(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
27 April 2017 (27.04.2017)

WIPO | PCT

(10) **International Publication Number**
WO 2017/068186 A1

(51) **International Patent Classification**:
C07K 16/28 (2006.01) ***A61K 39/00*** (2006.01)
A61K 39/395 (2006.01)

(21) **International Application Number**:
PCT/EP2016/075558

(22) **International Filing Date**:
24 October 2016 (24.10.2016)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/245,188 22 October 2015 (22.10.2015) US
62/276,352 8 January 2016 (08.01.2016) US

(71) **Applicant**: **ABLYNX NV** [BE/BE]; Technologiepark 21, 9052 Ghent-Zwijnaarde (BE).

(72) **Inventors**: **DESCHAGHT, Pieter**; Tuinstraat 6, 8310 Assebroek (BE). **LI, Sandra**; Smissenbosstraat 99, 1700 Dilbeek (BE). **SNOECK, Veerle**; Wannegemstraat 20, 9750 Zingem (BE). **PYPE, Jan**; Geraardsbergsesteenweg 48, 1540 Herne (BE).

(74) **Agents**: **STEFFERL, Andreas** et al.; Hoffmann Eitle Patent- und Rechtsanwälte PartmbB, Arabellastraße 30, 81925 Munich (DE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: GITR AGONISTS

(57) **Abstract**: The present invention relates to immunoglobulin single variable domains that bind GITR and more in particular to polypeptides that comprise or essentially consist of one or more such immunoglobulin single variable domains; to nucleic acids encoding such polypeptides; to methods for preparing such polypeptides; to compositions and in particular to pharmaceutical compositions that comprise such polypeptides, for prophylactic, therapeutic or diagnostic purposes. In particular, the polypeptides of the present invention enhance the biological activity of GITR.

WO 2017/068186 A1

GITR AGONISTS

FIELD OF THE INVENTION

The present invention relates to immunoglobulin single variable domains that bind GITR and more in particular to polypeptides, that comprise or essentially consist of one or more such immunoglobulin single variable domains (also referred to herein as *"ISVD(s) of the invention"*, and *"polypeptides of the invention"*, respectively).

The invention also relates to nucleic acids encoding such polypeptides (also referred to herein as "*nucleic acid(s) of the invention*"); to methods for preparing such polypeptides; to host cells expressing or capable of expressing such polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such polypeptides, nucleic acids and/or host cells; and to uses of polypeptides, nucleic acids, host cells and/or 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 from the further description herein.

BACKGROUND ART

Cancer takes an enormous human toll around the world. It is nowadays the world's leading cause of death, followed by heart disease and stroke. Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012. The number of new cases is expected to rise by about 70% over the next 2 decades (source: WHO Cancer). The total economic impact of premature death and disability from cancer worldwide was about $900 billion in 2008, representing 1.5% of the world's gross domestic product.

Chemotherapy has been a mainstay in cancer treatment for many years now. Despite some success, the cure rate with chemotherapy remains unsatisfactory, and severe side effects from these treatments are a concern. Improved therapies combatting cancer are eagerly awaited.

Considerable effort has recently been invested in cancer immunotherapy as a new treatment modality to eliminate cancer. Cancer immunotherapy attempts to stimulate the immune system to reject and destroy tumors.

The generation and maintenance of immune responses are controlled by both co-stimulatory and co-inhibitory signaling through T cell co-receptors. Immune activation is regulated by two major families of co-receptors expressed by T cells: the immunoglobulin-like (Ig) superfamily and the TNFR superfamily. The glucocorticoid-induced Tumor Necrosis Factor receptor-related protein (GITR) is a co-stimulatory member of the latter family. Human GITR exists as a trimer and signaling involves the recruitment of three receptor ectodomains by trimeric GITR ligand (GITRL), resulting in a 3:3 receptor:ligand complex formation [*Chattopadhyay et al. PNAS (2007) 104:19452-19457*]. A substantial level of GITR is constitutively expressed on $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) and plays a key role in the peripheral tolerance that is mediated by these cells. GITR is also expressed at low levels on CD4+ and CD8+ T cells (T effector cells) and its expression is enhanced rapidly after activation [*Nocentini et al. Br. J. Pharmacol. (2012) 165: 2089-2099*]. Additionally, expression of GITR has also been identified on dendritic cells, natural killer (NK) cells, B cells, macrophages and monocytes. Its ligand GITRL (TNFSF18) is expressed on the surface of various antigen presenting cells (such as dendritic cells, B-cells and macrophages) and on endothelial cells, triggering co-stimulation and leucocyte adhesion and transmigration, respectively [*Schaer et al J Immunother Cancer (2014) 2: 1-9; Lacal et al, J. Pharmacol. Exp. Ther. (2013) 347: 164-172*].

GITR activation has been implicated in a wide range of immune functions, involving both effector and regulatory T cells, and thus participating in the development of immune responses against tumors and infectious agents. In particular, preclinical evidence has been accumulating to indicate that GITR activation has effective anti-tumor properties. To date, agonistic monoclonal antibodies against GITR have been shown to promote anti-tumor immunity *[Turk et al., J. Exp. Med. (2004) 200:771-782; Ko et al., J. Exp. Med. (2005) 202:885-891; Ramirez-Montagut et al., J. Immunol. (2006) 176:6434-6442; Zhou et al., J. Immunol. (2007) 179:7365–7375; Cohen et al., PLoS One (2010) 5:e10436; Coe et al., Cancer Immunol. Immunother. (2010) 59:1367-1377; Zhou et al., J. Immunother. (2010) 33:789-797; Cote et al., J. Immunol. (2011) 186:275-283]*, to augment anti-tumor immunity in combination with vaccines against cancer antigens [*Cohen et al., Cancer Res. (2006) 66:4904-4912, Ko et al., Cancer Res. (2007) 67:7477-7486, Hoffman et al., J. Immunother. (2010) 33:136-145, Boczkowski et al., Cancer Gene Ther. (2009) 16:900-911]*, to synergize with other immune-modulatory therapies *[Ko et al., J. Exp. Med. (2005) 202:885-891, Houot et al., Blood (2009) 113:3546-3552, Mitsui, et al. Clin. Can. Res. (2010) 16:2781-2791]*, to enhance rejection of tumors expressing mutated self *[Duan et al., Cancer Res. (2009) 69:3545-3553]* and to enhance adoptive cell therapy [Liu et al., *Mol Ther.* (2009) 17:1274-81, Imai et al., *Can. Sci.* (2009) 100:1317-25]. Furthermore, targeting GITR *in vivo* has also produced some notable results in treating infectious diseases. During Friend virus infection in mice, treatment with agonist antibody to GITR reverses the effect of natural Treg cells, leading to enhanced Th1 and CD8+ T cell responses, reduction of viral load and pathology and restoration of CD8+ T cell mediated antitumor responses *[He et al., J. Virol. (2004) 78:11641-11647]*. Similarly, treatment of mice with agonist antibody to GITR diminishes herpetic keratitis *[Suvas et al., J. Virol. (2005) (18):11935-11942].*

Several mechanisms appear to contribute to GITR-mediated therapeutic effects. GITR activation *in vivo* for example, has been shown to impair expression of FoxP3 in intramural regulatory

T cells (Treg), resulting in a loss of Treg lineage stability with subsequent reduced suppression of effector T cells (Teff) [*Schaer et al Cancer Immunol. Res. (2013) 1: 320-331*]. Furthermore, GITR modulation is supporting Teff activity by inducing T cell proliferation and effector functions and by promoting T cell survival [*Mahoney et al Nat Rev Drug Discov. (2015) 14:561-84*].

Although GITR seems to be an attractive target for cancer immunotherapy, it remains unclear whether anti-GITR agonistic antibodies depend on their Fc function for anti-tumor effects. Ponte et al. demonstrated that FcR-mediated cross-linking of anti-GITR antibodies, is not required for enhancement of humoral and cellular immunity [*Ponte et al Immunol. (2010) 130, 231-242*]. Furthermore, Ponte showed that an Fc-disabled anti-GITR monoclonal antibody was effective in an *in vitro* lung tumor model as monotherapy and in combination with chemotherapeutic drugs enhanced anti-tumor immunity against established tumors in a s.c. tumor model (*Ponte et al. Keystone symposia, April 2011*). In contrast, Bulliard et al. found that activating FcγRs were essential for anti-tumor activities of anti-GITR antibodies [Bulliard et al J Exp Med. (2013) 210: 1685-1693].

Efficacious immunotherapies should inhibit Treg and simultaneously activate Teff, tipping the balance towards immuno-activation. However, while the results obtained to date establish GITR as a useful target for immunotherapy, it remains unclear which particular features of GITR agonists are especially advantageous for therapeutic purposes. As such, there is a need in the art for further insight into the specific functional properties that make GITR agonists therapeutically effective, as well as for improved therapeutic GITR agonists which are more effective in treating cancer and other conditions, such as infectious diseases.

SUMMARY OF THE INVENTION

The present invention provides GITR agonists with particular functional properties which are linked with improved and desirable therapeutic and/or pharmacological properties, in addition to other advantageous properties (such as, for example, improved ease of preparation, good stability, and/or reduced costs of goods), compared to the prior art amino acid sequences and antibodies.

Based on extensive screening, characterization and combinatory strategies, the present inventors surprisingly observed that polypeptides comprising immunoglobulin single variable domains binding GITR showed improved properties for modulating GITR activity compared to the GITR agonizing molecules described in the prior art. More specifically, the present inventors surprisingly observed that the polypeptides of the present invention exhibited higher efficacies at equipotent or even lower EC_{50} values as compared to the prior art antibodies. This is clinically very important as the effectiveness of a drug depends on its maximal efficacy.

Accordingly, the present invention relates to a polypeptide comprising at least one immunoglobulin single variable domain (ISVD) that specifically binds glucocorticoid-induced TNFR family-related receptor (GITR) with an EC_{50} value of less than 200 pM, and wherein the binding of said ISVD to said GITR enhances an immune response.

In particular, the polypeptides that can bind GITR, and in particular human GITR (SEQ ID NO: 231), are characterised by a biological potency, suitably measured and/or expressed as an EC_{50} value, as further described and defined herein, for instance, such as by a NF-κB luciferase reporter assay or a T-cell activation assay.

In one aspect, the polypeptides of the present invention are such that they bind (human) GITR with an EC_{50} of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 18, 16, 15, 14 or even less, such as less than 12 pM, as determined in a NF-κB luciferase reporter assay.

In another aspect, the polypeptides of the present invention are such that they bind (human) GITR with an EC_{50} of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40 or even less, such as less than 30 pM, as determined in a T-cell activation assay.

It will be appreciated that binding of polypeptides of the invention to (human) GITR may result in enhancing the proliferation or activation of T cells, B cells or natural killer cells as described herein.

It will further be appreciated that binding of the polypeptides of the invention to (human) GITR may result in inhibiting tumor cell growth, such as described herein.

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

Some preferred technical values for binding, enhancing an immune response, inhibiting tumor cell growth or other *in vivo* and/or *in vitro* potency of the polypeptides of the invention to (human) GITR will become clear from the further description and examples herein.

In one aspect the present invention provides a polypeptide as described herein, wherein said polypeptide has the structure FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, in which CDR1, CDR2 and CDR3 are as defined herein, and FR1, FR2, FR3 and FR4 are framework sequences. Accordingly, the present invention relates to polypeptides that (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

  (a) SEQ ID NOs: 73-88; and

  (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 73-88; and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 90-116; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 90-116; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NOs: 118-132 and 282-284; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 118-132 and 282-284.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NOs: 73-75; and
  - (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 73; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 90-98; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 90; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NOs: 118-119, 123 and 282-284; and
  - (f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 118.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NO: 73; and
  - (b) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 73, wherein
    - at position 2 the T has been changed into S;
    - at position 7 the D has been changed into N;
    - at position 8 the S has been changed into A; and/or
    - at position 10 the A has been changed into G;

and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NO: 90; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 90, wherein
    - at position 1 the A has been changed into H, T, or G;
    - at position 2 the I has been changed into M;
    - at position 3 the T has been changed into S;
    - at position 6 the G has been changed into S;
    - at position 7 the S has been changed into R, or G; and/or
    - at position 8 the P has been changed into S, T, or R

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NO: 118; and
  - (f) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 118, wherein
    - at position 9 the A has been changed into P;
    - at position 11 the M has been changed into L, K, R, or Q; and/or
    - at position 12 the D has been changed into N.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 118; or
- ii) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 123.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NOs: 76-78; and
  - (b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 76; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 99-103; and

- (d) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 99; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NOs: 120-123; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 120.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NO: 76; and
  - (b) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 76, wherein
    - at position 7 the D has been changed into N; and/or
    - at position 8 the S has been changed into A;

and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NO: 99; and
  - (d) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 99, wherein
    - at position 1 the A has been changed into S, or T;
    - at position 5 the S has been changed into T, G, or R;
    - at position 6 the T has been changed into K; and/or
    - at position 7 the N has been changed into I;

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NO: 120; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 120, wherein
    - at position 1 the E has been changed into K;
    - at position 4 the A has been changed into T;
    - at position 11 the I has been changed into M, or L; and/or
    - at position 12 the N has been changed into D.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3

complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 76, CDR2 is represented by SEQ ID NO: 99, and CDR3 is represented by SEQ ID NO: 120.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NOs: 79-84; and
  (b) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 79; and/or
(ii) CDR2 is chosen from the group consisting of:
  (c) SEQ ID NOs: 104-108; and
  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 104; and/or
(iii) CDR3 is chosen from the group consisting of:
  (e) SEQ ID NOs: 124-125; and
  (f) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 124.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
(a) SEQ ID NO: 79; and
(b) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 79, wherein
  - at position 2 the S has been changed into N;
  - at position 3 the V has been changed into I;
  - at position 7 the N has been changed into D;
  - at position 8 the D has been changed into S; and/or
  - at position 9 the M has been changed into V, or T;

and/or

(ii) CDR2 is chosen from the group consisting of:
(c) SEQ ID NO: 104; and
(d) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 104, wherein
  - at position 1 the D has been changed into G;

- at position 5 the R has been changed into A; and/or
- at position 6 the G has been changed into D;

and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NO: 124; and

(f) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 124, wherein

- at position 4 the T has been changed into M.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 79, CDR2 is represented by SEQ ID NO: 104, and CDR3 is represented by SEQ ID NO: 124.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NOs: 85-86; and

(b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 85; and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NOs: 109-110; and

(d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 109; and/or

(iii) CDR3 is SEQ ID NO: 126.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NO: 85; and

(b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 85, wherein

- at position 2 the S has been changed into N;

and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NO: 109; and

(d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 109, wherein

- at position 9 the T has been changed into S;

and/or

(iii) CDR3 is SEQ ID NO: 126.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 85, CDR2 is represented by SEQ ID NO: 109, and CDR3 is represented by SEQ ID NO: 126.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 87, CDR2 is represented by SEQ ID NO: 111, and CDR3 is represented by SEQ ID NO: 127.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
    - (c) SEQ ID NOs: 112-113; and
    - (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 112; and/or
- (iii) CDR3 is chosen from the group consisting of:
    - (e) SEQ ID NOs: 128-130; and
    - (f) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 128.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
    - (a) SEQ ID NO: 112; and
    - (b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 112, wherein
        - at position 4 the D has been changed into G;

and/or

- (iii) CDR3 is chosen from the group consisting of:
    - (c) SEQ ID NO: 128; and
    - (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 128, wherein
        - at position 9 the S has been changed into P; and/or

- at position 13 the T has been changed into A.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 77, CDR2 is represented by SEQ ID NO: 112, and CDR3 is represented by SEQ ID NO: 128.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is SEQ ID NO: 88; and/or

(ii) CDR2 is chosen from the group consisting of:

  (c) SEQ ID NOs: 114-116; and

  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 114; and/or

(iii) CDR3 is chosen from the group consisting of:

  (e) SEQ ID NOs: 131-132; and

  (f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 131.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is SEQ ID NO: 88; and/or

(ii) CDR2 is chosen from the group consisting of:

  (a) SEQ ID NO: 114; and

  (b) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 114, wherein

    - at position 1 the V has been changed into I, or A; and/or
    - at position 9 the M has been changed into I;

and/or

(iii) CDR3 is chosen from the group consisting of:

  (c) SEQ ID NO: 131; and

  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 131, wherein

    - at position 4 the G has been changed into E; and/or
    - at position 5 the R has been changed into Q.

In a further aspect the present invention provides a polypeptide as described herein, wherein said polypeptide (essentially) consist of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 88, CDR2 is represented by SEQ ID NO: 114, and CDR3 is represented by SEQ ID NO: 131.

In a preferred aspect, the at least one ISVD is chosen from the group of ISVDs, wherein:

- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 91; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 92; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 94; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 95; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 75, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 96; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 97; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 98; and CDR3 is SEQ ID NO: 119;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 282;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 283;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 284;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 120;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 100; and CDR3 is SEQ ID NO: 121;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 101; and CDR3 is SEQ ID NO: 122;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 102; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 103; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 79, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 105; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 80, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 81, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 82, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 84, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;

- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 107; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 108; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 125;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 109; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 86, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 87, CDR2 is SEQ ID NO: 111; and CDR3 is SEQ ID NO: 127;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 128;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 129;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 113; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 114; and CDR3 is SEQ ID NO: 131;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 115; and CDR3 is SEQ ID NO: 131; and
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 116; and CDR3 is SEQ ID NO: 132.

The polypeptides of the invention may (essentially) consist of an immunoglobulin single variable domain selected from a light chain variable domain sequence (*e.g.,* a V_L-sequence) and from a heavy chain variable domain sequence (*e.g.,* a V_H-sequence). The polypeptides of the invention may (essentially) consist of an immunoglobulin single variable domain selected from a heavy chain variable domain sequence that is derived from a conventional four-chain antibody and from a heavy chain variable domain sequence that is derived from heavy chain antibody. The polypeptides of the invention may (essentially) consist of an immunoglobulin single variable domain selected from a domain antibody (or an amino acid that is suitable for use as a domain antibody), a single domain antibody (or an amino acid that is suitable for use as a single domain antibody), a “dAb” (or an amino acid that is suitable for use as a dAb), a Nanobody , a VHH sequence, a camelized VH sequence, or a VHH sequence that has been obtained by affinity maturation. In a preferred aspect, the polypeptide of the invention (essentially) consists of a partially or fully humanized Nanobody, such as a partially or fully humanized VHH.

Preferred polypeptides of the invention are selected from any of SEQ ID NOs: 1-71 and 268-275 or polypeptides that have a sequence identity of more than 80%, preferably more than 90%, more preferably more than 95%, such as 96%, 97%, 98%, 99% or more sequence identity (as defined herein) with any of SEQ ID NOs: 1-71 and 268-275.

The polypeptide provided by the invention (also referred to as “polypeptide of the invention”) is preferably in essentially isolated form (as defined herein), which may comprise, or (essentially) consist of one or more ISVDs and which may optionally further comprise one or more further immunoglobulins (all optionally linked via one or more suitable linkers).

More particularly, the present invention provides multivalent polypeptides comprising, or (essentially) consisting of at least two, at least three, at least four or at least five ISVDs that can bind GITR, wherein said at least two, said at least three, said at least four, or said at least five ISVDs can be the same or different and wherein said at least two, said at least three, said at least four or said at least five ISVDs are directly linked to each other or linked to each other via a linker.

Without being limiting, suitable linkers may be selected from the group of linkers with SEQ ID NOs: 247-263, of which shorter linker lengths are preferred. Some particularly preferred linkers comprise between 1 and 20 amino acid residues, such as between 2 and 10 amino acid residues, such as 2, 3, 4, 5, 6, 7, 8 or 9 amino acid residues. In particular linker 9GS (SEQ ID NO: 251) or linker 3A (SEQ ID NO: 247) are especially preferred.

In another aspect, the invention relates to a compound or construct (also referred to herein as a "*compound of the invention*" or "*construct of the invention*", respectively) that comprises or (essentially) consists of one or more polypeptides of the invention (or suitable fragments thereof), and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers. As will become clear to the skilled person from the further disclosure herein, such further groups, residues, moieties or binding units may or may not provide further functionality to the polypeptides of the invention (and/or to the compound, construct or compositions in which it is present) and may or may not modify the properties of the polypeptide of the invention.

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

Examples of polypeptides of the invention that comprise such half-life extending moieties will become clear to the skilled person based on the further disclosure herein; and for example include, without limitation, polypeptides in which the one or more polypeptides of the invention are suitable linked to one or more serum proteins or fragments thereof (such as (human) serum albumin or suitable fragments thereof) or to one or more binding units that can bind to serum proteins (such as, for example, domain antibodies, amino acids that are suitable for use as a domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"'s, amino acids that are suitable for use as a dAb, Nanobodies, VHH sequences, humanized VHH sequences, or camelized VH sequences that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG); reference is made to the further description and references mentioned herein); polypeptides in which a polypeptide of the invention is linked to an Fc portion (such as a human Fc) or a suitable part or fragment thereof; or polypeptides in which the one or more polypeptides of the invention are suitable linked to one or more small proteins or peptides that can bind to serum proteins (such as, without limitation, the proteins and peptides described in WO 91/01743, WO 01/45746, WO 02/076489).

In one aspect, the compound or construct according to the invention that provides the polypeptide with increased half-life is chosen from the group consisting of an antibody constant region or fragments thereof, wherein the antibody constant region or fragments thereof are derived from human IgG, such as IgG1, IgG2, IgG3 or IgG4. In particular, such antibody constant region comprises a CH1 heavy chain domain, a CH2 heavy chain domain, a CH3 heavy chain domain and/or a CL light chain domain.

In one specific aspect of the invention, a compound or construct of the invention comprises

i) a monovalent polypeptide of the invention, wherein said monovalent polypeptide is linked to a CH1 heavy chain domain, which is followed by a CH2 heavy chain domain and a CH3 heavy chain domain respectively; and/or

ii) a monovalent polypeptide of the invention, wherein said monovalent polypeptide is linked to a CL light chain domain (such as Cκ or Cλ).

Preferred heavy chain and/or light chain domains of the invention are of the IgG type and comprise an amino acid sequence set forth in one of SEQ ID NO: 229, SEQ ID NO: 230, SEQ ID NO: 266, SEQ ID NO: 267, SEQ ID NO: 291 and SEQ ID NO: 292 or an amino acid sequence that has a sequence identity of more than 80%, preferably more than 90%, more preferably more than 95%, such as 96%, 97%, 98%, 99% or more sequence identity (as defined herein) with any of SEQ ID NOs: 229-230, SEQ ID NOs: 266-267 and SEQ ID NOs: 291-292.

Generally, the compounds or constructs of the invention with increased half-life preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding polypeptide of the invention per se.

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

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

In a preferred aspect, the invention relates to a compound or construct as defined above, which is selected from any of SEQ ID NOs: 206-223 and 285-290 or compounds or constructs that have a sequence identity of more than 80%, preferably more than 90%, more preferably more than 95%, such as 96%, 97%, 98%, 99% or more sequence identity (as defined herein) with any of SEQ ID NOs: 206-223 and 285-290 (see Table A-11).

The invention also relates to nucleic acids or nucleotide sequences that encode a polypeptide, a compound and/or construct of the invention. 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.

Nucleic acids encoding a polypeptide, a compound and/or construct of the invention can be linked to obtain a nucleic acid encoding a multivalent polypeptide of the invention. Accordingly, the present invention also relates to the use of a nucleic acid or nucleotide sequence that encodes a polypeptide, a compound and/or construct of the invention for the preparation of a genetic construct that encodes a multivalent polypeptide of the invention.

The invention further relates to a host or host cell that expresses (or that under suitable circumstances is capable of expressing) a polypeptide, a compound and/or 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 invention further relates to a composition containing or comprising at least one polypeptide, compound and/or 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 composition may for example be a pharmaceutical composition (as described herein) or a veterinary composition. Some preferred but non-limiting examples of such compositions will become clear from the further description herein.

The invention further relates to methods for preparing polypeptides, compounds and/or constructs, nucleic acids, host cells, and composition described herein. The method for producing a polypeptide, compound and/or construct, nucleic acid, host cell, and composition of the invention may comprise the following steps:

a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid or nucleotide sequence of the invention, or a genetic construct of the invention; optionally followed by:

b) isolating and/or purifying the polypeptide, compound and/or construct of the invention thus obtained.

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

The polypeptides, compounds and/or constructs and compositions of the present invention can be used for enhancing an immune response.

In particular, the polypeptides, compounds and/or constructs and compositions of the present invention can be used for enhancing the proliferation or activation of T cells, B cells or natural killer cells.

The polypeptides, compounds and/or constructs and compositions of the present invention can be used for inhibiting tumor growth.

The polypeptides, compounds and/or constructs and compositions of the present invention can be used for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

The polypeptides, compounds and/or constructs and compositions of the present invention can be used for prevention and/or treatment of infectious diseases. Infections can be broadly classified as bacterial, fungal, viral, or parasitic based on the category of infectious organism or agent involved. Accordingly, the polypeptides, compounds and/or constructs and compositions of the present invention can be used for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

The polypeptides, compounds and/or constructs and compositions of the present invention can be used for prevention and/or treatment of cancer. Exemplary cancers whose growth can be inhibited using the polypeptides, compounds and/or constructs and compositions of the present invention include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

As such, the polypeptides, compounds and/or constructs and compositions of the present invention can be used for the prevention and/or treatment of cancer, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

The methods for enhancing an immune response, in particular enhancing proliferation or activation of T cells, B cells or natural killer cells and the method for inhibiting tumor growth that are described herein can be used to treat and prevent a wide variety of GITR associated diseases, disorders or conditions. For example, in one aspect, the present invention provides a method for prevention and/or treatment of T cell, B cell or natural killer cell associated diseases comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide, compound and/or construct and composition as described herein.

In another aspect, the present invention provides a method for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide, compound and/or construct and composition as described herein.

In yet another aspect, the present invention provides a method for prevention and/or treatment of cancer comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide, compound and/or construct and composition as described herein. In particular, the present invention provides a method for prevention and/or treatment of cancer, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

It will be further appreciated that the methods and compositions described herein can be used in combination with other agents or therapeutic modalities. In one aspect, the methods and compositions described herein are administered in combination with chemotherapy, radiation therapy, cancer vaccines or one or more additional therapeutic agents, or a combination of any of the foregoing. Exemplary therapeutic agents that can be administered in combination with the methods and compositions of the invention include PD-1, PD-L1, PD-L2, CTLA-4, 4-1 BB (CD137), 4-1BB ligand, OX40, OX40 ligand, CD27, TNFRSF25, TL1A, CD40, CD40 ligand, LIGHT, LTA, HVEM, BTLA, CD160, CEACAM-1, CEACAM-5, LAIR1, 2B4, TGFR, LAG-3, TIM-3, Siglecs, ICOS (CD278), ICOS ligand, B7-H3, B7-H4, B7-1, B7-2, VISTA, HHLA2, TMIGD2, BTNL2, CD244, CD48, CD2, CDS, TIGIT, PVR family members, KIRs, ILTs, LIRs, NKG2D, NKG2A, MICA, MICB, CSF1R, IDO, TGFβ, Adenosine, ICAM-1, ICAM-2, ICAM-3, LFA-1 (CD11a/CD18), LFA-2, LFA-3, BAFFR, NKG2C, SLAMF7, NKp80, CD83 ligand, CD24, CD39, CD30, CD70, CD73, CD7, CXCR4, CXCL12, Phosphatidylserine, SIRPA, CD47, VEGF and Neuropilin.

The invention further relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for enhancing an immune response. In particular, the invention relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for enhancing proliferation or activation of T cells, B cells or natural killer cells.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for inhibiting tumor growth.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of at least one GITR associated diseases, disorders or conditions. Some preferred but non-limiting diseases, disorders or conditions will become clear from the further description herein.

In particular, the invention relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention or composition of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of cancer, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

Other aspects, advantages, applications and uses of the polypeptides and compositions will become clear from the further disclosure herein. Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety.

FIGURE LEGENDS

Figures 1A-1D: Quality control of the Flp-In™-293 cells stably transfected with mouse GITR (A), human GITR (B) or cyno GITR (C) and of activated T cells (D). The MFI value (mean fluorescence intensity) is plotted for each cell line. Detection with secondary antibody only (i.e. without the anti-GITR antibody) is indicated with "/".

Figures 2A-2B: Dose dependent binding of monovalent anti-GITR Nanobodies to human GITR expressed on activated human T cells (A-B). The MFI value (mean fluorescence intensity) is plotted against the concentration of the Nanobody.

Figure 3: Dose dependent binding of multivalent anti-GITR Nanobodies and an irrelevant Nanobody (IRR00077) to HEK293_NFkB-Nluc2P human GITR cells**.** The MFI value (mean fluorescence intensity) is plotted against the concentration of the Nanobody.

Figures 4A-4D: GITR activation in GloResponse™ NF-κB-Nluc2P HEK293 luciferase reporter cells expressing human GITR. Activation is assessed by measuring luminescence. The RLU value (Relative Light Units) is plotted against the concentration of the Nanobody.

Figures 5A-5F: Effect of GITR activation on T cell activation. On each plate, a range of concentrations of one Nanobody construct and human GITR-ligand (hGITRL) (R&D Systems 6987-GL-025/CF) were tested. Each graph represents that data retrieved from one plate. Activation is measured by monitoring the IFN-γ expression.

Figures 6A-6D: Effect of the linker length of the Nanobody constructs assessed in the GloResponse™ NF-κB-Nluc2P HEK293 luciferase reporter cells expressing human GITR (A-D). In constructs A023100032 and A023100035 are the A0231005A03 Nanobodies (Family 7) linked by a 9GS linker. In constructs A023100034 and A023100022 are the A0231005A03 Nanobodies linked by a 35GS linker. In constructs A023100045, A023100082 and A023100085 are the A0231004B01 Nanobodies (Family 26) linked by a 3A linker. In constructs A023100083 and A023100084 are the A0231004B01 Nanobodies linked by a 9GS linker. In construct A023100014 is the A0231004B01 Nanobody linked by a 35GS linker.

Figure 7: Schematic representation of a Nanobody-human IgG1 chimera.

Figures 8A-8N: Effect of different tested compounds, alone or in combination with anti-PD-1 mAb, on anti-tumor activity measured by changes in the tumor volume in groups of mice treated with DTA-1 (Figure 8A); DTA-1 + anti-PD-1 mAb (Figure 8B); anti-GITR NB dose level 1 (Figure 8C); irrelevant NB dose level 1 (Figure 8D); anti-GITR NB dose level 2 (Figure 8E); irrelevant NB dose level 2 (Figure 8F); anti-GITR NB dose level 1 + anti-PD-1 mAb (Figure 8G); anti-GITR NB dose level 2 + anti-PD-1 mAb (Figure 8H); irrelevant NB dose level 1 + anti-PD-1 mAb (Figure 8I); irrelevant NB dose level 2 + anti-PD-1 mAb (Figure 8J); anti-GITR Nb-rat IgG2b chimera (Figure 8K); anti-GITR Nb-human IgG1 chimera (Figure 8L); anti-GITR Nb-human IgG1 chimera + anti-PD-1 mAb (Figure 8M) and vehicle (Figure 8N). CR denotes complete regression.

Figures 9A-9D: Effect of different tested compounds, alone or in combination with anti-PD-1 mAb, on survival during the course of treatment.

Figure 10A-10C: *In vitro* benchmarking of the anti-GITR Nanobodies as assessed in the T cell activation assay. On each plate, a range of concentrations of one Nanobody construct and the clinical stage 36E5 mAb were tested. Each graph represents that data retrieved from one plate. Activation is measured by monitoring the IFN-γ expression.

Figure 11: Adjuvant effect of anti-GITR multivalent Nanobody A023100035 and anti-GITR Nanobody-huIgG1 chimera (A-0231-00_TP008) measured as anti-OVA total IgG titres on days 13 and day 21 after OVA prime and day 14 boost immunization. SDL1: single dose level 1 (on day 0 and day 14); SDL2: single dose level 2 (on day 0 and day 14); RD1: repeated dosing regimen 1 (3 injections with 1 injection every 2 days starting on day 0 and day 14, Q2Dx3); RD2: repeated dosing regimen 2 (11 injections with 1 injection every 2 days, Q2Dx11).

Figures 12A-12D: GITR activation by multivalent sequence optimized Nanobodies in GloResponse™ NF-κB-Nluc2P HEK293 luciferase reporter cells expressing human GITR. Activation is assessed by measuring luminescence. The RLU value (Relative Light Units) is plotted against the concentration of the Nanobody.

Figures 13A-13G: Effect of GITR activation by multivalent sequence optimized Nanobodies on T cell activation. On each plate, a range of concentrations of one Nanobody construct and human GITR-ligand (hGITRL) (R&D Systems 6987-GL-025/CF) were tested. Each graph represents that data retrieved from one plate. Activation is measured by monitoring the IFN-γ expression.

Figures 14A-14O: Effect of different tested compounds, alone or in combination with anti-PD-1 mAb, on anti-tumor activity measured by changes in the tumor volume over time in groups of mice treated with vehicle (Figure 14A); DTA-1 (Figure 14B); DTA-1 + anti-PD-1 mAb (Figure 14C); huIgG1 isotype control (Figure 14D); huIgG1 isotype control + anti-PD-1 (Figure 14E); irrelevant NB (Figure 14F); irrelevant NB + anti-PD-1 mAb (Figure 14G); anti-GITR NB A023100101 (Figure 14H); anti-GITR NB A023100101 + anti-PD-1 mAb (Figure 14I); anti-GITR NB A023100107 (Figure 14J); anti-GITR NB A023100107 + anti-PD-1 mAb (Figure 14K); anti-GITR NB A023100118 (Figure 14L); anti-GITR NB A023100118 + anti-PD-1 mAb (Figure 14M); anti-GITR Nanobody-huIgG1 chimera (Figure 14N); anti-GITR Nanobody-huIgG1 chimera + anti-PD-1 mAb (Figure 14O). CR denotes complete regression.

Figures 15A-15B: Effect of different tested compounds, alone or in combination with anti-PD-1 mAb, on survival during the course of treatment.

DETAILED DESCRIPTION

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. (Molecular Cloning: A Laboratory Manual (2nd.Ed.) Vols. 1-3, Cold Spring Harbor Laboratory Press, 1989), F. Ausubel et al. (Current protocols in molecular biology, Green Publishing and Wiley Interscience, New York, 1987), Lewin (Genes II, John Wiley & Sons, New York, N.Y., 1985), Old et al. (Principles of Gene Manipulation: An Introduction to Genetic Engineering (2nd edition) University of California Press, Berkeley, CA, 1981); Roitt et al. (Immunology (6th. Ed.) Mosby/Elsevier, Edinburgh, 2001), Roitt et al. (Roitt's Essential Immunology (10th Ed.) Blackwell Publishing, UK, 2001), and Janeway et al. (Immunobiology (6th Ed.) Garland Science Publishing/Churchill Livingstone, New York, 2005), as well as to the general background art cited herein.

Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known per se, as will be clear to the skilled person. Reference is for example again made to the standard handbooks and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews Presta (Adv. Drug Deliv. Rev. 58 (5-6): 640-56, 2006), Levin and Weiss (Mol. Biosyst. 2(1): 49-57, 2006), Irving et al. (J. Immunol. Methods 248(1-2): 31-45, 2001), Schmitz et al. (Placenta 21 Suppl. A: S106-12, 2000), Gonzales et al. (Tumour Biol. 26(1): 31-43, 2005), 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 (which may for example be by any suitable method described herein).

By means of a non-limiting example, when a polypeptide of the 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 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).

By “essentially consist of” is meant that the immunoglobulin single variable domain used in the method of the 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 terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of the immunoglobulin single variable domain.

For the purposes of comparing two or more nucleotide sequences, the percentage of “sequence identity” between a first nucleotide sequence and a second nucleotide sequence may be calculated by dividing [the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence] by [the total number of nucleotides in the first nucleotide sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of a nucleotide in the second nucleotide sequence - compared to the first nucleotide sequence - is considered as a difference at a single nucleotide (position). Alternatively, the degree of sequence identity between two or more nucleotide sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0967284, EP 1085089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2357768. Usually, for the purpose of determining the percentage of “sequence identity” between two nucleotide sequences in accordance with the calculation method outlined hereinabove, the nucleotide sequence with the greatest number of nucleotides will be taken as the “first” nucleotide sequence, and the other nucleotide sequence will be taken as the “second” nucleotide sequence.

For the purposes of comparing two or more amino acid sequences, the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid identity") may be calculated by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), *i.e.,* as an "amino acid difference" as defined herein. Alternatively, the degree of sequence identity between two amino acid sequences may be calculated using a known computer algorithm, such as those mentioned above for determining the degree of sequence identity for nucleotide sequences, again using standard settings. Usually, for the purpose of determining the percentage of "sequence identity" between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the "first" amino acid sequence, and the other amino acid sequence will be taken as the "second" amino acid sequence.

Also, in determining the degree of sequence identity between two amino acid sequences, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 04/037999, GB 2357768, WO 98/49185, WO 00/46383 and WO 01/09300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO 04/037999 as well as WO 98/49185 and from the further references cited therein.

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

Any amino acid substitutions applied to the polypeptides described herein may also be based on the analysis of the frequencies of amino acid variations between homologous proteins of different species developed by Schulz et al. ("Principles of Protein Structure", Springer-Verlag, 1978), on the analyses of structure forming potentials developed by Chou and Fasman (Biochemistry 13: 211, 1974; Adv. Enzymol., 47: 45-149, 1978), and on the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al. (Proc. Natl. Acad Sci. USA 81: 140-144, 1984), Kyte and Doolittle (J. Molec. Biol. 157: 105-132, 1981), and Goldman et al. (Ann. Rev. Biophys. Chem. 15: 321-353, 1986), all incorporated herein in their entirety by reference. Information on the primary, secondary and tertiary structure of Nanobodies is given in the description herein and in the general background art cited above. Also, for this purpose, the crystal structure of a V_{HH} domain from a llama is for example given by Desmyter et al. (Nature Structural Biology, 3: 803, 1996), Spinelli et al. (Natural Structural Biology, 3: 752-757, 1996) and Decanniere et al. (Structure, 7 (4): 361, 1999). Further information about some of the amino acid residues that in conventional V_H domains form the V_H/V_L interface and potential camelizing substitutions on these positions can be found in the prior art cited above.

Amino acid sequences and nucleic acid sequences are said to be "exactly the same" if they have 100% sequence identity (as defined herein) over their entire length.

When comparing two amino acid sequences, the term "amino acid difference" refers to an insertion, deletion or substitution of a single amino acid residue on a position of the first sequence, compared to the second sequence; it being understood that two amino acid sequences can contain one, two or more such amino acid differences.

The "amino acid difference" can be any one, two, three or maximal four substitutions, deletions or insertions, or any combination thereof, that either improve the properties of the polypeptide of the invention or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of the polypeptide of the invention. In this respect, the resulting polypeptide of the invention should at least bind GITR with the same, about the same, or a higher affinity compared to the polypeptide comprising the one or more CDR sequences without the one, two, three or maximal four substitutions, deletions or insertions, said affinity as e.g. measured by surface plasmon resonance (SPR).

For example, and depending on the host organism used to express the polypeptide of the invention, such deletions and/or substitutions may be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the art.

A "Nanobody family", "VHH family" or "family" as used in the present specification refers to a group of Nanobodies and/or VHH sequences that have identical lengths (i.e. they have the same number of amino acids within their sequence) and of which the amino acid sequence between position 8 and position 106 (according to Kabat numbering) has an amino acid sequence identity of 89% or more.

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 the target of specificity of an immunoglobulin.

The part of an antigen-binding molecule (such as an immunoglobulin, a conventional antibody, an immunoglobulin single variable domain and/or a polypeptide of the invention) that recognizes the epitope is called a “paratope”.

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

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 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) 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}) is generally considered to indicate non-specific binding. Preferably, a monovalent polypeptide of the invention will bind to the desired antigen with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as *e.g.,* between 10 and 5 nM or less. 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 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.

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 or more better than the affinity with which the 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 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.

The terms “(cross)-block”, “(cross)-blocked”, “(cross)-blocking”, “competitive binding”, “(cross)-compete”, “(cross)-competing” and “(cross)-competition” are used interchangeably herein to mean the ability of an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent to interfere with the binding of other immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or binding agents to a given target. The extent to which an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent is able to interfere with the binding of another to the target, and therefore whether it can be said to cross-block according to the invention, can be determined using competition binding assays. Particularly suitable quantitative cross-blocking assays are described in the Examples and include e.g. a fluorescence-activated cell sorting (FACS) binding assay with GITR expressed on cells. The extent of (cross)-blocking can be measured by the (reduced) channel fluorescence.

The following generally describes a suitable FACS assay for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent cross-blocks or is capable of cross-blocking according to the invention. It will be appreciated that the assay can be used with any of the immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or other binding agents described herein. The FACS instrument (e.g. FACS Canto; Becton Dickinson) is operated in line with the manufacturer's recommendations.

To evaluate the "(cross)-blocking" or "(cross)-competition" between two binding agents (such as e.g. two immunoglobulin single variable domains and/or Nanobodies) for binding GITR, a FACS competition experiment can be performed using cells (such as e.g. Flp-In™-293 cells) overexpressing human GITR and the parental cells as background cell line. Different detection reagents can be used including e.g. monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, cat# F1804), monoclonal anti-C-myc antibody (Sigma-Aldrich, cat# WH0004609M2), monoclonal ANTI-HIS TAG antibody (Sigma-Aldrich, cat# SAB1305538), each labeled differently. A wide range of fluorophores can be used as labels in flow cytometry (such as e.g PE (R-Phycoerythrin), 7-aminoactinomycin D (7-AAD), Acridine Orange, various forms of Alexa Fluor, Allophycocyanin (APC), AmCyan, Aminocoumarin, APC Cy5, APC Cy7, APC-H7, APC/Alexa Fluor 750, AsRed2, Azami-Green, Azurite, B ODIPY FL C5-ceramide, BCECF-AM, Bis-oxonol DiBAC2(3), BODIPY-FL, Calcein, Calcein AM, Caroxy-H2DCFDA, Cascade Blue, Cascade Yellow, Cell Tracker Green, Cerulean, CFSE, Chromomycin A3, CM-H2DCFDA, Cy2, Cy3, Cy3.5, Cy3B, Cy5, Cy5.5, Cy7, CyPet, DAF-FM DAF-FM diacetate, DAPI, DCFH (2'7'Dichorodihydrofluorescein), DHR, Dihydrocalcein AM, Dihydrorhoadamine, Dihydrothidium, DiLC1(5), DiOC6(3), DiOC7(3), dKeima-Red, DRAQ5, Dronpa-Green, various forms of DsRed dTomato, various forms of DyLight, *E.coli* BioParticles AF488, E2-Crimson, E2-Orange, EBFP2, ECFP, various forms of eFluor, EGFP, EGFP*, Emerald, eqFP650, eqFP670, ER-Tracker Blue-White DPX, Ethidium Bromide, Express2, EYFP, Fc OxyBurst Green, Fc OxyBurst Green 123, FITC, Fluo-3, Fluo-4, Fluorescein, Fura-2, Fura-Red, GFPuv, H2DCFDA, HcRed1, Hoechst Blue (33258), Hoechst Red (33342), Hydroxycoumarin, HyPer, Indo-1, Indo-1 Blue (Low Ca2+), Indo-1 Violet (High Ca2+), iRFP, J-Red, JC-1, JC-9, Katushka (TurboFP635), Katushka2 Kusabira-Orange, LDS 751, Lissamine Rhodamine B, various forms of Live/Dead, Lucifer yellow, Lucifer Yellow CH, Lyso Tracker Blue, Lyso Tracker Green, Lyso Tracker Red, mAmertrine, Marina Blue, mBanana, mCFP, mCherry, mCitrine, Methoxycoumarin, mHoneyDew, Midoriishi-Cyan, Mithramycin, Mito Tracker Deep Red, Mito Tracker Green, Mito Tracker Orange, Mito Tracker Red, MitoFluor Green, mKate (TagFP635), mKate2, mKeima, mKeima-Red, mKO, mKOk, mNeptune, Monochlorobimane, mOrange, mOrange2, mRaspberry, mPlum, mRFP1, mStrawberry, mTangerine, mTarquoise, mTFP1, mTFP1 (Teal), NBD, OxyBurst Green H2DCFDA, OxyBurst Green H2HFF BSA, Pacific Blue, PE (R-Phycoerythrin), PE Cy5, PE Cy5.5, PE Cy7, PE Texas Red, PE-Cy5 conjugates, PE-Cy7 conjugates, PerCP (Peridinin chlorphyll protein), PerCP Cy5.5, PhiYFP, PhiYFP-m, Propidium Iodide (PI), various forms of Qdot, Red 613, RFP Tomato, Rhod-2, S65A, S65C, S65L, S65T, Singlet Oxygen Sensor Green, Sirius, SNARF, Superfolder GFP, SYTOX Blue, SYTOX Green, SYTOX Orange, T-Sapphire, TagBFP, TagCFP, TagGFP, TagRFP, TagRFP657, TagYFP, tdTomato, Texas Red, Thiazole Orange, TMRE, TMRM, Topaz, TOTO-1, TO-PRO-1, TRITC, TRITC TruRed, TurboFP602, TurboFP635, TurboGFP, TurboRFP, TurboYFP, Venus, Vybrant CycleDye Violet, Wild Type GFP, X-Rhodamin, Y66F, Y66H, Y66W, YOYO-1, YPet, ZsGreen1, ZsYellow1, Zymosan A BioParticles AF488 (see more at: http://www.thefcn.org/flow-fluorochromes). Fluorophores, or simply "fluors", are typically attached to the antibody (e.g. the immunoglobulin single variable domains, such as Nanobodies) that recognizes GITR or to the antibody that is used as detection reagent. Various conjugated antibodies are available, such as (without being limiting) for example antibodies conjugated to Alexa Fluor®, DyLight®, Rhodamine, PE, FITC, and Cy3. Each fluorophore has a characteristic peak excitation and emission wavelength. The combination of labels which can be used will depend on the wavelength of the lamp(s) or laser(s) used to excite the fluorophore and on the detectors available.

To evaluate the competition between two test binding agents (termed A and B) for binding to GITR, a dilution series of cold (without any label) binding agent A is added to (e.g. 200 000) cells together with the labeled binding agent B*. The concentration of binding agent B* in the test mix should be high enough to readily saturate the binding sites on GITR expressed on the cells. The concentration of binding agent B* that saturates the binding sites for that binding agent on GITR expressed on the cells can be determined with a titration series of binding agent B* on the GITR cells and determination of the EC_{50} value for binding. In order to work at saturating concentration, binding agent B* can be used at 100x the EC_{50} concentration.

After incubation of the cells with the mixture of binding agent A and binding agent B* and cells wash, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded.

A separate solution of binding agent B* is also prepared. Binding agent B* in this solutions should be in the same buffer and at the same concentration as in the test mix (with binding agent A and B*). This separate solution is also added to the cells. After incubation and cells wash, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded.

A reduction of fluorescence for the cells incubated with the mixture of binding agent A and B* compared to the fluorescence for the cells incubated with the separate solution of binding agent B* indicates that binding agent A (cross)-blocks binding by binding agent B* for binding to GITR expressed on the cells.

A cross-blocking immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent according to the invention is one which will bind to the GITR in the above FACS cross-blocking assay such that during the assay and in the presence of a second immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent the recorded fluorescence is between 80% and 0.1% (e.g. 80% to 4%) of the maximum fluorescence (measured for the separate labelled immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent), specifically between 75% and 0.1% (e.g. 75% to 4%) of the maximum fluorescence, and more specifically between 70% and 0.1% (e.g. 70% to 4%) of maximum fluorescence (as just defined above).

The competition between two test binding agents (termed A* and B*) for binding to GITR can also be evaluated by adding both binding agents, each labeled with a different fluorophore, to the GITR expressing cells. After incubation and cells wash, read out can be performed on a FACS. A gate is set for each fluorophore and the total amount of channel fluorescence is recorded. Reduction and/or absence of fluorescence of one of the fluorophore indicate (cross)-blocking by the binding agents for binding to GITR expressed on the cells.

Other methods for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent directed against a target (cross)-blocks, is capable of (cross)-blocking, competitively binds or is (cross)-competitive as defined herein are described e.g. in Xiao-Chi Jia et al. (Journal of Immunological Methods 288: 91–98, 2004), Miller et al. (Journal of Immunological Methods 365: 118–125, 2011) and/or the methods described herein (see e.g. Example 7).

An amino acid sequence 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.,* GITR from different species of mammal, such as e.g., human GITR, cyno GITR and rat GITR) if it is specific for (as defined herein) these different antigens or antigenic determinants.

The term “glucocorticoid-induced TNF receptor”, hereinafter referred to as “GITR” is also known as Tumor Necrosis Receptor Superfamily 18 (TNFRSF18), Activation-Inducible TNFR Family Receptor (AITR), TEASR, CD357 and 312C2. GITR is constitutively expressed in all T cell subtypes and mostly in regulatory T cells (Treg), is up-regulated in CD4$^+$CD25$^-$ and CD8$^+$CD25$^-$ effector cells following TCR stimulation and cell activation (Nocentini et al. 2007, Eur J Immunol. 37:1165-1169). GITR acts as a costimulatory molecule in effector T cell activation and regulates Treg cell suppressor activity (Esparza et al. 2005, J Immunol. 174:7869-7874).

In the context of the present invention, “enhancing” or “to enhance” generally means increasing, potentiating or stimulating the activity of GITR, as measured using a suitable *in vitro*, cellular or *in vivo* assay (such as those mentioned herein). In particular, increasing or enhancing the activity of GITR, as measured using a suitable *in vitro*, cellular or *in vivo* assay (such as those mentioned herein), by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as

100%, compared to the activity of GITR in the same assay under the same conditions but without the presence of the polypeptide of the invention.

A "synergistic effect" of two compounds is one in which the effect of the combination of the two agents is greater than the sum of their individual effects and is preferably statistically different from the controls and the single drugs.

As used herein, the term “T cell, B cell or natural killer cell mediated disease” refers to any disease mediated by T cells (including effector T cells (e.g. , $CD8^+$ cells) and helper T cells (e.g., $CD4^+$ cells)), B cells or natural killer cells.

A “GITR associated disease, disorder or condition” refers to disease or symptom associated with the disease that is treatable by inducing, stimulating, or enhancing GITR activity, e.g. via the use of an agonist GITR polypeptide as described herein. Exemplary GITR associated diseases, disorders or conditions include, but are not limited to, cancer and infectious diseases.

As used herein, an "agonist" refers to a compound that partially or fully increases, enhances, induces or stimulates one or more biological activities of a corresponding target (*e.g.*, GITR) *in vitro* or *in vivo*. Examples of such biological activities of GITR include promoting CD4+ and CD8+ T cell survival, proliferation, NF-κB signaling, interleukin-2 production and effector functions and abrogate Treg cell suppressive effects or the generation of Treg cells. As will be clear to the skilled person, such an increase in biological activity may be determined in any suitable manner and/or using any suitable (*in vitro*, cellular or *in vivo*) assay known per se, such as the assays described herein or in the prior art cited herein. In particular, the biological activity may be increased, by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100%, compared to the biological activity in the same assay under the same conditions but without the presence of the polypeptide of the invention.

Full agonists are capable of maximal receptor stimulation (functional response), i.e. they elicit substantially the same level of full response as the endogenous ligand of the receptor (E= Emax= 100%). Here, the term “substantially the same”, means that the efficacy of a test compound ranges from 70% to 150%, more preferably from 80% to 140%, such as 90% to 120% compared to the maximal efficacy of said endogenous ligand measured in the same experimental setup and set at a 100%.

Partial agonists are unable to elicit maximal activity of the receptor, even at saturating concentrations. In other words, the maximum magnitude of the functional response produced by a full agonist of a target molecule (*e.g.*, GITR) cannot be produced by a partial agonist of the same target molecule, even by increasing the dosage of the partial agonist.

The terms “enhancing an immune response” and “inducing an immune response” are used interchangeably herein and refer to a process that results in the activation, stimulation or proliferation of one or more cellular response(s) of either T cells, B cells and/or natural killer (NK) cells. The polypeptides of the invention are capable of inducing proliferation or activation of T cells, B cells or natural killer cells. Suitable assays to measure T cell, B cell and natural killer cell activation are known in the art described herein, for instance as described in Buillard et al. 2013, J. Exp. Med. Vol. 210, 9: 1685-1693; Zhou et al. October 2010, J. Immunother. Vol. 33, No 8; and Hanabuchi 2006, Blood, Vol. 107, No 9: 3617–3623, respectively, or as exemplified in the examples below.

As used herein, the term "inhibits tumor cell growth" is intended to include any measurable decrease in the proliferation of tumor cells *in vitro* or tumor growth *in vivo*, e.g., decrease by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100%.

As used herein, the term "potency" is a measure of an agent, such as a polypeptide, ISVD or Nanobody, its biological activity. Potency of an agent can be determined by any suitable method known in the art, such as for instance as described in the experimental section. Cell culture based potency assays are often the preferred format for determining biological activity since they measure the physiological response elicited by the agent and can generate results within a relatively short period of time. Various types of cell based assays, based on the mechanism of action of the product, can be used, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays (e.g. NF-κB luciferase reporter assay), T cell activation assay, cell surface receptor binding assays and assays to measure expression of known markers of activation or cytokine secretion, all well known in the art.

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 efficacy of a polypeptide of the invention can be evaluated using *in vivo* models, such as the OVA immunization model or the syngeneic CT-26 colon carcinoma model (for instance as set out in the Examples section).

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 t1/2-alpha, t1/2-beta and the area under the curve (AUC). Reference is for example made to the standard handbooks, such as Kenneth et al (Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists, John Wiley & Sons Inc, 1986) and M Gibaldi and D Perron ("Pharmacokinetics", Marcel Dekker, 2nd Rev. Edition, 1982). 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 t1/2-beta, either with or without an increase in the t1/2-alpha and/or the AUC or both.

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

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

The term “immunoglobulin domain” as used herein refers to a globular region of an antibody chain (such as *e.g.*, a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a 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 “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 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 “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 this case, 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 (associated) 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).

In one embodiment of the invention, the immunoglobulin single variable domains are heavy chain variable domain sequences (*e.g.,* a VH-sequence); more specifically, the immunoglobulin single variable domains can be heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.

For example, the immunoglobulin single variable domain may be a (single) domain antibody (or an amino acid that is suitable for use as a (single) domain antibody), a "dAb" or dAb (or an amino acid that is suitable for use as a dAb) or a Nanobody (as defined herein, and including but not limited to a VHH); other single variable domains, or any suitable fragment of any one thereof.

In particular, the immunoglobulin single variable domain may be a Nanobody® (as defined herein) or a suitable fragment thereof. [Note: Nanobody®, Nanobodies® and Nanoclone® are registered trademarks of Ablynx N.V.] For a general description of Nanobodies, reference is made to the further description below, as well as to the prior art cited herein, such as e.g. described in WO 08/020079 (page 16).

“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. Nature 363: 446-448, 1993). The term “VHH domain” has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as “V_H domains” or “VH domains”) and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as “V_L domains” or “VL domains”). For a further description of VHH’s and Nanobodies, reference is made to the review article by Muyldermans (Reviews in Molecular Biotechnology 74: 277-302, 2001), as well as to the following patent applications, which are mentioned as general background art: WO 94/04678, WO 95/04079 and WO 96/34103 of the Vrije Universiteit Brussel; WO 94/25591, WO 99/37681, WO 00/40968, WO 00/43507, WO 00/65057, WO 01/40310, WO 01/44301, EP 1134231 and WO 02/48193 of Unilever; WO 97/49805, WO 01/21817, WO 03/035694, WO 03/054016 and WO 03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO 03/050531 of Algonomics N.V. and Ablynx N.V.; WO 01/90190 by the National Research Council of Canada; WO 03/025020 (= EP 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 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 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 description of Nanobodies, reference is made to the prior art cited herein, such as *e.g.,* described in WO 08/020079 (page 16).

"Domain antibodies", also known as "Dab"s, "Domain Antibodies", and "dAbs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in *e.g.,* EP 0368684, Ward et al. (Nature 341: 544-546, 1989), Holt et al. (Tends in Biotechnology 21: 484-490, 2003) and WO 03/002609 as well as for example WO 04/068820, WO 06/030220, WO 06/003388 and other published patent applications of Domantis Ltd. Domain antibodies essentially correspond to the VH or VL domains of non-camelid mammalians, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, *i.e.,* without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences. Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutical use in humans.

It should also be noted that, although less preferred in the context of the present invention because they are not of mammalian origin, single variable domains can be derived from certain species of shark (for example, the so-called "IgNAR domains", see for example WO 05/18629).

Thus, in the meaning of the present invention, the term "immunoglobulin single variable domain" or "single variable domain" comprises polypeptides which are derived from a non-human source, preferably a camelid, preferably a camelid heavy chain antibody. They may be humanized, as previously described. Moreover, the term comprises polypeptides derived from non-camelid sources, e.g. mouse or human, which have been "camelized", as *e.g.,* described in Davies and Riechmann (FEBS 339: 285-290, 1994; Biotechnol. 13: 475-479, 1995; Prot. Eng. 9: 531-537, 1996) and Riechmann and Muyldermans (J. Immunol. Methods 231: 25-38, 1999).

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

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

Determination of CDR regions may also be done according to different methods. 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.

In the present application, however, CDR sequences were determined according to Kontermann and Dübel (Eds., Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51, 2010). According to this method, FR1 comprises the amino acid residues at positions 1-25, CDR1 comprises the amino acid residues at positions 26-35, FR2 comprises the amino acids at positions 36-49, CDR2 comprises the amino acid residues at positions 50-58, FR3 comprises the amino acid residues at positions 59-94, CDR3 comprises the amino acid residues at positions 95-102, and FR4 comprises the amino acid residues at positions 103-113 (according to Kabat numbering).

Immunoglobulin single variable domains such as Domain antibodies and Nanobodies (including VHH domains) can be subjected to humanization. In particular, humanized immunoglobulin single variable domains, such as Nanobodies (including VHH domains) may be immunoglobulin single variable domains that are as generally defined for in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, at least one framework residue) that is and/or that corresponds to a humanizing substitution (as defined herein). 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.

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

The process of designing/selecting and/or preparing a polypeptide, starting from an immunoglobulin single variable domain such as a Domain antibody or a Nanobody, is also referred to herein as “formatting” said immunoglobulin single variable domain; and an immunoglobulin single variable domain that is made part of a polypeptide is said to be “formatted” or to be “in the format of” said polypeptide. 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; and such formatted immunoglobulin single variable domain form a further aspect of the invention.

For example, and without limitation, one or more immunoglobulin single variable domains may be used as a “binding unit”, “binding domain” or “building block” (these terms are used interchangeable) for the preparation of a polypeptide, which may optionally contain one or more further immunoglobulin single variable domains that can serve as a binding unit (*i.e.,* against the same or another epitope on GITR and/or against one or more other antigens, proteins or targets than GITR).

Monovalent polypeptides comprise or essentially consist of only one binding unit (such as *e.g.,* immunoglobulin single variable domains). Polypeptides that comprise two or more binding units (such as *e.g.,* immunoglobulin single variable domains) will also be referred to herein as "multivalent" polypeptides, and the binding units/immunoglobulin single variable domains present in such polypeptides will also be referred to herein as being in a "multivalent format". For example a "bivalent" polypeptide may comprise two immunoglobulin single variable domains, optionally linked via a linker sequence, whereas a "trivalent" polypeptide may comprise three immunoglobulin single variable domains, optionally linked via two linker sequences; whereas a "tetravalent" polypeptide may comprise four immunoglobulin single variable domains, optionally linked via three linker sequences; whereas a "pentavalent" polypeptide may comprise five immunoglobulin single variable domains, optionally linked via four linker sequences; whereas a "hexavalent" polypeptide may comprise six immunoglobulin single variable domains, optionally linked via five linker sequences, etc.

In a multivalent polypeptide, the two or more immunoglobulin single variable domains may be the same or different, and may be directed against the same antigen or antigenic determinant (for example against the same part(s) or epitope(s) or against different parts or epitopes) or may alternatively be directed against different antigens or antigenic determinants; or any suitable combination thereof. Polypeptides that contain at least two binding units (such as *e.g.,* immunoglobulin single variable domains) in which at least one binding unit is directed against a first antigen (*i.e.,* GITR) and at least one binding unit is directed against a second antigen (*i.e.,* different from GITR) will also be referred to as "multispecific" polypeptides, and the binding units (such as *e.g.,* immunoglobulin single variable domains) present in such polypeptides will also be referred to herein as being in a "multispecific format". Thus, for example, a "bispecific" polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain directed against a first antigen (*i.e.,* GITR) and at least one further immunoglobulin single variable domain directed against a second antigen (*i.e.,* different from GITR), whereas a "trispecific" polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain directed against a first antigen (*i.e.,* GITR), at least one further immunoglobulin single variable domain directed against a second antigen (*i.e.,* different from GITR) and at least one further immunoglobulin single variable domain directed against a third antigen (*i.e.,* different from both GITR and the second antigen); etc.

"Multiparatopic polypeptides", such as *e.g.,*" biparatopic polypeptides" or "triparatopic polypeptides", comprise or essentially consist of two or more binding units that each have a different paratope (as will be further described herein).

GITR agonists

The present invention provides polypeptides (also referred to herein as "polypeptides of the invention") that have specificity for and/or that bind GITR, preferably human GITR. GITR also known as TNFRSF18, AITR, CD357, TEASR or 312C2, is a protein that, in humans, is encoded by the TNFRSF18 gene, which maps on chromosome 1, at 1p36.3 according to Entrez Gene. The polypeptides of the invention preferably bind to human GITR (SEQ ID NO: 231).

The polypeptides provided by the present invention are GITR agonists and can thus induce, increase, stimulate or enhance GITR signaling. Activating the GITR biological pathway modulates T cell activation and enhances immune responses. Accordingly, the polypeptides provided by the present invention can be used in a variety of immunotherapeutic applications, such as in the treatment of a variety of cancers, immune disorders and infectious diseases, as will be further defined herein.

Based on extensive screening, characterization and combinatory strategies, the present inventors surprisingly observed that polypeptides comprising immunoglobulin single variable domains binding GITR showed improved properties for modulating GITR activity compared to the GITR agonizing molecules described in the prior art. More specifically, the present inventors surprisingly observed that the polypeptides of the present invention exhibited higher efficacies at equipotent or even lower EC_{50} values as compared to the prior art antibodies. This is clinically very important as the effectiveness of a drug depends on its maximal efficacy.

Accordingly, the present invention provides GITR agonists with particular functional properties which are linked with improved and desirable therapeutic and/or pharmacological properties, in addition to other advantageous properties (such as, for example, improved ease of preparation, good stability, and/or reduced costs of goods), compared to the prior art amino acid sequences and antibodies.

Binding of the polypeptides of the invention to GITR can be measured in binding assays. Typical assays include (without being limiting) assays in which GITR is exposed on a cell surface (such as e.g. Flp-In™-293 cells or GloResponse™ NF-κB-Nluc2P HEK293 cells). A preferred assay for measuring binding of the polypeptides of the invention to GITR is a FACS assay, such as e.g. the FACS assay as described in the examples, wherein binding of the polypeptides of the invention to GITR expressed on Flp-In™-293 cells and/or activated T cells is determined. Some preferred EC_{50} values for binding of the polypeptides of the invention to GITR will become clear from the further description and examples herein.

In such FACS binding assay, the polypeptides of the present invention may have EC_{50} values in binding human GITR of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, or even of 10^{-10} M or lower, such as 10^{-11} M. For example, in such FACS binding assay, the polypeptides of the present invention may have EC_{50} values in binding human GITR between 10^{-11} M and 10^{-8} M, such as between 10^{-9} M and 10^{-8} M, between 10^{-10} M and 10^{-9} M or between 10^{-11} M and 10^{-10} M.

The polypeptides of the invention bind GITR and can modulate (i.e. increase, enhance, stimulate or potentiate) the activity of GITR. More particularly, the polypeptides of the present invention may enhance an immune response.

Accordingly, in one aspect, the present invention relates to a polypeptide that specifically binds GITR with an EC_{50} of less than 200 pM, and wherein the binding of said polypeptide to said GITR enhances an immune response. More particularly, the polypeptides of the present invention enhance proliferation or activation of T cells, B cells or natural killer cells.

Proliferation or activation of T cell, B cells or natural killer cells can be determined by a variety of assays, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays (e.g. NF-κB luciferase reporter assay), T cell activation assay, cell surface receptor binding assays and assays to measure expression of known markers of activation or cytokine secretion, which are all well known in the art.

For example, any one of several conventional assays for monitoring cytokine production (such as IFN-γ and interleukins) as a measure of immune cells activation can be used. For example, for tracking T cell activation, interleukin-2 can be employed as a marker, which can be assayed as described in Proc. Natl. Acad. Sci. USA. 86:1333 (1989).

One can also employ immunofluorescence and flow cytometry to monitor cytokine production on a cellular basis, and to monitor cell surface markers that reflect cellular activation states. A host of such markers are known, detecting antibodies are broadly commercially available, and the markers are well known in the art.

A common assay for T cell proliferation entails measuring tritiated thymidine incorporation. The proliferation of T cells can be measured *in vitro* by determining the amount of ^{3}H-labeled thymidine incorporated into the replicating DNA of cultured cells. Therefore, the rate of DNA synthesis and, in turn, the rate of cell division can be quantified.

Some preferred EC_{50} values for activating GITR by the polypeptides of the invention will become clear from the further description and examples herein.

In some embodiments, the polypeptides of the invention enhance IFN-gamma production in a T-cell activation assay with activated $CD4^+$ T cells stimulated with anti-CD3 antibody OKT3, as described in Example 10. In this T cell activation assay, the polypeptides of the present invention have EC_{50} values for enhancing IFN-gamma production of 10^{-7} M or lower, preferably of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, 10^{-10} M or lower, or even of 10^{-11} M or lower. More particularly, in this T-cell activation assay, the polypeptides of the present invention enhance IFN-gamma production with EC_{50} values of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40 or even less, such as less than 30 pM.

Accordingly, the present invention relates to a polypeptide that specifically binds to GITR, and wherein the binding of said polypeptide to said GITR enhances IFN-gamma production in T cells with an EC_{50} of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40 or even less, such as less than 30 pM, as measured in a T-cell activation assay with activated $CD4^+$ T cells stimulated with anti-CD3 antibody OKT3 (as described in Example 10).

In some embodiments, the polypeptides of the invention enhance the activity of nuclear factor-kappa B (NF-κB) in a NF-κB luciferase reporter assay, as described in Example 9 and 18. NF-κB luciferase reporter assays have been described in Buillard et al. 2013, J. Exp. Med. Vol. 210, 9: 1685-1693. Some preferred EC_{50} values for activating GITR by the polypeptides of the invention will become clear from the further description and examples herein.

NF-κB plays a key role in inflammation, immune response and cell proliferation. This assay is specifically designed to monitor the activity of NF-κB regulated signal transduction pathways in cultured cells. In this NF-κB luciferase reporter assay, the polypeptides of the present invention enhance NF-κB activity as measured by luminescence after addition of Nano-Glo™ Reagent (Promega #N1120) with EC_{50} values of 10^{-7} M or lower, preferably of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, 10^{-10} M or lower, or even of 10^{-11} M or lower. More particularly, in this NF-κB luciferase reporter assay, the polypeptides of the present invention enhance NF-κB activity with EC_{50} values of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40, 35, 30, 25, 20, 18, 16, 15, 14 or even less, such as less than 12 pM.

Accordingly, the present invention relates to a polypeptide that specifically binds to GITR, and wherein the binding of said polypeptide to said GITR enhances NF-κB activity with an EC_{50} of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40, 35, 30, 25, 20, 18, 16, 15, 14 or even less, such as less than 12 pM, as measured in a NF-κB luciferase reporter assay (as described in Example 9 and 18).

Therapeutic effects of the polypeptides of the invention can further be evaluated in *in vivo* models, such as e.g. in mice, rats, pigs and/or primates. The CT26 model in BALB/c mice provides a syngeneic *in vivo* test system, which is frequently used for developing and testing immunotherapeutic concepts (Fearon et al. Cancer Res. 48: 2975-2980, 1988). For example, in the syngeneic CT-26 colon carcinoma model as described in Examples 13, 14 and 21, the polypeptides of the invention may inhibit tumor cell growth. In some embodiments, the polypeptides of the invention inhibit tumor cell growth, inhibit or prevent an increase in tumor weight or volume, and/or cause a decrease in tumor weight or volume by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least

55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100%.

Accordingly, the present invention relates to a polypeptide that specifically binds to GITR, and wherein the binding of said polypeptide to said GITR inhibits tumor cell growth, in a syngeneic CT-26 colon carcinoma model (as described in Examples 13, 14 and 21).

Monovalent polypeptides of the invention

The present invention provides stretches of amino acid residues (SEQ ID NOs: 73-88, SEQ ID NOs: 90-116, and SEQ ID NOs: 118-132 and 282-284; Table A-10) that are particularly suited for binding GITR. In particular, the invention provides stretches of amino acid residues which bind GITR and wherein the binding of said stretches to said GITR enhances an immune response (as described above). These stretches of amino acid residues may be present in, and/or may be incorporated into, a polypeptide of the invention, in particular in such a way that they form (part of) the antigen binding site of the polypeptide of the invention. These stretches of amino acid residues have been generated as CDR sequences of heavy chain antibodies or V_{HH} sequences that were raised against GITR. These stretches of amino acid residues are also referred to herein as "*CDR sequence(s) of the invention*" (*i.e.,* as "CDR1 sequence(s) of the invention", "CDR2 sequence(s) of the invention" and "CDR3 sequence(s) of the invention", respectively).

It should however be noted that the invention in its broadest sense is not limited to a specific structural role or function that these stretches of amino acid residues may have in a polypeptide of the invention, as long as these stretches of amino acid residues allow the polypeptide of the invention to bind to GITR with a certain affinity and potency (as defined herein). Thus, generally, the invention in its broadest sense provides monovalent polypeptides (also referred to herein as "*monovalent polypeptide(s) of the invention*") that are capable of binding to GITR with a certain specified affinity, avidity, efficacy and/or potency and that comprises one or more CDR sequences as described herein and, in particular a suitable combination of two or more such CDR sequences, that are suitably linked to each other via one or more further amino acid sequences, such that the entire polypeptide forms a binding domain and/or binding unit that is capable of binding to GITR. It should however also be noted that the presence of only one such CDR sequence in a monovalent polypeptide of the invention may by itself already be sufficient to provide the monovalent polypeptide of the invention the capacity of binding to GITR; reference is for example made to the so-called "Expedite fragments" described in WO 03/050531.

In a specific, but non-limiting aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequences:

- (a) SEQ ID NOs: 73-88; and
- (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 73-88; and/or

(ii) CDR2 sequences:

- (c) SEQ ID NOs: 90-116; and
- (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 90-116; and/or

(iii) CDR3 sequences:

- (e) SEQ ID NOs: 118-132 and 282-284; and
- (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 118-132 and 282-284.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequences:

- (a) SEQ ID NOs: 73-75; and
- (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 73; and/or

(ii) CDR2 sequences:

- (c) SEQ ID NOs: 90-98; and
- (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 90; and/or

(iii) CDR3 sequences:

- (e) SEQ ID NOs: 118-119, 123 and 282-284; and
- (f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 118.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequences:

- (a) SEQ ID NO: 73; and
- (b) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 73, wherein
  - at position 2 the T has been changed into S;
  - at position 7 the D has been changed into N;
  - at position 8 the S has been changed into A; and/or
  - at position 10 the A has been changed into G;

and/or

- (ii) CDR2 sequences:
  - (c) SEQ ID NO: 90; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 90, wherein
    - at position 1 the A has been changed into H, T, or G;
    - at position 2 the I has been changed into M;
    - at position 3 the T has been changed into S;
    - at position 6 the G has been changed into S;
    - at position 7 the S has been changed into R, or G; and/or
    - at position 8 the P has been changed into S, T, or R

and/or

- (iii) CDR3 sequences:
  - (e) SEQ ID NO: 118; and
  - (f) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 118, wherein
    - at position 9 the A has been changed into P;
    - at position 11 the M has been changed into L, K, R, or Q; and/or
    - at position 12 the D has been changed into N.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequence SEQ ID NO: 73; and/or
- (ii) CDR2 sequence SEQ ID NO: 90; and/or
- (iii) CDR3 sequence SEQ ID NO: 118, or

- (i) CDR1 sequence SEQ ID NO: 73; and/or
- (ii) CDR2 sequence SEQ ID NO: 90; and/or
- (iii) CDR3 sequence SEQ ID NO: 123.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequences:
  - (a) SEQ ID NOs: 76-78; and
  - (b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 76; and/or

- (ii) CDR2 sequences:
  - (c) SEQ ID NOs: 99-103; and
  - (d) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 99; and/or
- (iii) CDR3 sequences:
  - (e) SEQ ID NOs: 120-123; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 120.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequences:
  - (a) SEQ ID NO: 76; and
  - (b) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 76, wherein
    - at position 7 the D has been changed into N; and/or
    - at position 8 the S has been changed into A;

and/or

- (ii) CDR2 sequences:
  - (c) SEQ ID NO: 99; and
  - (d) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 99, wherein
    - at position 1 the A has been changed into S, or T;
    - at position 5 the S has been changed into T, G, or R;
    - at position 6 the T has been changed into K; and/or
    - at position 7 the N has been changed into I;

and/or

- (iii) CDR3 sequences:
  - (e) SEQ ID NO: 120; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 120, wherein
    - at position 1 the E has been changed into K;
    - at position 4 the A has been changed into T;
    - at position 11 the I has been changed into M, or L; and/or
    - at position 12 the N has been changed into D.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequence SEQ ID NO: 76; and/or
- (ii) CDR2 sequence SEQ ID NO: 99; and/or
- (iii) CDR3 sequence SEQ ID NO: 120.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequences:
  - (a) SEQ ID NOs: 79-84; and
  - (b) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 79; and/or
- (ii) CDR2 sequences:
  - (c) SEQ ID NOs: 104-108; and
  - (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 104; and/or
- (iii) CDR3 sequences:
  - (e) SEQ ID NOs: 124-125; and
  - (f) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 124.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

- (i) CDR1 sequences:
  - (a) SEQ ID NO: 79; and
  - (b) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 79, wherein
    - at position 2 the S has been changed into N;
    - at position 3 the V has been changed into I;
    - at position 7 the N has been changed into D;
    - at position 8 the D has been changed into S; and/or
    - at position 9 the M has been changed into V, or T;

and/or

- (ii) CDR2 sequences:
  - (c) SEQ ID NO: 104; and
  - (d) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 104, wherein

- at position 1 the D has been changed into G;
- at position 5 the R has been changed into A; and/or
- at position 6 the G has been changed into D;

and/or

(iii) CDR3 sequences:

(e) SEQ ID NO: 124; and

(f) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 124, wherein

- at position 4 the T has been changed into M.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 79; and/or

(ii) CDR2 sequence SEQ ID NO: 104; and/or

(iii) CDR3 sequence SEQ ID NO: 124.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequences:

(a) SEQ ID NOs: 85-86; and

(b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 85; and/or

(ii) CDR2 sequences:

(c) SEQ ID NOs: 109-110; and

(d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 109; and/or

(iii) CDR3 sequence SEQ ID NO: 126.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequences:

(a) SEQ ID NO: 85; and

(b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 85, wherein

- at position 2 the S has been changed into N;

and/or

(ii) CDR2 sequences:

(c) SEQ ID NO: 109; and

   (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 109, wherein
      - at position 9 the T has been changed into S;

and/or

(iii) CDR3 sequence SEQ ID NO: 126.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 85; and/or
(ii) CDR2 sequence SEQ ID NO: 109; and/or
(iii) CDR3 sequence SEQ ID NO: 126.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 87; and/or
(ii) CDR2 sequence SEQ ID NO: 111; and/or
(iii) CDR3 sequence SEQ ID NO: 127.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 77; and/or
(ii) CDR2 sequence:
   (a) SEQ ID NOs: 112-113; and
   (b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 112; and/or
(iii) CDR3 sequence:
   (c) SEQ ID NOs: 128-130; and
   (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 128.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 77; and/or
(ii) CDR2 sequences:
   (a) SEQ ID NO: 112; and
   (b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 112, wherein
      - at position 4 the D has been changed into G;

and/or

(iii) CDR3 sequences:

  (c) SEQ ID NO: 128; and

  (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 128, wherein

    - at position 9 the S has been changed into P; and/or
    - at position 13 the T has been changed into A.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 77; and/or

(ii) CDR2 sequence SEQ ID NO: 112; and/or

(iii) CDR3 sequence SEQ ID NO: 128.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 88; and/or

(ii) CDR2 sequences:

  (a) SEQ ID NOs: 114-116; and

  (b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 114; and/or

(iii) CDR3 sequences:

  (c) SEQ ID NOs: 131-132; and

  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 131.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 88; and/or

(ii) CDR2 sequences:

  (a) SEQ ID NO: 114; and

  (b) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 114, wherein

    - at position 1 the V has been changed into I, or A; and/or
    - at position 9 the M has been changed into I;

and/or

(iii) CDR3 sequences:

  (c) SEQ ID NO: 131; and

(d) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 131, wherein
  - at position 4 the G has been changed into E; and/or
  - at position 5 the R has been changed into Q.

In a further aspect, the monovalent polypeptide of the invention, may comprise at least one stretch of amino acid residues that is chosen from the group consisting of:

(i) CDR1 sequence SEQ ID NO: 88; and/or
(ii) CDR2 sequence SEQ ID NO: 114; and/or
(iii) CDR3 sequence SEQ ID NO: 131.

In particular, a monovalent polypeptide of the invention may be a monovalent polypeptide that comprises one antigen binding site, wherein said antigen binding site comprises at least one stretch of amino acid residues that is chosen from the group consisting of the CDR1 sequences, CDR2 sequences and CDR3 sequences as described above (or any suitable combination thereof). In a preferred aspect, however, the monovalent polypeptide of the invention comprises more than one, such as two or more stretches of amino acid residues chosen from the group consisting of the CDR1 sequences of the invention, the CDR2 sequences of the invention and/or the CDR3 sequences of the invention. Preferably, the monovalent polypeptide of the invention comprises three stretches of amino acid residues chosen from the group consisting of the CDR1 sequences of the invention, the CDR2 sequences of the invention and the CDR3 sequences of the invention, respectively. The combinations of CDR's that are mentioned herein as being preferred for the monovalent polypeptides of the invention are listed in Table A-10.

It should be further noted that the invention is not limited as to the origin of the monovalent polypeptide of the invention (or of the nucleic acid of the invention used to express it), nor as to the way that the monovalent polypeptide or nucleic acid of the invention is (or has been) generated or obtained. Thus, the monovalent polypeptides of the invention may be naturally occurring monovalent polypeptides (from any suitable species) or synthetic or semi-synthetic monovalent polypeptides.

Furthermore, it will also be clear to the skilled person that it is possible to "graft" one or more of the CDR's mentioned above onto other "scaffolds", including but not limited to human scaffolds or non-immunoglobulin scaffolds. Suitable scaffolds and techniques for such CDR grafting will be clear to the skilled person and are well known in the art, see for example US 7,180,370, WO 01/27160, EP 0605522, EP 0460167, US 7,054,297, Nicaise et al. (Protein Science 13: 1882-1891, 2004), Ewert et al. (Methods 34: 184-199, 2004), Kettleborough et al. (Protein Eng. 4: 773-783, 1991), O'Brien and Jones (Methods Mol. Biol. 207: 81-100, 2003), Skerra (J. Mol. Recognit. 13: 167-187, 2000) and Saerens et al. (J. Mol. Biol. 352: 597-607, 2005) and the further references cited therein. For example, techniques known per se for grafting mouse or rat CDR's onto human frameworks and scaffolds can be used in an analogous manner to provide chimeric proteins comprising one or more of the CDR sequences defined herein for the monovalent polypeptides of the invention and one or more human framework regions or sequences. Suitable scaffolds for presenting amino acid sequences will be clear to the skilled person, and for example comprise, without limitation, the binding scaffolds based on or derived from immunoglobulins (i.e. other than the immunoglobulin sequences already described herein), protein scaffolds derived from protein A domains (such as Affibodies™), tendamistat, fibronectin, lipocalin, CTLA-4, T-cell receptors, designed ankyrin repeats, avimers and PDZ domains (Binz et al. Nat. Biotech., 23: 1257, 2005), and binding moieties based on DNA or RNA including but not limited to DNA or RNA aptamers (Ulrich et al. Comb. Chem. High Throughput Screen 9: 619-32, 2006).

In said monovalent polypeptides of the invention, the CDR's may be linked to further amino acid sequences and/or may be linked to each other via amino acid sequences, in which said amino acid sequences are preferably framework sequences or are amino acid sequences that act as framework sequences, or together form a scaffold for presenting the CDR's.

According to a preferred, but non-limiting embodiment, the monovalent polypeptides of the invention comprise at least three CDR sequences linked to at least two framework sequences, in which preferably at least one of the three CDR sequences is a CDR3 sequence, with the other two CDR sequences being CDR1 or CDR2 sequences, and preferably being one CDR1 sequence and one CDR2 sequence. According to one specifically preferred, but non-limiting embodiment, the monovalent polypeptides of the invention have the structure FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, in which CDR1, CDR2 and CDR3 are as defined herein for the monovalent polypeptides of the invention, and FR1, FR2, FR3 and FR4 are framework sequences. In such a monovalent polypeptide of the invention, the framework sequences may be any suitable framework sequence, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis of the standard handbooks and the further disclosure and prior art mentioned herein.

Accordingly, a monovalent polypeptide of the present invention essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
    - (a) SEQ ID NOs: 73-88; and
    - (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 73-88; and/or
- (ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NOs: 90-116; and

(d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 90-116; and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NOs: 118-132 and 282-284; and

(f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 118-132 and 282-284.

Further preferred CDR sequences are depicted in Table A-10.

Sequence analysis of the resulting binders further resulted in the identification of 8 distinct families, i.e. Family 7, Family 26, Family 82, Family 109, Family 85, Family 38, Family 110 and Family 108. Corresponding alignments are provided in Table A-1, Table A-2, Table A-3, Table A-4, Table A-5, Table A-6, Table A-7 and Table A-8, respectively. Classification into different families was based on sequence similarities and differences in the CDRs. Family 7 comprises 21 clones (SEQ ID NOs: 1-21), Family 26 comprises 11 clones (SEQ ID NOs: 22-32), Family 82 comprises 23 clones (SEQ ID NOs: 33-55), Family 109 comprises 6 clones (SEQ ID NOs: 56-61), Families 85 and 108 are each represented by only 1 clone (SEQ ID NO: 62 and SEQ ID NO: 72, respectively), Family 38 comprises 6 clones (SEQ ID NOs: 63-68) and Family 110 comprises 3 clones (SEQ ID NOs: 69-71). Representatives of all families were isolated based on high affinity binding to GITR and human T cell activation (Example 5). In general Family 7, Family 26 and Family 109 representatives demonstrated the best EC_{50} values.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NOs: 73-75; and

(b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 73; and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NOs: 90-98; and

(d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 90; and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NOs: 118-119, 123 and 282-284; and

(f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 118.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NO: 73; and
  (b) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 73, wherein
    - at position 2 the T has been changed into S;
    - at position 7 the D has been changed into N;
    - at position 8 the S has been changed into A; and/or
    - at position 10 the A has been changed into G;

and/or

(ii) CDR2 is chosen from the group consisting of:
  (c) SEQ ID NO: 90; and
  (d) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 90, wherein
    - at position 1 the A has been changed into H, T, or G;
    - at position 2 the I has been changed into M;
    - at position 3 the T has been changed into S;
    - at position 6 the G has been changed into S;
    - at position 7 the S has been changed into R, or G; and/or
    - at position 8 the P has been changed into S, T, or R

and/or

(iii) CDR3 is chosen from the group consisting of:
  (e) SEQ ID NO: 118; and
  (f) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 118, wherein
    - at position 9 the A has been changed into P;
    - at position 11 the M has been changed into L, K, R, or Q; and/or
    - at position 12 the D has been changed into N.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 118; or
ii) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 123.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NOs: 76-78; and
  (b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 76; and/or
(ii) CDR2 is chosen from the group consisting of:
  (c) SEQ ID NOs: 99-103; and
  (d) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 99; and/or
(iii) CDR3 is chosen from the group consisting of:
  (e) SEQ ID NOs: 120-123; and
  (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 120.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
(a) SEQ ID NO: 76; and
(b) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 76, wherein
  - at position 7 the D has been changed into N; and/or
  - at position 8 the S has been changed into A;

and/or

(ii) CDR2 is chosen from the group consisting of:
(c) SEQ ID NO: 99; and

(d) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 99, wherein
  - at position 1 the A has been changed into S, or T;
  - at position 5 the S has been changed into T, G, or R;
  - at position 6 the T has been changed into K; and/or
  - at position 7 the N has been changed into I;

and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NO: 120; and

(f) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 120, wherein
  - at position 1 the E has been changed into K;
  - at position 4 the A has been changed into T;
  - at position 11 the I has been changed into M, or L; and/or
  - at position 12 the N has been changed into D.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 76, CDR2 is represented by SEQ ID NO: 99, and CDR3 is represented by SEQ ID NO: 120.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NOs: 79-84; and
  (b) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 79; and/or

(ii) CDR2 is chosen from the group consisting of:
  (c) SEQ ID NOs: 104-108; and
  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 104; and/or

(iii) CDR3 is chosen from the group consisting of:
  (e) SEQ ID NOs: 124-125; and

(f) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 124.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NO: 79; and

(b) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 79, wherein

- at position 2 the S has been changed into N;
- at position 3 the V has been changed into I;
- at position 7 the N has been changed into D;
- at position 8 the D has been changed into S; and/or
- at position 9 the M has been changed into V, or T;

and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NO: 104; and

(d) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 104, wherein

- at position 1 the D has been changed into G;
- at position 5 the R has been changed into A; and/or
- at position 6 the G has been changed into D;

and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NO: 124; and

(f) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 124, wherein

- at position 4 the T has been changed into M.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 79, CDR2 is represented by SEQ ID NO: 104, and CDR3 is represented by SEQ ID NO: 124.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
    - (a) SEQ ID NOs: 85-86; and
    - (b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 85; and/or
- (ii) CDR2 is chosen from the group consisting of:
    - (c) SEQ ID NOs: 109-110; and
    - (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 109; and/or
- (iii) CDR3 is SEQ ID NO: 126.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NO: 85; and

(b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 85, wherein

- at position 2 the S has been changed into N;

and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NO: 109; and

(d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 109, wherein

- at position 9 the T has been changed into S;

and/or

(iii) CDR3 is SEQ ID NO: 126.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 85, CDR2 is represented by SEQ ID NO: 109, and CDR3 is represented by SEQ ID NO: 126.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3

complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 87, CDR2 is represented by SEQ ID NO: 111, and CDR3 is represented by SEQ ID NO: 127.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (a) SEQ ID NOs: 112-113; and
  - (b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 112; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 128-130; and
  - (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 128.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (a) SEQ ID NO: 112; and
  - (b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 112, wherein
    - at position 4 the D has been changed into G;

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (c) SEQ ID NO: 128; and
  - (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 128, wherein
    - at position 9 the S has been changed into P; and/or
    - at position 13 the T has been changed into A.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 77, CDR2 is represented by SEQ ID NO: 112, and CDR3 is represented by SEQ ID NO: 128.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 88; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 114-116; and
  - (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 114; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NOs: 131-132; and
  - (f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 131.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 88; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (a) SEQ ID NO: 114; and
  - (b) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 114, wherein
    - at position 1 the V has been changed into I, or A; and/or
    - at position 9 the M has been changed into I;

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (c) SEQ ID NO: 131; and
  - (d) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 131, wherein
    - at position 4 the G has been changed into E; and/or
    - at position 5 the R has been changed into Q.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3

complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 88, CDR2 is represented by SEQ ID NO: 114, and CDR3 is represented by SEQ ID NO: 131.

According to a preferred but non-limiting aspect, the present invention relates to a monovalent polypeptide as described herein, wherein said at least one immunoglobulin single variable domain is chosen from the group of ISVDs, wherein:

- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 91; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 92; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 94; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 95; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 75, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 96; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 97; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 98; and CDR3 is SEQ ID NO: 119;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 282;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 283;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 284;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 120;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 100; and CDR3 is SEQ ID NO: 121;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 101; and CDR3 is SEQ ID NO: 122;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 102; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 103; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 79, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 105; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 80, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 81, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 82, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 84, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;

- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 107; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 108; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 125;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 109; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 86, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 87, CDR2 is SEQ ID NO: 111; and CDR3 is SEQ ID NO: 127;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 128;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 129;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 113; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 114; and CDR3 is SEQ ID NO: 131;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 115; and CDR3 is SEQ ID NO: 131; and
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 116; and CDR3 is SEQ ID NO: 132.

Representative polypeptides of the present invention having the CDRs described above are shown in Table A-10.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 1-21. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 1-21. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 1-21 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 1-21. In another preferred aspect, the monovalent polypeptide belongs to Family 7, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 1-21.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 22-32. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 22-32. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 22-32 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 22-32. In another preferred aspect, the monovalent polypeptide belongs to Family 26, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 22-32.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 33-55. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 33-55. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 33-55 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 33-55. In another preferred aspect, the monovalent polypeptide belongs to Family 82, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 33-55.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 56-61. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 56-61. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 56-61 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 56-61. In another preferred aspect, the monovalent polypeptide belongs to Family 109, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 56-61.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 63-68. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 63-68. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 63-68 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 63-68. In another preferred aspect, the monovalent polypeptide belongs to Family 38, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 63-68.

In one aspect, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 69-71. In another aspect, the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 69-71. Preferably, the monovalent polypeptide has the same number of amino acids within its sequence compared to any one of SEQ ID NOs: 69-71 and the monovalent polypeptide has an amino acid sequence between position 8 and position 106 (according to Kabat numbering) that has 89% or more sequence identity compared to any one of SEQ ID NOs: 69-71. In another preferred aspect, the monovalent polypeptide belongs to Family 110, such as e.g. a monovalent polypeptide selected from any one of SEQ ID NOs: 69-71.

Monovalent polypeptides comprising one or more of the above specified stretches of amino acid residues may modulate (i.e. increase, enhance, stimulate or potentiate) the activity of GITR. More particularly, the monovalent polypeptides of the present invention may enhance an immune response. As such, these polypeptides of the invention may enhance proliferation or activation of T cells, B cells or natural killer cells.

Proliferation or activation of T cell, B cells or natural killer cells can be determined by a variety of assays, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays (e.g. NF-κB luciferase reporter assay), T cell activation assay, cell surface receptor binding assays and assays to measure expression of known markers of activation or cytokine secretion, which are all well known in the art.

For example, any one of several conventional assays for monitoring cytokine production (such as IFN-γ and interleukins) as a measure of immune cells activation can be used. For example, for tracking T cell activation, interleukin-2 can be employed as a marker, which can be assayed as described in Proc. Natl. Acad. Sci. USA. 86:1333 (1989).

One can also employ immunofluorescence and flow cytometry to monitor cytokine production on a cellular basis, and to monitor cell surface markers that reflect cellular activation states. A host of such markers are known, detecting antibodies are broadly commercially available, and the markers are well known in the art.

A common assay for T cell proliferation entails measuring tritiated thymidine incorporation. The proliferation of T cells can be measured *in vitro* by determining the amount of ^{3}H-labeled thymidine incorporated into the replicating DNA of cultured cells. Therefore, the rate of DNA synthesis and, in turn, the rate of cell division can be quantified.

Binding of the monovalent polypeptides of the invention to GITR can be measured in binding assays. Typical assays include (without being limiting) assays in which GITR is exposed on a cell surface (such as e.g. Flp-In™-293 cells or GloResponse™ NF-κB-Nluc2P HEK293 cells). A preferred assay for measuring binding of the polypeptides of the invention to GITR is a FACS assay, such as e.g. the FACS assay as described in the examples, wherein binding of the polypeptides of the invention to GITR expressed on Flp-In™-293 cells and/or activated T cells is determined. Some preferred EC_{50}

values for binding of the polypeptides of the invention to GITR will become clear from the further description and examples herein.

In such FACS binding assay, the monovalent polypeptides of the present invention may have EC_{50} values in binding human GITR of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, or even of 10^{-10} M or lower. For example, in such FACS binding assay, the monovalent polypeptides of the present invention may have EC_{50} values in binding human GITR between 10^{-10} M and 10^{-8} M, such as between 10^{-9} M and 10^{-8} M or between 10^{-10} M and 10^{-9} M.

The invention also relates to a monovalent polypeptide which has at least 80% amino acid identity (or sequence identity as defined herein), preferably at least 85% amino acid identity, more preferably at least 90% amino acid identity, such as 95%, 96%, 97%, 98%, 99% amino acid identity or more or even (essentially) 100% amino acid identity with at least one of the amino acid sequences of SEQ ID NOs: 1-71.

In one specific, but non-limiting aspect, the monovalent polypeptide of the invention may be a monovalent polypeptide that comprises an immunoglobulin fold or a monovalent polypeptide that, under suitable conditions (such as physiological conditions) is capable of forming an immunoglobulin fold (*i.e.,* by folding). Reference is *inter alia* made to the review by Halaby et al. (J. Protein Eng. 12: 563-71, 1999). Preferably, when properly folded so as to form an immunoglobulin fold, the stretches of amino acid residues may be capable of properly forming the antigen binding site for binding GITR. Accordingly, in a preferred aspect the monovalent polypeptide of the invention is an immunoglobulin, such as e.g. an immunoglobulin single variable domain.

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

The framework sequences may preferably be such that the monovalent polypeptide of the invention is an immunoglobulin single variable domain such as a Domain antibody (or an amino acid sequence that is suitable for use as a domain antibody); a single domain antibody (or an amino acid that is suitable for use as a single domain antibody); a "dAb" (or an amino acid that is suitable for use as a dAb); a Nanobody®; a V_{HH} sequence; a humanized V_{HH} sequence; a camelized V_H sequence; or a V_{HH} sequence that has been obtained by affinity maturation. Again, suitable framework sequences will be clear to the skilled person, for example on the basis of the standard handbooks and the further disclosure and prior art mentioned herein.

In particular, the framework sequences present in the monovalent polypeptides of the invention may contain one or more of Hallmark residues (as defined in WO 08/020079 (Tables A-3 to A-8)), such that the monovalent polypeptide of the invention is a Nanobody. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein (see *e.g.,* Table A-10). Generally, Nanobodies (in particular V_{HH} sequences and partially humanized Nanobodies) can in particular be characterized by the presence of one or more "*Hallmark residues*" in one or more of the framework sequences (as *e.g.,* further described in WO 08/020079, page 61, line 24 to page 98, line 3).

More in particular, the invention provides polypeptides comprising at least one immunoglobulin single variable domain that is an amino acid sequence with the (general) structure

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

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

i) have at least 80%, more preferably 90%, even more preferably 95% amino acid identity with at least one of the amino acid sequences of SEQ ID NOs: 1-71 (see Table A-9), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded. In this respect, reference is also made to Table A-10, which lists the framework 1 sequences (SEQ ID NOs: 134-152), framework 2 sequences (SEQ ID NOs: 153-162), framework 3 sequences (SEQ ID NOs: 163-200) and framework 4 sequences (SEQ ID NOs: 201-205) of the immunoglobulin single variable domains of SEQ ID NOs: 1-71 (see Table A-9); or

ii) combinations of framework sequences as depicted in Table A-10;

and in which:

iii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

The present invention also provides a number of sequence optimized immunoglobulin single variable domains.

In particular, sequence optimized immunoglobulin single variable domains may be amino acid sequences that are as generally defined for immunoglobulin single variable domains in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Some preferred, but non-limiting humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring VHH sequence with the corresponding framework sequence of one or more closely related human VH sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said VHH sequence (in any manner known per se, as further described herein) and the resulting humanized VHH 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 domains may be partially humanized or fully humanized.

The present invention also provides a number of sequence optimized immunoglobulin single variable domains that may show improved expression and/or increased stability upon storage during stability studies. The amino acid sequences of the present invention may show reduced pyroglutamate post-translational modification of the N-terminus and hence have increased product stability. In addition, the amino acid sequences of the present invention may show other improved properties such as e.g. less immunogenicity, improved binding characteristics (suitably measured and/or expressed as a KD-value (actual or apparent), a KA-value (actual or apparent), a kon-rate and/or a koff-rate, or alternatively as an IC_{50} value, as further described herein) for GITR, improved affinity and/or improved avidity for GITR and/or improved efficacy and/or potency for agonizing GITR, compared to their corresponding parental amino acid sequences.

Some particularly preferred sequence optimized immunoglobulin single variable domains of the invention are sequence optimized variants of the immunoglobulin single variable domains of SEQ ID NOs: 1-71; the amino acid sequences of SEQ ID NOs: 268-275 are some especially preferred examples.

Thus, some other preferred immunoglobulin single variable domains of the invention are Nanobodies which can bind (as further defined herein) to GITR and which:

i) are a sequence optimized variant of one of the immunoglobulin single variable domains of SEQ ID NOs: 1-71; and/or

ii) have at least 80% amino acid identity with at least one of the immunoglobulin single variable domains of SEQ ID NOs: 1-71 and/or at least one of the the immunoglobulin single variable domains of SEQ ID NOs: 268-275 (see Table A-9), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; In this respect, reference is also made to Table A-10, which lists the framework 1 sequences (SEQ ID NOs: 134-152, 276 and 278), framework 2 sequences (SEQ ID NOs: 153-162), framework 3 sequences (SEQ ID NOs: 163-200, 277 and 279-281) and framework 4 sequences (SEQ ID NOs: 201-205) of the immunoglobulin single variable domains of SEQ ID NOs: 1-71 and 268-275 (see Table A-9); or

iii) combinations of framework sequences as depicted in Table A-10;

and in which:

iv) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

The immunoglobulins (and in particular immunoglobulin single variable domains) of the invention may also contain the specific mutations/amino acid residues described in the following co-pending US provisional applications, all entitled “Improved immunoglobulin variable domains”: US 61/994552 filed May 16, 2014; US 61/014,015 filed June 18, 2014; US 62/040,167 filed August 21, 2014; and US 62/047,560, filed September 8, 2014 (all assigned to Ablynx N.V.) as well as the International application WO 2015/173325 which was based on these provisional applications and which was published on November 19, 2015.

In particular, the immunoglobulins (and in particular immunoglobulin single variable domains) of the invention may suitably contain (i) a K or Q at position 112; or (ii) a K or Q at position 110 in combination with a V at position 11; or (iii) a T at position 89; or (iv) an L on position 89 with a K or Q at position 110; or (v) a V at position 11 and an L at position 89; or any suitable combination of (i) to (v).

As also described in said co-pending US provisional applications, when the immunoglobulins of the invention contain the mutations according to one of (i) to (v) above (or a suitable combination thereof):

- the amino acid residue at position 11 is preferably chosen from L, V or K (and is most preferably V); and
- the amino acid residue at position 14 is preferably suitably chosen from A or P; and
- the amino acid residue at position 41 is preferably suitably chosen from A or P; and
- the amino acid residue at position 89 is preferably suitably chosen from T, V or L; and
- the amino acid residue at position 108 is preferably suitably chosen from Q or L; and
- the amino acid residue at position 110 is preferably suitably chosen from T, K or Q; and
- the amino acid residue at position 112 is preferably suitably chosen from S, K or Q.

As mentioned in said co-pending US provisional applications, said mutations are effective in preventing or reducing binding of so-called "pre-existing antibodies" to the immunoglobulins and compounds of the invention. For this purpose, the immunoglobulins of the invention may also contain (optionally in combination with said mutations) a C-terminal extension (X)n (in which n is 1 to 10, preferably 1 to 5, such as 1, 2, 3, 4 or 5 (and preferably 1 or 2, such as 1); and each X is an (preferably naturally occurring) amino acid residue that is independently chosen, and preferably independently chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I)), for which reference is again made to said US provisional applications as well as to WO 12/175741. In particular, an immunoglobulin of the invention may contain such a C-terminal extension when it forms the C-terminal end of a protein, polypeptide or other compound or construct comprising the same (again, as further described in said US provisional applications as well as WO 12/175741).

Some specifically preferred, but non-limiting examples of immunoglobulins of the invention that contain such mutations and/or such a C-terminal extension are given in SEQ ID NOs: 268-275 and 285-290.

In a preferred aspect, the present invention provides an immunoglobulin or monovalent polypeptide that is selected from any of SEQ ID NOs: 1-71 and 268-275.

The present invention also relates to monovalent polypeptides and/or immunoglobulin single variable domains directed against GITR, that cross-blocks the binding to GITR of at least one of the immunoglobulins with SEQ ID NOs: 1-71 and 268-275 and/or that are cross-blocked from binding to GITR by at least one of the immunoglobulins with SEQ ID NOs: 1-71 and 268-275.

The invention further relates to monovalent polypeptides and/or immunoglobulin single variable domains directed against GITR that bind the same epitope as is bound by the monovalent polypeptides of the present invention, more particularly by the monovalent polypeptides with SEQ ID NOs: 1-71 and 268-275.

In a particular aspect, the invention relates to monovalent polypeptides and/or immunoglobulin single variable domains directed against GITR that bind the same epitope as is bound by the monovalent polypeptides of the present invention that belong to Family 7, Family 26, Family 82, Family 85 and Family 38, more particularly by the monovalent polypeptides with SEQ ID NOs: 1-55, 62-68 and 269-275.

In another particular aspect, the invention relates to monovalent polypeptides and/or immunoglobulin single variable domains directed against GITR that bind the same epitope as is bound by the monovalent polypeptides of the present invention that belong to Family 109 and Family 110, more particularly by the monovalent polypeptides with SEQ ID NOs: 56-61, 69-71 and 268.

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

These monovalent polypeptides of the invention, and in particular the immunoglobulins comprising the CDR sequences of the invention are particularly suited for use as building block or binding unit for the preparation of multivalent polypeptides.

Accordingly, the monovalent polypeptides of the invention that bind GITR can be in essentially isolated form (as defined herein), or they may form part of a protein or polypeptide, which may comprise or essentially consist of one or more monovalent polypeptides that bind GITR and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). The present invention also relates to a protein or polypeptide that comprises or essentially consists of one or more monovalent polypeptides of the invention (or suitable fragments thereof).

The one or more monovalent polypeptides of the invention are thus used as a binding unit or building block in such a protein or polypeptide, so as to provide a monovalent, multivalent or multiparatopic polypeptide of the invention, respectively, all as described herein. The present invention thus also relates to a polypeptide which is a monovalent construct comprising or essentially consisting of one monovalent polypeptide of the invention. The present invention thus also relates to a polypeptide which is a multivalent polypeptide, such as *e.g.,* a bivalent, trivalent, tetravalent, pentavalent or hexavalent polypeptide comprising or essentially consisting of two or more monovalent polypeptides of the invention (for multivalent and multispecific polypeptides containing one or more VHH domains and their preparation, reference is also made to Conrath et al. (J. Biol. Chem. 276: 7346-7350, 2001), as well as to for example WO 96/34103, WO 99/23221 and WO 2010/115998).

Multivalent polypeptides of the invention

The invention further relates to a multivalent polypeptide (also referred to herein as a "*multivalent polypeptide(s) of the invention*") that comprises or (essentially) consists of at least one immunoglobulin single variable domain (or suitable fragments thereof) directed against GITR, preferably human GITR, and one additional immunoglobulin single variable domain.

In a preferred aspect, the multivalent polypeptide of the invention comprises or essentially consists of two or more immunoglobulin single variable domains directed against GITR. The two or more immunoglobulin single variable domains may optionally be linked via one or more peptidic linkers.

In the multivalent polypeptide of the invention, the two or more immunoglobulin single variable domains or Nanobodies may be the same or different, and may be directed against the same antigen or antigenic determinant (for example against the same part(s) or epitope(s) or against different parts or epitopes) or may alternatively be directed against different antigens or antigenic determinants; or any suitable combination thereof. For example, a bivalent polypeptide of the invention may comprise (a) two identical immunoglobulin single variable domains or Nanobodies; (b) a first immunoglobulin single variable domain or Nanobody directed against a first antigenic determinant of a protein or antigen and a second immunoglobulin single variable domain or Nanobody directed against the same antigenic determinant of said protein or antigen which is different from the first immunoglobulin single variable domain or Nanobody; (c) a first immunoglobulin single variable domain or Nanobody directed against a first antigenic determinant of a protein or antigen and a second immunoglobulin single variable domain or Nanobody directed against another antigenic determinant of said protein or antigen, different from said first antigenic determinant; or (d) a first immunoglobulin single variable domain or Nanobody directed against a first protein or antigen and a second immunoglobulin single variable domain or Nanobody directed against a second protein or antigen (i.e. different from said first protein or antigen). Similarly, a trivalent polypeptide of the invention may, for example and without being limited thereto. comprise (a) three identical immunoglobulin single variable domains or Nanobodies; (b) two identical immunoglobulin single variable domains or Nanobodies against a first antigenic determinant of a protein or antigen and a third immunoglobulin single variable domain or Nanobody directed against a different antigenic determinant of the same protein or antigen; (c) two identical immunoglobulin single variable domains or Nanobodies against a first antigenic determinant of a protein or antigen and a third immunoglobulin single variable domain or Nanobody directed against a second protein or antigen different from said first protein or antigen; (d) a first immunoglobulin single variable domain or Nanobody directed against a first antigenic determinant of a first protein or antigen, a second immunoglobulin single variable domain or Nanobody directed against a second antigenic determinant of said first protein or antigen, different from said first antigenic determinant and a third immunoglobulin single variable domain or Nanobody directed against a second protein or antigen different from said first protein or antigen; or (e) a first immunoglobulin single variable domain or Nanobody directed against a first protein or antigen, a second immunoglobulin single variable domain or Nanobody directed against a second protein or antigen different from said first protein or antigen, and a third immunoglobulin single variable domain or Nanobody directed against a third protein or antigen different from said first and second protein or antigen.

Polypeptides of the invention that contain at least two immunoglobulin single variable domains and/or Nanobodies, in which at least one immunoglobulin single variable domain or Nanobody is directed against a first antigen (*i.e.* against GITR) and at least one immunoglobulin single variable domain or Nanobody is directed against a second antigen (*i.e.* different from GITR), will also be referred to as "multispecific" polypeptides of the invention, and the immunoglobulin single variable domains or Nanobodies present in such polypeptides will also be referred to herein as being in a "multispecific format". Thus, for example, a "bispecific" polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain or Nanobody directed against a first antigen (*i.e.* GITR) and at least one further immunoglobulin single variable domain or Nanobody directed against a second antigen (*i.e.* different from GITR), whereas a "trispecific" polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain or Nanobody directed against a first antigen (*i.e.* GITR), at least one further immunoglobulin single variable domain or Nanobody directed against a second antigen (*i.e.* different from GITR) and at least one further immunoglobulin single variable domain or Nanobody directed against a third antigen (*i.e.* different from both GITR, and the second antigen); etc.

Accordingly, in one aspect, in its simplest form, the multivalent polypeptide of the invention is a bivalent polypeptide of the invention comprising a first immunoglobulin single variable domain or Nanobody directed against GITR, and an identical second immunoglobulin single variable domain or Nanobody directed against GITR, wherein said first and second immunoglobulin single variable domain or Nanobody may optionally be linked via a linker sequence (as defined herein); in its simplest form a multivalent polypeptide of the invention may be a trivalent polypeptide of the invention, comprising a first immunoglobulin single variable domain or Nanobody directed against GITR, an identical second immunoglobulin single variable domain or Nanobody directed against GITR and an identical third immunoglobulin single variable domain or Nanobody directed against GITR, in which said first, second and third immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular two, linker sequences.

In another aspect, the multivalent polypeptide of the invention may be a bispecific polypeptide of the invention, comprising a first immunoglobulin single variable domain or Nanobody directed against GITR, and a second immunoglobulin single variable domain or Nanobody directed against a second antigen, in which said first and second immunoglobulin single variable domain or Nanobody may optionally be linked via a linker sequence (as defined herein); whereas a multivalent polypeptide of the invention may also be a trispecific polypeptide of the invention, comprising a first immunoglobulin single variable domain or Nanobody directed against GITR, a second immunoglobulin single variable domain or Nanobody directed against a second antigen and a third immunoglobulin single variable domain or Nanobody directed against a third antigen, in which said first, second and third immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular two, linker sequences.

In a preferred aspect, the polypeptide of the invention is a trivalent, bispecific polypeptide. A trivalent, bispecific polypeptide of the invention in its simplest form may be a trivalent polypeptide of the invention (as defined herein), comprising two identical immunoglobulin single variable domains or Nanobodies against GITR and a third immunoglobulin single variable domain or Nanobody directed against another antigen (e.g. serum albumin), in which said first, second and third immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular two, linker sequences. Particularly preferred trivalent, bispecific polypeptides in accordance with the invention are those shown in the Examples described herein and in Table A-11.

In another preferred aspect, the polypeptide of the invention is a tetravalent, bispecific polypeptide. A tetravalent, bispecific polypeptide of the invention in its simplest form may be a tetravalent polypeptide of the invention (as defined herein), comprising three identical immunoglobulin single variable domains or Nanobodies against GITR and a fourth immunoglobulin single variable domain or Nanobody directed against another antigen (e.g. serum albumin), in which said first, second, third and fourth immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular three, linker sequences. Particularly preferred tetravalent, bispecific polypeptides in accordance with the invention are those shown in the Examples described herein and in Table A-11.

In another preferred aspect, the polypeptide of the invention is a pentavalent, bispecific polypeptide. A pentavalent, bispecific polypeptide of the invention in its simplest form may be a pentavalent polypeptide of the invention (as defined herein), comprising four identical immunoglobulin single variable domains or Nanobodies against GITR and a fifth immunoglobulin single variable domain or Nanobody directed against another antigen (e.g. serum albumin), in which said first, second, third, fourth and fifth immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular four, linker sequences.

In another preferred aspect, the polypeptide of the invention is a hexavalent, bispecific polypeptide. A hexavalent, bispecific polypeptide of the invention in its simplest form may be a hexavalent polypeptide of the invention (as defined herein), comprising five identical immunoglobulin single variable domains or Nanobodies against GITR and a sixth immunoglobulin single variable domain or Nanobody directed against another antigen (e.g. serum albumin), in which said first, second, third, fourth, fifth and sixth immunoglobulin single variable domain or Nanobody may optionally be linked via one or more, and in particular five, linker sequences.

In a further aspect, the polypeptide of the invention is a multiparatopic polypeptide (also referred to herein as "*multiparatopic polypeptide(s) of the invention*"), such as *e.g.,* (a) "*biparatopic polypeptide(s) of the invention*" or "*triparatopic polypeptide(s) of the invention*". The term "*multiparatopic*" (antigen-) binding molecule or "*multiparatopic*" polypeptide as used herein shall mean a polypeptide comprising at least two (*i.e.* two or more) immunoglobulin single variable domains, wherein a "first" immunoglobulin single variable domain is directed against GITR and a "second" immunoglobulin single variable domain is directed against GITR, and wherein these "first" and "second" immunoglobulin single variable domains have a different paratope. Accordingly, the multiparatopic polypeptide comprises or consists of two or more immunoglobulin single variable domains that are directed against GITR, wherein at least one "first" immunoglobulin single variable domain is directed against a first epitope on GITR and at least one "second" immunoglobulin single variable domain is directed against a second epitope on GITR different from the first epitope on GITR.

In a further aspect, the polypeptide of the invention is a biparatopic polypeptide. The term "*biparatopic*" (antigen-)binding molecule or "*biparatopic*" polypeptide as used herein shall mean a polypeptide comprising a "first" immunoglobulin single variable domain directed against GITR and a "second" immunoglobulin single variable domain directed against GITR, wherein these "first" and "second" immunoglobulin single variable domains have a different paratope. Accordingly, the biparatopic polypeptide comprises or consists of two or more immunoglobulin single variable domains that are directed against GITR, wherein a "first" immunoglobulin single variable domain is directed against a first epitope on GITR and a "second" immunoglobulin single variable domain is directed against a second epitope on GITR different from the first epitope on GITR.

In another further aspect, the polypeptide of the invention is a triparatopic polypeptide. The term "*triparatopic*" (antigen-)binding molecule or "*triparatopic*" polypeptide as used herein shall mean a polypeptide comprising a "first" immunoglobulin single variable domain directed against GITR, a "second" immunoglobulin single variable domain directed against GITR and a "third" immunoglobulin single variable domain directed against GITR, wherein these “first”, “second” and “third” immunoglobulin single variable domains have a different paratope. Accordingly, the triparatopic polypeptide comprises or consists of three or more immunoglobulin single variable domains that are directed against GITR, wherein a “first” immunoglobulin single variable domain is directed against a first epitope on GITR, a “second” immunoglobulin single variable domain is directed against a second epitope on GITR different from the first epitope on GITR, and a “third” immunoglobulin single variable domain is directed against a third epitope on GITR different from the first and second epitope on GITR.

The two or more (such as two, three, four, five or six) immunoglobulin single variable domains present in the multivalent polypeptide of the invention may consist of a light chain variable domain sequence (e.*g.,* a V_L-sequence) or of a heavy chain variable domain sequence (*e.g.,* a V_H-sequence); they may consist of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or of a heavy chain variable domain sequence that is derived from a heavy chain antibody. In a preferred aspect, they consist of a Domain antibody (or an amino acid that is suitable for use as a domain antibody), of a single domain antibody (or an amino acid that is suitable for use as a single domain antibody), of a “dAb” (or an amino acid that is suitable for use as a dAb), of a Nanobody® (including but not limited to V_{HH}), of a humanized V_{HH} sequence, of a camelized V_H sequence; or of a V_{HH} sequence that has been obtained by affinity maturation. The two or more immunoglobulin single variable domains may consist of a partially or fully humanized Nanobody or a partially or fully humanized VHH. In a preferred aspect of the invention, the immunoglobulin single variable domains encompassed in the multivalent polypeptide of the invention are one or more monovalent polypeptides of the invention, as defined herein.

Binding of the multivalent polypeptides of the invention to GITR can be measured in binding assays. Typical assays include (without being limiting) assays in which GITR is exposed on a cell surface (such as e.g. Flp-In™-293 cells or GloResponse™ NF-κB-Nluc2P HEK293 cells). A preferred assay for measuring binding of the multivalent polypeptides of the invention to GITR is a FACS assay, such as e.g. the FACS assay as described in the examples, wherein binding of the multivalent polypeptides of the invention to GITR expressed on Flp-In™-293 cells and/or activated T cells is determined. Some preferred EC_{50} values for binding of the polypeptides of the invention to GITR will become clear from the further description and examples herein.

In such FACS binding assay, the multivalent polypeptides of the present invention may have EC_{50} values in binding human GITR of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, or even of 10^{-10} M or lower, such as 10^{-11} M. For example, in such FACS binding assay, the multivalent polypeptides of the present invention may have EC_{50} values in binding human GITR between 10^{-11} M and 10^{-8} M, such as between 10^{-11} M and 10^{-10} M, between 10^{-10} M and 10^{-9} M or between 10^{-11} M and 10^{-10} M. More particularly, multivalent polypeptides of the present invention that comprise 2 or more monovalent polypeptides belonging to Families 7, 26, 82, 85 and 109 may have EC_{50} values in binding human GITR between 10^{-11} M and 10^{-9} M, such as between 10^{-11} M and 10^{-10} M.

The multivalent polypeptides of the invention bind GITR and can modulate (i.e. increase, enhance, stimulate or potentiate) the activity of GITR. More particularly, the polypeptides of the present invention may enhance an immune response, such as enhance proliferation or activation of T cells, B cells or natural killer cells.

Proliferation or activation of T cell, B cells or natural killer cells can be determined by a variety of assays, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays (e.g. NF-κB luciferase reporter assay), T cell activation assay, cell surface receptor binding assays and assays to measure expression of known markers of activation or cytokine secretion, which are all well known in the art.

For example, any one of several conventional assays for monitoring cytokine production (e.g., IFN-γ, TNF-α, IL-6, IL-2, IL-4 and IL-10) as a measure of immune cells activation can be used. For example, for tracking T cell activation, interleukin-2 can be employed as a marker, which can be assayed as described in Proc. Natl. Acad. Sci. USA. 86:1333 (1989).

One can also employ immunofluorescence and flow cytometry to monitor cytokine production on a cellular basis, and to monitor cell surface markers that reflect cellular activation states. A host of such markers are known, detecting antibodies are broadly commercially available, and the markers are well known in the art.

A common assay for T cell proliferation entails measuring tritiated thymidine incorporation. The proliferation of T cells can be measured *in vitro* by determining the amount of ^{3}H-labeled thymidine incorporated into the replicating DNA of cultured cells. Therefore, the rate of DNA synthesis and, in turn, the rate of cell division can be quantified.

Some preferred EC_{50} values for activating GITR by the multivalent polypeptides of the invention will become clear from the further description and examples herein.

In some embodiments, the multivalent polypeptides of the invention enhance IFN-gamma production in a T-cell activation assay with activated $CD4^+$ T cells stimulated with anti-CD3 antibody OKT3, as described in Example 10. In this T cell activation assay, the multivalent polypeptides of the present invention have EC_{50} values for enhancing IFN-gamma production of 10^{-7} M or lower, preferably of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, 10^{-10} M or lower, or even of 10^{-11} M or lower. More particularly, multivalent polypeptides of the present invention that comprise 2 or more monovalent polypeptides belonging to Families 7, 26 and 109 may have EC_{50} values for enhancing IFN-gamma production between 10^{-11} M and 10^{-9} M, such as between 10^{-11} M and 10^{-10} M. Preferably, in this T-cell activation assay, the multivalent polypeptides of the present invention enhance IFN-gamma production with EC_{50} values of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40 or even less, such as less than 30 pM.

In some embodiments, the multivalent polypeptides of the invention enhance the activity of nuclear factor-kappa B (NF-κB) in a NF-κB luciferase reporter assay, as described in Example 9. NF-κB luciferase reporter assays have been described in Buillard et al. 2013, J. Exp. Med. Vol. 210, 9: 1685-1693. Some preferred EC_{50} values for activating GITR by the polypeptides of the invention will become clear from the further description and examples herein.

NF-κB plays a key role in inflammation, immune response and cell proliferation. This assay is specifically designed to monitor the activity of NF-κB regulated signal transduction pathways in cultured cells. In this NF-κB luciferase reporter assay, the multivalent polypeptides of the present invention enhance NF-κB activity as measured by luminescence after addition of Nano-Glo™ Reagent (Promega #N1120) with EC_{50} values of 10^{-7} M or lower, preferably of 10^{-8} M or lower, more preferably of 10^{-9} M or lower, 10^{-10} M or lower, or even of 10^{-11} M or lower. More particularly, multivalent polypeptides of the present invention that comprise 2 or more monovalent polypeptides belonging to Families 7, 26, 38, 82, 85 and 109 may have EC_{50} values for enhancing NF-κB activity between 10^{-11} M and 10^{-9} M, such as between 10^{-11} M and 10^{-10} M. Preferably, in this NF-κB luciferase reporter assay, the multivalent polypeptides of the present invention enhance NF-κB activity with EC_{50} values of 200 pM or less, such as less than 190, 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40, 35, 30, 25, 20, 18, 16, 15, 14 or even less, such as less than 12 pM.

Therapeutic effects of the multivalent polypeptides of the invention can further be evaluated in *in vivo* models, such as e.g. in mice, rats, pigs and/or primates. The CT26 model in BALB/c mice provides a syngeneic *in vivo* test system, which is frequently used for developing and testing immunotherapeutic concepts (Fearon et al. Cancer Res. 48: 2975-2980, 1988). For example, in the syngeneic CT-26 colon carcinoma model as described in Examples 13, 14 and 21, the multivalent polypeptides of the invention may inhibit tumor cell growth. In some embodiments, the multivalent polypeptides of the invention inhibit tumor cell growth, inhibit or prevent an increase in tumor weight or volume, and/or cause a decrease in tumor weight or volume by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100%.

Compounds, constructs and/or polypeptides of the invention

The monovalent polypeptide of the invention and the multivalent polypeptide of the invention, may or may not further comprise one or more other groups, residues, moieties or binding units (these monovalent polypeptides as well as multivalent polypeptides (with or without additional groups, residues, moieties or binding units) are all referred to as *"compound(s) of the invention"*, *"construct(s) of the invention"* and/or *"polypeptide(s) of the invention"*). If present, such further groups, residues, moieties or binding units may or may not provide further functionality to the immunoglobulin single variable domain (and/or to the polypeptide in which it is present) and may or may not modify the properties of the immunoglobulin single variable domain.

For example, such further groups, residues, moieties or binding units may be one or more additional amino acid sequences, such that the polypeptide is a (fusion) protein or (fusion) polypeptide. In a preferred but non-limiting aspect, said one or more other groups, residues, moieties or binding units are immunoglobulins. Even more preferably, said one or more other groups, residues, moieties or binding units are immunoglobulin single variable domains chosen from the group consisting of Domain antibodies, amino acids that are suitable for use as a domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"'s, amino acids that are suitable for use as a dAb, Nanobodies (such as e.g. VHH, humanized VHH or camelized VH sequences).

As described above, additional binding units, such as immunoglobulin single variable domains having different antigen specificity can be linked to form multispecific polypeptides. By combining immunoglobulin single variable domains of two or more specificities, bispecific, trispecific etc. constructs can be formed. For example, a polypeptide according to the invention may comprise one, two, three, four, five or more immunoglobulin single variable domains directed against GITR and one immunoglobulin single variable domain against another target. Such constructs and modifications thereof, which the skilled person can readily envisage, are all encompassed by the term *"compound of the invention, construct of the invention and/or polypeptide of the invention"* as used herein.

In the compounds, constructs and/or polypeptides described above, the one, two, three, four, five, six, or more immunoglobulin single variable domains 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 polypeptide is a fusion (protein) or fusion (polypeptide).

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

Examples of such amino acid sequences will be clear to the skilled person, and may generally comprise all amino acid sequences that are used in peptide fusions based on conventional antibodies and fragments thereof (including but not limited to ScFv's and single domain antibodies). Reference is for example made to the review by Holliger and Hudson (Nature Biotechnology 23: 1126-1136, 2005).

For example, such an amino acid sequence may or may not be an amino acid sequence that increases the half-life, the solubility, or the absorption, reduces the immunogenicity or the toxicity, eliminates or attenuates undesirable side effects, and/or confers other advantageous properties to and/or reduces the undesired properties of the compound, construct and/or polypeptide of the invention, compared to polypeptide of the invention per se. Some non-limiting examples of such amino acid sequences are serum proteins, such as human serum albumin (see for example WO 00/27435) or haptenic molecules (for example haptens that are recognized by circulating antibodies, see for example WO 98/22141).

In one specific aspect of the invention, a compound or construct is prepared that has an increased half-life, compared to the corresponding polypeptide of the invention. Examples of polypeptides of the invention that comprise such half-life extending moieties for example include, without limitation, polypeptides in which the immunoglobulin single variable domains are suitable linked to one or more serum proteins or fragments thereof (such as (human) serum albumin or suitable fragments thereof) or to one or more binding units that can bind to serum proteins (such as, for example, Domain antibodies, amino acids that are suitable for use as a domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"'s, amino acids that are suitable for use as a dAb, Nanobodies, VHH sequences, humanized VHH sequences or camelized VH sequences) that can bind to serum proteins (such as serum albumin (such as human serum albumin)), serum immunoglobulins (such as IgG), transferrin or one of the other serum proteins listed in WO 04/003019; polypeptides in which the immunoglobulin single variable domain is linked to an Fc portion (such as a human Fc), an antibody constant region (such as an antibody constant region from an IgG) or a suitable part or fragment thereof; or polypeptides in which the one or more immunoglobulin single variable domains are suitably linked to one or more small proteins or peptides that can bind to serum proteins (such as, without limitation, the proteins and peptides described in WO 91/01743, WO 01/45746 or WO 02/076489). Reference is also made to the dAb's described in WO 03/002609 and WO 04/003019 and to Harmsen et al. (Vaccine 23: 4926-42, 2005);

to EP 0368684, as well as to WO 08/028977, WO 08/043821, WO 08/043822 by Ablynx N.V. and WO 08/068280.

According to a specific, but non-limiting aspect of the invention, the polypeptides of the invention may contain, besides the one or more immunoglobulin single variable domains and/or monovalent polypeptides of the invention against GITR, at least one immunoglobulin single variable domain against human serum albumin. These immunoglobulin single variable domains against human serum albumin may be as generally described in the applications by Ablynx N.V. cited above (see for example WO 04/062551). Some particularly preferred Nanobodies that provide for increased half-life and that can be used in the polypeptides of the invention include the Nanobodies ALB-1 to ALB-10 disclosed in WO 06/122787 (see Tables II and III) of which ALB-8 (SEQ ID NO: 62 in WO 06/122787) is particularly preferred, as well as the Nanobodies disclosed in WO 2012/175400 (SEQ ID NOs: 1-11 of WO 2012/175400), the Nanobody with SEQ ID NO: 109 disclosed in the co-pending US provisional application No 62/047,560 entitled *"Improved immunoglobulin single variable domains"* (date of filing: September 8, 2014; assignee: Ablynx N.V.), and the Nanobodies disclosed in the co-pending US provisional application No 62/256,841 entitled *"Improved serum albumin binders"* (date of filing: November 18, 2015; assignee: Ablynx N.V.) of which Alb92 and Alb223 are particularly preferred (SEQ ID NO: 10 and SEQ ID NO: 63 in US 62/256,841, respectively).

In a particularly preferred but non-limiting aspect of the invention, the invention provides a polypeptide of the invention comprising at least one immunoglobulin single variable domain (ISVD); and further comprising one or more (preferably one) serum albumin binding immunoglobulin single variable domain as described herein, e.g. the serum albumin binding immunoglobulin single variable domain of Alb11, Alb23, Alb129, Alb132, Alb8, Alb11 (S112K)-A, Alb82, Alb82-A, Alb82-AA, Alb82-AAA, Alb82-G, Alb82-GG, Alb82-GGG, Alb92 or Alb223 (see Table A-14).

Accordingly, the polypeptide of the invention may, for example, be a tetravalent, bispecific polypeptide, comprising three immunoglobulin single variable domains, preferably monovalent polypeptides of the invention against GITR and a fourth immunoglobulin single variable domain directed against (human) serum albumin, in which said first, second, third and fourth immunoglobulin single variable domain may optionally be linked via one or more, and in particular three, linker sequences.

According to another aspect, one or more polypeptides of the invention may be linked (optionally via a suitable linker or hinge region) to one or more constant domains (for example, 2 or 3 constant domains that can be used as part of/to form an Fc portion), to an Fc portion, to an antibody constant region of an IgG type and/or to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the invention and/or may confer the ability to bind to one or more Fc receptors. For example, for this purpose, and without being limited thereto, the one or more further amino acid sequences may comprise one or more C_H2 and/or C_H3 domains of an antibody, such as from a heavy chain antibody (as described herein) and more preferably from a conventional human 4-chain antibody; and/or may form (part of) a Fc region, for example from IgG (e.g. from IgG1, IgG2, IgG3 or IgG4), from IgE or from another human Ig such as IgA, IgD or IgM. For example, WO 94/04678 describes heavy chain antibodies comprising a Camelid V_{HH} domain or a humanized derivative thereof (i.e. a Nanobody), in which the Camelidae C_H2 and/or C_H3 domain have been replaced by human C_H2 and C_H3 domains, so as to provide an immunoglobulin that consists of 2 heavy chains each comprising a Nanobody and human C_H2 and C_H3 domains (but no C_H1 domain), which immunoglobulin has the effector function provided by the C_H2 and C_H3 domains and which immunoglobulin can function without the presence of any light chains. In a more preferred aspect of the invention, the one or more further amino acid sequences may comprise one or more C_H1, C_H2, C_H3 and/or C_L domains of an antibody or fragments thereof, preferably from a conventional 4-chain antibody; and/or may form (part of) a human antibody constant region, for example from IgG (e.g. from IgG1, IgG2, IgG3 or IgG4), from IgE or from another human Ig such as IgA, IgD or IgM, so as to provide a compound or construct (such as a Nanobody-IgG chimera) that consists of i) 2 heavy chains each comprising a Nanobody and human C_H1, C_H2 and C_H3 heavy chain domains, wherein the C_H1 heavy chain domain is directly linked to the C-terminal part of the Nanobody and ii) 2 light chains each comprising a Nanobody and human C_L light chain domains (such as Cκ or Cλ), wherein the C_L light chain domain is directly linked to the C-terminal part of the Nanobody (see Figure 7). More particular, such compounds or constructs are of the IgG type and comprise an amino acid sequence set forth in one of SEQ ID NO: 229, 230, 291 and SEQ ID NO: 292 or an amino acid sequence that has a sequence identity of more than 80%, preferably more than 90%, more preferably more than 95%, such as 96%, 97%, 98%, 99% or more sequence identity (as defined herein) with any of SEQ ID NOs: 229-230 and 291-292.

Other amino acid sequences that can be suitably linked to the polypeptides of the invention so as to provide an effector function will be clear to the skilled person, and may be chosen on the basis of the desired effector function(s). Reference is for example made to WO 04/058820, WO 99/42077, WO 02/056910 and WO 05/017148, as well as the review by Holliger and Hudson, *supra*; and to WO 09/068628. Coupling of a polypeptide of the invention to an Fc portion or an antibody constant region may also lead to an increased half-life, compared to the corresponding polypeptide of the invention.

Other suitable constructs comprising one or more polypeptides of the invention and one or more constant domains with increased half-life *in vivo* will be clear to the skilled person, and may for example comprise polypeptides linked to a C_H3 domain, optionally via a linker sequence. Generally, any fusion protein or derivatives with increased half-life will preferably have a molecular weight of more than 50 kD, the cut-off value for renal absorption.

In another specific, but non-limiting, aspect, the polypeptides of the invention may be linked (optionally via a suitable linker or hinge region) to naturally occurring, synthetic or semi-synthetic constant domains (or analogs, variants, mutants, parts or fragments thereof) that have a reduced (or essentially no) tendency to self-associate into dimers (*i.e.* compared to constant domains that naturally occur in conventional 4-chain antibodies). Such monomeric (*i.e.* not self-associating) Fc chain variants, or fragments thereof, will be clear to the skilled person. For example, Helm et al. (J. Biol. Chem. 271: 7494, 1996), describe monomeric Fc chain variants that can be used in the polypeptide chains of the invention.

Also, such monomeric Fc chain variants are preferably such that they are still capable of binding to the complement or the relevant Fc receptor(s) (depending on the Fc portion from which they are derived), and/or such that they still have some or all of the effector functions of the Fc portion from which they are derived (or at a reduced level still suitable for the intended use). Alternatively, in such a polypeptide chain of the invention, the monomeric Fc chain may be used to confer increased half-life upon the polypeptide chain, in which case the monomeric Fc chain may also have no or essentially no effector functions.

Generally, the polypeptides of the invention with increased half-life preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin single variable domain or polypeptide of the invention *per se.*

Generally, the polypeptides of the invention with increased half-life preferably have a half-life that is increased with more than 1 hour, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the half-life of the corresponding immunoglobulin single variable domain or polypeptide of the invention *per se.*

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

The further amino acid residues may or may not change, alter or otherwise influence other (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:

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 (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). Suitable secretory leader peptides will be clear to the skilled person, and may be as further described herein. Usually, such a leader sequence will be linked to the N-terminus of the polypeptide, although the invention in its broadest sense is not limited thereto;

c) may form a “tag”, for example an amino acid sequence or residue that allows or facilitates the 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 amino acid sequence or 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, glutathione residues and a myc-tag such as AAAEQKLISEEDLNGAA;

d) 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.

The multivalent polypeptides of the invention can generally be prepared by a method which comprises at least the step of suitably linking the immunoglobulin single variable domain and/or monovalent polypeptide of the invention to one or more further immunoglobulin single variable domains and/or monovalent polypeptides of the invention, optionally via the one or more suitable linkers, so as to provide the multivalent polypeptide of the invention. Polypeptides of the invention can also be prepared by a method which generally comprises at least the steps of providing a nucleic acid that encodes a polypeptide of the invention, expressing said nucleic acid in a suitable manner, and recovering the expressed polypeptide of the invention. Such methods can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the methods and techniques further described herein.

A method for preparing multivalent polypeptides of the invention may comprise at least the steps of linking two or more immunoglobulin single variable domains and/or monovalent polypeptides of the invention and for example one or more linkers together in a suitable manner. The immunoglobulin single variable domains and/or monovalent polypeptides of the invention (and linkers) can be coupled by any method known in the art and as further described herein. Preferred techniques include the linking of the nucleic acid sequences that encode the immunoglobulin single variable domains and/or monovalent polypeptides of the invention (and linkers) to prepare a genetic construct that expresses the multivalent polypeptide. Techniques for linking amino acids or nucleic acids will be clear to the skilled person, and reference is again made to the standard handbooks, such as Sambrook et al. and Ausubel et al., mentioned above, as well as the Examples below.

Accordingly, the present invention also relates to the use of an immunoglobulin single variable domain and/or monovalent polypeptide of the invention in preparing a multivalent polypeptide of the invention. The method for the preparation of a multivalent polypeptide will comprise the linking of an immunoglobulin single variable domain and/or monovalent polypeptide of the invention to at least one further immunoglobulin single variable domain and/or monovalent polypeptide of the invention, optionally via one or more linkers. The immunoglobulin single variable domain and/or monovalent polypeptide of the invention is then used as a binding domain or binding unit in providing and/or preparing the multivalent polypeptide comprising two (*e.g.,* in a bivalent polypeptide), three (*e.g.,* in a trivalent polypeptide), four (e.g., in a tetravalent polypeptide), five (e.g., in a pentavalent polypeptide), six (e.g., in a hexavalent polypeptide) or more (*e.g.,* in a multivalent polypeptide) binding units. In this respect, the immunoglobulin singe variable domain and/or the monovalent polypeptide of the invention may be used as a binding domain or binding unit in providing and/or preparing a multivalent, such as bivalent, trivalent, tetravalent, pentavalent or hexavalent polypeptide of the invention comprising two, three, four, five, six or more binding units.

Accordingly, the present invention also relates to the use of an immunoglobulin single variable domain and/or particularly, a monovalent polypeptide of the invention (as described herein) in preparing a multivalent polypeptide. The method for the preparation of the multivalent polypeptide will comprise the linking of the immunoglobulin single variable domain and/or monovalent polypeptide of the invention to at least one further immunoglobulin single variable domain and/or monovalent polypeptide of the invention, optionally via one or more linkers.

Suitable spacers or linkers for use in multivalent 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 acid sequences. Preferably, said linker or spacer is suitable for use in constructing 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 sequence, and in particular amino acid sequences of between 1 and 50, preferably between 1 and 30, such as between 1 and 10 amino acid residues. Some preferred examples of such amino acid sequences include gly-ser linkers, for example of the type $(gly_xser_y)_z$, 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).

Some other particularly preferred linkers are mentioned in Table A-15, of which GS9 (SEQ ID NO: 251) is particularly preferred.

Other suitable linkers generally comprise organic compounds or polymers, in particular those suitable for use in proteins for pharmaceutical use. For instance, poly(ethyleneglycol) moieties have been used to link antibody domains, see for example WO 04/081026.

It is encompassed within the scope of the invention that the length, the degree of flexibility and/or other properties of the linker(s) used (although not critical, as it usually is for linkers used in ScFv fragments) may have some influence on the properties of the final polypeptide of the invention, including but not limited to the affinity, specificity or avidity for GITR, or for one or more of the other antigens. Based on the disclosure herein, the skilled person will be able to determine the optimal linker(s) for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

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.

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

Usually, for ease of expression and production, a polypeptide of the invention will be a linear polypeptide. However, the invention in its broadest sense is not limited thereto. For example, when a polypeptide of the invention comprises three of more amino acid sequences or Nanobodies, it is possible to link them by use of a linker with three or more “arms”, which each “arm” being linked to an amino acid sequence or Nanobody, so as to provide a “star-shaped” construct. It is also possible, although usually less preferred to use circular constructs.

Also encompassed in the present invention are compounds, constructs and/or polypeptides that comprise an immunoglobulin or polypeptide of the invention and further comprising tags or other functional moieties, *e.g.,* toxins, labels, radiochemicals, etc..

Alternatively, the additional groups, residues, moieties or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the one or more immunoglobulin single variable domains or monovalent polypeptides of the invention so as to provide a “derivative” of the polypeptide of the invention.

Accordingly, the invention in its broadest sense also comprises compounds, constructs and/or polypeptides that are derivatives of the polypeptides 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 of the invention and/or of one or more of the amino acid residues that form polypeptide of the invention.

Examples of such modifications, as well as examples of amino acid residues within the polypeptide 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 (see also Zangi et al., Nat Biotechnol 31(10):898-907, 2013).

For example, such a modification may involve the introduction (*e.g.,* by covalent linking or in any other suitable manner) of one or more functional groups, residues or moieties into or onto the polypeptide 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 of the invention. Examples of such functional groups will be clear to the skilled person.

For example, such modification may comprise the introduction (*e.g.,* by covalent binding or in any other suitable manner) of one or more functional groups that increase the half-life, the solubility and/or the absorption of the polypeptide of the invention, that reduce the immunogenicity and/or the toxicity of the polypeptide of the invention, that eliminate or attenuate any undesirable side effects of the polypeptide of the invention, and/or that confer other advantageous properties to and/or reduce the undesired properties of the polypeptide 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 (Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA, 1980). Such functional groups may for example be linked directly (for example covalently) to a polypeptide of the invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

One specific example is a derivative polypeptide of the invention wherein the polypeptide of the invention has been chemically modified to increase the half-life thereof (for example, by means of pegylation). This is one of the most widely used techniques for increasing the half-life and/or reducing the immunogenicity of pharmaceutical proteins and comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv's); reference is made to for example Chapman (Nat. Biotechnol. 54: 531-545, 2002), Veronese and Harris (Adv. Drug Deliv. Rev. 54: 453-456, 2003), Harris and Chess (Nat. Rev. Drug. Discov. 2: 214-221, 2003) and WO 04/060965. Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA.

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

Preferably, for the polypeptides of the invention, a PEG is used with a molecular weight of more than 5000 Dalton, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the range of 20,000-80,000 Dalton.

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 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 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 ^{152}Eu or others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes (such as ^{3}H, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, and ^{75}Se), metals, metals chelates or metallic cations (for example metallic cations such as ^{99m}Tc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, and ^{68}Ga or other metals or metallic cations that are particularly suited for use in *in vivo*, *in vitro* or *in situ* diagnosis and imaging, such as (^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe)), as well as chromophores and enzymes (such as malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels will be clear to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy.

Such labelled polypeptides of the invention may for example be used for *in vitro*, *in vivo* or *in situ* assays (including immunoassays known per se such as ELISA, RIA, EIA and other “sandwich assays”, etc.) as well as *in vivo* diagnostic and imaging purposes, depending on the choice of the specific label.

As will be clear to the skilled person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable chelating groups for example include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the polypeptide 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 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 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 of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh (Journal of Drug Targeting 8: 257, 2000). Such binding pairs may also be used to link a therapeutically active agent to the polypeptide 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 (Biotechnol. Appl. Biochem. 26: 143-151, 1997).

Preferably, the compounds, constructs, polypeptides and/or derivatives are such that they bind to GITR, with an affinity (suitably measured and/or expressed as a K_D-value (actual or apparent), a K_A-value (actual or apparent), a k_{on}-rate and/or a k_{off}-rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein (i.e. as defined for the polypeptides of the invention). Such derivatives will usually also have a GITR efficacy and/or potency as defined herein.

Such compounds, constructs and/or polypeptides of the invention and derivatives thereof may also be in essentially isolated form (as defined herein).

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

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

The method for producing a polypeptide 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 of the invention (also referred to herein as a "*nucleic acid of the invention*"),

optionally followed by:

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

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

- cultivating and/or maintaining a host of the invention under conditions that are such that said host of the invention expresses and/or produces at least one polypeptide of the invention;

optionally followed by:

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

Accordingly, the present invention also relates to a nucleic acid or nucleotide sequence that encodes a polypeptide of the invention (also referred to as "*nucleic acid 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 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 two nucleic acids encoding an immunoglobulin single variable domain or a monovalent polypeptide 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 above, as well as to 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.

Preferably, the regulatory and further elements of the genetic constructs of the invention are such that they are capable of providing their intended biological function in the intended host cell or host organism.

For instance, a promoter, enhancer or terminator should be “operable” in the intended host cell or host organism, by which is meant that (for example) said promoter should be capable of initiating or otherwise controlling/regulating the transcription and/or the expression of a nucleotide sequence - *e.g.,* a coding sequence - to which it is operably linked (as defined herein).

Some particularly preferred promoters include, but are not limited to, promoters known per se for the expression in the host cells mentioned herein; and in particular promoters for the expression in the bacterial cells, such as those mentioned herein and/or those used in the Examples.

A selection marker should be such that it allows - *i.e.,* under appropriate selection conditions - host cells and/or host organisms that have been (successfully) transformed with the nucleotide sequence of the invention to be distinguished from host cells/organisms that have not been (successfully) transformed. Some preferred, but non-limiting examples of such markers are genes that provide resistance against antibiotics (such as kanamycin or ampicillin), genes that provide for temperature resistance, or genes that allow the host cell or host organism to be maintained in the absence of certain factors, compounds and/or (food) components in the medium that are essential for survival of the non-transformed cells or organisms.

A leader sequence should be such that - in the intended host cell or host organism - it allows for the desired post-translational modifications and/or such that it directs the transcribed mRNA to a desired part or organelle of a cell. A leader sequence may also allow for secretion of the expression product from said cell. As such, the leader sequence may be any pro-, pre-, or prepro-sequence operable in the host cell or host organism. Leader sequences may not be required for expression in a bacterial cell. For example, leader sequences known per se for the expression and production of antibodies and antibody fragments (including but not limited to single domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

An expression marker or reporter gene should be such that - in the host cell or host organism - it allows for detection of the expression of (a gene or nucleotide sequence present on) the genetic construct. An expression marker may optionally also allow for the localisation of the expressed product, *e.g.,* in a specific part or organelle of a cell and/or in (a) specific cell(s), tissue(s), organ(s) or part(s) of a multicellular organism. Such reporter genes may also be expressed as a protein fusion with the amino acid sequence or polypeptide of the invention. Some preferred, but non-limiting examples include fluorescent proteins such as GFP.

Some preferred, but non-limiting examples of suitable promoters, terminator and further elements include those that can be used for the expression in the host cells mentioned herein; and in particular those that are suitable for expression in bacterial cells, such as those mentioned herein and/or those used in the Examples below. For some (further) non-limiting examples of the promoters, selection markers, leader sequences, expression markers and further elements that may be present/used in the genetic constructs of the invention - such as terminators, transcriptional and/or translational enhancers and/or integration factors - reference is made to the general handbooks such as Sambrook et al. and Ausubel et al. mentioned above, as well as to the examples that are given in WO 95/07463, WO 96/23810, WO 95/07463, WO 95/21191, WO 97/11094, WO 97/42320, WO 98/06737, WO 98/21355, US 7,207,410, US 5,693,492 and EP 1085089. Other examples will be clear to the skilled person. Reference is also made to the general background art cited above and the further references cited herein.

The genetic constructs of the invention may generally be provided by suitably linking the nucleotide sequence(s) of the invention to the one or more further elements described above, for example using the techniques described in the general handbooks such as Sambrook et al. and Ausubel et al., mentioned above.

Often, the genetic constructs of the invention will be obtained by inserting a nucleotide sequence of the invention in a suitable (expression) vector known per se. Some preferred, but non-limiting examples of suitable expression vectors are those used in the Examples below, as well as those mentioned herein.

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

- a bacterial strain, including but not limited to gram-negative strains such as strains of *Escherichia coli*; of *Proteus*, for example of *Proteus mirabilis*; of *Pseudomonas*, for example of *Pseudomonas fluorescens*; and gram-positive strains such as strains of *Bacillus*, for example of *Bacillus subtilis* or of *Bacillus brevis*; of *Streptomyces*, for example of *Streptomyces lividans*; of *Staphylococcus*, for example of *Staphylococcus carnosus*; and of *Lactococcus*, for example of *Lactococcus lactis*;
- a fungal cell, including but not limited to cells from species of *Trichoderma*, for example from *Trichoderma reesei*; of *Neurospora*, for example from *Neurospora crassa*; of *Sordaria*, for example from *Sordaria macrospora*; of *Aspergillus*, for example from *Aspergillus niger* or from *Aspergillus sojae*; or from other filamentous fungi;
- a yeast cell, including but not limited to cells from species of *Saccharomyces*, for example of *Saccharomyces cerevisiae*; of *Schizosaccharomyces*, for example of *Schizosaccharomyces pombe*; of *Pichia*, for example of *Pichia pastoris* or of *Pichia methanolica*; of *Hansenula*, for example of *Hansenula polymorpha*; of *Kluyveromyces*, for example of *Kluyveromyces lactis*; of *Arxula*, for example of *Arxula adeninivorans*; of *Yarrowia*, for example of *Yarrowia lipolytica*;
- an amphibian cell or cell line, such as *Xenopus oocytes*;

- an insect-derived cell or cell line, such as cells/cell lines derived from lepidoptera, including but not limited to *Spodoptera* SF9 and Sf21 cells or cells/cell lines derived from *Drosophila*, such as Schneider and Kc cells;
- a plant or plant cell, for example in tobacco plants; and/or
- a mammalian cell or cell line, for example a cell or cell line derived from a human, a cell or a cell line from mammals including but not limited to CHO-cells (for example CHO-K1 cells), BHK-cells and human cells or cell lines such as HeLa, COS, Caki and HEK293H cells;

as well as all other host cells or (non-human) hosts known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments), which will be clear to the skilled person. Reference is also made to the general background art cited hereinabove, as well as to for example WO 94/29457; WO 96/34103; WO 99/42077; Frenken et al. (Res Immunol. 149: 589-99, 1998); Riechmann and Muyldermans (1999), supra; van der Linden (J. Biotechnol. 80: 261-70, 2000); Joosten et al. (Microb. Cell Fact. 2: 1, 2003); Joosten et al. (Appl. Microbiol. Biotechnol. 66: 384-92, 2005); and the further references cited therein.

The polypeptides of the invention may also be expressed as so-called “intrabodies”, as for example described in WO 94/02610, WO 95/22618 and US 7,004,940; WO 03/014960; in Cattaneo and Biocca (“Intracellular Antibodies: Development and Applications” Landes and Springer-Verlag, 1997); and in Kontermann (Methods 34: 163-170, 2004).

The polypeptides of the invention can for example also be produced in the milk of transgenic mammals, for example in the milk of rabbits, cows, goats or sheep (see for example US 6,741,957, US 6,304,489 and US 6,849,992 for general techniques for introducing transgenes into mammals), in plants or parts of plants including but not limited to their leaves, flowers, fruits, seed, roots or tubers (for example in tobacco, maize, soybean or alfalfa) or in for example pupae of the silkworm *Bombix mori*.

Furthermore, the polypeptides of the invention can also be expressed and/or produced in cell-free expression systems, and suitable examples of such systems will be clear to the skilled person. Some preferred, but non-limiting examples include expression in the wheat germ system; in rabbit reticulocyte lysates; or in the *E. coli* Zubay system.

Preferably, in the invention, an (*in vivo* or *in vitro*) expression system, such as a bacterial expression system, is used that provides the polypeptides of the invention in a form that is suitable for pharmaceutical use, and such expression systems will again be clear to the skilled person. As also will be clear to the skilled person, polypeptides of the invention suitable for pharmaceutical use can be prepared using techniques for peptide synthesis.

For production on industrial scale, preferred heterologous hosts for the (industrial) production of immunoglobulin single variable domains or immunoglobulin single variable domain-containing polypeptide therapeutics include strains of *E. coli*, *Pichia pastoris*, *S. cerevisiae* that are suitable for large scale expression/production/fermentation, and in particular for large scale pharmaceutical expression/production/fermentation. Suitable examples of such strains will be clear to the skilled person. Such strains and production/expression systems are also made available by companies such as Biovitrum (Uppsala, Sweden).

Alternatively, mammalian cell lines, in particular Chinese hamster ovary (CHO) cells, can be used for large scale expression/production/fermentation, and in particular for large scale pharmaceutical expression/production/fermentation. Again, such expression/production systems are also made available by some of the companies mentioned above.

The choice of the specific expression system would depend in part on the requirement for certain post-translational modifications, more specifically glycosylation. The production of an immunoglobulin single variable domain-containing recombinant protein for which glycosylation is desired or required would necessitate the use of mammalian expression hosts that have the ability to glycosylate the expressed protein. In this respect, it will be clear to the skilled person that the glycosylation pattern obtained (*i.e.,* the kind, number and position of residues attached) will depend on the cell or cell line that is used for the expression. Preferably, either a human cell or cell line is used (*i.e.,* leading to a protein that essentially has a human glycosylation pattern) or another mammalian cell line is used that can provide a glycosylation pattern that is essentially and/or functionally the same as human glycosylation or at least mimics human glycosylation. Generally, prokaryotic hosts such as *E. coli* do not have the ability to glycosylate proteins, and the use of lower eukaryotes such as yeast usually leads to a glycosylation pattern that differs from human glycosylation. Nevertheless, it should be understood that all the foregoing host cells and expression systems can be used in the invention, depending on the desired polypeptide to be obtained.

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

According to one preferred, but non-limiting embodiment of the invention, the polypeptide of the invention is produced in a bacterial cell, in particular a bacterial cell suitable for large scale pharmaceutical production, such as cells of the strains mentioned above.

According to another preferred, but non-limiting embodiment of the invention, the polypeptide of the invention is produced in a yeast cell, in particular a yeast cell suitable for large scale pharmaceutical production, such as cells of the species mentioned above.

According to yet another preferred, but non-limiting embodiment of the invention, the polypeptide of the invention is produced in a mammalian cell, in particular in a human cell or in a cell of a human cell line, and more in particular in a human cell or in a cell of a human cell line that is suitable for large scale pharmaceutical production, such as the cell lines mentioned hereinabove.

When expression in a host cell is used to produce the polypeptides of the invention, the polypeptides of the invention can be produced either intracellullarly (*e.g.,* in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or can be produced extracellularly (*e.g.,* in the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified. When eukaryotic host cells are used, extracellular production is usually preferred since this considerably facilitates the further isolation and downstream processing of the polypeptides obtained. Bacterial cells such as the strains of *E. coli* mentioned above normally do not secrete proteins extracellularly, except for a few classes of proteins such as toxins and hemolysin, and secretory production in *E. coli* refers to the translocation of proteins across the inner membrane to the periplasmic space. Periplasmic production provides several advantages over cytosolic production. For example, the N-terminal amino acid sequence of the secreted product can be identical to the natural gene product after cleavage of the secretion signal sequence by a specific signal peptidase. Also, there appears to be much less protease activity in the periplasm than in the cytoplasm. In addition, protein purification is simpler due to fewer contaminating proteins in the periplasm. Another advantage is that correct disulfide bonds may form because the periplasm provides a more oxidative environment than the cytoplasm. Proteins overexpressed in *E. coli* are often found in insoluble aggregates, so-called inclusion bodies. These inclusion bodies may be located in the cytosol or in the periplasm; the recovery of biologically active proteins from these inclusion bodies requires a denaturation/refolding process. Many recombinant proteins, including therapeutic proteins, are recovered from inclusion bodies. Alternatively, as will be clear to the skilled person, recombinant strains of bacteria that have been genetically modified so as to secrete a desired protein, and in particular a polypeptide of the invention, can be used.

Thus, according to one non-limiting embodiment of the invention, the polypeptide of the invention is a polypeptide that has been produced intracellularly and that has been isolated from the host cell, and in particular from a bacterial cell or from an inclusion body in a bacterial cell. According to another non-limiting embodiment of the invention, the polypeptide of the invention is a polypeptide that has been produced extracellularly, and that has been isolated from the medium in which the host cell is cultivated.

Some preferred, but non-limiting promoters for use with these host cells include:

- for expression in *E. coli*: lac promoter (and derivatives thereof such as the lacUV5 promoter); arabinose promoter; left- (PL) and rightward (PR) promoter of phage lambda; promoter of the trp operon; hybrid lac/trp promoters (tac and trc); T7-promoter (more specifically that of T7-phage gene 10) and other T-phage promoters; promoter of the Tn10 tetracycline resistance gene; engineered variants of the above promoters that include one or more copies of an extraneous regulatory operator sequence;
- for expression in *S. cerevisiae*: constitutive: ADH1 (alcohol dehydrogenase 1), ENO (enolase), CYC1 (cytochrome c iso-1), GAPDH (glyceraldehydes-3-phosphate dehydrogenase), PGK1 (phosphoglycerate kinase), PYK1 (pyruvate kinase); regulated: GAL1,10,7 (galactose metabolic enzymes), ADH2 (alcohol dehydrogenase 2), PHO5 (acid phosphatase), CUP1 (copper metallothionein); heterologous: CaMV (cauliflower mosaic virus 35S promoter);
- for expression in *Pichia pastoris*: the AOX1 promoter (alcohol oxidase I);
- for expression in mammalian cells: human cytomegalovirus (hCMV) immediate early enhancer/promoter; human cytomegalovirus (hCMV) immediate early promoter variant that contains two tetracycline operator sequences such that the promoter can be regulated by the Tet repressor; Herpes Simplex Virus thymidine kinase (TK) promoter; Rous Sarcoma Virus long terminal repeat (RSV LTR) enhancer/promoter; elongation factor 1α (hEF-1α) promoter from human, chimpanzee, mouse or rat; the SV40 early promoter; HIV-1 long terminal repeat promoter; β-actin promoter;

Some preferred, but non-limiting vectors for use with these host cells include:

- vectors for expression in mammalian cells: pMAMneo (Clontech), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC37199), pRSVneo (ATCC37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460) and 1ZD35 (ATCC 37565), as well as viral-based expression systems, such as those based on adenovirus;
- vectors for expression in bacterial cells: pET vectors (Novagen) and pQE vectors (Qiagen);
- vectors for expression in yeast or other fungal cells: pYES2 (Invitrogen) and Pichia expression vectors (Invitrogen);
- vectors for expression in insect cells: pBlueBacII (Invitrogen) and other baculovirus vectors
- vectors for expression in plants or plant cells: for example vectors based on cauliflower mosaic virus or tobacco mosaic virus, suitable strains of Agrobacterium, or Ti-plasmid based vectors.

Some preferred, but non-limiting secretory sequences for use with these host cells include:

- for use in bacterial cells such as *E. coli*: PelB, Bla, OmpA, OmpC, OmpF, OmpT, StII, PhoA, PhoE, MalE, Lpp, LamB, and the like; TAT signal peptide, hemolysin C-terminal secretion signal;
- for use in yeast: α-mating factor prepro-sequence, phosphatase (pho1), invertase (Suc), etc.;

- for use in mammalian cells: indigenous signal in case the target protein is of eukaryotic origin; murine Ig κ-chain V-J2-C signal peptide; etc.

Suitable techniques for transforming a host or host cell of the invention will be clear to the skilled person and may depend on the intended host cell/host organism and the genetic construct to be used. Reference is again made to the handbooks and patent applications mentioned above.

After transformation, a step for detecting and selecting those host cells or host organisms that have been 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 or a stable cell line) or host organisms (which may be in the form of a stable mutant line or strain) form further aspects of the present invention.

Preferably, these host cells or host organisms are such that they express, or are (at least) capable of expressing (*e.g.,* under suitable conditions), a polypeptide of the invention (and in case of a host organism: in at least one cell, part, tissue or organ thereof). The invention also includes further generations, progeny and/or offspring of the host cell or host organism of the invention, that may for instance be obtained by cell division or by sexual or asexual reproduction.

To produce/obtain expression of the polypeptides 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 of the invention is expressed/produced. Suitable conditions will be clear to the skilled person and will usually depend upon the host cell/host organism used, as well as on the regulatory elements that control the expression of the (relevant) nucleotide sequence of the invention. Again, reference is made to the handbooks and patent applications mentioned above in the paragraphs on the genetic constructs of the invention.

Generally, suitable conditions may include the use of a suitable medium, the presence of a suitable source of food and/or suitable nutrients, the use of a suitable temperature, and optionally the presence of a suitable inducing factor or compound (*e.g.,* when the nucleotide sequences of the invention are under the control of an inducible promoter); all of which may be selected by the skilled person. Again, under such conditions, the polypeptides of the invention may be expressed in a constitutive manner, in a transient manner, or only when suitably induced.

It will also be clear to the skilled person that the polypeptide of the invention may (first) be generated in an immature form (as mentioned above), which may then be subjected to post-translational modification, depending on the host cell/host organism used. Also, the polypeptide of the invention may be glycosylated, again depending on the host cell/host organism used.

The polypeptide of the invention may then be isolated from the host cell/host organism and/or from the medium in which said host cell or host organism was cultivated, using protein isolation and/or purification techniques known per se, such as (preparative) chromatography and/or electrophoresis techniques, differential precipitation techniques, affinity techniques (*e.g.,* using a specific, cleavable amino acid sequence fused with the polypeptide of the invention) and/or preparative immunological techniques (i.e. using antibodies against the polypeptide to be isolated).

Compositions of the invention

Generally, for pharmaceutical use, the polypeptides, compounds, and/or constructs of the invention may be formulated as a pharmaceutical preparation or composition comprising at least one polypeptide, compound, and/or construct of the invention and at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally one or more further pharmaceutically active polypeptides and/or compounds. By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (such as by intravenous, intramuscular or subcutaneous injection or intravenous infusion), for topical administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, etc, wherein the parenteral administration is preferred. 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. Such a pharmaceutical preparation or composition will generally be referred to herein as a “pharmaceutical composition”. A pharmaceutical preparation or composition for use in a non-human organism will generally be referred to herein as a “veterinary composition”.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that contains at least one polypeptide of the invention, at least one compound of the invention, at least one construct of the invention or at least one nucleic acid 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. In a particular aspect, the invention relates to a pharmaceutical composition that contains at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-292, 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, compounds and/or constructs of the invention can be formulated and administered in any suitable manner known per se. 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, 21st Edition, Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

The polypeptides, compounds and/or constructs of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments (including ScFv's and diabodies) and other pharmaceutically active proteins. Such formulations and methods for preparing the same will be clear to the skilled person, and for example include preparations suitable for parenteral administration (e.g. 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.

The polypeptides, compounds and/or constructs of the invention can also be administered using methods of delivery known from gene therapy, see, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference for its gene therapy delivery methods. Using a gene therapy method of delivery, primary cells transfected with the gene encoding a polypeptide, compound and/or construct of the invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells and can additionally be transfected with signal and stabilization sequences for subcellularly localized expression.

Thus, the polypeptides, compounds and/or constructs of the invention may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the polypeptides, compounds and/or 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, compound and/or construct of the invention. Their percentage in the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of the polypeptide, compound and/or construct of the invention in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain binders, excipients, disintegrating agents, lubricants and sweetening or flavoring 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, compounds and/or constructs of the invention, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the polypeptides, compounds and/or constructs of the invention may be incorporated into sustained-release preparations and devices.

Preparations and formulations for oral administration may also be provided with an enteric coating that will allow the constructs of the invention to resist the gastric environment and pass into the intestines. More generally, preparations and formulations for oral administration may be suitably formulated for delivery into any desired part of the gastrointestinal tract. In addition, suitable suppositories may be used for delivery into the gastrointestinal tract.

The polypeptides, compounds and/or constructs of the invention may also be administered intravenously or intraperitoneally by infusion or injection. Particular examples are as further described on pages 144 and 145 of WO 08/020079 or in PCT/EP2010/062975 (entire document).

For topical administration, the polypeptides, compounds and/or constructs of the invention may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologic acceptable carrier, which may be a solid or a liquid. Particular examples are as further described on page 145 of WO 08/020079.

Useful dosages of the polypeptides, compounds and/or constructs of the invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see US 4,938,949.

Generally, the concentration of the polypeptides, compounds and/or constructs of the invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the polypeptides, compounds and/or constructs of the invention required for use in treatment will vary not only with the particular polypeptide, compound and/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. Also the dosage of the polypeptides, compounds and/or constructs of the invention varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations.

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. The dosage can also be adjusted by the individual physician in the event of any complication.

Uses of the polypeptides, compounds and/or constructs of the invention

The invention further relates to applications and uses of the polypeptides, compounds and/or constructs, nucleic acids, host cells and compositions described herein, as well as to methods for the prevention and/or treatment of GITR associated diseases, disorders or conditions, such as various cancers and infectious diseases. Some preferred but non-limiting applications and uses will become clear from the further description herein.

The polypeptide, compound and/or construct of the invention can generally be used to enhance an immune response. In particular, the polypeptide, compound and/or construct of the invention can enhance the proliferation or activation of T cells, B cells or natural killer cells by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100% compared to the activation status of T cells, B cells or natural killer cells in the absence of the polypeptide, compound and/or construct of the invention, as determined by a suitable assay, such as those described herein.

In another aspect, the polypeptide, compound and/or construct of the invention can inhibit tumor growth, induce tumor regression, increase progression-free survival and/or extend overall survival in an individual that has a tumor by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more, such as 100% compared to the tumor, progression-free survival and/or overall survival in that individual in the absence of the polypeptide, compound and/or construct of the invention, as determined by a suitable assay, such as those described herein.

In a further aspect, the invention relates to a method for the prevention and/or treatment of at least one GITR associated disease, disorder or condition, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

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

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

The invention relates to a method for the prevention and/or treatment of at least one disease, disorder or condition that is associated with GITR, with its biological or pharmacological activity, and/or with the biological pathways or signaling in which GITR is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same. In particular, said pharmaceutically effective amount may be an amount that is sufficient to stimulate, enhance or agonize GITR, its biological or pharmacological activity, and/or the biological pathways or signaling in which GITR is involved; and/or an amount that provides a level of the polypeptide of the invention, of the compound of the invention, and/or of the construct of the invention in the circulation that is sufficient to stimulate, enhance or agonize GITR, its biological or pharmacological activity, and/or the biological pathways or signaling in which GITR is involved.

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

More in particular, the invention relates to a method for the prevention and/or treatment of at least one disease, disorder and/or condition chosen from the group consisting of the diseases, disorders and conditions listed herein, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for enhancing an immune response.

The invention also relates to a method for enhancing proliferation or activation of T cells, B cells or natural killer cells.

The invention also relates to a method for inhibiting tumor growth.

The invention also relates to a method for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

More in particular, the invention also relates to a method for enhancing proliferation or activation of T cells, B cells or natural killer cells, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for inhibiting tumor growth, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for prevention and/or treatment of cancer, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide of the invention, of a compound of the invention, of a construct of the invention and/or of a pharmaceutical composition comprising the same.

More in particular, the invention also relates to a method for enhancing proliferation or activation of T cells, B cells or natural killer cells, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-292, and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for inhibiting tumor growth, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-292, and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for prevention and/or treatment of T cell, B cell or natural killer mediated diseases, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-, and/or of a pharmaceutical composition comprising the same.

The invention also relates to a method for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-292, and/or of a pharmaceutical composition comprising the same. Infections can be broadly classified as bacterial, fungal, viral, or parasitic based on the category of infectious organism or agent involved. Examples of bacteria, fungi, viruses and parasites which cause infection are well known in the art.

Some preferred, but non-limiting examples of pathogenic bacteria causing infections treatable by the method of the invention include syphilis, chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lymes disease bacteria.

Some preferred, but non-limiting examples of pathogenic viruses causing infections treatable by the method of the invention include HIV, hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus, arboviral encephalitis virus, and ebolaviruses (e.g., BDBV, EBOV, RESTV, SUDV and TAFV).

Some preferred, but non-limiting examples of pathogenic fungi causing infections treatable by the method of the invention include *Candida (albicans, krusei, glabrata, tropicalis*, etc.), *Cryptococcus neoformans*, *Aspergillus* (*fumigatus, niger*, etc.), *Genus Mucorales* (*mucor, absidia, rhizophus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis* and *Histoplasma capsulatum.*

Some preferred, but non-limiting examples of pathogenic parasites causing infections treatable by the method of the invention include *Entamoeba histolytica*, *Balantidium coli*, *Naegleriafowleri*, *Acanthamoeba sp.*, *Giardia lambia*, *Cryptosporidium sp.*, *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondi*, and *Nippostrongylus brasiliensis*. Accordingly, the present invention relates to a method for the prevention and/or treatment of infectious diseases with these bacterial, fungal, viral, or parasitic agents.

The invention also relates to a method for prevention and/or treatment of cancer, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-71, 206-223, 229-230, 266-275 and 285-292, and/or of a pharmaceutical composition comprising the same.

In particular, the present invention relates to a method for the prevention and/or treatment of squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

In another particular aspect, the present invention relates to a method for the prevention and/or treatment of squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies, said method comprising administering a pharmaceutically active amount of at least one of SEQ ID NOs: 1-72, 206-223, 229-230, 266-275 and 285-292, and/or of a pharmaceutical composition comprising the same.

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

In the above methods, the polypeptides, compounds and/or constructs of the invention and/or the compositions comprising the same can be administered in any suitable manner, depending on the specific pharmaceutical formulation or composition to be used. Thus, the polypeptides, compounds and/or constructs of the invention and/or the compositions comprising the same can for example be administered orally, intraperitoneally (e.g. intravenously, subcutaneously, intramuscularly, or via any other route of administration that circumvents the gastrointestinal tract), intranasally, transdermally, topically, by means of a suppository, by inhalation, again depending on the specific pharmaceutical formulation or composition to be used. The clinician will be able to select a suitable route of administration and a suitable pharmaceutical formulation or composition to be used in such administration, depending on the disease, disorder or condition to be prevented or treated and other factors well known to the clinician.

The polypeptides, compounds and/or constructs of the invention and/or the compositions comprising the same are administered according to a regime of treatment that is suitable for preventing and/or treating the disease, disorder or condition to be prevented or treated. The clinician will generally be able to determine a suitable treatment regimen, depending on factors such as the disease, disorder or condition to be prevented or treated, the severity of the disease to be treated and/or the severity of the symptoms thereof, the specific polypeptides, compounds and/or constructs of the invention to be used, the specific route of administration and pharmaceutical formulation or composition to be used, the age, gender, weight, diet, general condition of the patient, and similar factors well known to the clinician.

Generally, the treatment regimen will comprise the administration of one or more polypeptides, compounds and/or constructs of the invention, or of one or more compositions comprising the same, in one or more pharmaceutically effective amounts or doses. The specific amount(s) or doses to be administered can be determined by the clinician, again based on the factors cited above.

Generally, depending on the specific disease, disorder or condition to be treated, the potency of the specific polypeptide, compound and/or construct of the invention to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the clinician will be able to determine a suitable daily dose.

Usually, in the above method, a polypeptide, compound and/or construct of the invention will be used. It is however within the scope of the invention to use two or more polypeptides, compounds and/or constructs of the invention in combination.

The polypeptides, compounds and/or constructs of the invention may be used in combination with one or more further pharmaceutically active compounds or principles, i.e., as a combined treatment regimen, which may or may not lead to a synergistic effect.

Again, the clinician will be able to select such further compounds or principles, as well as a suitable combined treatment regimen, based on the factors cited above and his expert judgment.

In particular, the polypeptides, compounds and/or constructs of the invention may be used in combination with other pharmaceutically active compounds or principles that are or can be used for the prevention and/or treatment of the diseases, disorders and conditions cited herein, as a result of which a synergistic effect may or may not be obtained. Examples of such compounds and principles, as well as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician.

More particular, the polypeptides, compounds and/or constructs of the invention may be co-administered with chemotherapy, radiation therapy, cancer vaccines and/or one or more additional therapeutic agents. Methods for co-administration or treatment with other such agents or therapeutic modalities are well known in the art, see, e.g. Hardman, et al. (eds.) (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, NY; Poole and Peterson (eds.) (2001) Pharmacotherapeutics for Advanced Practice: A Practical Approach, Lippincott, Williams & Wilkins, Phila., PA; Chabner and Longo (eds.) (2001) Cancer Chemotherapy and Biotherapy, Lippincott, Williams & Wilkins, Phila., PA.

For example, in one embodiment, the polypeptides, compounds and/or constructs of the invention are administered in combination with one or more additional therapeutic agents. Such agents can include for instance, PD-1, PD-L1, PD-L2, CTLA-4, 4-1 BB (CD137), 4-1BB ligand, OX40, OX40 ligand, CD27, TNFRSF25, TL1A, CD40, CD40 ligand, LIGHT, LTA, HVEM, BTLA, CD160, CEACAM-1, CEACAM-5, LAIR1, 2B4, TGFR, LAG-3, TIM-3, Siglecs, ICOS (CD278), ICOS ligand, B7-H3, B7-H4, B7-1, B7-2, VISTA, HHLA2, TMIGD2, BTNL2, CD244, CD48, CD2, CDS, TIGIT, PVR family members, KIRs, ILTs, LIRs, NKG2D, NKG2A, MICA, MICB, CSF1R, IDO, TGFβ, Adenosine, ICAM-1, ICAM-2, ICAM-3, LFA-1 (CD11a/CD18), LFA-2, LFA-3, BAFFR, NKG2C, SLAMF7, NKp80, CD83 ligand, CD24, CD39, CD30, CD70, CD73, CD7, CXCR4, CXCL12, Phosphatidylserine, SIRPA, CD47, VEGF and Neuropilin.

In another embodiment, the polypeptides, compounds and/or constructs of the invention are administered in combination with an anti-PD-1 antibody or an antigen-binding fragment thereof, wherein the anti-PD-1 antibody or antigen-binding fragment thereof is administered simultaneously with the polypeptides, compounds and/or constructs of the invention, or prior to or subsequently to the administration of the polypeptides, compounds and/or constructs of the invention. As shown in Examples 14 and 21, the administration of the polypeptides, compounds and/or constructs of the invention in combination with an anti-PD-1 antibody to mice had a synergistic effect in inhibiting tumor growth.

In another embodiment, the polypeptides, compounds and/or constructs of the invention are administered in combination with an anti-CTLA-4 antibody or an antigen-binding fragment thereof, wherein the anti-CTLA-4 antibody or antigen-binding fragment thereof is administered simultaneously with the polypeptides, compounds and/or constructs of the invention, or prior to or subsequently to the administration of the polypeptides, compounds and/or constructs of the invention.

In another embodiment, the polypeptides, compounds and/or constructs of the invention are administered in combination with an anti-PD-L1 antibody or an antigen-binding fragment thereof wherein the anti-PD-L1 antibody or antigen-binding fragment thereof is administered simultaneously with the polypeptides, compounds and/or constructs of the invention, or prior to or subsequently to the administration of the polypeptides, compounds and/or constructs of the invention.

In yet other embodiments, the polypeptides, compounds and/or constructs of the invention are administered in combination with an anti-PD-1 antibody (or antigen-binding fragments thereof) and 5-FU.

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

Also, when two or more active substances or principles are to be used as part of a combined treatment regimen, each of the substances or principles may be administered in the same amount and according to the same regimen as used when the compound or principle is used on its own, and such combined use may or may not lead to a synergistic effect. However, when the combined use of the two or more active substances or principles leads to a synergistic effect, it may also be possible to reduce the amount of one, more or all of the substances or principles to be administered, while still achieving the desired therapeutic action. This may for example be useful for avoiding, limiting or reducing any unwanted side-effects that are associated with the use of one or more of the substances or principles when they are used in their usual amounts, while still obtaining the desired pharmaceutical or therapeutic effect.

The effectiveness of the treatment regimen used according to the invention may be determined and/or followed in any manner known per se for the disease, disorder or condition involved, as will be clear to the clinician. The clinician will also be able, where appropriate and on a case-by-case basis, to change or modify a particular treatment regimen, so as to achieve the desired therapeutic effect, to avoid, limit or reduce unwanted side-effects, and/or to achieve an appropriate balance between achieving the desired therapeutic effect on the one hand and avoiding, limiting or reducing undesired side effects on the other hand.

Generally, the treatment regimen will be followed until the desired therapeutic effect is achieved and/or for as long as the desired therapeutic effect is to be maintained. Again, this can be determined by the clinician.

In another aspect, the invention relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of at least one disease, disorder and condition associated with GITR; and/or for use in one or more of the methods of treatment mentioned herein.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention, for the manufacture of a pharmaceutical composition for prevention and/or treatment of at least one of the diseases, disorders and conditions associated with GITR and/or with the signaling pathways and/or the biological functions and responses in which GITR are involved; and/or in one or more of the methods described herein.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture 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 stimulating, enhancing or agonizing GITR, its biological or pharmacological activity, and/or the biological pathways or signaling in which GITR is involved.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for the prevention and/or treatment of at least one disease, disorder or condition that can be prevented and/or treated by administering a polypeptide, compound and/or construct of the invention to a patient.

More in particular, the invention relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for enhancing an immune response

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for enhancing proliferation or activation of T cells, B cells or natural killer cells.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for inhibiting tumor growth.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

The invention also relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of cancer.

More in particular, the invention relates to the use of a polypeptide, compound and/or construct of the invention for the manufacture of a pharmaceutical composition for the prevention and/or treatment of squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

The invention further relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in the prevention and/or treatment of at least one GITR related disease, disorder and/or condition.

The invention further relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in the prevention and/or treatment of at least one disease, disorder and/or condition associated with GITR, with its biological or pharmacological activity, and/or with the biological pathways or signaling in which GITR is involved.

The invention further relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in the prevention and/or treatment of at least one disease, disorder and/or condition that can be prevented and/or treated by stimulating, enhancing or agonizing GITR, its biological or pharmacological activity, and/or the biological pathways or signaling in which GITR is involved.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in the prevention and/or treatment of at least one disease, disorder and/or condition that can be prevented and/or treated by administering of a polypeptide, compound and/or construct of the invention to a patient. More in particular, the invention also relates to a polypeptide, compound and/or construct of the invention or pharmaceutical compositions comprising the same for use in enhancing an immune response.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in enhancing proliferation or activation of T cells, B cells or natural killer cells.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in inhibiting tumor growth.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

The invention also relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in prevention and/or treatment of cancer.

More in particular, the invention relates to a polypeptide, compound and/or construct of the invention or a pharmaceutical composition comprising the same for use in the prevention and/or treatment of squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

The subject to be treated may be any warm-blooded animal, but is in particular a mammal, and more in particular a human being. In veterinary applications, the subject to be treated includes any animal raised for commercial purposes or kept as a pet. 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, disorders and conditions mentioned herein.

Again, in such a pharmaceutical composition, the one or more polypeptides, compounds and/or constructs of the invention, or nucleotide encoding the same, and/or a pharmaceutical composition comprising the same, may also be suitably combined with one or more other active principles, such as those mentioned herein.

The invention also relates to a composition (such as, without limitation, a pharmaceutical composition or preparation as further described herein) for use, either *in vitro* (e.g. in an *in vitro* or cellular assay) or *in vivo* (e.g. in an a single cell or multi-cellular 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, disorder or condition of the invention).

The polypeptides, compounds and/or constructs of the present invention inhibit tumor cell growth, in a syngeneic CT-26 colon carcinoma model. Based on their mode of action, the polypeptides, compounds and/or constructs of the present invention may be useful in the treatment of other GITR associated diseases, including but not limited to various types of cancer and infectious diseases.

It is to be understood that reference to treatment includes both treatment of established symptoms and prophylactic treatment, unless explicitly stated otherwise.

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

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.

EXAMPLES

Example 1: Creation of GITR expression cell lines and recombinant cyno GITR and cyno GITR-Fc

1.1 GITR expressing cell lines

Stable Flp-In™-293 cells (Life technologies R750-07) and GloResponse™ NF-κB-Nluc2P HEK293 (Promega CS188801) cell lines with recombinant overexpression of human GITR, cynomologus GITR and mouse GITR were generated. For this, the coding sequences of GITR were cloned in a pcDNA3.1-derived vector, downstream of a CMV promotor. The sequences for human GITR and mouse GITR were retrieved from UniprotKB (humanGITR: Q9Y5U5 [SEQ ID NO: 231], mouseGITR: O35714 [SEQ ID NO: 232]). The sequence for the cynomolgus GITR was retrieved from the NCBI database (XP_005545180, SEQ ID NO: 233). Cell surface expression of human GITR was confirmed using the humanized IgG1 anti-human GITR antibody (HuQ6C8-Agly, see WO06105021) and mouse IgG1 anti-human GITR Clone #110416 (R&D Systems MAB689), cyno GITR expression with HuQ6C8-Agly and mouse GITR expression was confirmed using rat IgG2b anti-mouse GITR clone DTA-1 (eBioscience #16-5874) (Figure 1A-C).

1.2 Recombinant cyno GITR and cyno GITR-Fc

The extracellular domain of cyno GITR was extended either with a HIS tag or with human IgG1 Fc and the respective cDNA sequences were cloned into a mammalian expression vector. The resulting plasmids were transfected into HEK.EBNA cells and proteins were purified from the harvested cell supernatant respectively by IMAC and Protein A chromatography followed by a desalting step to PBS buffer.

Example 2: Immunization of llamas with human GITR, cloning of the heavy chain-only antibody fragment repertoires and preparation of phage

2.1 Immunization

After approval of the Ethical Committee (Ablynx NV, Belgium - EC2012#2), 6 camelids were immunized with a CMV-promoter based DNA vector encoding human GITR. Additionally, one camelid was immunized with recombinant mouse GITR-Fc (R&D Systems, 524-GR-050).

2.2 Cloning of the heavy chain-only antibody fragment repertoires and preparation of phages.

Per animal, blood samples were collected after the injection of one type of immunization antigen. From these blood samples, PBMC were prepared using Ficoll-Hypaque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). For each immunized llama, libraries were constructed by pooling the total RNA isolated from samples originating from a certain subset of the immunization schedule, *i.e.* after one type of immunization antigen. In short, the PCR-amplified VHH repertoire was cloned via specific restriction sites into a vector designed to facilitate phage display of the VHH library. The vector was derived from pUC119. In frame with the VHH coding sequence, the vector encodes a C-terminal 3xFLAG and HIS6 tag. Phages were prepared according to standard protocols (see for example WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 3: Selection of GITR specific VHHs via phage display

VHH repertoires obtained from all llamas and cloned as phage library were used in different selection strategies, applying a multiplicity of selection conditions. Variables included: i) the presentation form of GITR (on different backgrounds), ii) alternation of species source (human/cynomolgus/mouse GITR), iii) the antigen concentration, iv) the number of selection rounds, v) shielding of specific GITR epitopes. In brief, cells (see Example 1) or the soluble antigen (human GITR-Fc (Enzo Life Sciences, ALX-522-061-C050), cyno GITR (in-house), cyno GITR-Fc (in-house), mouse GITR (R&D Systems, 524-GR-050) were incubated for 1h-2h with the phage libraries followed by extensive washing; bound phages were eluted with trypsin (1 mg/mL) for 15 minutes and then the protease activity was immediately neutralized by applying 0.8 mM protease inhibitor ABSF. As control, selections with parental cell line or without antigen were performed in parallel.

Phage outputs were used to infect *E. coli* for analysis of individual VHH clones. Periplasmic extracts were prepared according to standard protocols (see for example WO 03/035694, WO 04/041865, WO 04/041863, WO 04/062551 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 4: Construction of Nanobody- IgG chimeras

4.1 Construction of Nanobody-human IgG1 chimeras

Nanobody-human IgG1 chimeras were composed of two heavy chains and two light chains. The heavy chain comprised an anti-GITR Nanobody fused to human IgG1 constant domains CH1- CH3. The light chain consisted of the same anti-GITR Nanobody fused to the constant domain of the human light chain CL (kappa). A schematic representation of a Nanobody-human IgG1 chimera is depicted in Figure 7.

The respective cDNA sequences were cloned in a mammalian expression vector in two separate expression cassettes. The resulting plasmids were transfected into HEK.EBNA cells and proteins were purified from the harvested cell supernatant by Protein A chromatography and desalting to PBS buffer.

4.2 Construction of Nanobody-rat IgG2b chimeras

Nanobody-rat IgG2b chimeras were composed of two heavy chains and two light chains. The heavy chain comprised an anti-GITR Nanobody fused to rat IgG2b constant domains CH1- CH3. The light chain consisted of the same anti-GITR Nanobody fused to the constant domain of the rat light chain CL (lambda).

The respective cDNA sequences were cloned in a mammalian expression vector in two separate expression cassettes. The resulting plasmids were transfected into HEK.EBNA cells and proteins were purified from the harvested cell supernatant by Protein A chromatography and desalting to PBS buffer.

Example 5: Screening

5.1 Screening for GITR binding Nanobodies in a binding ELISA

Periplasmic extracts were screened in a binding ELISA on human GITR (Enzo Life Sciences, ALX-522-061-C050) and cynomolgus GITR (in-house). To this end, a microtiter plate was coated with human or cynomolgus GITR (0.5 µg/ml), overnight incubated at 4 °C. Plates were blocked for one hour at room temperature with 75 µl 1% casein in PBS. The plates were washed with PBS-Tween. The periplasmic extracts (1/5 or 1/8000 diluted in PBS with 0.1% casein + 0.05% Tween) were incubated for at least 1 hour at RT. Plates were washed six times with PBS-Tween, after which binding of VHH was detected with anti-FLAG-HRP (Sigma-Aldrich, A8592) mAb 1/5000 in PBS with 0.1% casein + 0.05% Tween20. Staining was performed with the substrate esTMB (SDT reagents) and the signals were measured after 15 minutes at 450 nm.

Nanobodies which scored positive in the binding ELISA were sequenced. The sequence analysis resulted in the identification of 8 distinct families, i.e. Family 7, Family 26, Family 82, Family 109, Family 85, Family 38, Family 110 and Family 108. Corresponding alignments are provided in Table A-1, Table A-2, Table A-3, Table A-4, Table A-5, Table A-6, Table A-7 and Table A-8, respectively. The classification into different families was based on sequence similarities and differences in the CDRs. The sequence variability against a representative of each family is depicted in the tables below.

For **Family 7,** the amino acid sequence of the CDRs of clone A0231005A03 was used as a reference, against which the CDRs of all other Family 7 clones were compared. The sequence variability against A0231005A03 is shown below.

A0231005A03	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2*	3	4	5	6	7	8	9	10
wildtype sequence	E	T	I	F	S	I	D	S	M	A
variations		S						A		G
variations		S					N	A		G

* in case position 2 is an S, then position 8 is also A, and position 10 is also G

A0231005A03	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
wildtype sequence	A	I	T	G	G	G	S	P	N
variations	H						R	S	
variations	T							T	
variations	T						R	R	
variations	T	M						T	
variations	H						G	S	
variations	T					S		T	
variations	H						R		

variations	G		S				R	T	

A0231005A03	CDR3												
Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	101	102
absolute numbering	1	2	3	4	5	6	7	8	9	10	11	12	13
wildtype sequence	E	G	Q	A	G	W	G	T	A	L	M	D	Y
variations									P			N	
variations											L*		
variations											K*		
variations											R*		
variations											Q*		

* variations were introduced to replace Methionine in order to avoid oxidation of this residue

For **Family 26,** the amino acid sequence of the CDRs of clone A0231004B01 was used as a reference, against which the CDRs of all other Family 26 clones were compared. The sequence variability against A0231004B01 is shown below.

A0231004B01	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	G	S	I	F	S	I	D	S	M	G
variations								A		
variations							N	A		

A0231004B01	CDR2						
Kabat numbering	50	51	52	53	56	57	58
absolute numbering	1	2	3	4	5	6	7
wildtype sequence	A	I	T	S	S	T	N
variations	S				T		
variations					G		
variations					R		I
variations	T				G	K	

A0231004B01	CDR3

Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	101	102
absolute numbering	1	2	3	4	5	6	7	8	9	10	11	12	13
wildtype sequence	E	G	Q	A	G	W	G	T	A	L	I	N	Y
variations	K			T							M	D	
variations				T							M	D	
variations											M	D	
											L	D	

For **Family 82,** the amino acid sequence of the CDRs of clone A0231034A08 was used as a reference, against which the CDRs of all other Family 82 clones were compared. The sequence variability against A0231034A08 is shown below.

A0231034A08	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	G	S	V	F	S	I	N	D	M	G
variations			I				D	S		
variations			I				D			
variations			I						V	
variations		N	I							
variations			I							
variations			I						T	

A0231034A08	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
wildtype sequence	D	I	I	S	R	G	V	T	N
variations					A	D			
variations					A				
variations	G								
variations						D			

A0231034A08	CDR3												
Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	101	102

absolute numbering	1	2	3	4	5	6	7	8	9	10	11	12	13
wildtype sequence	H	I	S	T	G	W	G	R	P	H	N	N	Y
variations				M									

For **Family 109,** the amino acid sequence of the CDRs of clone A0231052E08 was used as a reference, against which the CDRs of all other Family 109 clones were compared. The sequence variability against A0231052E08 is shown below.

A0231052E08	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	R	S	I	F	S	T	Y	A	M	A
variations		N								

A0231052E08	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
wildtype sequence	F	I	Y	W	G	G	T	T	T
variations									S

A0231052E08	CDR3						
Kabat numbering	95	96	97	98	99	101	102
absolute numbering	1	2	3	4	5	6	7
wildtype sequence	Y	G	S	Y	A	L	P

For **Family 85,** the amino acid sequence of the CDRs of clone A0231033B02 is depicted in the tables below.

A0231033B02	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10

wildtype sequence	G	T	I	F	S	I	S	T	M	G

A0231033B02	CDR2							
Kabat numbering	50	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8
wildtype sequence	V	T	S	G	F	S	T	N

A0231033B02	CDR3												
Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	101	102
absolute numbering	1	2	3	4	5	6	7	8	9	10	11	12	13
wildtype sequence	Y	L	S	L	A	W	R	D	P	D	R	D	Y

For **Family 38,** the amino acid sequence of the CDRs of clone A0231003D11 was used as a reference, against which the CDRs of all other Family 38 clones were compared. The sequence variability against A0231003D11 is depicted in the tables below.

A0231003D11	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	G	S	I	F	S	I	D	A	M	G

A0231003D11	CDR2						
Kabat numbering	50	51	52	54	55	56	57
absolute numbering	1	2	3	4	5	6	7
wildtype sequence	E	I	S	D	H	T	T
variations				G			

A0231003D11	CDR3												
Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	101	102

absolute numbering	1	2	3	4	5	6	7	8	9	10	11	12	13
wildtype sequence	H	H	Q	R	G	W	G	T	S	I	T	V	T
variations													A
variations									P				

For **Family 110,** the amino acid sequence of the CDRs of clone A0231052A08 was used as a reference, against which the CDRs of all other Family 110 clones were compared. The sequence variability against A0231052A08 is depicted in the tables below.

A0231052A08	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	G	S	I	S	S	I	T	A	M	G

A0231052A08	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
wildtype sequence	V	I	S	R	S	G	A	T	M
variations	I								
variations	A								I

A0231052A08	CDR3						
Kabat numbering	95	96	97	98	99	101	102
absolute numbering	1	2	3	4	5	6	7
wildtype sequence	I	T	Q	G	R	T	Y
variations				E	Q		

For **Family 108,** the amino acid sequence of the CDRs of clone A0231051E01 is depicted in the tables below.

A0231051E01	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35

absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	G	S	I	F	S	F	I	V	M	G

A0231051E01	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
wildtype sequence	T	V	T	S	G	G	D	T	F

A0231051E01	CDR3									
Kabat numbering	95	96	97	98	99	100	100a	100b	101	102
absolute numbering	1	2	3	4	5	6	7	8	9	10
wildtype sequence	T	K	V	S	P	Y	K	E	T	T

5.2 Purification of monovalent Nanobodies

Representative Nanobodies were selected and expressed in *E. coli* TG1 as triple Flag, HIS6-tagged proteins. Expression was induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing and resuspension of the pellets in D-PBS. The periplasmic extracts were filtered (0.20 µm) and imidazole was added to a final concentration of 20 mM. The Nanobodies in these periplasmic extracts were purified via IMAC and desalted via Zebaspin (Fisher Scientific).

Example 6: Kinetic analysis of monovalent anti-GITR Nanobodies

Affinity was determined on the Proteon XPR36. Briefly, after human GITR-Fc (Enzo Life Sciences, ALX-522-061-C050) was immobilized on a GLC chip via amine coupling, a range of 6 concentrations of different monovalent Nanobodies (representing each family) were injected. The K_D values were determined by using the calculated association rate and dissociation rate constants (Table 1) (ProteOn Manager 3.1.0, Version 3.1.0.6 (2011)).

Table 1: K_D values of monovalent anti-GITR Nanobodies

	antigen: human GITR-Fc		
	Ka **1/Ms**	**kd** **1/s**	**K_D** **M**
A0231034A08 (Family 82)	2.7E+05	6.7E-04	2.5E-09
A0231004B01 (Family 26)	1.1E+06	2.7E-03	2.6E-09
A0231005A03 (Family 7)	6.8E+05	2.3E-03	3.4E-09
A0231003D11 (Family 38)	6.3E+05	2.9E-03	4.6E-09
A0231033B02 (Family 85)	4.9E+05	8.0E-03	1.6E-08
A0231052A08 (Family 110)	4.1E+04	9.7E-04	2.4E-08
A0231051E01 (Family 108)	1.9E+05	1.5E-02	7.8E-08
A0231052E08 (Family 109)	7.5E+05	9.0E-02	1.2E-07

Example 7: Binding of anti-GITR Nanobodies to GITR expressed on Flp-In™-293 cells and activated T cells

Binding of purified monovalent anti-GITR Nanobodies to human GITR expressed on activated T cells was evaluated in flow cytometry. For this purpose, PBMCs from healthy donors were cultured with Dynabeads® Human T-Activator CD3/CD28 (Gibco - Life Technologies #11131D) for 7 days in a 1/2 bead/cell ratio. After activation, a cell population was obtained consisting out of >98% T cells. These T cells were highly activated (CD25high, CD45RO+ T cells) and showed a good expression of GITR (Figure 1D).

Dilution series of Nanobodies starting from 1 µM were applied to the cells. Nanobodies were allowed to associate for 30 minutes at 4°C in FACS buffer (PBS supplemented with 10% FBS and 0.05 % azide). Cells were washed by centrifugation and probed with anti-FLAG antibodies (Sigma F1804) for 30 minutes at 4°C, to detect bound Nanobody. Detection was done with Goat anti-Mouse IgG-PE (Jackson ImmunoResearch #115-116-071) for 30 minutes at 4°C. Cells were washed and incubated with TOPRO3 to stain for dead cells which were subsequently removed during the gating procedure. The cells were then analysed via a BD FACSArray. The results are shown in Figures 2A-2B.

The EC_{50} values obtained from the dose response curve are represented in Table 2.

Table 2: EC$_{50}$ (M) values of anti-GITR monovalent Nanobodies to activated T cells as determined in flow cytometry

	activated human T cells		
ID	EC$_{50}$ (M)	95% LCI	95% UCI
A0231005A03 (Family 7)	7.9E-10	6.7E-10	9.2E-10
A0231004B01 (Family 26)	6.1E-10	5.2E-10	7.1E-10
A0231034A08 (Family 82)	5.4E-10	4.8E-10	6.1E-10
A0231052E08 (Family 109)	> 1.0E-7	/	/
A0231033B02 (Family 85)	2.1E-8	9.6E-9	4.7E-8
A0231003D11 (Family 38)	1.3E-9	1.0E-9	1.6E-9
A0231052A08 (Family 110)	2.3E-8	1.9E-8	2.7E8
A0231051E01 (Family 108)	> 1.0E-7	/	/

Example 8: Generation and binding of multivalent constructs

8.1 Construction of multivalent Nanobody constructs

In order to increase potency and/or efficacy, multivalent molecules were constructed by genetic engineering. Multiple Nanobodies were genetically linked together with 3A, 9GS or 35GS linkers. All multivalent GITR Nanobody constructs carried a C-terminal Albumin binding Nanobody and were subsequently expressed in *Pichia pastoris* according to standard conditions. Constructs with a FLAG3-HIS6 tag were purified using IMAC, while the tagless construct were purified via protein A binding. Different multivalent GITR constructs were made as listed in Table A-11.

8.2 Binding of multivalent anti-GITR Nanobodies to human GITR expressed on NFkB-Nluc2P HEK293 cells as determined by FACS.

Binding of the multivalent constructs to human GITR was determined by FACS. Briefly, Nanobodies were allowed to associate for 30 minutes at 4°C in FACS buffer (PBS supplemented with 10% FBS and 0.05 % azide). Cells were washed by centrifugation and probed with an in-house anti-ALB11 antibody for 30 minutes at 4°C, to detect bound Nanobody. Detection was done with Goat anti-Mouse IgG-PE (Jackson ImmunoResearch #115-116-071) for 30 minutes at 4°C. Cells were washed and incubated with TOPRO3 to stain for dead cells, which were subsequently removed during the gating procedure.

The cells were then analysed via a BD FACSArray. Binding curves are shown in Figure 3. The EC_{50} values obtained from the dose response curve are depicted in Table 3.

Table 3: EC_{50} (M) values of anti-GITR multivalent Nanobodies, Nanobody-human IgG1 chimera A-0231-00_TP008 and reference compounds for binding to HEK293_NFkB-Nluc2P human GITR cells as determined in flow cytometry

Construct ID	HEK293_NFkB-Nluc2P human GITR
A023100001 (Family 26)	2.17E-10
A023100014 (Family 26)	1.26E-10
A023100022 (Family 7)	1.51E-10
A023100025 (Family 109)	1.97E-10
A023100029 (Family 82)	3.26E-10
A023100030 (Family 85)	2.46E-10
A023100034 (Family 7)	2.04E-10
A-0231-00_TP008 (Family 7)	6.75E-11
Reference compounds	**HEK293_NFkB-Nluc2P human GITR**
HuQ6C8-Agly (see WO06105021)	4.00E-10
36E5 (see US8709424)	1.96E-10

Example 9: Activation of GITR by anti-GITR Nanobodies assessed in a NF-κB luciferase reporter assay

The functionality of the purified multivalent Nanobodies was assessed in a NF-κB luciferase reporter assay. To this end, the GloResponse™ NF-κB-Nluc2P HEK293 (Promega CS188801) cell line was stably transfected with human GITR. To evaluate the activation capacity of anti-GITR Nanobodies, the GITR expressing cells were seeded at 10,000 cells/well in white, tissue culture-treated 96-well plates (Costar #3917). Serial dilutions of anti-GITR Nanobodies were added and allowed to interact for 5 hours in a 37°C incubator at 5% CO_2. NF-kB activity was assessed by measuring luminescence after addition of Nano-Glo™ Reagent (Promega #N1120). Results shown in Table 4A illustrate the effect of anti-GITR Nanobodies and HuQ6C8-Agly on a pool of cells with a heterogeneous GITR expression. Results shown in Table 4B-C illustrate the effect of anti-GITR Nanobodies, HuQ6C8-Agly and 36E5 on single cell clones. Illustrative activation curves are shown in Figure 4A-D.

Table 4A: EC_{50} (M) and efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobodies and reference compound

Construct ID	HEK293_NFkB-Nluc2P human GITR EC_{50} (M)	HEK293_NFkB-Nluc2P human GITR Efficacy (%)

A023100003 (Family 7)	1.0E-10	101
A023100015 (Family 110)	1.8E-10	105
A023100020 (Family 108)	4.5E-10	105
A023100021 (Family 38)	2.6E-11	116
Reference compound		
HuQ6C8-Agly	2.2E-10	51

Table 4B: EC_{50} (M) and efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobodies, Nanobody-human IgG1 chimera A-0231-00_TP008 and reference compounds

Construct ID	HEK293 human GITR NF-KB luc reporter EC_{50} (M)	HEK293 human GITR NF-KB luc reporter Efficacy (%)
A023100001 (Family 26)	7.82E-11	60
A023100014 (Family 26)	2.96E-11	72
A023100022 (Family 7)	2.46E-11	85
A023100025 (Family 109)	3.17E-11	76
A023100029 (Family 82)	9.53E-11	77
A023100030 (Family 85)	8.72E-11	90
A023100032 (Family 7)	2.63E-11	108
A023100034 (Family 7)	5.60E-11	62
A023100035 (Family 7)	1.55E-11	112
A-0231-00_TP008 (Family 7)	1.52E-11	86.8
Reference compound		
HuQ6C8-Agly	3.93E-10	30.4
36E5	3.94E-11	ND

Table 4C: EC_{50} (M) and efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobodies

Construct ID	HEK293 human GITR NF-KB luc reporter EC_{50} (M)	HEK293 human GITR NF-KB luc reporter Efficacy (%)
A-0231-00_TP008 (Family 7)	1.89E-11	87
A023100035 (Family 7)	3.66E-11	129
Reference compound		
36E5	3.26E-11	37

Example 10: Human T cell activation capacity of anti-GITR Nanobodies

Functionality of purified multivalent monospecific anti-GITR Nanobodies was evaluated in a human T cell activation assay. Human CD4+ T cells were isolated from Buffy Coats from healthy donors using CD4+ T Cell Isolation Kit (Miltenyi Biotec #130-096-533) and cultured in RPMI-1640 (Life Technologies - Gibco #72400) supplemented with 10% FBS and 1% P/S. The isolated CD4+ T cells were subsequently activated for 10 days with Dynabeads® Human T-Activator CD3/CD28 (Life Technologies - Gibco #11131D) at a 1/5 bead/cell ratio and 4 additional days at a 1/1 ratio. The activation status was evaluated by flow cytometry by measuring CD25 expression (anti-CD25-PE – BD Bioscience #557138), CD45RA expression (anti-CD45RA-APC – BD Bioscience #550855) and CD45RO expression (anti-CD45RO-PE – BD Bioscience #555493). GITR expression was confirmed by detection with mouse anti-human GITR Clone 110416 (R&D Systems #MAB689).

To evaluate the activation capacity of anti-GITR Nanobodies the activated CD4+ T cells were cultured in RPMI-1640 supplemented with 10% Human AB serum and 1% P/S. Cells were added to plates coated with anti-CD3 clone OKT3 (eBioscience #16-0037-85) at 125ng/ml (IFN-γ production) or 8ng/ml (proliferation) in presence or absence of anti-GITR Nanobodies. IFN-γ production was measured by ELISA after 3 days incubation in a 37°C incubator at 5% CO_2. For proliferation measurement cells were incubated for 3 days and pulsed with ^{3}H-thymidine 18 hours before the cells were harvested and counted.

The effect of multivalent anti-GITR Nanobodies on the IFN-γ production is exemplified in Figures 5A-D. Nanobody A023100035, a tetravalent Nanobody (Family 7) with 9GS linker between the individual building blocks, acts as a full agonist, displaying substantially the same efficacy as the natural ligand (defined as Emax) throughout the tested concentration range. Figure 5E and 5F show the data obtained for the reference compounds 36E5 and HuQ6C8-Agly, respectively. These antibodies acts as a partial agonist displaying a lower maximal efficacy, that even further decreases with increasing concentrations.

Table 5 shows the effect on IFN-γ production of multivalent anti-GITR Nanobodies and anti-GITR antibodies in comparison to the natural ligand. EC_{50} values reflect the potency of the Nanobodies. The maximal efficacy as percentage of the Emax (= maximal response induced by the natural ligand) is also presented. Nanobody A023100035 clearly shows an efficacy comparable to the natural ligand, which was set at 100%.

Table 5: EC_{50} values (M) and the maximal efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobody constructs and reference compounds (IFN-γ read-out).

Construct ID	**EC_{50} (M)**	**Maximal efficacy (%)**
A023100032 (Family 7)	1,52E-10	74,1
A023100035 (Family 7)	4,10E-11	105,9
A023100014 (Family 26)	7,67E-11	64,2
A023100025 (Family 109)	1,30E-10	52,1
Reference compound		
36E5	2,94E-11	39,1
HuQ6C8-Agly	1,67E-09	45,6

Example 11: Linker length multivalent Nanobody constructs

The impact of the linker length was evaluated in the human GITR NF-κB luciferase reporter assay, as described in Example 9. Nanobody constructs with a 9GS linker showed a higher efficacy than Nanobody constructs with a 35GS linker (see Table 6A). This was confirmed using different Nanobody constructs (see Table 6B). In addition it was shown that Nanobody constructs with a 3A linker have comparable efficacies to the 9GS linked constructs.

The effect of multivalent anti-GITR Nanobody constructs with a different linker length on the NF-κB activity is also exemplified in Figures 6A-D. Unexpectedly, the inventors additionally showed that shortening the length of the linker can even further improve the functional properties of the GITR Nanobody constructs.

Table 6A: EC_{50} (M) and maximal efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobodies

Construct ID	EC_{50} (M)	Maximal efficacy (%)
A023100012 (Family 108) (A0231051E01-35GS-A0231051E01-35GS-ALB11)	1.72E-09	18.2
A023100031 (Family 108) (A0231051E01-9GS-A0231051E01-9GS-ALB11)	2.69E-09	51.7
A023100020 (Family 108) (A0231051E01-35GS-A0231051E01-35GS-A0231051E01-35GS-ALB11)	1.53E-09	85.4
A023100036 (Family 108) (A0231051E01-9GS-A0231051E01-9GS-A0231051E01-9GS-ALB11)	5.41E-10	89.9
A023100034 (Family 7) (A0231005A03-35GS-A0231005A03-35GS-ALB11)	8.74E-11	83.0
A023100032 (Family 7) (A0231005A03-9GS-A0231005A03-9GS-ALB11)	5.11E-11	137.9
A023100022 (Family 7) (A0231005A03-35GS-A0231005A03-35GS-A0231005A03-35GS-ALB11)	4.11E-11	105.5
A023100035 (Family 7) (A0231005A03-9GS-A0231005A03-9GS-A0231005A03-9GS-ALB11)	2.99E-11	130.5

Table 6B: EC_{50} (M) and maximal efficacy values (human GITR ligand = 100%) of anti-GITR multivalent Nanobodies

Construct ID	EC_{50} (M)	Maximal efficacy (%)
A023100083 (Family 26) A0231004B01-9GS-A0231004B01-9GS-ALB11	3.16E-11	126
A023100045 (Family 26) A0231004B01-3A-A0231004B01-9GS-ALB11	2.99E-11	133
A023100082 (Family 26)	3.13E-11	136

Construct ID	**EC_{50} (M)**	**Maximal efficacy (%)**
A0231004B01-3A-A0231004B01-3A-ALB11		
A023100014 (Family 26) A0231004B01-35GS-A0231004B01-35GS-A0231004B01-35GS-ALB11	3.30E-11	86
A023100084 (Family 26) A0231004B01-9GS-A0231004B01-9GS-A0231004B01-9GS-ALB11	2.27E-11	127
A023100085 (Family 26) A0231004B01-3A-A0231004B01-3A-A0231004B01-3A-ALB11	2.63E-11	146
A023100035 (Family 7) (A0231005A03-9GS-A0231005A03-9GS-A0231005A03-9GS-ALB11)	2.30E-11	132
Reference compound		
36E5	2.25E-11	33

Example 12: *In vivo* proof-of-concept in an OVA immunization model

In the OVA immunization model, BALB/c mice were immunized on day zero (D0) by subcutaneous (s.c.) administration of 100 µg ovalbumin in saline or adjuvant followed by a boost with the same s.c. dose in saline or adjuvant on D14. Mice that receive OVA in adjuvant are administered OVA in a 1:1 mixture with alum or Incomplete Freund's adjuvant (IFA).

The adjuvant effect of anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera on the humoral immune response to OVA is evaluated, reflecting the immune-enhancing effect of the anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera

To assess the effect of anti-GITR and anti-GITR Nanobody-ratIgG2b chimera on the humoral response to OVA, the anti-GITR Nanobody or irrelevant control Nanobody is administered intraperitoneally at the primary (D0) or boost (D14) immunisation for 3 consecutive days with a 1x 15 mg/kg loading dose on the first dosing day and a maintenance dose of 2x 10 mg/kg on the next two dosing days. Anti-GITR Nanobody-ratIgG2b chimera is administered intraperitoneally at D0 or D14 for 3 consecutive days at 25mg/kg. Anti-OVA serum titres are determined by ELISA on D-7, D13 and D21. The OVA mixed with Alum or IFA is included for comparison. Significant increase of total anti-OVA IgG levels in anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera treated animals are expected compared to untreated and irrelevant control animals.

Example 13: *In vivo* proof-of-concept in a syngeneic CT-26 colon carcinoma model

Tumour efficacy of anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera is demonstrated in a syngeneic CT-26 colon carcinoma model.

In this model, BALB/c mice are inoculated with BALB/c-derived colorectal carcinoma cells (CT26 cells). On day 0 (D0) tumours are induced by subcutaneous injection of $1x10^6$ of CT26 cells in 200 µL of RPMI 1640 into the right flank of BALB/c mice. When tumors reach a mean volume of 50-100mm^3 animals are treated according to the treatment regimen presented in Table 7.

In vivo efficacy of the anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera on tumour growth is evaluated and compared to the irrelevant control Nanobody. Mice are treated with the anti-GITR Nanobody or anti-GITR Nanobody-ratIgG2b chimera either in monotherapy or in combination therapy with anti-PD-1 antibody (Ab) or anti-PD-1 Ab + 5-fluoro-uracil (5FU). Mice treated with anti-CTLA-4 Ab are included as positive control group. Efficacy of anti-GITR Nanobody and anti-GITR Nanobody-ratIgG2b chimera is further compared to DTA-1 as monotherapy or in combination therapy with anti-PD-1 antibody.

Table 7: Treatment regimen

Group	No animals	Treatment	Dose	Adm. Route	Treatment schedule
1	10	Vehicle	-	IP	Q1Dx1
2	10	Anti-CTLA-4	10 mg/kg	IP	TWx2*
3	10	DTA-1	25 mg/kg	IP	Q1Dx1
4	10	DTA-1	25 mg/kg	IP	Q1Dx1
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
5	10	Anti-GITR NB	1x 15 mg/kg loading dose 9x 10 mg/kg maintenance dose	IP	Q2Dx10
6	10	Irrelevant NB	1x 15 mg/kg loading dose 9x 10mg/kg maintenance dose	IP	Q2Dx10
7	10	Anti-GITR NB	1x 15 mg/kg loading dose 9x 10 mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
8	10	Irrelevant NB	1x 15 mg/kg loading dose 9x 10 mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
9	10	Anti-GITR NB	1x 15 mg/kg loading dose 2x 2.5 mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
		5-FU	25 mg/kg	IV	Q7Dx3
10	10	Irrelevant NB	1x 15 mg/kg loading dose 2x 2.5 mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3

Group	No animals	Treatment	Dose	Adm. Route	Treatment schedule
		5-FU	25 mg/kg	IV	Q7Dx3
11	10	Anti-GITR NB-ratIgG2b chimera	25 mg/kg	IP	Q1Dx1
12	10	Anti-GITR NB-ratIgG2b chimera	1x 25mg/kg loading dose 9x 10mg/kg maintenance dose	IP	Q2Dx10
13	10	Anti-GITR NB-ratIgG2b chimera	25 mg/kg	IP	Q1Dx1
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
14	10	Anti-GITR NB-ratIgG2b chimera	1x 25mg/kg loading dose 9x 10mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
15	10	Anti-GITR NB-ratIgG2b chimera	25 mg/kg	IP	Q1Dx1
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
		5-FU	25 mg/kg	IV	Q7Dx3
16	10	Anti-GITR NB-ratIgG2b chimera	1x 25mg/kg loading dose 9x 10mg/kg maintenance dose	IP	Q2Dx10
		Anti-PD-1	10 mg/kg	IP	Q5Dx3
		5-FU	25 mg/kg	IV	Q7Dx3
Total	160				

**TWx2: total of 4 injections with 2 injections per week: on Monday/Thursday or on Tuesday/Friday. Q5Dx3: 3 injections with 1 injection every 5 days; Q7Dx3: 3 injections with 1 injection every 7 days; Q2Dx10: 10 injections with 1 injection every 2 days. In case of combinations: DTA-1, anti-GITR NB, anti-GITR NB-ratIgG2b chimera or irrelevant NB is injected after the anti-PD-1. The anti-PD-1 is injected after 5-FU.*

The viability, behavior and body weights are recorded. The length and width of the tumour is measured twice a week with calipers and tumour volume is estimated by the formula:

$$\boxed{\text{Tumor volume} = \frac{\text{width}^2 \times length}{2}}$$

Significant anti-tumour activity with GITR targeting Nanobody or Nanobody-ratIgG2b chimera reflect a markedly delay in onset of tumour development or complete tumour rejection.

A re-challenge with CT26 cells is done to evaluate establishment of anti-tumor memory in BALB/c mice that have rejected subcutaneous CT26 tumors.

On day 49 (D49, i.e. 49 days after the first SC injection of CT26 cells), $1x10^6$ of CT26 cells are injected subcutaneously in 200 µL of RPMI 1640 into the left flank of animals from groups 2, 3, 4, 5, 7 and 9. Only mice whose first CT26 tumour has been eliminated by the effect of the treatment are injected with the second CT26 tumour cells.

In addition, in order to demonstrate the CT26 tumour growth in absence of an immunological memory, $1x10^6$ of CT26 cells will be injected subcutaneously in 200 µL of RPMI 1640 into the left flank of nine (9) naive mice.

The monitoring of animals is performed as described above.

Markedly delay in onset of tumour development or complete tumour rejection with GITR targeting Nanobody or Nanobody-ratIgG2b chimera is expected.

Example 14: Agonistic anti-GITR Nanobodies alone or in combination with an anti-PD-1 antibody cause tumor regression and increase survival time in a syngeneic CT-26 colon carcinoma mouse model

Anti-tumor efficacy of agonistic anti-GITR Nanobodies (Nb) was demonstrated in a syngeneic CT-26 colon carcinoma model, in which BALB/c mice were inoculated with BALB/c-derived colorectal carcinoma cells (CT-26 cells). CT-26 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. BALB/c mice were subcutaneously injected with $1x10^6$ CT-26 cells in 200 µL volume of RPMI 1640 into the right flank. Tumor length and width was measured using calipers and tumor volume determined using the formula Tumor Volume (mm^3) = 0.5 x length x $width^2$, where length is the longer dimension. On day 8 after tumor challenge mice were randomized according to their individual tumor volume into 14 treatment groups with an equivalent mean tumor size of 89 mm^3 and dosing was started according to the treatment regimen presented in Table 8. Tumor volumes were recorded twice weekly. Survival was recorded every day. Mice were euthanized when tumors exceeded 10% of normal body weight.

Mice were treated with an anti-GITR Nanobody or with anti-GITR Nanobody-IgG chimeras of rat IgG2b or human IgG1 isotypes, either as a monotherapy or in combination with an anti-PD-1 monoclonal antibody (mAb; BioXCell clone RPM1-14). Mice treated with DTA-1 (BioXCell), an anti-GITR mAb, alone or in combination with anti-PD-1 mAb were included as positive reference control groups. One group of control mice (vehicle group 14) was left untreated. All treatments were administered via intraperitoneal injection (IP) starting on day 8 after tumor challenge. The groups of mice receiving DTA-1 were treated with a single dose of 25mg/kg (groups 1 and 2). The groups of mice receiving anti-GITR Nanobody were treated repeatedly with one IP injection every two days for 18 days, either at a dose level of 15 mg/kg single loading dose and 10 mg/kg maintenance dose (dose level 1; groups 3 and 7) or at a 30 mg/kg single loading dose and a 20 mg/kg maintenance dose (dose level 2; groups 5 and 8). Irrelevant control Nanobodies were dosed at the respective dose level 1 (groups 4 and 9) and dose level 2 (groups 6 and 10) multiple dosing regimens. The anti-GITR NB-IgG chimera of the rat IgG2b and human IgG1 isotype were administered as a single dose of 5 mg/kg (group 11) and 25 mg/kg (groups 12 and 13), respectively. Groups of mice receiving a combination therapy were administered a total of three IP injections with anti-PD-1 mAb with one injection every five days at a dose level of 10 mg/kg (groups 2, 7, 8, 9, 10 and 13).

The anti-tumor efficacy of the anti-GITR Nanobody and anti-GITR Nanobody-IgG chimeras was evaluated and compared with respective control groups. Tumor growth inhibition, delay of tumor growth and tumor regression together with survival of animals served as read-outs for anti-tumor efficacy.

As depicted in Figure 8, combination of anti-GITR Nanobody with anti-PD-1 mAb (groups 7 and 8; Figures 8G and 8H) resulted in a synergistic efficacy with 7/10 and 8/10 animals showing a delayed tumor development for Nanobody dose level 1 and 2, respectively, against 1/10 and 2/10 animals in the respective control groups (groups 9 and 10; Figures 8I and 8J) when compared with the vehicle group 14 (Figure 8N).

Monotherapy with anti-GITR Nanobody resulted in a delayed tumor development in 3/10 and in 4/10 animals at dose level 1 and 2, respectively, when compared to the respective control groups 5 and 6. Combination of anti-GITR Nanobody-IgG chimera of the human IgG1 isotype with anti-PD-1 mAb resulted in a robust synergistic effect shown by a delay in tumor development in 8/10 animals and 50 % complete regression (CR) (group 13). Monotherapy with the human IgG1 isotype chimera resulted in a delayed tumor growth in 6/10 animals with 1/10 animals showing complete tumor regression (group 12). Monotherapy with the rat IgG2b isotype chimera resulted in a delayed tumor growth in 6/10 animals (group 11) compared to the vehicle group. Treatment with the reference anti-GITR mAb, DTA-1, resulted in a delayed tumor development for 8/10 and 6/10 animals for monotherapy (group 1) and in combination with anti-PD-1 mAb (group 2), respectively, compared to their respective control groups. Both monotherapy and combination therapy resulted in a 20 % complete regression.

In addition, as depicted in Figure 9, the median survival time (start calculated from the day of tumor injection) of the vehicle group was 23 days (group 14). Median survival times for groups treated with the reference anti-GITR mAb, DTA-1, alone was 44.5 days with 40 % of animals still alive on day 40 post treatment, whereas combination therapy with DTA-1 and anti-PD-1 mAb resulted in a median survival of 34 days with 30 % of animals still alive on day 40 post treatment. The median survival for control groups receiving irrelevant Nanobody in combination with anti-PD-1 mAb was 21.5 and 23 days at irrelevant Nanobody dose levels 1 and 2, respectively. Monotherapy with anti-GITR Nanobody corresponded to median survival times of 23 days for both dose levels (groups 3 and 5), whereas the respective control groups treated with irrelevant Nanobody showed a median survival of 20 days for both dose levels (groups 4 and 6). Median survival of groups treated with the combination of anti-GITR Nanobody and anti-PD-1 mAb was 30 days at Nanobody dose level 1 (group 7), with 20 % still alive on day 40 post treatment, and 28.5 days at Nanobody dose level 2 (group 8). Animals receiving a monotherapy with anti-GITR Nanobody chimera of the rat IgG2b isotype (group 11) presented with a median survival of 27 days, with 10 % still alive on day 40 post treatment.

Animals treated with monotherapy of anti-GITR Nanobody chimera of the human IgG1 isotype (group 12) had a median survival of 27 days, with 30 % surviving on day 40 post treatment, whereas 60 % of animals treated with the combination of the human IgG1 isotype chimera and anti-PD-1 mAb were still alive on day 40 post treatment (group 13). In sum, the agonistic anti-GITR Nanobodies of the present invention show statistically significant effects in a relevant tumor model. These results were further confirmed by a separate and independent statistical analysis of the data obtained, which was an extended version of the so-called tobit regression model (Dagne & Huang, 2012).

Table 8: Treatment regimen

Group	No. Animals	Treatment	Dose	Route	Treatment Schedule
1	10	DTA-1	25mg/kg	IP	Q1Dx1
2	10	DTA-1	25mg/kg	IP	Q1Dx1
		anti-PD-1 Ab	10 mg/kg	IP	Q5Dx3
3	10	anti-GITR NB (A023100035)	1x 15 mg/kg loading dose, then 9x 10 mg/kg maintenance dose	IP	Q2Dx10
4	10	irrelevant NB	1x 15 mg/kg loading dose, then 9x 10 mg/kg maintenance dose	IP	Q2Dx10
5	10	anti-GITR NB (A023100035)	1x 30 mg/kg loading dose, then 9x 20 mg/kg maintenance dose	IP	Q2Dx10

Group	No. Animals	Treatment	Dose	Route	Treatment Schedule
6	10	irrelevant NB	1x 30 mg/kg loading dose, then 9x 20 mg/kg maintenance dose	IP	Q2Dx10
7	10	anti-GITR NB (A023100035)	1x 15 mg/kg loading dose, then 9x 10 mg/kg maintenance dose	IP	Q2Dx10
		anti-PD-1 Ab	10 mg/kg	IP	Q5Dx3
8	10	anti-GITR NB (A023100035)	1x 30 mg/kg loading dose, then 9x 20 mg/kg maintenance dose	IP	Q2Dx10
		anti-PD-1 Ab	10 mg/kg	IP	Q5Dx3
9	10	irrelevant NB	1x 15 mg/kg loading dose, then 9x 10 mg/kg maintenance dose	IP	Q2Dx10
		anti-PD-1	10 mg/kg	IP	Q5Dx3
10	10	irrelevant NB	1x 30 mg/kg loading dose, then 9x 20 mg/kg maintenance dose	IP	Q2Dx10
		anti-PD-1	10 mg/kg	IP	Q5Dx3
11	10	anti-GITR NB-rat IgG2b (A-0231-00_TP010)	5mg/kg	IP	Q1Dx1
12	10	anti-GITR NB-human IgG1 (A-0231-00_TP008)	25mg/kg	IP	Q1Dx1
13	10	anti-GITR NB-human IgG1 (A-0231-00_TP008)	25mg/kg	IP	Q1Dx1
		anti-PD-1	10 mg/kg	IP	Q5Dx3
14	10	vehicle	-	IP	Q1Dx1

Q1Dx1: Single injection on the day of start of treatment; Q5Dx3: 3 injections with 1 injection every 5 days; Q2Dx10: 10 injections with 1 injection every 2 days. In case of combinations: DTA-1, anti-GITR

NB, anti-GITR NB-ratIgG2b chimera, anti-GITR NB-human IgG1 chimera, or irrelevant NB is injected after the anti-PD-1.

Example 15: *In vitro* benchmarking of the anti-GITR Nanobodies in the T cell activation assay

The T cell experiments were performed as described in Example 10. Results are shown in Figures 10A-10C. These experiments show that the tested anti-GITR compounds have a similar potency (EC50) as the clinical stage 36E5 mAb. However, Nanobody constructs A023100035 and A-0231-00_TP008 clearly show a higher maximal efficacy compared to 36E5. This is clinically very important as the effectiveness of a drug depends on its maximal efficacy. Nanobody A023100014 shows an equal maximal efficacy compared to 36E5, which is maintained at higher concentrations, in contrast to 36E5.

Example 16: *In vivo* proof-of-concept of A023100035 and A-0231-00_TP008 in an OVA immunization model

The adjuvant effect of an anti-GITR multivalent Nanobody (A023100035) and anti-GITR Nanobody-huIgG1 chimera (A-0231-00_TP008) on the humoral immune response to OVA was demonstrated, reflecting the immune-enhancing effect of anti-GITR agonist Nanobodies and an anti-GITR Nanobody-huIgG1 chimera.

In the OVA immunization model, BALB/c mice were immunized on day zero (day 0) by subcutaneous (s.c.) administration of 100 µg OVA in saline or in a 1:1 mixture with Incomplete Freund's Adjuvant (IFA) followed by a boost with the same s.c. dose in saline or IFA on day 14.

To assess the effect of the anti-GITR Nanobody on the humoral response to OVA, the anti-GITR Nanobody was administered intraperitoneally at different dosing regimens, i.e. a single dosing regimen with administration on the day of primary (day 0) and of boost (day 14) immunization at 15 mg/kg (single dose level 1, SDL1) or 30 mg/kg (SDL2); a repeated dosing regimen with administration starting on day 0 and on day 14 with a loading dose level of 15 mg/kg and two maintenance dose levels of 10 mg/kg (Q2Dx3; repeated dosing regimen 1, RD1); a repeated dosing regimen with administration starting on day 0 with a loading dose level of 15 mg/kg and ten maintenance dose levels of 10 mg/kg (Q2Dx11; RD2). Irrelevant control Nanobody was administered at dosing regimens corresponding to the respective dosing regimen of anti-GITR Nanobody. Anti-GITR Nanobody-huIgG1 chimera was administered as a single dose on day 0 and day 14 at a dose level of 20 mg/kg and compared to an irrelevant human IgG1 mAb (Synagis) given at the same dosing regimen and level. An anti-mouse GITR agonist mAb (DTA-1; BioXCell) was included as a positive reference control and compared to its respective isotype control (anti-KLH, clone LTF-2, BioXCell), both administered at a single dosing regimen on day 0 and on day 14 at a dose level of 20 mg/kg. On day 0 and day 14 treatments were given prior to OVA immunization. OVA mixed with IFA was included as a reference comparison.

Anti-OVA total IgG serum titres were determined by ELISA on day 13 and day 21 after primary immunization. As depicted in Figure 11, on day 13 OVA titres significantly increased in animals subjected to the continuous repeated dosing regimen RD2 with a mean log10 titre of 4.273 (± 0.284) versus its respective control (3.325 ± 0.104; p<0.0001). On day 21 OVA titres further increased to 6.071 (± 0.277), significantly different versus control (4.962 ± 0.225; p<0.0001). In animals receiving anti-GITR Nanobody at SDL1, SDL2 and RD1 the highest titres on day 21 were induced by RD1 (6.744 ± 0.151) versus its respective control (5.090 ± 0.206; p<0.0001) and SDL1 (6.628 ± 0.125) versus its respective control (5.156 ± 0.368; p<0.0001). Similarly to SDL1, anti-GITR Nanobody at SDL2 resulted in increased OVA titres (6.225 ± 0.473), significantly different compared to its respective control (4.799 ± 0.081; p<0.0001). On day 21 anti-GITR Nanobody-huIgG1 chimera resulted in an OVA titre increase of 5.903 (± 0.249), significantly different compared to its isotype control (5.326 ± 0.237). Similarly, the difference in titre of treated versus control animals was significant upon treatment with DTA-1 (5.530 ± 0.343 versus 4.971 ± 0.281, respectively; p=0.0002).

Example 17: Sequence optimization of anti-GITR Nanobodies

17.1 Sequence optimization: sequence analysis

Parental wild type Nanobody sequences were mutated to yield Nanobody sequences that are more identical to human VH3-JH germline consensus sequences. Specific amino acids in the framework regions that differ between the Nanobody and the human VH3-JH germline consensus were altered to the human counterpart in such a way that the protein structure, activity and stability were kept intact. For Nanobody A0231004B01, a variant (A023100061, SEQ ID NO: 269) including the 5 mutations L11V, A74S, K83R, V89L, K105Q was generated (numbering according to Kabat). For A0231005A03, a variant (A023100078, SEQ ID NO: 271) with the same 5 mutations (L11V, A74S, K83R, V89L, K105Q) was generated. In addition, the methionine on position 100e was further substituted by a leucine (A023100090, SEQ ID NO: 272), a lysine (A023100091, SEQ ID NO: 282), an arginine (A023100092, SEQ ID NO: 283) or a glutamine (A023100093, SEQ ID NO: 284). For A0231034A08, a variant (A023100063, SEQ ID NO: 270) with the 5 mutations D10G, L11V, A74S, K83R, V89L was generated. And finally for Nanobody A0231052E08, a variant (A023100050, SEQ ID NO: 268) carrying the 9 following mutations was generated: L11V, A14P, S60A, A74S, M78L, K83R, A87T, G88A, V89L.

17.2 Construction, expression and purification of multivalent sequence optimized anti-GITR Nanobody constructs

For variants A023100061 and A023100090 different constructs were made as listed in Table A-11 (SEQ ID NOs: 285-290). All multivalent Nanobody constructs had C-terminal Albumin binding Nb (Alb92) and were subsequently expressed in *Pichia pastoris* according to standard conditions. The constructs were purified via protein A binding.

Example 18: Activation of GITR by sequence optimized anti-GITR multivalent Nanobodies assessed in a NF-κB luciferase reporter assay.

The functionality of the sequence optimized formatted Nanobodies was determined in a NF-κB luciferase reporter assay as described in Example 9.

EC_{50} values and efficacies are shown in Table 9. Illustrative activation curves are shown in Figure 12A-D.

Table 9: EC_{50} (M) and efficacy values (human GITR ligand = 100%) of anti-GITR sequence optimized multivalent Nanobodies and reference compound

Construct ID	HEK293_NF-κB luc reporter EC_{50} (M)	HEK293_NF-κB luc reporter Efficacy (%)
A023100101 A023100090(E1D)-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	2.00E-11	135.5
A023100107 A023100090(E1D)-9GS-A023100090-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	1.47E-11	147.3
A023100118 A023100090(E1D)-9GS-A023100090-9GS-A023100090-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	1.28E-11	140.2
A023100105 A023100061(E1D) -9GS-A023100061-9GS-A023100061-35GS-Alb92-A	N.D.	N.D.
A023100127 A023100061(E1D)-9GS-A023100061-9GS-A023100061-9GS-A023100061-35GS-Alb92-A	1.57E-11	149.6
A023100129 A023100061(E1D)-9GS-A023100061-9GS-A023100061-9GS-A023100061-9GS-A023100061-35GS-Alb92-A	1.18E-11	145.8
A-0231-00_TP011	1.80E-11	66.6
Reference compound		
36E5	2.56E-11	20.4

Example 19: Human T cell activation capacity of sequence optimized anti-GITR Nanobodies

The functionality of the sequence optimized formatted Nanobodies was also evaluated in a human T cell activation assay. The experiments were performed as described in Example 10.

Table 10 shows the effect on IFN-γ production of multivalent anti-GITR Nanobodies and anti-GITR antibodies in comparison to the natural ligand. EC_{50} values reflect the potency of the Nanobodies. The maximal efficacy as percentage of the Emax (= maximal response induced by the natural ligand) is also presented. All Nanobody constructs show an efficacy comparable to the natural ligand, which was set at 100%. Illustrative activation curves are shown in Figure 13A-G.

Table 10: EC_{50} values (M) and the maximal efficacy values (human GITR ligand = 100%) of anti-GITR sequence optimized multivalent Nanobody constructs and reference compounds (IFN-γ read-out).

Construct ID	EC_{50} (M)	Maximal Efficacy (%)
A023100101 A023100090(E1D)-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	6.42E-11	92.6
A023100107 A023100090(E1D)-9GS-A023100090-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	6.46E-11	99.8
A023100118 A023100090(E1D)-9GS-A023100090-9GS-A023100090-9GS-A023100090-9GS-A023100090-35GS-Alb92-A	4.26E-11	95.6
A023100105 A023100061(E1D)-9GS-A023100061-9GS-A023100061-35GS-Alb92-A	6.00E-11	97.0
A023100127 A023100061(E1D)-9GS-A023100061-9GS-A023100061-9GS-A023100061-35GS-Alb92-A	5.87E-11	86.8
A023100129 A023100061(E1D)-9GS-A023100061-9GS-A023100061-9GS-A023100061-9GS-A023100061-35GS-Alb92-A	5.15E-11	107.6
A-0231-00_TP011	4.62E-11	86.0
Reference compound		
36E5	4.37E-11	82.3

Example 20: *In vivo* proof-of-concept of sequence optimized anti-GITR multivalent Nanobodies in an OVA immunization model

The adjuvant effect of anti-GITR multivalent Nanobodies (A023100101, A023100107, A023100118) on the humoral immune response to OVA is evaluated, reflecting the immune-enhancing effect of anti-GITR agonist Nanobodies.

In the OVA immunization model, BALB/c mice are immunized on day zero (day 0) by subcutaneous (s.c.) administration of 100 µg OVA in saline or in a 1:1 mixture with Incomplete Freund's Adjuvant (IFA) followed by a boost with the same s.c. dose in saline or IFA on day 14.

To assess the effect of anti-GITR multivalent Nanobodies on the humoral response to OVA, the anti-GITR Nanobodies are administered intraperitoneally on the day of primary (day 0) and of boost (day

14) OVA immunization at a single dosing regimen with dose levels ranging from 0.5 to 20 mg/kg and prior to OVA immunization. Irrelevant control Nanobody is administered at a similar single dosing regimen at a dose level of 15 mg/kg. An anti-mouse GITR agonist mAb (DTA-1; BioXCell) is included as a positive reference control and is compared to its respective isotype control (anti-KLH, clone LTF-2, BioXCell), both administered at a single dosing regimen on day 0 and on day 14 at a dose level of 20 mg/kg. OVA mixed with IFA is included as a reference comparison.

Anti-OVA total IgG serum titres are determined by ELISA on day 13 and day 21 after primary immunization. Significant increase of total anti-OVA IgG levels in anti-GITR Nanobody-treated animals are expected compared to untreated and control animals.

Example 21: Anti-tumor efficacy of agonistic sequence optimized anti-GITR multivalent Nanobodies alone or in combination with an anti-PD-1 antibody in a syngeneic CT-26 colon carcinoma mouse model

Anti-tumor efficacy of anti-GITR agonistic multivalent Nanobodies (Nb) and an anti-GITR Nanobody-huIgG1 chimera was demonstrated in a syngeneic CT-26 colon carcinoma model, in which BALB/c mice were inoculated with BALB/c-derived colorectal carcinoma cells (CT-26 cells). CT-26 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. BALB/c mice were subcutaneously injected with $1x10^6$ CT-26 cells in 200 µL volume of RPMI 1640 into the right flank. Tumor length and width was measured using calipers and tumor volume determined using the formula Tumor Volume (mm^3) = 0.5 x length x $width^2$ where length is the longer dimension. On day 7 after tumor challenge mice were randomized according to their individual tumor volume into 15 treatment groups of 10 animals each with a mean tumor volume of 55 ± 19 mm^3. Dosing was started according to the treatment regimen presented in Table 11. Tumor volumes were recorded twice weekly. Survival, behavior and body weight were recorded every day. Animals were monitored until day 42 after treatment start.

Mice were treated with anti-GITR multivalent Nanobodies (A023100101, A023100107, A023100118) or with an anti-GITR Nanobody-huIgG1 chimera (A-0231-00_TP011), either as a monotherapy or in combination therapy with an anti-PD-1 monoclonal antibody (mAb). Mice treated with the anti-GITR mAb DTA-1, alone or in combination with anti-PD-1 mAb were included as positive control groups. One group of control mice (group 1) was left untreated. All treatments were administered via intraperitoneal injection (IP) starting on day 7 after tumor challenge. The groups of mice receiving DTA-1 were treated with a single dose of 25 mg/kg (groups 2 and 3). The groups of mice receiving anti-GITR Nanobodies were treated via a multiple dosing regimen with one IP injection every two days during 18 days, consisting of one loading dose and nine maintenance doses. Groups treated with anti-GITR Nanobody A023100101 received a loading dose of 15 mg/kg and a maintenance dose of 10 mg/kg (groups 8 and 9). Anti-GITR Nanobody A023100107 was dosed at 18.7 mg/kg loading dose and 12.5 mg/kg maintenance dose (groups 10 and 11). Anti-GITR Nanobody A023100118 was dosed at 22.5 mg/kg loading dose and 14 mg/kg maintenance dose (groups 12 and 13). Dose levels of the latter two anti-GITR Nanobodies A023100107 and A023100118 were set to be equimolar to anti-GITR Nanobody A023100101. Irrelevant control Nanobody was dosed similarly as anti-GITR Nanobody A023100101. Anti-GITR Nanobody-huIgG1 chimera was administered as a single dose of 25 mg/kg (groups 14 and 15), respectively. Groups of mice receiving combination therapy were administered a total of three IP injections with anti-PD-1 mAb, with one injection every five days at a dose level of 10 mg/kg (groups 3, 5, 7, 9, 11, 13, 15).

The anti-tumor efficacy of anti-GITR multivalent Nanobodies and anti-GITR Nanobody-huIgG1 chimera was evaluated and compared with respective control groups. Tumor growth and survival served as read-outs for anti-tumor efficacy. Animals were monitored until day 49 after tumour challenge, corresponding to day 42 after treatment start.

As shown in Figure 14, anti-GITR Nanobodies A023100101 (group 8) and A023100118 (group 12) showed a delayed trend in tumor growth for 1/10 animals compared to their respective control group (irrelevant Nanobody, group 6), whereas anti-GITR Nanobody A023100107 (group 10) significantly inhibited tumor growth development ($p= 0.323$) compared to irrelevant Nanobody. Anti-GITR Nanobody-huIgG1 chimera (group 14) resulted in a significant inhibition of tumour growth ($p < 0.0001$) compared to its respective control (huIgG1 isotype, group 4) with 5/10 and 3/10 animals showing delayed tumor growth development and a complete tumor regression, respectively. Similarly, anti-tumor efficacy was confirmed for DTA-1 mAb, showing significant inhibition of tumor growth development ($p < 0.0001$), with 3/10 animals with complete regression. A synergistic tumor growth inhibition was observed with anti-GITR Nanobody A023100101 (group 9; $p < 0.0022$) and with A023100107 (group 11; $p < 0.0007$) in combination with anti-PD-1 mAb, compared to the corresponding anti-PD-1 mAb control group (group 7). In group 9, in 1 out of 6 animals with delayed tumor growth a complete regression was observed, whereas in group 11 two animals out of 5 with delayed tumor growth showed a complete regression. The combination of anti-GITR Nanobody-huIgG1 chimera with anti-PD-1 mAb (group 15) resulted in a significant tumor growth inhibition ($p < 0.0001$) compared to huIgG1 isotype control (group 5). Similarly, DTA-1 mAb in combination with anti-PD-1 mAb showed a significant anti-tumor effect compared to group 7, as 5 animals showed a complete regression ($p < 0.0001$).

As depicted in Figure 15, the median survival time (from treatment start) of vehicle group 1 was 15 days, with 100 % mortality from day 21. Median survival time upon treatment with DTA-1 alone was 34.5 days with 40 % survival on day 42 post treatment ($p < 0.0001$), whereas combination therapy with DTA-1 and anti-PD-1 mAb resulted in a median survival of 34 days with 60 % of animals still alive on day 42 post treatment (p = 0.0003). The median survival for anti-PD-1 mAb control groups 5 and 7 was 19.5 days. Survival analysis of anti-GITR Nanobody monotherapy was significant for A023100107 (p < 0.0391) with a median survival of 19.5 days and 20 % survival on day 42. In combination with anti-PD-1 mAb survival analysis was significant for anti-GITR Nanobodies A023100101 (group 9; p = 0.0026) and A023100107 (group 10; p= 0.0056) showing 50 % and 30 % survival on day 42, respectively. Anti-GITR Nanobody A023100118 in combination with anti-PD-1 mAb (group 13) resulted in a median survival of 28 days. Monotherapy with anti-GITR Nanobody-huIgG1 chimera (group 14) showed a median survival time of 34.5 days and 40 % survival on day 42 (p<0.0001), whereas combination therapy with anti-PD-1 mAb (group 15) resulted in a median survival time of 29.5 days and 30 % survival on day 42 post treatment.

Table 11: Treatment regimen

Group	No. Animals	Treatment	Dose	Route	Treatment Schedule
1	10	vehicle	-	IP	Q1Dx1
2	10	DTA-1	25mg/kg	IP	Q1Dx1
3	10	DTA-1	25mg/kg	IP	Q1Dx1
		anti-PD-1	10mg/kg	IP	Q5Dx3
4	10	huIgG1 isotype control	25mg/kg	IP	Q1Dx1
5	10	huIgG1 isotype control	25mg/kg	IP	Q1Dx1
		anti-PD-1	10mg/kg	IP	Q5Dx3
6	10	Irrelevant NB	1x15mg/kg, then 9x10mg/kg	IP	Q2Dx10
7	10	Irrelevant NB	1x15mg/kg, then 9x10mg/kg	IP	Q2Dx10
		anti-PD-1	10mg/kg	IP	Q5Dx3
8	10	anti-GITR NB (A023100101)	1x15mg/kg, then 9x10mg/kg	IP	Q2Dx10
9	10	anti-GITR NB (A023100101)	1x15mg/kg, then 9x10mg/kg	IP	Q2Dx10
		anti-PD-1	10mg/kg	IP	Q5Dx3
10	10	anti-GITR NB (A023100107)	1x18.7mg/kg, then 9x12.5mg/kg	IP	Q2Dx10
11	10	anti-GITR NB (A023100107)	1x18.7mg/kg, then 9x12.5mg/kg	IP	Q2Dx10
		anti-PD-1	10mg/kg	IP	Q5Dx3
12	10	anti-GITR NB (A023100118)	1x22.5mg/kg, then 9x14mg/kg	IP	Q2Dx10
13	10	anti-GITR NB (A023100118)	1x22.5mg/kg, then 9x14mg/kg	IP	Q2Dx10
		anti-PD-1	10mg/kg	IP	Q5Dx3
14	10	anti-GITR NB-huIgG1 chimera (A-0231-00-TP011)	25mg/kg	IP	Q1Dx1
15	10	anti-GITR NB-huIgG1 chimera (A-0231-00-TP011)	25mg/kg	IP	Q1Dx1
		anti-PD-1	10mg/kg	IP	Q5Dx3

Q1Dx1: Single injection on the day of start of treatment; Q5Dx3: 3 injections with 1 injection every 5 days; Q2Dx10: 10 injections with 1 injection every 2 days. In case of combinations: DTA-1, anti-GITR NB, anti-GITR NB-huIgG1 chimera, irrelevant NB or isotype control mAb is injected after anti-PD-1 mAb.

TABLES

Table A- 1: Sequence alignment of GITR Family 7 binders

```
PA0231PMP005A03.1 : evqlvesggglvqpggslrlscaasETIFSIDSMAwyrqapgkqrelvaAITGGGSPNyadsvkgrftissdvakrtvylqmnslkpedtavyycnaEGQAGWGTALMDYwgkgtlvtvss
PA0231PMP004A03.1 : ......................t...S.....A.G.h............H.....RS.............g.s.......................................................
PA0231PMP004A12.1 : ..........................S.....A.G..h...........T......T.............g.s.........d...........................................
PA0231PMP004F05.1 : .............s............S.....A.G..............T.....RR......m...s..g.n..................................................q.....
PA0231PMP005A01.1 : ..........................S.....A.G.h............H.....RS.............g.s..................................................q.....
PA0231PMP005A02.1 : ......................t...S.....A.G.h............H.....RS.............g.s............r..........................................
PA0231PMP005A10.1 : ............r.............S.....A.G..............TM.....T.................................................................q.....
PA0231PMP005B02.1 : ......................t...S.....A.G.h............H.....GS.............g.s..................................................q.....
PA0231PMP005B08.1 : ...................................................r.........................................................................
PA0231PMP005C01.1 : ..........................................................................a....................................................
PA0231PMP005G10.1 : ............r.............S.....A.G..............TM.....T.......r...........................................................q.....
PA0231PMP006E02.1 : ......................t...S.....A.G.h............H.....RS..........a..g.s..................................................q.....
PA0231PMP006E08.1 : ..........................S.....A.G..............T......T.............g.s.........d...........................................
PA0231PMP006G04.1 : ............r.......p.....S.....A.G..............TM.....T......................................................................
PA0231PMP012E03.1 : .............s............S....NA.G..............T.....RR......m...s..g.n..................................................q.....
PA0231PMP012G07.1 : ..........................S.....A.G..............T....S.T.............g.n..................................................q.....
PA0231PMP020B05.1 : ..........................S.....A.G..............T......T.............g.n..................a...................................
PA0231PMP020D02.1 : ......................t...S.....A.G.h............H.....RS.............g.s..............g.........................q.....
PA0231PMP020E12.1 : ..........................S.....A.G..............T......T.............g.n..................................................q.....
PA0231PMP021B02.1 : ......................t...S.....A.G.h............H.....R...............g.s.......................................................
PA0231PMP023G01.1 : .......................g..S.....A.G..............G.S...RT.............g.n..n.............................P..N............
```

Table A- 2: Sequence alignment of GITR Family 26 binders

```
PA0231PMP004B01.1 : evqlvesggglvqpggslrlscaasGSIFSIDSMGwyrqapgkqrelvaAITSSTNyadsvkgrftisrdnakntvylqmnslkpedtavyycnlEGQAGWGTALINYwgkgtlvtvss
PA0231PMP004B02.1 : ....................t....................A................S...T....e..........a........................vK..T......MD......q.....
PA0231PMP012F03.1 : ....................t....................A................S...T....e..........a.......................svK..T......MD...........
PA0231PMP012D08.1 : ...............................NA.....................G......a.........h.........................T......MD...........
PA0231PMP006C07.1 : ...............................NA.....................G..............h.........................T......MD......q.....
PA0231PMP005G01.1 : ....................f..........NA.....................G.......r........h.........................T......MD......q.....
PA0231PMP005A08.1 : ...............................NA.....................G..............h.........................T......MD...........
PA0231PMP004B03.1 : .....................................................R.I.......................................................MD...........
PA0231PMP004A05.1 : ................................A................T...GK..................a......................................MD......q.....
PA0231PMP004A01.1 : ..........................................................................t...............................MD......q.....
PA0231PMP005B05.1 : ...............................NA..........................................a.......................h..........LD...........
```

Table A- 3: Sequence alignment of GITR Family 82 binders

```
PA0231PMP034A08.1 : evqlvesggdlvqpggslrlscaasGSVFSINDMGwfrqapgkqrelvaDIISRGVTNyadsvkgrftisgdpakntvylqmnslkpedtavyycnaHISTGWGRPHNNYwgqgtqvtvss
PA0231PMP033B12.1 : ...........................I...DS................AD.................h.....................................................
PA0231PMP033F01.1 : ...........................I...DS................AD.................h.q...................................................
PA0231PMP034B06.1 : ...........................I...DS................A..................h.....................................................
PA0231PMP048A08.1 : ...........................I...D.................A..................h.......................................l.....
PA0231PMP034A03.1 : ...........................I.....V..............................................................a........................
PA0231PMP034A02.1 : ................p.........NI.............................................................................................
PA0231PMP034B09.1 : ................p..........I.............................................................................................
PA0231PMP034A01.1 : ...........................I.............................................................................................
PA0231PMP034A09.1 : ...........................I.....T.......................................................................................
PA0231PMP034A10.1 : ...........................I.....................A.......................................................................
PA0231PMP034C06.1 : ...........................I..................G..........................................................................
PA0231PMP034D02.1 : ...........................I................................................a............................................
PA0231PMP034F01.1 : ..................g........I.............................................................................................
PA0231PMP048D06.1 : ............r..............I.............................................................................................
PA0231PMP048D12.1 : ...........................I...........................c.................................................................
PA0231PMP048C07.1 : ...........................I........l....................................................................................
PA0231PMP036C11.1 : ...........................I..................................r..........................................................
PA0231PMP036A04.1 : .........g.................I.............................................................................................
PA0231PMP034F11.1 : ...........................I.......................................v.....................................................
PA0231PMP034E12.1 : ...........................I.........................................h.....................................................
PA0231PMP047G08.1 : ...........................I.......................D.................h.....................................................
PA0231PMP034F10.1 : ...........................I............................................................................M.............l.....
```

Table A- 4: Sequence alignment of GITR Family 109 binders

```
PA0231PMP052E08.1 : evqlvesggglvqaggslrlsctgsRSIFSTYAMAwhrqapgkqrelvgFIYWGGTTTysdsvkgrftisrdnakntmylqmnslkpedagvyycniYGSYALPwgqgtlvtvss
PA0231PMP052A03.1 : ..........................N.............................S.v..............................a..........................
PA0231PMP052B05.1 : ..............................................................................................................q..........
PA0231PMP052C04.1 : ..........m...............N.............................S.v..............................a...............q.....
PA0231PMP052D06.1 : ........................................................S.v..............................a..........................
PA0231PMP052F03.1 : ..........................................................................s............................................
```

Table A- 5: Sequence alignment of GITR Family 85 binders

```
PA0231PMP033B02.1 : EVQLVESGGGLVQPGGSLRLSCAASGTIFSISTMGWYRQAPGKQREVVAVTSGFSTNYSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNAYLSLAWRDPDRDYWGQGTQVTVSS
```

Table A- 6: Sequence alignment of GITR Family 38 binders

```
PA0231PMP003D11.1 : evqlvesggglvqageslrlscaasGSIFSIDAMGwyrqapgkqrelvaEISDHTTygdsvkgrftisrgnaentvalqmnslkpedtgvyycnvHHQRGWGTSITVTwgqgtqvtvss
PA0231PMP017E06.1 : ......................................................................................................A...........
PA0231PMP017G05.1 : ..............................................................m....................................................
PA0231PMP010C09.1 : .........r...............................G.........................................................P..............
PA0231PMP017C01.1 : .........r..........................................................................................P..............
PA0231PMP017B08.1 : ............r..................................................................................................l.....
```

Table A- 7: Sequence alignment of GITR Family 110 binders

```
PA0231PMP052A08.1 : evqlvesggglvqaggslrlscvasGSISSITAMGwhrqapgaqregvaVISRSGATMlvdsvkgrftivqdnakntvylqmnslkvedtavygcsaITQGRTYwgqgtlvtvss
PA0231PMP052A01.1 : .................................................I...........................................................q.....
PA0231PMP052A05.1 : .................................................A.......I.a..........................................EQ.......q.....
```

Table A- 8: Sequence alignment of GITR Family 108 binders

```
PA0231PMP051E01.1 : EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSS
```

Table A-9: Amino acid sequences of monovalent anti-GITR Nanobodies ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
A0231PMP005A03	1	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP004A03	2	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGRSNYADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP004A12	3	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWYHQAPGKQRELVATITGGGSTNYADSVKGRFTISGDSAKRTVYLQMDSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP004F05	4	EVQLVESGGGLVQSGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATITGGGRRNYADSVMGRFSISGDNAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP005A01	5	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWHRQAPGKQRELVAHITGGGRSNYADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP005A02	6	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGRSNYADSVKGRFTISGDSAKRTVYLQMNSLRPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP005A10	7	EVQLVESGGGLVRPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATMTGGGSTNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP005B02	8	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGGSNYADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP005B08	9	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGRQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP005C01	10	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTAYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS

Name	ID	Amino acid sequence
A0231PMP005G10	11	EVQLVESGGGLVRPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATMTGGGSTNYADSVRGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP006E02	12	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGRSNYADSVKGRFAISGDSAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP006E08	13	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATITGGGSTNYADSVKGRFTISGDSAKRTVYLQMDSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP006G04	14	EVQLVESGGGLVRPGGSLRLPCAASESIFSIDAMGWYRQAPGKQRELVATMTGGGSTNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP012E03	15	EVQLVESGGGLVQSGGSLRLSCAASESIFSINAMGWYRQAPGKQRELVATITGGGRRNYADSVMGRFSISGDNAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP012G07	16	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATITGGSSTNYADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP020B05	17	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATITGGGSTNYADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAAYYCNAEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP020D02	18	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGRSNYADSVKGRFTISGDSAKRTVYLQMNSLKPGDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP020E12	19	EVQLVESGGGLVQPGGSLRLSCAASESIFSIDAMGWYRQAPGKQRELVATITGGGSTNYADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP021B02	20	EVQLVESGGGLVQPGGSLRLSCTASESIFSIDAMGWHRQAPGKQRELVAHITGGGRPNYADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSS

Name	ID	Amino acid sequence
A0231PMP023G01	21	EVQLVESGGGLVQPGGSLRLSCAGSESIFSIDAMGWYRQAPGKQRELVAGISGGGRTNYADSVKGRFTISGDNAKNTVYLQMNSLKPEDTAVYYCNAEGQAGWGTPLMNYWGKGTLVTVSS
A0231PMP004B01	22	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSS
A0231PMP004B02	23	EVQLVESGGGLVQPGGSLRLTCAASGSIFSIDAMGWYRQAPGKQRELVASITSTTNYAESVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCNVKGQTGWGTALMDYWGKGTQVTVSS
A0231PMP012F03	24	EVQLVESGGGLVQPGGSLRLTCAASGSIFSIDAMGWYRQAPGKQRELVASITSTTNYAESVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCSVKGQTGWGTALMDYWGKGTLVTVSS
A0231PMP012D08	25	EVQLVESGGGLVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGTNYADSAKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNLEGQTGWGTALMDYWGKGTLVTVSS
A0231PMP006C07	26	EVQLVESGGGLVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGTNYADSVKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNLEGQTGWGTALMDYWGKGTQVTVSS
A0231PMP005G01	27	EVQLVESGGGLVQPGGSLRLFCAASGSIFSINAMGWYRQAPGKQRELVAAITSGTNYADSVRGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNLEGQTGWGTALMDYWGKGTQVTVSS
A0231PMP005A08	28	EVQLVESGGGLVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGTNYADSVKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNLEGQTGWGTALMDYWGKGTLVTVSS
A0231PMP004B03	29	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSRTIYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALMDYWGKGTLVTVSS
A0231PMP004A05	30	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDAMGWYRQAPGKQRELVATITSGKNYADSVKGRFTISRDNAKNAVYLQMNSLKPEDTAVYYCNLEGQAGWGTALMDYWGKGTQVTVSS

Name	ID	Amino acid sequence
A0231PMP004A01	31	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQTNSLKPEDTAVYYCNLEGQAGWGTALMDYWGKGTQVTVSS
A0231PMP005B05	32	EVQLVESGGGLVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCHLEGQAGWGTALLDYWGKGTLVTVSS
A0231PMP034A08	33	EVQLVESGGDLVQPGGSLRLSCAASGSVFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP033B12	34	EVQLVESGGDLVQPGGSLRLSCAASGSIFSIDSMGWFRQAPGKQRELVADIISADVTNYADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP033F01	35	EVQLVESGGDLVQPGGSLRLSCAASGSIFSIDSMGWFRQAPGKQRELVADIISADVTNYADSVKGRFTISGDHAQNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034B06	36	EVQLVESGGDLVQPGGSLRLSCAASGSIFSIDSMGWFRQAPGKQRELVADIISAGVTNYADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP048A08	37	EVQLVESGGDLVQPGGSLRLSCAASGSIFSIDDMGWFRQAPGKQRELVADIISAGVTNYADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTLVTVSS
A0231PMP034A03	38	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDVGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAAYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034A02	39	EVQLVESGGDLVQPGGPLRLSCAASGNIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034B09	40	EVQLVESGGDLVQPGGPLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS

Name	ID	Amino acid sequence
A0231PMP034A01	41	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034A09	42	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDTGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034A10	43	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISAGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034C06	44	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVAGIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034D02	45	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTAYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034F01	46	EVQLVESGGDLVQPGGSLGLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP048D06	47	EVQLVESGGDLVRPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP048D12	48	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNCADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP048C07	49	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWLRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP036C11	50	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVRGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS

Name	ID	Amino acid sequence
A0231PMP036A04	51	EVQLVESGGGLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034F11	52	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTVSGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034E12	53	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP047G08	54	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRDVTNYADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTQVTVSS
A0231PMP034F10	55	EVQLVESGGDLVQPGGSLRLSCAASGSIFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISMGWGRPHNNYWGQGTLVTVSS
A0231PMP052E08	56	EVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNIYGSYALPWGQGTLVTVSS
A0231PMP052A03	57	EVQLVESGGGLVQAGGSLRLSCTGSRNIFSTYAMAWHRQAPGKQRELVGFIYWGGTTSYVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNIYGSYALPWGQGTLVTVSS
A0231PMP052B05	58	EVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNIYGSYALPQGQGTLVTVSS
A0231PMP052C04	59	EVQLVESGGGMVQAGGSLRLSCTGSRNIFSTYAMAWHRQAPGKQRELVGFIYWGGTTSYVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNIYGSYALPWGQGTQVTVSS
A0231PMP052D06	60	EVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTSYVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNIYGSYALPWGQGTLVTVSS

Name	ID	Amino acid sequence
A0231PMP052F03	61	EVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKSTMYLQMNSLKPEDAGVYYCNIYGSYALPWGQGTLVTVSS
A0231PMP033B02	62	EVQLVESGGGLVQPGGSLRLSCAASGTIFSISTMGWYRQAPGKQREVVAVTSGFSTNYSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNAYLSLAWRDPDRDYWGQGTQVTVSS
A0231PMP003D11	63	EVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTQVTVSS
A0231PMP017E06	64	EVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVAWGQGTQVTVSS
A0231PMP017G05	65	EVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSMKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTQVTVSS
A0231PMP010C09	66	EVQLVESGGRLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISGHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTPITVTWGQGTQVTVSS
A0231PMP017C01	67	EVQLVESGGRLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTPITVTWGQGTQVTVSS
A0231PMP017B08	68	EVQLVESGGGLVRAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTLVTVSS
A0231PMP052A08	69	EVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSS
A0231PMP052A01	70	EVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAIISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTQVTVSS

Name	**ID**	**Amino acid sequence**
A0231PMP052A05	71	EVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAAISRSGATILADSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQEQTYWGQGTQVTVSS
A0231PMP051E01	72	EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSS
A023100050	268	EVQLVESGGGVVQPGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCNIYGSYALPWGQGTLVTVSS
A023100061	269	EVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSS
A023100063	270	EVQLVESGGGVVQPGGSLRLSCAASGSVFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPSKNTVYLQMNSLRPEDTALYYCNAHISTGWGRPHNNYWGQGTLVTVSS
A023100078	271	EVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALMDYWGQGTLVTVSS
A023100090	272	EVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSS
A023100091	273	EVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALKDYWGQGTLVTVSS
A023100092	274	EVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALRDYWGQGTLVTVSS
A023100093	275	EVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALQDYWGQGTLVTVSS

Table A-10: Sequences for CDRs and frameworks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 (the following terms: "ID" refers to the given SEQ ID NO)

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
1	A0231PMP005A03	134	EVQLVESGGGLVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	163	YADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
2	A0231PMP004A03	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	91	HITGGGRSN	164	YADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
3	A0231PMP004A12	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	155	WYHQAPGKQRELVA	92	TITGGGSTN	165	YADSVKGRFTISGDSAKRTVYLQMDSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
4	A0231PMP004F05	136	EVQLVESGGGLVQSGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	93	TITGGGRRN	166	YADSVMGRFSISGDNAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
5	A0231PMP005A01	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	91	HITGGGRSN	164	YADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
6	A0231PMP005A02	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	91	HITGGGRSN	167	YADSVKGRFTISGDSAKRTVYLQMNSLRPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
7	A0231PMP005A10	137	EVQLVESGGGLVRPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	94	TMTGGGSTN	163	YADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
8	A0231PMP005B02	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	95	HITGGGGSN	164	YADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
9	A0231PMP005B08	134	EVQLVESGGGLVQPGGSLRLSCAAS	73	ETIFSIDSMA	156	WYRQAPGRQRELVA	90	AITGGGSPN	163	YADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
10	A0231PMP005C01	134	EVQLVESGGGLVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	168	YADSVKGRFTISSDVAKRTAYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
11	A0231PMP005G10	137	EVQLVESGGGLVRPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	94	TMTGGGSTN	169	YADSVRGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
12	A0231PMP006E02	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	91	HITGGGRSN	170	YADSVKGRFAISGDSAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
13	A0231PMP006E08	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	92	TITGGGSTN	165	YADSVKGRFTISGDSAKRTVYLQMDSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
14	A0231PMP006G04	138	EVQLVESGGGLVRPGGSLRLPCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	94	TMTGGGSTN	163	YADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
15	A0231PMP012E03	136	EVQLVESGGGLVQSGGSLRLSCAAS	75	ESIFSINAMG	153	WYRQAPGKQRELVA	93	TITGGGRRN	166	YADSVMGRFSISGDNAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
16	A0231PMP012G07	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	96	TITGGSSTN	171	YADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
17	A0231PMP020B05	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	92	TITGGGSTN	172	YADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAAYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
18	A0231PMP020D02	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	91	HITGGGRSN	173	YADSVKGRFTISGDSAKRTVYLQMNSLKPGDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
19	A0231PMP020E12	134	EVQLVESGGGLVQPGGSLRLSCAAS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	92	TITGGGSTN	171	YADSVKGRFTISGDNAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
20	A0231PMP021B02	135	EVQLVESGGGLVQPGGSLRLSCTAS	74	ESIFSIDAMG	154	WHRQAPGKQRELVA	97	HITGGGRPN	164	YADSVKGRFTISGDSAKRTVYLQMNSLKPEDTAVYYCNA	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
21	A0231PMP023G01	139	EVQLVESGGGLVQPGGSLRLSCAGS	74	ESIFSIDAMG	153	WYRQAPGKQRELVA	98	GISGGGRTN	174	YADSVKGRFTISGDNAKNTVYLQMNSLKPEDTAVYYCNA	119	EGQAGWGTPLMNY	201	WGKGTLVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
22	A0231PMP004B01	134	EVQLVESGGGLVQPGGSLRLSCAAS	76	GSIFSIDSMG	153	WYRQAPGKQRELVA	99	AITSSTN	175	YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNL	120	EGQAGWGTALINY	201	WGKGTLVTVSS
23	A0231PMP004B02	140	EVQLVESGGGLVQPGGSLRLTCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	100	SITSTTN	176	YAESVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCNV	121	KGQTGWGTALMDY	202	WGKGTQVTVSS
24	A0231PMP012F03	140	EVQLVESGGGLVQPGGSLRLTCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	100	SITSTTN	177	YAESVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCSV	121	KGQTGWGTALMDY	201	WGKGTLVTVSS
25	A0231PMP012D08	134	EVQLVESGGGLVQPGGSLRLSCAAS	78	GSIFSINAMG	153	WYRQAPGKQRELVA	101	AITSGTN	178	YADSAKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNL	122	EGQTGWGTALMDY	201	WGKGTLVTVSS
26	A0231PMP006C07	134	EVQLVESGGGLVQPGGSLRLSCAAS	78	GSIFSINAMG	153	WYRQAPGKQRELVA	101	AITSGTN	179	YADSVKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNL	122	EGQTGWGTALMDY	202	WGKGTQVTVSS
27	A0231PMP005G01	141	EVQLVESGGGLVQPGGSLRLFCAAS	78	GSIFSINAMG	153	WYRQAPGKQRELVA	101	AITSGTN	180	YADSVRGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNL	122	EGQTGWGTALMDY	202	WGKGTQVTVSS
28	A0231PMP005A08	134	EVQLVESGGGLVQPGGSLRLSCAAS	78	GSIFSINAMG	153	WYRQAPGKQRELVA	101	AITSGTN	179	YADSVKGRFTISRDHAKNTVYLQMNSLKPEDTAVYYCNL	122	EGQTGWGTALMDY	201	WGKGTLVTVSS
29	A0231PMP004B03	134	EVQLVESGGGLVQPGGSLRLSCAAS	76	GSIFSIDSMG	153	WYRQAPGKQRELVA	102	AITSRTI	175	YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNL	118	EGQAGWGTALMDY	201	WGKGTLVTVSS
30	A0231PMP004A05	134	EVQLVESGGGLVQPGGSLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	103	TITSGKN	181	YADSVKGRFTISRDNAKNAVYLQMNSLKPEDTAVYYCNL	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
31	A0231PMP004A01	134	EVQLVESGGGLVQPGGSLRLSCAAS	76	GSIFSIDSMG	153	WYRQAPGKQRELVA	99	AITSSTN	182	YADSVKGRFTISRDNAKNTVYLQTNSLKPEDTAVYYCNL	118	EGQAGWGTALMDY	202	WGKGTQVTVSS
32	A0231PMP005B05	134	EVQLVESGGGLVQPGGSLRLSCAAS	78	GSIFSINAMG	153	WYRQAPGKQRELVA	99	AITSSTN	183	YADSVKGRFTISRANAKNTVYLQMNSLKPEDTAVYYCHL	123	EGQAGWGTALLDY	201	WGKGTLVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
33	A0231PMP034A08	142	EVQLVESGGDLVQPGGSLRLSCAAS	79	GSVFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
34	A0231PMP033B12	142	EVQLVESGGDLVQPGGSLRLSCAAS	76	GSIFSIDSMG	157	WFRQAPGKQRELVA	105	DIISADVTN	185	YADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
35	A0231PMP033F01	142	EVQLVESGGDLVQPGGSLRLSCAAS	76	GSIFSIDSMG	157	WFRQAPGKQRELVA	105	DIISADVTN	186	YADSVKGRFTISGDHAQNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
36	A0231PMP034B06	142	EVQLVESGGDLVQPGGSLRLSCAAS	76	GSIFSIDSMG	157	WFRQAPGKQRELVA	106	DIISAGVTN	185	YADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
37	A0231PMP048A08	142	EVQLVESGGDLVQPGGSLRLSCAAS	80	GSIFSIDDMG	157	WFRQAPGKQRELVA	106	DIISAGVTN	185	YADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	204	WGQGTLVTVSS
38	A0231PMP034A03	142	EVQLVESGGDLVQPGGSLRLSCAAS	81	GSIFSINDVG	157	WFRQAPGKQRELVA	104	DIISRGVTN	187	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAAYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
39	A0231PMP034A02	143	EVQLVESGGDLVQPGGPLRLSCAAS	82	GNIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
40	A0231PMP034B09	143	EVQLVESGGDLVQPGGPLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
41	A0231PMP034A01	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
42	A0231PMP034A09	142	EVQLVESGGDLVQPGGSLRLSCAAS	84	GSIFSINDTG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
43	A0231PMP034A10	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	106	DIISAGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
44	A0231PMP034C06	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	107	GIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
45	A0231PMP034D02	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	188	YADSVKGRFTISGDPAKNTAYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
46	A0231PMP034F01	144	EVQLVESGGDLVQPGGSLGLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
47	A0231PMP048D06	145	EVQLVESGGDLVRPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
48	A0231PMP048D12	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	189	CADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
49	A0231PMP048C07	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	158	WLRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
50	A0231PMP036C11	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	190	YADSVRGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
51	A0231PMP036A04	134	EVQLVESGGGLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
52	A0231PMP034F11	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	191	YADSVKGRFTVSGDPAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
53	A0231PMP034E12	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	185	YADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS
54	A0231PMP047G08	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	108	DIISRDVTN	185	YADSVKGRFTISGDHAKNTVYLQMNSLKPEDTAVYYCNA	124	HISTGWGRPHNNY	203	WGQGTQVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
55	A0231PMP034F10	142	EVQLVESGGDLVQPGGSLRLSCAAS	83	GSIFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	184	YADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNA	125	HISMGWGRPHNNY	204	WGQGTLVTVSS
56	A0231PMP052E08	146	EVQLVESGGGLVQAGGSLRLSCTGS	85	RSIFSTYAMA	159	WHRQAPGKQRELVG	109	FIYWGGTTT	192	YSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNI	126	YGSYALP	204	WGQGTLVTVSS
57	A0231PMP052A03	146	EVQLVESGGGLVQAGGSLRLSCTGS	86	RNIFSTYAMA	159	WHRQAPGKQRELVG	110	FIYWGGTTS	193	YVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNI	126	YGSYALP	204	WGQGTLVTVSS
58	A0231PMP052B05	146	EVQLVESGGGLVQAGGSLRLSCTGS	85	RSIFSTYAMA	159	WHRQAPGKQRELVG	109	FIYWGGTTT	192	YSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNI	126	YGSYALP	205	QGQGTLVTVSS
59	A0231PMP052C04	147	EVQLVESGGGMVQAGGSLRLSCTGS	86	RNIFSTYAMA	159	WHRQAPGKQRELVG	110	FIYWGGTTS	193	YVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNI	126	YGSYALP	203	WGQGTQVTVSS
60	A0231PMP052D06	146	EVQLVESGGGLVQAGGSLRLSCTGS	85	RSIFSTYAMA	159	WHRQAPGKQRELVG	110	FIYWGGTTS	193	YVDSVKGRFTISRDNAKNTMYLQMNSLKPEDAAVYYCNI	126	YGSYALP	204	WGQGTLVTVSS
61	A0231PMP052F03	146	EVQLVESGGGLVQAGGSLRLSCTGS	85	RSIFSTYAMA	159	WHRQAPGKQRELVG	109	FIYWGGTTT	194	YSDSVKGRFTISRDNAKSTMYLQMNSLKPEDAGVYYCNI	126	YGSYALP	204	WGQGTLVTVSS
62	A0231PMP033B02	134	EVQLVESGGGLVQPGGSLRLSCAAS	87	GTIFSISTMG	160	WYRQAPGKQREVVA	111	VTSGFSTN	195	YSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNA	127	YLSLAWRDPDRDY	203	WGQGTQVTVSS
63	A0231PMP003D11	148	EVQLVESGGGLVQAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	112	EISDHTT	196	YGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	128	HHQRGWGTSITVT	203	WGQGTQVTVSS
64	A0231PMP017E06	148	EVQLVESGGGLVQAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	112	EISDHTT	196	YGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	129	HHQRGWGTSITVA	203	WGQGTQVTVSS
65	A0231PMP017G05	148	EVQLVESGGGLVQAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	112	EISDHTT	197	YGDSMKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	128	HHQRGWGTSITVT	203	WGQGTQVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
66	A0231PMP010C09	149	EVQLVESGGRLVQAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	113	EISGHTT	196	YGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	130	HHQRGWGTPITVT	203	WGQGTQVTVSS
67	A0231PMP017C01	149	EVQLVESGGRLVQAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	112	EISDHTT	196	YGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	130	HHQRGWGTPITVT	203	WGQGTQVTVSS
68	A0231PMP017B08	150	EVQLVESGGGLVRAGESLRLSCAAS	77	GSIFSIDAMG	153	WYRQAPGKQRELVA	112	EISDHTT	196	YGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNV	128	HHQRGWGTSITVT	204	WGQGTLVTVSS
69	A0231PMP052A08	151	EVQLVESGGGLVQAGGSLRLSCVAS	88	GSISSITAMG	161	WHRQAPGAQREGVA	114	VISRSGATM	198	LVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSA	131	ITQGRTY	204	WGQGTLVTVSS
70	A0231PMP052A01	151	EVQLVESGGGLVQAGGSLRLSCVAS	88	GSISSITAMG	161	WHRQAPGAQREGVA	115	IISRSGATM	198	LVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSA	131	ITQGRTY	203	WGQGTQVTVSS
71	A0231PMP052A05	151	EVQLVESGGGLVQAGGSLRLSCVAS	88	GSISSITAMG	161	WHRQAPGAQREGVA	116	AISRSGATI	199	LADSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSA	132	ITQEQTY	203	WGQGTQVTVSS
72	A0231PMP051E01	152	EVQLVESGGGLVQAGGSLRLSCAAS	89	GSIFSFIVMG	162	WYRQAPGEQRALVA	117	TVTSGGDTF	200	YVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYF	133	TKVSPYKETT	204	WGQGTLVTVSS
268	A023100050	276	EVQLVESGGGVVQPGGSLRLSCTGS	85	RSIFSTYAMA	159	WHRQAPGKQRELVG	109	FIYWGGTTT	277	YADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCNI	126	YGSYALP	204	WGQGTLVTVSS
269	A023100061	278	EVQLVESGGGVVQPGGSLRLSCAAS	76	GSIFSIDSMG	153	WYRQAPGKQRELVA	99	AITSSTN	279	YADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNL	120	EGQAGWGTALINY	204	WGQGTLVTVSS
270	A023100063	278	EVQLVESGGGVVQPGGSLRLSCAAS	79	GSVFSINDMG	157	WFRQAPGKQRELVA	104	DIISRGVTN	280	YADSVKGRFTISGDPSKNTVYLQMNSLRPEDTALYYCNA	124	HISTGWGRPHNNY	204	WGQGTLVTVSS
271	A023100078	278	EVQLVESGGGVVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	281	YADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNA	118	EGQAGWGTALMDY	204	WGQGTLVTVSS

ID	Nanobody	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
272	A023100090	278	EVQLVESGGGVVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	281	YADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNA	123	EGQAGWGTALLDY	204	WGQGTLVTVSS
273	A023100091	278	EVQLVESGGGVVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	281	YADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNA	282	EGQAGWGTALKDY	204	WGQGTLVTVSS
274	A023100092	278	EVQLVESGGGVVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	281	YADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNA	283	EGQAGWGTALRDY	204	WGQGTLVTVSS
275	A023100093	278	EVQLVESGGGVVQPGGSLRLSCAAS	73	ETIFSIDSMA	153	WYRQAPGKQRELVA	90	AITGGGSPN	281	YADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNA	284	EGQAGWGTALQDY	204	WGQGTLVTVSS

Table A-11: Amino acid sequences of selected multivalent anti-GITR Nanobodies

Name	ID	Amino acid sequence
A023100001	206	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100003	207	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
PA023100013	208	EVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100014	209	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS

Name	ID	Amino acid sequence
A023100015	210	EVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCVASGSISSITAMGWHRQAPGAQREGVAVISRSGATMLVDSVKGRFTIVQDNAKNTVYLQMNSLKVEDTAVYGCSAITQGRTYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100021	211	EVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGESLRLSCAASGSIFSIDAMGWYRQAPGKQRELVAEISDHTTYGDSVKGRFTISRGNAENTVALQMNSLKPEDTGVYYCNVHHQRGWGTSITVTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100022	212	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS

Name	ID	Amino acid sequence
A023100025	213	EVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNIYGSYALPWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNIYGSYALPWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCTGSRSIFSTYAMAWHRQAPGKQRELVGFIYWGGTTTYSDSVKGRFTISRDNAKNTMYLQMNSLKPEDAGVYYCNIYGSYALPWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100029	214	EVQLVESGGDLVQPGGSLRLSCAASGSVFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGDLVQPGGSLRLSCAASGSVFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGDLVQPGGSLRLSCAASGSVFSINDMGWFRQAPGKQRELVADIISRGVTNYADSVKGRFTISGDPAKNTVYLQMNSLKPEDTAVYYCNAHISTGWGRPHNNYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100030	215	EVQLVESGGGLVQPGGSLRLSCAASGTIFSISTMGWYRQAPGKQREVVAVTSGFSTNYSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNAYLSLAWRDPDRDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGTIFSISTMGWYRQAPGKQREVVAVTSGFSTNYSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNAYLSLAWRDPDRDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGTIFSISTMGWYRQAPGKQREVVAVTSGFSTNYSSAVKGRFTLSRDPAKNTVFLQMNSLQPEDTATYYCNAYLSLAWRDPDRDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100032	216	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS

Name	ID	Amino acid sequence
A023100034	217	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100035	218	EVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100045	219	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100082	220	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100083	221	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS

Name	ID	Amino acid sequence
A023100084	222	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100085	223	EVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNLEGQAGWGTALINYWGKGTLVTVSSAAAEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100012	224	EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100020	225	EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS

Name	**ID**	**Amino acid sequence**
A023100031	226	EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100036	227	EVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCAASGSIFSFIVMGWYRQAPGEQRALVATVTSGGDTFYVDSVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYFCYFTKVSPYKETTWGQGTLVTVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
A023100101	285	DVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
A023100105	286	DVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA

Name	ID	Amino acid sequence
A023100107	287	DVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
A023100118	288	DVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
A023100127	289	DVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA

Name	ID	Amino acid sequence
A023100129	290	DVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFSIDSMGWYRQAPGKQRELVAAITSSTNYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTALYYCNLEGQAGWGTALINYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA

Table A-12: Various amino acid sequences

Name	ID	Amino acid sequence
IRR00077	228	EVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYYADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYASYYECGHGLSTGGYGYDSWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYYADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYASYYECGHGLSTGGYGYDSWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYYADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYASYYECGHGLSTGGYGYDSWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
5A03-human CL (kappa) (light chain of construct A-0231-00_TP008)	229	MSVPTQVLGLLLLWLTDARCEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Name	ID	Amino acid sequence
5A03-human CH1-3 (IgG1) (heavy chain of construct A-0231-00_TP008)	230	MEWSWVFLFFLSVTTGVHSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
5A03-rat CL (lambda)	266	MSVPTQVLGLLLLWLTDARCEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSQPKSTPQLTVFPPSTEELQGNKATLVCLISDFYPSDVEVAWKANGAPISQGVDTANPTKQGNKYIASSFLRLTAEQWRSRNSFTCQVTHEGNTVEKSLSPAECV
5A03-rat CH1-3 (IgG2b)	267	MEWSWVFLFFLSVTTGVHSEVQLVESGGGLVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVAKRTVYLQMNSLKPEDTAVYYCNAEGQAGWGTALMDYWGKGTLVTVSSAQTTAPSVYPLAPGCGDTTSSTVTLGCLVKGYFPEPVTVTWNSGALSSDVHTFPAVLQSGLYTLTSSVTSSTWPSQTVTCNVAHPASSTKVDKKVERRNGGIGHKCPTCPTCHKCPVPELLGGPSVFIFPPKPKDILLISQNAKVTCVVVDVSEEEPDVQFSWFVNNVEVHTAQTQPREEQYNSTFRVVSALPIQHQDWMSGKEFKCKVNNKALPSPIEKTISKPKGLVRKPQVYVMGPPTEQLTEQTVSLTCLTSGFLPNDIGVEWTSNGHIEKNYKNTEPVMDSDGSFFMYSKLNVERSRWDSRAPFVCSVVHEGLHNHHVEKSISRPPGK
A023100090-human CL (kappa)	291	MSVPTQVLGLLLLWLTDARCEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
A023100090-human CH1-3 (IgG1)	292	MEWSWVFLFFLSVTTGVHSEVQLVESGGGVVQPGGSLRLSCAASETIFSIDSMAWYRQAPGKQRELVAAITGGGSPNYADSVKGRFTISSDVSKRTVYLQMNSLRPEDTALYYCNAEGQAGWGTALLDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Table A-13: GITR sequences from various species ("ID" refers to the SEQ ID NO as used herein)

Prot ID	Species	ID	Sequence
Q9Y5U5	Homo sapiens	231	MAQHGAMGAFRALCGLALLCALSLGQRPTGGPGCGPGRLLLGTGTDARCCRVHTTRCCRDYPGEECCSEWDCMCVQPEFHCGDPCCTTCRHHPCPPGQGVQSQGKFSFGFQCIDCASGTFSGGHEGHCKPWTDCTQFGFLTVFPGNKTHNAVCVPGSPPAEPLGWLTVVLLAVAACVLLLTSAQLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWV
O35714	Mus musculus	232	MGAWAMLYGVSMLCVLDLGQPSVVEEPGCGPGKVQNGSGNNTRCCSLYAPGKEDCPKERCICVTPEYHCGDPQCKICKHYPCQPGQRVESQGDIVFGFRCVACAMGTFSAGRDGHCRLWTNCSQFGFLTMFPGNKTHNAVCIPEPLPTEQYGHLTVIFLVMAACIFFLTTVQLGLHIWQLRRQHMCPRETQPFAEVQLSAEDACSFQFPEEERGEQTEEKCHLGGRWP
XP_005545180	Macaca fascicularis	233	MCACGTLCCLALLCAASLGQRPTGGPGCGPGRLLLGTGKDARCCRVHPTRCCRDYQSEECCSEWDCVCVQPEFHCGNPCCTTCQHHPCPSGQGVQPQGKFSFGFRCVDCALGTFSRGHDGHCKPWTDCTQFGFLTVFPGNKTHNAVCVPGSPPAEPPGWLTIVLLAVAACVLLLTSAQLGLHIWQLGSQPTGPRETQLLLEVPPSTEDASSCQFPEEERGERLAEEKGRLGDLWV

Table A-14: Serum albumin binding ISVD sequences ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
Alb8	234	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
Alb23	235	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
Alb129	236	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTATYYCTIGGSLSRSSQGTLVTVSSA
Alb132	237	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTATYYCTIGGSLSRSSQGTLVTVSSA
Alb11	238	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS
Alb11 (S112K)-A	239	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVKVSSA
Alb82	240	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSS
Alb82-A	241	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
Alb82-AA	242	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSAA
Alb82-AAA	243	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSAAA
Alb82-G	244	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSG
Alb82-GG	245	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSGG
Alb82-GGG	246	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSGGG
Alb92	264	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSS
Alb223	265	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA

Table A-15: Linker sequences ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
3A linker	247	AAA
5GS linker	248	GGGGS
7GS linker	249	SGGSGGS
8GS linker	250	GGGGCGGGS
9GS linker	251	GGGGSGGGS
10GS linker	252	GGGGSGGGGS
15GS linker	253	GGGGSGGGGSGGGGS
18GS linker	254	GGGGSGGGGSGGGGGGGS

20GS linker	255	GGGGSGGGGSGGGGSGGGGS
25GS linker	256	GGGGSGGGGSGGGGSGGGGSGGGGS
30GS linker	257	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
35GS linker	258	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
40GS linker	259	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
G1 hinge	260	EPKSCDKTHTCPPCP
9GS-G1 hinge	261	GGGGSGGGSEPKSCDKTHTCPPCP
Llama upper long hinge region	262	EPKTPKPQPAAA
G3 hinge	263	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCP

Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as an illustration of certain aspects and embodiments of the invention. Other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

CLAIMS

1. A polypeptide comprising at least one immunoglobulin single variable domain (ISVD) that specifically binds glucocorticoid-induced TNFR family-related receptor (GITR) with an EC_{50} value of less than 200 pM, and wherein the binding of said ISVD to said GITR enhances an immune response.

2. The polypeptide according to claim 1, wherein said polypeptide enhances proliferation or activation of T cells, B cells or natural killer cells.

3. The polypeptide according to any one of claims 1 to 2, wherein said polypeptide inhibits tumor cell growth.

4. The polypeptide according to any one of claims 1 to 3, wherein said GITR is human GITR.

5. The polypeptide according to any one of claims 1 to 4, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

   (i) CDR1 is chosen from the group consisting of:
      (a) SEQ ID NOs: 73-88; and
      (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 73-88; and/or
   (ii) CDR2 is chosen from the group consisting of:
      (c) SEQ ID NOs: 90-116; and
      (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 90-116; and/or
   (iii) CDR3 is chosen from the group consisting of:
      (e) SEQ ID NOs: 118-132 and 282-284; and
      (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequences of SEQ ID NOs: 118-132 and 282-284.

6. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NOs: 73-75; and
  - (b) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 73; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 90-98; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 90; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NOs: 118-119, 123 and 282-284; and
  - (f) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 118.

7. The polypeptide according to any one of claims 5 to 6, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NO: 73; and
  - (b) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 73, wherein
    - at position 2 the T has been changed into S;
    - at position 7 the D has been changed into N;
    - at position 8 the S has been changed into A; and/or
    - at position 10 the A has been changed into G;

and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NO: 90; and
  - (d) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 90, wherein
    - at position 1 the A has been changed into H, T, or G;
    - at position 2 the I has been changed into M;
    - at position 3 the T has been changed into S;

- at position 6 the G has been changed into S;
- at position 7 the S has been changed into R, or G; and/or
- at position 8 the P has been changed into S, T, or R;

and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NO: 118; and

(f) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 118, wherein

- at position 9 the A has been changed into P;
- at position 11 the M has been changed into L, K, R, or Q; and/or
- at position 12 the D has been changed into N.

8. The polypeptide according to any one of claim 5 to 7, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 118; or

ii) CDR1 is represented by SEQ ID NO: 73, CDR2 is represented by SEQ ID NO: 90, and CDR3 is represented by SEQ ID NO: 123.

9. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NOs: 76-78; and

(b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 76; and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NOs: 99-103; and

(d) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 99; and/or

(iii) CDR3 is chosen from the group consisting of:

- (e) SEQ ID NOs: 120-123; and
- (f) amino acid sequences that have 4, 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 120.

10. The polypeptide according to any one of claims 5 or 9, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NO: 76; and
  - (b) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 76, wherein
    - at position 7 the D has been changed into N; and/or
    - at position 8 the S has been changed into A;

and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NO: 99; and
  - (d) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 99, wherein
    - at position 1 the A has been changed into S, or T;
    - at position 5 the S has been changed into T, G, or R;
    - at position 6 the T has been changed into K; and/or
    - at position 7 the N has been changed into I;

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (e) SEQ ID NO: 120; and
  - (f) amino acid sequences that have 4, 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 120, wherein
    - at position 1 the E has been changed into K;
    - at position 4 the A has been changed into T;
    - at position 11 the I has been changed into M, or L; and/or
    - at position 12 the N has been changed into D.

11. The polypeptide according to any one of claims 5, 9 or 10, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 76, CDR2 is represented by SEQ ID NO: 99, and CDR3 is represented by SEQ ID NO: 120.

12. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NOs: 79-84; and
  (b) amino acid sequences that have 3, 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 79; and/or
(ii) CDR2 is chosen from the group consisting of:
  (c) SEQ ID NOs: 104-108; and
  (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 104; and/or
(iii) CDR3 is chosen from the group consisting of:
  (e) SEQ ID NOs: 124-125; and
  (f) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 124.

13. The polypeptide according to any one of claims 5 or 12, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:
  (a) SEQ ID NO: 79; and
  (b) amino acid sequences that have 3, 2, or 1 amino acid difference(s) with SEQ ID NO: 79, wherein
    - at position 2 the S has been changed into N;
    - at position 3 the V has been changed into I;
    - at position 7 the N has been changed into D;

- at position 8 the D has been changed into S; and/or
- at position 9 the M has been changed into V, or T;

and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NO: 104; and

(d) amino acid sequences that have 2, or 1 amino acid difference(s) with SEQ ID NO: 104, wherein

- at position 1 the D has been changed into G;
- at position 5 the R has been changed into A; and/or
- at position 6 the G has been changed into D;

and/or

(iii) CDR3 is chosen from the group consisting of:

(e) SEQ ID NO: 124; and

(f) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 124, wherein

- at position 4 the T has been changed into M.

14. The polypeptide according to any one of claims 5, 12 or 13, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 79, CDR2 is represented by SEQ ID NO: 104, and CDR3 is represented by SEQ ID NO: 124.

15. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

(i) CDR1 is chosen from the group consisting of:

(a) SEQ ID NOs: 85-86; and

(b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 85; and/or

(ii) CDR2 is chosen from the group consisting of:

(c) SEQ ID NOs: 109-110; and

   (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 109; and/or

  (iii) CDR3 is SEQ ID NO: 126.

16. The polypeptide according to any one of claims 5 or 15, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is chosen from the group consisting of:
  - (a) SEQ ID NO: 85; and
  - (b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 85, wherein
    - at position 2 the S has been changed into N;

and/or

- (ii) CDR2 is chosen from the group consisting of:
  - (c) SEQ ID NO: 109; and
  - (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 109, wherein
    - at position 9 the T has been changed into S;

and/or

- (iii) CDR3 is SEQ ID NO: 126.

17. The polypeptide according to any one of claims 5, 15 or 16, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 85, CDR2 is represented by SEQ ID NO: 109, and CDR3 is represented by SEQ ID NO: 126.

18. The polypeptide according to claim 5, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 87, CDR2 is represented by SEQ ID NO: 111, and CDR3 is represented by SEQ ID NO: 127.

19. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
    - (a) SEQ ID NOs: 112-113; and
    - (b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 112; and/or
- (iii) CDR3 is chosen from the group consisting of:
    - (c) SEQ ID NOs: 128-130; and
    - (d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 128.

20. The polypeptide according to any one of claims 5 or 19, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 77; and/or
- (ii) CDR2 is chosen from the group consisting of:
    - (a) SEQ ID NO: 112; and
    - (b) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 112, wherein
        - at position 4 the D has been changed into G;

and/or

- (iii) CDR3 is chosen from the group consisting of:
    - (c) SEQ ID NO: 128; and
    - (d) amino acid sequences that have 1 amino acid difference with SEQ ID NO: 128, wherein
        - at position 9 the S has been changed into P; and/or
        - at position 13 the T has been changed into A.

21. The polypeptide according to one of claims 5, 19 or 20, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 77, CDR2 is represented by SEQ ID NO: 112, and CDR3 is represented by SEQ ID NO: 128.

22. The polypeptide according to claim 5, wherein said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 88; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (a) SEQ ID NOs: 114-116; and
  - (b) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 114; and/or
- (iii) CDR3 is chosen from the group consisting of:
  - (c) SEQ ID NOs: 131-132; and
  - (d) amino acid sequences that have 2, or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 131.

23. The polypeptide according to one of claims 5 or 22, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- (i) CDR1 is SEQ ID NO: 88; and/or
- (ii) CDR2 is chosen from the group consisting of:
  - (a) SEQ ID NO: 114; and
  - (b) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 114, wherein
    - at position 1 the V has been changed into I, or A; and/or
    - at position 9 the M has been changed into I;

and/or

- (iii) CDR3 is chosen from the group consisting of:
  - (c) SEQ ID NO: 131; and
  - (d) amino acid sequences that have 2, or 1 amino acid(s) difference with SEQ ID NO: 131, wherein
    - at position 4 the G has been changed into E; and/or
    - at position 5 the R has been changed into Q.

24. The polypeptide according to one of claims 5, 22 or 23, in which said at least one immunoglobulin single variable domain essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which: CDR1 is represented by SEQ ID NO: 88, CDR2 is represented by SEQ ID NO: 114, and CDR3 is represented by SEQ ID NO: 131.

25. The polypeptide according to any of the preceding claims, wherein said at least one immunoglobulin single variable domain is chosen from the group of immunoglobulin single variable domains, wherein:

- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 91; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 92; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 94; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 95; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 75, CDR2 is SEQ ID NO: 93; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 96; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 97; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 74, CDR2 is SEQ ID NO: 98; and CDR3 is SEQ ID NO: 119;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 282;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 283;
- CDR1 is SEQ ID NO: 73, CDR2 is SEQ ID NO: 90; and CDR3 is SEQ ID NO: 284;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 120;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 100; and CDR3 is SEQ ID NO: 121;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 101; and CDR3 is SEQ ID NO: 122;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 102; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 103; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 118;
- CDR1 is SEQ ID NO: 78, CDR2 is SEQ ID NO: 99; and CDR3 is SEQ ID NO: 123;
- CDR1 is SEQ ID NO: 79, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 105; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 76, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;

- CDR1 is SEQ ID NO: 80, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 81, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 82, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 84, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 106; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 107; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 108; and CDR3 is SEQ ID NO: 124;
- CDR1 is SEQ ID NO: 83, CDR2 is SEQ ID NO: 104; and CDR3 is SEQ ID NO: 125;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 109; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 86, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 85, CDR2 is SEQ ID NO: 110; and CDR3 is SEQ ID NO: 126;
- CDR1 is SEQ ID NO: 87, CDR2 is SEQ ID NO: 111; and CDR3 is SEQ ID NO: 127;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 128;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 129;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 113; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 77, CDR2 is SEQ ID NO: 112; and CDR3 is SEQ ID NO: 130;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 114; and CDR3 is SEQ ID NO: 131;
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 115; and CDR3 is SEQ ID NO: 131; and
- CDR1 is SEQ ID NO: 88, CDR2 is SEQ ID NO: 116; and CDR3 is SEQ ID NO: 132.

26. The polypeptide according to any one of the preceding claims, wherein the EC_{50} in a NF-κB luciferase reporter assay is 190 pM or less, such as less than 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40, 35, 30, 25, 20, 18, 16, 15, 14 or even less, such as less than 12 pM.

27. The polypeptide according to any one of the preceding claims, wherein the EC_{50} in a T-cell activation assay is 190 pM or less, such as less than 180, 170, 160, 150, 140, 130, 120, 110, 100 or even less, such as less than 90, 80, 70, 60, 50, 40 or even less, such as less than 30 pM.

28. The polypeptide according to any one of the preceding claims, wherein said at least one immunoglobulin single variable domain essentially consists of a dAb, an immunoglobulin that is suitable

for use as a dAb, a Nanobody, a VHH sequence, a humanized VHH sequence, a camelized VH sequence, or a VHH sequence that has been obtained by affinity maturation.

29. The polypeptide according to any one of the preceding claims, wherein said at least one immunoglobulin single variable domain is chosen from the group consisting of ISVDs with SEQ ID NOs: 1-71 and 268-275 or ISVDs that have a sequence identity of more than 80% with SEQ ID NOs: 1-71 and 268-275.

30. The polypeptide according to any one of the preceding claims, comprising at least two immunoglobulin single variable domains that can bind GITR.

31. The polypeptide according to any one of the preceding claims, comprising at least three, at least four or at least five immunoglobulin single variable domains that can bind GITR.

32. The polypeptide according to any one of claims 30 to 31, wherein said at least two, said at least three, said at least four or said at least five ISVDs can be the same or different.

33. The polypeptide according to any one of claims 30 to 32, wherein said at least two, said at least three, said at least four or said at least five ISVDs are directly linked to each other or linked to each other via a linker.

34. The polypeptide according to claim 33, in which the linker is selected from the group of linkers with SEQ ID NOs: 247-263.

35. The polypeptide according to claim 34, in which the linker comprises the linker 9GS (SEQ ID NO: 251) or the linker 3A (SEQ ID NO: 247).

36. A compound or construct that comprises or essentially consists of a polypeptide according to any one of the preceding claims, and which further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers.

37. The compound or construct according to claim 36, which has an increased half-life compared to the corresponding polypeptide according to any one of claims 1 to 35, per se.

38. The compound or construct according to claim 37, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide according to any one of claims 1 to 35.

39. The compound or construct according to claim 38, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of polyethylene glycol, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, an antibody constant region, and small proteins or peptides that can bind to serum proteins.

40. The compound or construct according to any one of claims 38 or 39, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of an antibody constant region or fragments thereof, wherein the antibody constant region or fragments thereof are derived from a human IgG, such as IgG1, IgG2, IgG3 or IgG4.

41. The compound or construct according to claim 40, wherein said antibody constant region comprises a CH1 heavy chain domain, a CH2 heavy chain domain, a CH3 heavy chain domain and/or a CL light chain domain.

42. A compound or construct comprising

i) a polypeptide according to any one of claims 1 to 29, wherein said polypeptide is linked to a CH1 heavy chain domain, which is followed by a CH2 heavy chain domain and a CH3 heavy chain domain respectively; and/or

ii) a polypeptide according to any one of claims 1 to 29, wherein said polypeptide is linked to a CL light chain domain.

43. The compound or construct according to claim 42, which comprises an amino acid sequence set forth in one of SEQ ID NO: 229, 230, 291 and SEQ ID NO:292 or an amino acid sequence that has a sequence identity of more than 80% with SEQ ID NOs: 229-230 and 291-292.

44. The compound or construct according to one of claims 42 or 43, which is of the IgG type.

45. The compound or construct according to any one of claims 38 or 39, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of human serum albumin or fragments thereof.

46. The compound or construct according to any one of claims 38 or 39, in which said one or more other binding units that provide the polypeptide with increased half-life are chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

47. The compound or construct according to claim 46 in which said one or more other binding units that provide the polypeptide with increased half-life are chosen from the group consisting of domain antibodies, amino acids that are suitable for use as a domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"'s, amino acids that are suitable for use as a dAb, Nanobodies, VHH sequences, humanized VHH sequences, or camelized VH sequences that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

48. The compound or construct according to claim 47, wherein said compound or construct is chosen from the group consisting of compounds or constructs with SEQ ID NOs: 206-223 and 285-290 or compounds or constructs that have a sequence identity of more than 80% with SEQ ID NOs: 206-223 and 285-290.

49. The compound or construct according to any one of claims 46 to 48, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding polypeptide according to any of claims 1 to 44, per se.

50. The compound or construct according to any one of claims 46 to 49, that has a serum half-life that is increased with more than 1 hour, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding polypeptide according to any one of claims 1 to 35, per se.

51. The compound or construct according to any one of claims 46 to 50, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

52. A nucleic acid encoding a polypeptide according to any one of claims 1 to 35, or a compound or construct according to any one of claims 36 to 51.

53. An expression vector comprising a nucleic acid according to claim 52.

54. A host or host cell comprising a nucleic acid according to claim 52, or an expression vector according to claim 53.

55. A composition comprising a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a nucleic acid according to claim 52.

56. The composition according to claim 55, which is a pharmaceutical composition.

57. The composition according to claim 56, which further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

58. The composition according to any one of claims 55 to 57, the polypeptide according to any one of claims 1 to 35, the compound or construct according to any one of claims 36 to 51, for use as a medicament.

59. The composition, the polypeptide, or the compound or construct according to claim 58, for use in enhancing an immune response.

60. The composition, the polypeptide, or the compound or construct according to claim 58, for use in enhancing proliferation or activation of T cells, B cells or natural killer cells.

61. The composition, the polypeptide, or the compound or construct according to claim 58, for use in inhibiting tumor growth.

62. The composition, the polypeptide, or the compound or construct according to claim 58, for use in prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

63. The composition, the polypeptide, or the compound or construct according to claim 58, for use in prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

64. The composition according to any one of claims 55 to 63, the polypeptide according to any one of claims 1 to 35, or the compound or construct according to any one of claims 36 to 51, for use in the treatment or prevention of a GITR associated disease, disorder or condition.

65. The composition, the polypeptide, or the compound or construct according to claim 58, for use in prevention and/or treatment of cancer.

66. The composition, the polypeptide, or the compound or construct according to claim 65, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

67. A method for producing a polypeptide according to any one of claims 1 to 35, 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 sequence according to claim 52; optionally followed by:

b) isolating and/or purifying the polypeptide according to any one of claims 1 to 35.

68. A method for enhancing an immune response, wherein said method comprises administering a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

69. A method for enhancing proliferation or activation of T cells, B cells or natural killer cells, wherein said method comprises administering a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

70. A method for inhibiting tumor growth, wherein said method comprises administering a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

71. A method for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

72. A method for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or

construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

73. A method for prevention and/or treatment of a GITR associated disease, disorder or condition, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

74. A method for prevention and/or treatment of cancer, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57.

75. The method according to claim 74, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

76. The method according to any one of claims 68 to 75, wherein the method further comprises one or more of the following:

a) administering chemotherapy;
b) administering radiation therapy;
c) administering cancer vaccines; and/or
d) administering one or more additional therapeutic agents.

77. The method according to claim 76, wherein the additional therapeutic agent is chosen from the list of PD-1, PD-L1, PD-L2, CTLA-4, 4-1 BB (CD137), 4-1BB ligand, OX40, OX40 ligand, CD27, TNFRSF25,

TL1A, CD40, CD40 ligand, LIGHT, LTA, HVEM, BTLA, CD160, CEACAM-1, CEACAM-5, LAIR1, 2B4, TGFR, LAG-3, TIM-3, Siglecs, ICOS (CD278), ICOS ligand, B7-H3, B7-H4, B7-1, B7-2, VISTA, HHLA2, TMIGD2, BTNL2, CD244, CD48, CD2, CDS, TIGIT, PVR family members, KIRs, ILTs, LIRs, NKG2D, NKG2A, MICA, MICB, CSF1R, IDO, TGFβ, Adenosine, ICAM-1, ICAM-2, ICAM-3, LFA-1 (CD11a/CD18), LFA-2, LFA-3, BAFFR, NKG2C, SLAMF7, NKp80, CD83 ligand, CD24, CD39, CD30, CD70, CD73, CD7, CXCR4, CXCL12, Phosphatidylserine, SIRPA, CD47, VEGF and Neuropilin.

78. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for enhancing an immune response.

79. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for enhancing proliferation or activation of T cells, B cells or natural killer cells.

80. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for inhibiting tumor growth.

81. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for prevention and/or treatment of T cell, B cell or natural killer cell mediated diseases.

82. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for prevention and/or treatment of bacterial, fungal, viral or parasitic infectious diseases.

83. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the

manufacture of a pharmaceutical composition for prevention and/or treatment of at least one GITR associated disease, disorder or condition.

84. Use of a polypeptide according to any one of claims 1 to 35, a compound or construct according to any one of claims 36 to 51, or a composition according to any one of claims 55 to 57, for the manufacture of a pharmaceutical composition for prevention and/or treatment of cancer.

85. Use of a polypeptide according to claim 84, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, melanoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, glioblastoma, glioma, prostate cancer, testicular cancer, gastrointestinal cancer, pancreatic cancer, biliary tract cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, small bowel or appendix cancer, uterine or endometrial cancer, multiple myeloma, salivary gland carcinoma, adrenal gland cancer, osteosarcoma, chondrosarcoma, nasopharyngeal carcinoma, basal cell carcinoma, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, head and neck cancer, leukemia, lymphomas, merkel cell cancer and other hematologic malignancies.

Figure 1

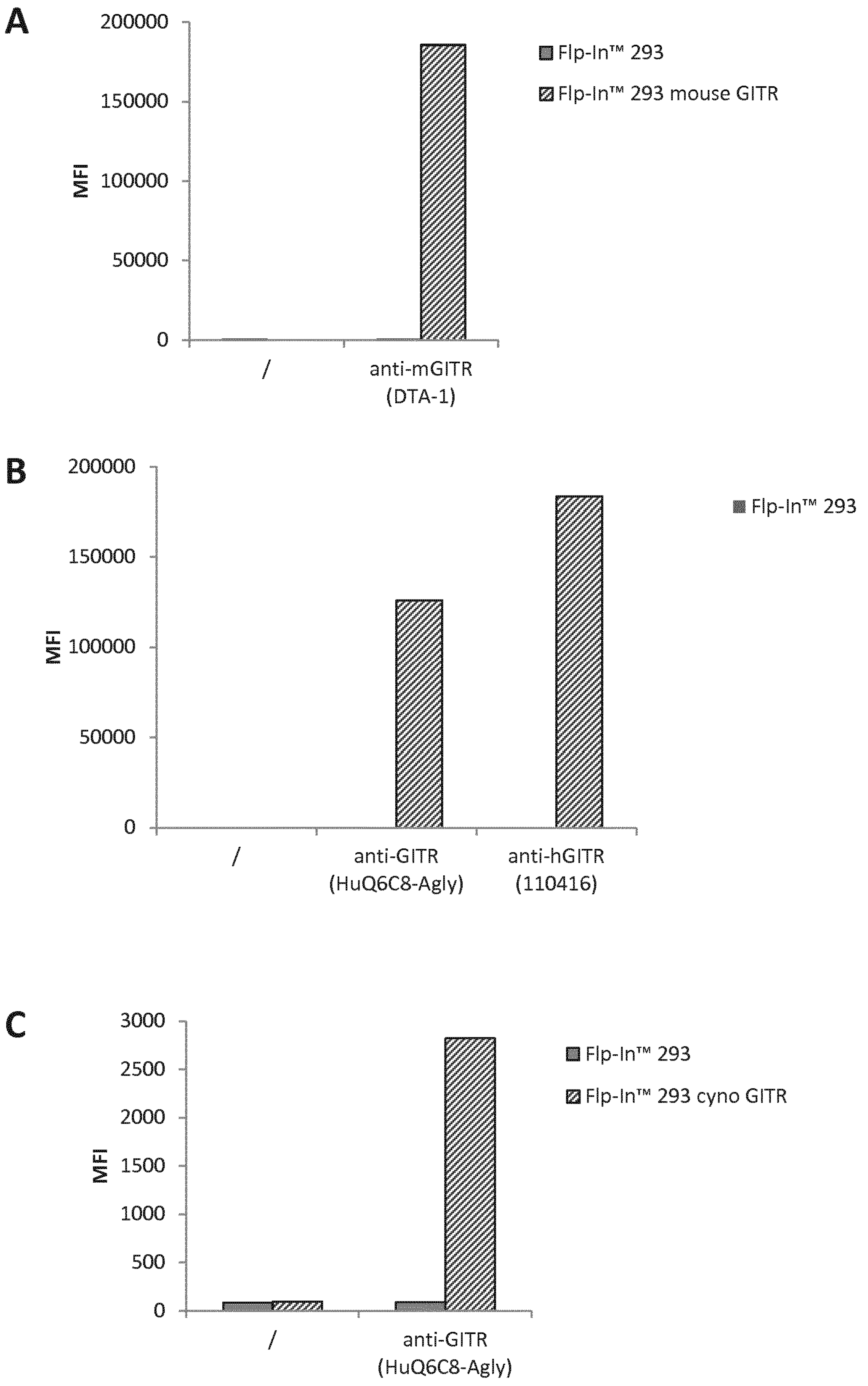

A
200000
150000
100000
50000
0
MFI
/
anti-mGITR (DTA-1)
▨ Flp-In™ 293
▨ Flp-In™ 293 mouse GITR
B
200000
150000
100000
50000
0
MFI
/
anti-GITR (HuQ6C8-Agly)
anti-hGITR (110416)
■ Flp-In™ 293
C
3000
2500
2000
1500
1000
500
0
MFI
/
anti-GITR (HuQ6C8-Agly)
■ Flp-In™ 293
▨ Flp-In™ 293 cyno GITR

Figure 1 Continued

D

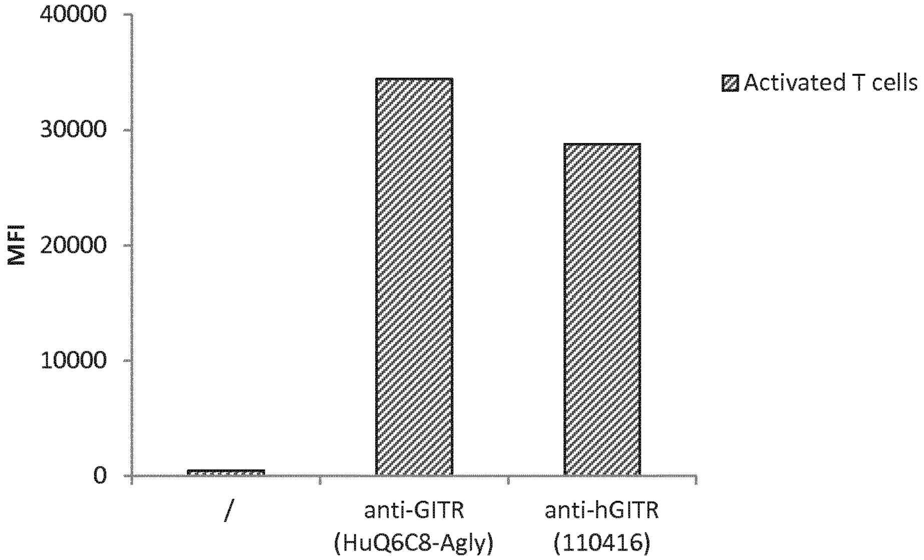
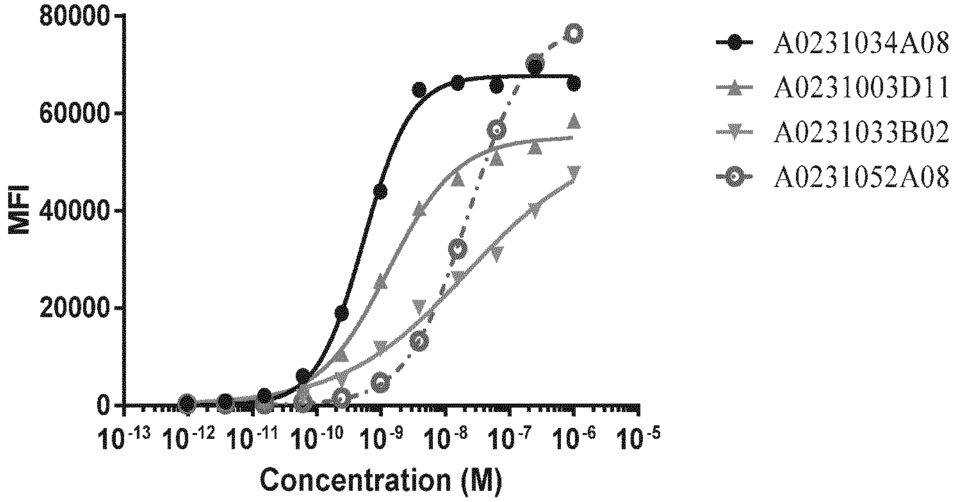


Figure 2

A



B

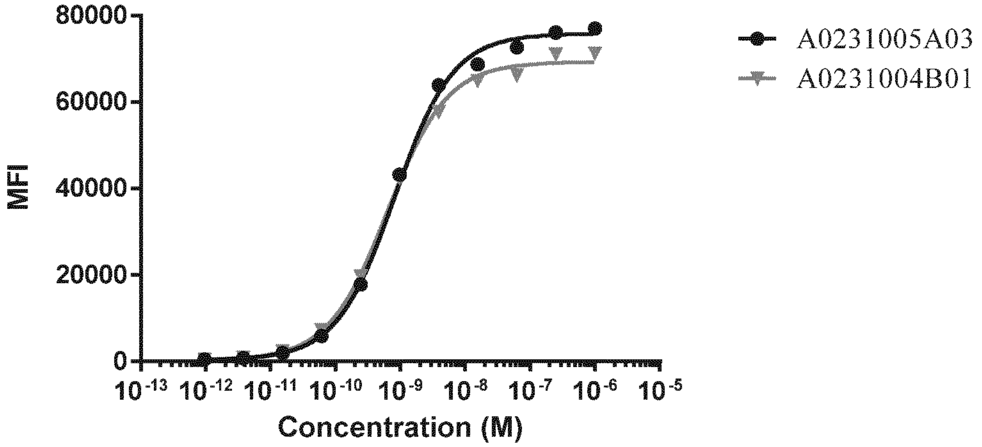


Figure 3

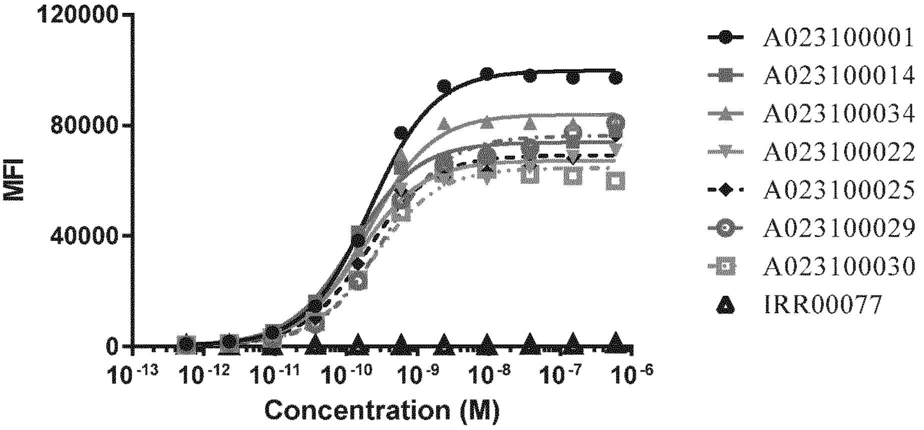
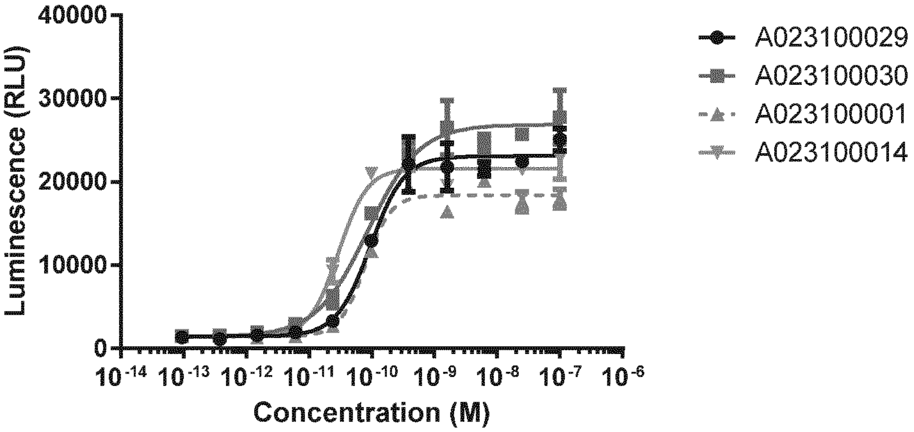


Figure 4

A



B

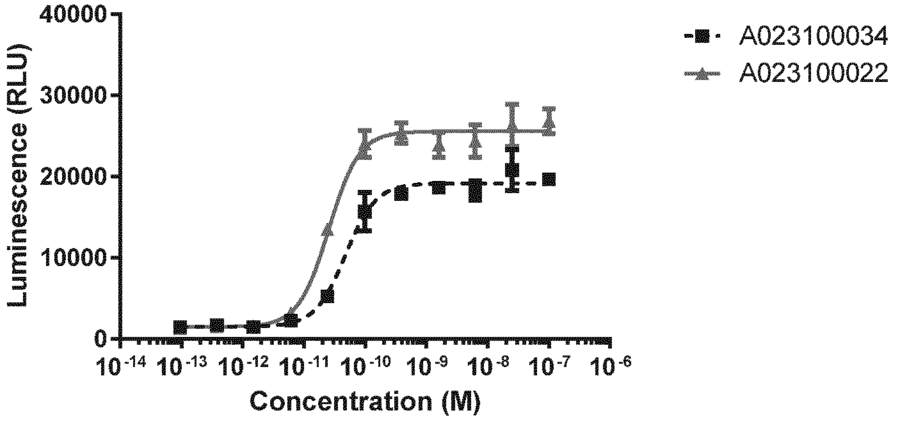


Figure 4 Continued

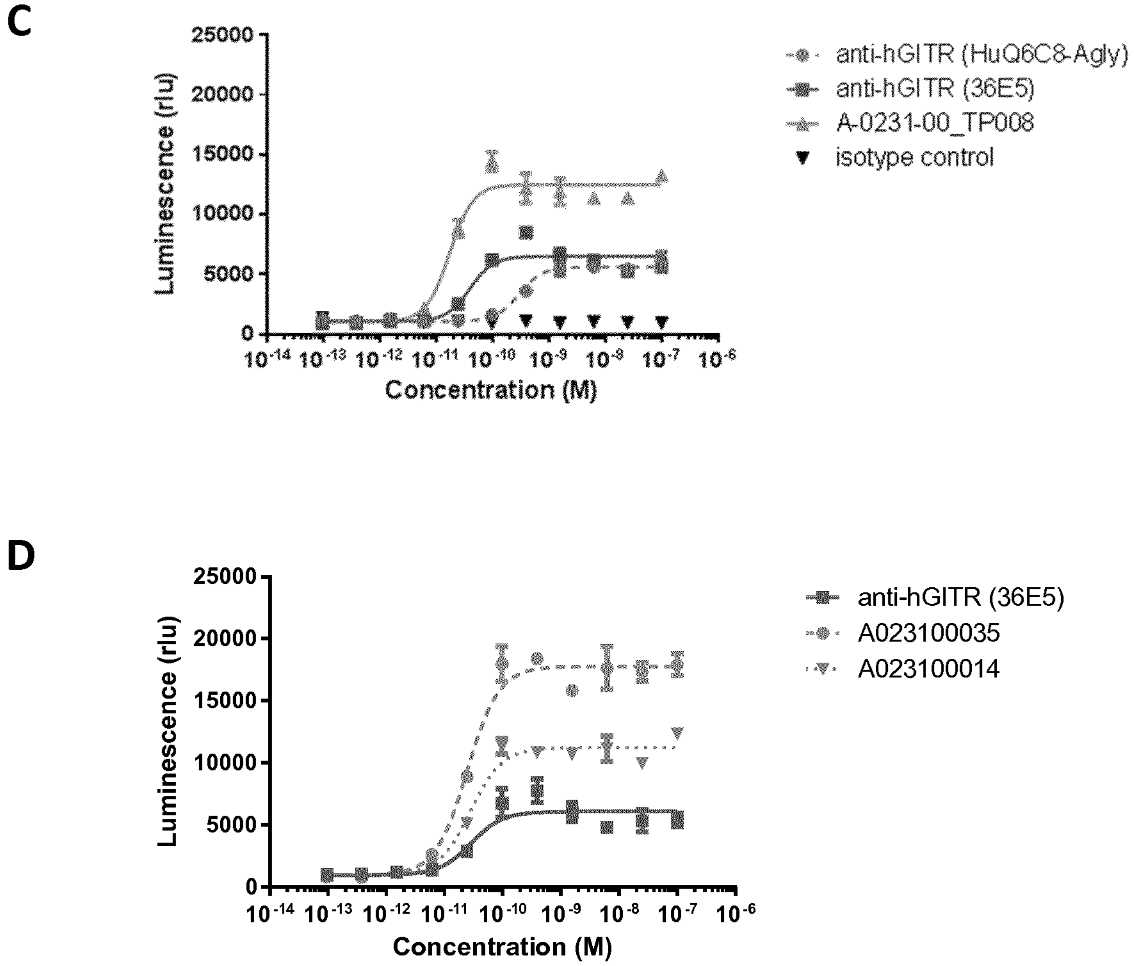


Figure 5

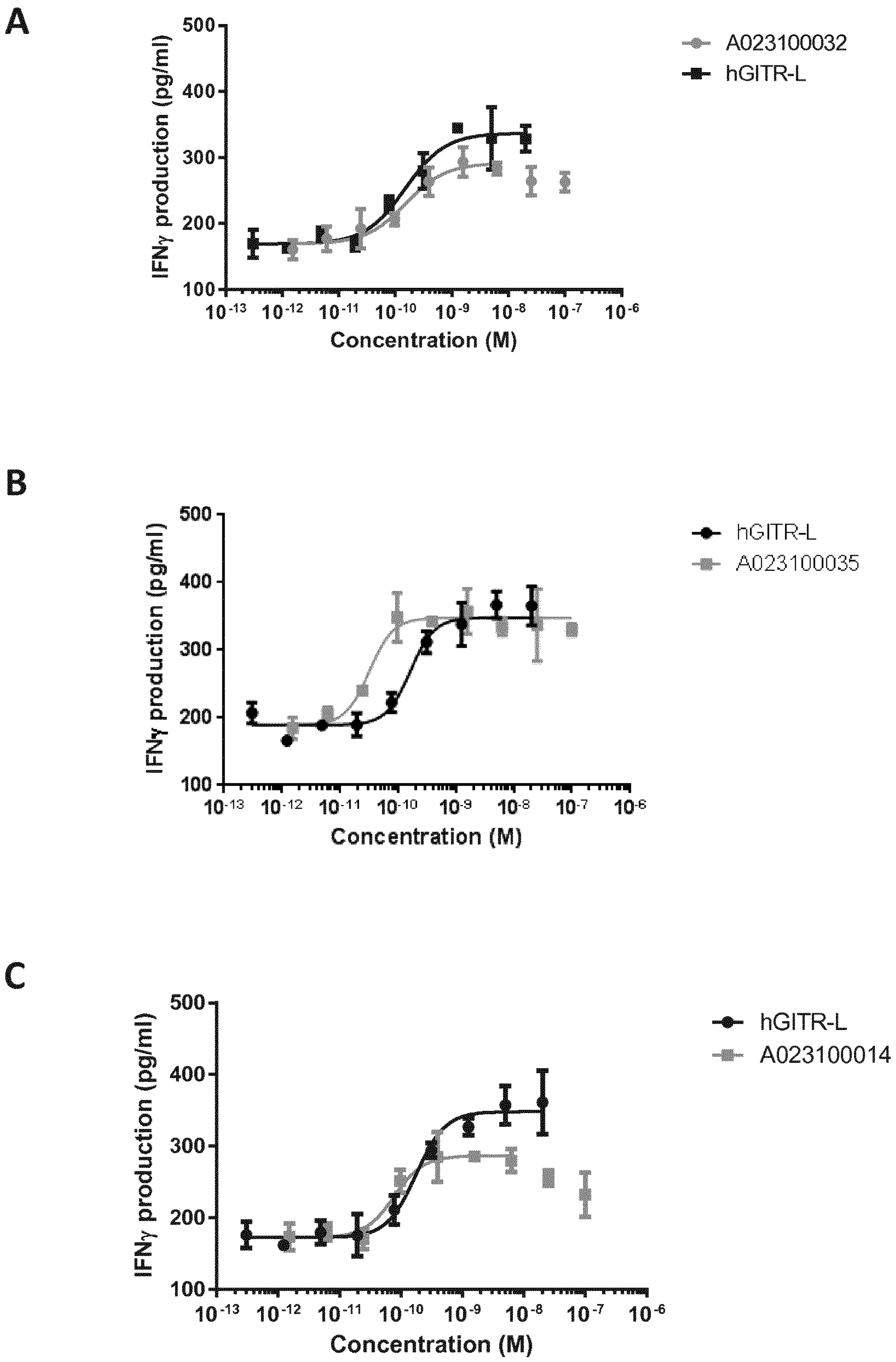

A
IFNγ production (pg/ml)
500
400
300
200
100
10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6
Concentration (M)
A023100032
hGITR-L
B
IFNγ production (pg/ml)
500
400
300
200
100
10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6
Concentration (M)
hGITR-L
A023100035
C
IFNγ production (pg/ml)
500
400
300
200
100
10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6
Concentration (M)
hGITR-L
A023100014

Figure 5 Continued

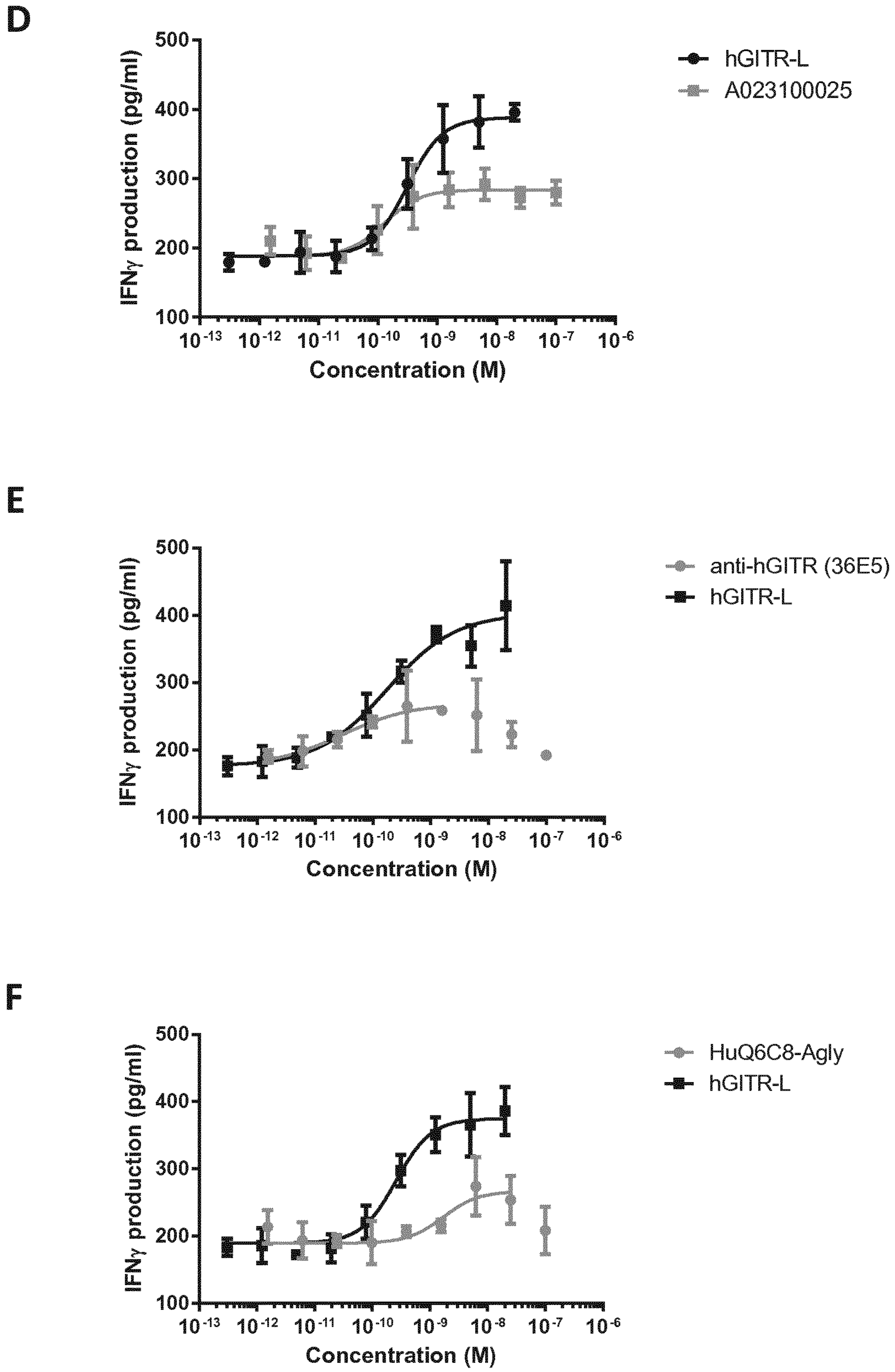


Figure 6

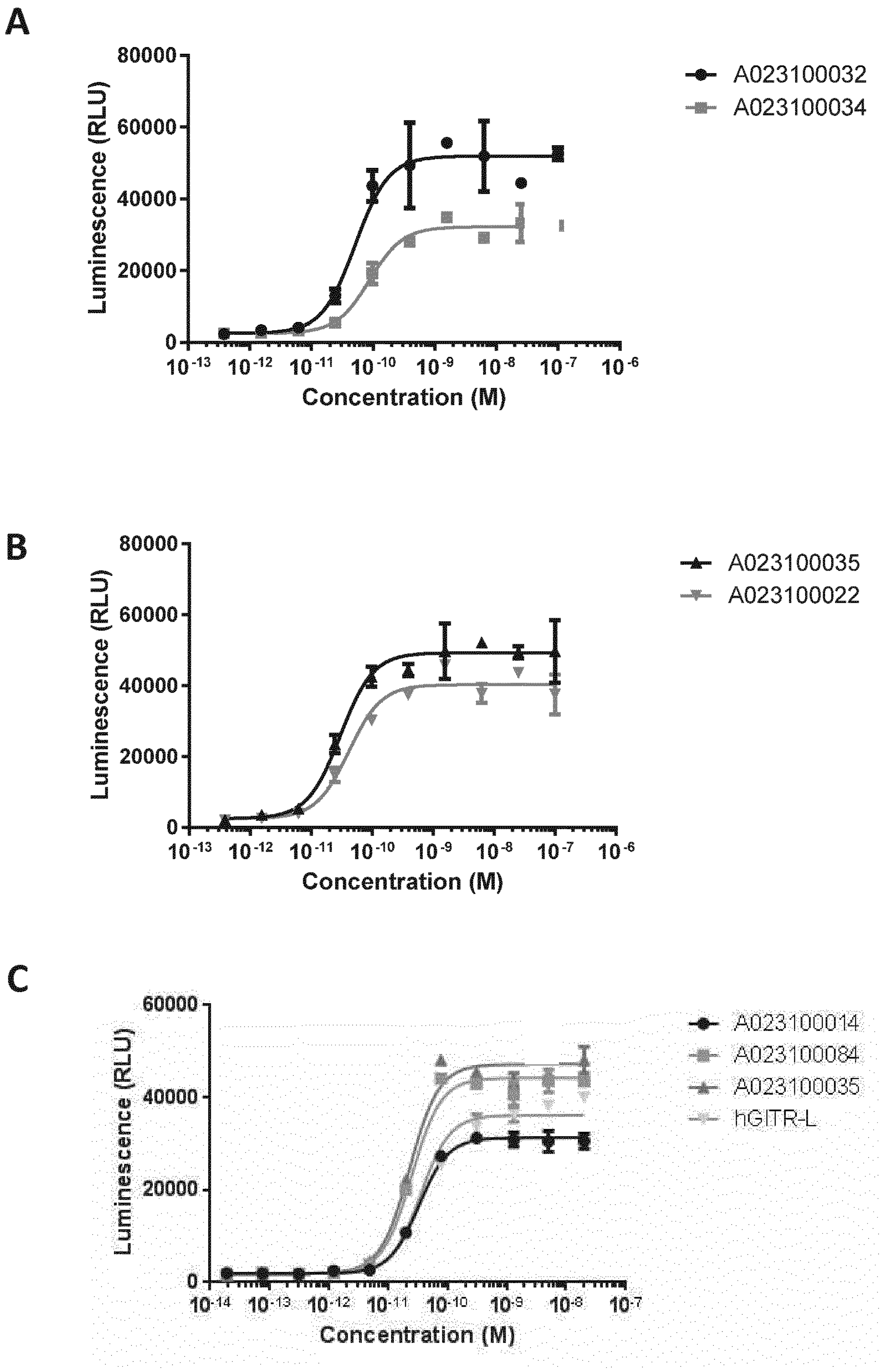

A
80000
60000
40000
20000
0
Luminescence (RLU)
10⁻¹³ 10⁻¹² 10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶
Concentration (M)
A023100032
A023100034
B
80000
60000
40000
20000
0
Luminescence (RLU)
10⁻¹³ 10⁻¹² 10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶
Concentration (M)
A023100035
A023100022
C
60000
40000
20000
0
Luminescence (RLU)
10⁻¹⁴ 10⁻¹³ 10⁻¹² 10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷
Concentration (M)
A023100014
A023100084
A023100035
hGITR-L

Figure 6 Continued

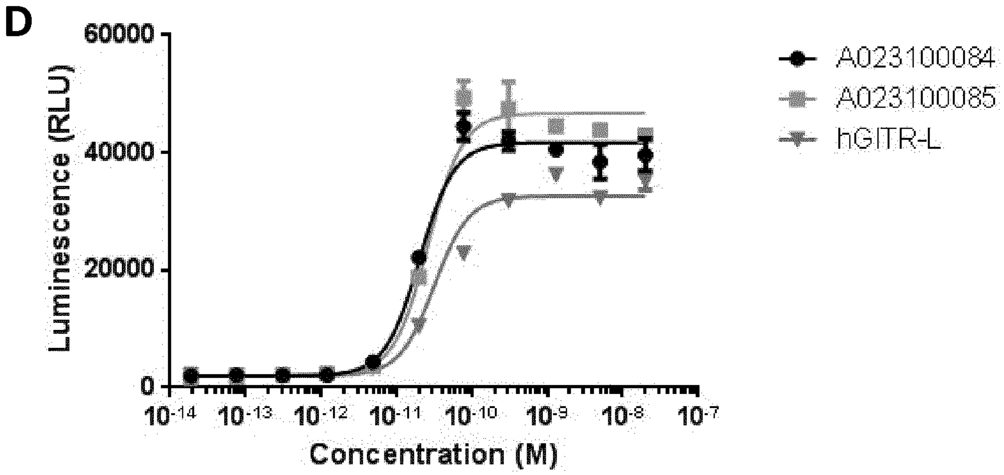


Figure 7

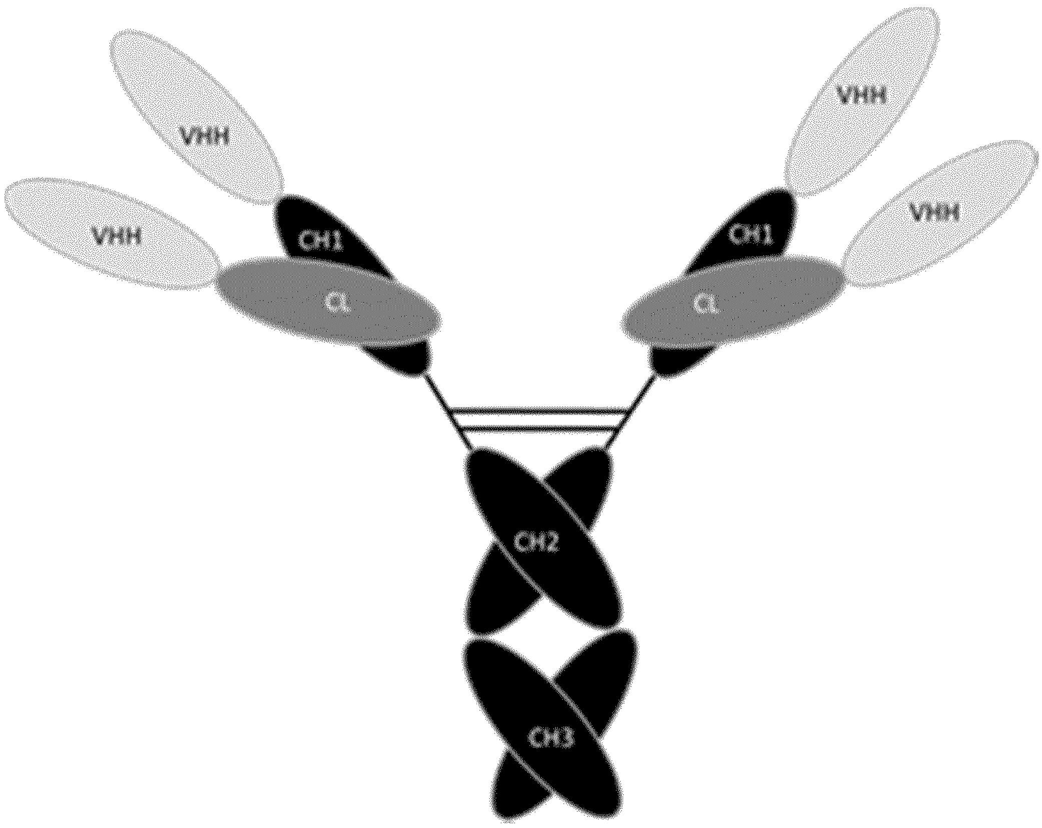


Figure 8

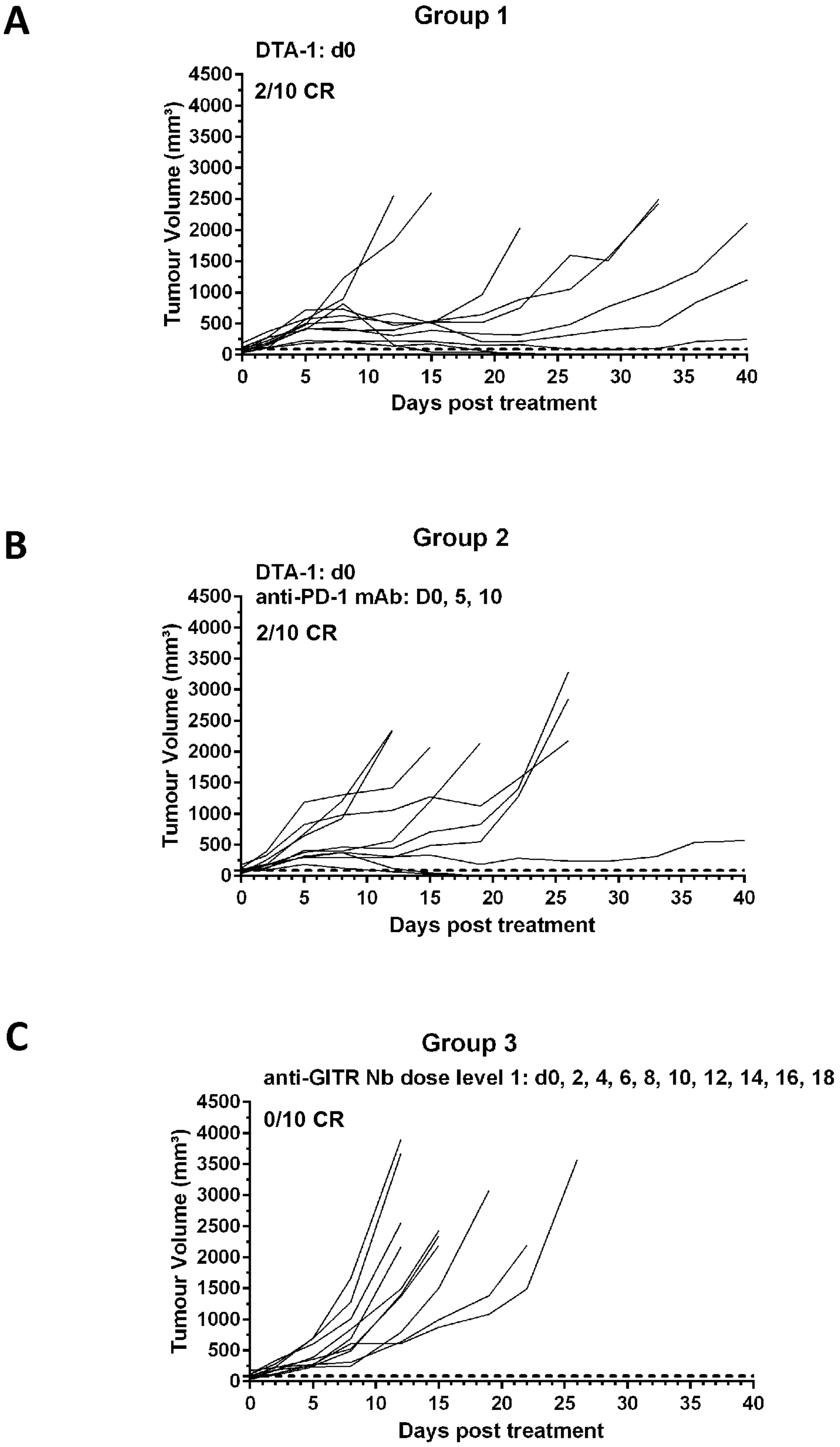
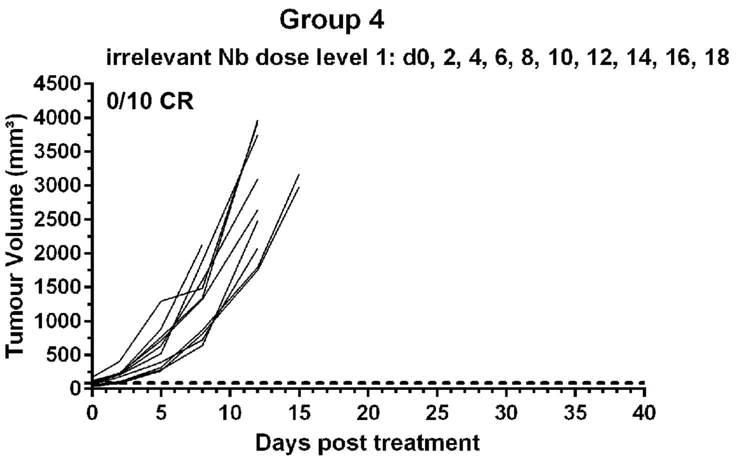
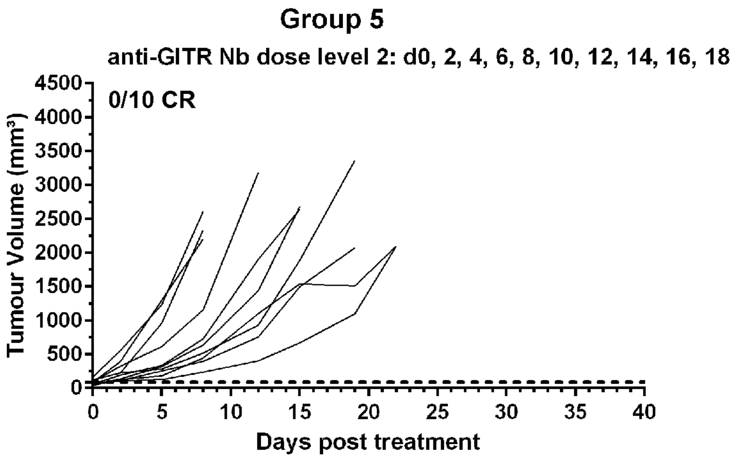

A
Group 1
DTA-1: d0
2/10 CR
Tumour Volume (mm³)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Days post treatment
B
Group 2
DTA-1: d0
anti-PD-1 mAb: D0, 5, 10
2/10 CR
Tumour Volume (mm³)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Days post treatment
C
Group 3
anti-GITR Nb dose level 1: d0, 2, 4, 6, 8, 10, 12, 14, 16, 18
0/10 CR
Tumour Volume (mm³)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Days post treatment

Figure 8 Continued

D



E



F

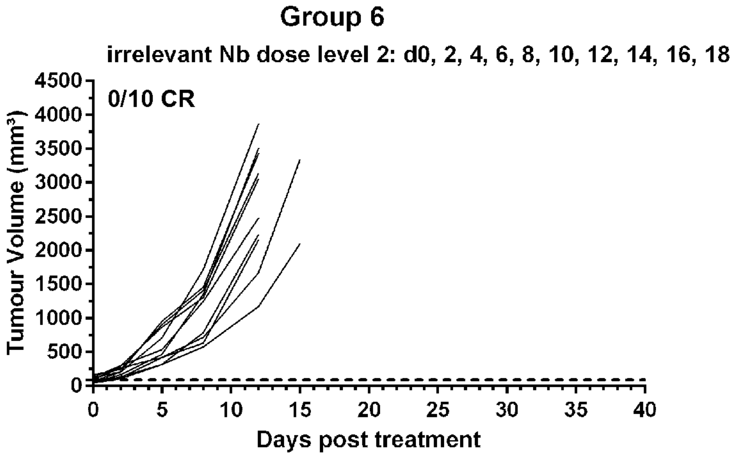
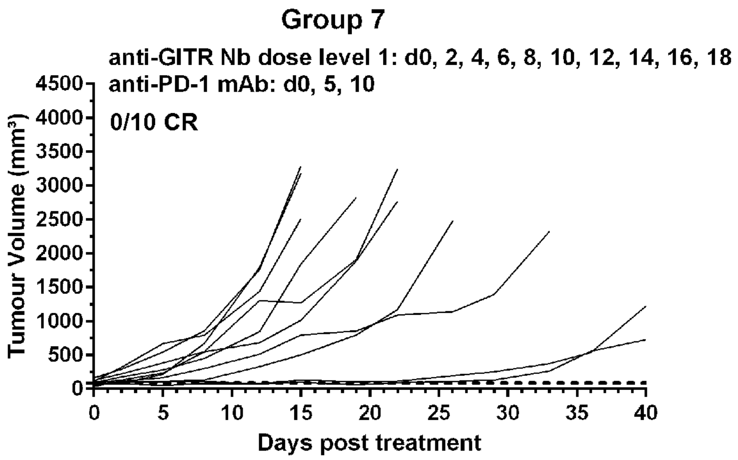
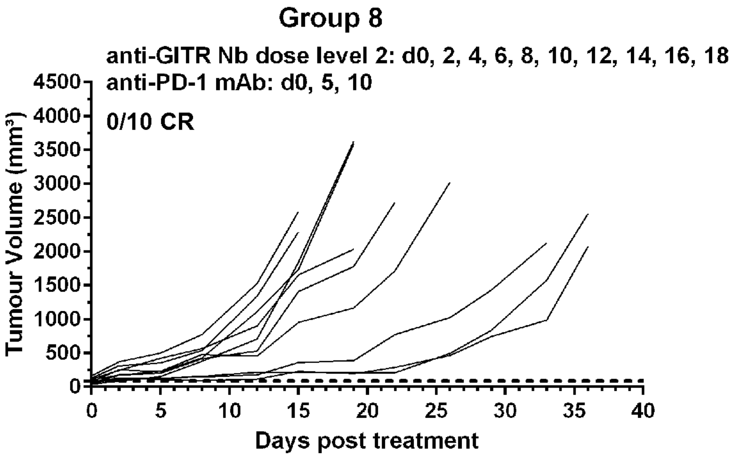


Figure 8 Continued

G



H



I

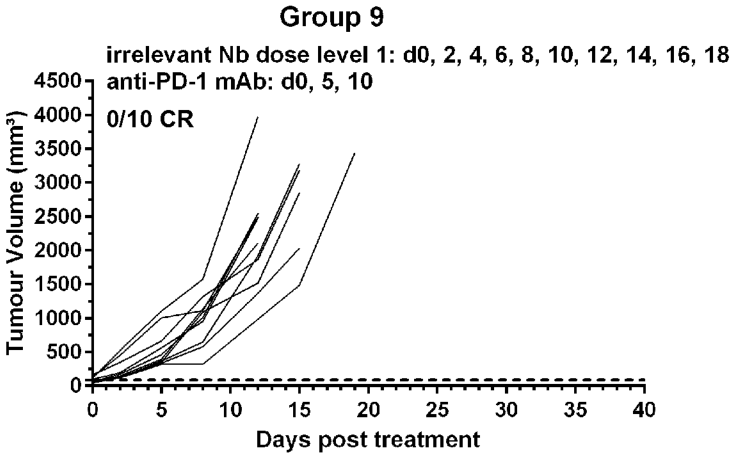
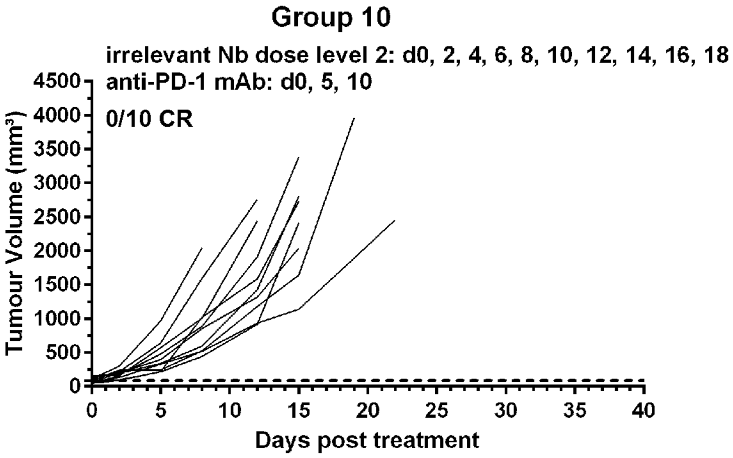
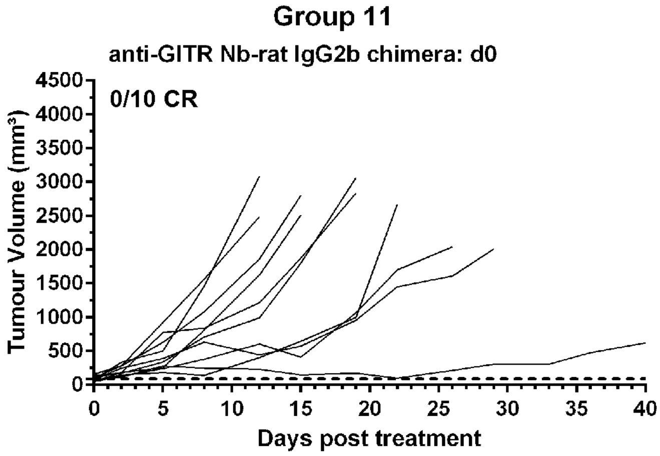


Figure 8 Continued

J



K



L

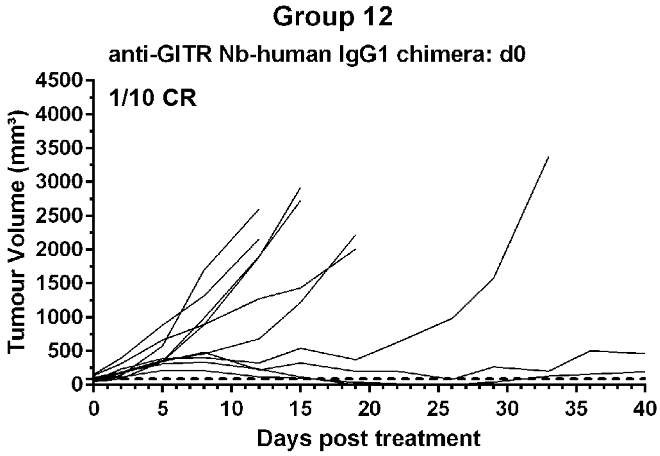
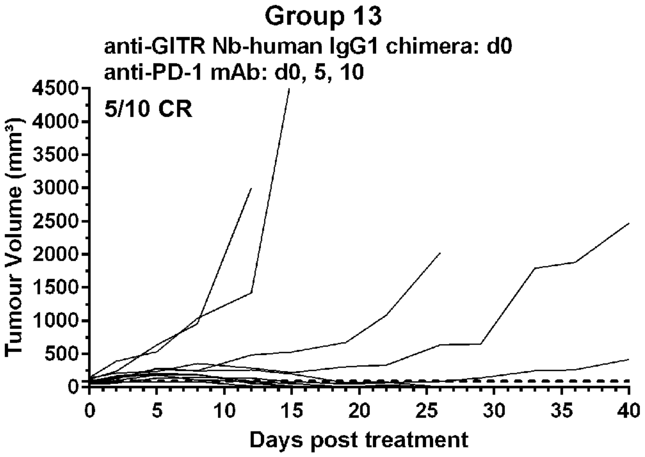


Figure 8 Continued

M



N

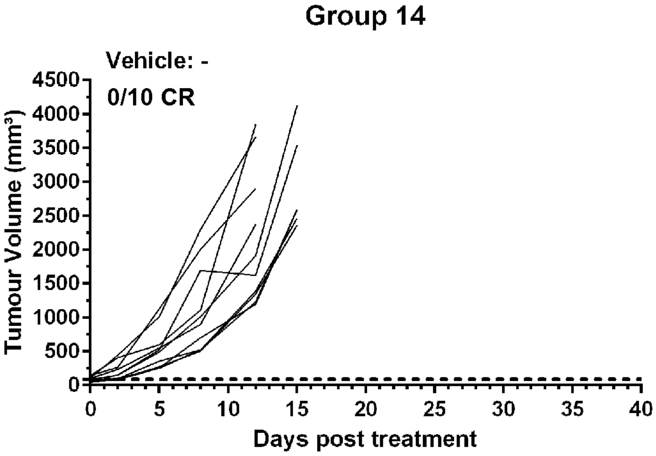
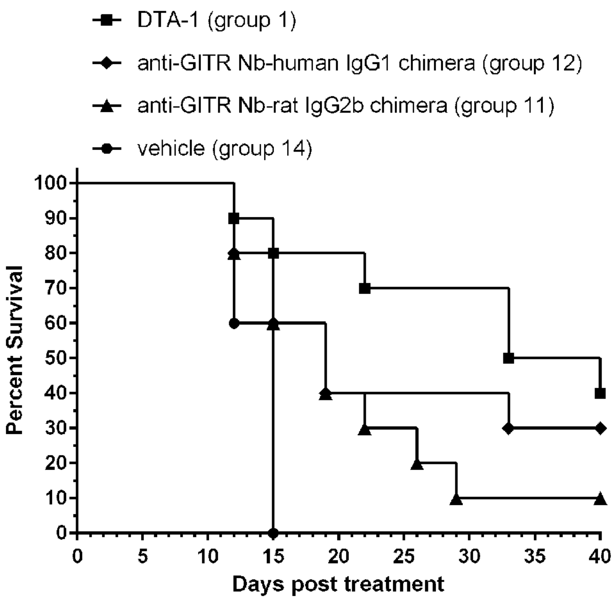


Figure 9

A


DTA-1 (group 1)
anti-GITR Nb-human IgG1 chimera (group 12)
anti-GITR Nb-rat IgG2b chimera (group 11)
vehicle (group 14)
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Days post treatment

B

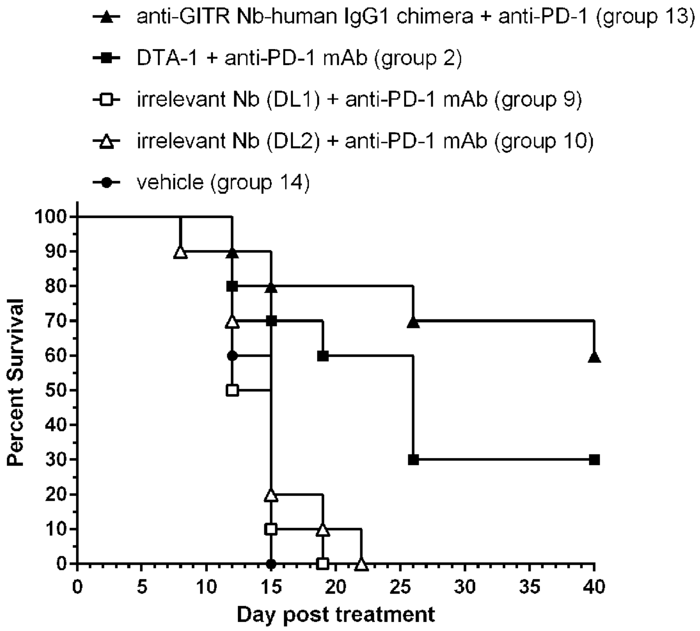
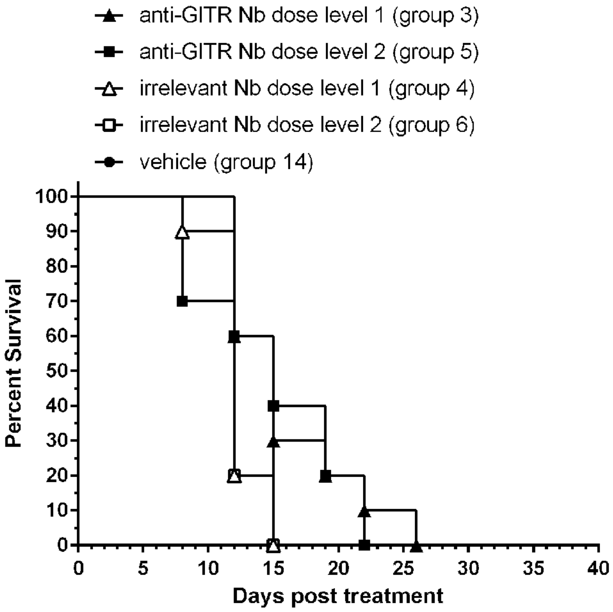

anti-GITR Nb-human IgG1 chimera + anti-PD-1 (group 13)
DTA-1 + anti-PD-1 mAb (group 2)
irrelevant Nb (DL1) + anti-PD-1 mAb (group 9)
irrelevant Nb (DL2) + anti-PD-1 mAb (group 10)
vehicle (group 14)
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Day post treatment

Figure 9 Continued

C



D

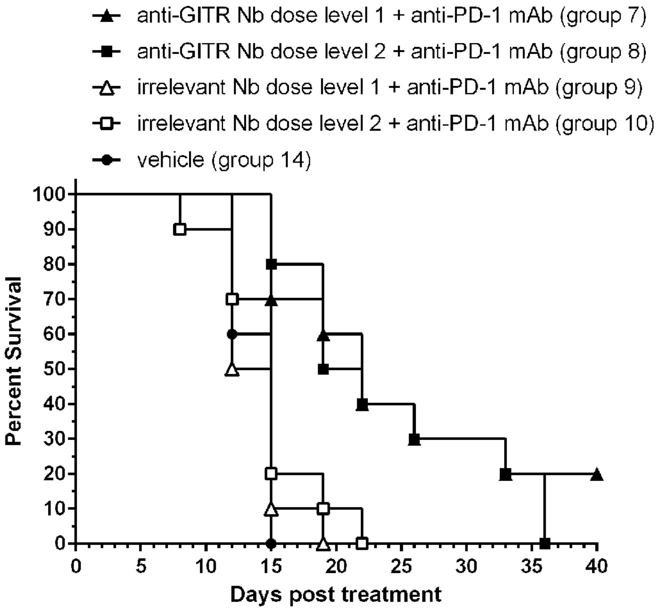


Figure 10

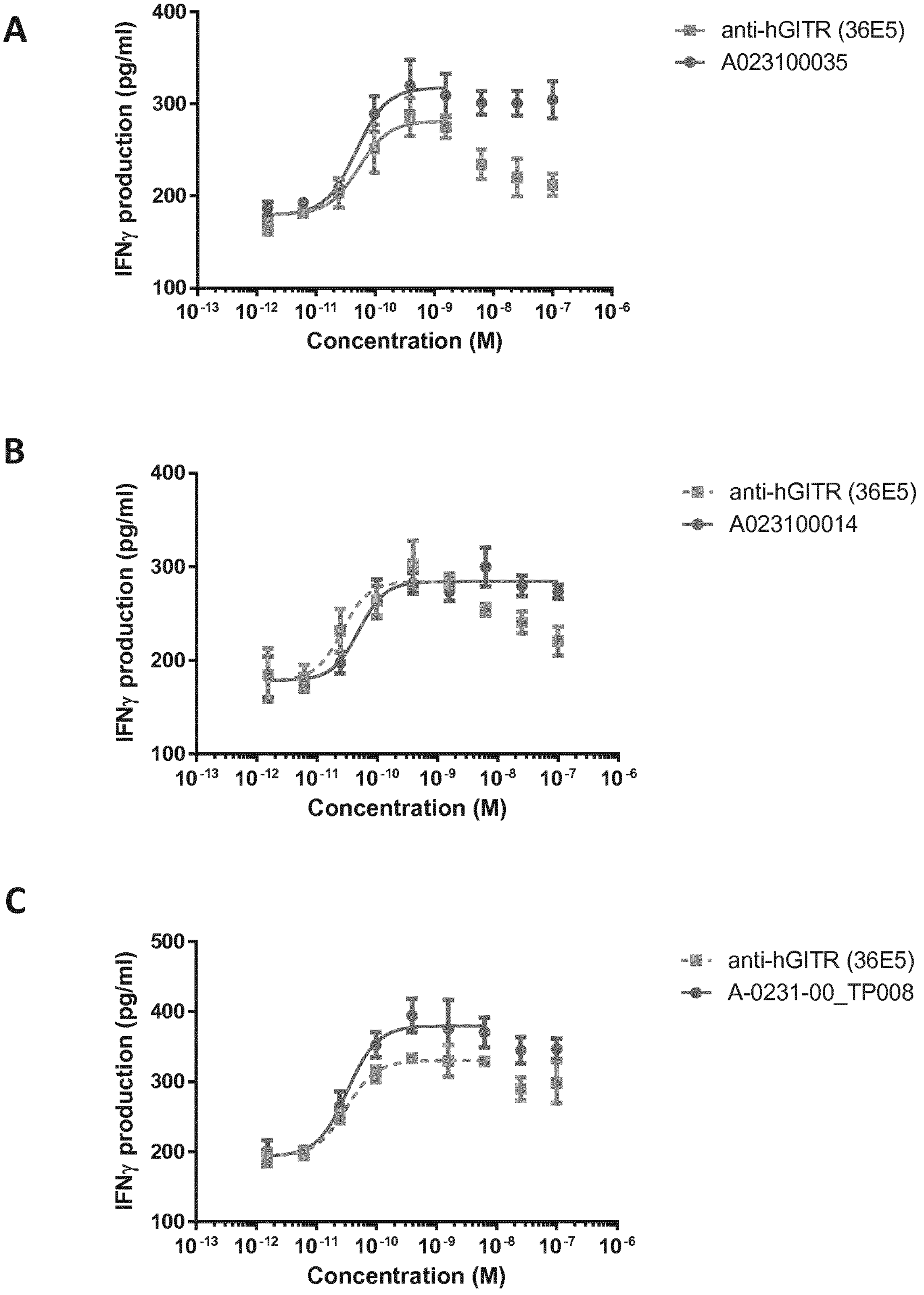
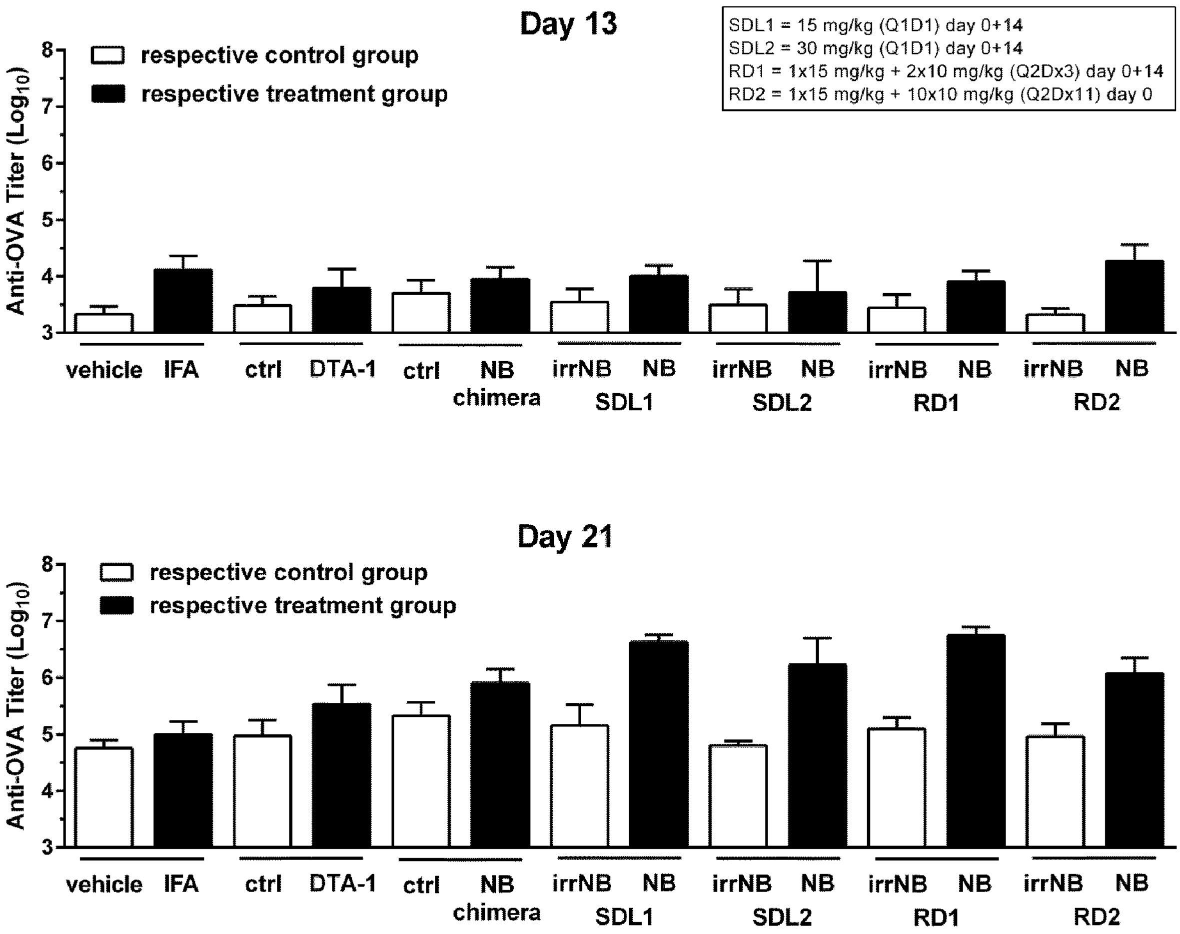

A
IFNγ production (pg/ml)
400
300
200
100
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}
Concentration (M)
anti-hGITR (36E5)
A023100035
B
IFNγ production (pg/ml)
400
300
200
100
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}
Concentration (M)
anti-hGITR (36E5)
A023100014
C
IFNγ production (pg/ml)
500
400
300
200
100
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}
Concentration (M)
anti-hGITR (36E5)
A-0231-00_TP008

Figure 11



SDL1 = 15 mg/kg (Q1D1) day 0+14
SDL2 = 30 mg/kg (Q1D1) day 0+14
RD1 = 1x15 mg/kg + 2x10 mg/kg (Q2Dx3) day 0+14
RD2 = 1x15 mg/kg + 10x10 mg/kg (Q2Dx11) day 0

Figure 12

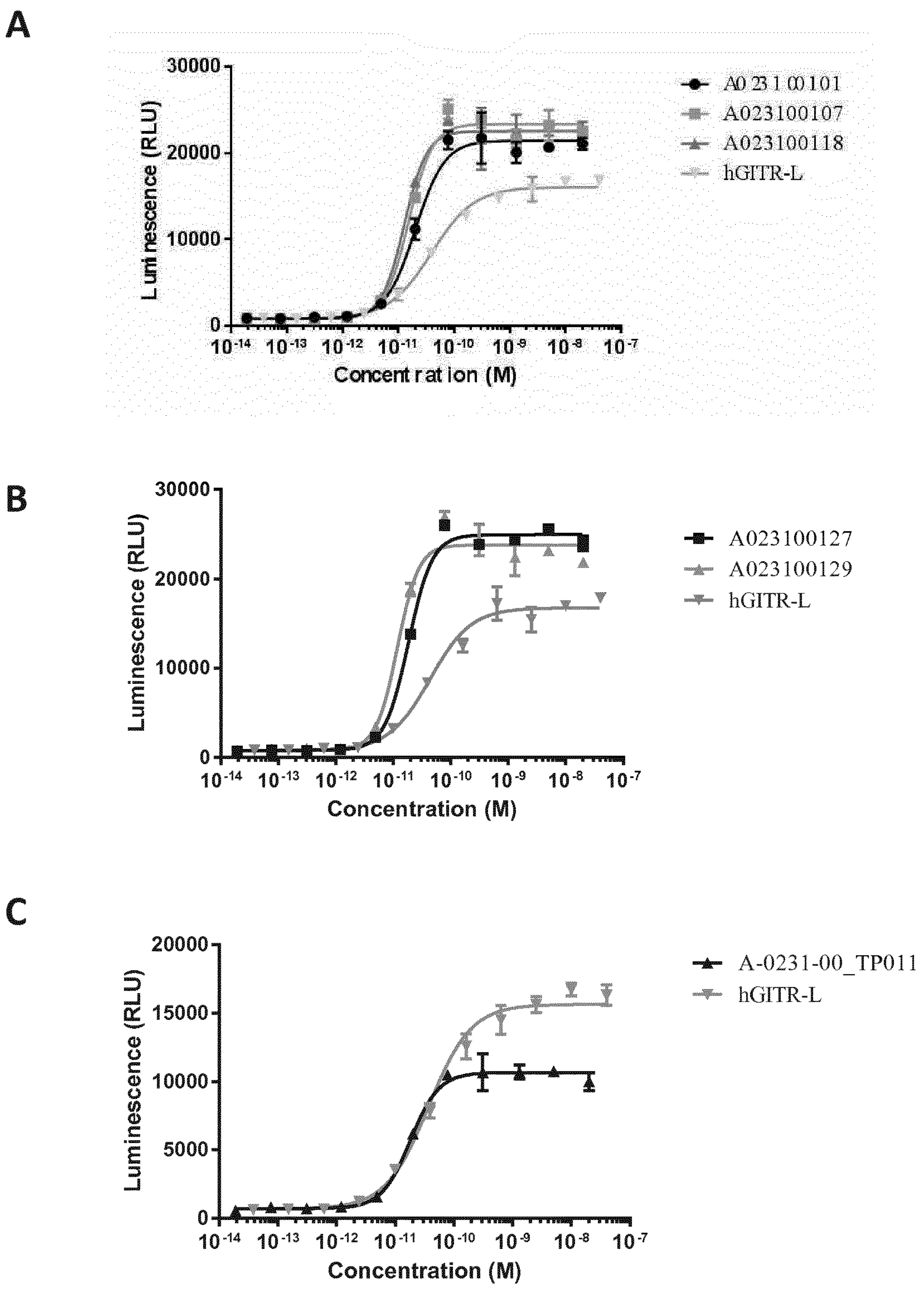

A
30000
20000
10000
0
Luminescence (RLU)
10^-14 10^-13 10^-12 10^-11 10^-10 10^-9 10^-8 10^-7
Concentration (M)
A023100101
A023100107
A023100118
hGITR-L
B
30000
20000
10000
0
Luminescence (RLU)
10^-14 10^-13 10^-12 10^-11 10^-10 10^-9 10^-8 10^-7
Concentration (M)
A023100127
A023100129
hGITR-L
C
20000
15000
10000
5000
0
Luminescence (RLU)
10^-14 10^-13 10^-12 10^-11 10^-10 10^-9 10^-8 10^-7
Concentration (M)
A-0231-00_TP011
hGITR-L

Figure 12 Continued

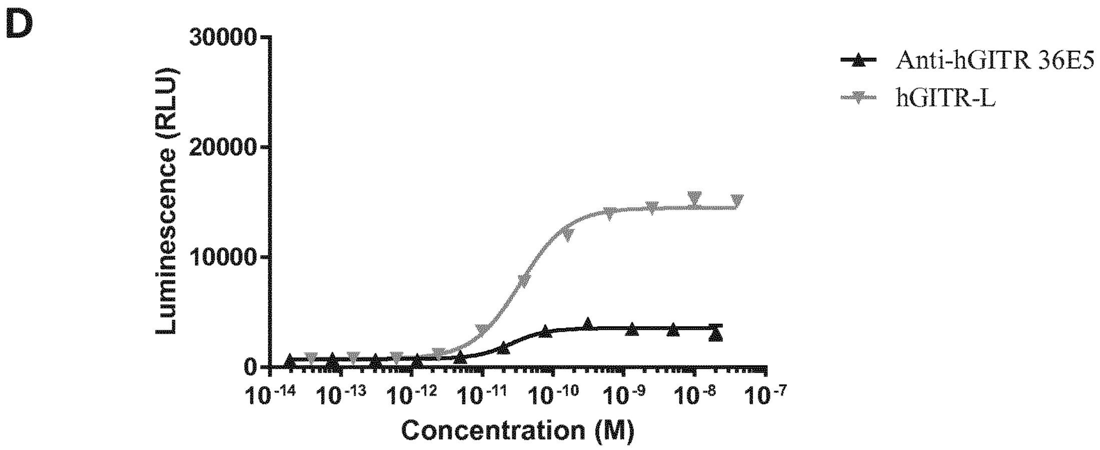
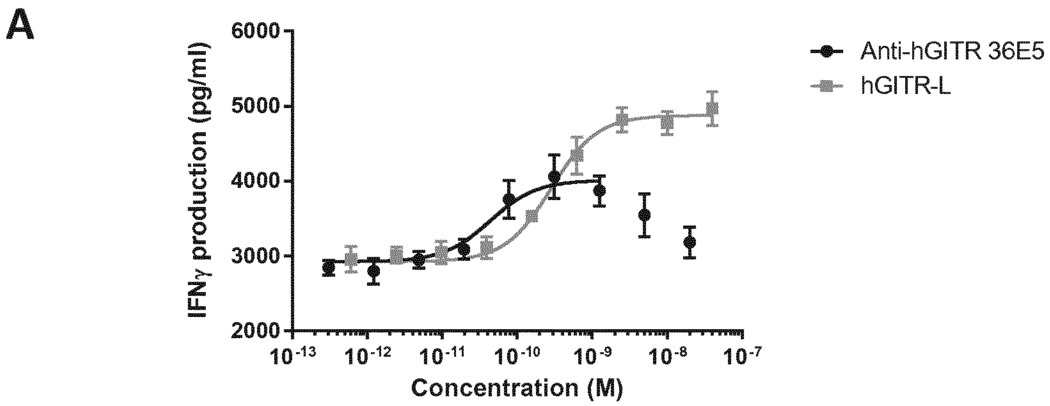


Figure 13



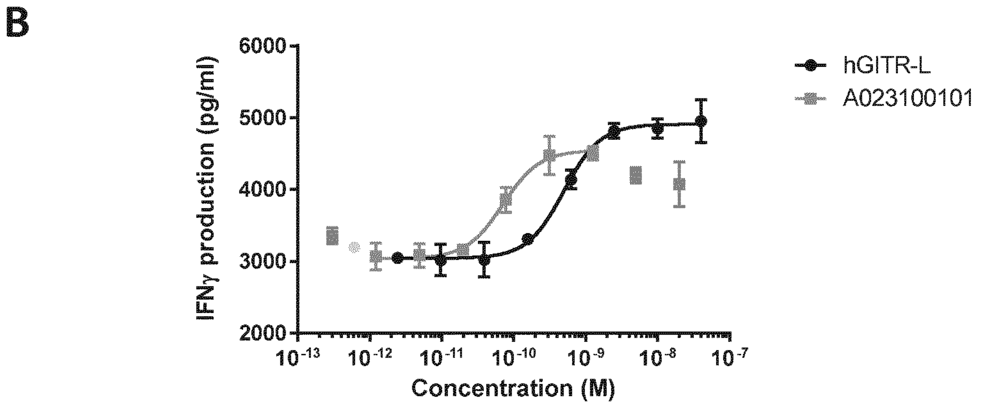
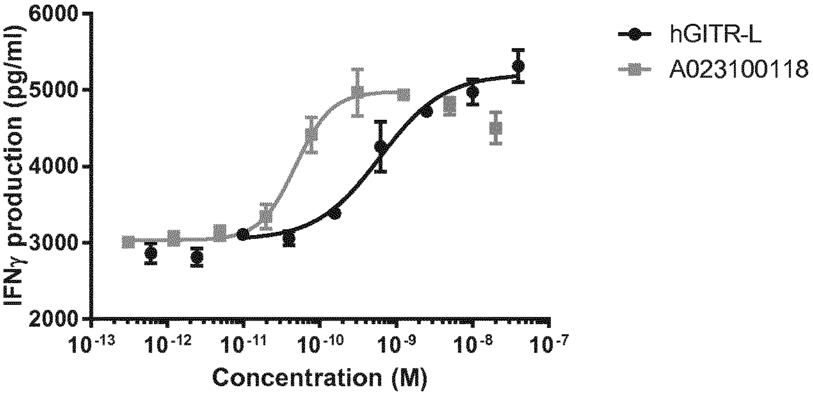
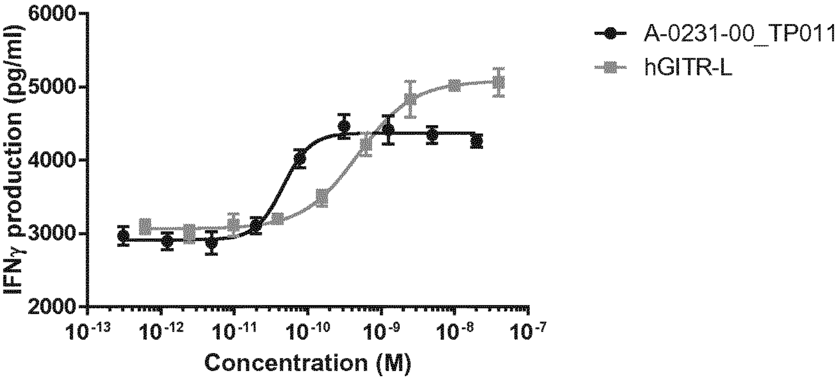


Figure 13 Continued

C



D



E

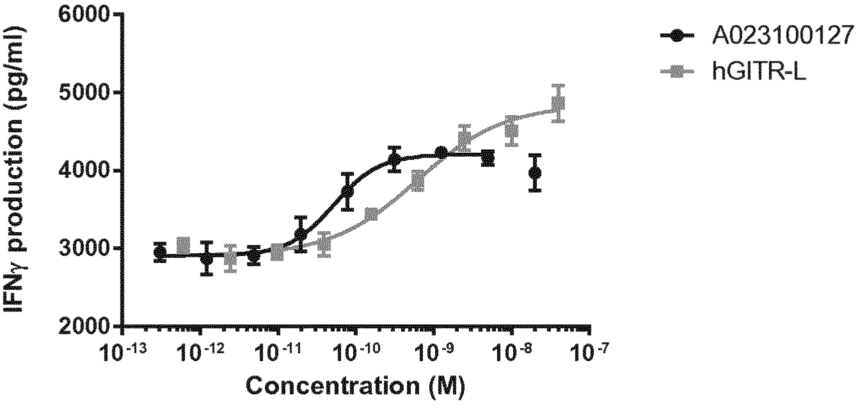
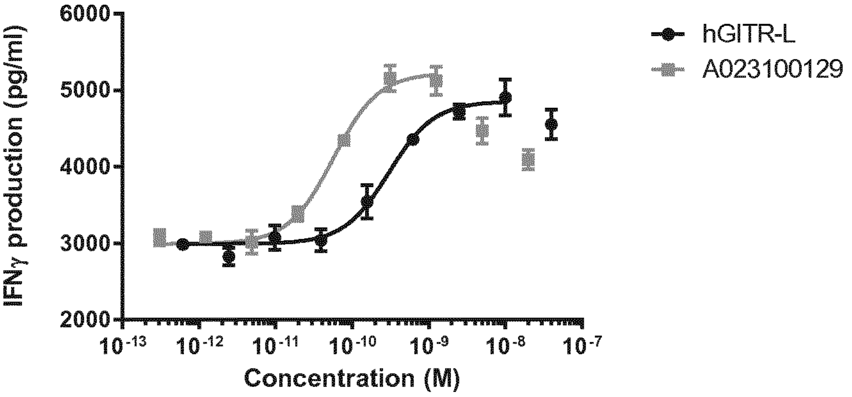


Figure 13 Continued

F



G

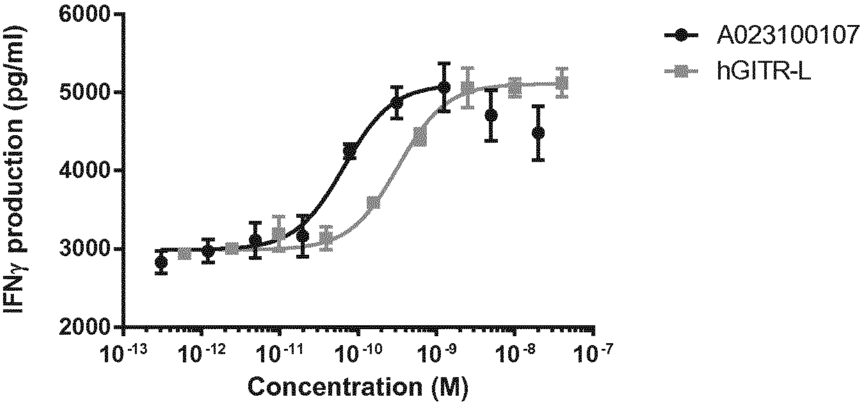
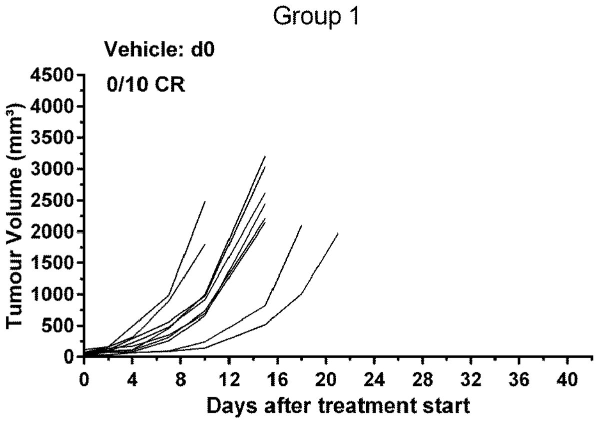
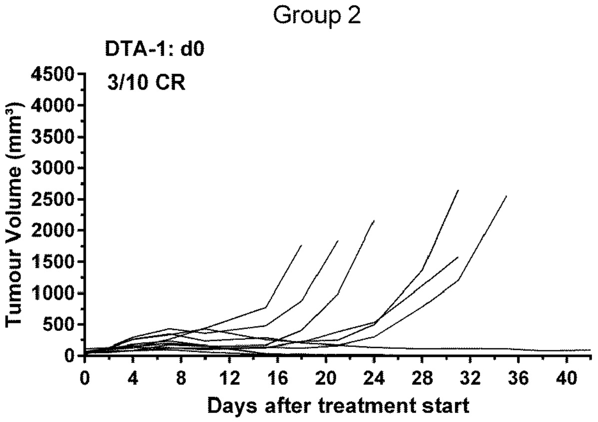


Figure 14

A



B



C

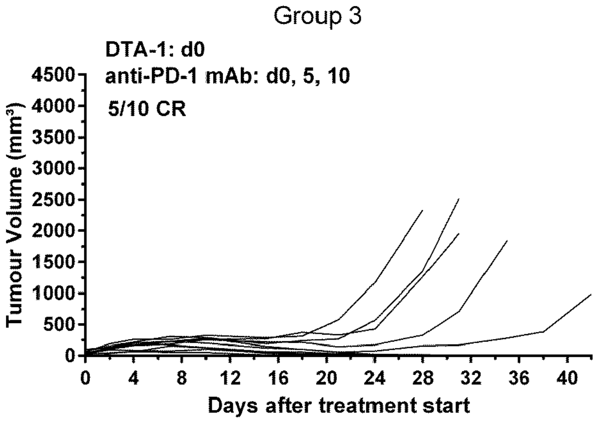


Figure 14 Continued

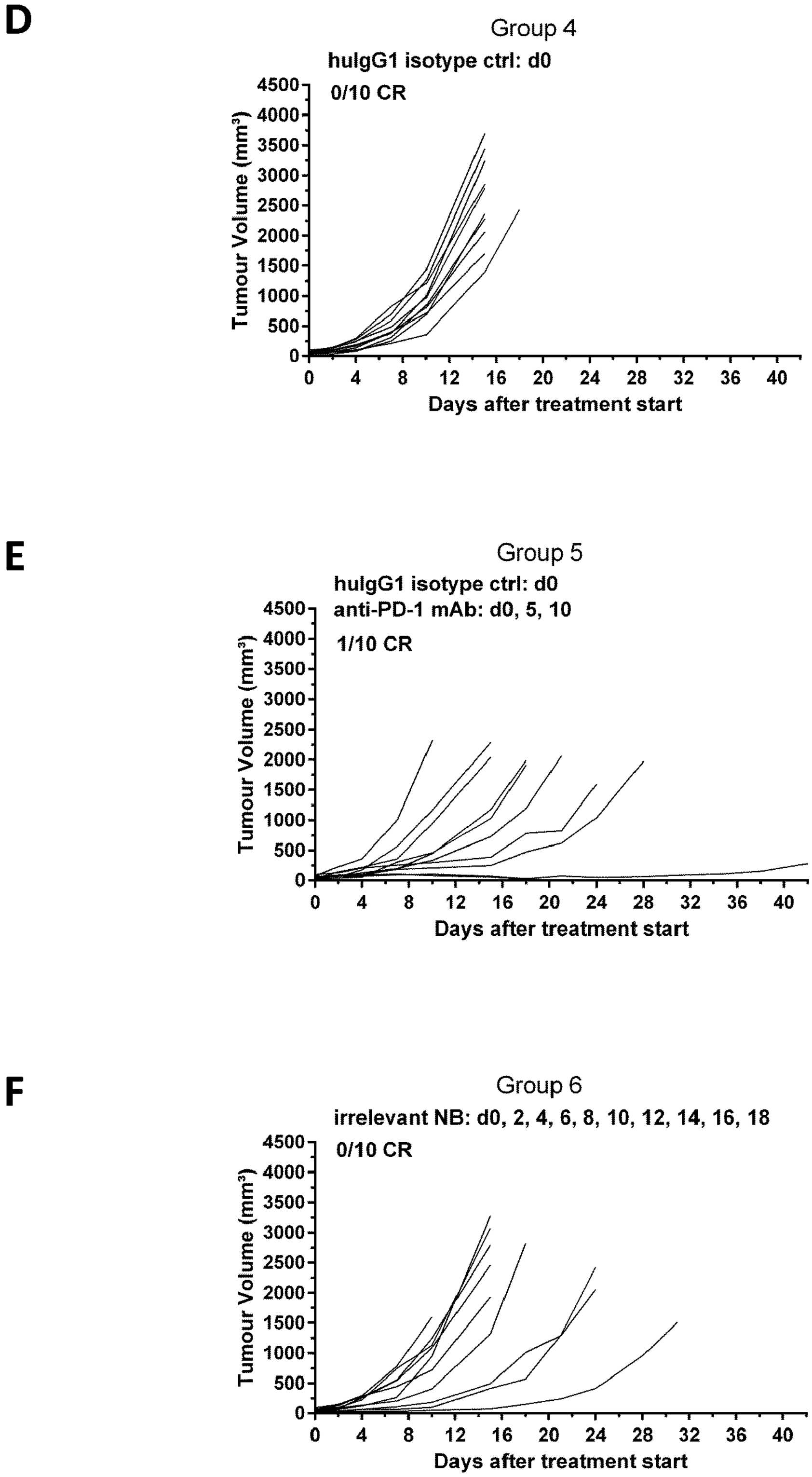
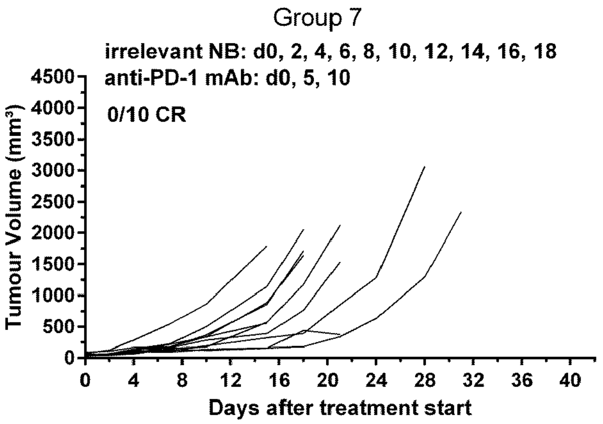
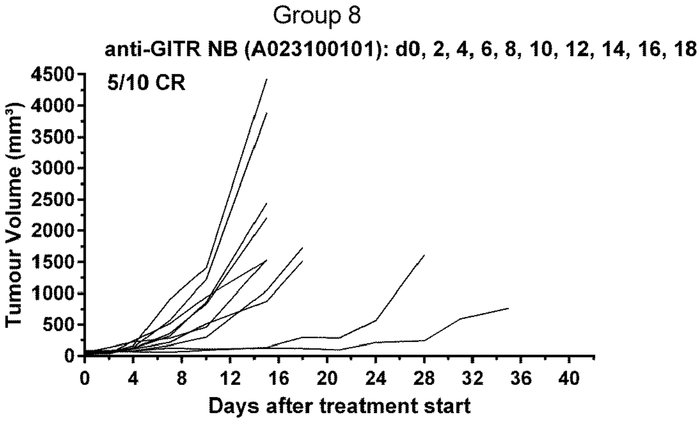


Figure 14 Continued

G



H



I

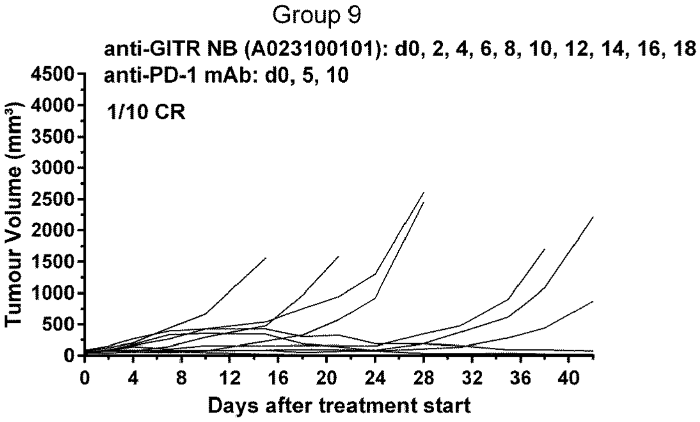
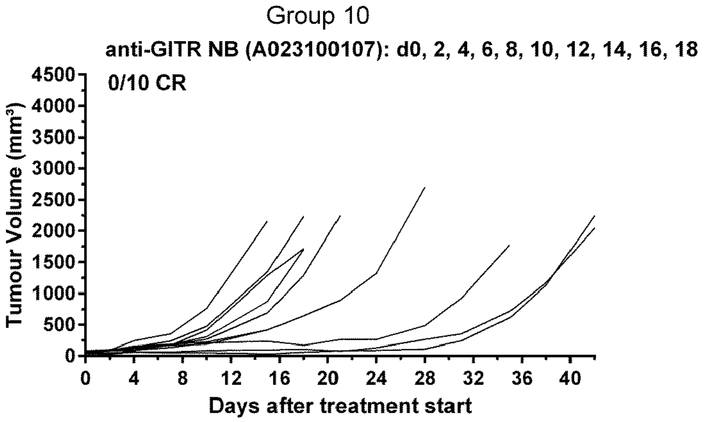
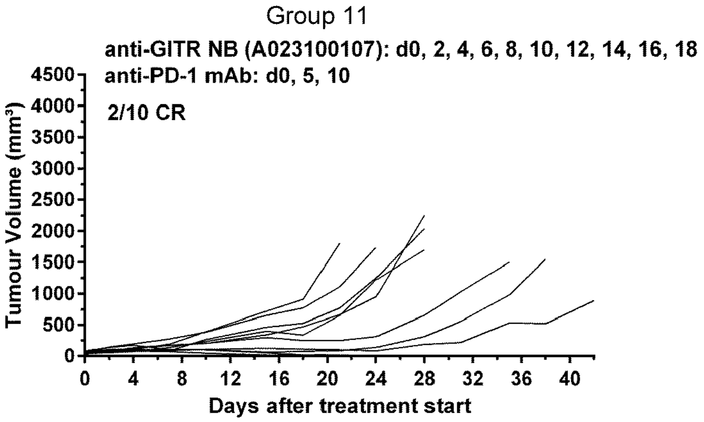


Figure 14 Continued

J


Group 10
anti-GITR NB (A023100107): d0, 2, 4, 6, 8, 10, 12, 14, 16, 18

K


Group 11
anti-GITR NB (A023100107): d0, 2, 4, 6, 8, 10, 12, 14, 16, 18
anti-PD-1 mAb: d0, 5, 10

L

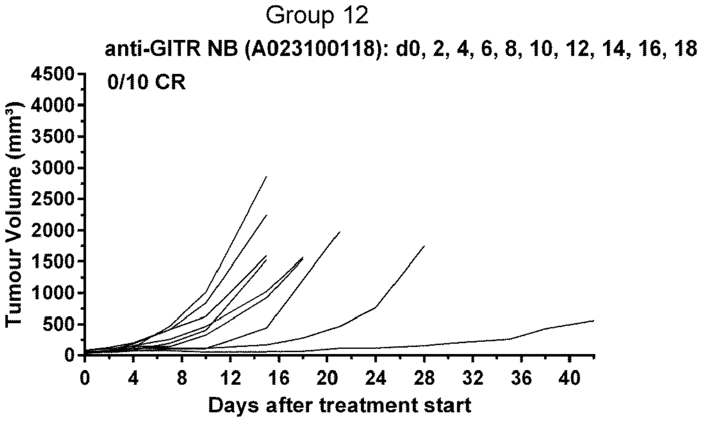
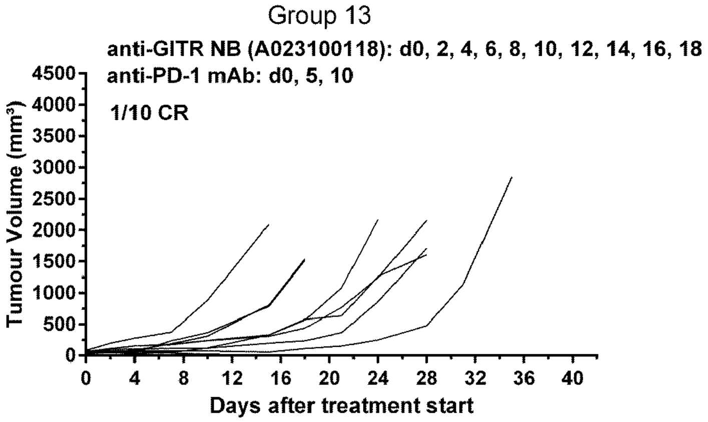
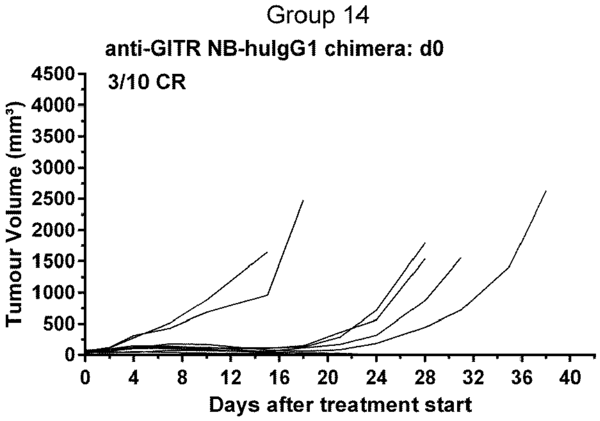

Group 12
anti-GITR NB (A023100118): d0, 2, 4, 6, 8, 10, 12, 14, 16, 18

Figure 14 Continued

M



N



O

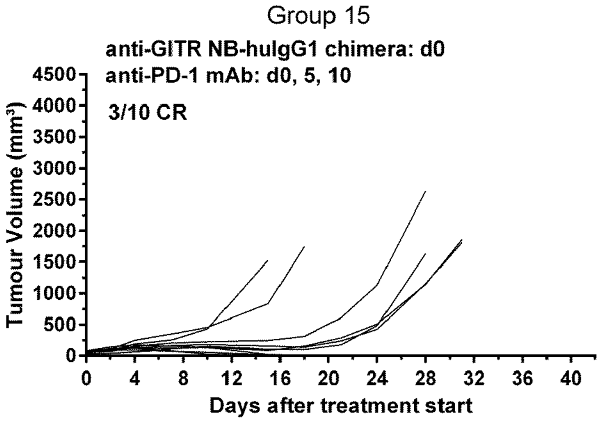
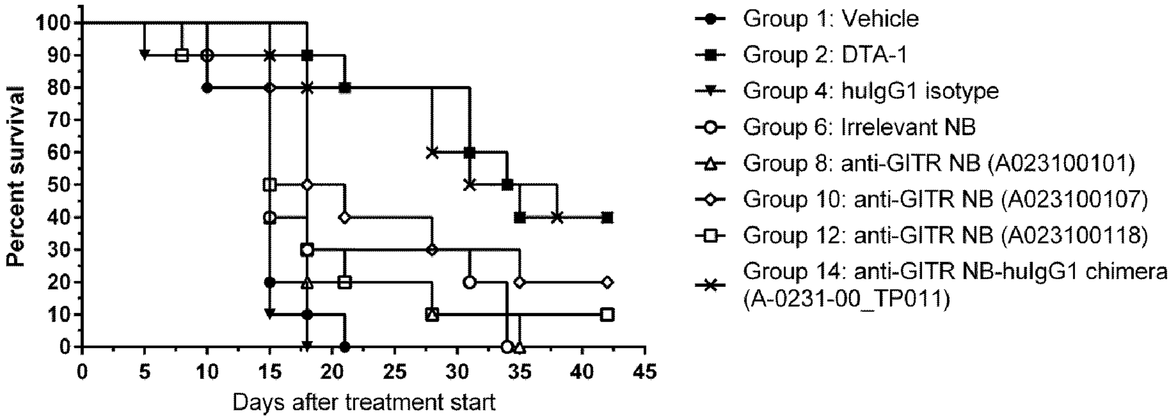
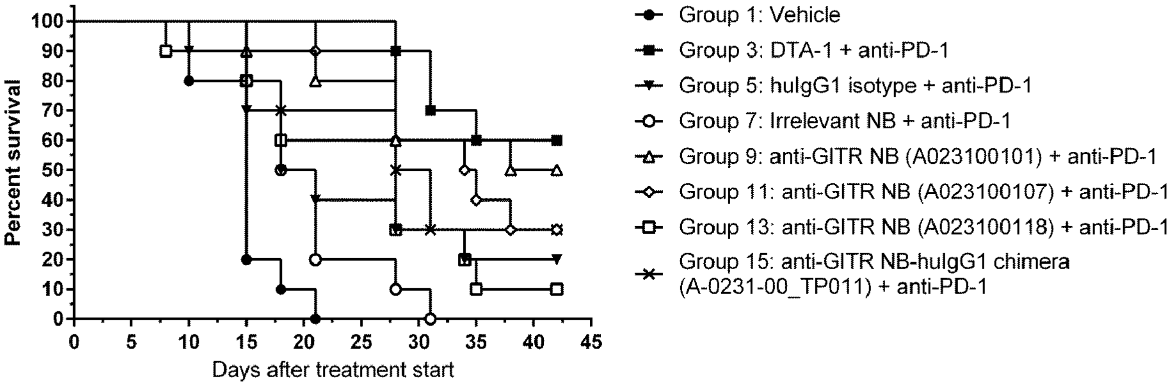


Figure 15

A


Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
Days after treatment start
Group 1: Vehicle
Group 2: DTA-1
Group 4: huIgG1 isotype
Group 6: Irrelevant NB
Group 8: anti-GITR NB (A023100101)
Group 10: anti-GITR NB (A023100107)
Group 12: anti-GITR NB (A023100118)
Group 14: anti-GITR NB-huIgG1 chimera (A-0231-00_TP011)

B


Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
Days after treatment start
Group 1: Vehicle
Group 3: DTA-1 + anti-PD-1
Group 5: huIgG1 isotype + anti-PD-1
Group 7: Irrelevant NB + anti-PD-1
Group 9: anti-GITR NB (A023100101) + anti-PD-1
Group 11: anti-GITR NB (A023100107) + anti-PD-1
Group 13: anti-GITR NB (A023100118) + anti-PD-1
Group 15: anti-GITR NB-huIgG1 chimera (A-0231-00_TP011) + anti-PD-1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/075558

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K39/395 A61K39/00
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 A61K

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M CHODORGE ET AL: "A series of Fas receptor agonist antibodies that demonstrate an inverse correlation between affinity and potency", CELL DEATH AND DIFFERENTIATION., vol. 19, no. 7, 20 January 2012 (2012-01-20), pages 1187-1195, XP055332053, GB ISSN: 1350-9047, DOI: 10.1038/cdd.2011.208 The whole document, in particular, p.1192, col.1, para.3 -----	1-85
Y	WO 2011/028683 A1 (SCHERING CORP [US]; SCHEBYE XIAO MIN [US]; ERMAKOV GRIGORY P [US]; HOD) 10 March 2011 (2011-03-10) The whole document, in particular Table 6 ----- -/--	1-85

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"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
3 February 2017

Date of mailing of the international search report
02/03/2017

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer
Chapman, Rob

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/075558

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/105021 A2 (TOLERRX INC [US]; SMITH L MARY [US]; SZYMANSKA GRAZYNA [US]; PONATH PA) 5 October 2006 (2006-10-05) The whole document, in particular the Examples and antibody 6C8 (TRX-518). -----	1-85
Y	ADAM D. COHEN ET AL: "Agonist Anti-GITR Monoclonal Antibody Induces Melanoma Tumor Immunity in Mice by Altering Regulatory T Cell Stability and Intra-Tumor Accumulation", PLOS ONE, vol. 5, no. 5, 3 May 2010 (2010-05-03), page e10436, XP055150996, DOI: 10.1371/journal.pone.0010436 The whole document, in particular, mAb DTA-1 -----	1-85
Y	DOROTA SMOLAREK ET AL: "Variable fragments of heavy chain antibodies (VHHs): a new magic bullet molecule of medicine?", POSTEPY HIGIENY I MEDYCYNY DOSWIADCZALNEJ, vol. 66, 14 June 2012 (2012-06-14), pages 348-358, XP055101869, DOI: 10.5604/17322693.1000334 The whole document, in particular section 4. -----	1-85
E	WO 2016/196792 A1 (BRISTOL-MYERS SQUIBB COMPANY [US]) 8 December 2016 (2016-12-08) the whole document -----	1-85
E	WO 2017/015623 A2 (INHIBRX LP [US]) 26 January 2017 (2017-01-26) The whole document, in particular the examples -----	1-85
X,P	WO 2015/184099 A1 (4ANTIBODY AG [CH]; SLOAN KETTERING INST CANCER [US]; LUDWIG INST FOR C) 3 December 2015 (2015-12-03) the whole document -----	1-85

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/075558

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-85(partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-85(partially)

The present application comprises 85 claims relating to several million possible antibody CDR combinations and 27 medical uses. According to Rule 6.1(a) PCT, the number of claims shall be reasonable in consideration of the nature of the invention claimed, which is not the case here. The examiner is unable to search the millions of combinations claimed without undue burden.

Furthermore, the application relates to sequence combinations which may or may not generate an antibody with defined technical properties.

In view of the importance of the role played by each single amino acid in the binding of an antibody to its target, especially at the level of its CDRs (see Rudikoff et.al., PNAS 1982, Vol.79, pp. 1979-1983), a claim to an antibody which is structurally characterised by less than the full sequence of all the CDRs, and their specific order, cannot be seen to sufficiently disclose an antibody with any particular properties. Thus, it is apparent that a search of all possible combinations of claimed sequences would not be useful, since only an antibody defined by at least 6 CDRs (HCDR1-3, LCDR1-3) has any defined technical properties of binding.

The Examiner has thus limited the scope of the search to mono- or multi-valent, immunoglobulin single variable domain polypeptides directed to GITR, and in addition, a specific embodiment, comprising SEQ ID NO:73, 90 and 123 (or 118) has been searched. Of the 27 various medical use claims, the use of the anti-GITR antibodies as a medicament and for the treatment of cancer has been searched, which is also the disease model utilised in the examples of the application.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/075558

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011028683 A1	10-03-2011	AU 2010289677 A1	05-04-2012
		BR 112012004823 A2	16-08-2016
		CA 2772613 A1	10-03-2011
		CN 102574924 A	11-07-2012
		CN 103951753 A	30-07-2014
		EP 2473531 A1	11-07-2012
		EP 3023438 A1	25-05-2016
		JP 2013503632 A	04-02-2013
		JP 2016146834 A	18-08-2016
		KR 20120059603 A	08-06-2012
		RU 2012112829 A	10-10-2013
		SG 178991 A1	27-04-2012
		US 2012189639 A1	26-07-2012
		US 2014348841 A1	27-11-2014
		WO 2011028683 A1	10-03-2011
WO 2006105021 A2	05-10-2006	AU 2006230099 A1	05-10-2006
		CA 2602777 A1	05-10-2006
		DK 1866339 T3	02-09-2013
		EP 1866339 A2	19-12-2007
		EP 2343320 A1	13-07-2011
		ES 2432091 T3	29-11-2013
		HK 1112005 A1	17-01-2014
		JP 5238936 B2	17-07-2013
		JP 2008533993 A	28-08-2008
		JP 2013091644 A	16-05-2013
		JP 2015232005 A	24-12-2015
		PT 1866339 E	03-09-2013
		US 2007098719 A1	03-05-2007
		US 2011059109 A1	10-03-2011
		US 2013183321 A1	18-07-2013
		US 2015259427 A1	17-09-2015
		WO 2006105021 A2	05-10-2006
WO 2016196792 A1	08-12-2016	NONE	
WO 2017015623 A2	26-01-2017	US 2017022284 A1	26-01-2017
		WO 2017015623 A2	26-01-2017
WO 2015184099 A1	03-12-2015	AU 2015266958 A1	08-12-2016
		SG 11201609721W A	29-12-2016
		TW 201607958 A	01-03-2016
		US 2015368349 A1	24-12-2015
		WO 2015184099 A1	03-12-2015