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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 July 2018 (12.07.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/127709 A1

(51) International Patent Classification:
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/GB2018/050035

(22) International Filing Date:
08 January 2018 (08.01.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

1700207.2	06 January 2017 (06.01.2017)	GB
1700208.0	06 January 2017 (06.01.2017)	GB
1700210.6	06 January 2017 (06.01.2017)	GB

(71) Applicant: **CRESCENDO BIOLOGICS LIMITED** [GB/GB]; Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB).

(72) Inventors: **EDWARDS, Bryan**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **EDWARDS, Carolyn**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **LEGG, James**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **LEWANDOWSKA, Martyna**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **SYDOREK, Daniela**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **JOHNSTON, Colette**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **ROSSANT, Christine**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB). **TENG, Yumin**; c/o Crescendo Biologics Limited, Meditrina Building 260, Babraham Research Campus, Cambridge, Cambridgeshire CB22 3AT (GB).

(74) Agent: **APPLEYARD LEES IP LLP**; 15 Clare Road, Halifax, Yorkshire HX1 2HY (GB).

(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, JO, JP, KE, KG, KH, 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,

(54) Title: SINGLE DOMAIN ANTIBODIES TO PROGRAMMED CELL DEATH (PD-1)

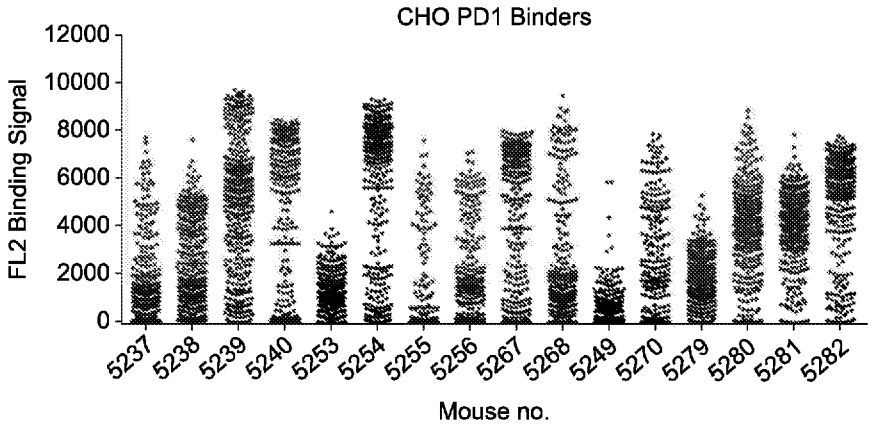

FIG. 1a

(57) Abstract: The invention relates to PD-1 binding agents that do not block the interaction of PD-1 with its ligands, and the use of such binding agents in the treatment, prevention and detection of disease.

WO 2018/127709 A1

[Continued on next page]

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

Single Domain Antibodies to Programmed Cell Death (PD-1)

Field of the Invention

The invention relates to PD-1 binding agents, in particular PD-1 binding V_H single domain antibodies (sdAb), and the use of such binding agents in the treatment, prevention and detection of disease.

Introduction

Antibody-based therapeutics have emerged as important components of therapies for an increasing number of human diseases in such fields as oncology, inflammatory and infectious diseases. Indeed, antibodies are one of the best-selling classes of drugs today; five of the top ten best selling drugs are antibodies.

The Programmed Death 1 (PD-1) protein is encoded by the PDCD1 gene and expressed as a 55kDa type I transmembrane protein (Agata 1996 Int Immunol 8(5):765-72). PD-1 is an immunoglobulin superfamily member (Ishida 1992 EMBO 11(11):3887-95) and it is an inhibitory member of the extended CD28/CTLA-4 family of T cell regulators. Other members of this family include CD28, CTLA-4, ICOS and BTLA. PD-1 exists as a monomer, lacking the unpaired cysteine residue characteristic of other CD28 family members (Zhang 2004 Immunity 20:337-47). Its cytoplasmic domain contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) that are phosphorylated during signal transduction (Riley 2009 Immunol Rev 229(1):114-25).

PD-1 is expressed on B cells, T cells, and monocytes (Agata 1996). The role of PD-1 in maintaining immunologic self-tolerance was demonstrated in PDCD1-/- mice, which develop autoimmune disorders (Nishimura 1999 Immunity 11:141-51, Nishimura 2001 Science 291(5502):319-22). The PD-1 pathway therefore regulates antigen responses, balancing autoimmunity and tolerance.

There are two ligands for PD-1 that mediate its regulatory function. PD-L1 (B7-H1) is normally expressed on dendritic cells, macrophages, resting B cells, bone marrow-derived mast cells and T cells as well as non-hematopoietic cell lineages (reviewed in Francisco 2010 Immunol Rev 236:219-42). PD-L2 (B7-DC) is largely expressed on dendritic cells and macrophages (Tseng 2001 J Exp Med 193(7):839-45). Ligand expression is influenced by local mediators and can be upregulated by inflammatory cytokines.

PD-1 is known as an immunoinhibitory protein that negatively regulates TCR signals. The interaction between PD-1 and PD-L1 can act as an immune checkpoint, which can lead to, e.g., a decrease in tumour infiltrating lymphocytes, a decrease in T- cell receptor mediated

proliferation, and/or immune evasion by cancerous cells. Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-L1 or PD-L2; the effect is additive when the interaction of PD-1 with both PD-L1 and PD-L2 is blockedl.

As T cells become activated and co-stimulated by antigen-presenting cells (APCs), T cell expression of PD-1 is induced. PD-1 engagement with ligand on the APC cross-links PD-1 and clusters it into the T cell receptor (TCR) complex within the immunological synapse (Yokosuka 2012 J Exp Med 209(9):1201-17). Within the T cell cytoplasm, PD-1 signalling domains ITIM and ITSM are phosphorylated. This induces Src-homology-2 domain-containing tyrosine phosphatase (SHP1/2) that attenuates various components of the T cell receptor (TCR) signalling. T cell activation is dampened, which leads to a reduction in cytokine response, proliferation and cytolytic activity. This downregulation of T cell function serves to prevent overstimulation, tolerising cells against weakly immunogenic self-antigen.

The PD-1 pathway can be exploited in cancer or infection, whereby tumours or viruses can evade effective immune recognition and T cells demonstrate an 'exhausted' phenotype. PD-L1 has also been shown to be expressed in many tumour types including urothelial, ovarian, breast, cervical, colon, pancreatic, gastric, melanoma, glioblastoma and non-small cell lung carcinoma (reviewed in Callahan 2014 J Leukoc Biol 94(1):41-53). The cytokines produced by cancer stromal cells can further upregulate PD-L1 in the tumour microenvironment (He 2015 Nature Scientific Reports 5:13110). As a result, tumour-specific T cells become unresponsive through PD-1 signalling and therefore fail to eliminate their target. T regulatory cells (T regs) have also been shown to express high levels of PD-1 and they suppress the anti-tumour response further (Lowther 2016 JCI Insight 1(5):85935).

Disruption of the PD-1:PD-L1 interaction enhances T cell activity. An anti-PD-1 monoclonal antibody demonstrates blockade of the interaction between PD-1 and its ligands (Wang 2014 Cancer Immunol Res 2(9):846-56). T cell function in-vitro can be enhanced by PD-1 blockade, as demonstrated by improved proliferation and cytokine responses in mixed lymphocyte reactions of T cells and dendritic cells. cytotoxic lymphocytes (CTLs) derived from melanoma patients has also been shown to be enhanced by PD-1 blockade in vitro using the antibody OPDIVO (nivolumab), and can become resistant to Treg suppression (Wang 2009 Int Immunol 21(9):1065-1077). This antibody has been tested in clinical dose escalation studies in melanoma, non-small cell lung carcinoma (NSCLC), renal cell cancer (RCC) and others. It shows improved overall survival rates compared to chemotherapy in NSCLC patients. Another PD-1 blocking antibody, KEYTRUDA® (pembrolizumab), demonstrates responses in NSCLC patients refractory to CTLA-4 blockade. OPDIVO® and KEYTRUDA® both functionally block the interaction of human PD-1 with its ligands.

It is possible to induce PD-1 signalling by cross-linking it on the membrane with a combination of anti-PD-1 plus anti-CD3 antibodies (Bennett 2003 J Immunol 170:711-18, Keir 2005 J Immunol 175:7372-7379). This function could be detrimental during an anti-tumour response because T cell activity would be suppressed. If suppression of T cell responses were desired, agonistic anti-PD-1 antibodies or those with effector functions could be used to treat immune-related diseases such as rheumatoid arthritis.

The aim of the present invention is to address the need of alternative antibody-based treatments for use in the treatment of disease, in particular in the treatment of cancer.

Summary of the Invention

The invention relates to an isolated human variable single domain antibody or an isolated human heavy chain only antibody that binds to human PD-1 and related methods for treating disease.

The inventors have surprisingly identified human variable single domain antibodies generated *in vivo* in transgenic mice that bind to human PD-1, but do not block the functional interaction between human PD-1 and its ligands.

Thus, the anti-PD-1 V_H single domain antibodies of the invention bind an epitope that is distant from the part of the PD-1 protein that interacts with its ligands PD-L1 and PD-L2 and that is therefore outside the region of binding of known therapeutics targeting PD-1. As further described below, this renders a human variable single domain antibodies of the invention particularly useful in anchoring binding molecules to human PD-1 when used in combination with other binding domains. For example, the human variable single domain antibodies can be used in combination therapies with antibodies or antibody fragments that bind to human PD-1 and block the interaction of PD-1 with its ligands PD-L1 and PDL-2. Furthermore, the human variable single domain antibodies can also be used in combination therapies with antibodies or antibody fragments that target other immune checkpoint inhibitors.

The small size of the V_H domains makes it possible to format the V_H domain in a multivalent format, for example by linking a V_H single domain antibody as described herein to another binding agent, such as another V_H single domain antibody. The second binding agent, such as another V_H single domain antibody, may bind to another epitope on PD-1, and may block the functional interaction PD-1 and PD-L1 and/or PD-L2. Alternatively, the second binding agent, such as another V_H single domain antibody, may bind another epitope on PD-1 and not block functional ligand interaction, may cross-link or cluster multiple PD-1 monomers to effect PD-1 signalling and functions.

As demonstrated herein, providing an isolated single domain antibody that binds to human PD-1 but does not block the interaction of human PD-1 with human PD-L1 and/or PD-L2 together with a inhibitor single domain antibody blocks the interaction of human PD-1 with human PD-L1 and/or PD-L2 in a bivalent format is advantageous. In this format, the inhibitory effect is increased by 10 to 25 fold compared to the blocker in monovalent format.

In one aspect, the invention relates to an isolated single domain antibody that binds to human PD-1 but does not block the interaction of human PD-1 with human PD-L1 and/or PD-L2.

In one aspect, the invention relates to an isolated V_H single domain antibody that binds to an epitope comprising one or more residue selected from R^{104}, D^{105}, F^{106}, H^{107}, M^{108}, S^{109} and V^{110} of human PD-1.

In one aspect, the invention relates to an isolated single V_H domain antibody that binds to human PD-1 comprising a CDR3 sequence as shown Table 1 or 2 below or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence identity thereto.

In one aspect, the invention relates to an isolated single domain antibody comprising a sequence selected from SEQ ID Nos. 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462 or a sequence with at least 60%, 70%, 80% or 90% homology thereto.

In preferred embodiments, the single domain is a human heavy chain variable domain. Human heavy chain variable domains are commonly designated V_H.

In one embodiment, the isolated single domain antibody wherein said single domain antibody is conjugated to a toxin, enzyme, radioisotope, half-life extending moiety, label, therapeutic molecule or other chemical moiety.

In another aspect, the invention relates to an isolated single domain antibody that does not compete with an antibody that blocks the functional interaction of PD-1 with PD-L1 and/or the interaction of PD-1 with PD-L2.

In another aspect, the invention relates to a binding agent that binds to essentially the same epitope as the single domain antibody described herein.

In another aspect, the invention relates to an isolated binding agent that competes for binding to human PD-1 with the single domain antibody as described herein.

In another aspect, the invention relates to an isolated binding agent comprising a single domain antibody as described herein. In one embodiment, said single domain antibody is linked to a second binding molecule that does not bind to PD-1. In one embodiment, said second single domain antibody binds to an immunooncology target. In one embodiment, said single domain antibody is linked to a second binding molecule that binds to PD-1. In one embodiment, said binding molecule blocks the interaction of PD-1 with PD-L1 and/or PD-L2.

In one embodiment, the isolated binding agent is conjugated to a toxin, enzyme, radioisotope, half-life extending moiety, therapeutic molecule or other chemical moiety.

In another aspect, the invention relates to the use of a single domain antibody described herein in a multispecific or multivalent binding agent.

In another aspect, the invention relates to an immunoconjugate comprising a single domain antibody as described herein or a binding molecule as described herein linked to a therapeutic agent.

In another aspect, the invention relates to a pharmaceutical composition comprising a single domain antibody, a binding agent or an immunoconjugate as described herein and a pharmaceutical carrier.

In another aspect, the invention relates to a method for treating a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency or other immune system-related disorder comprising administering a therapeutically effective amount of a comprising a single domain, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein.

In another aspect, the invention relates to the use of a single domain, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein in the manufacture of a medicament for the treatment of a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency or other immune system-related disorder.

In another aspect, the invention relates to a single domain antibody, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein for use as medicament.

In another aspect, the invention relates to a single domain antibody, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein for use in the treatment of a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency, and other immune system-related disorder.

In another aspect, the invention relates to a method of modulating an immune response comprising administering a single domain antibody, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein.

In another aspect, the invention relates to an isolated nucleic acid molecule comprising a nucleotide sequence selected from SEQ ID Nos. 81 to 100, 221 to 250 or 463 to 515.

In another aspect, the invention relates to a vector comprising a nucleic acid sequence selected from SEQ ID Nos. 81 to 100, 221 to 250, or 463 to 515.

In another aspect, the invention relates to a host cell comprising a vector comprising a nucleic acid sequence selected from SEQ ID Nos. 81 to 100, 221 to 250, or 463 to 515.

In another aspect, the invention relates to a method for producing a single domain antibody as described herein comprising expressing a nucleic acid encoding said binding molecule in a host cell and isolating the binding molecule from the host cell.

In another aspect, the invention relates to a kit comprising a single domain antibody, a binding agent, an immunoconjugate or a pharmaceutical composition as described herein.

In another aspect, the invention relates to a method for detecting the presence of human PD-1 in a test sample comprising contacting said sample with a single domain antibody as described herein and at least one detectable label and detecting binding of said single domain antibody to human PD-1.

In another aspect, the invention relates to a method for producing a V_H single domain antibody that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 said method comprising

a) immunising a transgenic animal that expresses a nucleic acid construct comprising human heavy chain V genes and that is not capable of making functional endogenous light or heavy chains with an PD-1 antigen,

b) generating a library from said animal

c) isolating V_H single domain antibodies from said libraries,

d) identifying a V_H single domain antibody that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 and

e) isolating said antibody.

In another aspect, the invention relates to a V_H single domain antibody obtained or obtainable by the method described above.

In another aspect, the invention relates to a human V_H single domain antibody that exhibits one or more of the following properties:

(a) binds to human PD-1 with a KD as shown in the examples;

(b) does not block the functional interaction of PD-1 with its ligands;

(c) binds to human PD-1 and cynomolgus monkey PD-1;

(d) does not bind to mouse PD-1;

(e) is capable of enhancing antagonistic action of an antagonistic human V_H single domain antibody when linked to such antibody;

(f) does not enhance T cell activation;

(g) has EC50 or IC50 values as shown in the examples.

In another aspect, the invention relates to an isolated heavy chain only antibody comprising a V_H domain that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2.

In another aspect, the invention relates to a transgenic rodent that produces a heavy chain only antibody as described herein.

In another aspect, the invention relates to a heavy chain only antibody comprising a V_H domain that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 obtained or obtainable from a transgenic mouse which expresses human V, D and J loci and does not produce functional endogenous lambda and kappa light chains and heavy chains.

Another aspect relates to an isolated heavy chain only antibody comprising a V_H domain that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 for use as an agonist.

Figures

The invention is further described in the following non-limiting figures.

Fig 1. a) Binding Assay and b) Inhibition Assay.

Fig 2. a) V_H single domain antibody 1.2 and 1.1 binding to human recombinant PD-1 protein b) V_H single domain antibody 1.2 and 1.1 binding to cynomolgus PD-1 recombinant protein c) V_H single domain antibody 1.2 and 1.1 do not bind to mouse PD-1.

Fig 3. Inhibition of human PD-L1 (3a) and PD-L2 (3b) binding to recombinant human PD-1 protein using V_H domain antibodies 1.2 and 1.1 in HTRF assay.

Fig 4. Binding of VHs to CHO human PD-1 cells and testing whether binding inhibits PD-L1 interaction. a) CHO-PD-1 binding b) CHO-PD-1 PD-L1 inhibition.

Fig 5. Functional reporter gene assay. Different single domain antibodies as well as biparatopic format were tested together with a control V_H in the functional reporter assay.

Fig 6. Stability of V_H single domain antibody 1.1 at 40°C for 0-14 days.

Fig 7. V_H single domain antibody 1.1 has no impact on IL-2 secretion from allogeneic dendritic cell/T cell co-culture. Positive control (POS) is a Humabody® V_H that functionally blocks PD-1:PD-L1 interaction. Negative control (NEG) is an irrelevant Humabody® V_H. IL-2 levels were determined after 2 days by Homogenous Time Resolved Fluorescence assay (HTRF).

Fig 8. a) 1.1 and 2.1 common epitope residues on human PD-1 (PDB code: 4ZQK) based on Pepscan analysis. The residues common to both the 1.1 and 2.1 epitope are shown in black. b) alignment of residues of human PD-1 involved in binding V_H single domain antibody 1.1 with PD-1 mouse sequence.

Fig.9. a) Mouse serum stability of V_H single domain antibody 2.12 and b) human serum stability of V_H single domain antibody 2.12.

Detailed description

Various aspects and embodiments will now be further described. In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, pathology, oncology, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are

generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Green and Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012); Therapeutic Monoclonal Antibodies: From Bench to Clinic, Zhiqiang An (Editor), Wiley, (2009); and Antibody Engineering, 2nd Ed., Vols 1 and 2, Ontermann and Dubel, eds., Springer-Verlag, Heidelberg (2010).

Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

The inventors have surprisingly identified human V_H single domain antibodies that bind to human PD-1, but do not block the functional interaction between human PD-1 and its ligands as demonstrated in the functional assays in the examples (see also Figures 5 and 7). The inventors have further shown that the human V_H single domain antibodies of the invention bind to an epitope that is distinct from the binding site of human PD-1 to PDL-1 or PDL-2.

The invention thus provides isolated single domain antibodies that bind human PD-1, but do not block the interaction of PD-1 with PD-L1 and/or the interaction of PD-1 with PD-L2, pharmaceutical compositions comprising such binding molecules, as well as isolated nucleic acids, isolated recombinant expression vectors and isolated host cells for making such binding proteins. Also provided are methods of using the single domain antibodies disclosed herein to detect human PD-1 and methods of treating disease. In another aspect, the invention provides binding molecules comprising a single domain antibody that binds human PD-1, but does not block the interaction of PD-1 with PD-L1 and/or the interaction of PD-1 with PD-L2 as described herein. In another aspect, the invention provides binding molecules comprising a single domain antibody that binds to an epitope on human PD-1 as defined herein.

In preferred embodiments, the single domain antibody is a single domain antibody wherein the domain is a human variable heavy chain (V_H) domain. Thus, in certain embodiments, the we provide isolated single domain antibodies that bind human PD-1, wherein the domain is a variable heavy chain domain, preferably a V_H domain and wherein said single domain

antibodies bind to human PD-1 and do not block the interaction of PD-1 with PD-L1 and/or the interaction of PD-1 with PD-L2.

As used herein, the term “do not block or do not inhibit the interaction of human PD-1 with its ligands” refers to the functional interaction of human PD-1 with its ligands. In other words, the binding of the single domain antibodies of the invention does not abolish or reduce the functional interaction of human PD-1 with its ligands. This can for example be measured in a PD-1 signaling assay and is not defined as blocking ligand binding. Thus, the binding of the single domain antibodies of the invention to human PD-1 does not affect the biological function of the interaction of human PD-1 with its ligands. In one embodiment, the ligand is PD-L1. In one embodiment, the ligand is PD-L2.

The single domain antibodies of the invention bind to PD-1 with high affinity and specificity.

The properties of the single domain antibodies of the invention as described above can be exploited in therapeutic methods and uses. Compounds of the invention are particularly useful in anchoring, associating or bringing into proximity a therapeutic molecule to human PD-1, for example in a targeted therapy to recruit the therapeutic compound to the cell or tissue of interest or to a region of the cell associated with PD-1 localisation. This makes the compounds of the invention particularly suitable for delivery together with other compounds, for example those that block the interaction of PD-1 with its ligands or other immunomoldulators. For example, the single domain antibodies of the invention can be linked, for example using peptide linkers, to a compound that antagonises human PD-1 binding to its ligands PD-L1 and/or PD-L2 thereby up-modulating the immune response. Such a compound can be selected from an antibody or fragment thereof, including a V_H single domain antibody.

Thus, one aspect relates to the use of a single domain antibody as disclosed herein in a multivalent binding agent, for example in combination with a V_H single domain antibody that blocks binding of PD-1 to its ligands PD-L1 and/or PD-L2. In one embodiment, this can be combined with a mAb or an Fc region of an antibody e.g. a Humabody® mAb fusion. This could enable longer half-life and or Fc effector function to deplete PD-1 positive cells for application in treating autoimmune diseases. In one embodiment, multivalent molecules find use as antagonists. In In one embodiment, multivalent molecules find use as agonists.

In another embodiment, the single domain antibodies can be co-administered together with a compound that blocks the interaction of PD-1 with its ligands or other immunomoldulators.

This can be done in the same medicament, or by sequential administration of separate compositions. In another aspect, the single domain can also be combined with another compound that down regulates immune response.

The single domain antibodies of the invention can further be used as depleting antibodies for example for targeting cells for depletion by NK cells or via delivery of a toxic payload. Furthermore, single domain antibodies of the invention can be used as imaging agents, for example in methods for diagnosing cancer or other biomarker related methods. Applications include a method of predicting clinical response of a patient affected by cancer to a treatment, for example with an immune checkpoint pathway inhibitor.

In particular, as explained below, the single domain antibodies of the invention can be used in a multivalent or multispecific format. Thus, the invention also relates to multifunctional binding agents comprising a single domain antibody as described herein.

Molecules of the invention bind specifically to wild type human PD-1 (UniProt Accession No. Q15116, GenBank Accession No. U64863, SEQ ID No. 518). Residues 1 – 20 correspond to the pre-sequence, residues 171 and beyond make up the transmembrane helix and the intracellular domain of PD-1.

Unless otherwise specified, the term PD-1 as used herein refers to human PD-1. The terms "Programmed Death 1," "Programmed Cell Death 1," "Protein PD-1," "PD-1," PD1," "PDCD1," "hPD-1" and "hPD-1" are used interchangeably, and include variants, isoforms, species homologs of human PD-1

The terms "PD-1 binding molecule/protein/polypeptide/agent", "PD-1 antigen binding molecule protein/polypeptide/agent", “anti-PD-1 single domain antibody”, “anti-PD-1 single immunoglobulin variable domain”, “anti-PD1 heavy chain only antibody” or “anti-PD-1 antibody” all refer to a molecule capable of specifically binding to the human PD-1 antigen. The binding reaction may be shown by standard methods, for example with reference to a negative control test using an antibody of unrelated specificity. The term "PD-1 binding molecule/agent" includes a PD-1 binding protein.

An antibody or binding molecule of the invention, including a single domain antibody and multivalent or multispecific binding agent described herein, "which binds" or is “capable of binding” an antigen of interest, e.g. PD-1, is one that binds the antigen with sufficient affinity such that the antibody is useful as a therapeutic agent in targeting a cell or tissue expressing the antigen.

Binding molecules of the invention, including the single domain antibodies and multivalent or multispecific binding agents described herein, bind specifically to human PD-1. In other words, binding to the PD-1 antigen is measurably different from a non-specific interaction. As demonstrated in the examples, the single domain antibodies of the invention do not cross react with mouse PD-1. Preferably, the single domain antibodies of the invention bind to human PD-1 and also bind to cyno PD-1.

The term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a KD for the target of at least about 10^{-4} M, alternatively at least about 10^{-5} M, alternatively at least about 10^{-6} M, alternatively at least about 10^{-7} M, alternatively at least about 10^{-8} M, alternatively at least about 10^{-9} M, alternatively at least about 10^{-10} M, alternatively at least about 10^{-11} M, alternatively at least about 10^{-12} M, or greater. In one embodiment, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

The term "antibody" broadly refers to any immunoglobulin (Ig) molecule, or antigen binding portion thereof, comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains, or any functional fragment, mutant, variant, or derivation thereof, which retains the essential epitope binding features of an Ig molecule. Such mutant, variant, or derivative antibody formats are known in the art.

In a full-length antibody, each heavy chain is comprised of a heavy chain variable region or domain (abbreviated herein as HCVR) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_H1, C_H2 and C_H3. Each light chain is comprised of a light chain variable region or domain (abbreviated herein as LCVR) and a light chain constant region. The light chain constant region is comprised of one domain, C_L.

The heavy chain and light chain variable regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each heavy chain and light chain variable region is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG 1, IgG2, IgG 3, IgG4, IgAl and IgA2) or subclass.

The term "CDR" refers to the complementarity-determining region within antibody variable sequences. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable regions.

The term "CDR set" refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs can be defined differently according to different systems known in the art.

The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., (1971) Ann. NY Acad. Sci. 190:382-391 and Kabat, et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Chothia refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901 -917 (1987)). The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1 -113 of the heavy chain).

The system described by Kabat is used herein unless otherwise specified. The terms "Kabat numbering", "Kabat definitions" and "Kabat labeling" are used interchangeably herein. These terms, which are recognized in the art, refer to a system of numbering amino acid residues which are more variable (i.e., hypervariable) than other amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen binding portion.

A chimeric antibody is a recombinant protein that contains the variable domains including the complementarity determining regions (CDRs) of an antibody derived from one species, preferably a rodent antibody, while the constant domains of the antibody molecule are derived from those of a human antibody. For veterinary applications, the constant domains of the chimeric antibody may be derived from that of other species, such as a cat or dog.

A humanized antibody is a recombinant protein in which the CDRs from an antibody from one species; e.g., a rodent antibody, are transferred from the heavy and light variable chains of the rodent antibody into human heavy and light variable domains (e.g., framework region sequences). The constant domains of the antibody molecule are derived from those of a human antibody. In certain embodiments, a limited number of framework region amino acid residues from the parent (rodent) antibody may be substituted into the human antibody framework region sequences.

The term "antigen binding site" refers to the part of the antibody or antibody fragment that comprises the area that specifically binds to an antigen. An antigen binding site may be provided by one or more antibody variable domains. Preferably, an antigen binding site is comprised within the associated V_H and V_L of an antibody or antibody fragment.

An antibody fragment is a portion of an antibody, for example as $F(ab')_2$, Fab, Fv, sFv and the like. Functional fragments of a full length antibody retain the target specificity of a full

length antibody. Recombinant functional antibody fragments, such as Fab (Fragment, antibody), scFv (single chain variable chain fragments) and single domain antibodies (dAbs) have therefore been used to develop therapeutics as an alternative to therapeutics based on mAbs.

scFv fragments (~25kDa) consist of the two variable domains, V_H and V_L. Naturally, V_H and V_L domain are non-covalently associated via hydrophobic interaction and tend to dissociate. However, stable fragments can be engineered by linking the domains with a hydrophilic flexible linker to create a single chain Fv (scFv).

The smallest antigen binding fragment is the single variable fragment, namely the V_H or V_L domain. Binding to a light chain/heavy chain partner respectively is not required for target binding. Such fragments are used in single domain antibodies. A single domain antibody (~12 to 15 kDa) therefore consists of or comprises either the V_H or V_L domain.

In one aspect, the invention relates to an isolated single domain antibody, an isolated variable single domain or an isolated immunoglobulin single variable domain wherein said isolated single domain antibody, isolated variable single domain or isolated immunoglobulin single variable domain binds to human PD-1, but does not block the interaction of PD-1 and PD-L1 or PD-L2.

The terms “single domain antibody, variable single domain or immunoglobulin single variable domain (ISV)” are all well known in the art and describe the single variable fragment of an antibody that binds to a target antigen. These terms are used interchangeably herein. As explained below, some embodiments relate to single heavy chain variable domain antibodies/immunoglobulin heavy chain single variable domains which bind a PD-1 antigen in the absence of light chain. Some embodiments relate to human heavy chain variable domain antibodies. Such binding molecules are also termed Humabody® herein. Humabody® is a registered trademark of Crescendo Biologics Ltd.

Thus, in some embodiments, the isolated binding agents/molecules of the invention comprise or consist of at least one single domain antibody wherein said domain is a human heavy chain variable domain. Thus, in one aspect, the binding agents of the invention comprise or consist of at least one immunoglobulin single variable heavy chain domain that has a V_H domain, and they are devoid of V_L domains.

The term "isolated" single domain antibody refers to a single domain antibody that is substantially free of other single domain antibodies, antibodies or antibody fragments having different antigenic specificities. Moreover, an isolated single domain antibody may be substantially free of other cellular material and/or chemicals.

Each single V_H domain antibody comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. Thus, in one embodiment of the invention, the domain is a human variable heavy chain (V_H) domain with the following formula FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4.

Modifications to the C or N-terminal V_H framework sequence may be made to the single domain antibodies of the invention to improve their properties. For example, the V_H domain may comprise C or N-terminal extensions or deletions. C-terminal extensions can be added to the C terminal end of a V_H domain which terminates with the residues VTVSS (SEQ ID No. 516).

In one embodiment, the single domain antibodies of the invention comprise C-terminal extensions or deletions of from 1 to 50, or more residues, for example 1 to 25, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acids. In one embodiment, the single domain antibodies of the invention comprise additional amino acids of the human C_H1 domain thus that the C terminal end extends into the C_H1 domain. In one embodiment, said extension comprises at least 1 alanine residue, for example a single alanine residue, a pair of alanine residues or a triplet of alanine residues.

Additional C or N-terminal residues can be linkers that are used to conjugate the single domain antibodies of the invention to another moiety, or tags that aid the detection of the molecule. Such tags are well known in the art and include for, example linker His tags, e.g., hexa-His (HHHHHH, SEQ ID No. 517) or myc tags.

As used herein, the term "homology" generally refers to the percentage of amino acid residues in a sequence that are identical with the residues of the reference polypeptide with which it is compared, after aligning the sequences and in some embodiments after introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Thus, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. Neither N- or C-terminal extensions, tags or insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known. The percent identity between two amino acid sequences can be determined using well known mathematical algorithms.

According to the various aspects and embodiments of the invention, the variable domain of the single domain antibodies of the invention is preferably a human variable domain (V_H). As used herein, a human V_H domain includes a fully human or substantially fully human V_H domain. As used herein, the term human V_H domain also includes V_H domains that are isolated from heavy chain only antibodies made by transgenic mice expressing fully human

immunoglobulin heavy chain loci, in particular in response to an immunisation with an antigen of interest, for example as described in WO2016/062990 and in the examples. In one embodiment, a human V_H domain can also include a V_H domain that is derived from or based on a human V_H domain amino acid or nucleic acid sequence encoding such V_H domain. Thus, the term includes variable heavy chain regions derived from or encoded by human germline immunoglobulin sequences. A substantially human V_H domain or V_H domain that is derived from or based on a human V_H domain may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced in vitro, e.g. by random or site-specific mutagenesis, or introduced by somatic mutation in vivo). The term “human V_H domain” therefore also includes a substantially human V_H domain wherein one or more amino acid residue has been modified. For example, a substantially human V_H domain may include up to 10, for example 1, 2, 3, 4 or 5 or up to 20 amino acid modifications compared to a fully human sequence.

However, the term "human V_H domain" or "substantially human V_H domain", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Preferably, the term "human V_H domain", as used herein, is also not intended to include camelized V_H domains, that is human V_H domains that have been specifically modified, for example in vitro by conventional mutagenesis methods to select predetermined positions in the V_H domains sequence and introduce one or more point mutation at the predetermined position to change one or more predetermined residue to a specific residue that can be found in a camelid V_{HH} domain.

As shown in the examples, the inventors have identified V_H domains that bind to PD-1 at common residues of PD-1, namely R^{104}, D^{105}, F^{106}, H^{107}, M^{108}, S^{109} and V^{110} of human PD-1. In one embodiment, the invention relates thus to an isolated single domain antibody that binds to an epitope, epitope part, domain, subunit or conformation of human PD-1 comprising one or more or all of the residues selected from R^{104}, D^{105}, F^{106}, H^{107}, M^{108}, S^{109} and V^{110} of human PD-1.

In one embodiment, said epitope, epitope part, domain, subunit or conformation further comprises one or more or all of G^{103}, V^{111}, R^{112} and A^{113}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises G^{103}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises R^{104}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises S^{109}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises V^{110}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises

V^{111}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises R^{112}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises A^{113}. As shown in the examples, the single domain antibodies described herein do have binding to these residues in common. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises all of G^{103}, R^{104}, S^{109}, V^{110}, V^{111}, R^{112} and A^{113} of human PD-1.

In one embodiment, said epitope, epitope part, domain, subunit or conformation described above further comprises one or more or all of residues N^{102}, D^{105}, F^{106}, H^{107}, M^{108}, R^{114} and R^{115} of human PD-1. Said epitope further comprises one or more or all of residues selected from N^{33}, P^{34}, P^{35}, T^{36}, F^{37}, S^{38}, C^{54}, F^{55}, S^{56}, N^{57}, T^{58}, S^{59}, E^{60}, S^{61}, F^{62}, V^{63}, L^{64}, N^{65}, W^{66}, P^{101} and G^{103}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises N^{102}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises D^{105}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises F^{106}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises H^{107}. In one embodiment, said epitope, epitope part, domain, subunit or conformation further comprises M^{108}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises R^{114}. In one embodiment, said epitope, epitope part, domain, subunit or conformation comprises R^{115}.

In another embodiment, the single domain antibody binds to an epitope comprising one or more or all residues selected from R^{104}, D^{105}, F^{106}, H^{107}, M^{108}, S^{109} and V^{110} of human PD-1 and further to one or more or all of S^{60}, E^{61}, S^{62}, F^{63}, V^{64}, L^{65}, N^{66}, W^{67}, Y^{68}, R^{69}, M^{70}, S^{71}, G^{90}, Q^{91}, D^{92}, C^{93}, R^{94}, F^{95}, R^{96}, V^{97}, T^{98}, V^{111}, R^{112}, A^{113} and R^{11}. As shown in the examples, the VHs of Family 2 bind to these residues.

The term “epitope” or “antigenic determinant” refers to a site on the surface of an antigen (e.g., PD-1) to which an immunoglobulin, antibody or antibody fragment, including a V_H single domain antibody specifically binds. Generally, an antigen has several or many different epitopes and reacts with many different antibodies. The term specifically includes linear epitopes and conformational epitopes.

Epitopes within protein antigens can be formed both from contiguous amino acids (usually a linear epitope) or non-contiguous amino acids juxtaposed by tertiary folding of the protein (usually a conformational epitope). Epitopes formed from contiguous amino acids are typically, but not always, retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in a unique spatial conformation. Methods for determining what epitopes are bound by a given antibody

or antibody fragment (i.e., epitope mapping) are well known in the art and include, for example, immunoblotting and immunoprecipitation assays, wherein overlapping or contiguous peptides from are tested for reactivity with a given antibody or antibody fragment.

An antibody binds "essentially the same epitope" as a reference antibody, when the two antibodies recognize identical or sterically overlapping epitopes. The most widely used and rapid methods for determining whether two epitopes bind to identical or sterically overlapping epitopes are competition assays, which can be configured in different formats, using either labelled antigen or labelled antibody.

In one embodiment, the invention relates to an isolated single V_H domain antibody that binds to human PD-1 comprising a CDR3 sequence as shown Table 1 below or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence identity thereto. In one embodiment, said sequence homology is at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. In one embodiment, said sequence homology is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

In one embodiment, the V_H single domain antibody has a CDR3 sequence comprising SEQ ID No. 3 or a sequence having at least 70%, at least 80%, at least 90%, or at least 95% homology to SEQ ID No. 3. In one embodiment, said sequence homology is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

In one embodiment, the V_H single domain antibody has a CDR1 as shown in SEQ ID No. 1 or SEQ ID No. 1 with 1 or 2 amino acid substitutions, a CDR2 as shown in SEQ ID No. 2 or SEQ ID No. 2 with 1 to 5 amino acid substitutions and a CDR3 as shown in SEQ ID No. 3 or SEQ ID No. 3 with 1 to 5 amino acid substitutions.

In one embodiment, the V_H single domain antibody comprises a combination of CDR1, 2 and 3 sequences selected from the CDR1, 2 and 3 sequences in Table 1 or combinations thereof. In one embodiment, the V_H single domain antibody comprises a set of CDR1, 2 and 3 sequences selected from the sets of CDR1, 2 and 3 sequences as shown for the any of the clones in Table 1. Thus, in one aspect, the isolated single domain antibody comprises a CDR1, CDR2 and CDR3 selected from CDRs1-3 of full length sequences SEQ ID No: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216 or 220. Accordingly, in one embodiment, the V_H single domain antibody comprises CDR1 having SEQ ID No. 1, CDR2 having SEQ ID No. 2 and CDR3 having SEQ ID No. 3 (CDRs of SEQ ID NO. 4), CDR1 having SEQ ID No. 5, CDR2 having

SEQ ID No. 6 and CDR3 having SEQ ID No. 7 (CDRs of SEQ ID NO. 8) and so forth. Thus, the V_H single domain antibody comprises one of the following CDR combinations: SEQ ID Nos. 1, 2, 3; SEQ ID Nos. 5, 6, 7; SEQ ID Nos. 9, 10, 11; SEQ ID Nos. 13, 14, 15; SEQ ID Nos. 17, 18, 19; SEQ ID Nos. 21, 22, 23; SEQ ID Nos. 25, 26, 27; SEQ ID Nos. 29, 30, 31; SEQ ID Nos.333, 34, 35; SEQ ID Nos. 37, 38, 39; SEQ ID Nos. 41, 42, 43, SEQ ID Nos. 45, 46, 47; SEQ ID Nos. 49, 50, 51; SEQ ID Nos. 53, 54, 55; SEQ ID Nos. 57, 58, 59; SEQ ID Nos. 61, 62, 63; SEQ ID Nos.65, 66, 67; SEQ ID Nos. 69, 70, 71; SEQ ID Nos.73. 74. 75; SEQ ID Nos. 77. 78, 79; SEQ ID Nos. 101, 102, 103; SEQ ID Nos. 105, 106, 107; SEQ ID Nos. 109, 110, 111; SEQ ID Nos. 113, 114, 115; SEQ ID Nos. 117, 118, 119; SEQ ID Nos. 121, 122, 123; SEQ ID Nos. 125, 126, 127; SEQ ID Nos. 129; 130; 131; SEQ ID Nos. 133, 134, 15; SEQ ID Nos. 137, 18, 139; SEQ ID Nos. 141, 142, 143; SEQ ID Nos. 145, 146, 147; SEQ ID Nos. 149, 150, 151; SEQ ID Nos. 153, 154, 155; SEQ ID Nos. 157, 158, 159; SEQ ID Nos. 161, 162, 163; SEQ ID Nos. 165, 166, 167; SEQ ID Nos. 169, 170, 171; SEQ ID Nos. 173, 174, 175; SEQ ID Nos. 177, 178, 179; SEQ ID Nos. 181, 182, 183; SEQ ID Nos. 185, 186, 187; SEQ ID Nos. 189, 190, 191; SEQ ID Nos. 193, 194, 195; SEQ ID Nos. 197, 198, 199; SEQ ID Nos. 201, 202, 203; SEQ ID Nos. 205, 206, 207; SEQ ID Nos. 209, 210, 211; SEQ ID Nos. 213, 214, 215; SEQ ID Nos. 217, 218, 219.

In another embodiment, said CDR1 comprises or consists of the amino acid sequence SEQ ID NO. 1 or a sequence with at least at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto. In one embodiment, said CDR2 comprises or consists of the amino acid sequence SEQ ID No. 2 or a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto. In one embodiment, said CDR3 comprises or consists of the amino acid sequence SEQ ID No. 3 or a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto.

In another embodiment, the V_H single domain antibody comprises or consists of a polypeptide sequence as shown for any one of V_H single domain antibodies 1.1 to 1.50 as shown in Table 1 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. Thus, the V_H single domain antibody comprises or consists of an amino acid sequence selected from SEQ ID Nos. 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216 or 220 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In

one embodiment, the V_H single domain antibody comprises or consists of SEQ ID No. 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216 or 220 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In one embodiment, the V_H single domain antibody comprises or consists of SEQ ID No. 4 or SEQ ID No. 176 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In one embodiment, said sequence homology is at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

Table 1 Full length sequences and CDR sequences of V_H single domain antibodies

Name	CDR1 sequence of V_H	CDR2 sequence of V_H	CDR3 sequence of V_H	Full length V_H sequence with formula 1: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4
1.1	SEQ ID NO: 1 DHAMH	SEQ ID NO: 2 GISWNS GSMGYA DSVKD	SEQ ID NO: 3 EKGPGLT GSTADYY GLDV	SEQ ID NO: 4 EVQLLESGGGSVQPGRSLRLSCAASG FTFDDHAMHWVRQAPGKGLEWVSGI SWNSGSMGYADSVKDRFTISRDNAKS SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDVWGQGTMVTVSS
1.2	SEQ ID NO: 5 DYAMH	SEQ ID NO: 6 GISWNG GSMGYA ASVKG	SEQ ID NO: 7 DKGPGLIG STADYYGL DV	SEQ ID NO: 8 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVKDKGPG LIGSTADYYGLDVWGQGTTVTVSS
1.3	SEQ ID NO:9 DYAMH	SEQ ID NO:10 GISWNS GSMGYA DSVKD	SEQ ID NO:11 DKGPGLIG STADYHGL DV	SEQ ID NO: 12 EVQLLESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGREWVSGI SWNSGSMGYADSVKDRFTISRDNAKN SLYLQMNSLRAEDTALYYCVKDKGPG LIGSTADYHGLDVWGQGTTVTVSS
1.4	SEQ ID NO: 13 DYAMH	SEQ ID NO: 14 GISWNG GSMGYA ESVKG	SEQ ID NO: 15 DKGPGLT GTTADYY GMDV	SEQ IS NO: 16 EVQLLESGGGLVQPGRSLRLSCAASG FTFADYAMHWVRQAPGKGREWVSGI SWNGGSMGYAESVKGRFTISRDNAK NSLYLQMNSLRAEDSALYYCVKDKGP GLTGTTADYYGMDVWGQGTTVTVSS
1.5	SEQ ID NO: 17 DYAMH	SEQ ID NO: 18 GISWNG GSMGYA DSVKD	SEQ ID NO: 19 DKGPGLIG STADYHGL DV	SEQ ID NO:20 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGLEWVSGI SWNGGSMGYADSVKDRFTISRDNAK NSLYLQMNRLRAEDTALYYCVKDKGP GLIGSTADYHGLDVWGQGTTVTVSS
1.6	SEQ ID NO: 21 SYAMH	SEQ ID NO: 22 GISWNS	SEQ ID NO: 23 DKGPGLT	SEQ ID NO: 24 EVQLVESGGGVVQPGRSLRLSCAASG FTFSSYAMHWVRQAPGKGREWVSGI

		GSMGYA ESVKG	GTTADYY GMDV	SWNSGSMGYAESVKGRFTISRDNAKN SLYLQMNSLRAEDSALYYCVKDKGPG LTGTTADYYGMDVWGQGTTVTVSS
1.7	SEQ ID NO: 25 DYAMH	SEQ ID NO: 26 GISWNG GSMGYA ESVKG	SEQ ID NO: 27 DKGPGLT GTTADYY GMDV	SEQ ID NO:28 EVQLVESGGGVIQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGREWVSGI SWNGGSMGYAESVKGRFTISRDNAQ NSLYLQMNSLRAEDSALYYCVKDKGP GLTGTTADYYGMDVWGQGTTVTVSS
1.8	SEQ ID NO: 29 DYAMH	SEQ ID NO: 30 GISWNS GSMGYA DSVKD	SEQ ID NO: 31 DKGPGLIG STADYHGL DV	SEQ ID NO: 32 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGLEWVSGI SWNSGSMGYADSVKDRFTISRDNAKN SLYLQMNSLRAEDTALYYCVKDKGPG LIGSTADYHGLDVWGQGTTVTVSS
1.9	SEQ ID NO: 33 DYAMH	SEQ ID NO: 34 GISWNG GSMGYA ESVKG	SEQ ID NO: 35 DKGPGLIG STADYYG MDV	SEQ ID NO:36 EVQLVESGGGCVQPGRSLRISCAASG FTFDDYAMHWVRQAPGKGREWVSGI SWNGGSMGYAESVKGRFTISRDNAK NSLYLQMNSLRAEDSALYYCVKDKGP GLIGSTADYYGMDVWGQGTTVTVSS
1.10	SEQ ID NO: 37 DYAMH	SEQ ID NO: 38 GISWNG GSMGYA ESVKG	SEQ ID NO: 39 DKGPGLT GTTADYY GMDV	SEQ ID NO:40 EVQLVESGGGLVQPGRSLRLSCAASG FTFADYAMHWVRQAPGKGREWVSGI SWNGGSMGYAESVKGRFTISRDNAK NSLYLQMNSLRAEDSALYYCVKDKGP GLTGTTADYYGMDVWGQGTTVTVSS
1.11	SEQ ID NO: 41 DYAMH	SEQ ID NO: 42 GISWNG GSMGYA ESVKG	SEQ ID NO: 43 DKGPGLIG STADYYGL DV	SEQ ID NO: 44 EVQLLESGGGFVQPGRSLRISCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAESVKGRFTISRDNAKN SLYLKMNSLRVEDTALYYCVKDKGPG LIGSTADYYGLDVWGQGTTVTVSS
1.12	SEQ ID NO: 45 DYAMH	SEQ ID NO: 46 GISWNG GSMGYA DSVKD	SEQ ID NO: 47 DKGPGLT GSTADYH GMDV	SEQ ID NO:48 QVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGREWVSGI SWNGGSMGYADSVKDRFTISRDNAK NSLYLQMNSLRAEDTALYYCVKDKGP GLTGSTADYHGMDVWGQGTTVTVSS
1.13	SEQ ID NO: 49 DYAMH	SEQ ID NO: 50 GISWNG GSMGYA ASVKG	SEQ ID NO: 51 DKGPGLIG STADYYGL DV	SEQ ID NO:52 QVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAQN SLYLQMNSLRAEDTALYYCVKDKGPG LIGSTADYYGLDVWGQGTTVTVSS
1.14	SEQ ID NO: 53 GYAMH	SEQ ID NO: 54 GISWNS GSMGYA ESVKG	SEQ ID NO: 55 DKGPGLT GSTADYY GMDV	SEQ ID NO: 56 EVQLVESGGGLVQPGRSLRLSCAASG FTFDGYAMHWVRQAPGKGREWVSGI SWNSGSMGYAESVKGRFTISRDNAKN SLYLQMNSLRAEDSALYYCVKDKGPG LTGSTADYYGMDVWGQGTTVTVSS
1.15	SEQ ID NO: 57 DYAMH	SEQ ID NO: 58 GISWNS	SEQ ID NO: 59 DKGPGLIG	SEQ ID NO: 60 EVQLLESGGGLVQPGRSLRLSCTASG FTFDDYAMHWVRQAPGKGREWVSGI

		GSMGYA ESVKG	STADYYG MDV	SWNSGSMGYAESVKGRFTISRDNAKN SLYLQMNSLRAEDSALYYCVKDKGPG LIGSTADYYGMDVWGQGTTVTVSS
1.16	SEQ ID NO: 61 DYAMH	SEQ ID NO: 62 GISWNG GSMGYA ESVKG	SEQ ID NO: 63 DKGPGLIG STADYYG MDV	SEQ ID NO: 64 QVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKGREWVSGI SWNGGSMGYAESVKGRFTISRDNAK NSLYLQMNSLRAEDSALYYCVKDKGP GLIGSTADYYGMDVWGQGTTVTVSS
1.17	SEQ ID NO: 65 DYAMH	SEQ ID NO: 66 GISWNS GSMGYA ASVKD	SEQ ID NO: 67 DKGPGLIG STADYHGL DV	SEQ ID NO: 68 EVQLLESGGGLVQPGGSLRLSCAASG FTFDDYAMHWVRQAPGKGLEWVSGI SWNSGSMGYAASVKDRFTISRDNAKN SLYLQMNSLTTEDTALYYCVKDKGPGL IGSTADYHGLDVWGQGTTVTVSS
1.18	SEQ ID NO: 69 DYAMH	SEQ ID NO: 70 GISWNG GSMGYA ASVKG	SEQ ID NO: 71 DKGPGLIG STADYYGL DV	SEQ ID NO: 72 QVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVKDKGPG LIGSTADYYGLDVWGQGTTVTVSS
1.19	SEQ ID NO: 73 DYAMH	SEQ ID NO: 74 GISWNG GSMGYA DSVKG	SEQ ID NO: 75 EKGPGLT GSTADYY GLDV	SEQ ID NO: 76 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDVWGQGTMVTVSS
1.20	SEQ ID NO: 77 DYAMH	SEQ ID NO: 78 GISWNG GSMGYA DSVKG	SEQ ID NO: 79 EKGPGLT GSTADYY GLDV	SEQ ID NO: 80 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDVWGQGTMVTVSS
1.21	SEQ ID NO: 101 DYAMH	SEQ ID NO: 102 GISWNG GSMGYA ASVKG	SEQ ID NO: 103 EKGPGLT GSTADYY GLDV	SEQ ID NO: 104 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDVWGQGTMVTVSS
1.22	SEQ ID NO: 105 DYAMH	SEQ ID NO: 106 GISWNG GSMGYA ASVKG	SEQ ID NO: 107 EKGPGLT GSTADYY GLDA	SEQ ID NO: 108 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.23	SEQ ID NO: 109 DYAMH	SEQ ID NO: 110 GISWNS GSMGYA ASVKG	SEQ ID NO: 111 EKGPGLT GSTADYY GLDV	SEQ ID NO: 112 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNSGSMGYAASVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDVWGQGTMVTVSS
1.24	SEQ ID NO: 113 DYAMH	SEQ ID NO: 114 GISWNS	SEQ ID NO: 115 EKGPGLT	SEQ ID NO: 116 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS

		GSMGYA ASVKG	GSTADYY GLDA	WNSGSMGYAASVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.25	SEQ ID NO: 117 DYAMH	SEQ ID NO: 118 GISWNG GSQGYA ASVKG	SEQ ID NO: 119 EKGPGLT GSTADYY GLDA	SEQ ID NO: 120 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSQGYAASVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.26	SEQ ID NO: 121 DYAMH	SEQ ID NO: 122 GISWNG GSMGYA DSVKG	SEQ ID NO: 123 EKGPGLT GSTADYY GLDA	SEQ ID NO: 124 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSMGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.27	SEQ ID NO: 125 DYAMH	SEQ ID NO: 126 GISWNG GSRGYA ASVKG	SEQ ID NO: 127 EKGPGLT GSTADYY GLDA	SEQ ID NO: 128 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSRGYAASVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.28	SEQ ID NO: 129 DYAMH	SEQ ID NO: 130 GISWNA GSMGYA ASVKG	SEQ ID NO: 131 EKGPGLT GSTADYY GLDA	SEQ ID NO: 132 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNAGSMGYAASVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.29	SEQ ID NO: 133 DYAMH	SEQ ID NO: 134 GISWNS GSMGYA DSVKG	SEQ ID NO: 135 EKGPGLT GSTADYY GLDV	SEQ ID NO: 136 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNSGSMGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDVWGQGTMVTVSS
1.30	SEQ ID NO: 137 DYAGH	SEQ ID NO: 138 GISWNG GSMGYA ASVKG	SEQ ID NO: 139 EKGPGLT GSTADYY GLDA	SEQ ID NO: 140 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAGHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.31	SEQ ID NO: 141 DYALH	SEQ ID NO: 142 GISWNG GSMGYA ASVKG	SEQ ID NO: 143 EKGPGLT GSTADYY GLDA	SEQ ID NO: 144 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNGGSMGYAASVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.32	SEQ ID NO: 145 DYAMH	SEQ ID NO: 146 GISWNS GSMGYA DSVKG	SEQ ID NO: 147 EKGPGLT GSTADYY GLDA	SEQ ID NO: 148 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNSGSMGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.33	SEQ ID NO: 149 DYAMH	SEQ ID NO: 150 GISWNG	SEQ ID NO: 151 EKGPGLT	SEQ ID NO: 152 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS

		GSYGYA DSVKG	GSTADYY GLDA	WNGGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.34	SEQ ID NO: 153 DYAMH	SEQ ID NO: 154 GISWNG GSQGYA DSVKG	SEQ ID NO: 155 EKGPGLT GSTADYY GLDA	SEQ ID NO: 156 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSQGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.35	SEQ ID NO: 157 DYAMH	SEQ ID NO: 158 GISWNG GSKGYA DSVKG	SEQ ID NO: 159 EKGPGLT GSTADYY GLDA	SEQ ID NO: 160 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNGGSKGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.36	SEQ ID NO: 161 DYAMH	SEQ ID NO: 162 GISWNA GSMGYA DSVKG	SEQ ID NO: 163 EKGPGLT GSTADYY GLDA	SEQ ID NO: 164 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAMHWVRQAPGKDLEWVSGIS WNAGSMGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.37	SEQ ID NO: 165 DYAFH	SEQ ID NO: 166 GISWNG GSMGYA DSVKG	SEQ ID NO: 167 EKGPGLT GSTADYY GLDA	SEQ ID NO: 168 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNGGSMGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.38	SEQ ID NO: 169 DYALH	SEQ ID NO: 170 GISWNG GSMGYA DSVKG	SEQ ID NO: 171 EKGPGLT GSTADYY GLDA	SEQ ID NO: 172 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNGGSMGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.39	SEQ ID NO: 173 DYALH	SEQ ID NO: 174 GISWNG GSYGYA DSVKG	SEQ ID NO: 175 EKGPGLT GSTADYY GLDA	SEQ ID NO: 176 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNGGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.40	SEQ ID NO: 177 DYALH	SEQ ID NO: 178 GISWNG GSQGYA DSVKG	SEQ ID NO: 179 EKGPGLT GSTADYY GLDA	SEQ ID NO: 180 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNGGSQGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.41	SEQ ID NO: 181 DYAFH	SEQ ID NO: 182 GISWNG GSYGYA DSVKG	SEQ ID NO: 183 EKGPGLT GSTADYY GLDA	SEQ ID NO: 184 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNGGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.42	SEQ ID NO: 185 DYAFH	SEQ ID NO: 186 GISWNG	SEQ ID NO: 187 EKGPGLT	SEQ ID NO: 188 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS

		GSQGYA DSVKG	GSTADYY GLDA	WNGGSQGYADSVKGRFTISRDNAKN SLYLQMNSLRAEDTALYYCVREKGPG LTGSTADYYGLDAWGQGTMVTVSS
1.43	SEQ ID NO: 189 DYAFH	SEQ ID NO: 190 GISWNA GSYGYA DSVKG	SEQ ID NO: 191 EKGPGLT GSTADYY GLDA	SEQ ID NO: 192 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNAGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.44	SEQ ID NO: 193 DYALH	SEQ ID NO: 194 GISWNS GSYGYA DSVKG	SEQ ID NO: 195 EKGPGLT GSTADYY GLDA	SEQ ID NO: 196 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNSGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.45	SEQ ID NO: 197 DYALH	SEQ ID NO: 198 GISWNA GSQGYA DSVKG	SEQ ID NO: 199 EKGPGLT GSTADYY GLDA	SEQ ID NO: 200 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNAGSQGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.46	SEQ ID NO: 201 DYALH	SEQ ID NO: 202 GISWNA GSYGYA DSVKG	SEQ ID NO: 203 EKGPGLT GSTADYY GLDA	SEQ ID NO: 204 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNAGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.47	SEQ ID NO: 205 DYAFH	SEQ ID NO: 206 GISWNS GSYGYA DSVKG	SEQ ID NO: 207 EKGPGLT GSTADYY GLDA	SEQ ID NO: 208 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNSGSYGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.48	SEQ ID NO: 209 DYALH	SEQ ID NO: 210 GISWNS GSQGYA DSVKG	SEQ ID NO: 211 EKGPGLT GSTADYY GLDA	SEQ ID NO: 212 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYALHWVRQAPGKDLEWVSGIS WNSGSQGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.49	SEQ ID NO: 213 DYAFH	SEQ ID NO: 214 GISWNS GSQGYA DSVKG	SEQ ID NO: 215 EKGPGLT GSTADYY GLDA	SEQ ID NO: 216 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNSGSQGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS
1.50	SEQ ID NO: 217 DYAFH	SEQ ID NO: 218 GISWNA GSQGYA DSVKG	SEQ ID NO: 219 EKGPGLT GSTADYY GLDA	SEQ ID NO: 220 EVQLVESGGGLVQPGRSLRLSCAASG FTFDDYAFHWVRQAPGKDLEWVSGIS WNAGSQGYADSVKGRFTISRDNAKNS LYLQMNSLRAEDTALYYCVREKGPGL TGSTADYYGLDAWGQGTMVTVSS

In one embodiment, the invention relates to an isolated single V_H domain antibody that binds to human PD-1 comprising a CDR3 sequence as shown Table 2 below or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence identity thereto. In one embodiment, said sequence homology is at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. In one embodiment, said sequence homology is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. In one embodiment, the V_H single domain antibody has a CDR3 sequence comprising SEQ ID No. 253 or a sequence having at least 70%, at least 80%, at least 90%, or at least 95% homology to SEQ ID No. 253. In one embodiment, said sequence homology is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

In one embodiment, the V_H single domain antibody has a CDR1 as shown in SEQ ID No. 251 or SEQ ID No. 251 with 1 or 2 amino acid substitutions, a CDR2 as shown in SEQ ID No. 252 or SEQ ID No. 252 with 1 to 5 amino acid substitutions and a CDR3 as shown in SEQ ID No. 253 or SEQ ID No. 253 with 1 to 5 amino acid substitutions.

In one embodiment, the V_H single domain antibody comprises a combination of CDR1, 2 and 3 sequences selected from the CDR1, 2 and 3 sequences in Table 2 or combinations thereof. In one embodiment, the V_H single domain antibody comprises a set of CDR1, 2 and 3 sequences selected from the sets of CDR1, 2 and 3 sequences as shown for the any of the clones in Table 2. Thus, in one aspect, the isolated single domain antibody comprises a CDR1, CDR2 and CDR3 selected from CDRs 1-3 of full length sequences SEQ ID Nos: 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462.

Accordingly, in one embodiment, the V_H single domain antibody comprises CDR1 having SEQ ID No. 251, CDR2 having SEQ ID No. 252 and CDR3 having SEQ ID No. 253 (CDRs of SEQ ID NO. 254) and so forth. Thus, the V_H single domain antibody comprises one of the following CDR combinations: SEQ ID Nos. 251, 252, 253; SEQ ID Nos. 255, 256, 257; SEQ ID Nos. 259, 260, 261; SEQ ID Nos. 263, 264, 265; SEQ ID Nos. 267, 268, 269; SEQ ID Nos. 271, 272, 273; SEQ ID Nos. 275, 276, 277; SEQ ID Nos. 279, 280, 281; SEQ ID Nos. 283, 284, 285; SEQ ID Nos. 287, 288, 289; SEQ ID Nos. 291, 292, 293; SEQ ID Nos. 295, 296, 297; SEQ ID Nos. 299, 300, 301; SEQ ID Nos. 303, 304, 305; SEQ ID Nos. 307, 308, 309; SEQ ID Nos. 311, 312, 313; SEQ ID Nos. 315, 316, 317; SEQ ID Nos. 319, 320, 321, SEQ ID Nos. 323, 324, 325; SEQ ID Nos. 327, 328, 329; SEQ ID Nos. 331, 332, 333; SEQ ID Nos. 335, 336, 337; SEQ ID Nos. 339, 340, 341; SEQ ID Nos. 343, 344, 345; SEQ ID

Nos. 347, 348, 349; SEQ ID Nos. 351, 352, 353; SEQ ID Nos. 355, 356, 357; SEQ ID Nos. 359, 360, 361; SEQ ID Nos. 363, 364, 365; SEQ ID Nos. 367, 368, 369; SEQ ID Nos. 371. 372, 373; SEQ ID Nos. 275, 376, 377; SEQ ID Nos. 379, 380, 381; SEQ ID Nos. 383, 384, 385; SEQ ID Nos. 387, 388, 389; SEQ ID Nos. 391, 392, 393; SEQ ID Nos. 395, 396, 397; SEQ ID Nos. 399, 400, 401; SEQ ID Nos. 403, 404, 405; SEQ ID Nos. 407, 408, 409; SEQ ID Nos. 411, 412, 413; SEQ ID Nos. 415, 416, 417; SEQ ID Nos. 419, 420, 421; SEQ ID Nos. 423, 424, 425; SEQ ID Nos. 427, 428, 429; SEQ ID Nos. 431, 432, 433; SEQ ID Nos. 435, 436, 437; SEQ ID Nos. 439, 440, 441; SEQ ID Nos. 443, 444, 445; SEQ ID Nos. 447, 448, 449; SEQ ID Nos. 451, 452, 453; SEQ ID Nos. 455, 456, 457 or SEQ ID Nos. 459, 460, 461.

In another embodiment, said CDR1 comprises or consists of the amino acid sequence SEQ ID NO. 251 or a sequence with at least at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto. In one embodiment, said CDR2 comprises or consists of the amino acid sequence SEQ ID No. 252 or a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto. In one embodiment, said CDR3 comprises or consists of the amino acid sequence SEQ ID No. 253 or a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% 96%, 97%, 98% or 99% homology thereto.

In another embodiment, the V_H single domain antibody comprises or consists of a polypeptide sequence as shown for any one of V_H single domain antibodies 2.1 to 2.53 as shown in Table 2 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. Thus, the V_H single domain antibody comprises or consists of an amino acid sequence selected from SEQ ID Nos. 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In one embodiment, the V_H single domain antibody comprises or consists of SEQ ID No. 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In one embodiment, the V_H single domain antibody comprises or consists of 254 or 298 or a sequence with at least 60%, 70%, 80%, 90%, 95% or more sequence homology thereto. In one embodiment,

said sequence homology is at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

Table 2 Full length sequences and CDR sequences of V_H single domain antibodies

Name	CDR1 sequence of VH	CDR2 sequence of VH	CDR3 sequence of VH	Full length VH sequence
2.1	SEQ ID NO: 251 DYAMS	SEQ ID NO: 252 GITWNGGSTGYADSVKD	SEQ ID NO: 253 DKYSYAWSYDGFDI	SEQ ID NO: 254 EVQLVESGGGVVRPGGSLRLSCAASGFTFDDYAMSWVRQAPGKGLEWVSGITWNGGSTGYADSVKDRFTISRDNAKNSLYLQMNSLRAEDTALYYCVRDKYSYAWSYDGFDIWGQGTMVTVSS
2.2	SEQ ID NO: 255 DYGMS	SEQ ID NO: 256 GISRNGGSAGYSDSAKD	SEQ ID NO: 257 EKYSSGWSYDDFDI	SEQ ID NO: 258 EVQLLESGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQPPGKGLEWVSGISRNGGSAGYSDSAKDRFTISRDNAKNSLYLQMNSLRADDTAMYYCAREKYSSGWSYDDFDIWGQGTMVTVSS
2.3	SEQ ID NO: 259 DYGMS	SEQ ID NO: 260 GISRNGGSAGYSDSAKD	SEQ ID NO: 261 EKYSSGWSYDDFDI	SEQ ID NO: 262 QVQLVESGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQSPGKGLEWVSGISRNGGSAGYSDSAKDRFTISRDNAKNSLYLQMNSLRADDTAMYYCAREKYSSGWSYDDFDIWGQGTMVTVSS
2.4	SEQ ID NO: 263 DYGMS	SEQ ID NO: 264 GISRNGGSAGYSDSAKD	SEQ ID NO: 265 EKYSSGWSYDDFDI	SEQ ID NO: 266 QVQLVESGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQPPGKGLEWVSGISRNGGSAGYSDSAKDRFTISRDNAKNSLYLQMNSLRADDTAMYYCAREKYSSGWSYDDFDIWGQGTMVTVSS
2.5	SEQ ID NO: 267 DYGMS	SEQ ID NO: 268 GISRNGGSAGYSDSAKD	SEQ ID NO: 269 EKYSSGWSYDDFDI	SEQ ID NO: 270 EVQLVESGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQPPGKGLEWVSGISRNGGSAGYSDSAKDRFTISRDNAKNSLYLQMNSLRADDTAMYYCAREKYSSGWSYDDFDIWGQGTMVTVSS
2.6	SEQ ID NO: 271 DYGMS	SEQ ID NO: 272 GISRNGGSTGYADSVKD	SEQ ID NO: 273 DPYSSGWSYDSFDI	SEQ ID NO: 274 QVQLVESGGGVVRLGGSLRLSCAASGFSFVDYGMSWVRQAPGQGLEWVSGISRNGGSTGYADSVKDRFTISRDNAKNTLYLQMNSLRAEDTALYYCARDPYSSGWSYDSFDIWGQGTMVTVSS
2.7	SEQ ID NO: 275 DYGMS	SEQ ID NO: 276 GISRNGGSTGYTASVKD	SEQ ID NO: 277 EKYSSGWSYDDFDI	SEQ ID NO: 278 QVQLVESGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQAPGKGLEWVSGISRNGGSTGYTASVKDRFTISRDNAKNSLYLQMNSLRADDTAMYYCAREKYSSGWSYDDFDIWGQGTMVTVSS
2.8	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 282

	NO: 279 DYAMS	NO: 280 GISWNG GSAGYA DSVKD	NO: 281 DPHSSA WSYDAF DI	QVQLVESGGGLVQPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGISWNGG SAGYADSVKDRFTISRDNAKNSLYLQMNS LRAEDTALYYCARDPHSSAWSYDAFDIWG QGTMVTVSS
2.9	SEQ ID NO: 283 DYAMS	SEQ ID NO: 284 GISWNG GSKGYA DSVKD	SEQ ID NO: 285 DPYSGA WSYDAF DI	SEQ ID NO: 286 QVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGISWNGG SKGYADSVKDRFTISRDNAKNSLYLQMNS LRAEDTALYYCARDPYSGAWSYDAFDIWG QGTMVTVSS
2.10	SEQ ID NO: 287 DYAMS	SEQ ID NO: 288 GISWNG GSTGYA DSVKD	SEQ ID NO: 289 DPYSGA WSYDAF DI	SEQ ID NO: 290 QVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGISWNGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCARDPYSGAWSYDAFDIWG QGTMVTVSS
2.11	SEQ ID NO: 291 NYAMS	SEQ ID NO: 292 GITWNG GSTGYA DSVKD	SEQ ID NO: 293 DKYSYA WSYDTF DI	SEQ ID NO: 294 QVQLVESGGGVVRPGGSLRLSCAASGFTF DNYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCARDKYSYAWSYDTFDIRGQ GTMVTVSS
2.12	SEQ ID NO: 295 DYAMS	SEQ ID NO: 296 GITWNAG STGYADS VKG	SEQ ID NO: 297 DKYSYA WSYDDF DI	SEQ ID NO: 298 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNAGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.13	SEQ ID NO: 299 DYAMS	SEQ ID NO: 300 GITWNR GSTGYA DSVKG	SEQ ID NO: 301 DKYSYA WSYDDF DI	SEQ ID NO: 302 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNRGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.14	SEQ ID NO: 303 DYAFS	SEQ ID NO: 304 GITWNG GSTGYA DSVKG	SEQ ID NO: 305 DKYSYA WSYDGF DI	SEQ ID NO: 306 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAFSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.15	SEQ ID NO: 307 DYAQS	SEQ ID NO: 308 GITWNG GSTGYA DSVKG	SEQ ID NO: 309 DKYSYA WSYDGF DI	SEQ ID NO: 310 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAQSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.16	SEQ ID NO: 311 DYANS	SEQ ID NO: 312 GITWNG GSTGYA DSVKG	SEQ ID NO: 313 DKYSYA WSYDGF DI	SEQ ID NO: 314 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYANSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.17	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 318

	NO: 315 DYAGS	NO: 316 GITWNG GSTGYA DSVKG	NO: 317 DKYSYA WSYDGF DI	EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAGSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.18	SEQ ID NO: 319 DYAES	SEQ ID NO: 320 GITWNG GSTGYA DSVKG	SEQ ID NO: 321 DKYSYA WSYDGF DI	SEQ ID NO: 322 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAESWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.19	SEQ ID NO: 323 DYAWS	SEQ ID NO: 324 GITWNG GSTGYA DSVKG	SEQ ID NO: 325 DKYSYA WSYDGF DI	SEQ ID NO: 326 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAWSWVRQAPGKGLEWVSGITWNGG STGYADSVKGRFTISRDNAKNSLYLQMNS LRAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.20	SEQ ID NO: 327 DYAVS	SEQ ID NO: 328 GITWNG GSTGYA DSVKG	SEQ ID NO: 329 DKYSYA WSYDGF DI	SEQ ID NO: 330 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAVSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.21	SEQ ID NO: 331 DYALS	SEQ ID NO: 332 GITWNG GSTGYA DSVKG	SEQ ID NO: 333 DKYSYA WSYDGF DI	SEQ ID NO: 334 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYALSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.22	SEQ ID NO: 335 DYASS	SEQ ID NO: 336 GITWNG GSTGYA DSVKG	SEQ ID NO: 337 DKYSYA WSYDGF DI	SEQ ID NO: 338 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYASSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.23	SEQ ID NO: 339 DYARS	SEQ ID NO: 340 GITWNG GSTGYA DSVKG	SEQ ID NO: 341 DKYSYA WSYDGF DI	SEQ ID NO: 342 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYARSWVRQAPGKGLEWVSGITWNGGS TGYADSVKGRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.24	SEQ ID NO: 343 DYAMS	SEQ ID NO: 344 GITWNSG STGYADS VKD	SEQ ID NO: 345 DKYSYA WSYDDF DI	SEQ ID NO: 346 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNSGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.25	SEQ ID NO: 347 DYAMS	SEQ ID NO: 348 GITWNQ GSTGYA DSVKD	SEQ ID NO: 349 DKYSYA WSYDDF DI	SEQ ID NO: 350 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNQG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.26	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 354

	NO: 351 DYAMS	NO: 352 GITWNH GSTGYA DSVKD	NO: 353 DKYSYA WSYDVF DI	EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNHGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDVFDIWGQ GTMVTVSS
2.27	SEQ ID NO: 355 DYAMS	SEQ ID NO: 356 GITWNAG STGYADS VKD	SEQ ID NO: 357 DKYSYA WSYDDF DI	SEQ ID NO: 358 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNAGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.28	SEQ ID NO: 359 DYAMS	SEQ ID NO: 360 GITWNG GSTGYA DSVKG	SEQ ID NO: 361 DKYSYA WSYDVF DI	SEQ ID NO: 362 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKGRFTISRDNAKNSLYLQMNS LRAEDTALYYCVRDKYSYAWSYDVFDIWG QGTMVTVSS
2.29	SEQ ID NO: 363 DYAMS	SEQ ID NO: 364 GITWNG GSTGYA DSVKG	SEQ ID NO: 365 DKYSYA WSYDDF DI	SEQ ID NO: 366 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKGRFTISRDNAKNSLYLQMNS LRAEDTALYYCVRDKYSYAWSYDDFDIWG QGTMVTVSS
2.30	SEQ ID NO: 367 DYAMS	SEQ ID NO: 368 GITWNKG STGYADS VKD	SEQ ID NO: 369 DKYSYA WSYDDF DI	SEQ ID NO: 370 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNKGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.31	SEQ ID NO: 371 DYAMS	SEQ ID NO: 372 GITWNR GSTGYA DSVKD	SEQ ID NO: 373 DKYSYA WSYDDF DI	SEQ ID NO: 374 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNRGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS
2.32	SEQ ID NO: 375 DYAIS	SEQ ID NO: 376 GITWNG GSTGYA DSVKD	SEQ ID NO: 377 DKYSYA WSYDGF DI	SEQ ID NO: 378 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAISWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.33	SEQ ID NO: 379 DYATS	SEQ ID NO: 380 GITWNG GSTGYA DSVKD	SEQ ID NO: 381 DKYSYA WSYDGF DI	SEQ ID NO: 382 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYATSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.34	SEQ ID NO: 383 DYANS	SEQ ID NO: 384 GITWNG GSTGYA DSVKD	SEQ ID NO: 385 DKYSYA WSYDGF DI	SEQ ID NO: 386 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYANSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.35	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 390

	NO: 387 DYADS	NO: 388 GITWNG GSTGYA DSVKD	NO: 389 DKYSYA WSYDGF DI	EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYADSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.36	SEQ ID NO: 391 DYASS	SEQ ID NO: 392 GITWNG GSTGYA DSVKD	SEQ ID NO: 393 DKYSYA WSYDGF DI	SEQ ID NO: 394 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYASSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.37	SEQ ID NO: 395 DYALS	SEQ ID NO: 396 GITWNG GSTGYA DSVKD	SEQ ID NO: 397 DKYSYA WSYDGF DI	SEQ ID NO: 398 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYALSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.38	SEQ ID NO: 399 DYAGS	SEQ ID NO: 400 GITWNG GSTGYA DSVKD	SEQ ID NO: 401 DKYSYA WSYDGF DI	SEQ ID NO: 402 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAGSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.39	SEQ ID NO: 403 DYAES	SEQ ID NO: 404 GITWNG GSTGYA DSVKD	SEQ ID NO: 405 DKYSYA WSYDGF DI	SEQ ID NO: 406 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAESWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.40	SEQ ID NO: 407 DYAWS	SEQ ID NO: 408 GITWNG GSTGYA DSVKD	SEQ ID NO: 409 DKYSYA WSYDGF DI	SEQ ID NO: 410 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAWSWVRQAPGKGLEWVSGITWNGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.41	SEQ ID NO: 411 DYARS	SEQ ID NO: 412 GITWNG GSTGYA DSVKD	SEQ ID NO: 413 DKYSYA WSYDGF DI	SEQ ID NO: 414 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYARSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.42	SEQ ID NO: 415 DYAFS	SEQ ID NO: 416 GITWNG GSTGYA DSVKD	SEQ ID NO: 417 DKYSYA WSYDGF DI	SEQ ID NO: 418 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAFSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.43	SEQ ID NO: 419 DYAVS	SEQ ID NO: 420 GITWNG GSTGYA DSVKD	SEQ ID NO: 421 DKYSYA WSYDGF DI	SEQ ID NO: 422 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAVSWVRQAPGKGLEWVSGITWNGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.44	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 426

	NO: 423 DYAMS	NO: 424 GITWTGG STGYADS VKD	NO: 425 DKYSYA WSYDGF DI	EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWTGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.45	SEQ ID NO: 427 DYAMS	SEQ ID NO: 428 GITWSG GSTGYA DSVKD	SEQ ID NO: 429 DKYSYA WSYDGF DI	SEQ ID NO: 430 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWSGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.46	SEQ ID NO: 431 DYAMS	SEQ ID NO: 432 GITWPG GSTGYA DSVKD	SEQ ID NO: 433 DKYSYA WSYDGF DI	SEQ ID NO: 434 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWPGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.47	SEQ ID NO: 435 DYAMS	SEQ ID NO: 436 GITWIGG STGYADS VKD	SEQ ID NO: 437 DKYSYA WSYDGF DI	SEQ ID NO: 438 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWIGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.48	SEQ ID NO: 439 DYAMS	SEQ ID NO: 440 GITWLGG STGYADS VKD	SEQ ID NO: 441 DKYSYA WSYDGF DI	SEQ ID NO: 442 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWLGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.49	SEQ ID NO: 443 DYAMS	SEQ ID NO: 444 GITWNG GSTGYA DSVKG	SEQ ID NO: 445 DKYSYA WSYDGF DI	SEQ ID NO: 446 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKGRFTISRDNAKNSLYLQMNS LRAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.50	SEQ ID NO: 447 DYAMS	SEQ ID NO: 448 GITWKG GSTGYA DSVKD	SEQ ID NO: 449 DKYSYA WSYDGF DI	SEQ ID NO: 450 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWKGGS TGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.51	SEQ ID NO: 451 DYAMS	SEQ ID NO: 452 GITWRG GSTGYA DSVKD	SEQ ID NO: 453 DKYSYA WSYDGF DI	SEQ ID NO: 454 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWRGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDGFDIWG QGTMVTVSS
2.52	SEQ ID NO: 455 DYAMS	SEQ ID NO: 456 GITWNG GSTGYA DSVKD	SEQ ID NO: 457 DKYSYA WSYDVF DI	SEQ ID NO: 458 EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDVFDIWGQ GTMVTVSS
2.53	SEQ ID	SEQ ID	SEQ ID	SEQ ID NO: 462

	NO: 459 DYAMS	NO: 460 GITWNG GSTGYA DSVKD	NO: 461 DKYSYA WSYDDF DI	EVQLVESGGGVVRPGGSLRLSCAASGFTF DDYAMSWVRQAPGKGLEWVSGITWNGG STGYADSVKDRFTISRDNAKNSLYLQMNSL RAEDTALYYCVRDKYSYAWSYDDFDIWGQ GTMVTVSS

In some embodiments, the invention provides a V_H single domain antibody that is a variant of any of the above single V_H domain antibodies having one or more amino acid substitutions, deletions, insertions or other modifications, and which retains a biological function of the single domain antibody. Thus, variant V_H single domain antibody can be sequence engineered. Modifications include at least one substitution, deletion or insertion of one or more codons encoding the single domain antibody or polypeptide that results in a change in the amino acid sequence as compared with the native sequence V_H single domain antibody or polypeptide. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 10, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids or 1 to 20 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence. A variant of a V_H single domain antibody described herein has at least 75%, 76%, 77%, 78%, 79%, 8 0%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology to the non-variant molecule, preferably at least 95%, 96%, 97%, 98% or 99% sequence homology.

In one embodiment, the modification is a conservative sequence modification. As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine,

methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of a single domain antibody of the invention can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function (i.e., the functions set forth in (c) through (l) above) using the functional assays described herein.

In some embodiments, the invention provides a V_H single domain antibody that is a variant of a single domain antibody selected from those shown in Table 1 or 2 that comprises one or more sequence modification and has improvements in one or more of a property such as binding affinity, specificity, thermostability, expression level, effector function, glycosylation, reduced immunogenicity, or solubility as compared to the unmodified single domain antibody.

In one embodiment, modifications can be made to decrease the immunogenicity of the single domain antibody. For example, one approach is to revert one or more framework residues to the corresponding human germline sequence. More specifically, a single domain antibody that has undergone somatic mutation may contain framework residues that differ from the germline sequence from which the single domain antibody is derived. Such residues can be identified by comparing the single domain antibody framework sequences to the germline sequences from which the single domain antibody is derived.

To return one or more of the amino acid residues in the framework region sequences to their germline configuration, the somatic mutations can be "backmutated" to the germline sequence by, for example, site-directed mutagenesis or PCR-mediated mutagenesis.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody.

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycoslated antibody can be made (i.e., the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen.

A skilled person will know that there are different ways to identify, obtain and optimise the antigen binding molecules as described herein, including in vitro and in vivo expression libraries. This is further described in the examples. Optimisation techniques known in the art, such as display (e.g., ribosome and/or phage display) and / or mutagenesis (e.g., error-prone mutagenesis) can be used. The invention therefore also comprises sequence optimised variants of the single domain antibodies described herein.

In one embodiment, the variant V_H single domain antibody is selected from any one of SEQ ID Nos. 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220 or 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462, but comprises one or more amino acid substitutions, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid substitutions compared to these sequences. In one embodiment, the one or more amino acid substitution is in one or more of the framework areas. In another embodiment, the one or more amino acid substitution is in one or more of the CDRs. In one embodiment, the amino acid substitutions are in the framework and in the CDR sequences. In one embodiment, the single domain antibody comprises or consists of SEQ ID No. 4 or 136 or a sequence which comprises one or more amino acid substitutions, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid substitutions.

In one embodiment, the V_H single domain antibody comprises SEQ ID No. 4 with amino acid substitutions at one or more or all of the following positions: 5L, 32H, 44G, 55S, 66D, 77S and/or 105T. In one embodiment, the V_H single domain antibody comprises SEQ ID No. 4 with amino acid substitutions selected from one of the following:

a) 5L→V, 11S→L, 32H→Y, 44G→D, 55S→G, 62D→A, 66D→G and 77S→N or
b) 5L→V, 11S→L, 32H→Y, 44G→D, 55S→G, 66D→G and 77S→N or
c) 1E→Q, 5L→V, 11S→L, 32H→Y, 44G→D, 55S→G, 66D→G, 77S→N, 98R→K, 99E→D, 105T→I and 102M→T.

In one embodiment, the V_H single domain antibody comprises SEQ ID No. 136 with amino acid substitutions at one or more or all of the following positions: M34, M58, V102, V116. In one embodiment, the V_H single domain antibody comprises SEQ ID No 136 with amino acid substitutions selected from one of the following:

a) M34→L, M58→Y, V116A (Humabody® 1.39);
b) M34→F, G54→ A, M58→Q, V102A (Humabody®1.50);

c) V116A (Humabody®1.26);

d) M34→L, M58→Q, V116A (Humabody®1.40).

In one embodiment, the V_H single domain antibody comprises SEQ ID No. 254 with amino acid substitutions at one or more or all of the following positions: G 109, D66, G55. In one embodiment, the V_H single domain antibody comprises SEQ ID No 254 with amino acid substitutions selected from one of the following:

a) G109→D, D66→G, G55→A (Humabody® 2.12);

b) G109→D, G55→A (Humabody® 2.27);

c) G109→D (Humabody® 2.53);

d) G109→D, D66→G (Humabody® 2.29);

e) G109→V, D66→G (Humabody® 2.28).

In one embodiment, when Q is found at position 1, it is changed to E or another residue.

The numbering used above is based on the actual position of the residue in the molecule.

Thus, these amino acid changes can typically be made without altering the biological activity, function, or other desired property of the polypeptide, such as its affinity or its specificity for antigen. In general, single amino acid substitutions in nonessential regions of a polypeptide do not substantially alter biological activity. Furthermore, substitutions of amino acids that are similar in structure or function are less likely to disrupt the polypeptides' biological activity. Abbreviations for the amino acid residues that comprise polypeptides and peptides described herein, and conservative substitutions for these amino acid residues are shown in the Table below.

Amino Acid Residues and Examples of Conservative Amino Acid Substitutions

Original residue **Three letter code, single letter code**	**Conservative substitution**
Alanine, Ala, A	Gly, Ser
Arginine, Arg, R	Lys, His
Asparagine, Asn, N	Gln, His
Aspartic acid Asp, D	Glu, Asn
Cysteine, Cys, C	Ser, Ala
Glutamine, Gln, Q	Asn
Glutamic acid, Glu, E	Asp, Gln
Glycine, Gly, G	Ala
Histidein, His, H	Asn, Gln

Isoleucine, Ile, I	Leu, Val
Leucine, Leu, L	Ile, Val
Lysine, lys, K	Ar, His
Methionine, Met, M	Leu, Ile, Tyr
Phenylalanine, Phe, F	Tyr, Met, Leu
Proline, Pro, P	Ala
Serine, Ser, S	Thr
Threonine, Thr, T	Ser
Tryptophan, Trp, W	Tyr, Phe
Tyrosine, Tyr, Y	Try, Phe
Valine, Val, V	Ile, Leu

V_H single domain antibodies of the invention have shown excellent stability. Furthermore, V_H single domain antibodies of the invention also show high specificity for human PD-1 and fast on rates (see examples).

The V_H single domain antibodies of the invention preferably have KD and EC_{50} values as further described herein and as shown in the examples. In one embodiment, the V_H single domain antibodies specifically binding to a human PD-1 with a binding affinity of KD 10^{-8} to 10^{-10}. In another embodiment, the single domain antibody has an EC50 value in the subnanomolar range as determined in binding to CHO-PD-1 cell line.

The term "KD" refers to the "equilibrium dissociation constant" and refers to the value obtained in a titration measurement at equilibrium, or by dividing the dissociation rate constant (Koff) by the association rate constant (Kon). “KA” refers to the affinity constant. The association rate constant, the dissociation rate constant and the equilibrium dissociation constant are used to represent the binding affinity of an antibody to an antigen. Methods for determining association and dissociation rate constants are well known in the art. Using fluorescence-based techniques offers high sensitivity and the ability to examine samples in physiological buffers at equilibrium. Other experimental approaches and instruments such as a BIAcore® (biomolecular interaction analysis) assay can be used. In one embodiment, a single domain antibody has a KD value in the nanomolar or subnanomaolar range.

The present invention further provides an isolated nucleic acid encoding a single domain antibody of the present invention. Nucleic acid may include DNA and/or RNA. In one aspect, the present invention provides a nucleic acid that codes for a CDR, for example CDR3, a set of two or three CDRs or a V_H single domain antibody of the invention as shown in Table 1 or 2.

In one aspect, the invention thus also relates to a nucleic acid sequences comprising or consisting of a sequence selected from SEQ ID Nos. 81 to 100 or 221 to 250. These encode V_H single domain antibody as shown in Table 1.

In one aspect, the invention thus also relates to a nucleic acid sequences comprising or consisting of a sequence selected from SEQ ID Nos. 463 to 515. These encode V_H single domain antibody as shown in Table 2.

In one embodiment, the nucleic acid sequence has at least 60%, 70%, 80%, 90%, 95% or more sequence homology to one of the nucleic acid sequences described above selected from Table 2. In one embodiment, said sequence homology is at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%.

A nucleic acid according to the present invention may comprise DNA or RNA and may be wholly or partially synthetic or recombinantly produced. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

Furthermore, the invention relates to a nucleic acid construct comprising at least one nucleic acid as defined above. The construct may be in the form of a plasmid, vector, transcription or expression cassette.

The invention also relates to an isolated recombinant host cell comprising one or more nucleic acid construct as described above. The host cell may be a bacterial, viral, insect, plant, mammalian or other suitable host cell. In one embodiment, the cell is an E. coli cell. In another embodiment, the cell is a yeast cell. In another embodiment, the cell is a Chinese Hamster Ovary (CHO) cell.

In an embodiment, a method of making an anti-PD-1 single domain antibody as described herein is provided, wherein the method comprises culturing the host cell under conditions suitable for expression of the polynucleotide encoding the single domain antibody, and isolating the single domain antibody.

In another aspect, the invention provides antibodies or fragments thereof that bind to the same epitope on human PD-1 as any of the PD-1 single domain antibodies of the invention (i.e., antibodies that have the ability to cross-compete for binding to PD-1 with any of the single domain antibodies of the invention). In preferred embodiments, the reference antibody

for cross-competition studies can be single domain antibody 1.1 (SEQ ID No. 4) or 2.1 (SEQ ID No. 254).

Such cross-competing antibodies can be identified based on their ability to cross-compete with any of single domain antibodies 1.1 to 1.50 or 2.1 to 2.53 in standard PD-1 binding assays. For example, BIAcore analysis, ELISA assays or flow cytometry may be used to demonstrate cross-competition with the single domain antibodies of the current invention.

In one embodiment, the invention provides a binding agent capable of binding human PD-1 wherein any one of the single domain antibodies described above displaces the binding agent in a competitive assay. In one embodiment, said is single domain antibody SEQ ID No. 4. In some embodiments, the binding agent is an antibody, a functional fragment thereof, for example a single domain antibody, or an antibody mimetic protein. In another aspect, invention provides a binding agent capable of binding human PD-1 wherein the binding agent displaces any one of the single domain antibodies described above in a competitive assay. In one embodiment, said single domain antibody comprises SEQ ID No. 4, 176, 254 or 298. In another aspect, invention provides a binding agent capable of binding human PD-1 wherein the binding agent binds to essentially the same epitope as the single domain antibody of the invention.

In another aspect, the invention provides an isolated heavy chain only antibody comprising a V_H domain as described herein and set out in Table 1 or 2.

In one aspect, the invention relates to a binding agent comprising a single domain antibody according to the invention and at least a second moiety. Thus, the invention provides multifunctional molecules. In one embodiment, the at least second moiety is a binding molecule, for example selected from an antibody or antibody fragment (e.g., a Fab, F(ab')2, Fv, a single chain Fv fragment (scFv) or single domain antibody, for example a V_H domain) or antibody mimetic protein. In one embodiment, the at least second moiety is a V_H domain. In one embodiment, the single domain antibody of the invention can be linked to an antibody Fc region or fragment thereof, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region.

The binding agent may be multivalent, for example bivalent, or multiparatopic, for example biparatopic. Thus, the binding molecule may comprise a first V_H single domain antibody and $V_H(A)$ and a second V_H single domain antibody and $V_H(B)$ and thus has the following formula: $V_H(A)$- $V_H(B)$.

Each V_H comprises CDR and FR regions. Thus, the binding molecule may have the following formula: FR1(A)-CDR1(A)-FR2(A)-CDR2(A)-FR3(A)-CDR3(A)-FR4(A)-FR1(B)-

CDR1(B)-FR2(B)-CDR2(BA)-FR3(B)-CDR3(B)-FR4(B). The order of the immunoglobulin single variable domains A and B is not particularly limited, so that, within a polypeptide of the invention, immunoglobulin single variable domain A may be located N-terminally and immunoglobulin single variable domain B may be located C-terminally, or vice versa. The V_H domain antibodies are typically connected via a linker.

In one embodiment, the binding molecule is biparatopic. In one embodiment, the binding molecule is bispecific. Thus, in one aspect, the invention relates to a bispecific molecule comprising the single domain antibody described herein linked to a second functional moiety having a different binding specificity than said single domain antibody.

In one embodiment, biparatopic binding molecules are provided that comprise a first and a second binding molecule that bind to the target protein PD-1, but on different or overlapping sites. Complete or partial blocking can be seen in epitope binning studies. The first binding molecule is a single domain antibody according to the invention. In one embodiment, the second binding molecule is a PD-1 inhibitor that blocks the interaction of human PD-1 with one of its ligands. In one embodiment, the second binding molecule blocks the interaction of PD-1 with PD-L1. In one embodiment, the second binding molecule blocks the interaction of PD-1 with PD-L2. In one embodiment, the second binding molecule blocks the interaction of PD-1 with PD-L1 and PD-L2. The order of the first and second is binding molecule not particularly limited and can be reversed.

In one embodiment, the PD-1 inhibitor is an anti-PD-1 antibody chosen from Nivolumab®, Pembrolizumab® or Pidilizumab®. In some embodiments, the anti-PD-1 antibody is Nivolumab®. Alternative names for Nivolumab® include MDX- 1106, MDX-1106-04, ONO-4538, or BMS-936558. In other embodiments, the anti-PD-1 antibody is Pembrolizumab®. Pembrolizumab® (Trade name KEYTRUDA® formerly Lambrolizumab®, also known as Merck 3745, MK-3475 or SCH-900475) is a humanized IgG4 monoclonal antibody that binds to PD-1. In some embodiments, the anti-PD-1 antibody is Pidilizumab®. Pidilizumab® (CT-011; Cure Tech) is a humanized IgG1k monoclonal antibody that binds to PD-1.

In one embodiment, the PD-1 inhibitor is a V_H single domain antibody. Thus, another aspect relates to a binding molecule has the following formula: V_H(A)- L-V_H(B) or V_H(B)-L-V_H(A) wherein V_H(A)- is a V_H single domain antibody as disclosed herein and wherein V_H(B) is a V_H single domain antibody that blocks binding of PD-1 to PD-L1 and/or PD-L2. L is a linker. Suitable linkers include for example a linker with GS residues such as $(Gly_4Ser)n$, where n=from 1 to 20, e.g. 1 to 10, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In one embodiment, the linker is $(Gly_4Ser)n$, where n=4 or more.

As shown in the examples, we have surprisingly shown that a binding molecule has the following formula: V_H(A)- L-V_H(B) as described above provides an enhanced inhibitory effect compared to a V_H single domain antibody that blocks binding of PD-1 to PD-L1 and/or PD-L2 and is not linked to a non-blocking V_H single domain antibody. Thus, the V_H single domain antibodies described herein find particular use for combination with a V_H single domain antibody that blocks binding of PD-1 to PD-L1 and/or PD-L2 or that binds to another epitope in a biparatopic molecule. In another embodiment, the orientation is V_H(B)-L-V_H(A). In one embodiment, L is $(Gly_4Ser)n$ wherein n is 4 or more.

In one embodiment, the binding molecule is multivalent, for example bivalent. Bivalent binding molecules comprise two V_H single domain antibodies that bind to the same target protein; e.g. human PD-1, at the same sites. In one embodiment, such molecules may comprise the same Humabody® V_H. In another embodiment, such molecules may comprise two V_H single domain antibodies that are part of the same family, i.e. selected from the sequences shown in Table 1 or 2. In another embodiment, such molecules may comprise two V_H single domain antibodies that are not part of the same family, but bind to the same site on human PD-1, for example a V_H single domain antibody as shown in table 1 linked to a V_H single domain antibody as shown in table 2.

Biparatopic and bivalent binding molecules of the present invention can be constructed using methods known in the art.

In certain embodiments, the binding agent is in the form of a multispecific, for example bispecific, binding agent providing multiple functionalities. Such multispecific agent comprises a single domain antibody according to the invention that has a first binding specificity to PD-1 and at least one further binding molecule with a second binding specificity. Said further binding molecule can be selected from an antibody, an antibody fragment or antibody mimetic. In one embodiment, said antibody fragment is selected from $F(ab')_2$, Fab, Fv, sFv or domain antibody. In one embodiment, said antibody fragment is a V_H single domain antibody.

In one embodiment, the binding agent is bispecific and comprises a single domain antibody according to the invention that has a first binding specificity to PD-1 and a second binding molecule with a second binding specificity. In one embodiment, the second binding molecule binds to an immunomodulatory agent, a checkpoint modulator, an agent involved in T-cell activation, a tumor microenvironment modifier (TME) or a tumour-specific target.

For example, the immunomodulator can be an inhibitor of an immune checkpoint molecule selected from an inhibitor of one or more of PD-L1, PD-L2, CTLA-4, TIM-3, LAG-3, CEACAM, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR beta. In another embodiment,

the immunomodulator can be an activator of a costimulatory molecule selected from an agonist of one or more of IL-2, Il-12, OX40, OX40L, CD2, CD3, CD27, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD30, CD40, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3, B7-H4 or CD83 ligand, CD3, CD8, CD28, CD4 or ICAM-1.

In one embodiment, the binding agent described above comprises further binding molecules. Thus, the binding agent can be trispecific or tetraspecific. Additional specificities are also envisaged. Any combination of the aforesaid molecules can be made in a multispecific binding agent, for example, a trispecific binding agent that includes a single domain antibody of the invention and a second and third binding specificity.

In another embodiment, the at least second moiety may serve to prolong the half-life of the binding molecule. The second moiety may comprise a protein, for example an antibody, or part thereof that binds a serum albumin, e.g., human serum albumin (HSA) or mouse serum albumin (MSA). The second moiety may comprise a V_H domain that binds serum albumin, e.g., human serum albumin (HSA) or mouse serum albumin (MSA).

The second moiety may comprise a serum albumin, e.g. a human serum albumin (HSA) or a variant thereof such as HSA C34S. Further provided is a binding molecule as described herein comprising a V_H domain and an Fc domain, e.g., wherein the V_H domain is fused to an Fc domain. Further provided is a binding molecule that comprises a second variable domain that specifically binds a second antigen, where the second antigen is an antigen other than human PD-1. The second antigen may be a cluster of differentiation (CD) molecule or a Major Histocompatibility Complex (MHC) Class II molecule.

In one embodiment, the anti-PD-1 single domain antibodies or multivalent binding agents of the invention are labelled with a detectable or functional label. A label can be any molecule that produces or can be induced to produce a signal, including but not limited to fluorophores, fluorescers, radiolabels, enzymes, chemiluminescers, a nuclear magnetic resonance active label or photosensitizers. Thus, the binding may be detected and/or measured by detecting fluorescence or luminescence, radioactivity, enzyme activity or light absorbance.

In still other embodiments, the anti-PD-1 single domain antibodies or multivalent binding agents of the invention are coupled to at least one therapeutic moiety, such as a drug, an enzyme or a toxin. In one embodiment, the therapeutic moiety is a toxin, for example a cytotoxic radionuclide, chemical toxin or protein toxin.

In another aspect, the anti-PD-1 single domain antibodies or multivalent binding agents of the invention are modified to increase half-life, for example by a chemical modification, especially by PEGylation, or by incorporation in a liposome or using a serum albumin protein.

Half-life may be increased by 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 V_H single domain antibodies of the invention. For example, increased half-life may be more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding V_H single domain antibodies of the invention.

To generate a multivalent binding agents as described above, two binding molecules are connected by a linker, for example a polypeptide linker. Suitable linkers include for example a linker with GS residues such as $(Gly_4Ser)n$, where n=from 1 to 20, e.g., 1 to 10, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

In one embodiment, the anti-PD1 single domain antibodies can also be formatted to induce PD-1 agonism, for example in a bivalent or biparatopic format.. PD-1 signalling is induced by PD-L1 and leads to downregulation of T cell activity. One could say that something that induces PD-1 signalling is a 'PD1 agonist', which has contrasting effect to a T cell agonist. We provide evidence of a PD-1 engager that is capable of causing PD-1 agonism in absence of a CD3 or T cell receptor clustering antibody

An exemplary system to measure PD-1 agonism is a reporter cell line that shows a response when PD-1 signalling occurs. Ligand drives this response (PD-L1). Multivalent Humabody® V_H are capable of a mild agonism and biparatopic format enhances this. Thus, a V_H single domain antibody selected from table 1 can be combined with another V_H single domain antibody selected from table 1 to provide agonistic function. In another embodiment, a V_H single domain antibody selected from table 2 can be combined with another V_H single domain antibody selected from table 2 to provide agonistic function. In another embodiment, a V_H single domain antibody selected from table 1 can be combined with another V_H single domain antibody selected from table 2 to provide agonistic function.

A biparatopic format has the formula: $V_H(A)$-L-$V_H(B)$ or $V_H(B)$-L-$V_H(A)$ wherein $V_H(A)$- is a V_H single domain antibody as disclosed herein (i.e. selected from table 1 or 2) and wherein $V_H(B)$ is a V_H single domain antibody that blocks binding of PD-1 to PD-L1 and/or PD-L2. In another embodiment, the orientation is $V_H(B)$-L- $V_H(A)$. L is a linker. Suitable linkers include for example a linker with GS residues such as (Gly4Ser)n, where n=from 1 to 10, e.g., 1, 2,

3, 4, 5, 6, 7, 8, 9 or 10. In one embodiment, the linker is Gly4Ser)n, where n=less than 4, for example 1, 2 or 3.

A biparatopic molecule can enhance receptor cross-linking. A biparatopic molecule binds two epitopes on a single PD1 monomer. Alternatively, it can engage one arm with one PD1 molecule and the other arm with a second molecule. The second molecule still has an available epitope for a further biparatopic to engage and so a 'chain' of PD1 molecules can be clustered together. A shorter linker than $(Gly_4Ser)_4$ can prevent binding to a single molecule and can encourage cross-linking, thereby permitting PD1 agonism. The preferred embodiment for this function is a multivalent molecule, ideally with non-antagonistic function, joined by linkers of less than $(Gly_4Ser)_4$, preferably with at least 2 epitopes. In one embodiment, L $(Gly_4Ser)_n$ wherein n is 1, 2, 3 or 4.

Our data shows a biparatopic format containing an antagonist and an anchor. Although this can induce PD1 signalling, there is sufficient masking of the ligand-binding epitope to allow it to block ligand-induced signalling and have an antagonistic function.

A PD-1 agonist as described above is useful in the treatment of autoimmune and/or inflammatory and/or infectious diseases. Thus, another aspect relates to a PD-1 agonist as described above for use in the treatment of autoimmune and/or inflammatory and/or infectious diseases as well as methods for use in the treatment of autoimmune and/or inflammatory and/or infectious diseases comprising administration of a PD-1 agonist as described above. Another aspect thus relates to an agonistic multivalent or multiparatopic molecule comprising single domain antibody as described herein combined with another single domain antibody as described herein (for example one of the single domain antibody of table 1 combined with another single domain antibody of table 1) or combined with a PD-1 blocker.

A single domain antibody described herein can be obtained from a transgenic rodent that expresses heavy chain only antibodies upon stimulation with a PD-1 antigen. The transgenic rodent, for example a mouse, preferably has a reduced capacity to express endogenous antibody genes. Thus, in one embodiment, the rodent has a reduced capacity to express endogenous light and/or heavy chain antibody genes. The rodent may therefore comprise modifications to disrupt expression of endogenous kappa and lambda light and/or heavy chain antibody genes so that no functional light and/or heavy chains are produced, for example as further explained below.

The invention also relates to a method for producing human heavy chain only antibodies capable of binding human PD-1 which do not block the interaction of PD-1 with PD-L1 and/or PD-L2 said method comprising

a) immunising a transgenic rodent with an PD-1 antigen wherein said rodent expresses a nucleic acid construct comprising un-rearranged human heavy chain V genes and is not capable of making functional endogenous light or heavy chains,
b) isolating human heavy chain only antibodies
c) identifying heavy chain only antibodies that bind to human PD-1 but do not block the interaction of PD-1 with PD-L1 and/or PD-L2.

Further steps may include isolating a V_H domain from said human heavy chain only antibodies or generating a library of sequences comprising V_H domain sequences from said mouse and isolating sequences comprising V_H domain sequences from said libraries.

The invention also relates to a method for producing a single V_H domain antibody capable of binding human PD-1 said method comprising

a) immunising a transgenic rodent with an PD-1 antigen wherein said rodent expresses a nucleic acid construct comprising un-rearranged human heavy chain V genes and is not capable of making functional endogenous light or heavy chains,
b) generating a library of sequences comprising V_H domain sequences from said mouse
c) isolating sequences comprising V_H domain sequences from said libraries
d) identifying V_H domains that bind to human PD-1 but do not block the interaction of PD-1 with PD-L1 and/or PD-L2.

Functional assays for example as shown in the examples may be used to assess if a heavy chain only antibody or V_H domain binds to PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2.

Methods for preparing or generating the polypeptides, nucleic acids, host cells, products and compositions described herein using in vitro expression libraries can comprise the steps of:

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

b) screening said set, collection or library for amino acid sequences that can bind to / have affinity for PD-1 and does not block the interaction of PD-1 with PD-L1 and/or PD-L2

c) isolating the amino acid sequence(s) that can bind to / have affinity for PD-1.

In the above method, the set, collection or library of amino acid sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) amino acid sequences will be clear to the person skilled in the art (see for example Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press; 1st edition (October 28, 1996) Brian K. Kay, Jill Winter, John McCafferty).

Libraries, for example phage libraries, are generated by isolating a cell or tissue expressing an antigen-specific, heavy chain-only antibody, cloning the sequence encoding the VH domain(s) from mRNA derived from the isolated cell or tissue and displaying the encoded protein using a library. The V_H domain(s) can be expressed in bacterial, yeast, insect, plant mammalian or other expression systems.

The invention also relates to an isolated V_H single domain antibody or an isolated heavy chain only antibody comprising a V_H domain binding to PD-1 comprising an amino acid product of or derived from a human V_H germline sequence. The heavy chain only antibody may be fully human or comprise mouse sequences.

In the various aspects and embodiment of the invention as out herein, the term rodent may relate to a mouse or a rat.

In one embodiment, the rodent is a mouse. The mouse may comprise a non-functional endogenous lambda light chain locus. Thus, the mouse does not make a functional endogenous lambda light chain. In one embodiment, the lambda light chain locus is deleted in part or completely or rendered non-functional through insertion, inversion, a recombination event, gene editing or gene silencing. For example, at least the constant region genes C1, C2 and C3 may be deleted or rendered non-functional through insertion or other modification as described above. In one embodiment, the locus is functionally silenced so that the mouse does not make a functional lambda light chain.

Furthermore, the mouse may comprise a non-functional endogenous kappa light chain locus. Thus, the mouse does not make a functional endogenous kappa light chain. In one embodiment, the kappa light chain locus is deleted in part or completely or rendered non-functional through insertion, inversion, a recombination event, gene editing or gene silencing. In one embodiment, the locus is functionally silenced so that the mouse does not make a functional kappa light chain.

The mouse having functionally-silenced endogenous lambda and kappa L-chain loci may, for example, be made as disclosed in WO 2003/000737, which is hereby incorporated by reference in its entirety.

Furthermore, the mouse may comprise a non-functional endogenous heavy chain locus. Thus, the mouse does not make a functional endogenous heavy chain. In one embodiment, the heavy chain locus is deleted in part or completely or rendered non-functional through insertion, inversion, a recombination event, gene editing or gene silencing. In one embodiment, the locus is functionally silenced so that the mouse does not make a functional heavy chain.

For example, as described in WO 2004/076618 (hereby incorporated by reference in its entirety), all 8 endogenous heavy chain constant region immunoglobulin genes (μ, δ, γ3, γ1, γ2a, γ2b, ε and α) are absent in the mouse, or partially absent to the extent that they are non-functional, or genes δ, γ3, γ1, γ2a, γ2b and ε are absent and the flanking genes μ and α are partially absent to the extent that they are rendered non-functional, or genes μ, δ, γ3, γ1, γ2a, γ2b and ε are absent and α is partially absent to the extent that it is rendered non-functional, or δ, γ3, γ1, γ2a, γ2b, ε and α are absent and μ is partially absent to the extent that it is rendered non-functional. By deletion in part is meant that the endogenous locus gene sequence has been deleted or disrupted, for example by an insertion, to the extent that no functional endogenous gene product is encoded by the locus, *i.e.*, that no functional product is expressed from the locus. In another embodiment, the locus is functionally silenced.

In one embodiment, the mouse comprises a non-functional endogenous heavy chain locus, a non-functional endogenous lambda light chain locus and a non-functional endogenous kappa light chain locus. The mouse therefore does not produce any functional endogenous light or heavy chains. Thus, the mouse is a triple knockout (TKO) mouse.

The transgenic mouse may comprise a vector, for example a Yeast Artificial Chromosome (YAC) for expressing a heterologous, preferably a human, heavy chain locus. YACs are vectors that can be employed for the cloning of very large DNA inserts in yeast. As well as comprising all three cis-acting structural elements essential for behaving like natural yeast chromosomes (an autonomously replicating sequence (ARS), a centromere (CEN) and two telomeres (TEL)), their capacity to accept large DNA inserts enables them to reach the minimum size (150 kb) required for chromosome-like stability and for fidelity of transmission

in yeast cells. The construction and use of YACs is well known in the art (*e.g.*, Bruschi, C.V. and Gjuracic, K. Yeast Artificial Chromosomes, Encyclopedia of Life Sciences, 2002 Macmillan Publishers Ltd, Nature Publishing Group).

For example, the YAC may comprise a plethora of unrearranaged human V_H, D and J genes in combination with mouse immunoglobulin constant region genes lacking C_H1 domains, mouse enhancer and regulatory regions. The human V_H, D and J genes are human V_H, D and J loci and they are unrearranged genes that are fully human. An example of such a YAC is provided in the example section.

Alternative methods known in the art may be used for deletion or inactivation of endogenous mouse or rat immunoglobulin genes and introduction of human V_H, D and J genes in combination with mouse immunoglobulin constant region genes lacking C_H1 domains, mouse enhancer and regulatory regions.

Transgenic mice can be created according to standard techniques as illustrated in the examples. The two most characterised routes for creating transgenic mice are via pronuclear microinjection of genetic material into freshly fertilised oocytes or via the introduction of stably transfected embryonic stem cells into morula or blastocyst stage embryos. Regardless of how the genetic material is introduced, the manipulated embryos are transferred to pseudo-pregnant female recipients where pregnancy continues and candidate transgenic pups are born.

The main differences between these broad methods are that ES clones can be screened extensively before their use to create a transgenic animal. In contrast, pronuclear microinjection relies on the genetic material integrating to the host genome after its introduction and, generally speaking, the successful incorporation of the transgene cannot be confirmed until after pups are born.

There are many methods known in the art to both assist with and determine whether successful integration of transgenes occurs. Transgenic animals can be generated by multiple means including random integration of the construct into the genome, site-specific integration, or homologous recombination. There are various tools and techniques that can be used to both drive and select for transgene integration and subsequent modification including the use of drug resistance markers (positive selection), recombinases, recombination-mediated cassette exchange, negative selection techniques, and nucleases to improve the efficiency of recombination. Most of these methods are commonly used in the

modification of ES cells. However, some of the techniques may have utility for enhancing transgenesis mediated via pronuclear injection.

Further refinements can be used to give more efficient generation of the transgenic line within the desired background. As described above, in preferred embodiments, the endogenous mouse immunoglobulin expression is silenced to permit sole use of the introduced transgene for the expression of the heavy-chain only repertoire that can be exploited for drug discovery. Genetically-manipulated mice, for example TKO mice that are silenced for all endogenous immunoglobulin loci (mouse heavy chain, mouse kappa chain and mouse lambda chain) can be used as described above. The transfer of any introduced transgene to this TKO background can be achieved via breeding, either conventional or with the inclusion of an IVF step to give efficient scaling of the process. However, it is also possible to include the TKO background during the transgenesis procedure. For example, for microinjection, the oocytes may be derived from TKO donors. Similarly, ES cells from TKO embryos can be derived for use in transgenesis.

Triple knock-out mice into which transgenes have been introduced to express immunoglobulin loci are referred to herein as TKO/Tg.

In one embodiment, the mouse is as described in WO2016/062990.

The invention also relates to a rodent, preferably a mouse which expresses a human heavy chain locus and which has been immunized with a PD-1 antigen. The invention also relates to a rodent as described above, preferably a mouse which expresses a heavy chain only antibody comprising a human V_H domain that binds to human PD-1. Preferably, said rodent is not capable of making functional endogenous kappa and lambda light and/or heavy chains. The human heavy chain locus is located on a transgene which can be as described above.

The invention also relates to an anti-human PD-1 heavy chain only antibody comprising a human V_H domain or an anti-human PD-1 single V_H domain antibody obtained or obtainable from a rodent, preferably a mouse, immunised with a human PD-1 antigen and which expresses a human heavy chain locus. Preferably, said rodent is not capable of making functional endogenous kappa and lambda light and/or heavy chains. The human heavy chain locus is located on a transgene which can be as described above.

In another aspect of the present invention, there is provided a pharmaceutical composition comprising a single domain antibody according to the present invention and optionally a pharmaceutically acceptable carrier. A single domain antibody of the present invention or the pharmaceutical composition of the invention can be administered by any convenient route, including but not limited to oral, topical, parenteral, sublingual, rectal, vaginal, ocular, intranasal, pulmonary, intradermal, intravitrial, intramuscular, intraperitoneal, intravenous, subcutaneous, intracerebral, transdermal, transmucosal, by inhalation, or topical, particularly to the ears, nose, eyes, or skin or by inhalation.

Parenteral administration includes, for example, intravenous, intramuscular, intraarterial, intraperitoneal, intranasal, rectal, intravesical, intradermal, topical or subcutaneous administration. Preferably, the compositions are administered parenterally.

The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The term "carrier" refers to a diluent, adjuvant or excipient, with which a drug antibody conjugate of the present invention is administered. Such pharmaceutical carriers can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents can be used. In one embodiment, when administered to an animal, the single domain antibody of the present invention or compositions and pharmaceutically acceptable carriers are sterile. Water is a preferred carrier when the drug antibody conjugates of the present invention are administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

The pharmaceutical composition of the invention can be in the form of a liquid, *e.g.*, a solution, emulsion or suspension. The liquid can be useful for delivery by injection, infusion (*e.g.*, IV infusion) or sub-cutaneously.

When intended for oral administration, the composition is preferably in solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more of the following can be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, corn starch and the like; lubricants such as magnesium stearate; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent. When the composition is in the form of a capsule (e. g. a gelatin capsule), it can contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrin or a fatty oil.

The composition can be in the form of a liquid, e. g. an elixir, syrup, solution, emulsion or suspension. The liquid can be useful for oral administration or for delivery by injection. When intended for oral administration, a composition can comprise one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition for administration by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent can also be included.

Compositions can take the form of one or more dosage units.

In specific embodiments, it can be desirable to administer the composition locally to the area in need of treatment, or by intravenous injection or infusion.

The amount of the single domain antibody of the present invention that is effective/active in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account.

Typically, the amount is at least about 0.01% of a single domain antibody of the present invention by weight of the composition. When intended for oral administration, this amount can be varied to range from about 0.1 % to about 80% by weight of the composition. Preferred oral compositions can comprise from about 4% to about 50% of the single domain antibody of the present invention by weight of the composition.

Preferred compositions of the present invention are prepared so that a parenteral dosage unit contains from about 0.01 % to about 2% by weight of the single domain antibody of the present invention.

For administration by injection, the composition can comprise from about typically about 0.1 mg/kg to about 250 mg/kg of the animal's body weight, preferably, between about 0.1 mg/kg and about 20 mg/kg of the animal's body weight, and more preferably about 1 mg/kg to about 10 mg/kg of the animal's body weight. In one embodiment, the composition is administered at a dose of about 1 to 30 mg/kg, e.g., about 5 to 25 mg/kg, about 10 to 20 mg/kg, about 1 to 5 mg/kg, or about 3 mg/kg. The dosing schedule can vary from e.g., once a week to once every 2, 3, or 4 weeks.

The invention provides methods of treating PD-1-mediated diseases or disorders in a mammal, e.g., a human patient, comprising administering an effective amount of an antibody of the present invention to a mammal in need thereof. In particular, the invention furthermore relates to a method for the prevention and/or treatment of a disorder selected from cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency, and other immune system-related disorder said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a single domain antibody or pharmaceutical composition of the invention, or of a pharmaceutical composition of the invention.

As used herein, "treat", "treating" or "treatment" means inhibiting or relieving a disease or disorder. For example, treatment can include a postponement of development of the symptoms associated with a disease or disorder, and/or a reduction in the severity of such symptoms that will, or are expected, to develop with said disease. The terms include ameliorating existing symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result is being conferred on at least some of the mammals, e.g., human patients, being treated. Many medical treatments are effective for some, but not all, patients that undergo the treatment.

The term "subject" or "patient" refers to an animal which is the object of treatment, observation, or experiment. By way of example only, a subject includes, but is not limited to, a mammal, including, but not limited to, a human or a non-human mammal, such as a non-human primate, murine, bovine, equine, canine, ovine, or feline.

As used herein, the term "effective amount" means an amount of an anti-PD-1 antibody, that when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to achieve the desired therapeutic or prophylactic effect under the conditions of administration

The invention also relates to a single domain antibody or pharmaceutical composition of the invention for use in the treatment or prevention of a disease.

In another aspect, the invention relates to a single domain antibody or pharmaceutical composition of the invention for use in the treatment or prevention of cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency, and other immune system-related disorder.

In another aspect, the invention relates to the use of a single domain antibody or pharmaceutical composition of the invention in the treatment or prevention of a disease.

In another aspect, the invention relates to the use of a single domain antibody or pharmaceutical composition of the invention in the manufacture of a medicament for the treatment or prevention of cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency, and other immune system-related disorder.

The cancer can be selected from a solid or non-solid tumor. For example, the cancer may be selected from bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, breast cancer, brain cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, kidney cancer, sarcoma of soft tissue, cancer of the urethra, cancer of the bladder, renal cancer, lung cancer, non-small cell lung cancer, thymoma, urothelial carcinoma leukemia, prostate cancer, mesothelioma, adrenocortical

carcinoma, lymphomas, such as such as Hodgkin's disease, non-Hodgkin's, gastric cancer, and multiple myelomas.

In one embodiment, the tumor is a solid tumor. Examples of solid tumors which may be accordingly treated include breast carcinoma, lung carcinoma, colorectal carcinoma, pancreatic carcinoma, glioma and lymphoma. Some examples of such tumors include epidermoid tumors, squamous tumors, such as head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, including small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, and liver tumors. Other examples include Kaposi's sarcoma, CNS, neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas and cerebral metastases, melanoma, gastrointestinal and renal carcinomas and sarcomas, rhabdomyosarcoma, glioblastoma, preferably glioblastoma multiforme, and leiomyosarcoma. Examples of vascularized skin cancers for which the antagonists of this invention are effective include squamous cell carcinoma, basal cell carcinoma and skin cancers that can be treated by suppressing the growth of malignant keratinocytes, such as human malignant keratinocytes.

In one embodiment, the tumor is a non-solid tumor. Examples of non-solid tumors include leukemia, multiple myeloma and lymphoma.

In one aspect, the cancer is identified as a PD-L1 positive cancer. In one aspect, the cancer is locally advanced unresectable, metastatic, or recurrent cancer.

Preferred cancers whose growth may be inhibited using the antibodies of the invention include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), breast cancer, colon cancer and lung cancer (e.g. non-small cell lung cancer).

In one embodiment, the cancer has progressed after another treatment, for example chemotherapy.

The single domain antibodies and pharmaceutical compositions of the present invention are particularly useful for the treatment of cancers that are associated with cells (e.g., exhausted T cells, B cells, monocytes, etc.) that express abnormally high levels of PD-1. Other preferred cancers include those characterized by elevated expression of PD-1 and/or its

ligands PD-L1 and/or PD-L2. In one embodiment, the cancer is selected from a cancer that has high levels of cancer-associated genetic mutations and/or high levels of expression of tumour antigens. In another embodiment, the cancer is selected from a cancer known to be immunogenic or that is able to become immunogenic upon treatment with other cancer therapies.

The immune disorder can be selected from auto-immune diseases, graft vs. host disease, arthritis, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erthematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, Neuromyelitis optica (NMO), type 1 or immune -mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/ giant cell arteritis, transverse myelitis, ulcerative colitis, uveitis, vasculitides such as dermatitis herpetiformis vasculitis, vitiligo, and Wegener's granulomatosis.

The neurological disease can be selected from Alzheimer’s disease, epilepsy, Parkinson's disease, dementia, multiple sclerosis, peripheral neuropathy or post-herpetic neuralgia.

The single domain antibody or pharmaceutical composition of the invention may be administered as the sole active ingredient or in combination with one or more other therapeutic agent. A therapeutic agent is a compound or molecule which is useful in the treatment of a disease. Examples of therapeutic agents include antibodies, antibody fragments, drugs, toxins, nucleases, hormones, immunomodulators, pro-apoptotic agents, anti-angiogenic agents, boron compounds, photoactive agents or dyes and radioisotopes. An antibody molecule includes a full antibody or fragment thereof (e.g., a Fab, F(ab')2, Fv, a

single chain Fv fragment (scFv) or a single domain antibody, for example a V_H domain) or antibody mimetic protein.

In one embodiment, the single domain antibody is used in combination with an existing therapy or therapeutic agent, for example an anti-cancer therapy. Thus, in another aspect, the invention also relates to a combination therapy comprising administration of a single domain antibody or pharmaceutical composition of the invention and an anti-cancer therapy. The anti-cancer therapy may include a therapeutic agent or radiation therapy and includes gene therapy, viral therapy, RNA therapy bone marrow transplantation, nanotherapy, targeted anti-cancer therapies or oncolytic drugs. Examples of other therapeutic agents include other checkpoint inhibitors, antineoplastic agents, immunogenic agents, attenuated cancerous cells, tumor antigens, antigen presenting cells such as dendritic cells pulsed with tumor-derived antigen or nucleic acids, immune stimulating cytokines (e.g., IL-2, IFNa2, GM-CSF), targeted small molecules and biological molecules (such as components of signal transduction pathways, e.g. modulators of tyrosine kinases and inhibitors of receptor tyrosine kinases, and agents that bind to tumor- specific antigens, including EGFR antagonists), an anti-inflammatory agent, a cytotoxic agent, a radiotoxic agent, or an immunosuppressive agent and cells transfected with a gene encoding an immune stimulating cytokine (e.g., GM-CSF), chemotherapy. In one embodiment, the single domain antibody is used in combination with surgery.

In one embodiment, the single domain antibody or pharmaceutical composition of the invention is administered together with an immunomodulator, a checkpoint modulator, an agent involved in T-cell activation, a tumor microenvironment modifier (TME) or a tumour-specific target. For example, the immunomodulator can be an inhibitor of an immune checkpoint molecule selected from an inhibitor of one or more of PD-1, PD-L1, PD-L2, CTLA-4, TIM-3, LAG-3, CEACAM, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR beta. In another embodiment, the immunomodulator can be an activator of a costimulatory molecule selected from an agonist of one or more of OX40, OX40L, CD2, CD27, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD30, CD40, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3 or CD83 ligand, CD3, CD8, CD28, CD4 or ICAM-1.

In one embodiment, the PD-1 inhibitor is an anti-PD-1 antibody chosen from Nivolumab®, Pembrolizumab® or Pidilizumab®.

In a specific embodiment of the present invention, the composition is administered concurrently with a chemotherapeutic agent or with radiation therapy. In another specific embodiment, the chemotherapeutic agent or radiation therapy is administered prior or subsequent to administration of the composition of the present invention, preferably at least an hour, five hours, 12 hours, a day, a week, a month, more preferably several months (e. g. up to three months), prior or subsequent to administration of composition of the present invention.

In some embodiments, the single domain antibodies of the invention may be administered with two or more therapeutic agents. In some embodiments, the binding agents of the invention may be administered with two or more therapeutic agents.

The single domain antibody or pharmaceutical composition of the invention may be administered at the same time or at a different time as the other therapy or therapeutic compound or therapy, e.g., simultaneously, separately or sequentially.

In another aspect, the invention relates to an immunoconjugate comprising a single domain antibody of the invention conjugated to at least one therapeutic and/or diagnostic agent.

In another aspect, the invention provides a kit for the treatment or prevention of a disease or an immune response and/or for detecting PD-1 for diagnosis, prognosis or monitoring disease comprising a single domain antibody of the invention. Such a kit may contain other components, packaging, instructions, or material to aid in the detection of PD-1 protein. The kit may include a labeled single domain antibody of the invention as described above and one or more compounds for detecting the label.

The invention in another aspect provides a single domain antibody of the invention packaged in lyophilized form, or packaged in an aqueous medium.

The invention also relates to a single domain antibody as described herein with reference to the figures, examples and/or tables 1 and 2.

In another aspect, antibodies of the invention are used for non-therapeutic purposes, such as diagnostic tests and assays. A method for detecting the presence of human PD-1 in a test sample comprises contacting said sample with a single domain antibody according to the invention and at least one detectable label and detecting binding of said single domain antibody to human PD-1.

In one embodiment, the invention relates to a method of diagnosing a PD-1-mediated adaptive immune resistance in a patient who has cancer. The method comprises contacting a sample with a compound disclosed herein that has been labelled with a detectable moiety; and detecting expression of PD-1 on immune cells, e.g., CD8+ T cells; B cells; and macrophages. The sample may be tumor tissue.

Modifications of antibodies for diagnostic purposes are well known in the art. For example, antibodies may be modified with a ligand group such as biotin, or a detectable marker group such as a fluorescent group, a radioisotope, or an enzyme. Compounds of the invention can be labelled using conventional techniques. Suitable detectable labels include but are not limited to fluorophores, chromophores, radioactive atoms, electron-dense reagents, enzymes, and ligands having specific binding partners.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. While the foregoing disclosure provides a general description of the subject matter encompassed within the scope of the present invention, including methods, as well as the best mode thereof, of making and using this invention, the following examples are provided to further enable those skilled in the art to practice this invention and to provide a complete written description thereof. However, those skilled in the art will appreciate that the specifics of these examples should not be read as limiting on the invention, the scope of which should be apprehended from the claims and equivalents thereof appended to this disclosure. Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

All documents mentioned in this specification are incorporated herein by reference in their entirety, including references to gene accession numbers and references to patent publications.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein. Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

The invention is further described in the non-limiting examples.

EXAMPLES

EXAMPLE 1. Construction of Tg/TKO mice

Mice carrying a human heavy-chain antibody transgenic locus in germline configuration within a background that is silenced for endogenous heavy and light chain antibody expression (triple knock-out, or TKO) were created as previously described (WO2004/076618, WO2003/000737, Ren *et al.*, Genomics, 84, 686, 2004; Zou *et al.*, J. Immunol., 170, 1354, 2003 and WO2016/062990). Briefly, transgenic mice were derived following pronuclear microinjection of freshly fertilised oocytes with a yeast artificial chromosome (YAC) comprising a plethora of human V_H, D and J genes in combination with mouse immunoglobulin constant region genes lacking C_H1 domains, mouse enhancer and regulatory regions. Yeast artificial chromosomes (YACs) are vectors that can be employed for the cloning of very large DNA inserts in yeast. As well as comprising all three cis-acting structural elements essential for behaving like natural yeast chromosomes (an autonomously replicating sequence (ARS), a centromere (CEN) and two telomeres (TEL)), their capacity to accept large DNA inserts enables them to reach the minimum size (150 kb) required for chromosome-like stability and for fidelity of transmission in yeast cells. The construction and use of YACs is well known in the art (*e.g.*, Bruschi, C.V. and Gjuracic, K. Yeast Artificial Chromosomes, Encyclopedia of Life Sciences, 2002, Macmillan Publishers Ltd., Nature Publishing Group / www.els.net).

The YAC used comprised multiple human heavy chain V genes, multiple human heavy chain D and J genes, a murine C_H1 gene and a murine 3' enhancer gene. It lacks the C_H1 exon.

The transgenic founder mice were back crossed with animals that lacked endogenous immunoglobulin expression to create the Tg/TKO lines used in the immunisation studies described.

EXAMPLE 2. Antigen for immunisation

The immunisations used recombinant human PD-1 Fc chimera purchased from R&D, catalogue number 1086-PD, lot number FVQ081502B.

EXAMPLE 3. Immunisation Protocol

Tg/TKO mice aged 8-12 weeks of age each received an initial prime dose of either 50ug or 10ug of recombinant purified human PD-1 protein emulsified in Complete Freund's Adjuvant and delivered subcutaneously, followed by three boosts of 10µg of the recombinant protein, emulsified in Incomplete Freund's Adjuvant, also administered subcutaneously, given at various intervals following the initial priming. A final dose of 10µg recombinant purified

human PD-1 protein antigen was administered intraperitoneally, in phosphate buffered saline, in the absence of adjuvant.

EXAMPLE 4. Serum ELISA

Serum was collected from mice before and after immunisation, and checked by ELISA for the presence of serum PD-1/Fc reactive heavy chain antibodies in response to PD-1/Fc immunisation using Nunc Maxisorp plates (Nunc cat. no. 443404) coated overnight with recombinant huPD-1-Fc solution in PBS. Plates were washed using PBS. To block non-specific protein interactions, a solution of 3% (w/v) skimmed milk powder (Marvel®) in PBS was added to the wells and the plate was incubated for at least one hour at room temperature, then discarded.

Whole blood samples were centrifuged at 13000rpm for 5 mins to separate blood from serum. Dilutions of serum were prepared in 3% Marvel™/PBS in polypropylene tubes or plates, pre-incubated for at least one hour at room temperature then transferred to the blocked ELISA plate and incubated for at least one hour. Unbound protein was removed by repetitive washing with PBS/Tween 20 followed by PBS. A 1:10000 solution of biotin-conjugated, goat anti-mouse IgG, Fcgamma subclass 1 specific antibody (Jackson cat. no.115-065-205), prepared in PBS/3% Marvel was added to each well and incubated at room temperature for at least one hour. Unbound detection antibody was removed by repeated washing using PBS/Tween 20 and PBS. Neutravidin-HRP solution (Pierce cat. no. 31030) in 3% Marvel/PBS was added to the ELISA plates and allowed to bind for 30 minutes, then washed as above.

Mice were checked by ELISA for the presence of antibody in serum. All mice showed a robust immune response.

EXAMPLE 5. Generation of Libraries from Immunised Mice

Generation of libraries from immunised mice described above followed standard protocols of library generation as summarised below.

Tissue collection and homogenisation

Total spleen, inguinal and brachial lymph nodes were used according to standard protocols.

RNA extraction and RT-PCR

Spleen: 400µl supernatant was used for preparation of total RNA. RNA was extracted from total spleen using Qiagen RNeasy® kit (cat. no. 74104) following the manufacturer's protocol.

Lymph nodes: prepared by essentially the same process on the Kingfisher

V_H sequences were mined from the RNA samples using Superscript III RT-PCR high-fidelity kit (Invitrogen cat. no. 12574-035) according to the manufacturer's protocol. For each spleen and LN RNA sample, RT-PCR reactions were performed using a single J_H primer in combination with primers for V_H1, V_H2, V_H3, V_H4 or V_H6 families.

Products in the range of 370bp were confirmed by gel electrophoresis

RT-PCR products were pooled so that products from lymph nodes and spleen were combined. Amplified material was purified using the GeneJet™ purification kit (cat# K0702) according to the manufacturer's protocol.

Cloning into phagemid vector

The phagemid vector, pUCG3, was employed in these studies. A conventional PCR-based method was used to construct the V_H phagemid libraries from the amplified V_H sequences. Purified V_H RT-PCR products were used to prime a PCR reaction from the linearised pUCG3 resulting in a heterogeneous population of V_H cloned into pUCG3.

PCR products were analysed on a 1% (w/v) agarose gel.

Generation of phagemid library

V_H/phagemid PCR products were pooled by animal-of-origin and purified using Fermentas PCR purification kit (cat. no. K0702) according to the manufacturer's instructions. Eluted DNA was used to transform TG1 *E. coli* (Lucigen, cat. no. 60502-2) by electroporation using the Bio-Rad GenePulser Xcell. Electroporated cells were pooled.

A 10-fold dilution series of the transformations was plated on 2xTY agar petri plates with 2% (w/v) glucose and 100µg/ml ampicillin. Resulting colonies on these dishes were used to estimate library size. The remainder of the transformation was plated on large format 2xTY agar Bioassay dishes supplemented with 2% (w/v) glucose and 100µg/ml ampicillin. All agar plates were incubated overnight at 30°C.

Libraries were harvested by adding 10 ml of 2xTY broth to the large format bioassay dishes. Bacterial colonies were gently scraped and OD600 recorded. Aliquots were stored at -80oC in cryovials after addition of an equal volume of 50% (v/v) glycerol solution or used directly in a phage selection process

EXAMPLE 6. Selection strategies for isolation of PD-1 binding V_H

Preparation of library phage stocks and phage display selections were performed according to published methods (Antibody Engineering, edited by Benny Lo, chapter 8, p161-176,

2004). In most cases, phage display combined with a panning approach was used to isolate binding V_H domains. However, a variety of different selection methods are well described in the art, including soluble selection and selections performed under stress (e.g., heat).

EXAMPLE 7. Screening of Periplasmic Extracts for Binding to CHO human PD-1 Cells and Inhibition of PD-L1 Binding PD-1

Following selections of the libraries, specific V_H that bound to CHO cells expressing human PD-1 and either partially inhibited or did not inhibit the interaction between recombinant human PD-1 protein and recombinant human PD-L1 protein were identified by single point screening of bacterial periplasmic extracts. Small-scale bacterial periplasmic extracts were prepared from 1ml cultures, grown in deep well plates. Starter cultures were used to inoculate 96-well deep well plates (Fisher, cat. no. MPA-600-030X) containing 2XTY broth (Melford cat. no. M2130), supplemented with 0.1% (w/v) glucose and 100µg/ml ampicillin at 37°C with 250rpm shaking. When OD_{600} had reached 0.6-1, V_H production was induced by adding 100µl of 2XTY, supplemented with 0.5mM IPTG and ampicillin and the cultures were grown overnight at 30°C with shaking at 220rpm. *E. coli* were pelleted by centrifugation at 3200rpm for 10 mins and supernatants discarded. Cell pellets were resuspended in 120µl of ice cold extraction buffer (50mM MOPs, 0.5mM EDTA, 0.5M Sucrose), then 180µl of 1:5 diluted ice cold extraction buffer added. Cells were incubated on ice for 30 minutes and then centrifuged at 4500rpm for 15 mins at 4°C. Supernatants were transferred to polypropylene plates for testing in assays.

Binding of His-tagged V_H in the supernatants to CHO cell expressed human PD-1 was assessed using Fluorescence Microvolume Assay Technology (FMAT), a fluorescence-based platform that detects fluorescence localized to beads or cells settled at the bottom of microwells (Dietz *et al., Cytometry 23*:177-186 (1996), Miraglia *et al., J. Biomol. Screening 4*:193-204 (1999). A CHO TREX human PD1 cell line was generated in-house using full-length human PD-1 sequence by standard procedures. All reagents were prepared in FMAT assay buffer (pH 7.4) containing PBS, 0.1% Bovine Serum Albumin, 0.01% Sodium Azide. Peripreps were transferred into 384 well black clear-bottomed assay plates (Costar cat. no. 3655) and incubated for a minimum of 2 hours at room temperature with 1.5nM Anti-His (Millipore cat. no. 05-949)/3nM Goat Anti-Mouse Alexa Fluor-488 (Jackson Immunolabs cat. no. 115-545-071) and 2000 CHO human PD-1 cells pre-stained with DRAQ5 (Thermo Scientific cat. no. 62251). Plates were read in the FL2 (502nm-537nm) and FL5 (677-800nm) channels on the TTP Mirrorball plate reader following excitation at 488nm and

640nm. Data was gated on FL5 perimeter and peak intensity and the FL2 median mean fluorescence intensity of the gated data used for determination of V_H binding.

In parallel to the CHO PD-1 binding assay periplasmic extracts were tested for inhibition of the interaction of PD-L1 protein with PD-1 protein by single point screening in an HTRF inhibition assay. All samples and reagents were prepared in HTRF assay buffer containing PBS, 0.1% (w/v) BSA and 0.4M Potassium Fluoride. Periplasmic extracts were incubated with 25nM strep tagged human PD-L1 (Acro Biosystems cat no. PD1-H5282), 1.5nM Anti human-Fc Cryptate PAb (Cisbio cat. no. 61HFCKLB), 10nM StrepMAB- Oyster 645 conjugate in black 384-shallow-well plates (Costar cat. no. 3676) for a minimum of 3 hours at room temperature. Total binding controls containing periplasmic extract sample buffer and non-specific binding controls containing excess untagged competitor were set up on each plate for data normalisation. Time-resolved fluorescent emission at 620nm and 665nm was measured following excitation at 337nm on the BMG PHERAstar plate reader. Data was expressed as a % of the total binding control (% control) after subtraction of the background signal determined from the non-specific binding control wells. V_H were identified that bound to the CHO human PD-1 cells with FL2 fluorescence >1000 and showed partial or no inhibition of PD-1 binding to PD-L1 (Figure 1a and b).

EXAMPLE 8. Sequencing

Each individual V_H clone as identified above was sequenced from the phagemid and grouped based on V_H germline and CDR3 amino acid similarity. Representative clones were further characterised. Further clones were generated by sequence optimisation of clone 1.1 or 2.1. Standard methods were used for optimisation. Clones 1.1 to 1.18 as shown in table 1 were isolated as above and grouped into a single family. Clones 1.19-1.50 are sequence optimised clones of clone 1.1. Clones 2.1 to 2.11 as shown in table 2 were isolated as above and grouped into a single family. Clones 2.12-2.53 are sequence optimised clones of clone 2.1.

EXAMPLE 9. Preparation and Characterisation of Purified V_H single domain antibody

a) Preparation of purified V_H

Purified V_H were obtained by using the V_H C-terminal 6xHIS tag for nickel-agarose affinity chromatographic purification of the periplasmic extracts. A starter culture of each V_H was grown overnight in 2XTY media (2XTY broth (Melford cat. no. M2103) supplemented with

2% (w/v) glucose and 100µg/ml ampicillin at 30°C with 250rpm shaking. This overnight culture was then used to inoculate 50ml-200ml 2XTY media and incubated at 37°C with 250rpm shaking for approximately 6-8 hours (until OD_{600} = 0.6-1.0). Cultures were centrifuged at 3200rpm for 10 mins and the cell pellets resuspended in fresh 2XTY broth containing 100µg/ml ampicillin/1mM IPTG. Shake flasks were incubated overnight at 30°C and 250rpm. Cultures were again centrifuged at 3200rpm for 10mins and supernatants discarded. Cell pellets were resuspended in ice cold extraction buffer (20% (w/v) sucrose, 1mM EDTA, 50mM Tris-HCl pH 8.0 or 50mM MOPS) by gently pipetting then diluted further with 1:5 diluted ice cold extraction buffer. Cells were incubated on ice for 30 minutes then centrifuged at 4500rpm for 15 mins at 4°C. Supernatants were transferred to tubes containing 10mM imidazole (Sigma cat. no. I2399) and pre-equilibrated nickel agarose beads (Qiagen, Ni-NTA 50% soln, cat. no. 30210). V_H binding was allowed to proceed for 2 hours at 4°C with gentle shaking. The beads were transferred to a polyprep column (BioRad cat. no. 731-1550) and the supernatant discarded by gravity flow. Columns were washed 3 times with PBS/0.05% Tween® followed by 3 washes with 5ml of PBS/20mM Imidazole. V_H were eluted from the columns using PBS/250mM imidazole. The imidazole was removed from the purified V_H preparations by buffer exchange with NAP-5 columns (GE Healthcare, 17-0853-01) and elution with PBS. Yields of purified V_H were estimated spectrophotometrically and purity was assessed using SDS PAGE.

Alternatively, V_H were purified from the supernatants of W3110 *E coli* with pJExpress vector. For this procedure up to 400ml cultures were grown at 37°C with 250rpm shaking in TB media before being induced overnight with 1mM IPTG overnight. The resulting supernatants were harvested and V_H purified on AKTA Pure using a Ni-Sepharose excel column (HiScale 16, GE Healthcare). Yields of purified V_H were estimated spectrophotometrically and purity was assessed using SDS PAGE.

b) Species Cross Reactivity Testing

Purified V_H were tested for their ability to bind to human PD-1 (R&D Systems cat no. 1086-PD), cynomolgus PD-1 (Acro Biosystems cat no. PD1-C5254) and mouse PD1 (R&D Systems cat no. 1021-PD) in an HTRF Binding assay format. All reagents and serially diluted V_H were prepared in assay buffer containing PBS, 0.1% BSA and 0.4M Potassium Fluoride. Samples or assay buffer (non-specific binding) were incubated with 2nM human/cynomolgus or mouse PD-1, 1nM Anti human-Fc Cryptate PAb (Cisbio cat. no. 61HFCKLB) and 30nM anti His-D2 (CisBio cat no 61HISDLA) in black 384-shallow well assay plates for a minimum of 3 hours at room temperature. Time-resolved fluorescent

emission at 620nm and 665nm was measured following excitation at 337nm on the BMG PHERAstar plate reader. The HTRF ratio were calculated ((665nm emission/620nm emission)*10000) and the data corrected for (non-specific binding) to give the specific binding signal.

V_H single domain antibodies generated as explained above showed binding to human (Figure 2a) and cynomolgus PD-1 (Figure 2b) recombinant protein, but no binding to mouse PD-1 protein (Figure 2c).

Table 3 shows EC50 values obtained.

V_H single domain antibodies	human PD-1 EC_{50} (M)	cyno PD-1 EC_{50} (M)	mouse PD-1 EC_{50} (M)
1.2	9.0E-10	1.1E-9	No binding
1.1	5.0E-10	6.0E-10	No binding
2.1	3.3E-09	6.3E-09	No binding
1.39	2.6E-09	6.0E-09	No binding
2.12	1.3E-08	1.8E-08	No binding

c) Inhibition of human PD-L1 and PD-L2 binding to recombinant human PD-1 Protein

Purified V_H were serially diluted in HTRF assay buffer and tested in the HTRF PD-1:PD-L1 Inhibition assay as described above.

For the PD-L2 inhibition assay, recombinant human PD-1 protein was labelled with Europium Trisbipyridine Cryptate (Cisbio cat no. 62EUSPEA) according to the manufacturer's protocol and PD-L2-Fc (Acro Biosystems cat no. PD2-H882R) was biotinylated according to EZ-link kit protocol (Thermo 21327). Serial dilutions of V_H were incubated with 10nM Streptavidin AlexaFluor-647 (Life Technologies cat no. S32357), 3nM biotinylated PD-L2-Fc and Europium Cryptate labelled PD-1-Fc (167-fold dilution) for a minimum of 3 hours at room temperature. V_H showed a partial inhibition profile in the PD-L1

inhibition assay, but did not inhibit binding of PD-L2 to human PD-1 protein (Figure 3 showing V_H single domain antibodies 1.1 and 1.2).

d) Inhibition of human PD-L1 and human PD-L2 to CHO human PD-1 cells

Purified V_H were serially diluted in FMAT assay buffer and tested for binding to CHO human PD-1 cells as described above and for inhibition of human PD-L1/PD-L2 binding to CHO human PD-1 cells.

For the inhibition assay all reagents were prepared in FMAT assay buffer. V_H, buffer (total binding controls) or excess competitor (non-specific binding control) were incubated with 400pM human Fc tagged human PDL-1 (or 100pM human Fc tagged human PD-L2), 4nM anti human Fc-Alexa Fluor-488 and 2000 per well CHO human PD1 DRAQ5 stained cells in 384 well black clear-bottomed assay plates. Plates were incubated for 2 hours at room temperature then fluorescence measured in the FL2 (502nm-537nm) and FL5 (677-800nm) channels on the Mirrorball plate reader (TTP) following excitation at 488nm and 640nm. Data was expressed as a % of the total binding control (i.e. % control) after subtraction of the background signal determined from the non-specific binding control wells.

Example data for binding to CHO-human PD-1 cells and inhibition of binding of human PDL-1 to the CHO human PD-1 cells is shown in Figure 4a and 4b respectively. The Humabody® 1.1 and 1.20 showed concentration dependent binding to CHO human PD1 cells (figure 4a) but did not inhibit PD-L1 (figure 4b) and PD-L2 binding to CHO human PD-1 cells (Table 4).

Table 4

Humabody® VH	CHO PD-1 binding EC50 (M)	CHO PD-1:PD-L1 IC_{50} (M)	CHO PD-1:PD-L2 IC_{50} (M)
1.39	0.6E-09	No inhibition	No inhibition
2.12	1.3E-09	No inhibition	No inhibition
2.1	0.7E-09	No inhibition	

e) Reporter Gene Assays

The ability of V_H single domain antibodies to inhibit functional responses in transfected Jurkat cells as a result of PD-1:PD-L1 blockade was assessed using an NFAT-Luciferase Reporter Gene assay. A Jurkat reporter cell line expressing human PD-1 and a luciferase reporter gene under the control of a promoter with an NFAT response element and a CHO cell line expressing a T-Cell Receptor activator and human PD-L1 under the control of a tetracycline inducible promotor were generated by standard methods. Cells were prepared in bulk, then frozen and stored in liquid nitrogen.

CHO human PD-L1/TCR activator cells were thawed in in a 37°C water bath, washed once with PBS, resuspended in (Hams F12/10% FBS/1µg/ml tetracycline) and plated at 10000 cells/well in a 96 well white TC treated assay plate. Plates were incubated at 37°C overnight in a CO2 incubator.

Samples were serially diluted in assay medium (RPMI + 2% FBS). Jurkat PD-1 reporter cells were thawed in a 37°C water bath, washed once with medium, then diluted into assay medium at 5e5 cells/ml. The media was removed from the CHO cells and 50µl diluted sample or assay media (background control) added to the plates followed by 50µl of the diluted Jurkat reporter cells. The plates were incubated for 6 hours at 37°C overnight in a CO2 incubator, then removed from the incubator and equilibrated to room temperature for 20mins. NanoGlo substrate (100µl of substrate diluted 1:50 in NanGlo buffer (Promega cat no. N1120) was added and the plates incubated for 20mins at room temperature prior to measurement of luminescence signal (RLU). Data was expressed as fold/background signal. Humabody® V_H 1.39, 2.12, 2.1 and 1.1 were tested together with control PD-1 single blocking V_H VH(A) (SEQ ID NO. 528) and biparatopic (Blocking VH(A)-4GS-1.39, Blocking VH(A)-4GS-2.12) (PD-1 antagonists).

Example data for activity of V_H in the reporter assay is shown in Figure 5a, b and c. Tested Humabody® V_H showed no activity in the assay demonstrating that Humabody® V_H 1.39, 2.12 and 1.1 do not block PD-1: PD-L1 functional interaction. The biparatopic molecule shows an increased blocking activity (10 to 25 fold compared to monovalent blocker).

f) Measurement of Binding Kinetics using Octet

Binding kinetics of clones to human PD-1-huFc were measured in real-time bio-layer interferometer based biosensor Octet (ForteBio). Recombinant human PD-1-huFc was either immobilized by standard amine coupling to amine reactive biosensors in 10 mM sodium acetate at pH 5.0 or capture by protein G biosensors (ForteBio). All the binding studies were performed in HBS-ET Octet kinetics buffer. Biosensors were always washed in

Octet kinetics buffer in between different steps. A seven point, two-fold dilution series of each Humabody® V_H was made with a top concentration in range 100- 30nM. The contact time for each of the association steps was varied between 180-300 seconds and the dissociation step was varied between 400-600 seconds. Kinetic association (k_a) and dissociation (k_d) rate constants were determined by processing and fitting the data to a 1:1 binding model using ForteBio Analysis software. The calculated affinity and kinetic constants are shown in Table 5 and the KD is in the nanomolar range.

Table 5

Humabody® V_H	ka (1/Ms)	kd (1/s)	KD (M)
1.1	4.5E+05	9.01E-04	1.9E-9
2.1	5.9E+05	1.5E-03	2.6E-9
2.39	3.7E+5	5.9E-04	1.6E-9
2.52	6.7E+05	2.2E-03	3.3E-09
2.53	1.0E+06	1.7E-02	1.6E-08
2.13	1.3E+06	3.5E-02	2.5E-08
2.37	4.5E+05	1.5E-03	3.4E-09
1.34	1.9E+05	7E-04	3.6E-09
1.33	3.7E+05	7.4E-04	2.0E-09
1.37	2.4E+05	1.3E-05	5.5E-09

g) Binding kinetics to human PD-1 using surface plasmon resonance

Binding kinetics of certain V_H single domain antibodies binding to human PD-1-huFc were measured by surface plasmon resonance (SPR) technology using Biacore T200 instrument (GE Healthcare). Recombinant human PD-1-huFc was immobilized by standard amine coupling to CM5 sensorschip (GE Healthcare) using 0.01 mg/ml solution of antigen in 10 mM sodium acetate at pH 5.5. For the reference flow cell, a blank immobilisation was carried out. Single cycle kinetics assays were used to study the interaction, a five point, three-fold dilution series of each Humabody® was made with a top concentration of 30 nM. The binding kinetics were followed by flowing the Humabody® over the chip surface in HBS EP+ buffer at a flow rate of 30 µl/min. The contact time for each of the association steps was 180 seconds and the dissociation step was varied between 1200- 3600 seconds. The data was fitted to a 1:1 binding model after double reference subtraction using the Biacore T200 Evaluation software. The calculated affinity and kinetic constants are shown in Table 6 below

Table 6

Humabody® V_H	ka (1/Ms)	kd (1/s)	KD (M)
1.48	1.80E+05	2.91E-03	1.62E-08
1.39	3.16E+05	1.34E-03	4.24E-09
1.40	2.43E+05	1.45E-03	5.98E-09
1.44	2.24E+05	2.04E-03	9.10E-09
1.26	2.65E+05	2.11E-03	7.94E-09
1.47	2.48E+05	2.54E-03	1.02E-08
1.50	1.60E+05	2.29E-03	1.43E-08
2.12	5.5E+6	0.03848	6.89E-9

h) Serum stability

Serum stability of V_H was assessed by measurement of its activity following incubation for 0, 1, 4 or 7 days in both human and mouse serum (Sigma M5905). The pre-incubated samples were serially diluted and tested in the 2.1 epitope competition assay. On incubation at 37°C in the presence of either mouse or human serum no significant change in the activity was seen (Table 7). Example binding curve data is shown in Figure 9a and 9b.

Table 7

Constructs	IC 50 (M)							
	Day 0		Day 1		Day 4		Day 7	
	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse
1.39	12.9E-09	8.7E-09	10.1E-09	9.3E-09	10.2E-09	11.1E-09	9.3E-09	11.6E-09

i) V_H single domain antibodies demonstrate good stability

Purified V_H were subjected to size exclusion chromatography. Briefly, purified V_H were stored at 1 or 5 mg/ml in PBS buffer for 0-14 days at either 4°C or 40°C, and then analysed at various time points using a Waters H-Class Bio UPLC containing a PDA detector (detection at 280nm) with separation on a Waters ACQUITY BEH 125Å SEC column. Samples were injected in 10µl volumes and were run in a mobile phase containing 200 mM NaCl, 100 mM sodium phosphate, pH 7.4 + 5% propan-1-ol at a flow rate of 0.4ml/min. Data were collected for 6 minutes and the area of the monomer peak remaining after storage as compared to that present at the start (T=0) was calculated. Examples of an anti-PD-1 V_H single domain antibody 1.1 incubated at 40°C for 14 days is illustrated in Figure 6 and in Table 8. At incubation at 4°C for 14 days, no significant change was seen.

Table 8: Stability of V_H single domain antibody. This shows the percentage of monomer present after 0, 1, 3, 7 and 14 days.

Name	0 days	1 days	4 days	7 days	14 days
1.1	100	90.19	87.04	94.93	100.10
1.1	100	95.01	91.79	92.55	90.46
1.21	100.00	100.44	104.46	106.95	111.95
1.29	100.00	99.20	101.18	108.35	115.06
1.39	100.00	102.25	101.71	104.41	96.61
2.12	100.00	99.26	99.10	93.94	90.35
2.1	100.00	99.20	100.15	100.40	100.78
2.29	100.00	99.99	101.03	93.96	96.03
2.52	100.00	100.80	100.79	96.70	99.58
2.28	100.00	101.41	101.04	99.40	100.46
2.53	100.00	99.79	101.12	100.00	101.06

j) 1.1 and 2.1 Epitope competition assays

Sequence optimised single domain antibodies with improved activity and/or expression levels over that of the parental (non-optimised) single domain antibodies were initially identified by testing of bacterial periplasmic extracts for their ability to compete with the binding of the parental clone 1.1 or a 2.1 to CHO human PD-1 cells in an FMAT epitope competition assay.

Humabody® 1.1 or 2.1 V_H sequence was amplified by PCR and sub-cloned into a vector enabling expression with a C terminally fused Strep tag. TG1 bacterial cultures transformed with the expression vector were cultured, periplasmic extracts prepared using extraction buffer (20% w/v sucrose, 1mM EDTA, 50mM Tris-HCl pH8.0) then Strep-tagged V_H purified from the periplasm using Strep-Tactin affinity resin (Qiagen 30002).

For the epitope competition assay reagents were prepared in FMAT assay buffer. Bacterial periplasmic extracts, buffer (total binding controls) or excess His tagged V_H competitor (non-specific binding control) were incubated with 2nM 1.1-Strep tagged protein or 2nM 2.1-Strep tagged protein, 1.5nM Strep-Tag® II monoclonal antibody (Millipore 71590), 2.5nM Goat anti mouse Fc-Alexa Fluor 488 and 2000 CHO human PD-1 DRAQ5 stained cells per well in a 384 well black clear-bottomed assay plate. Plates were incubated for a minimum of 1.5 hours at room temperature then fluorescence measured in the FL2 (502nm-537nm) and FL5 (677-800nm) channels on the Mirrorball plate reader (TTP) following excitation at 488nm and

640nm. Data was expressed as a % of the total binding control (i.e. % control) after subtraction of the background signal determined from the non-specific binding control wells. Clones that showed improved activity compared to the parent V_H were purified and tested multipoint in the Epitope Competition Assay for IC_{50} determination or were tested directly in the reporter gene assay described below (data shown in Table 10).

Table 10

Names	1.1 EC IC50 (M)	2.1 EC IC50 (M)
1.39	8.9E-09	9.7E-09
1.34	6.5E-09	
1.26	8E-09	
1.33	2.5E-09	
1.37	7.5E-09	
2.27	9E-09	1.3E-08
2.53	7E-09	1.4E-08
2.52	6.5E-09	2.1E-08
1.1	3.5E-09	3.6E-09
2.1	9.2E-09	9.4E-09

k) Effects of PD-1 specific V_H single domain antibody on human T cell activation in a Mixed Lymphocyte Reaction

PD-1 specific V_H single domain antibody V_H 1.1 does not block the functional interaction of PD-1 with PD-L1 and therefore does not affect T cell activation in a Mixed Lymphocyte Reaction. Monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) and differentiated into dendritic cells for 7 days using GM-CSF and IL-4. Dendritic cells were cultured with allogeneic CD4+ T cells, isolated from PBMCs via magnetic separation. Co-cultures were incubated for 2-7 days in the presence of PD-1-specific V_H single domain antibody or control. T cell stimulation was measured by proliferation assay or cytokine quantification from the cell supernatant. IL-2 levels were determined after 3 days by Homogenous Time Resolved Fluorescence assay (HTRF, CisBio).

V_H single domain antibody 1.1 does not enhance T cell activation (Figure 7).

EXAMPLE 10. Epitope mapping of V_H single domain antibodies

The binding epitopes on PD-1 of V_H single domain antibodies 1.1 and 2.1 were determined using peptide scanning analysis (PepScan). 1.1 in monovalent format was screened against

arrays of PD-1 linear peptides and PD-1 peptides constrained to mimic loops and β-strands, with one residue offset between peptides in each set. 1.1 and 2.1 in bivalent format were screened against arrays combining discontinuous peptides.

Synthesis of peptides: To reconstruct continuous epitopes of huPD-1 extracellular domain, a library of peptides was synthesized. An amino functionalized polypropylene support was obtained by grafting with a proprietary hydrophilic polymer formulation, followed by reaction with t-butyloxycarbonyl-hexamethylenediamine (BocHMDA) using dicyclohexylcarbodiimide (DCC) with Nhydroxybenzotriazole (HOBt) and subsequent cleavage of the Boc-groups using trifluoroacetic acid (TFA). Standard Fmoc-peptide synthesis was used to synthesize peptides on the amino-functionalized solid support by custom modified JANUS liquid handling stations (Perkin Elmer). Synthesis of structural mimics was done using Pepscan's proprietary Chemically Linked Peptides on Scaffolds (CLIPS) technology. CLIPS technology allows to structure peptides into single loops, double loops, triple loops, sheet-like folds, helix-like folds and combinations thereof. CLIPS templates are coupled to cysteine residues. The side-chains of multiple cysteines in the peptides are coupled to one or two CLIPS templates. For example, a 0.5 mM solution of the P2 CLIPS (2,6- bis(bromomethyl)pyridine) is dissolved in ammonium bicarbonate (20 mM, pH 7.8)/acetonitrile (1:3(v/v)). This solution is added onto the peptide arrays. The CLIPS template will bind to side-chains of two cysteines as present in the solid-phase bound peptides of the peptide-arrays (455 wells plate with 3 µl wells). The peptide arrays are gently shaken in the solution for 30 to 60 minutes while completely covered in solution. Finally, the peptide arrays are washed extensively with excess of H2O and sonicated in disrupt-buffer containing 1% SDS/0.1% beta-mercaptoethanol in PBS (pH 7.2) at 70°C for 30 minutes, followed by sonication in H2O for another 45 minutes. The T3 CLIPS carrying peptides were made in a similar way but now with three cysteines.

ELISA screening: The binding of antibody to each of the synthesized peptides was tested in a PEPSCAN-based ELISA. The peptide arrays were incubated with anti-His Tag monoclonal antibody (R&D) (overnight at 4°C). After washing, the peptide arrays were incubated with a 1/1000 dilution of rabbit anti-mouse IgG (H+L) HRP conjugate (Southern Biotech) for one hour at 25°C. After washing, the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 20 µl/ml of 3 percent H2O2 were added. After one hour, the colour development was measured. The colour development was quantified with a charge coupled device (CCD) - camera and an image processing system.

Data processing: The values obtained from the CCD camera range from 0 to 3000 mAU, similar to a standard 96-well plate ELISA-reader. The results are quantified and stored into

the Peplab database. Occasionally a well contains an air-bubble resulting in a false-positive value, the cards are manually inspected, and any values caused by an air-bubble are scored as 0.

Synthesis quality control: To verify the quality of the synthesized peptides, a separate set of positive and negative control peptides was synthesized in parallel. These were screened with antibody 57.9 (Posthumus et al., J. Virology, 1990, 64:3304-3309).

When screened against linear peptides and peptides constrained to mimic loops and β-strands peptides, 1.1 shows consistent binding to a subset of peptides comprising the motif 102-NGRDFHMSVVRARR-115 (SEQ ID No. 519). Binding to this epitope was observed over all sets of peptides tested and was independent of the structural restraints imposed upon the peptides.

Screened against discontinuous peptides, 1.1 displays binding to a subset of peptides comprising the motifs 33-NPPTFS-38 (SEQ ID No. 520), 54-CSFSNTSESFVLNW-67 (SEQ ID No. 521) and 101-PNGRDFHMSV-110 (SEQ ID No. 522). The data is consistent with binding to the epitope identified in the linear peptides and also identifies additional residues which are proximal to the linear epitope in the tertiary structure.

2.1 displayed affinity for a discontinuous set of peptides comprising the motifs 60-SESFVLNWYRMS-71 (SEQ ID No. 523), 90-GQDCRFRVT-98 (SEQ ID No. 524) and 104-RDFHMSVVRAR-114 (SEQ ID No. 525). The identified sequences are proximal in the tertiary structure consistent with a discontinuous epitope.

Analysis of the huPD-1 structure (PDB code: 4ZQK) indicates that the identified epitopes for both 1.1 and 2.1 are located on the opposing face of the huPD-1 extracellular domain to that of the PD-L1 binding interface, consistent with binding to huPD-1 without interrupting native ligand binding (see Figure 8A).

The sequence 104-RDFHMSV-110 (SEQ ID No.526) (see figure 8A) is consistent within the epitopes identified for both Humabody® VH. The partial overlap of epitopes is consistent with the two Humabody® VH displaying competitive binding.

Example 11. PD-1 stimulation

Experiments were performed using DiscoverX PathHunter Checkpoint assay, which measures PD-1 signalling. Cells expressed PD-1 linked to an intracellular enzyme fragment and SH2-domain containing phosphatase linked to another enzyme fragment. Upon dimerization of receptor, complementation of the enzyme occurs. Substrate addition leads to chemiluminescent signal. PD-1 dimerization occurs in response to a PD-L1+ cell line. In absence of ligand, this dimerization could be induced by antibodies. PD-1+ cells were

incubated with a dilution series of Humabody® VH for 3 hours before addition of detection reagent. Chemiluminescence was read as relative light units and EC50 was calculated from curve fit. Maximum response was calculated as percentage increase in RLU above basal response. Table 11 Biparatopic Humabody (1GS) enhances PD-1 signalling.

Bivalent 1.1 induces low level PD-1 signalling, when formatted as a biparatopic it is capable of PD1 agonism. A shorter linker increases this further, which is likely to be caused by enhanced cross-linking. V_H(B) is a blocking single V_H domain that blocks ligand binding (SEQ ID No. 529). When measured in this assay, a bivalent molecule using 2.1 shows no agonistic effect.

Table 11

	EC50 uM	Max. % increase in response over baseline
VH (B)-1GS-1.21	0.00059	1332
VH (B)-4GS-1.21	0.0005	253.2
1.1a-4GS-1.1a	0.001	57.77

Claims

1. An isolated single domain antibody that binds to human PD-1 but does not block the interaction of human PD-1 with human PD-L1 and/or PD-L2.
2. An isolated single domain antibody according to claim 1 wherein said single domain antibody binds to an epitope comprising one or more residue selected from R^{104}, D^{105}, F^{106}, H^{107}, M^{108}, S^{109} and V^{110} of human PD-1.
3. An isolated single domain antibody according to claim 1 or 2 wherein said single domain antibody binds to an epitope further comprising one or more residue selected from G^{103}, V^{111}, R^{112} and A^{113} of human PD-1.
4. An isolated single domain antibody according to a preceding claim wherein said epitope further comprises one, more than one or all residues selected from N^{102}, D^{105}, F^{106}, H^{107}, M^{108}, R^{114} and R^{115}.
5. An isolated single domain antibody according to a preceding claim wherein said epitope further comprises one, more or all residues selected from N^{33}, P^{34}, P^{35}, T^{36}, F^{37}, S^{38}, C^{54}, F^{55}, S^{56}, N^{57}, T^{58}, S^{59}, E^{60}, S^{61}, F^{62}, V^{63}, L^{64}, N^{65}, W^{66}, P^{101}, N^{102} and G^{103}.
6. An isolated single domain antibody according to any of claims 1 to 4 wherein said epitope further comprises one, more than one or all residues selected from S^{60}, E^{61}, S^{62}, F^{63}, V^{64}, L^{65}, N^{66}, W^{67}, Y^{68}, R^{69}, M^{70}, S^{71}, G^{90}, Q^{91}, D^{92}, C^{93}, R^{94}, F^{95}, R^{96}, V^{97}, T^{98}, V^{111}, R^{112}, A^{113} and R^{114}.
7. An isolated single domain antibody according to a preceding claim wherein said single domain is a human heavy chain variable domain (V_H).
8. An isolated single domain antibody according to a preceding claim comprising a CDR1 as shown in SEQ ID No. 1 or SEQ ID No. 1 with 1 or 2 amino acid substitutions, a CDR2 as shown in SEQ ID No. 2 or SEQ ID No. 2 with 1 to 5 amino acid substitutions and a CDR3 as shown in SEQ ID No. 3 or SEQ ID No. 3 with 1 to 5 amino acid substitutions.
9. An isolated single domain antibody according to a preceding claim comprising a CDR1, CDR2 and CDR3 selected from CDRs 1 to 3 of the following V_H sequences SEQ ID Nos: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216 or 220.
10. A single domain antibody according to a preceding claim comprising a sequence selected from SEQ ID Nos. 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 104, 108, 112, 116, 120, 124, 128, 132, 136, 140, 144, 148, 152, 156,

160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216 or 220 or a sequence with at least 60%, 70%, 80% or 90% homology thereto.

11. An isolated single domain antibody according to any of claims 1 to 7 comprising a CDR1 as shown in SEQ ID No. 251 or SEQ ID No. 251 with 1 or 2 amino acid substitutions, a CDR2 as shown in SEQ ID No. 252 or SEQ ID No. 252 with 1 to 5 amino acid substitutions and a CDR3 as shown in SEQ ID No. 253 or SEQ ID No. 253 with 1 to 5 amino acid substitutions.
12. An isolated single domain antibody according to claim 11 comprising a CDR1, CDR2 and CDR3 selected from CDRs1-3 of the following V_H sequences SEQ ID Nos: 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462.
13. A single domain antibody according to claim 12 comprising a sequence selected from SEQ ID Nos. 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, 314, 318, 322, 326, 330, 334, 338, 342, 346, 350, 354, 358, 362, 366, 370, 374, 378, 382, 386, 390, 394, 398, 402, 406, 410, 414, 418, 422, 426, 430, 434, 438, 442, 446, 450, 454, 458 or 462 or a sequence with at least 60%, 70%, 80% or 90% homology thereto.
14. An isolated single domain antibody according to a preceding claim wherein said single domain antibody is conjugated to a toxin, enzyme, radioisotope, half-life extending moiety, label, therapeutic molecule or other chemical moiety.
15. An isolated single domain antibody according to claim 14 wherein said half-life extending moiety is selected from the group consisting of an albumin binding moiety, a transferrin binding moiety, a polyethylene glycol molecule, a recombinant polyethylene glycol molecule, human serum albumin, a fragment of human serum albumin, and an albumin binding peptide or single domain antibody that binds to human serum albumin.
16. An isolated single domain antibody according to a preceding claim that does not compete with an antibody that blocks the interaction of PD-1 with PD-L1 and/or the interaction of PD-1 with PD-L2.
17. An isolated single domain antibody according to a preceding claim obtained or obtainable from a transgenic rodent that expresses a transgene comprising human V, D and J regions.
18. An isolated single domain antibody according to claim 17 wherein said rodent does not produce any functional endogenous light and heavy chains.

19. An isolated binding agent that binds to essentially the same epitope as a single domain antibody of any of claims 1 to 18.
20. An isolated binding agent that competes for binding to human PD-1 with a single domain antibody of any one of claims 1 to 18.
21. An isolated binding agent according to claim 19 or 20 wherein said binding molecule is an antibody or fragment thereof.
22. An isolated binding agent according to claim 21 wherein said fragment is a human heavy chain variable domain (V_H).
23. An isolated binding agent comprising a single domain antibody according any of claims 1 to 18.
24. An isolated binding agent according to claim 23 wherein said single domain antibody is linked to a second binding molecule that does not bind to PD-1.
25. An isolated binding agent according to claim 24 wherein said second single domain antibody binds to an immonooncology target.
26. An isolated binding agent according to claim 23 wherein said single domain antibody is linked to a second binding molecule that binds to PD-1.
27. An isolated binding agent according to claim 26 wherein said binding molecule blocks the interaction of PD-1 with PDL-1 and/or PDL-2.
28. An isolated binding agent according to any of claims 23 to 27 wherein said binding molecule is an antibody or a fragment thereof.
29. An isolated binding agent according to claim 28 wherein said binding molecule is a human heavy chain variable domain (V_H).
30. An isolated binding agent according to any of claims 19 to 29 conjugated to a toxin, enzyme, radioisotope, half-life extending moiety, therapeutic molecule or other chemical moiety.
31. An isolated binding agent according to claim 30 wherein said half-life extending moiety is selected from the group consisting of an albumin binding moiety, a transferrin binding moiety, a polyethylene glycol molecule, a recombinant polyethylene glycol molecule, human serum albumin, a fragment of human serum albumin, and an albumin binding peptide or single domain antibody that binds to human serum albumin.
32. The use of a single domain antibody according to any of claims 1 to 18 in a multispecific or multivalent binding agent.
33. An immunoconjugate comprising a single domain antibody according to any one of claims 1 to 18 or a binding agent according to any one of claims 19 to 31 linked to a therapeutic agent.

34. An immunoconjugate according to claim 33 wherein said therapeutic agent is a toxin, enzyme, radioisotope or other chemical moiety.
35. A pharmaceutical composition comprising a single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31 or an immunoconjugate according to claim 33 and 34 and a pharmaceutical carrier.
36. A method for treating a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency or other immune system-related disorder comprising administering a therapeutically effective amount of a comprising a single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31, an immunoconjugate according to claim 33 and 34 or a pharmaceutical composition according to claim 35.
37. Use of a single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31, an immunoconjugate according to claim 33 and 34 or a pharmaceutical composition according to claim 35 in the manufacture of a medicament for the treatment of a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency or other immune system-related disorder.
38. A single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31, an immunoconjugate according to claim 33 and 34 or a pharmaceutical composition according to claim 35 for use as a medicament.
39. A single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31, an immunoconjugate according to claim 33 and 34 or a pharmaceutical composition according to claim 35 for use in the treatment of a cancer, an immune disorder, neurological disease, inflammatory disorder, allergy, transplant rejection, viral infection, immune deficiency, and other immune system-related disorder.
40. A method according to claim 36, a use according to claim 37, or a single domain antibody, a binding agent or pharmaceutical composition according to claim 35 wherein said cancer is selected from bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, breast cancer, brain cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, kidney cancer, sarcoma of soft tissue,

cancer of the urethra, cancer of the bladder, renal cancer, lung cancer, non-small cell lung cancer, thymoma, urothelial carcinoma leukemia, prostate cancer, mesothelioma, adrenocortical carcinoma, lymphomas, such as such as Hodgkin's disease, non-Hodgkin's, gastric cancer, and multiple myelomas.

41. A method of modulating an immune response comprising administering a single domain antibody according to any one of claims 1 to 18, a binding agent according to any of claims 19 to 31, an immunoconjugate according to claim 33 and 34 or a pharmaceutical composition according to claim 35.
42. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a single domain antibody according to any of claims 1 to 18.
43. An isolated nucleic acid molecule according to claim 42 selected from SEQ ID NO. 81 to 100, 221 to 250, or 463 to 515.
44. A vector comprising a nucleic acid according to claim 43 or 44.
45. A host cell comprising a nucleic acid according to claim 42 or 43 or a vector according to claim 44.
46. A host cell according to claim 45 wherein said host cell is a bacterial, insect, plant, viral or mammalian cell.
47. A method for producing a single domain antibody according to any one of claims 1 to 18 comprising expressing a nucleic acid encoding said binding molecule in a host cell and isolating the binding molecule from the host cell.
48. A kit comprising a single domain antibody according to any one of claims 1 to 18 or a binding agent according to any of claims 19 to 31, an immunoconjugate according to claims 33 and 34 or a pharmaceutical composition according to claim 35.
49. A method for detecting the presence of human PD-1 in a test sample comprising contacting said sample with a single domain antibody according to any of claims 1 to 18 and at least one detectable label and detecting binding of said single domain antibody to human PD-1.
50. A method for producing a single V_H domain antibody that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 said method comprising
    a) immunising a transgenic animal that expresses a nucleic acid construct comprising human heavy chain V genes and that is not capable of making functional endogenous light or heavy chains with an PD-1 antigen,
    b) generating a library from said animal,
    c) isolating single V_H domain antibodies from said libraries,
    d) identifying a single V_H domain antibody that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2 and

e) isolating said antibody.

51. A single V_H domain antibody obtained or obtainable by the method of claim 50.
52. An isolated heavy chain only antibody comprising a V_H domain that binds to human PD-1 but does not block the interaction of PD-1 with PD-L1 and/or PD-L2.
53. A transgenic rodent that produces a heavy chain only antibody of claim 52.
54. A bispecific molecule comprising the single domain antibody of any of claims 1 to 18 linked to a second functional moiety having a different binding specificity than said single domain antibody.
55. A bispecific molecule according to claim 54 wherein said second moiety is an antibody, an antibody fragment or antibody mimetic.
56. A bispecific molecule according to claim 54 or 55 wherein said second moiety binds to an immunooncology target.
57. A multivalent binding agent comprising a single domain antibody according to any of claims 1 to 18 for use as an agonist.
58. A multivalent binding agent according to claim 57 wherein said binding molecule comprises two or more single domain antibody according to any of claims 1 to 18.
59. A multivalent binding agent according to claim 57 wherein said binding molecule comprises a single domain antibody according to any of claims 1 to 18 and a single domain antibody that binds to PD-1 and blocks the interaction with PD-L1 and/or PD-L2.

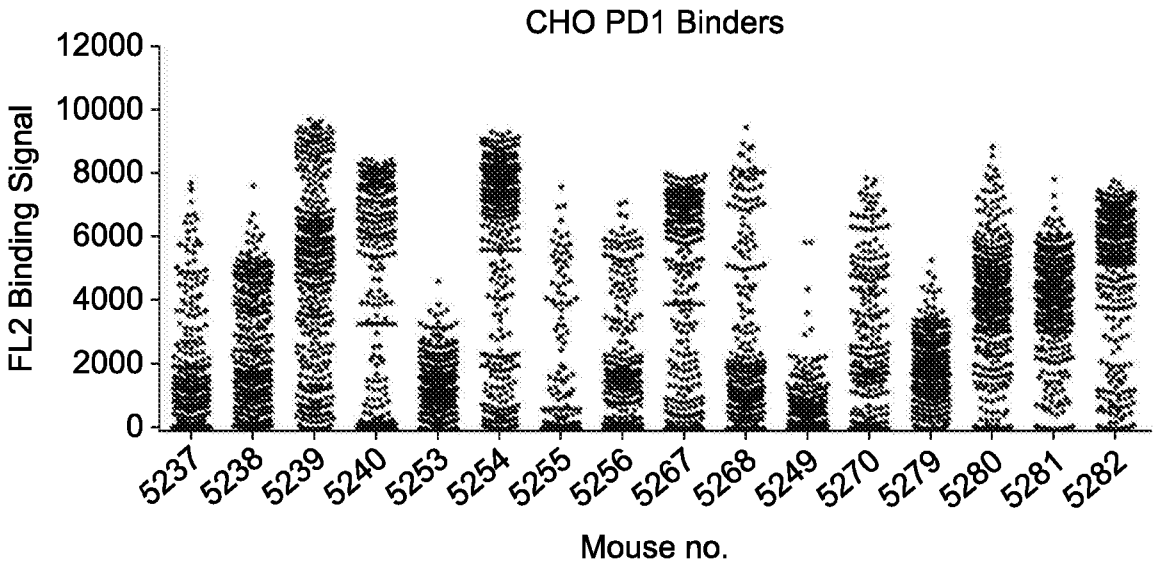
CHO PD1 Binders
FL2 Binding Signal
12000
10000
8000
6000
4000
2000
0
5237
5238
5239
5240
5253
5254
5255
5256
5267
5268
5249
5270
5279
5280
5281
5282
Mouse no.

FIG. 1a

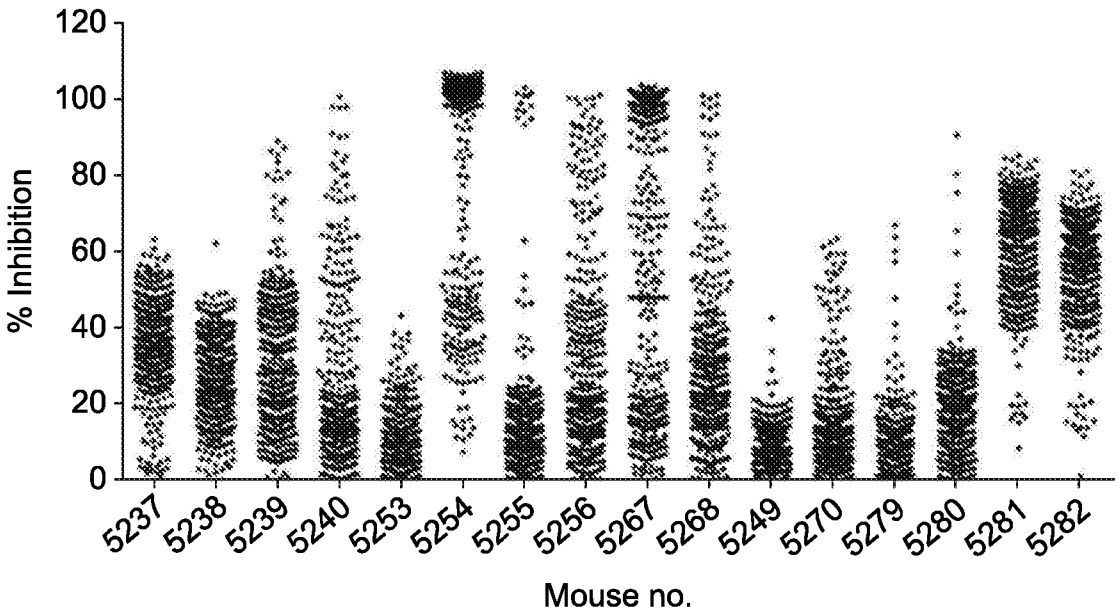
% Inhibition
120
100
80
60
40
20
0
5237
5238
5239
5240
5253
5254
5255
5256
5267
5268
5249
5270
5279
5280
5281
5282
Mouse no.

FIG. 1b

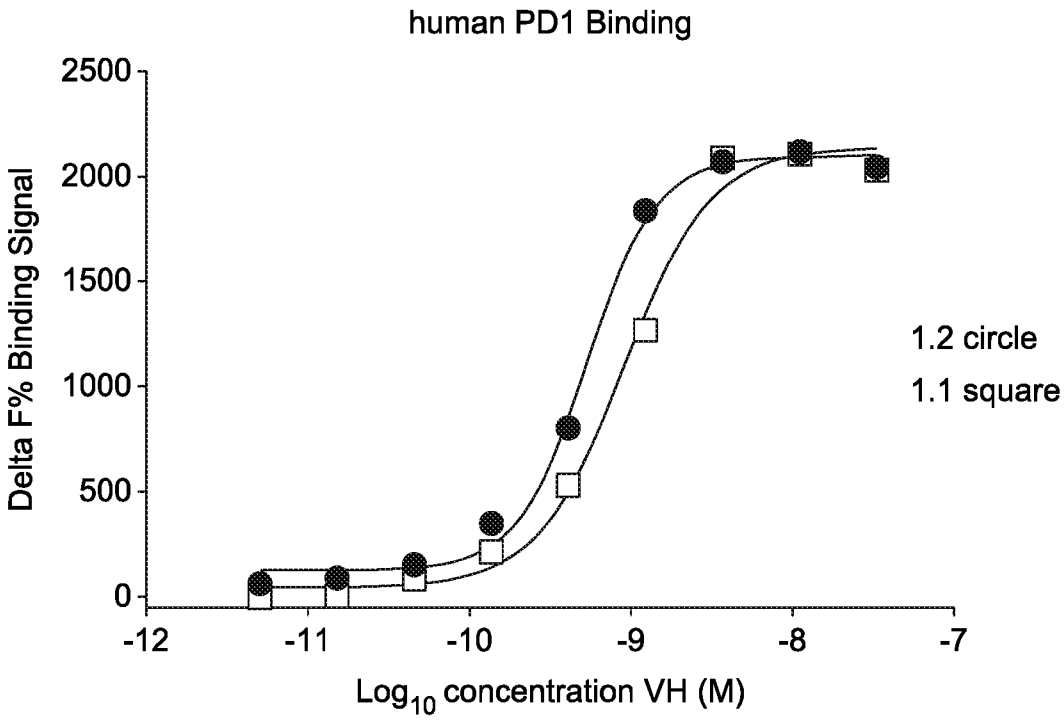
human PD1 Binding
Delta F% Binding Signal
2500
2000
1500
1000
500
0
-12
-11
-10
-9
-8
-7
Log10 concentration VH (M)
1.2 circle
1.1 square

FIG. 2a

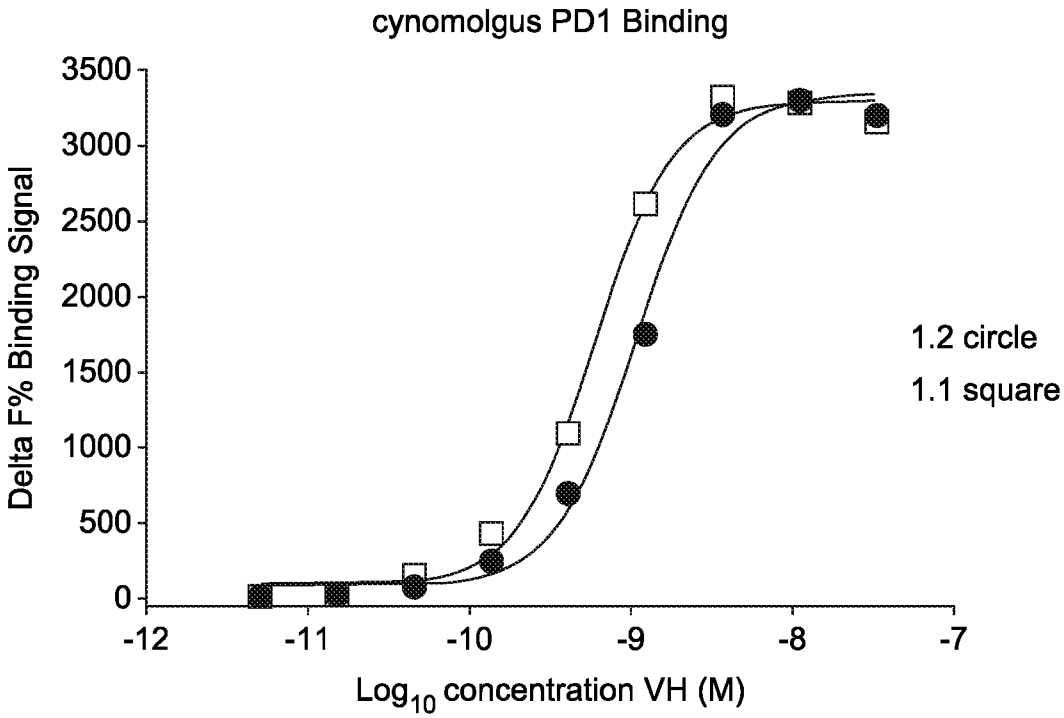
cynomolgus PD1 Binding
Delta F% Binding Signal
3500
3000
2500
2000
1500
1000
500
0
-12
-11
-10
-9
-8
-7
Log10 concentration VH (M)
1.2 circle
1.1 square

FIG. 2b

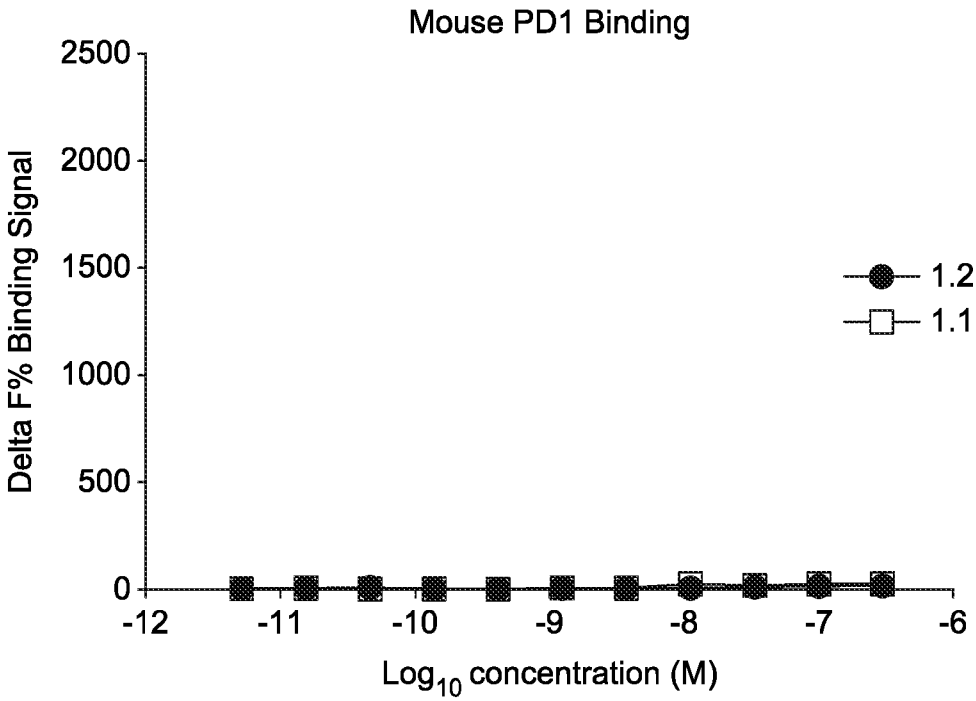
Mouse PD1 Binding
Delta F% Binding Signal
2500
2000
1500
1000
500
0
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)
1.2
1.1

FIG. 2c

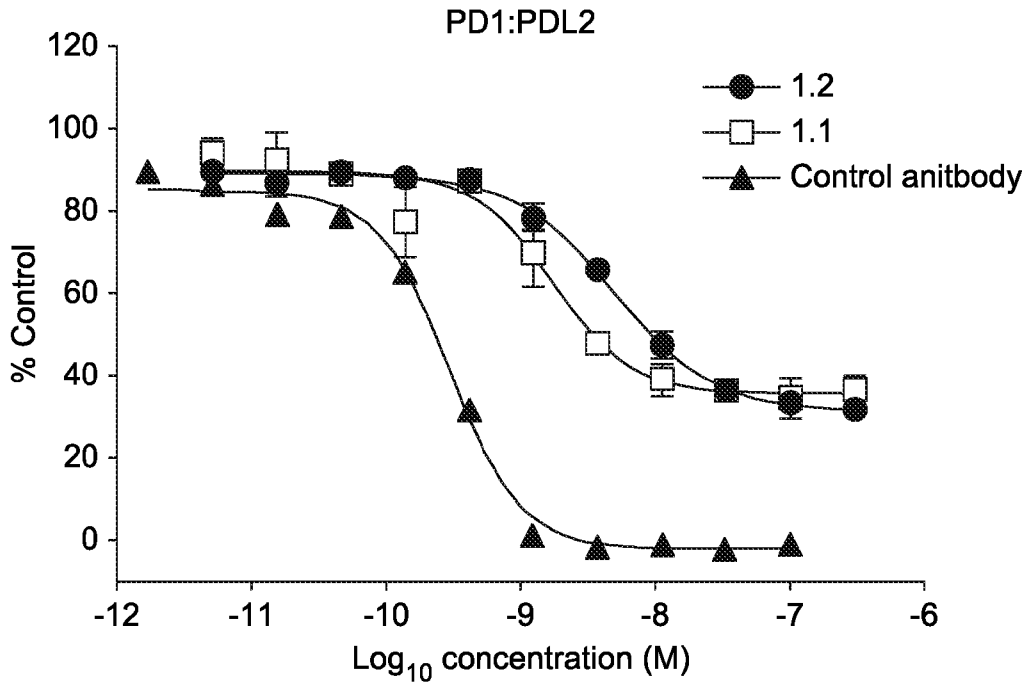
PD1:PDL2
% Control
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)
1.2
1.1
Control anitbody

FIG. 3a

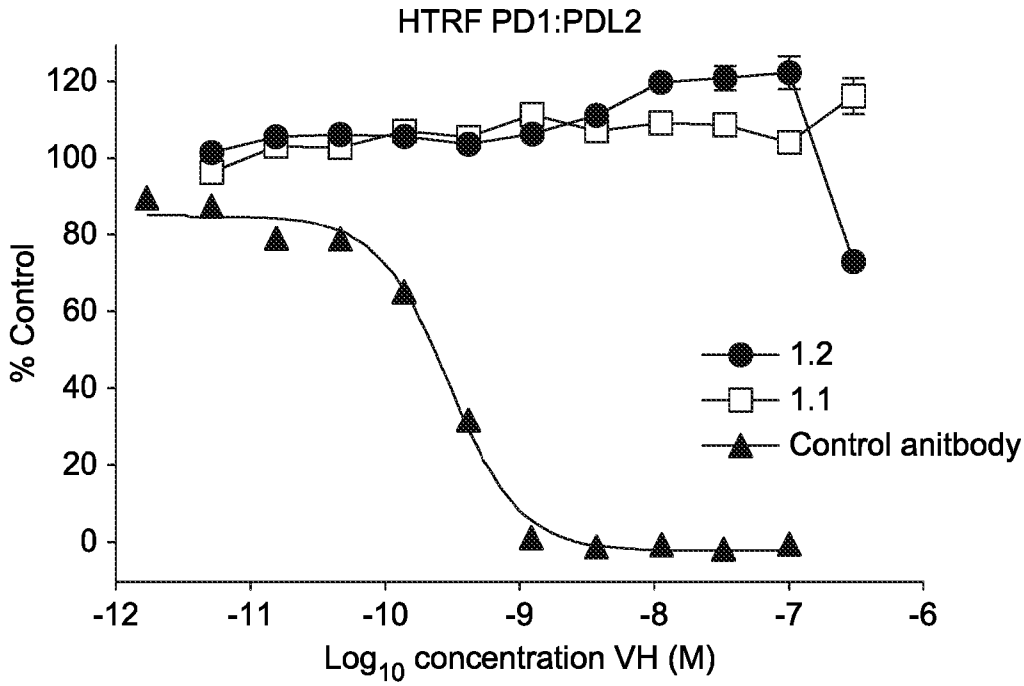
HTRF PD1:PDL2
% Control
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
Log10 concentration VH (M)
1.2
1.1
Control anitbody

FIG. 3b

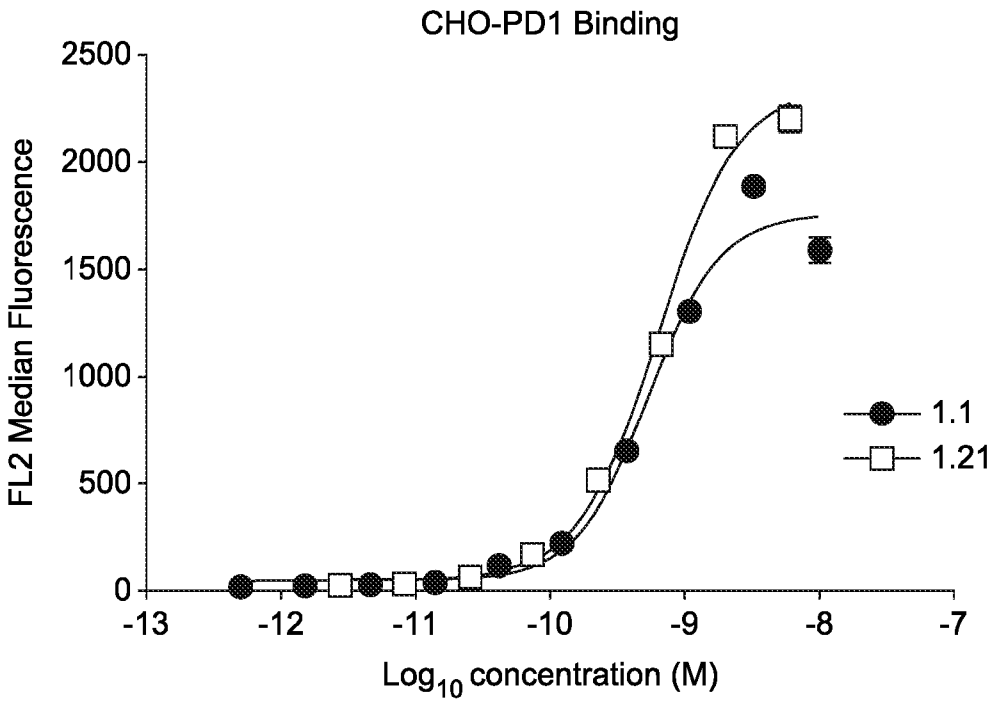
CHO-PD1 Binding
FL2 Median Fluorescence
2500
2000
1500
1000
500
0
-13
-12
-11
-10
-9
-8
-7
Log10 concentration (M)
1.1
1.21

FIG. 4a

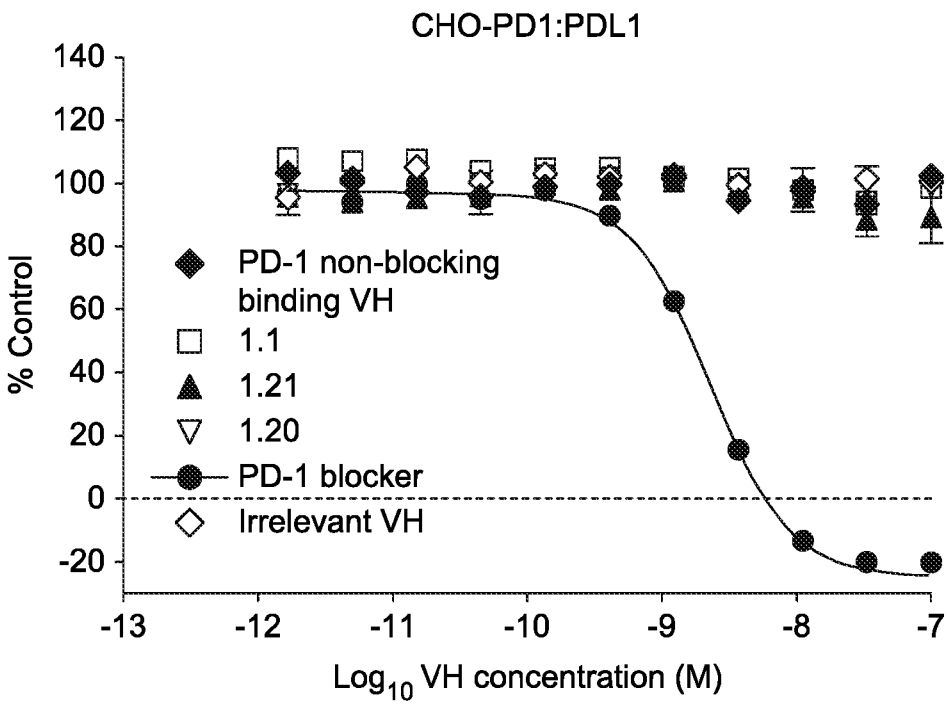
CHO-PD1:PDL1
% Control
140
120
100
80
60
40
20
0
-20
PD-1 non-blocking binding VH
1.1
1.21
1.20
PD-1 blocker
Irrelevant VH
-13
-12
-11
-10
-9
-8
-7
Log10 VH concentration (M)

FIG. 4b

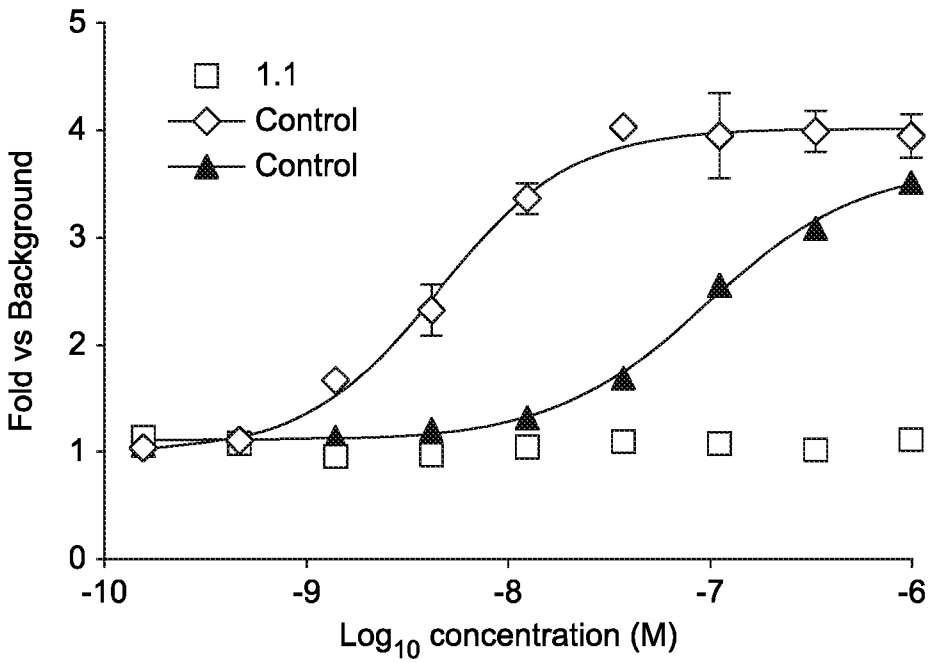
Fold vs Background
5
4
3
2
1
0
1.1
Control
Control
-10
-9
-8
-7
-6
Log10 concentration (M)

FIG. 5a

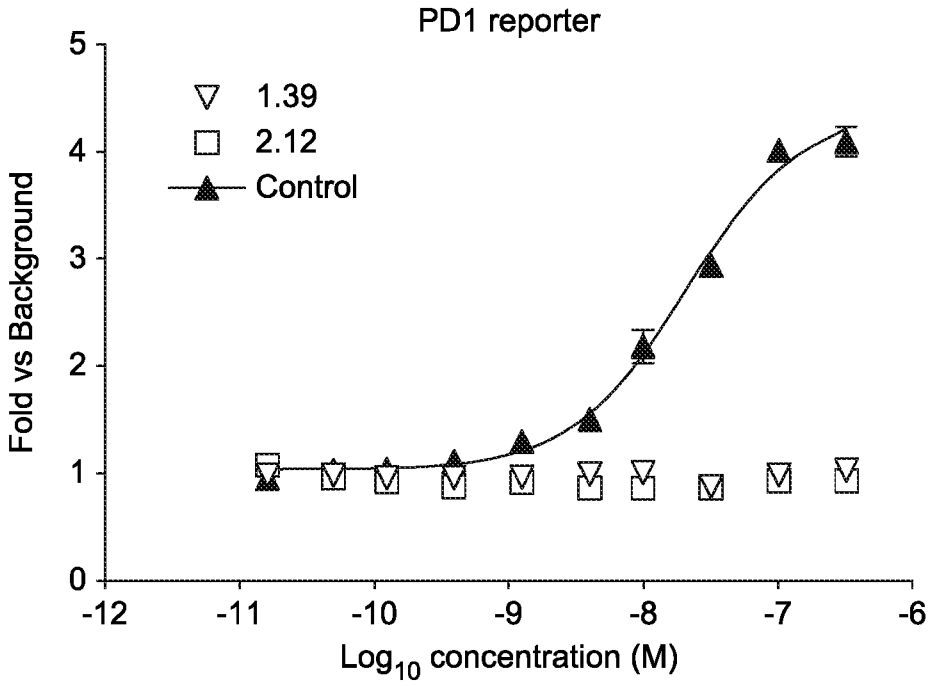
PD1 reporter
1.39
2.12
Control
Fold vs Background
0
1
2
3
4
5
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)

FIG. 5b

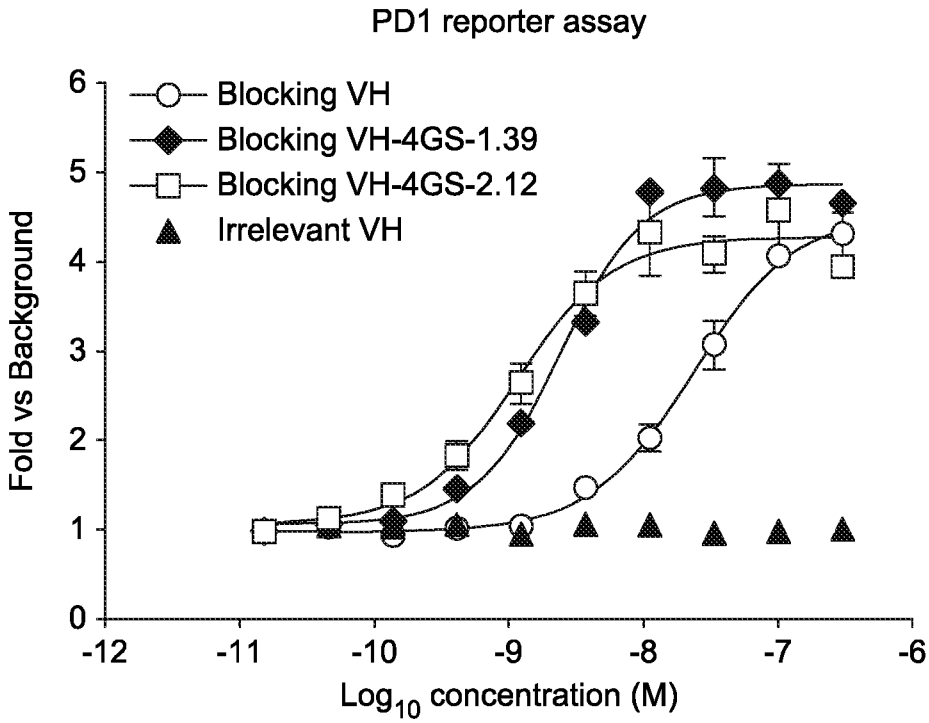
PD1 reporter assay
Blocking VH
Blocking VH-4GS-1.39
Blocking VH-4GS-2.12
Irrelevant VH
Fold vs Background
0
1
2
3
4
5
6
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)

FIG. 5c

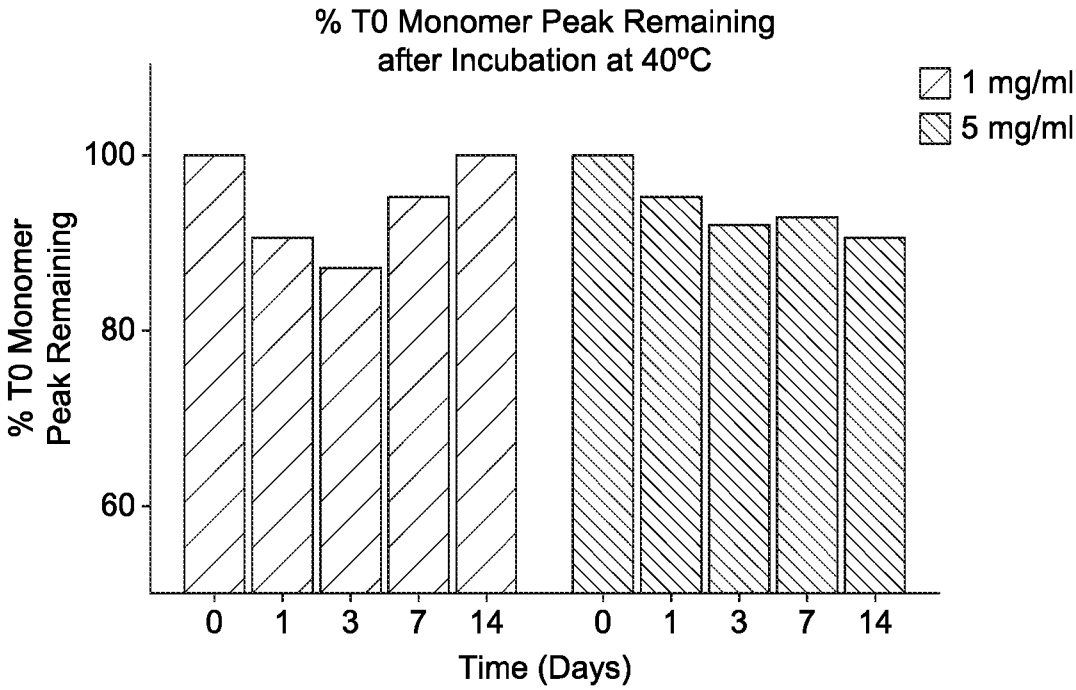
% T0 Monomer Peak Remaining
after Incubation at 40ºC
1 mg/ml
5 mg/ml
% T0 Monomer
Peak Remaining
100
80
60
0
1
3
7
14
0
1
3
7
14
Time (Days)

FIG. 6

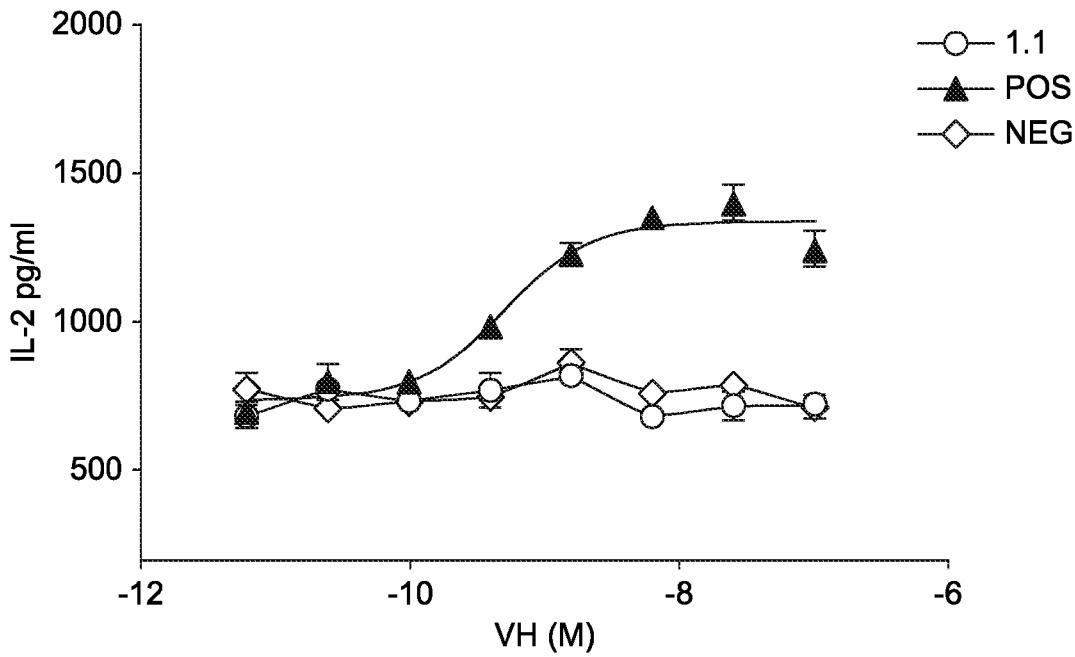
2000
1500
1000
500
IL-2 pg/ml
1.1
POS
NEG
-12
-10
-8
-6
VH (M)

FIG. 7

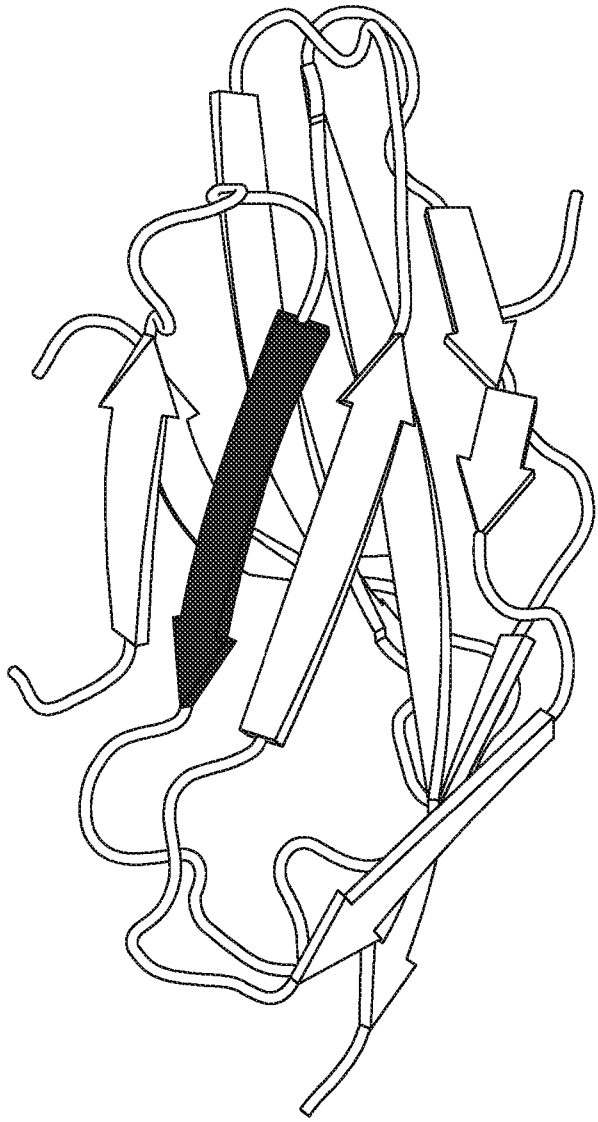

FIG. 8a

```
Human:  NGRDFHMSVVRARR
            |  ||||     ||
Mouse:  NRHDFHMNILDTRR
```

FIG. 8b

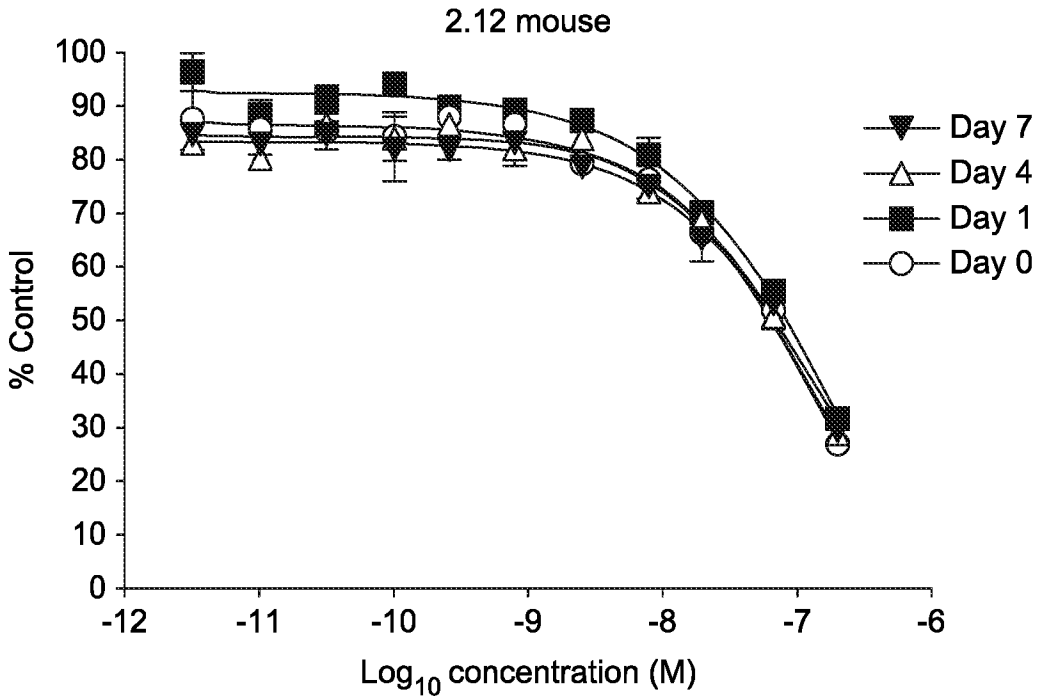
2.12 mouse
% Control
100
90
80
70
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)
Day 7
Day 4
Day 1
Day 0

FIG. 9a

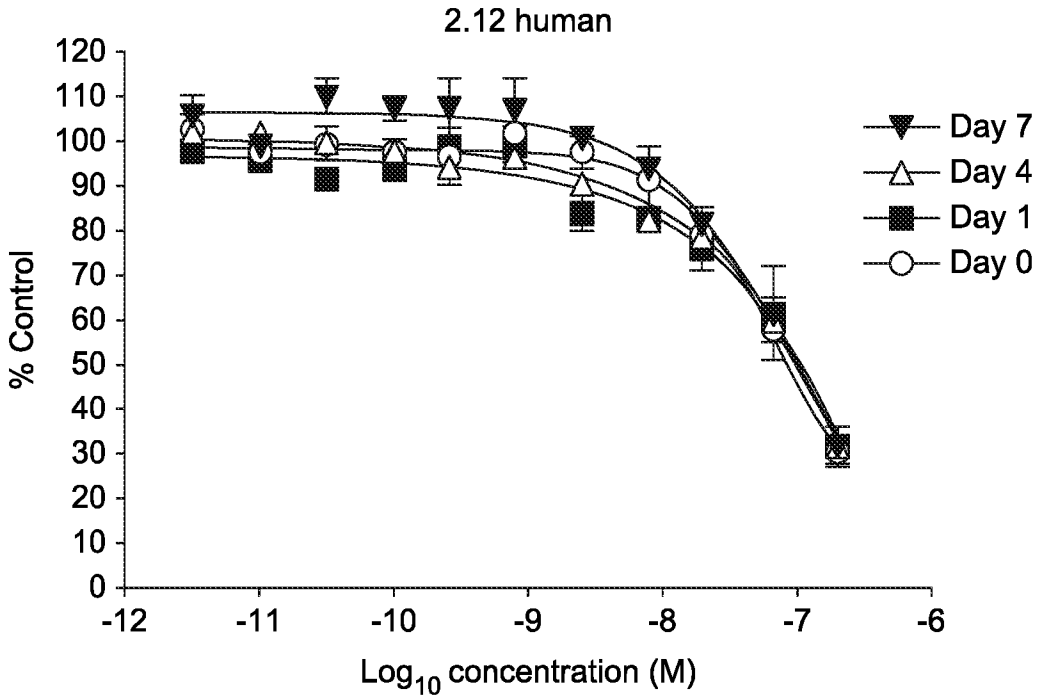
2.12 human
% Control
120
110
100
90
80
70
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
Log10 concentration (M)
Day 7
Day 4
Day 1
Day 0

FIG. 9b

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/050035

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/110621 A1 (UCB PHARMA SA [BE]; TYSON KERRY LOUISE [GB]) 15 September 2011 (2011-09-15)	1-10, 14-59
Y	page 12, paragraphs 3,4; example 2	1-10, 14-59

X	WO 2009/114335 A2 (MERCK & CO INC [US]; FINNEFROCK ADAM C [US]; FU TONG-MING [US]; FREED) 17 September 2009 (2009-09-17)	1-10, 14-59
Y	page 3, paragraph 6	1-10, 14-59

X	WO 2016/106159 A1 (ENUMERAL BIOMEDICAL HOLDING INC [US]) 30 June 2016 (2016-06-30)	1-10, 14-59
Y	paragraphs [0062], [0063]; table 43	1-10, 14-59

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
7 February 2018	25/04/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fellows, Edward

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2016/020856 A2 (MABQUEST SA [CH]) 11 February 2016 (2016-02-11) paragraphs [0017], [0020]	1-10, 14-59 1-10, 14-59

Y	YU GENG, ANDY LEE AND XIN WANG: "Single doman antibodies against immune checkpoint targets PD-1 and PD-L1", ANTIBODES AND THERAPEUTICS CONFERANCE 2016, December 2016 (2016-12), XP055447941, DOI: 10.4172/1745-7580.C1.005 abstract	1-10, 14-59

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2018/050035

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.:
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:

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:

see additional sheet

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.:

1-10, 14-59(all partially)

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

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10, 14-59(all partially)

VH sdAb 1.1 defined by its SEQ ID number as seen in present claim 9 and related subject-matter.

2-50. claims: 1-7, 11-59(all partially)

VH sdAb 1.2-1.50 defined respectively by their SEQ ID numbers as seen in present claim 9 and related subject-matter.

51-103. claims: 1-10, 14-59(all partially)

VH sdAb 2.1-2.53 defined respectively by their SEQ ID numbers as seen in present claim 12 and related subject-matter.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2018/050035

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011110621 A1	15-09-2011	AR 080501 A1	11-04-2012
		CA 2791944 A1	15-09-2011
		CN 102892785 A	23-01-2013
		EP 2545076 A1	16-01-2013
		JP 6174321 B2	02-08-2017
		JP 2013521769 A	13-06-2013
		JP 2016155827 A	01-09-2016
		TW 201134488 A	16-10-2011
		US 2011229461 A1	22-09-2011
		US 2016068586 A1	10-03-2016
		WO 2011110621 A1	15-09-2011
WO 2009114335 A2	17-09-2009	EP 2262837 A2	22-12-2010
		US 2011008369 A1	13-01-2011
		WO 2009114335 A2	17-09-2009
WO 2016106159 A1	30-06-2016	CA 2971734 A1	30-06-2016
		EP 3237446 A1	01-11-2017
		US 2016251436 A1	01-09-2016
		US 2016319019 A1	03-11-2016
		WO 2016106159 A1	30-06-2016
WO 2016020856 A2	11-02-2016	AU 2015298356 A1	16-02-2017
		CA 2957258 A1	11-02-2016
		CN 107074947 A	18-08-2017
		EP 3177644 A2	14-06-2017
		JP 2017531028 A	19-10-2017
		KR 20170069996 A	21-06-2017
		SG 11201700672Y A	27-02-2017
		US 2017166642 A1	15-06-2017
		US 2017226210 A1	10-08-2017
		WO 2016020856 A2	11-02-2016