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(54) Title: ANTIGEN-BINDING PROTEINS

(57) Abstract: The present invention relates to antigen binding proteins comprising a receptor-Fc fusion which is linked to one or more epitope-binding domains, methods for making such proteins, and uses thereof.

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Antigen-binding Proteins

Background

Receptor-Fc fusions (soluble receptors) are well known as therapeutic proteins which are capable of binding to and neutralising a target. Examples of Receptor-Fc fusions which are currently on the market or in clinical development are abatacept, etanercept and atacicept.

Abatacept (marketed as Orencia) is a fusion protein composed of an immunoglobulin fused to the extracellular domain of CTLA-4, a molecule capable of binding B7. Abatacept is a selective costimulation modulator as it inhibits the costimulation of T cells. It is licensed in the United States for the treatment of rheumatoid arthritis in the case of inadequate response to anti-TNFa therapy.

Etanercept (Enbrel) is a soluble recombinant human p75 tumour necrosis factor TNF receptor (TNFR2) and human IgG1 Fc portion fusion protein produced in a mammalian cell expression system, which is being developed for use in treating rheumatoid arthritis (RA) and other inflammatory conditions.

Atacicept is a recombinant fusion protein that comprises the receptor portion of the B lymphocyte TACI receptor, which binds to and is activated by the cytokines BlyS and APRIL. The soluble protein comprises the fusion of the extracellular domain of the TACI receptor with the Fc portion of human IgG1. The TACI receptor is a member of the TNF receptor family. Atacicept binds to excess BLyS and APRIL, preventing their binding to B-cells, thereby regulating B-cell maturity and antibody production. It is being developed for the treatment of autoimmune disease.

Summary of invention

The present invention in particular relates to an antigen-binding protein comprising a receptor-Fc fusion which is linked to one or more epitope-binding domains.

The invention also provides a polynucleotide sequence encoding a heavy chain of any of the antigen-binding protein described herein, and a polynucleotide encoding a light chain of any of the antigen-binding proteins described herein. Such polynucleotides represent the coding sequence which corresponds to the equivalent polypeptide sequences, however it will be understood that such polynucleotide sequences could be cloned into an expression vector along with a start codon, an appropriate signal sequence and a stop codon.

The invention also provides a recombinant transformed or transfected host cell comprising one or more polynucleotides encoding a heavy chain and a light chain of any of the antigen-binding proteins described herein.

The invention further provides a method for the production of any of the antigen-binding proteins described herein which method comprises the step of culturing a host cell comprising a vector, encoding any of the antigen-binding proteins described herein, for example in a serum- free culture media.

The invention further provides a pharmaceutical composition comprising an antigen-binding protein as described herein a pharmaceutically acceptable carrier.

The invention further provides the use of such antigen-binding proteins or pharmaceutical compositions such antigen-binding proteins in the treatment of immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases.

Definitions

The term 'receptor-Fc fusion' as used herein refers to a soluble ligand or extracellular domain of a receptor or cell surface protein linked to the Fc region of an antibody. Fragments of such soluble ligands or extracellular domains of a receptor or cell surface protein are included within this definition providing they retain the biological function of the full length protein, i.e. providing they retain antigen-binding ability.

A "domain" is a folded protein structure which has tertiary structure independent of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain. A "single antibody variable domain" is a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains and modified variable domains, for example, in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least the binding activity and specificity of the full-length domain.

The phrase "immunoglobulin single variable domain" refers to an antibody variable domain (V_H, V_{HH}, V_L) that specifically binds an antigen or epitope independently of a different V region or domain. An immunoglobulin single variable domain can be present in a format (*e.g.,* homo- or hetero-multimer) with other, different variable regions or variable domains where the other regions or domains are not required for antigen binding by the single immunoglobulin variable domain (*i.e.*, where the immunoglobulin single variable domain binds antigen independently of the additional variable domains). A "domain antibody" or "dAb" is the same as an "immunoglobulin single variable domain" which is capable of binding to an antigen as the term is used herein. An immunoglobulin single variable domain may be a human antibody variable domain, but also includes single antibody variable domains from other species such as rodent (for example, as disclosed in WO 00/29004), nurse shark and *Camelid* V_{HH} dAbs. Camelid V_{HH} are immunoglobulin single variable domain polypeptides that are derived from species including camel, llama, alpaca, dromedary, and guanaco, which produce heavy chain antibodies naturally devoid of light chains. Such V_{HH} domains may be humanised according to standard techniques available in the art, and such domains are still considered to be "domain antibodies" according to the invention. As used herein "V_H includes camelid V_{HH} domains. NARV are another type of immunoglobulin single variable domain which were identified in cartilaginous fish including the nurse shark. These domains are also known as Novel Antigen Receptor variable region (commonly abbreviated to V(NAR) or NARV). For further details see Mol. Immunol. **44**, 656-665 (2006) and US20050043519A.

The term “Epitope-binding domain” refers to a domain that specifically binds an antigen or epitope independently of a different V region or domain, this may be a domain antibody (dAb), for example a human, camelid or shark immunoglobulin single variable domain or it may be a non-Immunoglobulin (non-Ig) domain which has been subjected to protein engineering in order to obtain binding to a ligand other than its natural ligand, for example a domain which is a derivative of a scaffold selected from the group consisting of CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody); Heat shock proteins such as GroEl and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human γ-crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin); which has been subjected to protein engineering in order to obtain binding to a ligand other than its natural ligand.

CTLA-4 (Cytotoxic T Lymphocyte-associated Antigen 4) is a CD28-family receptor expressed on mainly CD4+ T-cells. Its extracellular domain has a variable domain-like immunoglobulin fold. Loops corresponding to CDRs of antibodies can be substituted with heterologous sequence to confer different binding properties. CTLA-4 molecules engineered to have different binding specificities are also known as Evibodies. For further details see Journal of Immunological Methods **248 (1-2)**, 31-45 (2001)

Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid β-sheet secondary structure with a numer of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are derived from lipocalins. For further details see Biochim Biophys Acta **1482**: 337-350 (2000), US7250297B1 and US20070224633

An affibody is a scaffold derived from Protein A of *Staphylococcus aureus* which can be engineered to bind to antigen. The domain consists of a three-helical bundle of approximately 58 amino acids. Libraries have been generated by randomisation of surface residues. For further details see Protein Eng. Des. Sel. **17**, 455-462 (2004) and EP1641818A1

Avimers are multidomain proteins derived from the A-domain scaffold family. The native domains of approximately 35 amino acids adopt a defined disulphide bonded structure. Diversity is generated by shuffling of the natural variation exhibited by the family of A-domains. For further details see Nature Biotechnology **23(12)**, 1556 - 1561 (2005) and Expert Opinion on Investigational Drugs **16(6)**, 909-917 (June 2007)

A transferrin is a monomeric serum transport glycoprotein. Transferrins can be engineered to bind different target antigens by insertion of peptide sequences in a permissive surface loop. Examples of engineered transferrin scaffolds include the Trans-body. For further details see J. Biol. Chem **274**, 24066-24073 (1999).

Designed Ankyrin Repeat Proteins (DARPins) are derived from Ankyrin which is a family of proteins that mediate attachment of integral membrane proteins to the cytoskeleton. A single ankyrin repeat is a 33 residue motif consisting of two α-helices and a β-turn. They can be engineered to bind different target antigens by randomising residues in the first α-helix and a β-turn of each repeat. Their binding interface can be increased by increasing the number of modules (a method of affinity maturation). For further details see J. Mol. Biol. **332**, 489-503 (2003), PNAS **100(4)**, 1700-1705 (2003) and J. Mol. Biol. 369, 1015-1028 (2007) and US20040132028A1.

Fibronectin is a scaffold which can be engineered to bind to antigen. Adnectins consists of a backbone of the natural amino acid sequence of the 10th domain of the 15 repeating units of human fibronectin type III (FN3). Three loops at one end of the β-sandwich can be engineered to enable an Adnectin to specifically recognize a therapeutic target of interest. For further details see Protein Eng. Des. Sel. **18**, 435-444 (2005), US20080139791, WO2005056764 and US6818418B1.

Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein, typically thioredoxin (TrxA) which contains a constrained variable peptide loop inserted at the active site. For further details see Expert Opin. Biol. Ther. 5, 783-797 (2005).

Microbodies are derived from naturally occurring microproteins of 25-50 amino acids in length which contain 3-4 cysteine bridges – examples of microproteins include KalataB1 and conotoxin and knottins. The microproteins have a loop which can be engineered to include upto 25 amino acids without affecting the overall fold of the microprotein. For further details of engineered knottin domains, see WO2008098796.

Other epitope binding domains include proteins which have been used as a scaffold to engineer different target antigen binding properties include human γ-crystallin and human ubiquitin (affilins), kunitz type domains of human protease inhibitors, PDZ-domains of the Ras-binding protein AF-6, scorpion toxins (charybdotoxin), C-type lectin domain (tetranectins) are reviewed in Chapter 7 – Non-Antibody Scaffolds from Handbook of Therapeutic Antibodies (2007, edited by Stefan Dubel) and Protein Science **15**:14-27 (2006). Epitope binding domains of the present invention could be derived from any of these alternative protein domains.

In one embodiment of the invention the antigen-binding site binds to antigen with a Kd of at least 1mM, for example a Kd of 10nM, 1nM, 500pM, 200pM, 100pM, to each

antigen as measured by Biacore™, such as the Biacore™ method as described in method 4 or 5.

As used herein, the term “antigen-binding site” refers to a site on a protein which is capable of specifically binding to antigen, this may be a single domain, for example an epitope-binding domain, or it may be the portion of the soluble ligand or extracellular domain of a receptor or cell surface protein which is capable of binding antigen.

Detailed description of Invention

The present invention provides an antigen-binding protein comprising a Receptor-Fc fusion which is linked to one or more epitope-binding domains.

Such antigen-binding proteins comprise an immunoglobulin scaffold, i.e. they comprise the Fc portion of an antibody, which is linked to
(i) a soluble ligand or extracellular domain of a receptor or cell surface protein, and
(ii) one or more epitope-binding domains.

The antigen-binding proteins of the present invention are also referred to as Receptor-Fc-epitope binding domain fusions or Receptor-Ig-epitope binding domain fusions.

The antigen binding proteins of the present invention comprise an Fc portion of an antibody. This Fc portion may be selected from antibodies of any isotype, for example IgG1, IgG2, IgG3, IgG4 or IgG4PE.

In one embodiment of the present invention the epitope binding domain is an immunoglobulin single variable domain.

It will be understood that any of the antigen-binding proteins described herein will be capable of neutralising one or more antigens.

The term “neutralises” and grammatical variations thereof as used throughout the present specification in relation to antigen-binding proteins of the invention means that a biological activity of the target is reduced, either totally or partially, in the presence of the antigen-binding proteins of the present invention in comparison to the activity of the target in the absence of such antigen-binding proteins. Neutralisation may be due to but not limited to one or more of blocking ligand binding, preventing the ligand activating the receptor, down regulating the receptor or affecting effector functionality.

Levels of neutralisation can be measured in several ways, for example by use of any of the assays as set out in the examples below, for example in an assay which measures inhibition of ligand binding to receptor which may be carried out for example as described in Example 3. The neutralisation of VEGFR2, in this assay is measured by assessing the decreased binding between the ligand and its receptor in the presence of neutralising antigen-binding protein.

Other methods of assessing neutralisation, for example, by assessing the decreased binding between the ligand and its receptor in the presence of neutralising antigen-binding protein are known in the art, and include, for example, Biacore™ assays.

In an alternative aspect of the present invention there is provided antigen-binding proteins which have at least substantially equivalent neutralising activity to the antigen-binding proteins exemplified herein.

In one embodiment the antigen-binding proteins of the invention have specificity for VEGF, for example they comprise a receptor-Fc fusion linked to an epitope binding domain which binds to VEGF, for example an immunoglobulin single variable domain, an anticalin, or an adnectin which binds to VEGF.

In one embodiment the antigen-binding proteins of the invention have specificity for VEGFR2, for example they comprise a receptor-Fc fusion linked to an epitope binding domain which binds to VEGFR2, for example an immunoglobulin single variable domain or an adnectin which binds to VEGFR2.

In one embodiment the antigen-binding proteins of the invention have specificity for TNFα, for example they comprise a receptor-Fc fusion linked to an epitope binding domain which binds to TNFα, for example an immunoglobulin single variable domain or an adnectin which binds to TNFα.

In one embodiment the antigen-binding proteins of the invention have specificity for IL-13, for example they comprise a receptor-Fc fusion linked to an epitope binding domain which binds to IL-13, for example an immunoglobulin single variable domain or an adnectin which binds to IL-13.

In one embodiment the antigen-binding proteins of the invention have specificity for HER2, for example they comprise a receptor-Fc fusion linked to an epitope binding domain which binds to HER2, for example an immunoglobulin single variable domain or an adnectin which binds to HER2.

In one embodiment the antigen-binding protein of the present invention has specificity for only one antigen, for example, the present invention provides a receptor-Fc fusion capable of binding TNFα linked to one or more epitope binding domains which are capable of binding TNFα, for example the receptor-Fc fusion set out in SEQ ID NO: 21 or SEQ ID NO: 28 linked to the epitope binding domain set out in SEQ ID NO:7.

In an alternative embodiment the antigen-binding protein of the present invention has specificity for more than one antigen, for example, the present invention provides a receptor-Fc fusion capable of binding B7-1 linked to one or more epitope binding

domains which are capable of binding to one or more antigens selected from VEGFR2, VEGF, TNFα, HER2, IL-13 for example, a receptor-Fc fusion capable of binding B7-1 linked to an epitope binding domain capable of binding VEGFR2, or a receptor-Fc fusion capable of binding B7-1 linked to an epitope binding domain capable of binding VEGF, or a receptor-Fc fusion capable of binding B7-1 linked to an epitope binding domain capable of binding TNFα, or a receptor-Fc fusion capable of binding B7-1 linked to an epitope binding domain capable of binding HER2.

In one embodiment the antigen-binding protein of the present invention is capable of binding B7-1 and VEGFR2 simultaneously, or B7-1 and VEGF simultaneously, or B7-1 and TNFα simultaneously, or B7-1 and HER2 simultaneously.

In one embodiment the antigen-binding protein of the present invention has specificity for more than one antigen, for example, the present invention provides a receptor-Fc fusion capable of binding BLys and/or APRIL linked to one or more epitope binding domains which are capable of binding to one or more antigens selected from VEGFR2, VEGF, TNFα, HER2, IL-13 for example, a receptor-Fc fusion capable of binding BLys and/or APRIL linked to an epitope binding domain capable of binding VEGFR2, or a receptor-Fc fusion capable of binding BLys and/or APRIL linked to an epitope binding domain capable of binding VEGF, or a receptor-Fc fusion capable of binding BLys and/or APRIL linked to an epitope binding domain capable of binding TNFα, or a receptor-Fc fusion capable of binding BLys and/or APRIL linked to an epitope binding domain capable of binding HER2.

In one embodiment the antigen-binding protein of the present invention is capable of binding BLys and/or APRIL and VEGFR2 simultaneously, or BLys and/or APRIL and VEGF simultaneously, or BLys and/or APRIL and TNFα simultaneously, or BLys and/or APRIL and HER2 simultaneously,

In one embodiment the antigen-binding protein of the present invention has specificity for more than one antigen, for example, the present invention provides a receptor-Fc fusion capable of binding TNFα linked to one or more epitope binding domains which are capable of binding to one or more antigens selected from VEGFR2, VEGF, HER2, IL-13 for example , a receptor-Fc fusion capable of binding TNFα linked to an epitope binding domain capable of binding VEGFR2, or a receptor-Fc fusion capable of binding TNFα linked to an epitope binding domain capable of binding VEGF, or a receptor-Fc fusion capable of binding TNFα linked to an epitope binding domain capable of binding HER2.

In one embodiment the antigen-binding protein of the present invention is capable of binding TNFα and VEGFR2 simultaneously, or TNFα and VEGF simultaneously, or TNFα and HER2 simultaneously,

It will be understood that any of the antigen-binding proteins described herein may be capable of binding two or more antigens simultaneously, for example, as determined by stochiometry analysis by using a suitable assay such as that described in Example 4.

Examples of such antigen-binding proteins include CTLA-4-Ig fusions linked to an epitope binding domain with a specificity for VEGFR2, for example an anti- VEGFR2 adnectin, linked to the c-terminus or the n-terminus of the CTLA-4 Ig fusion, for example an antigen-binding protein comprising the CTLA-4-Ig sequence set out in SEQ ID NO:19 or SEQ ID NO:20 which further comprises one or more epitope-binding domains which bind to VEGFR2, for example the adnectin set out in SEQ ID NO: 6. Examples of such a Receptor-Fc-adnectin fusion include the antigen binding protein set out in SEQ ID NO:23 or SEQ ID NO:4.

Other examples of such antigen-binding proteins include CTLA-4-Ig fusions linked to an epitope binding domain with a specificity for VEGF for example an anti- VEGF immunoglobulin single variable domain or anti-VEGF anticalin, linked to the c-terminus or the n-terminus of the CTLA-4 Ig fusion, for example a Receptor-Fc-epitope binding domain fusion comprising the CTLA-4-Ig sequence set out in SEQ ID NO: 19 or SEQ ID NO:20, which further comprises one or more epitope-binding domains which bind to VEGF, for example the dAb set out in SEQ ID NO: 13, or the anticalin set out in SEQ ID NO: 9. Examples of such a Receptor-Fc-dAb fusion include the antigen binding protein set out in SEQ ID NO:27 or SEQ ID NO:28.

Other examples of such antigen-binding proteins include CTLA-4-Ig fusions linked to an epitope binding domain with a specificity for TNFα, for example an anti- TNFα adnectin, linked to the c-terminus or the n-terminus of the CTLA-4 Ig fusion for example a Receptor-Fc-epitope binding domain fusion comprising the CTLA-4-Ig sequence set out in SEQ ID NO: 19 or SEQ ID NO:20, which further comprises one or more epitope-binding domains which bind to TNFα, for example the adnectin set out in SEQ ID NO:7. Examples of such a Receptor-Fc-adnectin fusion include the antigen binding protein set out in SEQ ID NO:25 or SEQ ID NO:26.

Other examples of such antigen-binding proteins include CTLA-4-Ig fusions linked to an epitope binding domain with a specificity for IL-13, for example an anti- IL-13 immunoglobulin single variable domain, linked to the c-terminus or the n-terminus of the CTLA-4 Ig fusion, for example a Receptor-Fc-dAb fusion comprising the CTLA-4-Ig sequence set out in SEQ ID NO: 19 or SEQ ID NO:20, which further comprises one or more epitope-binding domains which bind to IL-13, for example the dAb set out in SEQ ID NO:14. Examples of such a Receptor-Fc-dAb fusion include the antigen binding protein set out in SEQ ID NO:29 or SEQ ID NO:30.

Other examples of such antigen-binding proteins include CTLA-4-Ig fusions linked to an epitope binding domain with a specificity for HER2, for example an anti-HER2 affibody, linked to the c-terminus or the n-terminus of the CTLA-4 Ig fusion, for example a Receptor-Fc-epitope binding fusion comprising the CTLA-4-Ig sequence set out in SEQ ID NO: 19 or SEQ ID NO:20, which further comprises one or more epitope-binding domains which bind to HER2, for example the DARPin set out in SEQ ID NO: 8, or the affibody set out in SEQ ID NO:10.

Examples of such antigen-binding proteins include TNFR2-Ig fusions linked to an epitope binding domain with a specificity for VEGFR2, for example an anti- VEGFR2 adnectin, linked to the c-terminus or the n-terminus of the TNFR2-Ig fusion, for example an antigen-binding protein comprising the TNFR2-Ig sequence set out in SEQ ID NO:21 which further comprises one or more epitope-binding domains which bind to VEGFR2, for example the adnectin set out in SEQ ID NO: 6.

Other examples of such antigen-binding proteins include TNFR2-Ig fusions linked to an epitope binding domain with a specificity for VEGF for example an anti- VEGF immunoglobulin single variable domain or anti-VEGF anticalin, linked to the c-terminus or the n-terminus of the TNFR2-Ig fusion, for example a Receptor-Fc-epitope binding domain fusion comprising the TNFR2-Ig sequence set out in SEQ ID NO: 21, which further comprises one or more epitope-binding domains which bind to VEGF, for example the dAb set out in SEQ ID NO: 13, or the anticalin set out in SEQ ID NO: 9.

Other examples of such antigen-binding proteins include TNFR2-Ig fusions linked to an epitope binding domain with a specificity for TNFα, for example an anti- TNFα adnectin, linked to the c-terminus or the n-terminus of the TNFR2-Ig fusion for example a Receptor-Fc-epitope binding domain fusion comprising the TNFR2-Ig sequence set out in SEQ ID NO: 21, which further comprises one or more epitope-binding domains which bind to TNFα, for example the adnectin set out in SEQ ID NO:7.

Other examples of such antigen-binding proteins include TNFR2-Ig fusions linked to an epitope binding domain with a specificity for IL-13, for example an anti- IL-13 immunoglobulin single variable domain, linked to the c-terminus or the n-terminus of the TNFR2-Ig fusion, for example a Receptor-Fc-dAb fusion comprising the TNFR2-Ig sequence set out in SEQ ID NO: 21, which further comprises one or more epitope-binding domains which bind to IL-13, for example the dAb set out in SEQ ID NO:14.

Other examples of such antigen-binding proteins include TNFR2-Ig fusions linked to an epitope binding domain with a specificity for HER2, for example an anti-HER2 affibody, linked to the c-terminus or the n-terminus of the TNFR2-Ig fusion, for example a Receptor-Fc-epitope binding fusion comprising the TNFR2-Ig sequence

set out in SEQ ID NO: 21, which further comprises one or more epitope-binding domains which bind to HER2, for example the DARPin set out in SEQ ID NO: 8, or the affibody set out in SEQ ID NO:10.

Examples of such antigen-binding proteins include TACI-Ig fusions linked to an epitope binding domain with a specificity for VEGFR2, for example an anti- VEGFR2 adnectin, linked to the c-terminus or the n-terminus of the TACI-Ig fusion, for example an antigen-binding protein comprising the TACI-Ig sequence set out in SEQ ID NO:22 which further comprises one or more epitope-binding domains which bind to VEGFR2, for example the adnectin set out in SEQ ID NO: 6.

Other examples of such antigen-binding proteins include TACI-Ig fusions linked to an epitope binding domain with a specificity for VEGF for example an anti- VEGF immunoglobulin single variable domain or anti-VEGF anticalin, linked to the c-terminus or the n-terminus of the TACI-Ig fusion, for example a Receptor-Fc-epitope binding domain fusion comprising the TACI-Ig sequence set out in SEQ ID NO: 22, which further comprises one or more epitope-binding domains which bind to VEGF, for example the dAb set out in SEQ ID NO: 13, or the anticalin set out in SEQ ID NO: 9.

Other examples of such antigen-binding proteins include TACI-Ig fusions linked to an epitope binding domain with a specificity for TNFα, for example an anti-TNFα adnectin, linked to the c-terminus or the n-terminus of the TACI-Ig fusion for example a Receptor-Fc-epitope binding domain fusion comprising the TACI-Ig sequence set out in SEQ ID NO: 22, which further comprises one or more epitope-binding domains which bind to TNFα, for example the adnectin set out in SEQ ID NO:7.

Other examples of such antigen-binding proteins include TACI-Ig fusions linked to an epitope binding domain with a specificity for IL-13, for example an anti- IL-13 immunoglobulin single variable domain, linked to the c-terminus or the n-terminus of the TACI-Ig fusion, for example a Receptor-Fc-dAb fusion comprising the TACI-Ig sequence set out in SEQ ID NO: 22, which further comprises one or more epitope-binding domains which bind to IL-13, for example the dAb set out in SEQ ID NO:14.

Other examples of such antigen-binding proteins include TACI-Ig fusions linked to an epitope binding domain with a specificity for HER2, for example an anti-HER2 affibody, linked to the c-terminus or the n-terminus of the TACI-Ig fusion, for example a Receptor-Fc-epitope binding fusion comprising the TACI-Ig sequence set out in SEQ ID NO: 22, which further comprises one or more epitope-binding domains which bind to HER2, for example the DARPin set out in SEQ ID NO: 8, or the affibody set out in SEQ ID NO:10.

Such Receptor-Fc-immunoglobulin single variable domain fusions may also have one or more further epitope binding domains with the same or different antigen-specificity attached to its c-terminus or the n-terminus.

In one embodiment of the present invention there is provided a Receptor-Fc-immunoglobulin single variable domain fusion according to the invention described herein and comprising a constant region such that the Receptor-Fc-immunoglobulin single variable domain fusion has reduced ADCC and/or complement activation or effector functionality. In one such embodiment the heavy chain constant region may comprise a naturally disabled constant region of IgG2 or IgG4 isotype or a mutated IgG1 constant region. Examples of suitable modifications are described in EP0307434. One example comprises the substitutions of alanine residues at positions 235 and 237 (EU index numbering).

In one embodiment the antigen-binding proteins of the present invention will retain Fc functionality for example will be capable of one or both of ADCC and CDC activity.

The antigen-binding proteins of the invention may have some effector function. For example if the Immunoglobulin scaffold contains an Fc region derived from an antibody with effector function, for example if the Immunoglobulin scaffold comprises CH2 and CH3 from IgG1. Levels of effector function can be varied according to known techniques, for example by mutations in the CH2 domain, for example wherein the IgG1 CH2 domain has one or more mutations at positions selected from 239 and 332 and 330, for example the mutations are selected from S239D and I332E and A330L such that the antibody has enhanced effector function, and/or for example altering the glycosylation profile of the antigen-binding protein of the invention such that there is a reduction in fucosylation of the Fc region.

In one embodiment, the antigen-binding proteins comprise an epitope-binding domain which is a domain antibody (immunoglobulin single variable domain), for example the epitope binding domain may be a human VH or human VL, or a camelid V_{HH} or a shark immunoglobulin single variable domain (NARV).
In one embodiment the antigen-binding proteins comprise an epitope-binding domain which is a derivative of a non-Ig scaffold, for example a non-Ig scaffold selected from the group consisting of CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody); Heat shock proteins such as GroEl and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human γ-crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin); which has been subjected to protein engineering in order to obtain binding to a ligand other than its natural ligand.

In one embodiment of the present invention there are four epitope binding domains, for example four domain antibodies, two of the epitope binding domains may have specificity for the same antigen, or all of the epitope binding domains present in the antigen-binding protein may have specificity for the same antigen.

Receptor-Fc fusions of the present invention may be linked to epitope-binding domains by the use of linkers. Examples of suitable linkers include amino acid sequences which may be from 1 amino acid to 150 amino acids in length, or from 1 amino acid to 140 amino acids, for example, from 1 amino acid to 130 amino acids, or from 1 to 120 amino acids, or from 1 to 80 amino acids, or from 1 to 50 amino acids, or from 1 to 20 amino acids, or from 1 to 10 amino acids, or from 5 to 18 amino acids. Such sequences may have their own tertiary structure, for example, a linker of the present invention may comprise a single variable domain. The size of a linker in one embodiment is equivalent to a single variable domain. Suitable linkers may be of a size from 1 to 20 angstroms, for example less than 15 angstroms, or less than 10 angstroms, or less than 5 angstroms.

In one embodiment of the present invention at least one of the epitope binding domains is directly attached to the Receptor-Fc fusion with a linker comprising from 1 to 150 amino acids, for example 1 to 50, for example 1 to 20 amino acids, for example 1 to 10 amino acids. Such linkers may be selected from any one of those set out in SEQ ID NO: 15-18 or SEQ ID NO: 31-32 or SEQ ID NO: 41-42, or multiples of such linkers.

Linkers of use in the antigen-binding proteins of the present invention may comprise alone or in addition to other linkers, one or more sets of GS residues, for example 'GSTVAAPS' (SEQ ID NO: 41) or 'TVAAPSGS' (SEQ ID NO:32) or 'GSTVAAPSGS' (SEQ ID NO: 42).

In one embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(PAS)_n(GS)_m$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(GGGGS)_n(GS)_m$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(TVAAPS)_n(GS)_m$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(GS)_m(TVAAPSGS)_n$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(PAVPPP)_n(GS)_m$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(TVSDVP)_n(GS)_m$'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker '$(TGLDSP)_n(GS)_m$'. In all such embodiments, n = 1-10, and m = 0-4.

Examples of such linkers include $(PAS)_n(GS)_m$ wherein n=1 and m=1, $(PAS)_n(GS)_m$ wherein n=2 and m=1, $(PAS)_n(GS)_m$ wherein n=3 and m=1, $(PAS)_n(GS)_m$ wherein n=4

and m=1, $(PAS)_n(GS)_m$ wherein n=2 and m=0, $(PAS)_n(GS)_m$ wherein n=3 and m=0, $(PAS)_n(GS)_m$ wherein n=4 and m=0.

Examples of such linkers include $(GGGGS)_n(GS)_m$ wherein n=1 and m=1 $(GGGGS)_n(GS)_m$ wherein n=2 and m=1, $(GGGGS)_n(GS)_m$ wherein n=3 and m=1, $(GGGGS)_n(GS)_m$ wherein n=4 and m=1, $(GGGGS)_n(GS)_m$ wherein n=2 and m=0, $(GGGGS)_n(GS)_m$ wherein n=3 and m=0, $(GGGGS)_n(GS)_m$ wherein n=4 and m=0.

Examples of such linkers include $(TVAAPS)_n(GS)_m$ wherein n=1 and m=1 (SEQ ID NO:41), $(TVAAPS)_n(GS)_m$ wherein n=2 and m=1 (SEQ ID NO:48), $(TVAAPS)_n(GS)_m$ wherein n=3 and m=1 (SEQ ID NO:49), $(TVAAPS)_n(GS)_m$ wherein n=4 and m=1, $(TVAAPS)_n(GS)_m$ wherein n=2 and m=0, $(TVAAPS)_n(GS)_m$ wherein n=3 and m=0, $(TVAAPS)_n(GS)_m$ wherein n=4 and m=0.

Examples of such linkers include $(GS)_m(TVAAPSGS)_n$ wherein n=1 and m=1 (SEQ ID NO:42), $(GS)_m(TVAAPSGS)_n$ wherein n=2 and m=1 (SEQ ID NO:43), $(GS)_m(TVAAPSGS)_n$ wherein n=3 and m=1 (SEQ ID NO:44), or $(GS)_m(TVAAPSGS)_n$ wherein n=4 and m=1 (SEQ ID NO:45), $(GS)_m(TVAAPSGS)_n$ wherein n=5 and m=1 (SEQ ID NO:46), $(GS)_m(TVAAPSGS)_n$ wherein n=6 and m=1 (SEQ ID NO:47), $(GS)_m(TVAAPSGS)_n$ wherein n=1 and m=0 (SEQ ID NO:32), $(GS)_m(TVAAPSGS)_n$ wherein n=2 and m=10, $(GS)_m(TVAAPSGS)_n$ wherein n=3 and m=0, or $(GS)_m(TVAAPSGS)_n$ wherein n=0.

Examples of such linkers include $(PAVPPP)_n(GS)_m$ wherein n=1 and m=1, $(PAVPPP)_n(GS)_m$ wherein n=2 and m=1, $(PAVPPP)_n(GS)_m$ wherein n=3 and m=1, $(PAVPPP)_n(GS)_m$ wherein n=4 and m=1, $(PAVPPP)_n(GS)_m$ wherein n=2 and m=0, $(PAVPPP)_n(GS)_m$ wherein n=3 and m=0, $(PAVPPP)_n(GS)_m$ wherein n=4 and m=0.

Examples of such linkers include $(TVSDVP)_n(GS)_m$ wherein n=1 and m=1), $(TVSDVP)_n(GS)_m$ wherein n=2 and m=1, $(TVSDVP)_n(GS)_m$ wherein n=3 and m=1, $(TVSDVP)_n(GS)_m$ wherein n=4 and m=1, $(TVSDVP)_n(GS)_m$ wherein n=2 and m=0, $(TVSDVP)_n(GS)_m$ wherein n=3 and m=0, $(TVSDVP)_n(GS)_m$ wherein n=4 and m=0.

Examples of such linkers include $(TGLDSP)_n(GS)_m$ wherein n=1 and m=1 , $(TGLDSP)_n(GS)_m$ wherein n=2 and m=1, $(TGLDSP)_n(GS)_m$ wherein n=3 and m=1, $(TGLDSP)_n(GS)_m$ wherein n=4 and m=1, $(TGLDSP)_n(GS)_m$ wherein n=2 and m=0, $(TGLDSP)_n(GS)_m$ wherein n=3 and m=0, $(TGLDSP)_n(GS)_m$ wherein n=4 and m=0.

In another embodiment there is no linker between the epitope binding domain and the Receptor-Fc fusion. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker 'TVAAPS' (SEQ ID NO: 16). In another embodiment the epitope binding domain, is linked to the Receptor-Fc fusion by the linker 'TVAAPSGS' (SEQ ID NO: 32). In another embodiment the epitope binding

domain is linked to the Receptor-Fc fusion by the linker 'GS (SEQ ID NO: 31)'. In another embodiment the epitope binding domain is linked to the Receptor-Fc fusion by the linker 'ASTKGPT' (SEQ ID NO: 17).

In one embodiment, the antigen-binding protein of the present invention comprises at least one epitope binding domain, which is capable of binding human serum albumin.

The invention also provides the antigen-binding proteins for use in medicine, for example for use in the manufacture of a medicament for treating immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

The invention provides a method of treating a patient suffering from immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis, comprising administering a therapeutic amount of an antigen-binding protein of the invention.

The antigen-binding proteins of the invention may be used for the treatment of immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

Immunoglobulin scaffolds of use in the present invention comprise the Fc portion of a conventional antibody. Immunoglobulin scaffolds of the present invention may comprise the Fc region of a non-conventional antibody structure, such as a monovalent antibody. Such monovalent antibodies may comprise a heavy chain which dimerises with a second heavy chain which is lacking a functional variable region and CH1 region, wherein the first and second heavy chains are modified so that they will form heterodimers rather than homodimers, resulting in a monovalent antibody with two heavy chains and one light chain such as the monovalent antibody described in WO2006015371. The Fc region of such monovalent antibodies can provide the Immunoglobulin scaffold of the present invention to which soluble ligands, extracellular domains of a receptor or cell surface protein and epitope binding domains can be linked. In such a monovalent structure it is possible to have a soluble ligand or extracellular domain of a receptor or cell surface protein linked to the first heavy chain and one or more epitope binding domains linked to the second heavy chain.

Epitope-binding domains of use in the present invention are domains that specifically bind an antigen or epitope independently of a different V region or domain, this may be a domain antibody or may be a non-Ig domain, for example a domain which is a

derivative of a scaffold selected from the group consisting of CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody); Heat shock proteins such as GroEl and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human γ-crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin); which has been subjected to protein engineering in order to obtain binding to a ligand other than its natural ligand. In one embodiment this may be a domain antibody or other suitable domains such as a domain selected from the group consisting of CTLA-4, lipocallin, SpA, an Affibody, an avimer, GroEl, transferrin, GroES and fibronectin. In one embodiment this may be selected from an immunoglobulin single variable domain, an Affibody, an ankyrin repeat protein (DARPin) and an adnectin. In another embodiment this may be selected from an Affibody, an ankyrin repeat protein (DARPin) and an adnectin. In another embodiment this may be a domain antibody, for example a domain antibody selected from a human, camelid or shark (NARV) domain antibody.

Epitope-binding domains can be linked to the Receptor-Fc fusion at one or more positions. These positions include the C-terminus and the N-terminus of the Receptor-Fc fusion. For example they may be linked directly to the Fc portion of the Receptor-Fc fusion, or they may be linked to the soluble ligand or extracellular domain of a receptor or cell surface protein portion of the Receptor-Fc fusion. Where the soluble ligand or extracellular domain of a receptor or cell surface protein is linked to the N-terminus of the Fc portion, the epitope-binding domain may be linked directly to the c-terminus of the Fc portion or to the N-terminus of the soluble ligand or extracellular domain of a receptor or cell surface protein.

In one embodiment, a first epitope binding domain is linked to the Receptor-Fc fusion and a second epitope binding domain is linked to the first epitope binding domain, for example a first epitope binding domain may be linked to the c-terminus of the Receptor-Fc fusion, and that epitope binding domain can be linked at its c-terminus to a second epitope binding domain, or for example a first epitope binding domain may be linked to the n-terminus of the Receptor-Fc fusion, and that first epitope binding domain may be further linked at its n-terminus to a second epitope binding domain, When the epitope-binding domain is a domain antibody, some domain antibodies may be suited to particular positions within the scaffold.

In antigen binding proteins where the N-terminus of immunoglobulin single variable domains are fused to an antibody constant domain, a peptide linker between the immunoglobulin single variable domain and the Fc portion may help the immunoglobulin single variable domain to bind to antigen. Indeed, the N-terminal end of an immunoglobulin single variable domain is located closely to the complementarity-determining regions (CDRs) involved in antigen-binding activity.

Thus a short peptide linker acts as a spacer between the epitope-binding, and the Fc portion, which may allow the immunoglobulin single variable domain CDRs to more easily reach the antigen, which may therefore bind with high affinity.

The surroundings in which immunoglobulin single variable domains are linked to the IgG will differ depending on which antibody chain they are fused to:

When fused at the C-terminal end of the Fc portion, each immunoglobulin single variable domain is expected to be located in the vicinity of the C_H3 domains of the Fc portion. This is not expected to impact on the Fc binding properties to Fc receptors (e.g. FcγRI, II, III an FcRn) as these receptors engage with the C_H2 domains (for the FcγRI, II and III class of receptors) or with the hinge between the C_H2 and C_H3 domains (e.g. FcRn receptor). Another feature of such antigen-binding proteins is that both immunoglobulin single variable domains are expected to be spatially close to each other and provided that flexibility is provided by provision of appropriate linkers, these immunoglobulin single variable domains may even form homodimeric species, hence propagating the 'zipped' quaternary structure of the Fc portion, which may enhance stability of the protein.

Such structural considerations can aid in the choice of the most suitable position to link an epitope-binding domain, for example an immunoglobulin single variable domain, on to a Receptor-Fc fusion.

Understanding the solution state and mode of binding at the immunoglobulin single variable domain is also helpful. Evidence has accumulated that *in vitro* dAbs can predominantly exist in monomeric, homo-dimeric or multimeric forms in solution (Reiter et al. (1999) J Mol Biol 290 p685-698; Ewert et al (2003) J Mol Biol 325, p531-553, Jespers et al (2004) J Mol Biol 337 p893-903; Jespers et al (2004) Nat Biotechnol 22 p1161-1165; Martin et al (1997) Protein Eng. 10 p607-614; Sepulvada et al (2003) J Mol Biol 333 p355-365). This is fairly reminiscent to multimerisation events observed *in vivo* with Ig domains such as Bence-Jones proteins (which are dimers of immunoglobulin light chains (Epp et al (1975) Biochemistry 14 p4943-4952; Huan et al (1994) Biochemistry 33 p14848-14857; Huang et al (1997) Mol immunol 34 p1291-1301) and amyloid fibers (James *et al.* (2007) *J Mol Biol*. **367**:603-8).

For example, it may be desirable to link domain antibodies that tend to dimerise in solution to the C-terminal end of the Fc portion in preference to the N-terminal end of the Receptor-Fc fusion as linking to the C-terminal end of the Fc will allow those dAbs to dimerise more easily in the context of the antigen-binding protein of the invention.

The antigen-binding proteins of the present invention may comprise antigen-binding sites specific for a single antigen, or may have antigen-binding sites specific for two

or more antigens, or for two or more epitopes on a single antigen, or there may be antigen-binding sites each of which is specific for a different epitope on the same or different antigens.

The invention also provides the antigen-binding proteins for use in medicine, for example for use in the manufacture of a medicament for treating immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

In particular, the antigen-binding proteins of the present invention may be useful in treating immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

The invention provides a method of treating a patient suffering from immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis comprising administering a therapeutic amount of an antigen-binding protein of the invention.

Antigen binding proteins of the present invention comprising CTLA4-Ig fusions may be useful in the treatment of arthritic diseases such as rheumatoid arthritis.

Antigen binding proteins of the present invention comprising TNFR2-Ig fusions may be useful in the treatment of inflammatory diseases such as RA, crohns disease, and psoriasis.

Antigen binding proteins of the present invention comprising TACI-Fc fusions may be useful in the treatment of autoimmune diseases such as SLE, or MS, or in treating cancer, for example MM, or CLL.

The antigen-binding proteins of the present invention may be produced by transfection of a host cell with an expression vector comprising the coding sequence for the antigen-binding protein of the invention. An expression vector or recombinant plasmid is produced by placing these coding sequences for the antigen-binding protein in operative association with conventional regulatory control sequences capable of controlling the replication and expression in, and/or secretion from, a host cell. Regulatory sequences include promoter sequences, e.g., CMV promoter, and signal sequences which can be derived from other known antibodies.
A selected host cell is transfected by conventional techniques with the vector to create the transfected host cell of the invention comprising the recombinant or

synthetic heavy chains. The transfected cell is then cultured by conventional techniques to produce the engineered antigen-binding protein of the invention. The antigen-binding protein is screened from culture by appropriate assay, such as ELISA or RIA. Similar conventional techniques may be employed to construct other antigen-binding proteins.

Suitable vectors for the cloning and subcloning steps employed in the methods and construction of the compositions of this invention may be selected by one of skill in the art. For example, the conventional pUC series of cloning vectors may be used. One vector, pUC19, is commercially available from supply houses, such as Amersham (Buckinghamshire, United Kingdom) or Pharmacia (Uppsala, Sweden). Additionally, any vector which is capable of replicating readily, has an abundance of cloning sites and selectable genes (e.g., antibiotic resistance), and is easily manipulated may be used for cloning. Thus, the selection of the cloning vector is not a limiting factor in this invention.

The expression vectors may also be characterized by genes suitable for amplifying expression of the heterologous DNA sequences, e.g., the mammalian dihydrofolate reductase gene (DHFR). Other vector sequences include a poly A signal sequence, such as from bovine growth hormone (BGH) and the betaglobin promoter sequence (betaglopro). The expression vectors useful herein may be synthesized by techniques well known to those skilled in this art.

The components of such vectors, e.g. replicons, selection genes, enhancers, promoters, signal sequences and the like, may be obtained from commercial or natural sources or synthesized by known procedures for use in directing the expression and/or secretion of the product of the recombinant DNA in a selected host. Other appropriate expression vectors of which numerous types are known in the art for mammalian, bacterial, insect, yeast, and fungal expression may also be selected for this purpose.

The present invention also encompasses a cell line transfected with a recombinant plasmid containing the coding sequences of the antigen-binding proteins of the present invention. Host cells useful for the cloning and other manipulations of these cloning vectors are also conventional. However, cells from various strains of E. coli may be used for replication of the cloning vectors and other steps in the construction of antigen-binding proteins of this invention.

Suitable host cells or cell lines for the expression of the antigen-binding proteins of the invention include mammalian cells such as NS0, Sp2/0, CHO (e.g. DG44), COS, HEK, a fibroblast cell (e.g., 3T3), and myeloma cells, for example it may be expressed in a CHO or a myeloma cell. Human cells may be used, thus enabling the molecule to be modified with human glycosylation patterns. Alternatively, other eukaryotic cell lines may be employed. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Sambrook et al., cited above.

Bacterial cells may prove useful as host cells suitable for the expression of the recombinant Fabs or other embodiments of the present invention (see, e.g., Plückthun, A., Immunol. Rev., 130:151-188 (1992)). However, due to the tendency of proteins expressed in bacterial cells to be in an unfolded or improperly folded form or in a non-glycosylated form, any recombinant antigen binding protein produced in a bacterial cell would have to be screened for retention of antigen binding ability. If the molecule expressed by the bacterial cell was produced in a properly folded form, that bacterial cell would be a desirable host, or in alternative embodiments the molecule may express in the bacterial host and then be subsequently re-folded. For example, various strains of E. coli used for expression are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Streptomyces, other bacilli and the like may also be employed in this method.

Where desired, strains of yeast cells known to those skilled in the art are also available as host cells, as well as insect cells, e.g. Drosophila and Lepidoptera and viral expression systems. See, e.g. Miller et al., Genetic Engineering, 8:277-298, Plenum Press (1986) and references cited therein.

The general methods by which the vectors may be constructed, the transfection methods required to produce the host cells of the invention, and culture methods necessary to produce the antigen-binding protein of the invention from such host cell may all be conventional techniques. Typically, the culture method of the present invention is a serum-free culture method, usually by culturing cells serum-free in suspension. Likewise, once produced, the antigen-binding proteins of the invention may be purified from the cell culture contents according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Such techniques are within the skill of the art and do not limit this invention. For example, preparation of altered antibodies are described in WO 99/58679 and WO 96/16990.

Yet another method of expression of the antigen-binding proteins may utilize expression in a transgenic animal, such as described in U. S. Patent No. 4,873,316. This relates to an expression system using the animal's casein promoter which when transgenically incorporated into a mammal permits the female to produce the desired recombinant protein in its milk.

In a further aspect of the invention there is provided a method of producing an antigen binding protein of the invention which method comprises the step of culturing a host cell transformed or transfected with a vector comprising a polynucleotide encoding the antigen binding protein of the invention and recovering the antigen binding protein thereby produced.

In accordance with the present invention there is provided a method of producing an antigen-binding protein of the present invention which method comprises the steps of;

(a) providing a vector comprising a polynucleotide encoding the antigen-binding protein
(b) transforming a mammalian host cell (e.g. CHO) with said vector;

(c) culturing the host cell of step (c) under conditions conducive to the secretion of the antigen-binding protein from said host cell into said culture media;
(d) recovering the secreted antigen-binding protein of step (d).

Once expressed by the desired method, the antigen-binding protein is then examined for in vitro activity by use of an appropriate assay. Presently conventional ELISA assay formats are employed to assess qualitative and quantitative binding of the antigen-binding protein to its target. Additionally, other in vitro assays may also be used to verify neutralizing efficacy prior to subsequent human clinical studies performed to evaluate the persistence of the antigen-binding protein in the body despite the usual clearance mechanisms.

The dose and duration of treatment relates to the relative duration of the molecules of the present invention in the human circulation, and can be adjusted by one of skill in the art depending upon the condition being treated and the general health of the patient. It is envisaged that repeated dosing (e.g. once a week or once every two weeks) over an extended time period (e.g. four to six months) maybe required to achieve maximal therapeutic efficacy.

The mode of administration of the therapeutic agent of the invention may be any suitable route which delivers the agent to the host. The antigen-binding proteins, and pharmaceutical compositions of the invention are particularly useful for parenteral administration, i.e., subcutaneously (s.c.), intrathecally, intraperitoneally, intramuscularly (i.m.), intravenously (i.v.), or intranasally.

Therapeutic agents of the invention may be prepared as pharmaceutical compositions containing an effective amount of the antigen-binding protein of the invention as an active ingredient in a pharmaceutically acceptable carrier. In the prophylactic agent of the invention, an aqueous suspension or solution containing the antigen-binding protein, may be buffered at physiological pH, in a form ready for injection. The compositions for parenteral administration will commonly comprise a solution of the antigen-binding protein of the invention or a cocktail thereof dissolved in a pharmaceutically acceptable carrier, for example an aqueous carrier. A variety of aqueous carriers may be employed, e.g., 0.9% saline, 0.3% glycine, and the like. These solutions may be made sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antigen-binding protein of the invention in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and between about 1 ng to about

100 mg, e.g. about 50 ng to about 30 mg, or about 5 mg to about 25 mg, of an antigen-binding protein of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 to about 30, or about 5 mg to about 25 mg of an antigen-binding protein of the invention per ml of Ringer's solution. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania. For the preparation of intravenously administrable antigen-binding protein formulations of the invention see Lasmar U and Parkins D "The formulation of Biopharmaceutical products", Pharma. Sci.Tech.today, page 129-137, Vol.3 (3rd April 2000), Wang, W "Instability, stabilisation and formulation of liquid protein pharmaceuticals", Int. J. Pharm 185 (1999) 129-188, Stability of Protein Pharmaceuticals Part A and B ed Ahern T.J., Manning M.C., New York, NY: Plenum Press (1992), Akers,M.J. "Excipient-Drug interactions in Parenteral Formulations", J.Pharm Sci 91 (2002) 2283-2300, Imamura, K et al "Effects of types of sugar on stabilization of Protein in the dried state", J Pharm Sci 92 (2003) 266-274,Izutsu, Kkojima, S. "Excipient crystalinity and its protein-structure-stabilizing effect during freeze-drying", J Pharm. Pharmacol, 54 (2002) 1033-1039, Johnson, R, "Mannitol-sucrose mixtures-versatile formulations for protein lyophilization", J. Pharm. Sci, 91 (2002) 914-922.

Ha,E Wang W, Wang Y.j. "Peroxide formation in polysorbate 80 and protein stability", J. Pharm Sci, 91, 2252-2264,(2002) the entire contents of which are incorporated herein by reference and to which the reader is specifically referred.

In one embodiment the therapeutic agent of the invention, when in a pharmaceutical preparation, will be present in unit dose forms. The appropriate therapeutically effective dose will be determined readily by those of skill in the art. Suitable doses may be calculated for patients according to their weight, for example suitable doses may be in the range of 0.01 to 20mg/kg, for example 0.1 to 20mg/kg, for example 1 to 20mg/kg, for example 10 to 20mg/kg or for example 1 to 15mg/kg, for example 10 to 15mg/kg. To effectively treat conditions of use in the present invention in a human, suitable doses may be within the range of 0.01 to 1000 mg, for example 0.1 to 1000mg, for example 0.1 to 500mg, for example 500mg, for example 0.1 to 100mg, or 0.1 to 80mg, or 0.1 to 60mg, or 0.1 to 40mg, or for example 1 to 100mg, or 1 to 50mg, of an antigen-binding protein of this invention, which may be administered parenterally, for example subcutaneously, intravenously or intramuscularly. Such dose may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician.

The antigen-binding proteins described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed.

There are several methods known in the art which can be used to find epitope-binding domains of use in the present invention.

The term "library" refers to a mixture of heterogeneous polypeptides or nucleic acids. The library is composed of members, each of which has a single polypeptide or nucleic acid sequence. To this extent, "library" is synonymous with "repertoire." Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of polypeptides or nucleic acids, or may be in the form of organisms or cells, for example bacteria, viruses, animal or plant cells and the like, transformed with a library of nucleic acids. In one example, each individual organism or cell contains only one or a limited number of library members. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In a one aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member. Thus, the population of host organisms has the potential to encode a large repertoire of diverse polypeptides.

A "universal framework" is a single antibody framework sequence corresponding to the regions of an antibody conserved in sequence as defined by Kabat ("Sequences of Proteins of Immunological Interest", US Department of Health and Human Services) or corresponding to the human germline immunoglobulin repertoire or structure as defined by Chothia and Lesk, (1987) J. Mol. Biol. 196:910-917. There may be a single framework, or a set of such frameworks, which has been found to permit the derivation of virtually any binding specificity though variation in the hypervariable regions alone.

Amino acid and nucleotide sequence alignments and homology, similarity or identity, as defined herein are in one embodiment prepared and determined using the algorithm BLAST 2 Sequences, using default parameters (Tatusova, T. A. *et al.*, *FEMS Microbiol Lett*, *174*:187-188 (1999)).

When a display system (*e.g.*, a display system that links coding function of a nucleic acid and functional characteristics of the peptide or polypeptide encoded by the nucleic acid) is used in the methods described herein, eg in the selection of a dAb or other epitope binding domain, it is frequently advantageous to amplify or increase the copy number of the nucleic acids that encode the selected peptides or polypeptides. This provides an efficient way of obtaining sufficient quantities of nucleic acids and/or peptides or polypeptides for additional rounds of selection, using the methods described herein or other suitable methods, or for preparing additional repertoires (*e.g.*, affinity maturation repertoires). Thus, in some embodiments, the methods of selecting epitope binding domains comprises using a display system (*e.g.*, that links coding function of a nucleic acid and functional characteristics of the peptide or

polypeptide encoded by the nucleic acid, such as phage display) and further comprises amplifying or increasing the copy number of a nucleic acid that encodes a selected peptide or polypeptide. Nucleic acids can be amplified using any suitable methods, such as by phage amplification, cell growth or polymerase chain reaction.

In one example, the methods employ a display system that links the coding function of a nucleic acid and physical, chemical and/or functional characteristics of the polypeptide encoded by the nucleic acid. Such a display system can comprise a plurality of replicable genetic packages, such as bacteriophage or cells (bacteria). The display system may comprise a library, such as a bacteriophage display library. Bacteriophage display is an example of a display system.

A number of suitable bacteriophage display systems (*e.g.*, monovalent display and multivalent display systems) have been described. (See, *e.g.*, Griffiths *et al.*, U.S. Patent No. 6,555,313 B1 (incorporated herein by reference); Johnson *et al.*, U.S. Patent No. 5,733,743 (incorporated herein by reference); McCafferty *et al.*, U.S. Patent No. 5,969,108 (incorporated herein by reference); Mulligan-Kehoe, U.S. Patent No. 5,702,892 (Incorporated herein by reference); Winter, G. *et al.*, *Annu. Rev. Immunol. 12*:433-455 (1994); Soumillion, P. *et al., Appl. Biochem. Biotechnol.* 47(2-3):175-189 (1994); Castagnoli, L. *et al., Comb. Chem. High Throughput Screen,* 4(2):121-133 (2001).) The peptides or polypeptides displayed in a bacteriophage display system can be displayed on any suitable bacteriophage, such as a filamentous phage (*e.g.*, fd, M13, F1), a lytic phage (*e.g.*, T4, T7, lambda), or an RNA phage (*e.g.*, MS2), for example.

Generally, a library of phage that displays a repertoire of peptides or phagepolypeptides, as fusion proteins with a suitable phage coat protein (*e.g.*, fd pIII protein), is produced or provided. The fusion protein can display the peptides or polypeptides at the tip of the phage coat protein, or if desired at an internal position. For example, the displayed peptide or polypeptide can be present at a position that is amino-terminal to domain 1 of pIII. (Domain 1 of pIII is also referred to as N1.) The displayed polypeptide can be directly fused to pIII (*e.g.*, the N-terminus of domain 1 of pIII) or fused to pIII using a linker. If desired, the fusion can further comprise a tag (*e.g.*, myc epitope, His tag). Libraries that comprise a repertoire of peptides or polypeptides that are displayed as fusion proteins with a phage coat protein,can be produced using any suitable methods, such as by introducing a library of phage vectors or phagemid vectors encoding the displayed peptides or polypeptides into suitable host bacteria, and culturing the resulting bacteria to produce phage (*e.g.*, using a suitable helper phage or complementing plasmid if desired). The library of phage can be recovered from the culture using any suitable method, such as precipitation and centrifugation.

The display system can comprise a repertoire of peptides or polypeptides that contains any desired amount of diversity. For example, the repertoire can contain peptides or polypeptides that have amino acid sequences that correspond to naturally occurring polypeptides expressed by an organism, group of organisms,

desired tissue or desired cell type, or can contain peptides or polypeptides that have random or randomized amino acid sequences. If desired, the polypeptides can share a common core or scaffold. For example, all polypeptides in the repertoire or library can be based on a scaffold selected from protein A, protein L, protein G, a fibronectin domain, an anticalin, CTLA4, a desired enzyme (*e.g.*, a polymerase, a cellulase), or a polypeptide from the immunoglobulin superfamily, such as an antibody or antibody fragment (*e.g.*, an antibody variable domain). The polypeptides in such a repertoire or library can comprise defined regions of random or randomized amino acid sequence and regions of common amino acid sequence. In certain embodiments, all or substantially all polypeptides in a repertoire are of a desired type, such as a desired enzyme (*e.g.*, a polymerase) or a desired antigen-binding fragment of an antibody (*e.g.*, human V_H or human V_L). In some embodiments, the polypeptide display system comprises a repertoire of polypeptides wherein each polypeptide comprises an antibody variable domain. For example, each polypeptide in the repertoire can contain a V_H, a V_L or an Fv (*e.g.*, a single chain Fv).

Amino acid sequence diversity can be introduced into any desired region of a peptide or polypeptide or scaffold using any suitable method. For example, amino acid sequence diversity can be introduced into a target region, such as a complementarity determining region of an antibody variable domain or a hydrophobic domain, by preparing a library of nucleic acids that encode the diversified polypeptides using any suitable mutagenesis methods (*e.g.*, low fidelity PCR, oligonucleotide-mediated or site directed mutagenesis, diversification using NNK codons) or any other suitable method. If desired, a region of a polypeptide to be diversified can be randomized. The size of the polypeptides that make up the repertoire is largely a matter of choice and uniform polypeptide size is not required. The polypeptides in the repertoire may have at least tertiary structure (form at least one domain).

Selection/Isolation/Recovery

An epitope binding domain or population of domains can be selected, isolated and/or recovered from a repertoire or library (*e.g.*, in a display system) using any suitable method. For example, a domain is selected or isolated based on a selectable characteristic (*e.g.*, physical characteristic, chemical characteristic, functional characteristic). Suitable selectable functional characteristics include biological activities of the peptides or polypeptides in the repertoire, for example, binding to a generic ligand (*e.g.*, a superantigen), binding to a target ligand (*e.g.*, an antigen, an epitope, a substrate), binding to an antibody (*e.g.*, through an epitope expressed on a peptide or polypeptide), and catalytic activity. (See, *e.g.*, Tomlinson *et al.*, WO 99/20749; WO 01/57065; WO 99/58655.)

In some embodiments, the protease resistant peptide or polypeptide is selected and/or isolated from a library or repertoire of peptides or polypeptides in which substantially all domains share a common selectable feature. For example, the domain can be selected from a library or repertoire in which substantially all domains bind a common generic ligand, bind a common target ligand, bind (or are bound by) a

common antibody, or possess a common catalytic activity. This type of selection is particularly useful for preparing a repertoire of domains that are based on a parental peptide or polypeptide that has a desired biological activity, for example, when performing affinity maturation of an immunoglobulin single variable domain.

Selection based on binding to a common generic ligand can yield a collection or population of domains that contain all or substantially all of the domains that were components of the original library or repertoire. For example, domains that bind a target ligand or a generic ligand, such as protein A, protein L or an antibody, can be selected, isolated and/or recovered by panning or using a suitable affinity matrix.

Panning can be accomplished by adding a solution of ligand (*e.g.*, generic ligand, target ligand) to a suitable vessel (*e.g.*, tube, petri dish) and allowing the ligand to become deposited or coated onto the walls of the vessel. Excess ligand can be washed away and domains can be added to the vessel and the vessel maintained under conditions suitable for peptides or polypeptides to bind the immobilized ligand. Unbound domains can be washed away and bound domains can be recovered using any suitable method, such as scraping or lowering the pH, for example.

Suitable ligand affinity matrices generally contain a solid support or bead (*e.g.*, agarose) to which a ligand is covalently or noncovalently attached. The affinity matrix can be combined with peptides or polypeptides (*e.g.*, a repertoire that has been incubated with protease) using a batch process, a column process or any other suitable process under conditions suitable for binding of domains to the ligand on the matrix. domains that do not bind the affinity matrix can be washed away and bound domains can be eluted and recovered using any suitable method, such as elution with a lower pH buffer, with a mild denaturing agent (*e.g.*, urea), or with a peptide or domain that competes for binding to the ligand. In one example, a biotinylated target ligand is combined with a repertoire under conditions suitable for domains in the repertoire to bind the target ligand. Bound domains are recovered using immobilized avidin or streptavidin (*e.g.*, on a bead).

In some embodiments, the generic or target ligand is an antibody or antigen binding fragment thereof. Antibodies or antigen binding fragments that bind structural features of peptides or polypeptides that are substantially conserved in the peptides or polypeptides of a library or repertoire are particularly useful as generic ligands. Antibodies and antigen binding fragments suitable for use as ligands for isolating, selecting and/or recovering protease resistant peptides or polypeptides can be monoclonal or polyclonal and can be prepared using any suitable method.

LIBRARIES/REPERTOIRES

Libraries that encode and/or contain protease epitope binding domains can be prepared or obtained using any suitable method. A library can be designed to encode domains based on a domain or scaffold of interest (*e.g.*, a domain selected from a library) or can be selected from another library using the methods described herein. For example, a library enriched in domains can be prepared using a suitable polypeptide display system.

Libraries that encode a repertoire of a desired type of domain can readily be produced using any suitable method. For example, a nucleic acid sequence that encodes a desired type of polypeptide (*e.g.*, an immunoglobulin variable domain) can be obtained and a collection of nucleic acids that each contain one or more mutations can be prepared, for example by amplifying the nucleic acid using an error-prone polymerase chain reaction (PCR) system, by chemical mutagenesis (Deng *et al., J. Biol. Chem.*, 269:9533 (1994)) or using bacterial mutator strains (Low *et al., J. Mol. Biol.*, 260:359 (1996)).

In other embodiments, particular regions of the nucleic acid can be targeted for diversification. Methods for mutating selected positions are also well known in the art and include, for example, the use of mismatched oligonucleotides or degenerate oligonucleotides, with or without the use of PCR. For example, synthetic antibody libraries have been created by targeting mutations to the antigen binding loops. Random or semi-random antibody H3 and L3 regions have been appended to germline immunoblulin V gene segments to produce large libraries with unmutated framework regions (Hoogenboom and Winter (1992) supra; Nissim *et al.* (1994) supra; Griffiths *et al.* (1994) supra; DeKruif *et al.* (1995) supra). Such diversification has been extended to include some or all of the other antigen binding loops (Crameri *et al.* (1996) *Nature Med.*, 2:100; Riechmann *et al.* (1995) *Bio/Technology*, 13:475; Morphosys, WO 97/08320, supra). In other embodiments, particular regions of the nucleic acid can be targeted for diversification by, for example, a two-step PCR strategy employing the product of the first PCR as a "mega-primer." (See, *e.g.*, Landt, O. *et al., Gene 96*:125-128 (1990).) Targeted diversification can also be accomplished, for example, by SOE PCR. (See, *e.g.*, Horton, R.M. *et al., Gene 77*:61-68 (1989).)

Sequence diversity at selected positions can be achieved by altering the coding sequence which specifies the sequence of the polypeptide such that a number of possible amino acids (*e.g.*, all 20 or a subset thereof) can be incorporated at that position. Using the IUPAC nomenclature, the most versatile codon is NNK, which encodes all amino acids as well as the TAG stop codon. The NNK codon may be used in order to introduce the required diversity. Other codons which achieve the same ends are also of use, including the NNN codon, which leads to the production of the additional stop codons TGA and TAA. Such a targeted approach can allow the full sequence space in a target area to be explored.

Some libraries comprise domains that are members of the immunoglobulin superfamily (*e.g.,* antibodies or portions thereof). For example the libraries can comprise domains that have a known main-chain conformation. (See, *e.g.*, Tomlinson et al., WO 99/20749.) Libraries can be prepared in a suitable plasmid or vector. As used herein, vector refers to a discrete element that is used to introduce heterologous DNA into cells for the expression and/or replication thereof. Any suitable vector can be used, including plasmids (*e.g.*, bacterial plasmids), viral or bacteriophage vectors, artificial chromosomes and episomal vectors. Such vectors may be used for simple cloning and mutagenesis, or an expression vector can be

used to drive expression of the library. Vectors and plasmids usually contain one or more cloning sites (*e.g.*, a polylinker), an origin of replication and at least one selectable marker gene. Expression vectors can further contain elements to drive transcription and translation of a polypeptide, such as an enhancer element, promoter, transcription termination signal, signal sequences, and the like. These elements can be arranged in such a way as to be operably linked to a cloned insert encoding a polypeptide, such that the polypeptide is expressed and produced when such an expression vector is maintained under conditions suitable for expression (*e.g.*, in a suitable host cell).

Cloning and expression vectors generally contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (*e.g.* SV40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors, unless these are used in mammalian cells able to replicate high levels of DNA, such as COS cells.

Cloning or expression vectors can contain a selection gene also referred to as selectable marker. Such marker genes encode a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, *e.g.* ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

Suitable expression vectors can contain a number of components, for example, an origin of replication, a selectable marker gene, one or more expression control elements, such as a transcription control element (*e.g.*, promoter, enhancer, terminator) and/or one or more translation signals, a signal sequence or leader sequence, and the like. Expression control elements and a signal or leader sequence, if present, can be provided by the vector or other source. For example, the transcriptional and/or translational control sequences of a cloned nucleic acid encoding an antibody chain can be used to direct expression.

A promoter can be provided for expression in a desired host cell. Promoters can be constitutive or inducible. For example, a promoter can be operably linked to a nucleic acid encoding an antibody, antibody chain or portion thereof, such that it directs transcription of the nucleic acid. A variety of suitable promoters for procaryotic (*e.g.*, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system, lac, tac, T3, T7 promoters for *E. coli*) and eucaryotic (*e.g.*, simian virus 40 early or late promoter, Rous sarcoma virus

long terminal repeat promoter, cytomegalovirus promoter, adenovirus late promoter, EG-1a promoter) hosts are available.

In addition, expression vectors typically comprise a selectable marker for selection of host cells carrying the vector, and, in the case of a replicable expression vector, an origin of replication. Genes encoding products which confer antibiotic or drug resistance are common selectable markers and may be used in procaryotic (*e.g.*, β-lactamase gene (ampicillin resistance), *Tet* gene for tetracycline resistance) and eucaryotic cells (*e.g.*, neomycin (G418 or geneticin), gpt (mycophenolic acid), ampicillin, or hygromycin resistance genes). Dihydrofolate reductase marker genes permit selection with methotrexate in a variety of hosts. Genes encoding the gene product of auxotrophic markers of the host (*e.g.*, *LEU2, URA3, HIS3*) are often used as selectable markers in yeast. Use of viral (*e.g.*, baculovirus) or phage vectors, and vectors which are capable of integrating into the genome of the host cell, such as retroviral vectors, are also contemplated.

Suitable expression vectors for expression in prokaryotic (*e.g.*, bacterial cells such as *E. coli*) or mammalian cells include, for example, a pET vector (*e.g.*, pET-12a, pET-36, pET-37, pET-39, pET-40, Novagen and others), a phage vector (*e.g.*, pCANTAB 5 E, Pharmacia), pRIT2T (Protein A fusion vector, Pharmacia), pCDM8, pCDNA1.1/amp, pcDNA3.1, pRc/RSV, pEF-1 (Invitrogen, Carlsbad, CA), pCMV-SCRIPT, pFB, pSG5, pXT1 (Stratagene, La Jolla, CA), pCDEF3 (Goldman, L.A., *et al.*, *Biotechniques*, *21*:1013-1015 (1996)), pSVSPORT (GibcoBRL, Rockville, MD), pEF-Bos (Mizushima, S., *et al.*, *Nucleic Acids Res.*, *18*:5322 (1990)) and the like. Expression vectors which are suitable for use in various expression hosts, such as prokaryotic cells (*E. coli*), insect cells (*Drosophila* Schnieder S2 cells, Sf9), yeast (*P. methanolica*, *P. pastoris*, *S. cerevisiae*) and mammalian cells (eg, COS cells) are available.

Some examples of vectors are expression vectors that enable the expression of a nucleotide sequence corresponding to a polypeptide library member. Thus, selection with generic and/or target ligands can be performed by separate propagation and expression of a single clone expressing the polypeptide library member. As described above, a particular selection display system is bacteriophage display. Thus, phage or phagemid vectors may be used, for example vectors may be phagemid vectors which have an *E. coli*. origin of replication (for double stranded replication) and also a phage origin of replication (for production of single-stranded DNA). The manipulation and expression of such vectors is well known in the art (Hoogenboom and Winter (1992) supra; Nissim *et al.* (1994) supra). Briefly, the vector can contain a β-lactamase gene to confer selectivity on the phagemid and a lac promoter upstream of an expression cassette that can contain a suitable leader sequence, a multiple cloning site, one or more peptide tags, one or more TAG stop codons and the phage protein pIII. Thus, using various suppressor and non-suppressor strains of *E. coli* and with the addition of glucose, iso-propyl thio-β-D-galactoside (IPTG) or a helper phage, such as VCS M13, the vector is able to replicate as a plasmid with no expression, produce large quantities of the polypeptide

library member only or product phage, some of which contain at least one copy of the polypeptide-pIII fusion on their surface.

Antibody variable domains may comprise a target ligand binding site and/or a generic ligand binding site. In certain embodiments, the generic ligand binding site is a binding site for a superantigen, such as protein A, protein L or protein G. The variable domains can be based on any desired variable domain, for example a human VH (*e.g.*, V_H 1a, V_H 1b, V_H 2, V_H 3, V_H 4, V_H 5, V_H 6), a human Vλ (*e.g.*, VλI, VλII, VλIII, VλIV, VλV, VλVI or Vκ1) or a human Vκ (*e.g.*, Vκ2, Vκ3, Vκ4, Vκ5, Vκ6, Vκ7, Vκ8, Vκ9 or Vκ10).

A still further category of techniques involves the selection of repertoires in artificial compartments, which allow the linkage of a gene with its gene product. For example, a selection system in which nucleic acids encoding desirable gene products may be selected in microcapsules formed by water-in-oil emulsions is described in WO99/02671, WO00/40712 and Tawfik & Griffiths (1998) *Nature Biotechnol* **16**(7), 652-6. Genetic elements encoding a gene product having a desired activity are compartmentalised into microcapsules and then transcribed and/or translated to produce their respective gene products (RNA or protein) within the microcapsules. Genetic elements which produce gene product having desired activity are subsequently sorted. This approach selects gene products of interest by detecting the desired activity by a variety of means.

Characterisation of the epitope binding domains.

The binding of a domain to its specific antigen or epitope can be tested by methods which will be familiar to those skilled in the art and include ELISA. In one example, binding is tested using monoclonal phage ELISA.

Phage ELISA may be performed according to any suitable procedure: an exemplary protocol is set forth below.

Populations of phage produced at each round of selection can be screened for binding by ELISA to the selected antigen or epitope, to identify "polyclonal" phage antibodies. Phage from single infected bacterial colonies from these populations can then be screened by ELISA to identify "monoclonal" phage antibodies. It is also desirable to screen soluble antibody fragments for binding to antigen or epitope, and this can also be undertaken by ELISA using reagents, for example, against a C- or N-terminal tag (see for example Winter *et al*. (1994) Ann. Rev. Immunology 12, 433-55 and references cited therein.

The diversity of the selected phage monoclonal antibodies may also be assessed by gel electrophoresis of PCR products (Marks *et al*. 1991, *supra*; Nissim *et al*. 1994

supra), probing (Tomlinson *et al.*, 1992) J. Mol. Biol. 227, 776) or by sequencing of the vector DNA.

Structure of dAbs

In the case that the dAbs are selected from V-gene repertoires selected for instance using phage display technology as herein described, then these variable domains comprise a universal framework region, such that is they may be recognised by a specific generic ligand as herein defined. The use of universal frameworks, generic ligands and the like is described in WO99/20749.

Where V-gene repertoires are used variation in polypeptide sequence may be located within the structural loops of the variable domains. The polypeptide sequences of either variable domain may be altered by DNA shuffling or by mutation in order to enhance the interaction of each variable domain with its complementary pair. DNA shuffling is known in the art and taught, for example, by Stemmer, 1994, Nature 370: 389-391 and U.S. Patent No. 6,297,053, both of which are incorporated herein by reference. Other methods of mutagenesis are well known to those of skill in the art.

Scaffolds for use in Constructing dAbs

i. Selection of the main-chain conformation

The members of the immunoglobulin superfamily all share a similar fold for their polypeptide chain. For example, although antibodies are highly diverse in terms of their primary sequence, comparison of sequences and crystallographic structures has revealed that, contrary to expectation, five of the six antigen binding loops of antibodies (H1, H2, L1, L2, L3) adopt a limited number of main-chain conformations, or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, **196**: 901; Chothia *et al.* (1989) *Nature*, **342**: 877). Analysis of loop lengths and key residues has therefore enabled prediction of the main-chain conformations of H1, H2, L1, L2 and L3 found in the majority of human antibodies (Chothia *et al*. (1992) *J. Mol. Biol*., **227**: 799; Tomlinson *et al*. (1995) *EMBO J.*, **14**: 4628; Williams *et al*. (1996) *J. Mol. Biol.*, **264**: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, **263**: 800; Shirai *et al.* (1996) *FEBS Letters*, **399**: 1).

The dAbs are advantageously assembled from libraries of domains, such as libraries of V_H domains and/or libraries of V_L domains. In one aspect, libraries of domains are designed in which certain loop lengths and key residues have been chosen to ensure

that the main-chain conformation of the members is known. Advantageously, these are real conformations of immunoglobulin superfamily molecules found in nature, to minimise the chances that they are non-functional, as discussed above. Germline V gene segments serve as one suitable basic framework for constructing antibody or T-cell receptor libraries; other sequences are also of use. Variations may occur at a low frequency, such that a small number of functional members may possess an altered main-chain conformation, which does not affect its function.

Canonical structure theory is also of use to assess the number of different main-chain conformations encoded by ligands, to predict the main-chain conformation based on ligand sequences and to chose residues for diversification which do not affect the canonical structure. It is known that, in the human V_κ domain, the L1 loop can adopt one of four canonical structures, the L2 loop has a single canonical structure and that 90% of human V_κ domains adopt one of four or five canonical structures for the L3 loop (Tomlinson *et al*. (1995) supra); thus, in the V_κ domain alone, different canonical structures can combine to create a range of different main-chain conformations. Given that the Vλ domain encodes a different range of canonical structures for the L1, L2 and L3 loops and that V_κ and Vλ domains can pair with any V_H domain which can encode several canonical structures for the H1 and H2 loops, the number of canonical structure combinations observed for these five loops is very large. This implies that the generation of diversity in the main-chain conformation may be essential for the production of a wide range of binding specificities. However, by constructing an antibody library based on a single known main-chain conformation it has been found, contrary to expectation, that diversity in the main-chain conformation is not required to generate sufficient diversity to target substantially all antigens. Even more surprisingly, the single main-chain conformation need not be a consensus structure - a single naturally occurring conformation can be used as the basis for an entire library. Thus, in a one particular aspect, the dAbs possess a single known main-chain conformation.

The single main-chain conformation that is chosen may be commonplace among molecules of the immunoglobulin superfamily type in question. A conformation is commonplace when a significant number of naturally occurring molecules are observed to adopt it. Accordingly, in one aspect, the natural occurrence of the different main-chain conformations for each binding loop of an immunoglobulin domain are considered separately and then a naturally occurring variable domain is chosen which possesses the desired combination of main-chain conformations for the different loops. If none is available, the nearest equivalent may be chosen. The desired combination of main-chain conformations for the different loops may be created by selecting germline gene segments which encode the desired main-chain conformations. In one example, the selected germline gene segments are frequently expressed in nature, and in particular they may be the most frequently expressed of all natural germline gene segments.

In designing libraries the incidence of the different main-chain conformations for each of the six antigen binding loops may be considered separately. For H1, H2, L1, L2 and L3, a given conformation that is adopted by between 20% and 100% of the antigen binding loops of naturally occurring molecules is chosen. Typically, its observed incidence is above 35% (i.e. between 35% and 100%) and, ideally, above 50% or even above 65%. Since the vast majority of H3 loops do not have canonical structures, it is preferable to select a main-chain conformation which is commonplace among those loops which do display canonical structures. For each of the loops, the conformation which is observed most often in the natural repertoire is therefore selected. In human antibodies, the most popular canonical structures (CS) for each loop are as follows: H1 - CS 1 (79% of the expressed repertoire), H2 - CS 3 (46%), L1 - CS 2 of V_κ(39%), L2 - CS 1 (100%), L3 - CS 1 of V_κ(36%) (calculation assumes a κ:λ ratio of 70:30, Hood *et al*. (1967) *Cold Spring Harbor Symp. Quant. Biol*., **48**: 133). For H3 loops that have canonical structures, a CDR3 length (Kabat *et al*. (1991) *Sequences of proteins of immunological interest*, U.S. Department of Health and Human Services) of seven residues with a salt-bridge from residue 94 to residue 101 appears to be the most common. There are at least 16 human antibody sequences in the EMBL data library with the required H3 length and key residues to form this conformation and at least two crystallographic structures in the protein data bank which can be used as a basis for antibody modelling (2cgr and 1tet). The most frequently expressed germline gene segments that this combination of canonical structures are the V_H segment 3-23 (DP-47), the J_H segment JH4b, the V_κ segment O2/O12 (DPK9) and the J_κ segment J_κ1. V_H segments DP45 and DP38 are also suitable. These segments can therefore be used in combination as a basis to construct a library with the desired single main-chain conformation.

Alternatively, instead of choosing the single main-chain conformation based on the natural occurrence of the different main-chain conformations for each of the binding loops in isolation, the natural occurrence of combinations of main-chain conformations is used as the basis for choosing the single main-chain conformation. In the case of antibodies, for example, the natural occurrence of canonical structure combinations for any two, three, four, five, or for all six of the antigen binding loops can be determined. Here, the chosen conformation may be commonplace in naturally occurring antibodies and may be observed most frequently in the natural repertoire. Thus, in human antibodies, for example, when natural combinations of the five antigen binding loops, H1, H2, L1, L2 and L3, are considered, the most frequent combination of canonical structures is determined and then combined with the most popular conformation for the H3 loop, as a basis for choosing the single main-chain conformation.

Diversification of the canonical sequence

Having selected several known main-chain conformations or a single known main-chain conformation, dAbs can be constructed by varying the binding site of the molecule in order to generate a repertoire with structural and/or functional diversity. This means that variants are generated such that they possess sufficient diversity in their structure and/or in their function so that they are capable of providing a range of activities.

The desired diversity is typically generated by varying the selected molecule at one or more positions. The positions to be changed can be chosen at random or they may be selected. The variation can then be achieved either by randomisation, during which the resident amino acid is replaced by any amino acid or analogue thereof, natural or synthetic, producing a very large number of variants or by replacing the resident amino acid with one or more of a defined subset of amino acids, producing a more limited number of variants.

Various methods have been reported for introducing such diversity. Error-prone PCR (Hawkins *et al*. (1992) *J. Mol. Biol*., 226: 889), chemical mutagenesis (Deng *et al*. (1994) *J. Biol. Chem*., **269**: 9533) or bacterial mutator strains (Low *et al*. (1996) *J. Mol. Biol.,* **260**: 359) can be used to introduce random mutations into the genes that encode the molecule. Methods for mutating selected positions are also well known in the art and include the use of mismatched oligonucleotides or degenerate oligonucleotides, with or without the use of PCR. For example, several synthetic antibody libraries have been created by targeting mutations to the antigen binding loops. The H3 region of a human tetanus toxoid-binding Fab has been randomised to create a range of new binding specificities (Barbas *et al*. (1992) *Proc. Natl. Acad. Sci. USA*, **89**: 4457). Random or semi-random H3 and L3 regions have been appended to germline V gene segments to produce large libraries with unmutated framework regions (Hoogenboom & Winter (1992) *J. Mol. Biol.*, **227**: 381; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, **89**: 4457; Nissim *et al.* (1994) *EMBO J.*, **13**: 692; Griffiths *et al.* (1994) *EMBO J.*, **13**: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, **248**: 97). Such diversification has been extended to include some or all of the other antigen binding loops (Crameri *et al*. (1996) *Nature Med.,* **2**: 100; Riechmann *et al*. (1995) *Bio/Technology*, **13**: 475; Morphosys, WO97/08320, supra).

Since loop randomisation has the potential to create approximately more than 10^{15} structures for H3 alone and a similarly large number of variants for the other five loops, it is not feasible using current transformation technology or even by using cell free systems to produce a library representing all possible combinations. For example, in one of the largest libraries constructed to date, 6×10^{10} different antibodies, which is only a fraction of the potential diversity for a library of this design, were generated (Griffiths *et al*. (1994) supra).

In a one embodiment, only those residues which are directly involved in creating or modifying the desired function of the molecule are diversified. For many molecules, the function will be to bind a target and therefore diversity should be concentrated in the target binding site, while avoiding changing residues which are crucial to the overall packing of the molecule or to maintaining the chosen main-chain conformation.

In one aspect, libraries of dAbs are used in which only those residues in the antigen binding site are varied. These residues are extremely diverse in the human antibody repertoire and are known to make contacts in high-resolution antibody/antigen complexes. For example, in L2 it is known that positions 50 and 53 are diverse in naturally occurring antibodies and are observed to make contact with the antigen. In contrast, the conventional approach would have been to diversify all the residues in the corresponding Complementarity Determining Region (CDR1) as defined by Kabat *et al*. (1991, supra), some seven residues compared to the two diversified in the library.. This represents a significant improvement in terms of the functional diversity required to create a range of antigen binding specificities.

In nature, antibody diversity is the result of two processes: somatic recombination of germline V, D and J gene segments to create a naive primary repertoire (so called germline and junctional diversity) and somatic hypermutation of the resulting rearranged V genes. Analysis of human antibody sequences has shown that diversity in the primary repertoire is focused at the centre of the antigen binding site whereas somatic hypermutation spreads diversity to regions at the periphery of the antigen binding site that are highly conserved in the primary repertoire (see Tomlinson *et al*. (1996) *J. Mol. Biol.*, **256**: 813). This complementarity has probably evolved as an efficient strategy for searching sequence space and, although apparently unique to antibodies, it can easily be applied to other polypeptide repertoires. The residues which are varied are a subset of those that form the binding site for the target. Different (including overlapping) subsets of residues in the target binding site are diversified at different stages during selection, if desired.

In the case of an antibody repertoire, an initial 'naive' repertoire is created where some, but not all, of the residues in the antigen binding site are diversified. As used herein in this context, the term "naive" or "dummy" refers to antibody molecules that have no pre-determined target. These molecules resemble those which are encoded by the immunoglobulin genes of an individual who has not undergone immune diversification, as is the case with fetal and newborn individuals, whose immune systems have not yet been challenged by a wide variety of antigenic stimuli. This repertoire is then selected against a range of antigens or epitopes. If required, further diversity can then be introduced outside the region diversified in the initial repertoire. This matured repertoire can be selected for modified function, specificity or affinity.

It will be understood that the sequences described herein include sequences which are substantially identical, for example sequences which are at least 90% identical, for example which are at least 91%, or at least 92%, or at least 93%, or at least 94% or at least 95%, or at least 96%, or at least 97% or at least 98%, or at least 99% identical to the sequences described herein.

For nucleic acids, the term “substantial identity” indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, for example at least about 98% to 99.5% of the nucleotides. Alternatively, substantial identity exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

For nucleotide and amino acid sequences, the term “identical" indicates the degree of identity between two nucleic acid or amino acid sequences when optimally aligned and compared with appropriate insertions or deletions. Alternatively, substantial identity exists when the DNA segments will hybridize under selective hybridization conditions, to the complement of the strand.

The percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions times 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO: 33, that is be 100% identical, or it may include up to a certain integer number of nucleotide alterations as compared to

the reference sequence. Such alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO: 33 by the numerical percent of the respective percent identity(divided by 100) and subtracting that product from said total number of nucleotides in SEQ ID NO: 33, or:

$$nn \leq xn - (xn \bullet y),$$

wherein nn is the number of nucleotide alterations, xn is the total number of nucleotides in SEQ ID NO: 33, and y is 0.50 for 50%, 0.60 for 60%, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and wherein any non-integer product of xn and y is rounded down to the nearest integer prior to subtracting it from xn. Alterations of the polynucleotide sequence of SEQ ID NO: 33 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

Similarly, in another example, a polypeptide sequence of the present invention may be identical to the reference sequence encoded by SEQ ID NO: 30, that is be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in the polypeptide sequence encoded by SEQ ID NO: 30 by the numerical percent of the respective percent identity (divided by 100) and then subtracting that product from said total number of amino acids in the polypeptide sequence encoded by SEQ ID NO: 30, or:

$$na \leq xa - (xa \bullet y),$$

wherein na is the number of amino acid alterations, xa is the total number of amino acids in the polypeptide sequence encoded by SEQ ID NO: 30, and y is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and wherein any non-integer product of xa and y is rounded down to the nearest integer prior to subtracting it from xa.

Examples

Example 1 - Design and Construction of CTLA4-Ig fused to anti-VEGFR2 adnectin via a GS linker

A codon-optimised DNA sequence encoding CTLA4-Ig (a HindIII site at the N-terminus and BamHI site at the C-terminus were included to facilitate cloning) was constructed and cloned into a mammalian expression vector containing the CT01 adnectin. This allowed the adnectin to be fused onto the C-terminus of the CTLA4-Ig via a GS linker. The resulting antigen binding protein was named BPC1821. The DNA and protein sequences of BPC1821 are given in SEQ I.D. No. 33 and 23 respectively.

The expression plasmid encoding BPC1821 was transiently transfected into HEK 293-6E cells using 293fectin (Invitrogen, 12347019). A tryptone feed was added to the cell culture after 24 hours and the supernatant was harvested after 96 hours. BPC1821 was purified using a Protein A column before being tested in a binding assay.

Example 2 - VEGFR2 and B7-1 Binding ELISA

A 96-well high binding plate was coated with 0.4µg/ml of recombinant human VEGFR2 Fc Chimera (R&D Systems, 357-KD-050) in PBS and stored overnight at 4°C. The plate was washed twice with Tris-Buffered Saline with 0.05% of Tween-20. 200µL of blocking solution (5% BSA in DPBS buffer) was added to each well and the plate was incubated for at least 1hour at room temperature. Another wash step was then performed. BPC1821 and two negative control antibodies (Sigma I5154 and the bispecific IGF1R–VEGFR2 antigen binding construct BPC1801) were successively diluted across the plate in blocking solution. After 1 hour incubation, the plate was washed. Recombinant human B7-1 Fc Chimera (RnD Systems, 140-B1-100) was biotinylated using the ECL biotinylation module from GE Healthcare. The biotinylated B7-1 was diluted in blocking solution to 1µg/mL and 50µL was added to each well. The plate was incubated for one hour then washed. ExtrAvidin peroxidase (Sigma, E2886) was diluted 1 in 1000 in blocking solution and 50µL was added to each well. After another wash step, 50µl of OPD SigmaFast substrate solution was added to each well and the reaction was stopped 15 minutes later by addition of 25µL of 3M sulphuric acid. Absorbance was read at 490nm using the VersaMax Tunable Microplate Reader (Molecular Devices) using a basic endpoint protocol.

Figure 1 shows the results of the ELISA and confirms that bispecific BPC1821 shows binding to both VEGFR2 and B7-1. The negative control antibodies do not show binding to both VEGFR2 and B7-1.

Please note that Examples 3 and 4 are prophetic. They provide guidance for carrying out additional assays in which the antigen binding proteins of the invention can be tested,

Example 3 – VEGF Receptor Binding Assay

This assay measures the binding of $VEGF_{165}$ to VEGF R2 (VEGF receptor) and the ability of test molecules to block this interaction. ELISA plates are coated overnight with VEGF receptor (R&D Systems, Cat No: 357-KD-050) (0.5µg/ml final concentration in 0.2M sodium carbonate bicarbonate pH9.4), washed and blocked with 2% BSA in PBS. VEGF (R&D Systems, Cat No: 293-VE-050). The test molecules (diluted in 0.1%BSA in 0.05% Tween 20™ PBS) are pre-incubated with VEGF for one hour prior to addition to the plate (3ng/ml VEGF final concentration). Binding of VEGF to VEGF receptor is detected using biotinylated anti-VEGF antibody (0.5µg/ml final concentration) (R&D Systems, Cat No: BAF293) and a peroxidase conjugated anti-biotin secondary antibody (1:5000 dilution) (Stratech, Cat No: 200-032-096) and is visualised at OD450 using a colorimetric substrate (Sure Blue TMB peroxidase substrate, KPL) after stopping the reaction with an equal volume of 1M HCl.

Example 4 - Stoichiometry assessment of receptor-Fc bispecific antibodies (using Biacore™)

Anti-human IgG is immobilised onto a CM5 biosensor chip by primary amine coupling. Receptor-Fc fused to epitope binding domains can be captured onto this surface after which a single concentration of the ligands for the receptor-Fc fusions and epitope binding domains are passed over. The concentration is selected to be sufficient to saturate the binding surface and the binding signal observed reached full R-max. Stoichiometries can be calculated using the given formula:

Stoich=Rmax * Mw (ligand) / Mw (analyte)* R (ligand immobilised or captured)

Where the stoichiometries were calculated for more than one analyte binding at the same time, the different ligands were passed over sequentially at the saturating ligand concentration and the stoichometries calculated as above. The work is carried out on a BIAcore (for example a Biacore 3000 or T100), typically at 25°C using HBS-EP running buffer.

Example 5 - Design and Construction of CTLA4-Ig fused to either an anti-IL-13 dAb or an anti-VEGF dAb via a GS linker

The DNA plasmid containing the CTLA4-Ig fused to the anti-VEGFR2 adnectin was used as a base plasmid to construct the CTLA4-Ig-anti-IL-13 dAb and CTLA4-Ig-anti-VEGF dAb bispecifics. The vector was prepared by digesting the base plasmid with BamHI and EcoRI to remove the adnectin sequence. DNA sequences encoding the anti-IL-13 dAb and the anti-VEGF dAb were restricted with BamHI and EcoRI and ligated into the vector. The resulting CTLA4-Ig-anti-IL-13 dAb and CTLA4-Ig-anti-VEGF dAb bispecifics were named BPC1824 and BPC1825 respectively, where, in both cases, the dAb was fused onto the C-terminus of the CTLA4-Ig via a GS linker.

The DNA and protein sequences of BPC1824 and BPC1825 are given in Seq ID 27, 29, 34 and 35.

The expression plasmids encoding BPC1824 and BPC1825 were transiently transfected into HEK 293-6E cells using 293fectin (Invitrogen, 12347019). A tryptone feed was added to each cell culture after 24 hours and supernatants were harvested after 96 hours. The supernatants were used as the test articles in binding assays.

Example 6 - IL-13 and B7-1 Binding ELISA

A 96-well high binding plate was coated with 5µg/ml of human IL-13 (in-house material) in PBS and stored overnight at 4°C. The plate was washed twice with Tris-Buffered Saline with 0.05% of Tween-20. 200µL of blocking solution (5% BSA in DPBS buffer) was added to each well and the plate was incubated for at least 1hour at room temperature. Another wash step was then performed. BPC1824 and two negative control antibodies (Sigma I5154 and BPC1825) were successively diluted across the plate in blocking solution. After 1 hour incubation, the plate was washed. Recombinant human B7-1 Fc Chimera (RnD Systems, 140-B1-100) was biotinylated using the ECL biotinylation module from GE Healthcare. The biotinylated B7-1 was diluted in blocking solution to 1µg/mL and 50µL was added to each well. The plate was incubated for one hour then washed. ExtrAvidin peroxidase (Sigma, E2886) was diluted 1 in 1000 in blocking solution and 50µL was added to each well. After another wash step, 50µl of OPD SigmaFast substrate solution was added to each well and the reaction was stopped 15 minutes later by addition of 25µL of 3M sulphuric acid. Absorbance was read at 490nm using the VersaMax Tunable Microplate Reader (Molecular Devices) using a basic endpoint protocol.

Figure 2 shows the results of the ELISA and confirms that bispecific BPC1824 shows binding to both IL-13 and B7-1. The negative control antibodies do not show binding to both IL-13 and B7-1.

Example 7 - VEGF and B7-1 Binding ELISA

A 96-well high binding plate was coated with 0.4µg/ml of human VEGF165 (in-house material) in PBS and stored overnight at 4°C. The plate was washed twice with Tris-Buffered Saline with 0.05% of Tween-20. 200µL of blocking solution (5% BSA in DPBS buffer) was added to each well and the plate was incubated for at least 1hour at room temperature. Another wash step was then performed. BPC1825 and two negative control antibodies (Sigma I5154 and BPC1824) were successively diluted across the plate in blocking solution. After 1 hour incubation, the plate was washed. Recombinant human B7-1 Fc Chimera (RnD Systems, 140-B1-100) was biotinylated using the ECL biotinylation module from GE Healthcare. The biotinylated B7-1 was diluted in blocking solution to 1µg/mL and 50µL was added to each well. The plate was incubated for one hour then washed. ExtrAvidin peroxidase (Sigma, E2886) was diluted 1 in 1000 in blocking solution and 50µL was added to each well. After another wash step, 50µl of OPD SigmaFast substrate solution was added to each well and the reaction was stopped 15 minutes later by addition of 25µL of 3M

sulphuric acid. Absorbance was read at 490nm using the VersaMax Tunable Microplate Reader (Molecular Devices) using a basic endpoint protocol.
Figure 3 shows the results of the ELISA and confirms that bispecific BPC1825 shows binding to both VEGF and B7-1. The negative control antibodies do not show binding to both VEGF and B7-1.

Example 8

Design and construction of a TNFα receptor Fc fusion fused to a VEGF dAb via an STG or TVAAPPSTG linker

A codon-optimised DNA sequence encoding a human TNFα receptor Fc fusion (etanercept) was constructed and cloned into a mammalian expression vector (pTT5) along with the DOM15-26-593 anti VEGF dAb from another construct.

The Receptor Fc was flanked with additional sequences to provide an N-terminal Campath1 signal peptide, and provided either an STG linker or TVAAPSTVAAPSTVAAPSTVAAPSTG linker at the C-terminus for fusion to the dAb. The flanking sequences included an AgeI restriction site and a SalI restriction site to facilitate cloning into the vector with the dAb. The resulting antigen binding proteins were named EtanSTG593 and EtanTV4593, respectively. The DNA and protein sequences of EtanSTG593 are given in SEQ ID NO: 37 and 38, respectively, and of EtanTV4593 are given in SEQ ID NO: 39 and 40 respectively.

Example 9

EtanSTG593 and EtanTV4593 purification and VEGF and TNFα binding Analysis

The EtanSTG593 and EtanTV4593 plasmids were independently expressed in HEK 293-6E cells (National Research Council Canada) using 293Fectin (Invitrogen) for transfection. EtanSTG593 and EtanTV4593 were harvested after 5 days, and purified by MAb Select Sure (GE Healthcare) affinity chromatography to give batch samples M4004 and M4005 respectively. The proteins were formulated in F1 buffer (0.1M Citrate pH6, 10% PEG300, 5% Sucrose) or ET buffer (10mM Tris pH7.4, 4% D-Manitol, 1% Sucrose). The proteins were further purified by Size Exclusion Chromotography on a HiLoad Superdex S200 10/300 GL column (GE Healthcare) to reduce the level of aggregates.

Binding analysis was carried out on a ProteOn XPR36 machine (BioRad TM). Protein A was immobilised on a GLM chip by primary amine coupling. The constructs to be tested were captured on this Protein A surface. The analytes, TNFα and VEGF were used at 256 nM, 64 nM, 16 nM, 4 nM and 1nM. 0 nM (i.e. buffer alone)TNFα and VEGF was used to double reference binding curves.

The novel six by six flowcell set up of the ProteOn allows up to six constructs to be captured at the same time and also allows six concentrations of analyte to be flowed over the captured antibody(s), in all generating 36 interactions per cycle.

To regenerate the Protein A surface, 50 mM NaOH was used, this removed captured construct(s) and allowed another capture and binding cycle to begin. The data obtained was fitted to 1:1 model inherent to the ProteOn analysis software. The run was carried out using HBS-EP as running buffer and at a temperature of 25°C.

Table 1: VEGF Binding Results

Construct	Ka [1/Ms]	Kd [1/s]	KD(nM)
M4004 F1	1.18E+05	1.01E-04	0.850
M4005 F1	3.18E+05	1.85E-05	0.058
M4004 ET	1.24E+05	7.84E-05	0.631
M4005 ET	4.54E+05	4.44E-05	0.098

Table 2: TNFα Binding Results

Construct	Ka [1/Ms]	Kd [1/s]	KD(nM)
M4004 F1	5.10E+06	1.22E-04	0.024
M4005 F1	4.95E+06	1.05E-04	0.021
M4004 ET	4.81E+06	1.15E-04	0.024
M4005 ET	4.87E+06	1.38E-04	0.028

Table 3 (Sequences)

Description	Sequence identifier (SEQ ID NO)	
	amino acid sequence	DNA sequence
CTLA4 region from CTLA4-Ig	1	
CTLA4 L104EA29Y region from CTLA4-Ig	2	
Fc region from CTLA4-Ig	3	
Example signal peptide sequence	4	
Example signal peptide sequence	5	
Anti-VEGFR2 adnectin	6	
Anti-TNFalpha adnectin	7	
Anti-Her2 DARPin	8	
Anti-VEGF Anticalin	9	
Anti-Her2 Affibody	10	
Camelid VHH	11	
Anti-HEL shark NARV	12	
anti-VEGF dAb DOM15-26-593	13	
anti-IL-13 dAb DOM10-53-616	14	
GGGGS Linker	15	
TVAAPS Linker	16	
ASTKGPT Linker	17	
ASTKGPS Linker	18	
CTLA4-Ig, fusion of SEQ ID NO: 1 and 3	19	
CTLA4-Ig L104EA29Y version (fusion of SEQ ID NO: 2 and 3)	20	
TNFR2-Ig fusion	21	
TACI-Ig fusion	22	
CTLA4-Ig fused to VEGFR2 adnectin (GS linker)	23	33
CTLA4-Ig fused to VEGFR2 adnectin (TVAAPSGS linker)	24	
CTLA4-Ig-antiTNFα adnectin (GS linker)	25	
CTLA4-Ig-anti-TNFα adnectin (TVAAPSGS linker)	26	
CTLA4-Ig-anti-VEGF dAb (GS linker)	27	35
CTLA4-Ig-anti-VEGF dAb (TVAAPSGS linker)	28	
CTLA4-Ig-anti-IL-13 dAb (GS linker)	29	34
CTLA4-Ig-anti-IL-13 dAb (TVAAPSGS linker)	30	
GS Linker	31	

TVAAPSGS Linker	32	
TNFR2-Ig fusion alternative sequence	36	
EtanSTG593	38	37
EtanTV4593	40	39
GSTVAAPS Linker	41	
$GS(TVAAPSGS)_1$ Linker	42	
$GS(TVAAPSGS)_2$ Linker	43	
$GS(TVAAPSGS)_3$ Linker	44	
$GS(TVAAPSGS)_4$ Linker	45	
$GS(TVAAPSGS)_5$ Linker	46	
$GS(TVAAPSGS)_6$ Linker	47	
$(TVAAPS)_2(GS)_1$ Linker	48	
$(TVAAPS)_3(GS)_1$ Linker	49	

SEQ ID NO: 1 – CTLA4 region from CTLA4-Ig

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
Q

SEQ ID NO: 2 – CTLA4 L104EA29Y version from CTLA4-Ig

MHVAQPAVVLASSRGIASFVCEYASPGKYTEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYEGIGNGTQIYVIDPEPCPDSD
Q

SEQ ID NO: 3 – Fc region from CTLA4-Ig

EPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD
GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 4 – Example signal peptide sequence

MGVLLTQRTLLSLVLALLFPSMASMA

SEQ ID NO: 5 – Example signal peptide sequence

MGWSCIILFLVATATGVHS

SEQ ID NO: 6– anti-VEGFR2 adnectin

EVVAATPTSLLISWRHPHFPTRYYRITYGETGGNSPVQEFTVPLQPPTATISGLKPGVDYTITVYAVTD
GRNGRLLSIPISINYRT

SEQ ID NO: 7– anti-TNFalpha adnectin

VSDVPRDLEVVAATPTSLLISWDTHNAYNGYYRITYGETGGNSPVREFTVPHPEVTATISGL
KPGVDDTITVYAVTNHHMPLRIFGPISINHRT

SEQ ID NO: 8– anti-Her2 DARPin

DLGKKLLEAARAGQDDEVRILMANGADVNAKDEYGLTPLYLATAHGHLEIVEVLLKNGADVNAVDAIGF
TPLHLAAFIGHLEIAEVLLKHGADVNAQDKFGKTAFDISIGNGNEDLAEILQKL

SEQ ID NO: 9 – anti-VEGF Anticalin

DGGGIRRSMSGTWYLKAMTVDREFPEMNLESVTPMTLTLLKGHNLEAKVTMLISGRCQEVKAVLGRTKE
RKKYTADGGKHVAYIIPSAVRDHVIFYSEGQLHGKPVRGVKLVGRDPKNNLEALEDFEKAAGARGLSTE
SILIPRQSETCSPG

SEQ ID NO: 10 – anti-Her2 affibody

VDNKFNKELRQAYWEIQALPNLNWTQSRAFIRSLYDDPSQSANLLAEAKKLNDAQAPK

SEQ ID NO: 11– Camelid VHH

QVQLVESGGGLVQAGGSLRLSCAASGYAYTYIYMGWFRQAPGKEREGVAAMDSGGGGTLYADSVKGRFT
ISRDKGKNTVYLQMDSLKPEDTATYYCAAGGYELRDRTYGQWGQGTQVTVSS

SEQ ID NO: 12 – anti-HEL shark NARV

ARVDQTPRSVTKETGESLTINCVLRDASYALGSTCWYRKKSGEGNEESISKGGRYVETVNSGSKSFSLR
INDLTVEDGGTYRCGLGVAGGYCDYALCSSRYAECGDGTAVTVN

SEQ ID NO: 13– anti-VEGF dAb DOM15-26-593

EVQLLVSGGGLVQPGGSLRLSCAASGFTFKAYPMMWVRQAPGKGLEWVSEISPSGSYTYYADSVKGRFT
ISRDNSKNTLYLQMNSLRAEDTAVYYCAKDPRKLDYWGQGTLVTVSS

SEQ ID No:14 = anti-IL-13 dAb DOM10-53-616

GVQLLESGGGLVQPGGSLRLSCAASGFVFPWYDMGWVRQAPGKGLEWVSSIDWHGKITYYAD
SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATAEDEPGYDYWGQGTLVTVSS

SEQ ID No:15 = G4S linker

GGGGS

SEQ ID No:16 = linker

TVAAPS

SEQ ID NO:17 = linker

ASTKGPT

SEQ ID NO:18 = linker

ASTKGPS

SEQ ID NO: 19 – CTLA4-Ig

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 20– CTLA4-Ig L104EA29Y version

MHVAQPAVVLASSRGIASFVCEYASPGKYTEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYEGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:21 – TNFR2-Ig fusion

MAPVAVWAALAVGLQLWAAAHALPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQH
AKVFCTKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGW
YCALSKQEGCRLCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICN
VVAIPGNASMDAVCTSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPS
PPAEGSTGDEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:22 – TACI-Ig fusion

MDAMKRGLCCVLLLCGAVFVSLSQEIHAELRRFRRAMRSCPEEQYWDPLLGTCMSCKTICNH
QSQRTCAAFCRSLSCRKEQGKFYDHLLRDCISCASICGQHPKQCAYFCENKLRSEPKSSDKT
HTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH

NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSIEKTISKAKGQPREPQ
VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:23 - Protein Sequence of CTLA4-Ig-anti-VEGFR2 adnectin (GS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGT
SSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDQEPKSSDKTHTSPP
SPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK**GS**EVVAATPTSLLISWRHPHFPTRYYRITYGETGGNSPVQEFTVPLQPPTATISGLK
PGVDYTITVYAVTDGRNGRLLSIPISINYRT

SEQ ID NO: 24 – CTLA4-Ig-anti-VEGFR2 adnectin (TVAAPSGS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**TVAAPSGS**EVVAATP
TSLLISWRHPHFPTRYYRITYGETGGNSPVQEFTVPLQPPTATISGLKPGVDYTITVYAVTD
GRNGRLLSIPISINYRT

SEQ ID NO: 25– CTLA4-Ig-anti-TNFα adnectin (GS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GS**EVVAATPTSLLIS
WDTHNAYNGYYRITYGETGGNSPVREFTVPHPEVTATISGLKPGVDDTITVYAVTNHHMPLR
IFGPISINHRT

SEQ ID NO: 26 – CTLA4-Ig-anti-TNFα adnectin (TVAAPSGS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**TVAAPSGS**EVVAATP
TSLLISWDTHNAYNGYYRITYGETGGNSPVREFTVPHPEVTATISGLKPGVDDTITVYAVTN
HHMPLRIFGPISINHRT

SEQ ID NO: 27– CTLA4-Ig-anti-VEGF dAb (GS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS

DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GS**EVQLLVSGGGLVQ
PGGSLRLSCAASGFTFKAYPMMWVRQAPGKGLEWVSEISPSGSYTYYADSVKGRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCAKDPRKLDYWGQGTLVTVSS

SEQ ID NO: 28 – CTLA4-Ig-anti-VEGF dAb (TVAAPSGS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**TVAAPSGS**EVQLLVS
GGGLVQPGGSLRLSCAASGFTFKAYPMMWVRQAPGKGLEWVSEISPSGSYTYYADSVKGRFT
ISRDNSKNTLYLQMNSLRAEDTAVYYCAKDPRKLDYWGQGTLVTVSS

SEQ ID NO: 29 – CTLA4-Ig-anti-IL-13 dAb (GS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GS**GVQLLESGGGLVQ
PGGSLRLSCAASGFVFPWYDMGWVRQAPGKGLEWVSSIDWHGKITYYADSVKGRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCATAEDEPGYDYWGQGTLVTVSS

SEQ ID NO: 30 – CTLA4-Ig-anti-IL-13 dAb (TVAAPSGS linker)

MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD
DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSD
QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**TVAAPSGS**GVQLLES
GGGLVQPGGSLRLSCAASGFVFPWYDMGWVRQAPGKGLEWVSSIDWHGKITYYADSVKGRFT
ISRDNSKNTLYLQMNSLRAEDTAVYYCATAEDEPGYDYWGQGTLVTVSS

SEQ ID NO:31 – Linker

GS

SEQ ID NO:32 – Linker

TVAAPSGS

SEQ ID NO:33 DNA Sequence of CTLA4-Ig-anti-VEGFR2 adnectin (GS linker)

ATGCATGTCGCCCAGCCAGCGGTGGTGCTGGCCAGCTCCCGCGGCATTGCCTCCTTCGTGTG
CGAGTACGCCAGCCCCGGCAAGGCCACCGAGGTGCGCGTCACGGTGCTCCGCCAGGCCGATA
GCCAGGTGACCGAAGTGTGTGCCGCTACGTACATGATGGGGAACGAGCTGACCTTCCTGGAC
GACTCTATCTGCACCGGGACCTCGAGCGGGAACCAGGTGAACCTGACCATCCAGGGCCTGCG
CGCGATGGACACGGGCCTGTACATCTGCAAGGTGGAGTTGATGTACCCCCCCCCGTACTACC
TGGGGATCGGCAACGGCACGCAGATCTACGTCATCGACCCCGAACCTTGCCCTGACAGCGAC
CAGGAGCCCAAGTCTAGTGACAAGACCCATACCTCTCCCCCCAGCCCCGCTCCAGAGCTGCT
GGGGGGCTCCAGCGTGTTCCTGTTTCCCCCCAAGCCTAAGGACACCCTGATGATCTCCAGAA

CCCCCGAGGTGACCTGCGTGGTCGTGGATGTGAGTCACGAGGACCCTGAGGTGAAGTTCAAC
TGGTACGTGGACGGGGTGGAGGTGCATAACGCCAAGACCAAGCCTCGCGAGGAGCAGTACAA
CAGTACCTACCGCGTGGTGTCCGTGCTCACTGTGCTGCATCAGGACTGGCTGAACGGCAAGG
AGTATAAGTGCAAGGTGTCTAACAAGGCCTTGCCCGCCCCCATCGAGAAAACAATCTCCAAG
GCCAAAGGGCAGCCCAGGGAACCTCAGGTGTACACCCTCCCTCCAAGCCGTGACGAGCTGAC
CAAGAACCAGGTCTCTCTGACCTGCTTGGTGAAGGGCTTCTACCCTAGCGACATCGCTGTGG
AGTGGGAGTCCAACGGGCAGCCCGAGAACAACTACAAAACCACCCCGCCCGTGCTGGACTCT
GACGGCTCCTTCTTCCTGTACAGCAAACTGACCGTGGACAAGTCCAGGTGGCAGCAGGGAAA
CGTGTTCAGCTGCAGCGTCATGCATGAGGCCCTGCATAACCATTACACACAGAAGAGCCTGT
CCCTGAGCCCCGGCAAGGGATCCGAGGTGGTGGCCGCCACCCCCACCAGCCTGCTGATTTCC
TGGAGGCACCCCCACTTCCCCACACGCTACTACAGGATCACCTACGGCGAGACCGGCGGCAA
CAGCCCCGTGCAGGAGTTCACCGTGCCCCTGCAGCCTCCCACTGCCACCATCAGCGGCCTCA
AGCCCGGCGTGGACTACACCATCACCGTGTACGCCGTCACCGACGGAAGGAACGGCAGGCTG
CTGAGCATCCCCATCAGCATCAACTACAGGACC

SEQ ID NO: 34 - DNA Sequence of CTLA4-Ig-anti-IL-13 dAb (GS linker)

ATGCATGTCGCCCAGCCAGCGGTGGTGCTGGCCAGCTCCCGCGGCATTGCCTCCTTCGTGTG
CGAGTACGCCAGCCCCGGCAAGGCCACCGAGGTGCGCGTCACGGTGCTCCGCCAGGCCGATA
GCCAGGTGACCGAAGTGTGTGCCGCTACGTACATGATGGGGAACGAGCTGACCTTCCTGGAC
GACTCTATCTGCACCGGGACCTCGAGCGGGAACCAGGTGAACCTGACCATCCAGGGCCTGCG
CGCGATGGACACGGGCCTGTACATCTGCAAGGTGGAGTTGATGTACCCCCCCCCGTACTACC
TGGGGATCGGCAACGGCACGCAGATCTACGTCATCGACCCCGAACCTTGCCCTGACAGCGAC
CAGGAGCCCAAGTCTAGTGACAAGACCCATACCTCTCCCCCCAGCCCCGCTCCAGAGCTGCT
GGGGGGCTCCAGCGTGTTCCTGTTTCCCCCCAAGCCTAAGGACACCCTGATGATCTCCAGAA
CCCCCGAGGTGACCTGCGTGGTCGTGGATGTGAGTCACGAGGACCCTGAGGTGAAGTTCAAC
TGGTACGTGGACGGGGTGGAGGTGCATAACGCCAAGACCAAGCCTCGCGAGGAGCAGTACAA
CAGTACCTACCGCGTGGTGTCCGTGCTCACTGTGCTGCATCAGGACTGGCTGAACGGCAAGG
AGTATAAGTGCAAGGTGTCTAACAAGGCCTTGCCCGCCCCCATCGAGAAAACAATCTCCAAG
GCCAAAGGGCAGCCCAGGGAACCTCAGGTGTACACCCTCCCTCCAAGCCGTGACGAGCTGAC
CAAGAACCAGGTCTCTCTGACCTGCTTGGTGAAGGGCTTCTACCCTAGCGACATCGCTGTGG
AGTGGGAGTCCAACGGGCAGCCCGAGAACAACTACAAAACCACCCCGCCCGTGCTGGACTCT
GACGGCTCCTTCTTCCTGTACAGCAAACTGACCGTGGACAAGTCCAGGTGGCAGCAGGGAAA
CGTGTTCAGCTGCAGCGTCATGCATGAGGCCCTGCATAACCATTACACACAGAAGAGCCTGT
CCCTGAGCCCCGGCAAGGGATCCGGCGTGCAGCTCCTGGAGAGCGGCGGAGGCCTGGTCCAG
CCCGGCGGCAGCCTGAGGCTGAGCTGCGCCGCCAGCGGCTTCGTGTTCCCCTGGTATGATAT
GGGCTGGGTGAGGCAGGCCCCCGGCAAGGGCCTGGAGTGGGTGTCCAGCATCGACTGGCACG
GGAAGATCACCTACTACGCCGACAGCGTGAAGGGCAGGTTCACCATCAGCAGGGACAACAGC
AAGAACACCCTGTACCTGCAGATGAACAGCCTGAGGGCCGAGGACACCGCAGTGTACTACTG
CGCCACCGCCGAGGACGAACCCGGCTACGACTACTGGGGCCAGGGCACCCTGGTGACTGTGA
GCAGC

SEQ ID NO: 35 - DNA Sequence of CTLA4-Ig-anti-VEGF dAb (GS linker)

ATGCATGTCGCCCAGCCAGCGGTGGTGCTGGCCAGCTCCCGCGGCATTGCCTCCTTCGTGTG
CGAGTACGCCAGCCCCGGCAAGGCCACCGAGGTGCGCGTCACGGTGCTCCGCCAGGCCGATA
GCCAGGTGACCGAAGTGTGTGCCGCTACGTACATGATGGGGAACGAGCTGACCTTCCTGGAC
GACTCTATCTGCACCGGGACCTCGAGCGGGAACCAGGTGAACCTGACCATCCAGGGCCTGCG
CGCGATGGACACGGGCCTGTACATCTGCAAGGTGGAGTTGATGTACCCCCCCCCGTACTACC

TGGGGATCGGCAACGGCACGCAGATCTACGTCATCGACCCCGAACCTTGCCCTGACAGCGAC
CAGGAGCCCAAGTCTAGTGACAAGACCCATACCTCTCCCCCCAGCCCCGCTCCAGAGCTGCT
GGGGGGCTCCAGCGTGTTCCTGTTTCCCCCCAAGCCTAAGGACACCCTGATGATCTCCAGAA
CCCCCGAGGTGACCTGCGTGGTCGTGGATGTGAGTCACGAGGACCCTGAGGTGAAGTTCAAC
TGGTACGTGGACGGGGTGGAGGTGCATAACGCCAAGACCAAGCCTCGCGAGGAGCAGTACAA
CAGTACCTACCGCGTGGTGTCCGTGCTCACTGTGCTGCATCAGGACTGGCTGAACGGCAAGG
AGTATAAGTGCAAGGTGTCTAACAAGGCCTTGCCCGCCCCCATCGAGAAAACAATCTCCAAG
GCCAAAGGGCAGCCCAGGGAACCTCAGGTGTACACCCTCCCTCCAAGCCGTGACGAGCTGAC
CAAGAACCAGGTCTCTCTGACCTGCTTGGTGAAGGGCTTCTACCCTAGCGACATCGCTGTGG
AGTGGGAGTCCAACGGGCAGCCCGAGAACAACTACAAAACCACCCCGCCCGTGCTGGACTCT
GACGGCTCCTTCTTCCTGTACAGCAAACTGACCGTGGACAAGTCCAGGTGGCAGCAGGGAAA
CGTGTTCAGCTGCAGCGTCATGCATGAGGCCCTGCATAACCATTACACACAGAAGAGCCTGT
CCCTGAGCCCCGGCAAGGGATCCGAGGTGCAGCTCCTGGTCAGCGGCGGCGGCCTGGTCCAG
CCCGGAGGCTCACTGAGGCTGAGCTGCGCCGCTAGCGGCTTCACCTTCAAGGCCTACCCCAT
GATGTGGGTCAGGCAGGCCCCCGGCAAAGGCCTGGAGTGGGTGTCTGAGATCAGCCCCAGCG
GCAGCTACACCTACTACGCCGACAGCGTGAAGGGCAGGTTCACCATCAGCAGGGACAACAGC
AAGAACACCCTGTACCTGCAGATGAACTCTCTGAGGGCCGAGGACACCGCCGTGTACTACTG
CGCCAAGGACCCCAGGAAGCTGGACTATTGGGGCCAGGGCACTCTGGTGACCGTGAGCAGC

SEQ ID NO: 36 – TNFR2-Ig fusion alternative sequence

MAPVAVWAALAVGLELWAAAHALPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQH
AKVFCTKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGW
YCALSKQEGCRLCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICN
VVAIPGNASMDAVCTSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPS
PPAEGSTGDEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:27

CTGCCCGCTCAGGTGGCCTTCACTCCCTACGCCCCAGAGCCCGGCTCTACCTGCAGGCTGAG
GGAGTACTACGACCAGACCGCCCAGATGTGCTGCAGCAAGTGCAGCCCCGGCCAGCACGCCA
AAGTGTTCTGCACCAAGACCAGCGACACCGTGTGCGATAGCTGCGAGGACAGCACCTACACC
CAGCTGTGGAACTGGGTCCCCGAGTGCCTGAGCTGCGGCTCTAGGTGTAGCAGCGACCAGGT
CGAGACCCAGGCCTGCACCAGGGAACAGAACCGGATCTGCACATGCAGGCCCGGCTGGTACT
GCGCCCTCAGCAAACAGGAGGGCTGCAGGCTGTGTGCCCCCCTCAGGAAGTGCAGGCCCGGG
TTTGGCGTGGCCAGGCCCGGAACCGAGACTAGCGACGTGGTGTGCAAACCCTGCGCCCCCGG
CACCTTCAGCAATACCACTAGCAGCACCGACATCTGCAGGCCTCACCAGATCTGCAACGTGG
TGGCCATTCCCGGCAACGCAAGCATGGACGCCGTGTGCACCAGCACCAGCCCCACCAGGTCA
ATGGCCCCTGGAGCCGTGCATCTGCCCCAGCCCGTGAGCACCAGAAGCCAGCACACCCAGCC
TACCCCCGAGCCCAGCACCGCCCCTAGCACCAGCTTCCTGCTGCCTATGGGCCCCTCCCCTC
CCGCCGAGGGCTCAACCGGCGACGAACCCAAGAGCTGCGACAAGACCCACACCTGCCCCCCC
TGCCCCGCACCAGAACTCCTGGGCGGACCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGA
CACCCTGATGATCAGCAGGACCCCCGAGGTGACCTGTGTGGTGGTGGACGTGAGCCACGAGG
ACCCCGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAG
CCCAGGGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTCCTGACCGTGCTGCACCA
GGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCCGCCCCCA
TCGAGAAGACCATCAGCAAGGCCAAAGGCCAGCCCAGGGAGCCACAGGTGTACACACTGCCC
CCCAGCAGGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTA

TCCCAGCGATATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACCA
CCCCCCCCGTCCTGGACTCCGACGGGAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAG
AGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCACAACCA
CTACACCCAGAAGTCCCTGAGCCTGAGCCCCGGCAAGTCGACCGGTGAGGTGCAGCTGCTGG
TGTCTGGCGGCGGACTGGTGCAGCCTGGCGGCAGCCTGAGACTGAGCTGCGCCGCCAGCGGC
TTCACCTTCAAGGCCTACCCCATGATGTGGGTGCGGCAGGCCCCTGGCAAGGGCCTGGAATG
GGTGTCCGAGATCAGCCCCAGCGGCAGCTACACCTACTACGCCGACAGCGTGAAGGGCCGGT
TCACCATCAGCCGGGACAACAGCAAGAACACCCTGTACCTGCAGATGAACAGCCTGCGGGCC
GAGGACACCGCCGTGTACTACTGCGCCAAGGACCCCCGGAAGCTGGACTACTGGGGCCAGGG
CACCCTGGTGACCGTGAGCAGC

SEQ ID NO:38

LPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCEDSTYT
QLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGCRLCAPLRKCRPG
FGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICNVVAIPGNASMDAVCTSTSPTRS
MAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGDEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLP
PSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKSTGEVQLLVSGGGLVQPGGSLRLSCAASG
FTFKAYPMMWVRQAPGKGLEWVSEISPSGSYTYYADSVKGRFTISRDNSKNTLYLQMNSLRA
EDTAVYYCAKDPRKLDYWGQGTLVTVSS

SEQ ID NO:39

CTGCCCGCTCAGGTGGCCTTCACTCCCTACGCCCCAGAGCCCGGCTCTACCTGCAGGCTGAG
GGAGTACTACGACCAGACCGCCCAGATGTGCTGCAGCAAGTGCAGCCCCGGCCAGCACGCCA
AAGTGTTCTGCACCAAGACCAGCGACACCGTGTGCGATAGCTGCGAGGACAGCACCTACACC
CAGCTGTGGAACTGGGTCCCCGAGTGCCTGAGCTGCGGCTCTAGGTGTAGCAGCGACCAGGT
CGAGACCCAGGCCTGCACCAGGGAACAGAACCGGATCTGCACATGCAGGCCCGGCTGGTACT
GCGCCCTCAGCAAACAGGAGGGCTGCAGGCTGTGTGCCCCCCTCAGGAAGTGCAGGCCCGGG
TTTGGCGTGGCCAGGCCCGGAACCGAGACTAGCGACGTGGTGTGCAAACCCTGCGCCCCCGG
CACCTTCAGCAATACCACTAGCAGCACCGACATCTGCAGGCCTCACCAGATCTGCAACGTGG
TGGCCATTCCCGGCAACGCAAGCATGGACGCCGTGTGCACCAGCACCAGCCCCACCAGGTCA
ATGGCCCCTGGAGCCGTGCATCTGCCCCAGCCCGTGAGCACCAGAAGCCAGCACACCCAGCC
TACCCCCGAGCCCAGCACCGCCCCTAGCACCAGCTTCCTGCTGCCTATGGGCCCCTCCCCTC
CCGCCGAGGGCTCAACCGGCGACGAACCCAAGAGCTGCGACAAGACCCACACCTGCCCCCCC
TGCCCCGCACCAGAACTCCTGGGCGGACCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGA
CACCCTGATGATCAGCAGGACCCCCGAGGTGACCTGTGTGGTGGTGGACGTGAGCCACGAGG
ACCCCGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAG
CCCAGGGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTCCTGACCGTGCTGCACCA
GGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCCGCCCCCA
TCGAGAAGACCATCAGCAAGGCCAAAGGCCAGCCCAGGGAGCCACAGGTGTACACACTGCCC
CCCAGCAGGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTA
TCCCAGCGATATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACCA
CCCCCCCCGTCCTGGACTCCGACGGGAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAG
AGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCACAACCA
CTACACCCAGAAGTCCCTGAGCCTGAGCCCCGGCAAGACCGTGGCGGCGCCCAGCACGGTGG
CCGCCCCCTCCACCGTCGCCGCGCCAAGCACCGTGGCTGCTCCGTCGACCGGTGAGGTGCAG
CTGCTGGTGTCTGGCGGCGGACTGGTGCAGCCTGGCGGCAGCCTGAGACTGAGCTGCGCCGC

CAGCGGCTTCACCTTCAAGGCCTACCCCATGATGTGGGTGCGGCAGGCCCCTGGCAAGGGCC
TGGAATGGGTGTCCGAGATCAGCCCCAGCGGCAGCTACACCTACTACGCCGACAGCGTGAAG
GGCCGGTTCACCATCAGCCGGGACAACAGCAAGAACACCCTGTACCTGCAGATGAACAGCCT
GCGGGCCGAGGACACCGCCGTGTACTACTGCGCCAAGGACCCCCGGAAGCTGGACTACTGGG
GCCAGGGCACCCTGGTGACCGTGAGCAGC

SEQ ID NO:40

LPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCEDSTYT
QLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGCRLCAPLRKCRPG
FGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICNVVAIPGNASMDAVCTSTSPTRS
MAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGDEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLP
PSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKTVAAPSTVAAPSTVAAPSTVAAPSTGEVQ
LLVSGGGLVQPGGSLRLSCAASGFTFKAYPMMWVRQAPGKGLEWVSEISPSGSYTYYADSVK
GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDPRKLDYWGQGTLVTVSS

SEQ ID NO: 41

GSTVAAPS

SEQ ID NO: 42

GSTVAAPSGS

SEQ ID NO:43

GSTVAAPSGSTVAAPSGS

SEQ ID NO:44

GSTVAAPSGSTVAAPSGSTVAAPSGS

SEQ ID NO:45

GSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGS

SEQ ID NO:46

GSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGS

SEQ ID NO:47

GSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGSTVAAPSGS

SEQ ID NO:48

TVAAPSTVAAPSGS

SEQ ID NO:49

TVAAPSTVAAPSTVAAPSGS

Brief Description of Figures

Figure 1 - Bridging ELISA showing that bispecific BPC1821 binds to both VEGFR2 and B7-1.

Figure 2 –Bridging ELISA showing that bispecific BPC1824 binds to both IL-13 and B7-1.

Figure 3 - Bridging ELISA showing that bispecific BPC1825 binds to both VEGF and B7-1.

Claims

1. An antigen-binding protein comprising a receptor-Fc fusion which is linked to one or more epitope-binding domains.

2. The antigen-binding protein according to claim 1 wherein at least one epitope binding domain is an immunoglobulin single variable domain.

3. The antigen-binding protein according to claim 2 wherein the immunoglobulin single variable domain is a human dAb.

4. The antigen-binding protein according to claim 2 wherein the immunoglobulin single variable domain is a camelid VHH immunoglobulin single variable domain or a shark immunoglobulin single variable domain (NARV).

5. The antigen-binding protein according to any one of claims 1 to 4 wherein at least one epitope binding domain is derived from a scaffold selected from a non-Ig domain selected from CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody); Heat shock proteins such as GroEl and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human γ-crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin).

6. The antigen-binding protein according to claim 5 wherein the epitope binding domain is derived from a scaffold selected from an Affibody, an ankyrin repeat protein (DARPin) and an adnectin.

7. The antigen-binding protein of any preceding claim wherein the binding protein has specificity for more than one antigen.

8. The antigen-binding protein according to any preceding claim wherein the receptor-Fc fusion comprises CTLA-4-Ig.

9. The antigen-binding protein according to any preceding claim wherein the receptor-Fc fusion comprises TNFR2-Ig.

10. The antigen-binding protein according to any preceding claim wherein the receptor-Fc fusion comprises TACI-Ig.

11. The antigen-binding protein according to any preceding claim wherein at least one epitope binding domain is capable of binding VEGF or VEGFR2.

12. The antigen-binding protein according to any preceding claim wherein at least one epitope binding domain is capable of binding TNFα.

13. The antigen-binding protein according to any preceding claim wherein at least one epitope binding domain is capable of binding HER2.

14. The antigen-binding protein according to any preceding claim wherein at least one of the epitope binding domains is directly attached to the receptor-Fc fusion with a linker comprising from 1 to 150 amino acids.

15. The antigen-binding protein according to claim 14 wherein at least one of the epitope binding domains is directly attached to the receptor-Fc fusion with a linker comprising from 1 to 20 amino acids.

16. The antigen-binding protein according to claim 15 wherein at least one of the epitope binding domains is directly attached to the Receptor-Fc fusion with a linker selected from any one of those set out in SEQ ID NO: 15-19, SEQ ID NO: 31-32, or any multiple or combination thereof.

17. The antigen-binding protein according to any preceding claim wherein at least one of the epitope binding domains binds human serum albumin.

18. The antigen-binding protein according to any preceding claim comprising an epitope binding domain attached to the N-terminus of the Receptor-Fc fusion.

19. The antigen-binding protein according to any preceding claim comprising an epitope binding domain attached to the C-terminus of the Receptor-Fc fusion

20. A polynucleotide sequence encoding an antigen-binding protein according to any one of claims 1 to 19.

21. A recombinant transformed or transfected host cell comprising one or more polynucleotide sequences encoding an antigen-binding protein of any preceding claim.

22. A method for the production of an antigen-binding protein according to claims 1 to 19 which method comprises the step of culturing a host cell of claim 21 and isolating the antigen-binding protein.

23. A pharmaceutical composition comprising an antigen-binding protein of any one of claims 1 to 19 and a pharmaceutically acceptable carrier.

24. The antigen-binding protein according to any preceding claim for use in medicine.

25. The antigen-binding protein according to any preceding claim for use in the manufacture of a medicament for treating immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

26. A method of treating a patient suffering from immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis comprising administering a therapeutic amount of an antigen-binding protein according to any one of claims 1 to 19.

27. The antigen-binding protein according to any one of claims 1 to 19 for the treatment of immune diseases for example auto-immune diseases, or cancer, or inflammatory diseases, for example systemic lupus erythramatosis, multiple sclerosis, crohns disease, psoriasis, or arthritic diseases, for example rheumatoid arthritis.

Figures

Figure 1

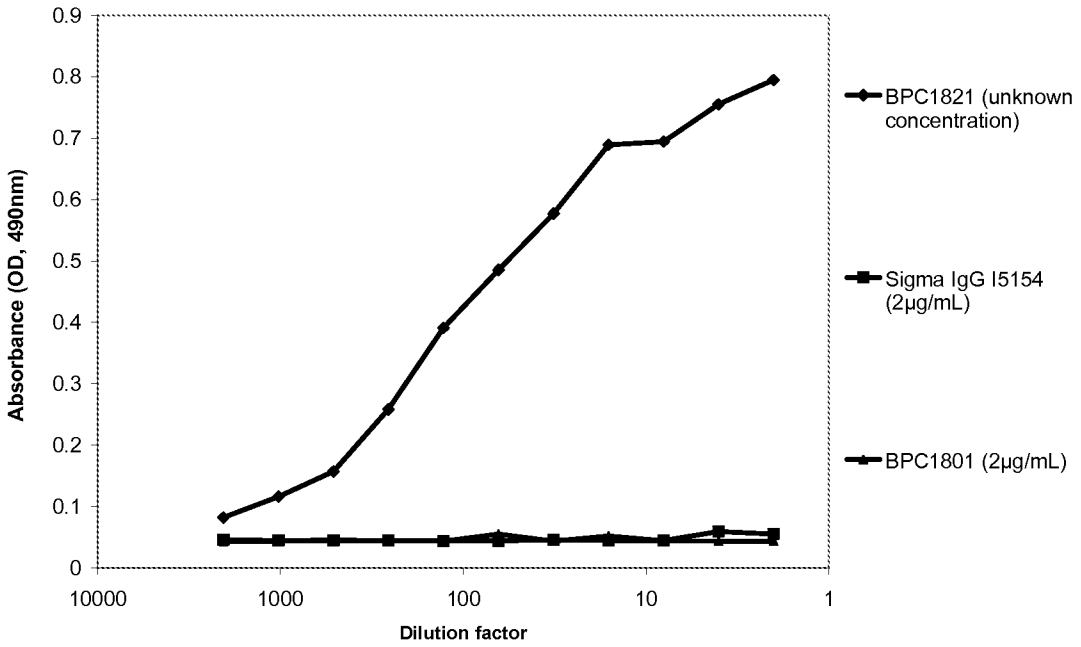


Figure 2

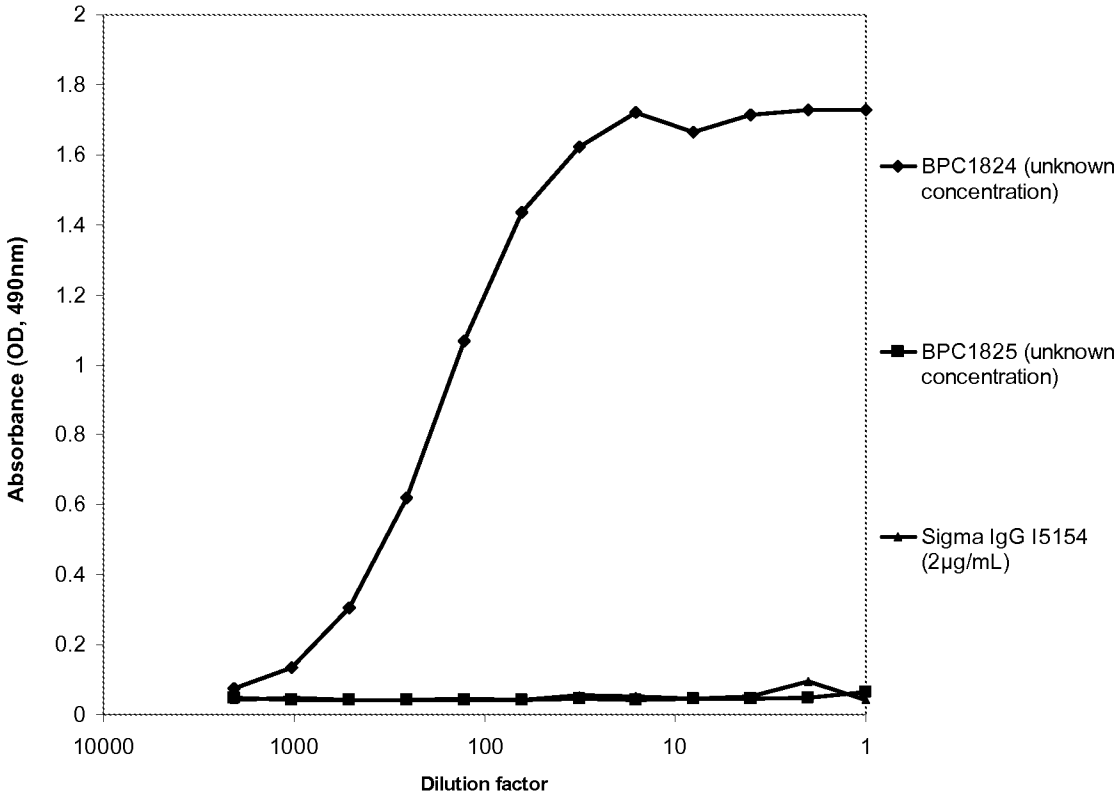
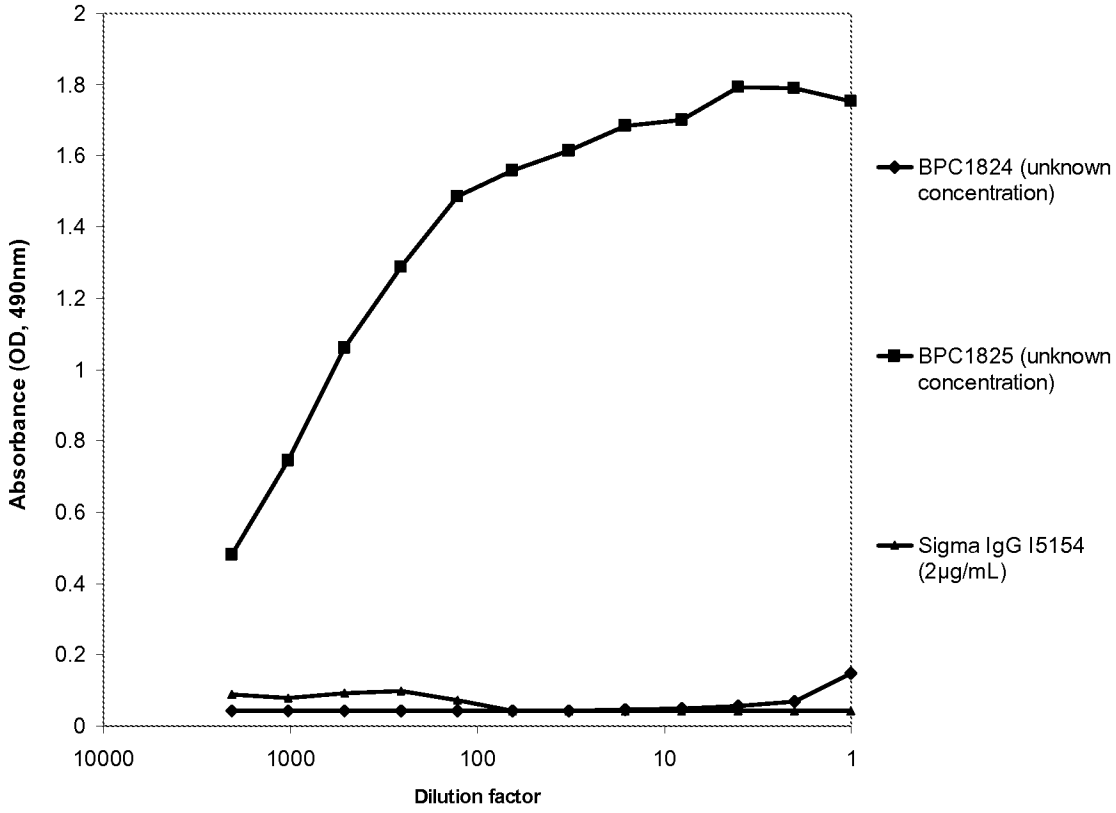


Figure 3


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (OD, 490nm)
10000
1000
100
10
1
Dilution factor
BPC1824 (unknown concentration)
BPC1825 (unknown concentration)
Sigma IgG I5154 (2µg/mL)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/057227

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/22 C07K16/24 C07K16/28 C07K16/32 A61K38/17
A61K47/48 C07K14/705 C07K14/715 C07K19/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/005638 A2 (REGENERON PHARMA [US]; STAHL NEIL [US]; YANCOPOULOS GEORGE D [US]; KAR) 20 January 2005 (2005-01-20) e.g. page 1. paragraph 1; page 5, paragraph 27; example 1 the whole document -----	1-4,7,8, 11, 14-16, 18-27
A	BUCH MAYA H ET AL: "Abatacept in the treatment of rheumatoid arthritis." ARTHRITIS RESEARCH & THERAPY 2008 LNKD-PUBMED:19007425, vol. 10 Suppl 1, 2008, page S5, XP002592942 ISSN: 1478-6362 the whole document -----	1-4,7,8, 11, 14-16, 18-27

-/--

[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 document 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
21 July 2010	30/09/2010

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	Gruber, Andreas

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.
A	DANILA MARIA I ET AL: "Pharmacogenetics of etanercept in rheumatoid arthritis." PHARMACOGENOMICS AUG 2008 LNKD-PUBMED:18681777, vol. 9, no. 8, August 2008 (2008-08), pages 1011-1015, XP002592943 ISSN: 1744-8042 the whole document	1-4,7,8, 11, 14-16, 18-27
A	CARBONATTO MICHELA ET AL: "Nonclinical safety, pharmacokinetics, and pharmacodynamics of atacicept." TOXICOLOGICAL SCIENCES : AN OFFICIAL JOURNAL OF THE SOCIETY OF TOXICOLOGY SEP 2008 LNKD- PUBMED:18522929, vol. 105, no. 1, September 2008 (2008-09), pages 200-210, XP002592944 ISSN: 1096-0929 the whole document	1-4,7,8, 11, 14-16, 18-27
A	EP 1 878 750 A2 (DOMANTIS LTD [GB]) 16 January 2008 (2008-01-16) the whole document	1-4,7,8, 11, 14-16, 18-27
T	HUANG ET AL: "Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology" CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB LNKD- DOI:10.1016/J.COPBIO.2009.10.010, vol. 20, no. 6, 1 December 2009 (2009-12-01), pages 692-699, XP026778880 ISSN: 0958-1669 [retrieved on 2009-11-04] the whole document	
A	US 2002/197254 A1 (BROWNING JEFFREY L [US] ET AL) 26 December 2002 (2002-12-26) the whole document	1-4,7,8, 11, 14-16, 18-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/057227

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.:

1-4, 7, 8, 11, 14-16, 18-27(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:

Invention: 1; Claims: 1-4, 7, 8, 11, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to immunoglobulin single variable domain capable of binding VEGF, polynucleotide sequence, host cell, method, composition

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Invention: 2; Claims: 1-4, 7, 8, 11, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to immunoglobulin single variable domain capable of binding VEGFR2, polynucleotide sequence, host cell, method, composition

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Invention: 3; Claims: 1-4, 7, 8, 12, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to immunoglobulin single variable domain capable of binding TNFa, polynucleotide sequence, host cell, method, composition

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Invention: 4; Claims: 1-4, 7, 8, 13-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to immunoglobulin single variable domain capable of binding HER2, polynucleotide sequence, host cell, method, composition

---

Invention: 5; Claims: 1-4, 7, 8, 14-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to immunoglobulin single variable domain capable of binding human serum albumin, polynucleotide sequence, host cell, method, composition

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Invention: 6; Claims: 1-4, 7, 9, 11, 14-16, 18-27(all partially)

antigen-binding protein comprising TNFR2-Ig linked to immunoglobulin single variable domain capable of binding VEGF, polynucleotide sequence, host cell, method, composition

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Invention: 7; Claims: 1-4, 7, 9, 11, 14-16, 18-27(all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

antigen-binding protein comprising TNFR2-Ig linked to immunoglobulin single variable domain capable of binding VEGFR2, polynucleotide sequence, host cell, method, composition

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Invention: 8; Claims: 1-4, 9, 12, 14-16, 18-27(all partially)

antigen-binding protein comprising TNFR2-Ig linked to immunoglobulin single variable domain capable of binding TNFa, polynucleotide sequence, host cell, method, composition

---

Invention: 9; Claims: 1-4, 7, 9, 13-16, 18-27(all partially)

antigen-binding protein comprising TNFR2-Ig linked to immunoglobulin single variable domain capable of binding HER2, polynucleotide sequence, host cell, method, composition

---

Invention: 10; Claims: 1-4, 7, 9, 14-27(all partially)

antigen-binding protein comprising TNFR2-Ig linked to immunoglobulin single variable domain capable of binding human serum albumin, polynucleotide sequence, host cell, method, composition

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Invention: 11; Claims: 1-4, 7, 10, 11, 14-16, 18-27(all partially)

antigen-binding protein comprising TACI-Ig linked to immunoglobulin single variable domain capable of binding VEGF, polynucleotide sequence, host cell, method, composition

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Invention: 12; Claims: 1-4, 7, 10, 11, 14-16, 18-27(all partially)

antigen-binding protein comprising TACI-Ig linked to immunoglobulin single variable domain capable of binding VEGFR2, polynucleotide sequence, host cell, method, composition

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Invention: 13; Claims: 1-4, 7, 10, 12, 14-16, 18-27(all partially)

antigen-binding protein comprising TACI-Ig linked to immunoglobulin single variable domain capable of binding TNFa, polynucleotide sequence, host cell, method, composition

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Invention: 14; Claims: 1-4, 7, 10, 13-16, 18-27(all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

antigen-binding protein comprising TACI-Ig linked to immunoglobulin single variable domain capable of binding HER2, polynucleotide sequence, host cell, method, composition

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Invention: 15; Claims: 1-4, 7, 10, 14-27(all partially)

antigen-binding protein comprising TACI-Ig linked to immunoglobulin single variable domain capable of binding human serum albumin, polynucleotide sequence, host cell, method, composition

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Invention: 16; Claims: 1, 5, 8, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to CTLA-4 (Evibody), polynucleotide sequence, host cell, method, composition

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Inventions: 17-24; Claims: 1, 5, 7, 8, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to any one of the following compounds (each compound being one invention):
a) lipocalin;
b) Heat shock proteins such as GroEl and GroES;
c) transferrin (trans-body);
d) peptide aptamer;
e) C-type lectin domain (Tetranectin);
f) human y-crystallin and human ubiquitin (affilins);
g) PDZ domains; or
h) scorpion toxinkunitz type domains of human protease inhibitors;
polynucleotide sequence, host cell, method, composition

Inventions: 25-27; Claims: 1, 5-8, 14-16, 18-27(all partially)

antigen-binding protein comprising CTLA4-Ig linked to any one of the following compounds (each compound being one invention):
a) Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody);
b) ankyrin repeat protein (DARPin);
c) fibronectin (adnectin);
polynucleotide sequence, host cell, method, composition

Inventions: 28-36; Claims: 1, 5, 7, 9, 14-16, 18-27(all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

antigen-binding protein comprising TNFR2-Ig linked to any one of the following compounds (each compound being one invention):
a) CTLA-4 (Evibody);
b) lipocalin;
c) Heat shock proteins such as GroEl and GroES;
d) transferrin (trans-body);
e) peptide aptamer;
f) C-type lectin domain (Tetranectin);
g) human y-crystallin and human ubiquitin (affilins);
h) PDZ domains; or
i) scorpion toxinkunitz type domains of human protease inhibitors;
polynucleotide sequence, host cell, method, composition

Inventions: 37-39; Claims: 1, 5-7, 9, 14-16, 18-27(all partially)

antigen-binding protein comprising TNFR2-Ig linked to any one of the following compounds (each compound being one invention):
a) Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody);
b) ankyrin repeat protein (DARPin);
c) fibronectin (adnectin);
polynucleotide sequence, host cell, method, composition

Inventions: 40-48; Claims: 1, 5, 7, 10, 14-16, 18-27(all partially)

antigen-binding protein comprising TACI-Ig linked to any one of the following compounds (each compound being one invention):
a) CTLA-4 (Evibody);
b) lipocalin;
c) Heat shock proteins such as GroEl and GroES;
d) transferrin (trans-body);
e) peptide aptamer;
f) C-type lectin domain (Tetranectin);
g) human y-crystallin and human ubiquitin (affilins);
h) PDZ domains; or
i) scorpion toxinkunitz type domains of human protease inhibitors;
polynucleotide sequence, host cell, method, composition

Inventions: 49-51; Claims: 1, 5-7, 10, 14-16, 18-27(all partially)

antigen-binding protein comprising TACI-Ig linked to any one of the following compounds (each compound being one invention):
a) Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody);
b) ankyrin repeat protein (DARPin);
c) fibronectin (adnectin);
polynucleotide sequence, host cell, method, composition

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/057227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005005638 A2	20-01-2005	US 2005032175 A1	10-02-2005
EP 1878750 A2	16-01-2008	EP 1878751 A2	16-01-2008
US 2002197254 A1	26-12-2002	US 7255854 B1	14-08-2007
		US 2008219967 A1	11-09-2008