vivo* model as described in the Example section (see *e.g.* Example 17)).

In contrast, the "efficacy" of the polypeptide of the invention measures the maximum strength of the effect itself, at saturating polypeptide concentrations. Efficacy indicates the maximum response achievable from the polypeptide of the invention. It refers to the ability of a polypeptide to produce the desired (therapeutic) effect.

The "half-life" of a polypeptide of the invention can generally be defined as described in paragraph o) on page 57 of WO 08/020079 and as mentioned therein refers to the time taken for the serum concentration of the polypeptide to be reduced by 50%, *in vivo*, for example due to degradation of the polypeptide and/or clearance or sequestration of the polypeptide by natural mechanisms. The *in vivo* half-life of a polypeptide of the invention can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may for example generally be as described in paragraph o) on page 57 of WO

08/020079. As also mentioned in paragraph o) on page 57 of WO 08/020079, the half-life can be expressed using parameters such as the $t_{1/2}$ -alpha, $t_{1/2}$ -beta and the area under the curve (AUC). Reference is for example made to the standard handbooks, such as Kenneth et al. 1986 (Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists, John Wiley & Sons Inc) and M Gibaldi and D Perron 1982 ("Pharmacokinetics", Marcel Dekker, 2nd Rev. Ed.). The terms "increase in half-life" or "increased half-life" are also as defined in paragraph o) on page 57 of WO 08/020079 and in particular refer to an increase in the $t_{1/2}$ -beta, either with or without an increase in the $t_{1/2}$ -alpha and/or the AUC or both.

10 The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove.

15 The invention will now be further described by means of the following non-limiting examples and figures:

EXAMPLES**Preparation of cyno IgE-Fc and Fc(epsilon)RI α -Fc**

A cyno IgE-Fc fragment was produced and used as a tool to identify IgE-specific Nanobodies which were human and cyno IgE cross-reactive. RNA was extracted from peripheral blood mononuclear cells from 2 male and 2 female cynomolgus monkeys. The cyno IgE sequence (SEQ ID NO: 2) was amplified by RT-PCR using different primers (SEQ ID NO's: 3-6). The obtained fragments were cloned in a cloning vector and sequenced. The cyno IgE c(epsilon)2-c(epsilon)3-c(epsilon)4 fragment was cloned in an expression vector derived from pCIneo which contained the human cytomegalovirus (CMV) immediate-early enhancer/promoter region, the SV40 late polyadenylation signal, a resistance gene for ampicillin or carbenicillin, a multicloning site and the murine Ig kappa light chain leader sequence. In frame with the cynomolgus IgE-Fc coding sequence, the vector coded for a C-terminal (His)10 tag. The cis-acting viral DNA element, oriP locus allowed for episomal replication in HEK-EBNA cells, expressing the Epstein-Barr virus nuclear antigen-1. Culture supernatant was harvested and cyno IgE Fc was purified using cation exchange (Source 30S) followed by affinity purification (His TrapTMFF) and desalting (Desalting 26/10 HiPrep, all columns from GE Healthcare).

An Fc-fusion of the human high affinity IgE receptor Fc(epsilon)RI α (gene bank P12319) was produced as a tool. The human Fc ϵ RI α was synthesized and cloned in an expression vector as a fusion with a human IgG Fc fragment. Stable transfected CHO cells (ATCC) were generated and culture supernatant was harvested. The human Fc(epsilon)RI(alpha)Fc fragment was then purified using protein A (Mabselect sure; GE Healthcare).

Example 1: Immunizations

Four llamas (No. 002, No. 004, No. 193, No. 197) were 4-6 times immunized with 50-100 microgram doses of human IgE (Scripps Laboratories, San Diego, CA, Cat# I0224) (llamas 002 and 004) or 50-100 microgram human IgE kappa chain (Diatec, Oslo, Norway). The intervals between the immunizations varied between 1-5 weeks. Proteins were formulated in Stimmune adjuvant (Cedi Diagnostics, Lelystad, The Netherlands) for animals 002 and 004, or Complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (Difco, Becton Dickinson, Franklin Lakes, NJ) for animals 193 and 197.

Sera from blood samples of llamas 002, 004, 193 and 197 were obtained prior to immunization, during the immunization protocol and after completion of the immunizations. Human IgE was coated onto Nunc Maxisorb plates at 2 microgram/ml, blocked with 1% casein in PBS and incubated with serial dilutions of pre- and post-immune llama sera. Plate-immobilized llama IgG was detected using

HRP conjugated goat-anti-llama IgG (Bethyl Labs, Montgomery, TX) and TMB chromogen according to standard methods. Comparison of optical density values clearly indicated immunization induced a humoral immune response against IgE in all four animals.

Peripheral blood mononuclear cells were prepared from the blood samples using Ficoll-Hypaque according to the manufacturer's instructions. Total RNA extracted from these cells and from lymph nodes was used as starting material for RT-PCR to amplify Nanobody encoding gene fragments. These fragments were cloned into phagemid vector derived of pUC119, containing the LacZ promoter, a coliphage pIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, and a multicloning site harbours several restriction sites. In frame with the Nanobody coding sequence, the vector codes for a C-terminal c-myc tag and a (His)6 tag. The signal peptide was the gen3 leader sequence which translocates the expressed Nanobody to the periplasm. Phage was prepared according to standard protocols (Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press; 1st edition (October 28, 1996)) and stored after filter sterilization at 4°C until further use. In total, 4 phage libraries were constructed (002, 004, 193 and 197), with library sizes between 3.5×10^6 and 28×10^6 , and a percentage of insert ranging from 96 to 100%.

Example 2: Selections of IgE binding Nanobodies

Phage display was used to enrich IgE-specific Nanobodies. Phages were prepared according to standard methods from libraries obtained from llamas No. 002, 004, 193 and 197. Phage libraries were used for two rounds (R1/R2) of selection on plate-immobilized cyno IgE Fc. Cyno IgE c(epsilon)2-c(epsilon)3-c(epsilon)4-Fc was immobilized at concentrations varying from 0.2 to 2 microgram/ml on Nunc Maxisorp ELISA plates. Plate-immobilized phages were retrieved using trypsin elution or Fc(epsilon)RIα elution. Outputs of R1/R2 selections were analyzed for enrichment factor (# phage present in eluate relative to control). Based on these parameters, the best selections were chosen for further analysis. Individual colonies were picked and grown in 96 deep well plates (1 ml volume) and induced by adding IPTG for Nanobody expression. Periplasmic extracts were prepared according to standard methods. Nanobodies were expressed as fusion proteins containing C-terminal both the c-myc as well as the 6His tags.

Example 3: Screening for IgE blocking Nanobodies in AlphaScreen assay

In order to screen for human/cyno cross-reactive IgE blocking Nanobodies, periplasmic extracts were analyzed for their ability to block the interaction of human IgE (Diatec, Oslo, Norway) or cyno IgE c(epsilon)2-c(epsilon)3-c(epsilon)4 with human Fc(epsilon)RI(alpha)-Fc. To this end, two alphascreen assays (Amplified Luminiscent Proximity Homogeneous Assay; Perkin Elmer, Waltman, MA) were set up. In brief, 0.05 nM biotinylated hIgE or 0.1 nM biotinylated cyno IgE c(epsilon)2-

c(epsilon)3-c(epsilon)4 was captured by streptavidin Donor beads and 0.05 nM Fc(epsilon)RI(alpha)-Fc was captured by anti-hFc-Acceptor beads.

Diluted periplasmic extracts of individual Nanobody clones were analysed. The omalizumab Fab fragment, known to inhibit the human IgE/Fc(epsilon)RI(alpha)Fc interaction, was used as a positive control. Assays were read in an Envision alphascreen option fitted multimode reader (Perkin Elmer, Waltman, MA). Individual clones were scored as putative HuIgE/HuFc(epsilon)RI(alpha)Fc interaction or cyno IgE c(epsilon)2-c(epsilon)3-c(epsilon)4/HuFc(epsilon)RI(alpha)Fc interaction inhibiting if the presence of the periplasmic extract decreased the fluorescent signal of the acceptor beads.

All four libraries contained both non-blocking and blocking clones when tested on human IgE, but only library 197 retrieved cyno cross-reactive neutralizing Nanobodies. Cyno cross-reactive inhibiting Nanobodies were selected and sequenced. Sequence analysis revealed 1 Family (named family 12) which was subdivided into two subfamilies 12.1 (comprising clones 39D11, 39D8, 42D7 and 42G5; SEQ ID NO's: 7, 8, 9, 10, respectively) and 12.2 (clone 36G5; SEQ ID NO: 11) and a unique clone (39B02; SEQ ID NO: 12). The corresponding DNA sequences are given in SEQ ID NO's: 13-18. This low diversity indicates that it is very difficult to generate and identify cyno-cross reactive Nanobodies which block the interaction of IgE with Fc(epsilon)RI(alpha).

Example 4: Off-rate determination of IgE binding Nanobodies

Off-rate constants (k_{off}) of individual inhibitory Nanobody clone periplasmic extracts were determined by surface plasmon resonance on a Biacore T100 instrument. Human IgE (Diatec, Oslo, Norway) was amine-coupled to a CM5 sensor chip at a density of 3100 RU. Remaining reactive groups were inactivated using ethanolamine. Nanobody binding and off-rate was assessed at a single dilution of periplasmic extract. The omalizumab Fab fragment (referred to herein as "Reference Fab"; obtained by papain digestion of commercially obtained omalizumab) was tested at a single concentration of 100nM. Each sample was injected 2 minutes at a flow rate of 45 µl/min to allow binding to chip-bound antigen. Next, binding buffer without periplasmic extracts was sent over the chip at the same flow rate to allow spontaneous dissociation of bound Nanobody. Analyte remaining bound after the monitored dissociation phase was removed by injecting regeneration solution (4.5M MgCl₂). Data of the best representatives per subfamily and the unique clone 39B2 (all selected for purification and more detailed analysis) are shown in Table 1.

Table 1: Off rate values of Nanobodies (periplasmic extracts).

Clone	Off rate Hu IgE (s^{-1})	Off rate cyno IgE (s^{-1})
39D8	2.24E-03	1.22E-03
42D7	2.25E-03	1.09E-03
39D11	2.39E-03	1.32E-03
36G5	1.11E-02	3.28E-03
42G5	2.83E-02	5.76E-03
39B2	7.96E-02	5.69E-04

Example 5: IgE-binding Nanobody expression and purification

Selected Nanobodies were expressed in the periplasmic space of *E.coli* as c-myc, His6-tagged proteins in a culture volume of 250 mL. Expression was induced in high density cultures by addition of 1 mM IPTG and allowed to continue for 4h at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts were used as starting material for affinity chromatography using HisTrap crude columns (GE Healthcare). Nanobodies were eluted from the column with 250 mM imidazole and subsequently desalted towards dPBS.

Example 6: Inhibition of HulGE or cyno IgE Fc (ce2-ce3-ce4) /HuFc(epsilon)RI(alpha)Fc interaction in Alphascreen

Serial dilutions of purified Nanobodies were then analyzed for their ability to block the interaction of HulGE or cyno IgE Fc(ce2-ce3-ce4) with HuFc(epsilon)RI(alpha)Fc using the same alphascreen assays as described above. Data are shown in Tables 2 and 3 and indicate all Nanobodies except 39B2 have a similar potency compared to the Reference Fab (obtained by papain digestion of commercially obtained omalizumab).

Table 2: IC50 values for competition between the human IgE receptor Fc(epsilon)RI(alpha) and anti-IgE Nanobodies to bind human IgE, their 95% confidence intervals (CI95) and percentage inhibition at 250 nM Nanobody as determined by Alphascreen.

Clone	IC50 (M)	CI95	% inhibition at 250 nM Nanobody
39D8	4.191e-010	3.985e-010 to 4.408e-010	97.1
39D11	4.412e-010	4.087e-010 to 4.764e-010	97.1
42D7	5.977e-010	5.190e-010 to 6.885e-010	97.2
Human IgE	3.008e-010	2.311e-010 to 3.915e-010	98.6
Reference Fab	9.223e-010	8.634e-010 to 9.853e-010	99.0
36G5	1.423e-009	1.133e-009 to 1.789e-009	94.7
42G5	4.346e-009	3.192e-009 to 5.917e-009	96.0
39B2	No bottom level reached at 250 nM Nb	NA	51.8

NA: not applicable

5

Table 3: IC50 values for competition between the human IgE receptor Fc(epsilon)RI(alpha) and anti-IgE Nanobodies to bind cyno IgE Fc (Ce2-ce3-ce4), their 95% confidence intervals (CI95) and percentage inhibition at 250 nM Nanobody as determined by Alphascreen.

Clone	IC50 (M)	CI95	% inhibition at 250 nM Nanobody
36G5	5,835e-010	4.648e-010 to 7.325e-010	88.1
39B2	No bottom level reached at 250 nM Nb	NA	38.0
42G5	9,595e-010	8.047e-010 to 1.144e-009	91.2
cyno-IgE	9,197e-010	7.528e-010 to 1.123e-009	99.5
Reference Fab	1,385e-009	1.190e-009 to 1.611e-009	99.3
39D8	7,420e-011	6.598e-011 to 8.345e-011	93.6
39D11	1,194e-010	9.836e-011 to 1.450e-010	93.5
42D7	1,265e-010	1.013e-010 to 1.579e-010	92.7
cyno-IgE	2,822e-010	2.133e-010 to 3.733e-010	96.5
Reference Fab	1,303e-009	8.692e-010 to 1.954e-009	97.7

NA: not applicable

10

Example 7: Inhibition of HulGE or cyno IgE/ HuFc(epsilon)RI interaction in ELISA

Nanobodies were analyzed for their ability to block the interaction of HulGE (Diatec, Oslo, Norway) or cyno IgE (plasma) with Fc(epsilon)RI. To this end, ELISA plates were coated with Fc(epsilon)RI (0.1 microgram/ml), then blocked. Serial Nanobody dilutions were pre-incubated with 50 pM HulGE or 1/50 cyno plasma dilution, followed by addition of the pre-incubated mixture to the Fc(epsilon)RI coated ELISA plates. Binding of HulGE or cyno IgE to the immobilized Fc(epsilon)RI was detected by using goat anti-human IgE-HRP (KPL, Gaithersburg, MD). Presence of putative IgE/ Fc(epsilon)RI interaction inhibitors would result in decreased OD signals. The results are shown in Tables 4 and 5.

20

Table 4: IC50 values for competition between Fc(epsilon)RI(alpha) and anti-IgE Nanobodies to bind human IgE Fc (Ce2-ce3-ce4), their 95% confidence intervals and percentage inhibition at 500 nM Nanobody as determined by ELISA.

Clone	HulGE		
	IC50 (M)	CI95	% inhibition by 500 nM Nanobody
39D8	5.504e-010	4.312e-010 to 7.025e-010	99.4
42D7	9.588e-010	8.439e-010 to 1.089e-009	99.6
39D11	5.107e-010	4.302e-010 to 6.063e-010	99.5
36G5	2.962e-009	2.534e-009 to 3.463e-009	98.6
39B2	3.942e-008	2.694e-008 to 5.768e-008	69.3
Reference Fab	1.168e-009	1.005e-009 to 1.358e-009	99.1
Omalizumab	2.568e-010	1.588e-010 to 4.154e-010	97.7
42G5	8.377e-009	6.729e-009 to 1.043e-008	98.1

5 Table 5: IC50 values for competition between the human IgE receptor Fc(epsilon)RI(alpha) and anti-IgE Nanobodies to bind cyno IgE Fc (Ce2-ce3-ce4), their 95% confidence intervals and percentage inhibition at 500 nM Nanobody as determined by ELISA.

Clone	CynolGE		
	IC50 (M)	CI95	% inhibition by 500 nM Nanobody
39D8	1.800e-009	1.445e-009 to 2.242e-009	99.7
39D11	5.199e-009	3.541e-009 to 7.633e-009	99.8
42D7	4.564e-009	4.046e-009 to 5.149e-009	92.0
42G5	7.536e-009	4.591e-009 to 1.237e-008	92.5
Omalizumab	5.085e-009	4.097e-009 to 6.311e-009	90.5
Reference Fab	3.782e-008	2.900e-008 to 4.932e-008	87.4
36G5	3.005e-008	2.564e-008 to 3.521e-008	80.3
39B2	No competition	NA	0

NA: not applicable

10 **Example 8: Inhibition of HulGE/HuFc(epsilon)RII interaction in ELISA**

Nanobodies were analyzed for their ability to block the interaction of HulGE (Diatec, Oslo, Norway) with recombinant Fc(epsilon)RII (R&D Systems, Minneapolis, MN). To this end, ELISA plates were coated with Fc(epsilon)RII, then blocked. Serial Nanobody dilutions were pre-incubated with 20 nM HulGE, followed by addition of the pre-incubated mixture to the Fc(epsilon)RII coated ELISA plates. Binding of HulGE to the immobilized Fc(epsilon)RII was detected by using goat anti-human IgE-HRP (KPL, Gaithersburg, MD). Presence of putative HulGE/ Fc(epsilon)RII interaction inhibitors would result in decreased OD signals. The data are shown in Table 6 and indicate that all three tested Nanobodies block IgE binding to Fc(epsilon)RII. Nanobodies 39D11 and 36G5 had a similar potency and efficacy (100% block) as compared to Reference Fab, whereas Nanobody 39B2 was 10-fold less potent and only reached around 80% block at 500 nM Nanobody concentration.

Table 6: IC50 values for competition between the human IgE receptor Fc(epsilon)RII and anti-IgE Nanobodies to bind human IgE, their 95% confidence intervals as determined by ELISA.

Clone	IC50 (M)	CI95
39D11	1.291e-008	6.846e-009 to 2.436e-008
36G5	1.996e-008	1.115e-008 to 3.571e-008
39B2	1.527e-007	6.977e-008 to 3.344e-007
Reference Fab	1.547e-008	9.281e-009 to 2.580e-008

Example 9: Inhibition of HulgE/omalizumab interaction in Alphascreen

- 5 Nanobodies were analyzed for their ability to block the interaction of HulgE (Diatec, Oslo, Norway) with omalizumab. To this end, an alphascreen assay (Perkin Elmer, Waltham, MA) was set up. In brief, serial dilution series of individual Nanobodies were incubated with 0.1nM biotinylated hulgE, 0.1nM omalizumab, streptavidin coated donor beads and anti-human Fc Nanobody coupled acceptor beads. The Reference Fab fragment, known to inhibit the HulgE/omalizumab interaction,
- 10 was used as a positive control. Assays were read in an Envision alphascreen option fitted multimode reader (Perkin Elmer, Waltham, MA). Individual clones were scored as putative HulgE/omalizumab interaction inhibitors if their presence decreased the fluorescent signal of the acceptor beads. The results are shown in Table 7 and indicate that all tested Nanobodies except 39B2 compete with omalizumab for IgE binding. The bottom plateau levels of the Nanobody inhibition curves are
- 15 significantly higher (% inhibition maximum 79.9%) in comparison to that of Reference Fab or IgE (% inhibition maximum 96.9 and 99.6, respectively), suggesting that the omalizumab en Nanobody epitopes are different.

Table 7: IC50 values for competition between omalizumab and anti-IgE Nanobodies to bind human IgE, their 95% confidence intervals and percentage inhibition at 250 nM Nanobody as determined by Alphascreen.

Clone	IC50 (M)	CI95	% inhibition by 250 nM Nanobody
36G5	6,363e-009	3.854e-009 to 1.051e-008	62.9
39B2	NA	NA	9.3
42G5	2.486e-008	1.520e-008 to 4.065e-008	61.5
39D8	1.482e-009	1.300e-009 to 1.691e-009	79.9
39D11	1.511e-009	1.342e-009 to 1.702e-009	79.6
42D7	1.723e-009	1.511e-009 to 1.964e-009	79.1
IgE	5.189e-010	4.388e-010 to 6.137e-010	96.9
Reference Fab	2.686e-009	2.548e-009 to 2.832e-009	99.6

NA: not applicable

Example 10: Inhibition of HulgE/HuFc(epsilon)RI and HuFc(epsilon)RII interaction in FACS

- 25 Nanobodies 39D11 and 36G5 were tested in FACS competition assay for their ability to inhibit IgE-binding to Fc(epsilon)RI on RBL Fc(epsilon)RI(alpha) stable transfectants and Fc(epsilon)RII on Raji

cells. To this end serial dilutions of Nanobody were added to the cells followed by addition of human IgE (7.5 nM for RBL Fc(epsilon)RI assay; 10 nM for Raji cell assay). Presence of putative interaction inhibitors would result in decreased MCF signals. The data in Table 8 indicate that both Nanobodies block IgE binding to Fc(epsilon)RI and Fc(epsilon)RII comparable to the positive control (Reference Fab). All three tested molecules reached more than 90% inhibition at 1 μ M concentrations.

Table 8: IC50 values for competition between anti-IgE Nanobodies and Fc(epsilon)RI or Fc(epsilon)RII (expressed on transfected RBL and endogenously expressed on Raji cells, respectively) to bind human IgE and their 95% confidence intervals as determined by FACS.

Clone	RBL- Fc(epsilon)RI(alpha)		Raji- Fc(epsilon)RII(alpha)	
	IC50 (M)	CI95	IC50 (M)	CI95
Reference Fab	8.386e-009	7.753e-009 to 9.071e-009	8.767e-009	6.131e-009 to 12.54e-009
39D11	4.952e-009	4.475e-009 to 5.479e-009	10.19e-009	3.660e-009 to 28.35e-009
36G05	9.543e-009	8.418e-009 to 10.82e-009	35.83e-009	13.27e-009 to 96.76e-009

Example 11: Inhibition in degranulation assay

Assay setup was based on a protocol supplied with xCELLigence RTCA instrument (Roche). The System measures electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. The real time impedance measurement provides quantitative information about the morphological status of the cells, including degranulation, without incorporation of labels. RBL cells transfected with Fc(epsilon)RIalpha (10000 cells/well) were overnight incubated in E-plates and sensitized with Chimaeric human anti-NP IgE (50ng/ml; Serotec; Catalog MCA333S) for 1 hr. Then, degranulation was triggered by adding NIP-BSA (50 ng/ml; Biosearch Technologies). The activation/degranulation of the basophils can be detected in real time on the xCELLigence System measuring impedance (measure every minute during 8 hrs). The maximum cell index signal per well was used to calculate EC50 and IC50 values. A correlation was shown with histamine release in supernatant (LDN histamine Research ELISA).

Nanobodies 39D08, 39D11, 36G5 and 39B2 were tested in degranulation assay for their ability to inhibit IgE-mediated degranulation. To this end, serial dilutions of Nanobody or Reference Fab were pre-incubated with human IgE and then added to the cells. Results are shown in Table 9. Nanobody 39B2 only reached 25% block of degranulation at a Nanobody concentration of 500 nM, whereas the other 3 Nanobodies and Reference Fab blocked more than 95% at this concentration. The potency of the Nanobodies was 3- to 40-fold weaker in comparison with the Reference Fab control.

Table 9: IC50 values for blocking IgE-mediated degranulation of RBL-Fc(epsilon)RI(alpha) transfected cells by anti-IgE Nanobodies or Reference Fab, and their 95% confidence intervals as determined by impedance measurement. Crosslinking was done using the allergen.

Clone	IC50 (nM)	CI95 (nM)
Omalizumab	2.858	1.662 to 4.917
39D08	18.52	14.00 to 24.50
39D11	18.72	12.38 to 28.32
36G5	112.2	43.61 to 288.4
Reference Fab	5.768	3.900 to 8.530

5 **Example 12: Affinity determination**

Affinity constants of individual Nanobodies were determined by surface plasmon resonance (SPR) on a Biacore 3000 instrument. In brief, Human IgE (Diatec, Oslo, Norway) or cyno IgE Fc (Ce2-ce3-ce4) was amine-coupled to a CM5 sensor chip. Remaining reactive groups were inactivated using ethanolamine. Nanobodies were injected at different concentrations. Each sample was injected at a flow rate of 45 µl/min to allow for binding to chip-bound antigen, followed by binding buffer without Nanobody at the same flow rate to allow for spontaneous dissociation. Analyte remaining bound after the monitored dissociation phase was removed by injecting regeneration solution (4.5M MgCl₂). Binding curves at different concentrations were used to calculate the kinetic parameters k_{on} -values (k_a), k_{off} -values (k_d) and K_D . Kinetic parameters were determined using heterogeneous ligand fit since a two phase interaction was observed. The obtained results are presented in Table 10.

Table 10: Kinetic parameters for binding of anti-IgE Nanobodies to human IgE and cyno IgE Fc.

Clone	IgE	Type	KD (nM)	k_a (1/Ms)	k_d (1/s)
39D11	Human IgE	KD1 (52%)	15.3	5.81^E5	8.9^E-3
		KD2 (48%)	0.7	5.58^E6	3.9^E-3
	Cyno IgE Fc	KD1 (46%)	26.1	5.34^E5	1.4^E-2
		KD2 (54%)	0.58	7.02^E6	4.1^E-3
36G5	Human IgE	KD1 (28%)	96	6.00^E5	5.7^E-2
		KD2 (72%)	10	4.76^E6	4.9^E-2
	Cyno IgE Fc	KD1 (46%)	54	1.69^E6	9.2^E-2
		KD2 (54%)	6	1.13^E7	6.7^E-2

Example 13: Sequence optimization and affinity maturation of 39D11

20 A. **Sequence optimization: sequence analysis**

The IgE-specific Nanobody 39D11 was the most potent cyno cross-reactive Nanobody identified during screening. It was selected and further pursued for sequence optimization and affinity maturation.

Based on alignment of 39D11 sequence with VH3-23/JH5 human germline, it was decided to generate 4 variants: IGE009, IGE010, IGE011 and IGE012 (SEQ ID NO's: 19-22, respectively). All variants include the three mutations V5L, K83R and Q108L. In variants IGE011 and IGE012, a unique

mutation W91Y is included. In all four variants, the methionine at position 77 is substituted by threonine (IGE009 and IGE011) or leucine (IGE010 and IGE012). Sequence alignment of sequence optimized variants with 39D11 is shown in Figure 1.

5 B. Characterization of four 39D11 humanization variants in potency assays

The four variants were first tested in the degranulation assay to evaluate the effect of introduced mutations on the ability to inhibit IgE-mediated degranulation. Table 11 shows obtained calculated IC50 values. These results indicated that the introduced mutation W91Y is detrimental for potency and results in a higher off rate, excluding variants IGE011 and IGE012. Variant IGE009 was
10 selected for affinity maturation as it has the highest percentage framework identity with human germline sequences.

Table 11: Potency analysis of 39D11 sequence optimized variants measured in a competition Alphascreen, off rate determination and a degranulation assay.

Clone	Alphascreen		Off rate (s ⁻¹)	Degranulation	
	IC50 (nM)	CI95		IC50 (nM)	CI95
39D11	0.417	0.491 to 0.698	k _d 1: 1.6 ^E -2 (73.3%) k _d 2: 1.1 ^E -2 (26.7%)	4.250	3.022 to 5.978
IGE009	0.343	0.211 to 0.557	k _d 1: 1.2 ^E -2 (77.2%) k _d 2: 1.2 ^E -2 (22.8%)	5.289	3.275 to 8.542
IGE010	0.351	0.229 to 0.536	k _d 1: 8.6 ^E -3 (79.6%) k _d 2: 1.4 ^E -2 (20.4%)	5.844	4.628 to 7.379
IGE011	1.336	0.879 to 2.029	k _d : 9.81 ^E -2	103.7	70.93 to 151.7
IGE012	1.236	0.772 to 1.977	k _d : 1.23 ^E -1	78.24	44.46 to 137.7

15 C. Affinity maturation of IGE009 by error prone PCR methodology

An error-prone library was generated by amplifying IGE009 under error-prone conditions and cloning the fragments into a phage display vector. The obtained library was subjected to multiple rounds of in solution panning using gradually decreasing concentrations (10-0.001 nM) of
20 biotinylated human IgE. Outputs from the phage selections were analyzed for off rate on ProteOn. Up to 6-fold improved off-rates relative to 39D11 were measured for the best clones. Sequence analysis identified mutations (both in the frameworks and the CDR's) with positive effect on off-rate. These were: E6Q; F29Y; G30D; S31N or S31P; S35G or S35A; G44R; N75K; D97E and Y100eF.

These mutations were included in a combinatorial library. The obtained library was again
25 subjected to in solution panning using 1 nM biotinylated human IgE. Outputs from the phage selections were analyzed for off rate on ProteOn and sequences were analysed. Sequence analysis and off rate analysis were combined and as a result, 7 variants (IGE025-IGE030, SEQ ID NO's: 26-31) containing different combinations of beneficial mutations were designed. These variants were

expressed in *Pichia* as fusions (SEQ ID NO's: 66-71) with the HSA-specific Nanobody Alb-8 to improve the *in vivo* half-life of the construct. As control, similar Nb-9GS-Alb-8 formats were made containing the parental IgE-specific Nanobody 39D11 or its humanized variant IGE009 (constructs IGE020 and IGE019 respectively, SEQ ID NO's: 65 and 64). Alignment of the affinity matured variants with 39D11 en IGE009 is shown in Figure 1.

D. Off rate determination on BIAcore of 10 affinity matured bispecific variants

Off rates were analysed on BIAcore. Binding profiles and off rates are shown in Table 12. Off rate analysis on BIAcore shows decreased off rates for affinity matured variants compared to IGE009 (up to 25-fold on human IgE and up to 15-fold on cyno IgE-Fc). The binding patterns are still biphasic but the percentage of the fast off rate is significantly reduced after affinity maturation (certainly on human IgE).

Table 12: Kinetic parameters for binding of affinity matured formatted anti-IgE Nanobodies to human IgE and cyno IgE-Fc.

Construct	Off rate (1/S)	
	Human IgE	Cyno IgE-Fc
IGE009	k_d 1: 1.32×10^{-2} (58%)	k_d 1: 1.54×10^{-2} (58.5%)
	k_d 2: 2.53×10^{-3}	k_d 2: 2.01×10^{-3}
IGE019 (IGE009/ALB8)	k_d 1: 1.34×10^{-2} (51.6%)	k_d 1: 1.52×10^{-2} (51.9%)
	k_d 2: 3.00×10^{-3}	k_d 2: 2.21×10^{-3}
IGE025/ALB8	k_d 1: 1.05×10^{-2} (20.4%)	k_d 1: 1.82×10^{-2} (27.9%)
	k_d 2: 1.02×10^{-3}	k_d 2: 9.74×10^{-4}
IGE026/ALB8	k_d 1: 9.78×10^{-3} (3.3%)	k_d 1: 8.9×10^{-3} (10.1%)
	k_d 2: 1.18×10^{-4}	k_d 2: 1.24×10^{-4}
IGE027/ALB8	k_d 1: 7.44×10^{-3} (4.8%)	k_d 1: 8.46×10^{-3} (13.2%)
	k_d 2: 1.14×10^{-4}	k_d 2: 1.82×10^{-4}
IGE028/ALB8	k_d 1: 9.65×10^{-3} (3.7%)	k_d 1: 6.55×10^{-3} (12.0%)
	k_d 2: 1.31×10^{-4}	k_d 2: 1.38×10^{-4}
IGE029/ALB8	k_d 1: 1.06×10^{-2} (7.8%)	k_d 1: 9.66×10^{-3} (23.5%)
	k_d 2: 3.13×10^{-4}	k_d 2: 4.8×10^{-4}
IGE030/ALB8	k_d 1: 1.31×10^{-2} (3.8%)	k_d 1: 8.57×10^{-3} (15.3%)
	k_d 2: 1.57×10^{-4}	k_d 2: 2.26×10^{-4}

E. In vitro potency analysis in degranulation assay of affinity matured bispecific variants

Affinity matured variants were tested in degranulation assay in the presence or absence of purified human serum albumin (Sigma, A8763). Calculated IC50s and CI95 are shown in Table 13. The obtained results clearly indicate that a number of variants outperform Omalizumab in degranulation assay. Binding of HSA does not influence the potency of the Nanobody constructs.

Table 13: IC50 values for blocking IgE-mediated degranulation of RBL-Fc(epsilon)RI(alpha) transfected cells by anti-IgE Nanobodies or control Mab/Fab, and their 95% confidence intervals as determined by impedance measurement.

Construct	Without HSA		With 25 µM HSA	
	IC50 (nM)	CI95		
Omalizumab	0.8179	0.4745 to 1.410	1,151	0.6501 to 2.037
Reference Fab	4.618	3.799 to 5.612	3.688	2.103 to 6.466
IGE009	8.883	6.610 to 11.94	9.803	6.520 to 14.74
IGE019 (IGE009/ALB8)	10.23	6.293 to 16.63	9.139	6.909 to 12.09
IGE025/ALB8	1.795	1.462 to 2.202	2.361	1.169 to 4.769
IGE026/ALB8	0.2480	0.1854 to 0.3316	0.3102	0.2727 to 0.3528
IGE027/ALB8	0.2473	0.2079 to 0.2941	0.1922	0.1409 to 0.2622
IGE028/ALB8	0.3814	0.3226 to 0.4510	0.5225	0.3684 to 0.7413
IGE029/ALB8	0.2646	0.2376 to 0.2947	0.2702	0.2058 to 0.3547
IGE030/ALB8	0.2161	0.1691 to 0.2761	0.2050	0.1865 to 0.2255

5 F. Affinity determination of IGE026 and IGE027 using KinExA approach

The affinity of Nanobodies IGE026, IGE027 and 39D11 was analyzed by in solution affinity determination on an automated KinExA 3200 instrument (Sapidyne Instruments Inc.). All experiments were performed with PBS supplemented with 0.02% sodium azide, 0.05% Tween 20 and 10mM Imidazole. Human IgE-c(epsilon)2-c(epsilon)3-c(epsilon)4 was prediluted to 60 µg/mL (100 µg/mL for 10 39D11) in PBS + 0.02% sodium azide and incubated with 200 µg polymethylmethacrylate (PMMA) beads (Sapidyne, 440198) at 15 rpm, overnight at 4°C. IgE coated beads were blocked with 1mL assay buffer + 1% casein while rotating at 15 rpm for 1 hour at RT. The beads were packed into the flow cell by drawing 675µL of bead suspension through the cell at a rate of 1.5mL/min. For equilibrium affinity determination, a constant Nanobody concentration of 10 pM (700pM for 39D11) was incubated for 15 45 hours (3 hours for the 39D11 assay) with titrating concentrations of human IgE-c(epsilon)2-c(epsilon)3-c(epsilon)4 of 20nM to 8.59 fM (100nM to 6.10pM in the 39D11 assay) in sample buffer (PBS + 0.02% Sodium Azide + 0.1% casein + 0.05% Tween 20 + 10mM Imidazole). The mixtures (9mL) (2.5 mL for the 39D11 assay) were flown over the human IgE-c(epsilon)2-c(epsilon)3-c(epsilon)4 coupled beads at a rate of 0.25mL/min. After washing, 1 µg/mL APC labeled anti-Flag antibody (M2- 20 Surelight-APC, PerkinElmer AD0059F), diluted in sample buffer was flown over the bead pack to bind captured Nanobody. Samples were run in duplicate. The theoretical binding curves were fit to the experimental signals with the 'Affinity, Standard' analysis method in the KinExA Pro Software v2.0.1.14, rendering an affinity K_D value.

25 The obtained affinity of the Nanobodies IGE026 and IGE027 was 30 to 50-fold improved compared to that of 39D11 (Table 14).

Table 14: KD values for IgE binding.

Construct	KD (pM)	CI95 (pM)
39D11	624	350-788
IGE026	19	13-25
IGE027	12	5-6

G. Binding affinity to serum albumin

Binding of formatted Nanobodies to serum albumin was analysed by SPR (Biacore 3000) in conditions with low density albumin immobilization (500 RU). A titration series of Nanobodies (2.5 to 200 nM) was injected at a flow rate 45 µl/min. Affinity constants were determined and are listed in Table 15. The HSA-specific Nanobody Alb-8 was included for comparison. In general, a lower affinity was observed.

Table 15: KD values of formatted Nanobodies to serum albumin.

	Human serum albumin			Cyno serum albumin		
	ka (1/Ms)	kd (1/s)	KD (nM)	ka (1/Ms)	kd (1/s)	KD (nM)
Alb-8	3.15E+05	1.93E-03	6.12	3.28E+05	1.89E-03	5.76
IGE025/ALB8	9.15E+04	5.94E-03	64.9	1.11E+05	5.87E-03	53
IGE026/ALB8	1.12E+05	5.86E-03	52.3	1.03E+05	5.78E-03	56.3
IGE027/ALB8	1.09E+05	5.42E-03	49.6	9.99E+04	5.37E-03	53.8
IGE030/ALB8	1.14E+05	5.31E-03	46.4	1.04E+05	5.27E-03	50.6

Example 14: Storage stability of constructs comprising Alb-23 compared to Alb-8.

Initial data on the stability of the IGE026/ALB8 construct (SEQ ID NO: 67) showed that the storage stability of this compounds leaves room for improvement. A construct was made which genetically fuses the IgE binding building block IGE045 (SEQ ID NO: 27) via a 9GS linker to another HSA building block Alb-23 (SEQ ID NO: 39).

Both bispecific Nanobody constructs comprising the same Nanobody against IgE (IGE045, SEQ ID NO: 27) linked to Alb-23 (IGE045-9GS-Alb-23/IGE047; SEQ ID NO: 72) or linked to Alb-8 (IGE045-9GS-Alb-8; SEQ ID NO: 67) were prepared, formulated in D-PBS buffer at a concentration of 50 mg/ml and stored in plastic PCR tubes in the dark for 1 month at different temperatures. After that, the amount of pre-peak (corresponding to dimer formation) was determined and compared using SE-HPLC. The SE-HPLC analysis was performed using a BioSep SEC-2000 column (Phenomenex) and D-PBS as running buffer at a flow rate of 0.2 ml/min. 10µg material was injected and data was analysed using Chromeleon software.

The results are shown in Table 16.

Table 16: Comparative storage stability of Alb-23 and Alb-8 constructs (% Pre peak on SE-HPLC after storage for the indicated storage period at the indicated temperatures).

Construct	Albumin binder	Storage period	Temperature (°C)		
			5	25	40
IGE047 (SEQ ID NO: 72)	Alb-23	1 month	0.0	3.7	7.6
IGE045-9GS-Alb-8 (SEQ ID NO: 67)	Alb-8	1 month	0.0	11.7	30.3

Example 15: Sequence optimization of IGE047

5 A. Sequence optimization

The construct with Alb-23, prepared in Example 14 (SEQ ID NO: 72) was recloned into an expression vector as such that the first amino acid (E) of the genetic fusion protein was replaced by an aspartic acid residue (D). In addition, this second cloning procedure added an Alanine residue (A) at the C-terminus of the fusion protein resulting in IGE122 (SEQ ID NO: 86).

10 For production of IGE122 (SEQ ID NO: 86), a *Pichia pastoris* expression system was developed, based on the commercially available system from Invitrogen using X-33 as a host strain, the AOX1 promoter controlling the expression and the alpha mating factor secretion peptide for secretion of the Nanobody into the medium. The Nanobody was cloned into a derivative of the pPicalpha expression vector where specific restriction sites had been removed or introduced. Transformed
15 *Pichia pastoris* clones were selected on zeocin containing plates and a qPCR was performed to rank the clones according to their copy numbers. Expression levels were compared between clones in shake flask experiments and the best expression clone was selected as final host.

B. In vitro potency analysis in degranulation assay

20 Using the degranulation assay (Example 11), we compared the potency of IGE122 (SEQ ID NO: 86) to Omalizumab. A serial dilution of both compounds was pre-incubated for 30min with Chimaeric human anti-NP IgE (135 ng/mL; Serotec). Afterwards the pre-incubated Nanobody-IgE mixtures were incubated for 4h on overnight incubated RBL cells transfected with Fc(epsilon)RIalpha (17600 cells/well). Degranulation of the cells was stimulated by adding NIP-BSA (22 ng/mL; Biosearch
25 Technologies) and the degranulation of the basophils was monitored in real time on the xCELLigence System measuring the impedance of each well every minute during 8h.

As shown in Table 17 and Figure 3, IGE122 was ± 3 -fold more potent than Omalizumab for inhibiting IgE-mediated degranulation of basophils *in vitro*.

Table 17: IC50 values and respective 95% confidence intervals(CI95) for blocking IgE-mediated degranulation of RBL-Fc(epsilon)RI(alpha) transfected cells by IGE122 (SEQ ID NO: 86), as determined in the degranulation assay. Crosslinking was done using the allergen.

Clone	IC50 (nM)	CI95 (nM)
IGE122	0.632	0.562 – 0.711
Omalizumab	1.777	1.463 – 2.158

5 C. In vitro potency analysis for HSA binding

Binding of the Alb-23 moiety of IGE122 to human serum albumin (HSA) was tested in the HSA binding potency assay (Figure 4). In brief, a serial dilution of the Nanobody ranging from 1000 nM to 1.25 pM was captured on a HSA coated plate (Sigma A7223, 3 µg/mL) and detected sequentially by a Flag/His tagged anti-Nanobody Nanobody (Nb; in-house produced; 0.1 µg/mL) and a monoclonal anti-Flag antibody (horseradish peroxidase labelled; Sigma A8592, 0.1 µg/mL). The bound and detected IGE122 was visualised using TMB as the colorimetric substrate. The calculated EC50 value and the 95% CI are shown in Table 18.

Table 18: EC50 value and CI95 of IGE122 in the HSA binding potency assay

Construct	EC50 (nM)	CI95 (nM)
IGE122	1.608	1.232 to 2.100

15 **Example 16: Species cross-reactivity for serum albumin and IgE**

A. Species cross-reactivity of IGE047 for binding serum albumin in ELISA

Species cross reactivity of the albumin binding moiety Alb-23 for serum albumin of human, cynomolgus monkey, mouse, rat, guinea pig, dog, pig and rabbit was assessed in a binding ELISA using the Alb-23-containing polypeptide IGE047 (SEQ ID NO: 72). In brief, titration series of IGE047 were incubated on microtiter plates coated with the serum albumin of the above mentioned species. Next, bound IGE047 was detected with the biotinylated polyclonal rabbit antibody R345 directed against Nanobodies (in-house produced antibody, 2µg/ml), followed by a secondary detection with HRP-labeled streptavidin (Thermo Scientific, 0.25 µg/ml).

25 A dose-dependent binding was observed for serum albumin from human and cynomolgus monkey, and to a lesser extent for serum albumin from mouse and guinea pig. No binding of IGE047 was observed for rat, dog, pig and rabbit serum albumin (Figure 5).

B. Species cross-reactivity testing of IGE122 for binding IgE in competition ELISA

30 Cross-reactivity of IGE122 for IgE was assessed for cynomolgus monkey, mouse and guinea pig IgE in a competition ELISA. In brief, titration series of IGE123 (=Flag-tagged IGE122) were preincubated with either recombinant full-length human IgE (Abcam, 20nM), recombinant human IgE

Fc-fragment (in-house produced, 20nM), recombinant cynomolgus monkey IgE Fc-fragment (in-house produced, 20nM), recombinant full-length mouse IgE (ICL, 20nM) or recombinant guinea pig IgE Fc-fragment (in-house produced, 20nM). Next, these solutions were incubated on microtiter plates coated with recombinant full-length human IgE (Abcam, 0.625µg/ml), after which captured IgE123 was detected with a HRP-labeled monoclonal anti-Flag antibody (Sigma, A8592).

A dose-dependent inhibition of IGE122 binding to coated human IgE was observed for human and cynomolgus monkey IgE, while no inhibition was observed for mouse IgE and guinea pig IgE Fc fragment, indicating that IGE122 is cross-reactive with human and cynomolgus monkey IgE but not with mouse and guinea pig IgE (Figures 6A and 6B).

C. Species cross-reactivity of IGE122 for binding IgE in a functional assay

The competition binding ELISA indicated that IGE122 was only cross-reactive with human and cynomolgus monkey IgE. These observations were confirmed in the functional degranulation assay already described in Example 15, section B. In short, a titration series of IGE122 was preincubated with either human or cynomolgus monkey IgE Fc fragment for 30 min at room temperature and subsequently incubated on tissue culture E-plates seeded with RBL2H3 basophil cells stably expressing either human or cynomolgus monkey Fc(epsilon)RIalpha (17600 cells/well) for 4 hours at 37°C. Next, degranulation was induced via cross-linking of IgE-Fc(epsilon)RIalpha complexes at the surface of the basophils using a polyclonal antibody against human IgE (AbDSerotec, STAR147, 10µg/ml) and monitored using the xCELLigence system for 8 hours.

Results show no significant difference between the IC50 values for IGE122 inhibiting the binding of respectively human or cynomolgus monkey IgE Fc fragment to the Fc(epsilon)RI expressed at the surface of basophils (Table 19 and Figure 7).

Table 19. Average IC50 ± stdev (nM) of IGE122 in a degranulation assay using transfected RBL2H3 cells stably expressing human Fc(epsilon)RIalpha (n = 4) or cynomolgus monkey Fc(epsilon)RIalpha. A polyclonal antibody against IgE was used as a cross-linking tool.

	Human	cynomolgus monkey
IGE122	7.362 ± 2.016 (n = 4)	5.261 ± 2.001 (n = 3)

Example 17: PK/PD study in cynomolgus monkey

IGE122 was evaluated *in vivo* in a PK/PD study in cynomolgus monkey. Cynomolgus monkeys were screened for moderate to high IgE plasma levels. Following a single administration of vehicle, IGE122 or Omalizumab in these cynomolgus monkeys, PK, PD and immunogenicity markers were measured at different time points.

In this study, 6 groups of cynomolgus monkeys received a single administration of either vehicle, IGE122 or Omalizumab (according to Table 20). A single administration of vehicle, different amounts of IGE122 (0.03, 0.1 and 0.3 mg/kg) or Omalizumab (0.03 and 0.3 mg/kg) were administered either subcutaneously (s.c.) or intravenously (i.v.) respectively as indicated in Table 20.

5

Table 20: Animal treatment groups for the single dose PK/PD study in cynomolgus monkey

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Drug	Vehicle	IGE122			Omalizumab	
Number of animals	3	6	6	6	6	6
Amount (mg/kg)	NA	0.03	0.1	0.3	0.03	0.1
Route of administration	s.c.	s.c.	s.c.	s.c.	i.v.	i.v.

NA: not applicable; s.c.: subcutaneous injection; i.v.: intravenous injection.

After the single administration of vehicle, IGE122 or Omalizumab, the animals were monitored daily. Blood samples were collected from the femoral vein at the following different pre-determined time points: Predose, 30min, 60min, 120min, 1d, 3d, 5d, 7d, 9d, 13d, 17d, 22d, 29d, 36d, 50d and 70days after administration, of which 7 samples (Predose, 17d, 22d, 29d, 36d, 50d and 70 days after administration) were used for PK (free IGE122 and total IGE122 or total Omalizumab), PD (free IgE and total IgE) and ADA assessment. The remaining 9 samples were used for PK and PD assessment.

Free IgE cynomolgus monkey plasma levels were quantified using a validated free IgE biomarker ELISA-based assay. The principle of this assay is to capture free IgE (not bound to IGE122 or Omalizumab) via its high affinity receptor (FcεRI). In brief, neutravidin was first coated on a 96 well Maxisorp plate (Nunc) by adsorption, after which excess binding sites were blocked with PBS-1% Bovine Serum Albumin (BSA). After blocking, biotinylated recombinant cynomolgus monkey FcεRI-Fc was immobilized on the neutravidin coated plates. After incubation, detection was performed with a Horse Radish Peroxidase (HRP)-labeled affinity purified goat anti-human IgE antibody. In the presence of H₂O₂, the peroxidase catalyzes a chemical reaction with the soluble Tetramethylbenzidine (sTMB) resulting in a colorimetric change. After stopping the colorimetric reaction with 1M HCl, the optical density is measured at a wavelength of 450nm in a plate spectrophotometer.

The analysis results showed an immediate drop in free IgE after treatment administration.

The modeling analysis based on pharmacokinetics (PK, total IGE122 and total Omalizumab) and pharmacodynamics (PD, total IgE) markers allow the determination of the *in vivo* K_D of both IGE122 and Omalizumab. Modeling analysis will be performed based on the published (Mager and Krzyzanski 2005, Pharm. Res. 22: 1589-1596; Gibiansky and Gibiansky 2009, Expert Opinion Drug Metab. Toxicol.

5: 1-10) Quasi Equilibrium (QE) approximation to the published (Mager and Jusko 2001, J. Pharmacokinet. Pharmacodyn. 28: 507-532; Levy 1994, Clin. Pharmacol. Ther. 56: 248-252) Target Mediated Drug Disposition model (TMDD).

5 **Example 18: Binding of Nanobody to complexes of human Fc(epsilon)RI and IgE**

Binding measurements were performed with a BIAcore T100 using a CM5 sensorchip with running buffer HBS-EP+ at 25°C. To analyse the binding of Nanobody to complexed IgE, Fc-tagged Fc(epsilon)RI (Fc(epsilon)RIA:Fc; produced in CHO k1 cells and purified via MabSelect affinity chromatography (GE healthcare)) was immobilized using standard amine coupling according to the manufacturer's procedure. Human IgE (135µl human IgE (DIA HE1, batch 2929) at a concentration of 500 nM) was then captured via the immobilized receptor. Subsequently, a negative control (i.e. not binding IgE) Nanobody (1 µM) was injected followed by the injection of 1 µM IGE047 (SEQ ID NO: 72). Both were injected for 2 minutes at 45µl/min.

As shown in Figure 9, during the injection of the negative control Nanobody, no additional binding was seen and the dissociation rate of human IgE did not change. For IGE047 an increase in signal could be detected the first seconds upon injection. However, this increase in signal was immediately followed by a fast decrease in signal. As the signal reflects changes in mass, the decrease in signal during the association phase of the Nanobody could thus be interpreted as an increased dissociation of (Nanobody complexed) IgE from the receptor. Interaction of the IGE047 Nanobody with human IgE bound to Fc(epsilon)RI thus results in a faster dissociation of IgE from the IgE receptor surface. The simultaneous binding of Nanobody and Fc(epsilon) receptor IA to human IgE resulted in a faster dissociation of IgE from the receptor.

25 **Example 19: Displacement of Fc(epsilon)RI-bound IgE by IGE122 in an *in vitro* cell based degranulation assay**

Using a modified protocol used for the degranulation assay described in Example 11, the potential displacement of Fc(epsilon)RI-bound IgE by IGE122 was assessed. RBL2FH3 cells expressing human Fc(epsilon)RI were incubated with chimaeric human anti-NIP IgE (0.732nM, saturating concentration) for 1 hour. Cells were then washed and incubated for 4 hours in presence of a dose range concentrations of IGE122 or Omalizumab in presence or absence of HSA (1mg/ml). Degranulation of the cells was stimulated by adding NIP-BSA (22 ng/mL; Biosearch Technologies) and the degranulation of the basophils was monitored in real time on the xCELLigence System measuring the impedance of each well every minute during 8h. In the presence or absence of human serum albumin, increased concentrations of IGE122 induced a decrease in the measured impedance of RBL2H3 cells reflecting a decrease in cell degranulation (Figure 8). These data suggest the ability of

IGE122 to displace IgE-bound to Fc(epsilon)RI resulting in decreased degranulation of the cells with increasing concentrations of IGE122. Only at high concentrations of Omalizumab, a decrease in the degranulation of RBL2H3 cells was also observed, in particular in presence of HSA. MacGlashan D et al. (1998, Blood 91: 1633) demonstrated that Omalizumab is not able to induce the displacement of IgE already bound to Fc(epsilon)RI. Therefore, the decrease in degranulation observed at high concentrations of Omalizumab might probably not be due to displacement of IgE from Fc(epsilon)RI but rather an aspecific response of the cells to high protein concentrations. In line with these data, IGE122 binds to a different epitope than Omalizumab on IgE (Example 9), which could support this differential mode of action of IGE122 demonstrated *in vitro*.

In conclusion, IGE122 is not only able to neutralize soluble IgE, but can also displace Fc(epsilon)RI-bound IgE, as shown in the *in vitro* cell based degranulation assay described herein.

Example 20: Ex vivo study on human clinical blood samples of allergic asthma/rhinitis patients to assess displacement of Fc(epsilon)RI-bound IgE by IGE122

As described in Example 19, *in vitro* experiments have demonstrated that IGE122 has a dual mode of action: it neutralizes soluble IgE and it displaces preformed IgE-Fc(epsilon)RI complexes. To determine whether the displacement of Fc(epsilon)RI-bound IgE is biologically relevant, *ex vivo* experiments are performed on clinical blood samples of high IgE patients with allergic asthma or allergic rhinitis that are positive for house dust mite. First, three biomarker assays are developed and validated using flow cytometry as platform to determine following biomarkers: 1) the CD-sensitivity (CD-sens) of the basophils, 2) the number of Fc(epsilon)RI-bound IgE molecules and 3) the number of Fc(epsilon)RI receptors on the basophils. The CD-sens is a sensitivity measurement of basophils to a specific allergen. Johansson et al. (2005, Allergy 60: 1192-9), have shown proof of concept for the use of CD-sens in whole blood as a surrogate biomarker for airway allergen sensitivity in patients with allergic asthma and rhinitis. In a next step, upon incubation of the clinical human blood samples (or isolated human basophils from the blood samples) with IGE122, Omalizumab or medium, these three biomarkers (CD-sens, IgE molecules and Fc(epsilon)RI receptors) will be investigated at different timepoints.

SEQUENCES

Table A-1: IgE sequences.

Name	SEQ ID NO:	SEQUENCE
human IgE Fc	1	Dnktfsvcsrdftptvkiqlqsscdggghfpptiqlclvsytpgtinitwledgqvmdvdlst asttqegelastqseltsqkhwlsdrtytcqvtyqgghtfedstkkcadsnprgvsaylsrpspf dlfirkspititclvdlapskgtvnltsrasgkpvnhstrkeekqrngltltvtstlpvgtrdwie getyqcrvthphlpralmrsttktsgraaapevyafatpewpgsrdkrtlacliqnfmpeidis vqwlhnevqlpdarhsttqprktkgsgffvfrlevtraeweqkdeficravheaaspsqtqv ravsvnpgk
Cyno IgE Fc	2	VNKTFGVSSRNFTPTVKILQSSCDDGHFPPTIQLLCLISGYTPGAINVTWL ENGQVMKVNSTPPATQEGELASTQSEFTLAQKHWLSDRTYTCQVTYQGT TYNDSTKKCADSNPRGVSAYLSRPSFDLFISKSPITITCLVVDLAPSKETVNL WSRASGKPVPHIPATEKKQRNGTLTVTSILPVVTQDWIEGETYQCRVTHPH LPRALVRSMKTSGPRAAPEVYVFATPEKLESRDKRTLACLIQNFMPEDISV QWLHSDVQLPDARHSVTQPRKTKGSGFFVFRLEVTKAEWEQKDEFICRA VHEAASPSWIVQQAVSVNPGK

5

Table A-2: Primers used for the amplification of the cyno IgE sequence.

Name	SEQ ID NO:	SEQUENCE
O-2.09 IgE-Fc	3	GTCAACAAAACCTTTGGCGTC
O-2.10 IgE-Fc	4	TTTACCGGGATTACAGACAC
O-2.42 IgE-Fc	5	ACGACAGACCAAGAAGTGTGC
O-2.43 IgE-Fc	6	CGCCAGAGAAGCTAGAGAGC

Table A-3: Amino acid sequences of IgE binding Nanobodies.

Name	SEQ ID NO:	SEQUENCE
39D11	7	EVQLVESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGPEWVSSI DTGGDITHYADSVKGRFTISRDNANNMLYLQMNSLKPEDTAVYWCATDEDY ALGPNEYDYYGQGTQVTVSS
39D8	8	EVQLVESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGPEWVSSI DTGGDITHYADSVKGRFTISRDNANNMLYLQMNSLKPGDTAVYWCATDED YALGPNEYDYYGQGTQVTVSS
42D7	9	EVQLVESGGGLVQPGGPLRLSCAASGFTFGSYDMSWVRQAPGKGPEWVSSI DTGGDITHYADSVKGRFTISRDNANNMLYLQMNSLKPEDTAVYWCATDEDY ALGPNEYDYYGQGTQVTVSS
42G5	10	EVQLVESGGGLVQPGGSLRLSCAASGFTFGNYDMSWVRQAPGKGPEWVSSI DTGGDITHYADPVKGRFTISRDNAAANTLYLQMNSLKPEDTAVYYCATDEDYA LGPNEYDYYGQGTQVTVSS
36G5	11	EVQLVESGGGLVQPGGSLRLSCVASGFTFGSYDMSWVRQAPGKRPEWVSAI NSGGGSTYYADSVKGRFTISRDNANTLYLQMNSLKPEDTAVYYCATDEDYA LGPNEYDYYGQGTQVTVSS
39B02	12	EVQLVESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGPEWVSAI NSGGGSTYYADSVKGRFTISRDNANTLYLQMNSLKPEDTAVYYCSTDLGPW IEGTMEEYEEYWGQGTQVTVSS

10

Table A-4: DNA sequence of IgE binding Nanobodies.

Name	SEQ ID NO:	SEQUENCE
39D11	13	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGGGGCCCCGAGTGGGTCTCATCT ATTGATACTGGTGGTGTATATCACACACTACGCAGACTCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCAACAATATGTTGTATCTGCAAATGA ACAGCCTGAAACCTGAGGACACGGCCGTGTATTGGTGTGCAACAGATGAG GACTACGCGTTGGGACCGAATGAGTATGACTACTACGGCCAGGGGACCCA GGTCACCGTCTCCTCAGCGGCCGCA
39D8	14	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGGGGCCCCGAGTGGGTCTCATCT ATTGATACTGGTGGTGTATATCACACACTACGCAGACTCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCAACAATATGTTGTATCTGCAAATGA ACAGCCTGAAACCTGGGGACACGGCCGTGTATTGGTGTGCAACAGATGAG GACTACGCGTTGGGACCGAATGAGTATGACTACTACGGCCAGGGGACCCA GGTCACCGTCTCCTCAGCGGCCGCA
42D7	15	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGGGGCCCCGAGTGGGTCTCATCT ATTGATACTGGTGGTGTATATCACACACTACGCAGACTCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCAACAATATGTTGTATCTGCAAATGA ACAGCCTGAAACCTGAGGACACGGCCGTGTATTGGTGTGCAACAGATGAG GACTACGCGTTGGGACCGAATGAGTATGACTACTACGGCCAGGGGACCCA GGTCACCGTCTCCTCAGCGGCCGCA
42G5	16	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGGGGCCCCGAGTGGGTCTCATCT ATTGATACTGGTGGTGTATATCACACACTACGCAGACCCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCGCAACACGCTGTATCTGCAAATGA ACAGCCTGAAACCTGAGGACACGGCCGTGTATTACTGTGCAACAGACGAG GACTACGCGTTGGGACCGAATGAGTATGACTACTACGGCCAGGGGACCCA GGTCACCGTCTCCTCAGCGGCCGCA
36G5	17	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGTAGCCTCTGGATTACATTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGAGGCCCCGAGTGGGTCTCAGCT ATTAATAGTGGTGGTGGTAGCACATACTATGCAGACTCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCAAGAACACGCTGTATCTGCAGATGA ACAGCCTGAAACCTGAGGACACGGCCGTGTATTACTGTGCAACAGATGAG GACTACGCATTGGGACCGAATGAGTATGACTACTATGGCCAGGGGACCCA GGTCACCGTCTCCTCAGCGGCCGCA
39B02	18	GAGGTGCAATTGGTGGAGTCTGGGGGAGGCTTGGTGCAGCCTGGGGGTTC TCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCGGAAGCTATGACA TGAGCTGGGTCCGCCAGGCTCCAGGAAAGGGGCCCCGAGTGGGTCTCAGCT ATTAATAGTGGTGGTGGTAGCACATACTATGCAGACTCCGTGAAGGGCCG ATTACCATCTCCAGAGACAACGCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGAAACCTGAGGACACGGCCGTGTATTACTGTTCAACAGACCTC GGACCGTGGATAGAAGGTACCATGGAGGAATATGAGTATGAGTACTGGGG CCAGGGGACCCAGGTCACCGTCTCCTCAGCGGCCGCA

Table A-4: Sequences of sequence optimized IgE binding Nanobodies.

Name	SEQ ID NO:	SEQUENCE
IGE009	19	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE010	20	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE011	21	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE012	22	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE009+N75K	23	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE009+G44R IGE56F07	24	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE009+G44R+N 75K	25	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEDYALGPNEYDYYGQGT LVT VSS
IGE025	26	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPGKGP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE026 IGE0045 IgE66G02	27	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE027	28	EVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE028	29	EVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMGWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE029	30	EVQLQSGGGLVQPGGSLRLSCAASGFTFGSYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE030	31	EVQLQSGGGLVQPGGSLRLSCAASGFTYGNIDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IgE66G02 (E1D)	32	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE027 (E1D)	33	DVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE028 (E1D)	34	DVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMGWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE029 (E1D)	35	DVQLQSGGGLVQPGGSLRLSCAASGFTFGSYDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS
IGE030 (E1D)	36	DVQLQSGGGLVQPGGSLRLSCAASGFTYGNIDMAWVRQAPGKRP EWVS SIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYWCAT DEEYALGPNEFDYYGQGT LVT VSS

Table A-5: Sequence of albumin binding Nanobodies

Name	SEQ ID NO:	SEQUENCE
Alb-1	37	AVQLVESGGGLVQPGNLRLSCAASGFTFRSFGMSWVRQAPGKEPEWVSSISGSGSDT LYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-8	38	EVQLVESGGGLVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDT LYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-23	39	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-23A	40	AVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-23B	41	AVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-23C	42	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSS
Alb-23D	43	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSA
Alb-23E	44	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSAA
Alb-23F	45	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSAAA
Alb-23G	46	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSG
Alb-23H	47	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSGG
Alb-23I	48	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDT LYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGTQVTVSSGGG

Table A-6: Linker sequences.

Name	SEQ ID NO:	SEQUENCE
5GS	49	GGGGS
6GS	50	SGGSGGS
7GS	51	SGGSGGS
9GS	52	GGGGSGGGGS
10GS	53	GGGGSGGGGS
15GS	54	GGGGSGGGGS
18GS	55	GGGGSGGGGS
20GS	56	GGGGSGGGGS
25GS	57	GGGGSGGGGS
30GS	58	GGGGSGGGGS
35GS	59	GGGGSGGGGS
G1 hinge	60	EPKSCDKTHTCPPCP
9GS-G1 hinge	61	GGGGSGGGSEPKSCDKTHTCPPCP
G3 hinge	62	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCP
3Ala	63	AAA

Table A-10: Sequence of fusion proteins.

Name	SEQ ID NO:	SEQUENCE
IGE019 IGE009/ALB-8 IGE/ALB-8-019	64	EVQLLESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGK GPEWVSSIDTGGDITHYADSVKGRFTISRDNANNTLYLQMNSLR PEDTAVYWCATDEDYALGPNEYDYYGQGTTLTVSSGGGGSGGG SEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE020 39D11/ALB-8 IGE/ALB-8-020	65	EVQLVESGGGLVQPGGSLRLSCAASGFTFGSYDMSWVRQAPGK GPEWVSSIDTGGDITHYADSVKGRFTISRDNANNMLYLQMNSL KPEDTAVYWCATDEDYALGPNEYDYYGQGTQVTVSSGGGGSGG GGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQA PGKLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNS LRPEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE025/ALB-8 IGE/ALB-8-025	66	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KGPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSL RPEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGG GSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAP GKLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSL RPEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE026/ALB-8 IGE/ALB-8-026	67	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE027/ALB-8 IGE/ALB-8-027	68	EVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE028/ALB-8 IGE/ALB-8-028	69	EVQLQSGGGLVQPGGSLRLSCAASGFTFGNYDMGWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE029/ALB-8 IGE/ALB-8-029	70	EVQLQSGGGLVQPGGSLRLSCAASGFTFGSYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPG KLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE030/ALB-8 IGE/ALB-8-030	71	VQLQSGGGLVQPGGSLRLSCAASGFTYGNIDMAWVRQAPGK RPEWVSSIDTGGDITHYADSVKGRFTISRDNAKNTLYLQMNSLRP EDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGK GLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRP EDTAVYYCTIGGSLRSSQGTTLTVSS

IGE045-9GS-Alb-23 IGE047	72	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE045-9GS-Alb-23A	73	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SAVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IGE045-9GS-Alb-23B	74	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SAVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTQTVSS
IGE045-9GS-Alb-23C	75	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTQTVSS
IGE045-9GS-Alb-23D	76	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSA
IGE045-9GS-Alb-23E	77	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSAA
IGE045-9GS-Alb-23F	78	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSAAA
IGE045-9GS-Alb-23G	79	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSG
IGE045-9GS-Alb-23H	80	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNANTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG

		SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSGG
IGE045-9GS-Alb-23I	81	EVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSGGG
IGE045(E1D)-9GS-Alb-23	82	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IgE66G02(E1D)-9GS-Alb-23A	83	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SAVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSS
IgE66G02(E1D)-9GS-Alb-23B	84	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SAVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTQTVSS
IgE66G02(E1D)-9GS-Alb-23C	85	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTQTVSS
IgE66G02(E1D)-9GS-Alb-23D IGE122	86	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSA
IgE66G02(E1D)-9GS-Alb-23E	87	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSAA
IgE66G02(E1D)-9GS-Alb-23F	88	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSAAA

IgE66G02(E1D)-9GS-Alb-23G	89	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSG
IgE66G02(E1D)-9GS-Alb-23H	90	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSG
IgE66G02(E1D)-9GS-Alb-23I	91	DVQLLESGGGLVQPGGSLRLSCAASGFTFGNYDMAWVRQAPG KRPEWVSSIDTGGDITHYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYWCATDEEYALGPNEFDYYGQGTTLTVSSGGGGSGGG SEVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPG KGPEWVSSISGSGSDTYADSVKGRFTISRDNKNTLYLQMNSLR PEDTAVYYCTIGGSLRSSQGTTLTVSSGGG

CLAIMS

1. Polypeptide that comprises or essentially consists of one or more immunoglobulin single variable domain directed against IgE and an immunoglobulin single variable domain directed against human serum albumin wherein:
- 5 a) the one or more immunoglobulin single variable domain directed against IgE is selected from:
- a) SEQ ID NO: 32;
- b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:
- 10 i) the amino acid variant has an Aspartic acid (Asp, D) at position 1 (said position determined according to Kabat numbering); and
- ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32;
- 15 b) the immunoglobulin single variable domain directed against human serum albumin is selected from:
- a) SEQ ID NO: 39;
- b) amino acid variants that have no more than 6, preferably no more than 5, no more than 4, more preferably no more than 3, no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 39, provided that:
- 20 i) the amino acid variant binds human serum albumin with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to SEQ ID NO: 39.
- 25
2. Polypeptide according to claim 1, wherein IgE is selected from human IgE (full length) and constructs made from human IgE such as e.g. the a human IgE c(epsilon)2-c(epsilon)3-c(epsilon)4 fragment of IgE.
- 30
3. Polypeptide according to any of claims 1 or 2, which has one immunoglobulin single variable domain directed against IgE.

4. Polypeptide according to any of claims 1 or 3, wherein the one or more immunoglobulin single variable domain directed against IgE is located at the N-terminal side of the polypeptide.

5. Polypeptide according to any of claims 1 to 4, wherein the immunoglobulin single variable domain directed against human serum albumin is located at the C-terminal part of the polypeptide.

6. Polypeptide according to any of claims 1 to 5, wherein the one or more immunoglobulin single variable domain directed against IgE is selected from:

a) SEQ ID NO: 32;

b) amino acid variants that have no more than 4, preferably no more than 3, more preferably no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 32, provided that:

i) the amino acid difference is a substitution in the framework 1 and/or CDR1 region; and

ii) the amino acid variant binds IgE with the same, about the same, or a higher affinity (said affinity as measured by KinExA) and/or the amino acid variant has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to SEQ ID NO: 32.

7. Polypeptide according to claim 6, wherein the amino acid difference is a substitution at a position in the immunoglobulin single variable domain directed against IgE selected from positions 6, 29, 31 and 35 (said positions determined according to Kabat numbering).

8. Polypeptide according to claim 7, wherein the amino acid difference is a substitution selected from Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly.

9. Polypeptide according to any of claims 1 to 8, wherein the one or more immunoglobulin single variable domain directed against IgE is selected from any of SEQ ID NO's: 32-36.

10. Polypeptide according to any of claims 1 to 9, wherein the immunoglobulin single variable domain directed against human serum albumin is selected from:

a) SEQ ID NO: 39;

b) amino acid variants that have no more than 6, preferably no more than 5, or no more than 4, more preferably no more than 3, or no more than 2, most preferably no more than one amino acid difference with SEQ ID NO: 39, provided that:

- i) the amino acid difference is a substitution in one or more of the framework regions and/or the amino acid difference is an addition of 1 to 3 amino acid residues at the C-terminal part of the immunoglobulin single variable domain; and
- ii) the amino acid variant binds human serum albumin with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to SEQ ID NO: 39.

11. Polypeptide according to claim 10, wherein the amino acid difference is a substitution at a position selected from positions 1, 14, 30, 87 and 108 (said positions determined according to Kabat numbering).

12. Polypeptide according to claim 11, wherein the amino acid difference is a substitution selected from Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln.

13. Polypeptide according to any of claims 9 to 12, wherein the amino acid difference is an addition of 1 to 3 amino acid residues at the C-terminal part of the immunoglobulin single variable domain.

14. Polypeptide according to claim 13, wherein the amino acid difference is the addition of one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly at the C-terminal end of the immunoglobulin single variable domain.

15. Polypeptide according to any of claims 1 to 14, wherein the immunoglobulin single variable domain directed against human serum albumin is selected from SEQ ID NO's: 39-48.

16. Polypeptide according to any of claims 1 to 15, wherein the one or more immunoglobulin single variable domain directed against IgE and the immunoglobulin single variable domain directed against human serum albumin are directly linked to each other.

17. Polypeptide according to any of claims 1 to 15, wherein the one or more immunoglobulin single variable domain directed against IgE and the immunoglobulin single variable domain

directed against human serum albumin are linked to each other via one or more linkers or spacers.

18. Polypeptide according to claim 17, wherein the linker is SEQ ID NO: 52.

5

19. Polypeptide according to any of claims 1 to 18, selected from the following polypeptides:

a) SEQ ID NO's: 82;

b) polypeptides that have no more than 10, preferably no more than 9, no more than 8, no more than 7, no more than 6, more preferably no more than 5, no more than 4, no more than 3, no more than 2, most preferably no more than one amino acid difference with one of SEQ ID NO's: 82, provided that:

10

i) the polypeptide has an Aspartic acid (Asp, D) at position 1; and

ii) the polypeptide binds IgE with the same, about the same, or a higher affinity (said affinity as measured by KinExA) and/or the polypeptide has the same, about the same, or a higher potency (as determined in a degranulation assay as defined in Example 11) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference; and

15

iii) the polypeptide binds human serum albumin with the same, about the same, or a higher affinity (said affinity as measured by surface plasmon resonance or KinExA) compared to the polypeptide without the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid residue difference.

20

20. Polypeptide according to claim 19, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference is a substitution in the immunoglobulin single variable domain directed against IgE at a position selected from positions 6, 29, 31 and 35 (said positions determined according to Kabat numbering).

25

21. Polypeptide according to any of claims 19 or 20, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference is a substitution in the immunoglobulin single variable domain directed against IgE selected from Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering).

30

22. Polypeptide according to any of claims 19 to 21, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one amino acid difference is a substitution in the immunoglobulin single variable domain directed

against human serum albumin at a position selected from positions 1, 14, 30, 87 and 108 (said positions determined according to Kabat numbering).

23. Polypeptide according to any of claims 19 to 22, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one
5 amino acid difference is a substitution in the immunoglobulin single variable domain directed against human serum albumin selected from Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering).
24. Polypeptide according to any of claims 19 to 23, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one
10 amino acid difference is an addition of 1 to 3 amino acid residues at the C-terminal end of the immunoglobulin single variable domain against human serum albumin.
25. Polypeptide according to any of claims 19 to 24, wherein the 10, 9, 8, 7, 6, 5, 4, 3, 2 or one
15 amino acid difference is an addition of one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly at the C-terminal end of the immunoglobulin single variable domain against human serum albumin.
26. Polypeptide that is directed against IgE, comprising or essentially consisting of SEQ ID NO: 72, wherein:
- 20
- the first Glutamic acid has been changed into Aspartic acid;
 - in the immunoglobulin single variable domain directed against IgE, one or more (such as two, three or four) amino acid residues have been mutated selected from the following: Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering);
- 25
- in the immunoglobulin single variable domain directed against human serum albumin one or more (such as two, three, four or five) amino acid residues have been mutated selected from the following: Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering); and/or
 - at the C-terminal end of the immunoglobulin single variable domain against human serum
30 albumin one or more (such as two or three) amino acid residues have been added selected from the following: one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly.
27. Polypeptide according to claim 26, comprising or essentially consisting of SEQ ID NO: 72, wherein the first Glutamic acid has been changed into Aspartic acid.

28. Polypeptide according to any of claims 26 or 27, comprising or essentially consisting of SEQ ID NO: 72, wherein in the immunoglobulin single variable domain directed against IgE, one or more (such as two, three or four) amino acid residues have been mutated selected from the following: Glu6Gln, Phe29Tyr, Asn31Ser, Asn31Pro and Ala35Gly (said positions determined according to Kabat numbering).
29. Polypeptide according to any of claims 26 to 28, comprising or essentially consisting of SEQ ID NO: 72, wherein in the immunoglobulin single variable domain directed against human serum albumin one or more (such as two, three, four or five) amino acid residues have been mutated selected from the following: Glu1Ala, Pro14Ala, Arg30Ser, Arg87Lys and Leu108Gln (said positions determined according to Kabat numbering).
30. Polypeptide according to any of claims 26 to 29, comprising or essentially consisting of SEQ ID NO: 72, wherein at the C-terminal end of the immunoglobulin single variable domain against human serum albumin one or more (such as two or three) amino acid residues have been added selected from the following: one of Ala, Ala-Ala, Ala-Ala-Ala, Gly, Gly-Gly and Gly-Gly-Gly.
31. Polypeptide according to any of claim 26 to 30, comprising or essentially consisting of SEQ ID NO: 72, in which in the immunoglobulin single variable domain directed against IgE, following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):
- Glu6Gln;
 - Glu6Gln and Ala35Gly;
 - Glu6Gln and Asn31Ser;
 - Glu6Gln and Phe29Tyr;
 - Glu1Asp;
 - Glu1Asp and Glu6Gln;
 - Glu1Asp, Glu6Gln and Ala35Gly;
 - Glu1Asp, Glu6Gln and Asn31Ser; or
 - Glu1Asp, Glu6Gln and Phe29Tyr.

32. Polypeptide according to any of claims 26 to 31, in which, in the immunoglobulin single variable domain directed against human serum albumin, following amino acid residue(s) have been substituted (positions as determined according to Kabat numbering):

- Glu1Ala;
- Glu1Ala and Leu108Gln; or
- Leu108Gln.

33. Polypeptide according to any of claims 26 to 32, in which, at the C-terminal end of the immunoglobulin single variable domain against human serum albumin, following amino acid residue(s) have been added:

- Ala;
- Ala-Ala;
- Ala-Ala-Ala;
- Gly;
- Gly-Gly; or
- Gly-Gly-Gly.

34. Polypeptide according to any of claims 1 to 33, comprising or essentially consisting of one of SEQ ID NO's: 73-91.

35. Nucleic acid or nucleotide sequence that encodes a polypeptide according to any of claims 1 to 34.

36. Nucleic acid or nucleotide sequence according to claim 35, that is in the form of a genetic construct.

37. Host or host cell that expresses, or that under suitable circumstances is capable of expressing, a polypeptide according to any of claims 1 to 34 and/or that comprises a nucleic acid or nucleotide sequence according to claim 35, or a genetic construct according to claim 36.

38. Construct that comprises or essentially consists of one or more polypeptides according to any of claims 1 to 34, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

39. Construct according to claim 38, in which said one or more other groups, residues, moieties or binding units are amino acid sequences.
40. Construct according to any of claims 38 or 39, in which said one or more linkers, if present, are one or more amino acid sequences.
41. Construct according to any of claims 38 to 40, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.
42. Construct according to any of claims 38 to 41, in which said one or more other groups, residues, moieties or binding units are selected from domain antibodies, single domain antibodies, "dAb's", or Nanobodies.
43. Composition comprising at least one polypeptide according to any of claims 1 to 34, at least one construct according to any of claims 38 to 42, or a nucleic acid or nucleotide sequence according to claim 35.
44. Composition according to claim 43, which is a pharmaceutical composition.
45. Composition according to any of claims 43 or 44, additionally comprising at least one pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant.
46. Method for producing a polypeptide according to any of claims 1 to 34, or a composition according to any of claims 43 to 45, said method at least comprising the steps of:
- a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid or nucleotide sequence according to claim 35,
- or
- cultivating and/or maintaining a host or host cell according to claim 37 under conditions that are such that said host or host cell expresses and/or produces at least a polypeptide according to any of claims 1 to 34, or composition according to any of claims 43 to 45, optionally followed by:
- b) isolating and/or purifying the polypeptide according to any of claims 1 to 34, thus obtained.
47. Method for preparing a polypeptide according to any of claims 1 to 34, said method comprising at least the steps of linking one or more immunoglobulin single variable domain

against IgE and an immunoglobulin single variable domain against human serum albumin and, optionally, one or more linkers.

48. Method according to claim 47, comprising the steps of:

- 5 a) linking a nucleic acid sequence encoding one or more immunoglobulin single variable domain against IgE and a nucleic acid sequence encoding an immunoglobulin single variable domain against human serum albumin (and also for example nucleic acids encoding one or more linkers and further one or more further elements of genetic constructs known per se) to obtain a genetic construct according to claim 36;
- 10 b) expressing, in a suitable host cell or host organism or in another suitable expression system, the genetic construct obtained in a) optionally followed by:
- c) isolating and/or purifying the polypeptide according to any of claims 1 to 34, thus obtained.

15 49. The polypeptide according to any of claims 1 to 34 for use in therapy.

50. The polypeptide according to any of claims 1 to 34, for the prevention and/or treatment of at least one disease or disorder associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE.

20 51. The polypeptide according to any of claims 1 to 34, for the prevention and/or treatment of at least one disease or disorder that can be treated by modulating IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved.

25 52. The polypeptide according to any of claims 1 to 34, for the prevention and/or treatment of asthma, allergic rhinitis, hay fever, conjunctivitis, eczema, urticaria, food allergies and other allergies, including serious and/or life-threatening allergic reactions such as those to insect bites or stings, snake bites etc., as well as to allergic reaction to medication; and more generally any disease or disorder associated with anaphylactic hypersensitivity and/or (atopic) allergy.

30

53. Method for the prevention and/or treatment of at least one disease or disorder associated with IgE, with increased levels and/or overproduction of IgE or with abnormal sensitivity (such as hypersensitivity) for IgE, said method comprising administering, to a subject in need

thereof, a pharmaceutically active amount of a polypeptide according to any of claims 1 to 34, and/or of a pharmaceutical composition comprising the same.

54. Method for the prevention and/or treatment of at least one disease or disorder that can be
5 treated by modulating IgE, its biological or pharmacological activity, and/or the biological pathways or signalling in which IgE is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide according to any of claims 1 to 34, and/or of a pharmaceutical composition comprising the same.
- 10 55. Method for the prevention and/or treatment of asthma, allergic rhinitis, hay fever, conjunctivitis, eczema, urticaria, food allergies and other allergies, including serious and/or life-threatening allergic reactions such as those to insect bites or stings, snake bites etc., as well as to allergic reaction to medication; and more generally any disease or disorder
15 associated with anaphylactic hypersensitivity and/or (atopic) allergy, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide according to any of claims 1 to 34, and/or of a pharmaceutical composition comprising the same.

Figure 1

Kabat#	10	20	30	40	50
39D11					
IGE009	EVQLVESGGGLVQPGGSLRLS	CAASGFTFGSYDMSWVRQ	APGKGP	EWSSIDTGGD	ITHY
IGE010	...L.....				
IGE011	...L.....				
IGE012	...L.....				
IGE009+N75K	...L.....				
IGE009+G44R	...L.....			R.....	
IGE009+G44R+N75K	...L.....			R.....	
IGE025	...L.....		N..A..		
IGE026	...L.....		N..A..	R.....	
IGE027	...LQ.....		N..A..	R.....	
IGE028	...LQ.....		N..G..	R.....	
IGE029	...LQ.....			A.....	R.....
IGE030	...LQ.....		Y.N..A..	R.....	
IGE66G02 (E1D)	D...L.....		N..A..	R.....	
IGE027 (E1D)	D...LQ.....		N..A..	R.....	
IGE028 (E1D)	D...LQ.....		N..G..	R.....	
IGE029 (E1D)	D...LQ.....			A.....	R.....
IGE030 (E1D)	D...LQ.....		Y.N..A..	R.....	

Figure 1 continued

Kabat#	60	70	80	90	100	110	SEQ ID NO
39D11	ADSVKGRFTISRDNANMLYLQMN	SLKPEDTAVYWCATDE	DYALGPNEYDYGQGTQVT	VSS			13
IGE009	abc	 abcde					19
IGE010	abc	 abcde					20
IGE011	abc	 abcde					21
IGE012	abc	 abcde					22
IGE009+N75K	abc	 abcde					23
IGE009+G44R	abc	 abcde					24
IGE009+G44R+N75K	abc	 abcde					25
IGE025	abc	 abcde					26
IGE026	abc	 abcde					27
IGE027	abc	 abcde					28
IGE028	abc	 abcde					29
IGE029	abc	 abcde					30
IGE030	abc	 abcde					31
IGE66G02 (E1D)	abc	 abcde					32
IGE027 (E1D)	abc	 abcde					33
IGE028 (E1D)	abc	 abcde					34
IGE029 (E1D)	abc	 abcde					35
IGE030 (E1D)	abc	 abcde					36

Figure 2

Alb-23	AVQLLESGGG	LVQPGGSLRL	SCAASGFTFR	SFGMSWVRQA	PGKGPEWVSS	ISGSGSDTLY
Alb-23A	A.....					
Alb-23B	A.....					
Alb-23C						
Alb-23D						
Alb-23E						
Alb-23F						
Alb-23G						
Alb-23H						
Alb-23I						

	ADSVKGRFTI	SRDNSKNTLY	LQMNLSLRPD	TAVYCTIGG	SLSRSSQGTL	VTVSS	SEQ ID NO
Alb-23							39
Alb-23A							40
Alb-23B					 Q		41
Alb-23C					 Q		42
Alb-23D					 A		43
Alb-23E					 AA		44
Alb-23F					 AAA		45
Alb-23G					 G		46
Alb-23H					 GG		47
Alb-23I					 GGG		48

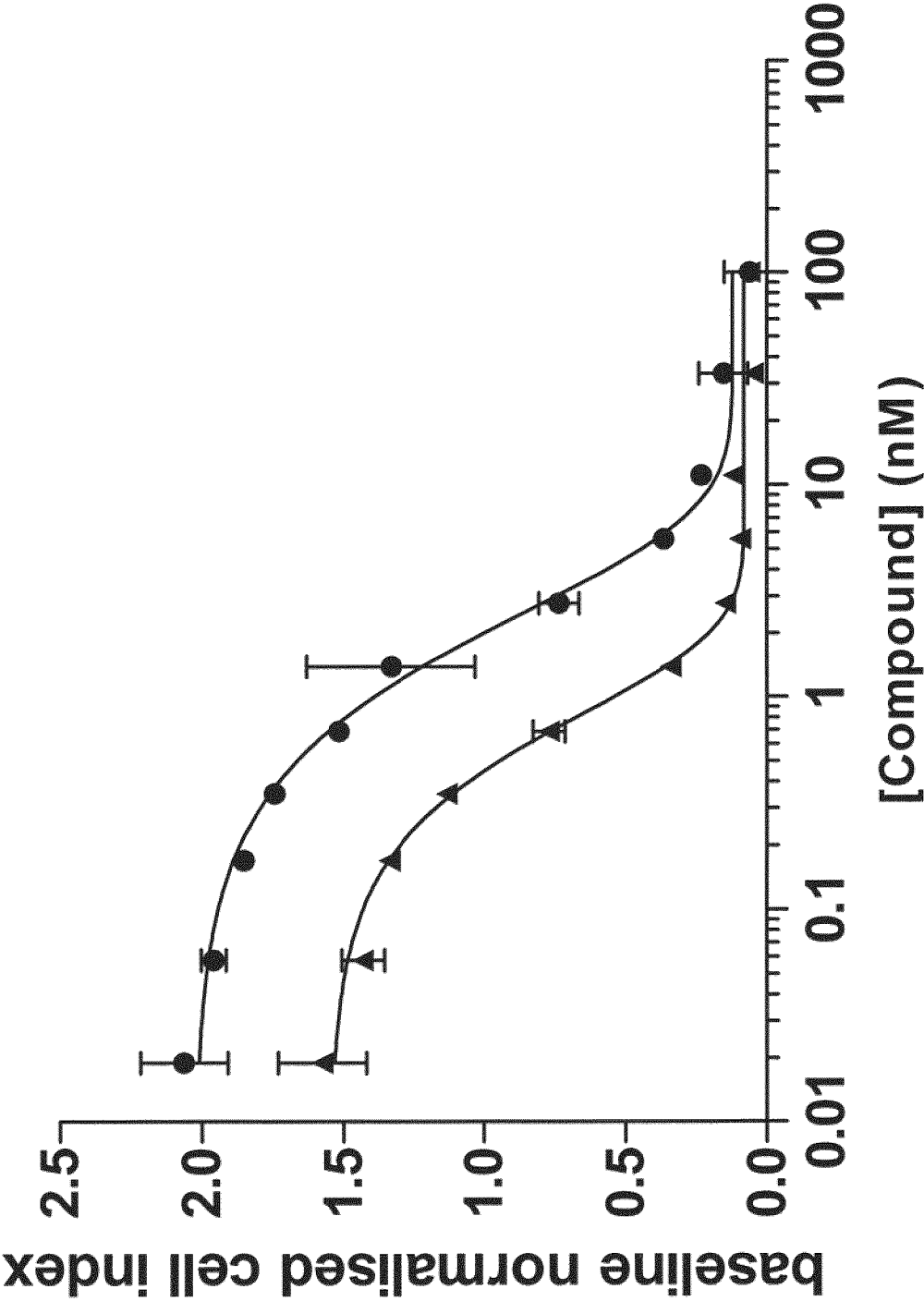


Figure 3

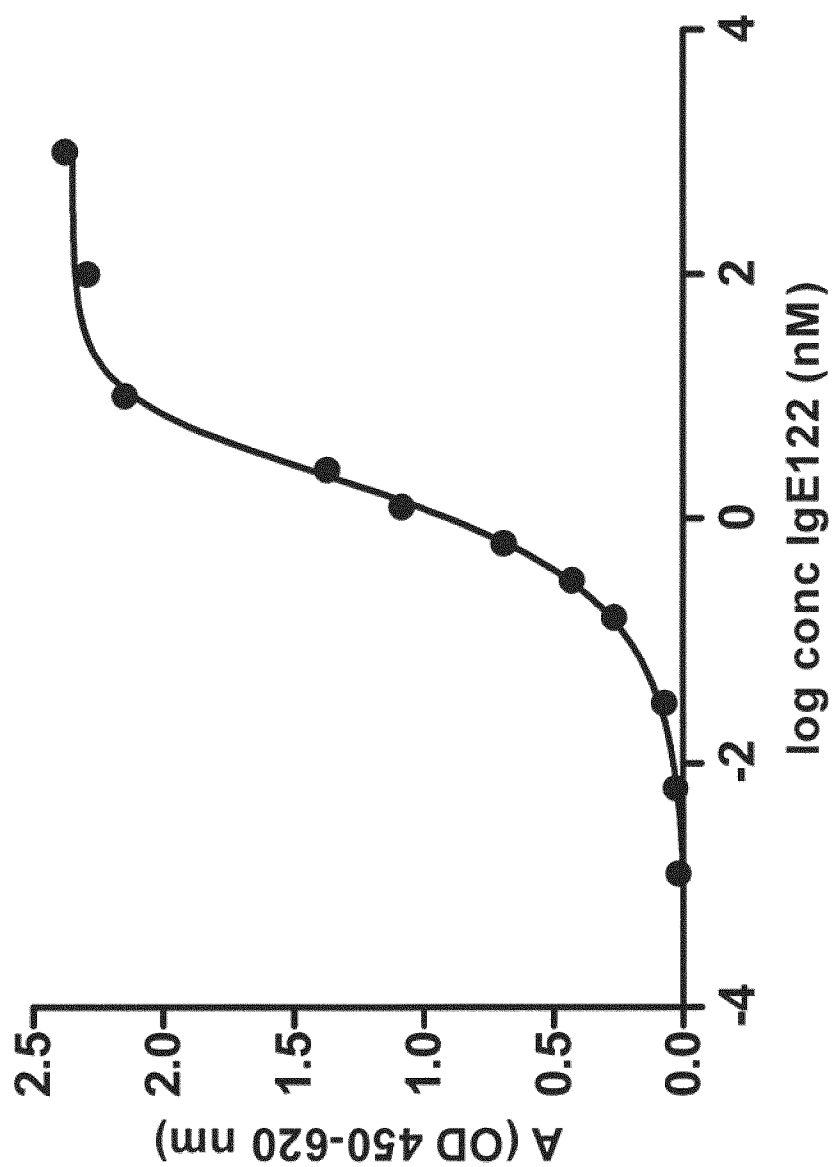


Figure 4

Figure 5

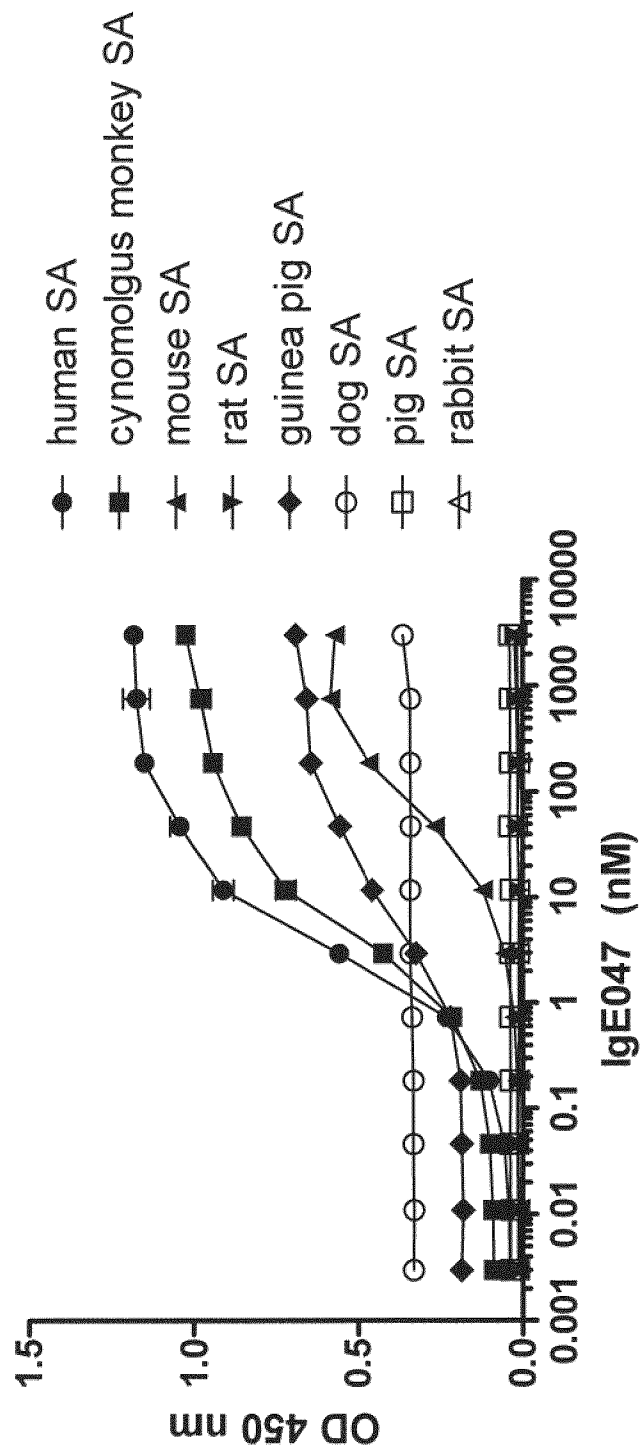


Figure 6A

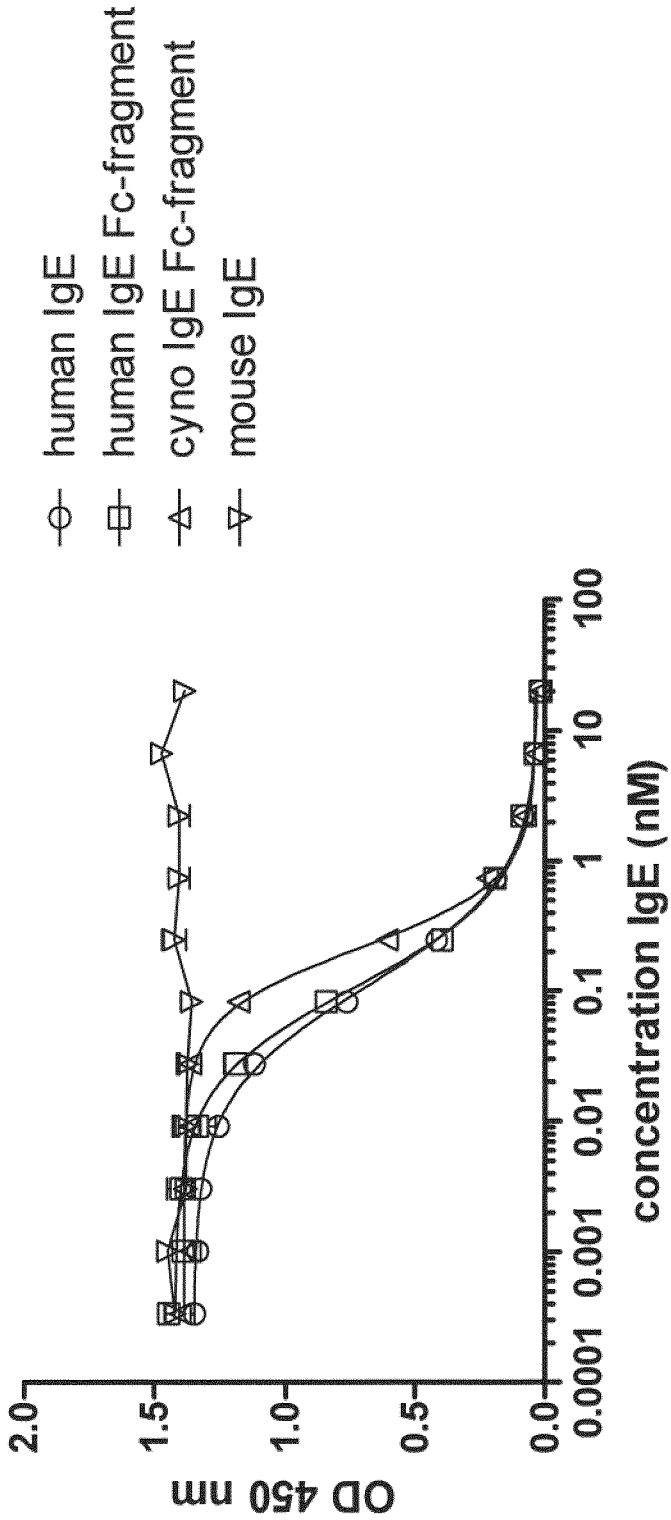


Figure 6B

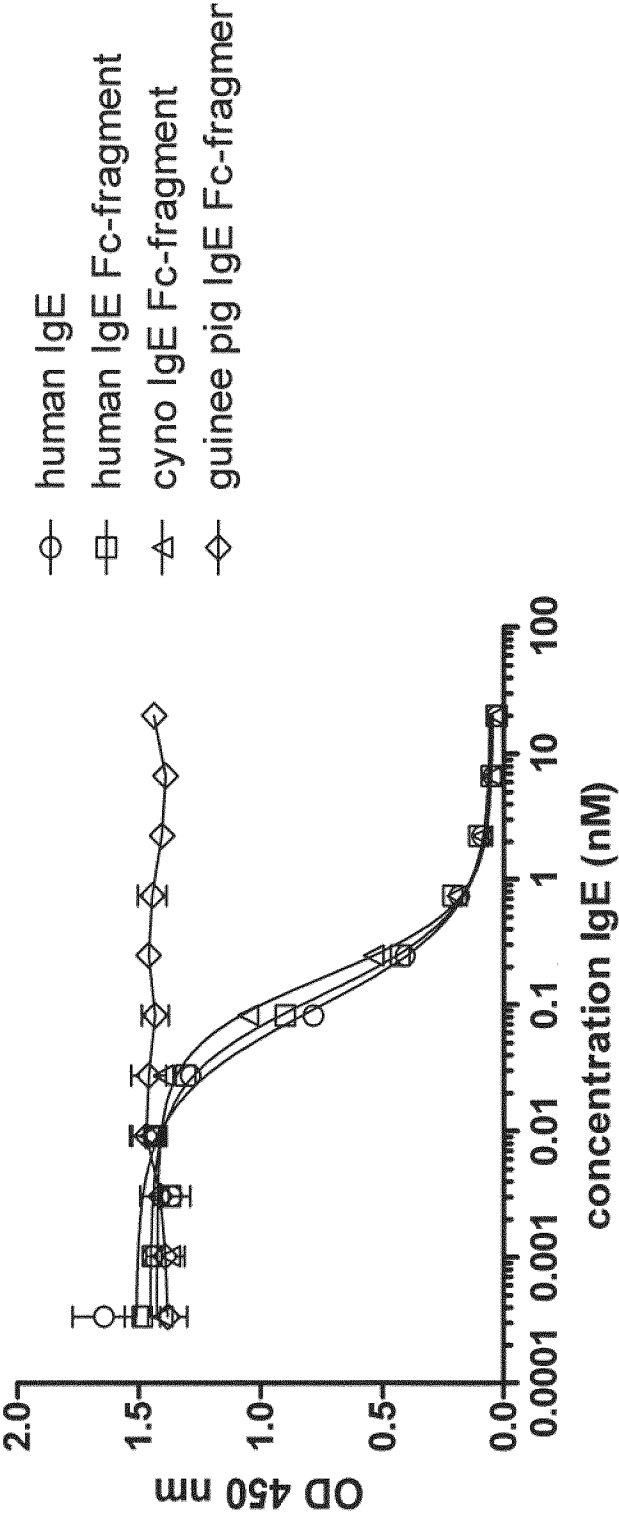


Figure 7

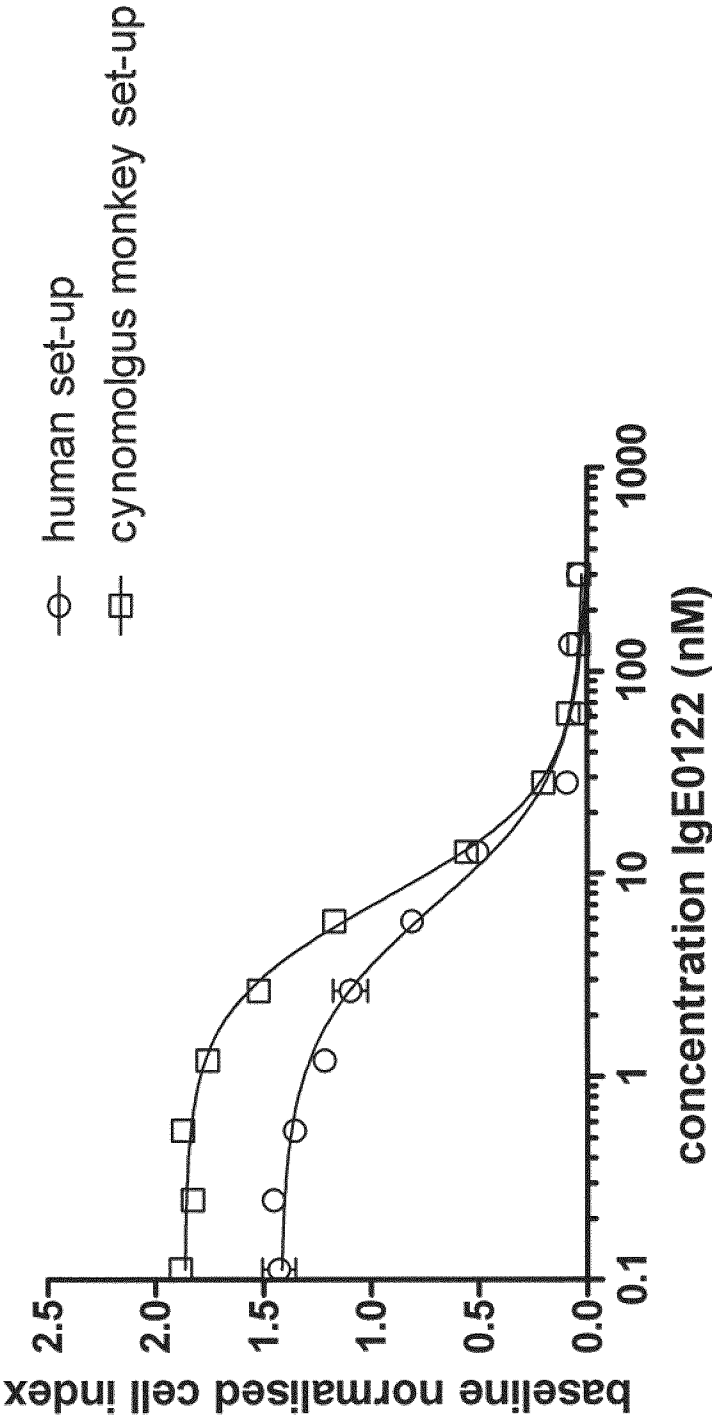
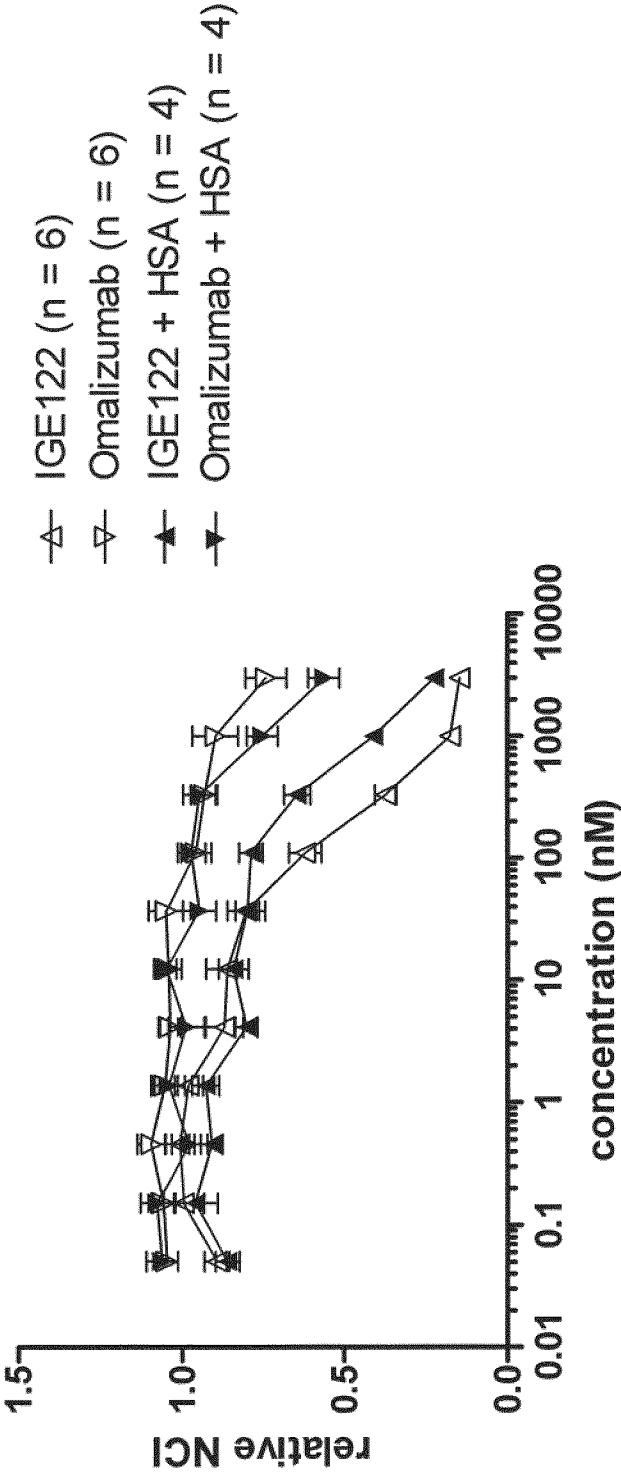
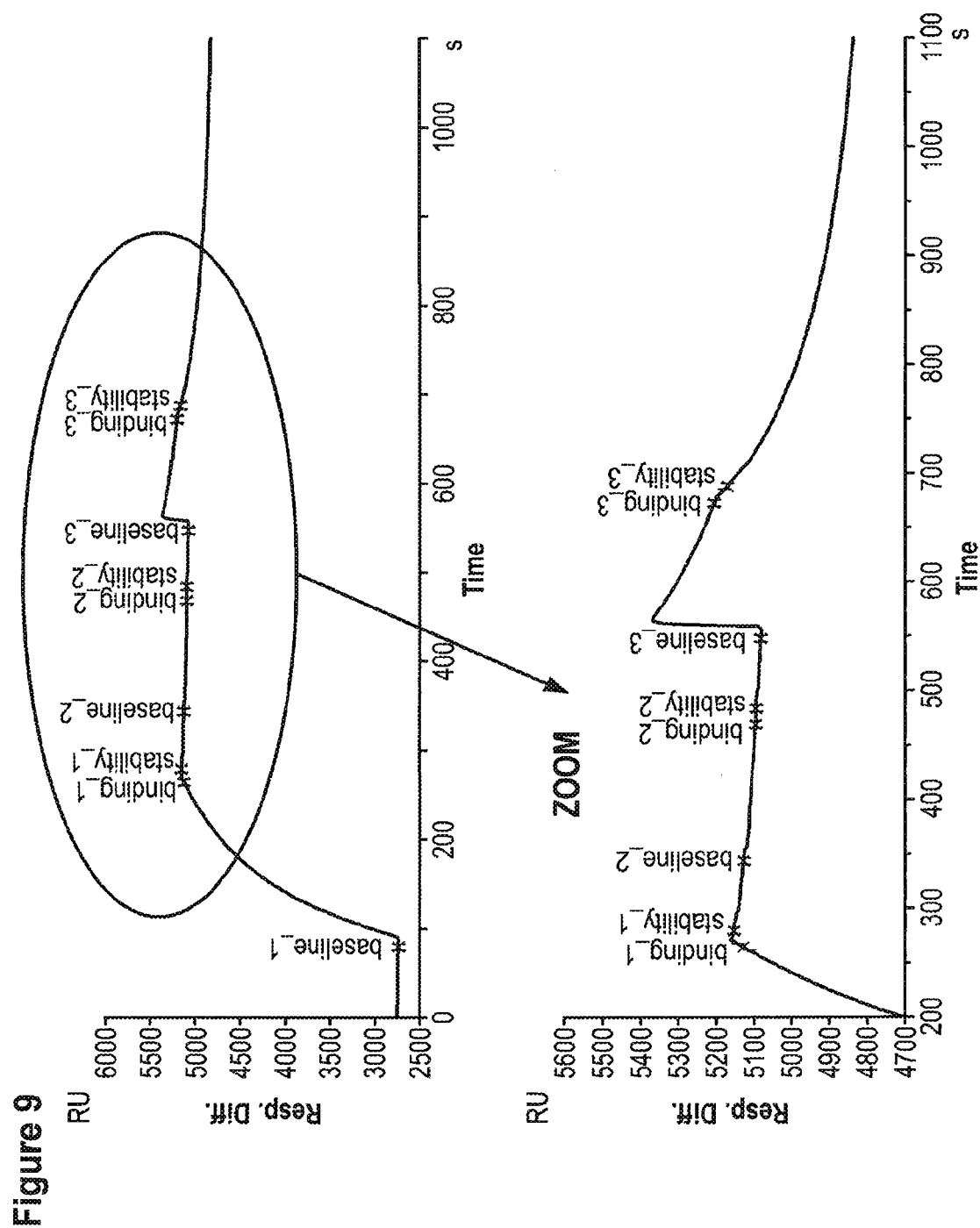


Figure 8





INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/075902

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/18 C07K16/42
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.



See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 February 2014

Date of mailing of the international search report

19/03/2014

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Authorized officer

Cilensek, Zoran

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/075902

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

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