Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3075367 A1 2019/04/04

(21) 3 075 367

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2018/09/27
(87) Date publication PCT/PCT Publication Date: 2019/04/04
(85) Entrée phase nationale/National Entry: 2020/03/09
(86) N° demande PCT/PCT Application No.: EP 2018/076333
(87) N° publication PCT/PCT Publication No.: 2019/063726
(30) Priorités/Priorities: 2017/09/27 (US62/563,948); 2018/05/04 (US62/667,126)

(51) Cl.Int./Int.Cl. *C07K 16/18* (2006.01), *A61K 39/395* (2006.01), *A61P 37/06* (2006.01), *C07K 16/24* (2006.01), *C07K 16/28* (2006.01), *C07K 16/46* (2006.01)

(71) Demandeur/Applicant:
ELASMOGEN LTD, GB

(72) Inventeurs/Inventors:
UBAH, OBINNA, GB;
BARELLE, CAROLINE, GB;
PORTER, ANDREW, GB

(74) Agent: LEUNG, JASON C.

(54) Titre : MOLECULES DE LIAISON SPECIFIQUES
(54) Title: SPECIFIC BINDING MOLECULES

(57) Abrégé/Abstract:
The present invention relates to the formation of multi-domain specific binding molecules comprising VNARs. Specific binding domains that bind to Tumour Necrosis Factor alpha (TNFa) are also provided.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
04 April 2019 (04.04.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/063726 A1

(51) International Patent Classification:
C07K 16/18 (2006.01) *A61K 39/395* (2006.01)
C07K 16/24 (2006.01) *A61P 37/06* (2006.01)
C07K 16/28 (2006.01) *C07K 16/46* (2006.01)

(21) International Application Number:
PCT/EP2018/076333

(22) International Filing Date:
27 September 2018 (27.09.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/563,948 27 September 2017 (27.09.2017) US
62/667,126 04 May 2018 (04.05.2018) US

(71) Applicant: **ELASMOGEN LTD** [GB/GB]; Liberty Building, Foresterhill Health Research Complex, Foresterhill Road, Aberdeen Aberdeenshire AB25 2ZP (GB).

(72) Inventors: **UBAH, Obinna**; c/o Elasmogen Ltd, Liberty Building, Foresterhill Health Research Complex, Foresterhill Road, Aberdeen Aberdeenshire AB25 2ZP (GB). **BARELLE, Caroline**; c/o Elasmogen Ltd, Liberty Building, Foresterhill Health Research Complex, Foresterhill Road, Aberdeen Aberdeenshire AB25 2ZP (GB). **PORTER, Andrew**; c/o Elasmogen Ltd, Liberty Building, Foresterhill Health Research Complex, Foresterhill Road, Aberdeen Aberdeenshire AB25 2ZP (GB).

(74) Agent: **BRISCOE, Paul**; Kilburn & Strode LLP, Lacon London, 84 Theobalds Road, London Greater London WC1X 8NL (GB).

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

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

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: SPECIFIC BINDING MOLECULES

(57) Abstract: The present invention relates to the formation of multi-domain specific binding molecules comprising VNARs. Specific binding domains that bind to Tumour Necrosis Factor alpha (TNFα) are also provided.

WO 2019/063726 A1

SPECIFIC BINDING MOLECULES

FIELD OF INVENTION

The present invention relates to the formation of multi-domain specific binding molecules comprising VNARs. Specific binding domains that bind to Tumour Necrosis Factor alpha (TNFα) are also provided.

BACKGROUND

The search for specific, increasingly efficacious, and diversified therapeutic weapons to combat diseases has utilised a myriad of distinct modalities. From the traditional small molecule to incrementally larger biologic pharmaceuticals, for example single binding domains (10-15 kDa) to full IgG (~150 kDa). Single domains currently under investigation as potential therapeutics include a wide variety of distinct protein scaffolds, all with their associated advantages and disadvantages.

Such single domain scaffolds can be derived from an array of proteins from distinct species. The Novel or New antigen receptor (IgNAR) is an approximately 160 kDa homodimeric protein found in the sera of cartilaginous fish (Greenberg A. S., *et al.*, Nature, 1995. **374**(6518): p. 168-173, Dooley, H., *et al*, Mol. Immunol, 2003. **40**(1): p. 25-33; Müller, M.R., *et al.*, mAbs, 2012. **4**(6): p. 673-685)). Each molecule consists of a single N-terminal variable domain (VNAR) and five constant domains (CNAR). The IgNAR domains are members of the immunoglobulin-superfamily. The VNAR is a tightly folded domain with structural and some sequence similarities to the immunoglobulin and T-cell receptor Variable domains and to cell adhesion molecules and is termed the VNAR by analogy to the N Variable terminal domain of the classical immunoglobulins and T Cell receptors. The VNAR shares limited sequence homology to immunoglobulins, for example 25-30% similarity between VNAR and human light chain sequences (Dooley, H. and Flajnik, M. F., Eur. J. Immunol., 2005. **35**(3): p. 936-945).

Kovaleva M. *et al* Expert Opin. Biol. Ther. 2014. **14**(10): p. 1527-1539 and Zielonka S. *et al* mAbs 2015. **7**(1): p. 15-25 have recently provided summaries of the structural characterization and generation of the VNARs which are hereby incorporated by reference.

The VNAR does not appear to have evolved from a classical immunoglobulin antibody ancestor. The distinct structural features of VNARs are the truncation of the sequences equivalent to the CDR2 loop present in conventional immunoglobulin variable domains and the lack of the hydrophobic VH/VL interface residues which would normally allow association with a light chain domain, which is not present in the IgNAR structure and the presence in some of the VNAR subtypes of additional Cysteine residues in the CDR regions that are observed to form additional disulphide bridges in addition to the canonical Immunoglobulin superfamily bridge between the Cysteines in the Framework 1 and 3 regions N terminally adjacent to CDRs 1 and 3.

To date, there are three defined types of shark IgNAR known as I, II and III (Figure 1). These have been categorized based on the position of non-canonical cysteine residues which are under strong selective pressure and are therefore rarely replaced.

All three types have the classical immunoglobulin canonical cysteines at positions 35 and 107 (numbering as in Kabat, E.A. *et al. Sequences of proteins of immunological interest. 5th ed*. 1991, Bethesda: US Dept. of Health and Human Services, PHS, NIH) that stabilize the standard immunoglobulin fold, together with an invariant tryptophan at position 36. There is no defined CDR2 as such, but regions of sequence variation that compare more closely to TCR HV2 and HV4 have been defined in framework 2 and 3 respectively. Type I has germline encoded cysteine residues in framework 2 and framework 4 and an even number of additional cysteines within CDR3. Crystal structure studies of a Type I IgNAR isolated against and in complex with lysozyme enabled the contribution of these cysteine residues to be determined. Both the framework 2 and 4 cysteines form disulphide bridges with those in CDR3 forming a tightly packed structure within which the CDR3 loop is held tightly down towards the HV2 region. To date Type I IgNARs have only been identified in nurse sharks - all other elasmobranchs, including members of the same order have only Type II or variations of this type.

Type II IgNAR are defined as having a cysteine residue in CDR1 and CDR3 which form intramolecular disulphide bonds that hold these two regions in close proximity, resulting in a protruding CDR3 (Figure 2) that is conducive to binding pockets or grooves. Type I sequences typically have longer CDR3s than type II with an average of 21 and 15 residues respectively. This is believed to be due to a strong selective pressure for two or more cysteine residues in Type I CDR3 to associate with their framework 2 and 4 counterparts. Studies into the accumulation of somatic mutations show that there are a greater number of mutations in CDR1 of type II than type I, whereas HV2 regions of Type I show greater sequence variation than Type II. This evidence correlates well with the determined positioning of these regions within the antigen binding sites.

A third IgNAR type known as Type III has been identified in neonates. This member of the IgNAR family lacks diversity within CDR3 due to the germline fusion of the D1 and D2 regions (which form CDR3) with the V-gene. Almost all known clones have a CDR3 length of 15 residues with little or no sequence diversity.

Another structural type of VNAR, termed type (IIIb or IV), has only two canonical cysteine residues. So far, this type has been found primarily in dogfish sharks (Liu, J.L., *et al. Mol. Immunol*. 2007. **44(7):** p. 1775–1783; Kovalenko O.V., *et al. J Biol Chem*. 2013**. 288(24):** p. 17408-19) and was also isolated from semisynthetic V-NAR libraries derived from wobbegong sharks (Streltsov, V.A. *et al*. (2004) *Proc. Natl. Acad. Sci. U.S.A*. **101**(34): p. 12444–12449).

It has been shown however specific VNARs isolated from synthetic libraries formed from the VNAR sequences can bind with high affinity to other proteins (Shao C.Y. *et al. Mol Immunol*. 2007. **44**(4): p. 656-65; WO2014/173959) and that the IgNAR is part of the adaptive immune system as cartilaginous fish can be immunized with antigen and responsive IgNARs obtained that bind to the antigen (Dooley, H., *et al*, *Mol. Immunol*, 2003. **40**(1): p. 25-33; WO2003/014161). It has been shown that the IgNAR has a mechanism for combinatorial joining of V like sequences with D and J sequences similar to that of immunoglobulins and the T cell receptor (summarized by Zielonka S. *et al mAbs* 2015. **7**(1): p. 15-25).

The VNAR binding surface, unlike the variable domains in other natural immunoglobulins, derives from four regions of diversity: CDR1, HV2, HV4 and CDR3 (see also Stanfield, R. L., *et al, Science*, 2004. **305**(5691): p. 1770-1773; Streltsov, V.A., *et al*, *Protein Sci.*, 2005. **14**(11): p. 2901-2909; Stanfield, R. L., *et al*., *J Mol. Biol.,* 2007. **367**(2): p. 358-372), joined by intervening framework sequences in the order: FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4. The combination of a lack of a natural light chain partner and lack of CDR2 make VNARs the smallest naturally occurring binding domains in the vertebrate kingdom.

The IgNAR shares some incidental features with the heavy chain only immunoglobulin (HCAb) found in camelidae (camels, dromedaries and llamas, Hamers-Casterman, C. *et al*. *Nature*, 1993. **363**, 446-448; Wesolowski, J., *et al*., *Med Microbiol Immunol*, 2009. 198(3): p. 157-74) Unlike the IgNAR the HCAb is clearly derived from the immunoglobulin family and shares significant sequence homology to standard immunogloblulins. Importantly one key distinction of VNARs is that the molecule has not had at any point in its evolution a partner light chain, unlike classical immunoglobulins or the HCAbs. Flajnik M.F. *et al PLoS Biol* 2011. **9**(8): e1001120 and Zielonka S. *et al mAbs* 2015. **7**(1): p. 15-25 have commented on the similarities and differences between, and the distinct evolutionary origins of, the VNAR and the immunoglobulin-derived V_{HH} single binding domain from the camelids.

The binding domains derived from light and heavy chains (VL and VH respectively) of classical immunoglobulins, have been shown to be able to be linked together to form bivalent or multivalent and bispecific binding entities whether in the scFv format (Bird et al., 1988; Huston et al., 1988), in which the immunoglobulin VL and VH domains are joined by a short peptide linker Traunecker *et al*. (Traunecker A, *et al. EMBO J*.1991. **10**, p. 3655-36, Traunecker A, *et al. Int J Cancer* Suppl.7, 51-52; Neri D. *J Mol Biol.* 1995. **246**(3): p. 367-73 or as diabodies (Holliger P. *et al*., *Proc. Natl. Acad. Sci. USA* 1993. **90**, 6444-6448; Holliger P. *et al*. *Nat. Biotechnol.* **15**, 632-636. See Mack M, et al *Proc. Natl. Acad. Sci. USA* 1995. **92**, p. 7021-7025, Jost CR, *et al Mol. Immunol*. 1996. **33**, p. 211-219 for other early examples). Tandabs comprise two pairs of VL and VH domains connected in a single polypeptide chain (Kipriyanov S.M. et al., *J. Mol. Biol*. **293**, 41-56 to form bispecific and bivalent for molecules).

Additionally V_{HH}s have been shown to be able to be linked together to form bivalent or multivalent and bispecific binding entities (Els Conrath *et al. J Biol Chem*. 2001. **276**(10) p.7346–7350). Similarly the variable domains from T cell receptors can be linked to immunoglobulin scFv to form bispecific formats (McCormack E. *et al. Cancer Immunol Immunother*. 2013. **62**(4): p. 773-85). Single antibody variable domains from classical immunoglobulins (dABs: Ward E.S. *et al. Nature* 1989, **341**, p. 544-546) can also be dimerized. The overall concept of bispecific binding molecules and current progress in their development has recently been reviewed by, for example, Kontermann R. *mAbs* 2012. **4**(2): 185-197; Jost C. and Pluckthun A. *Curr Opin Struct Biol.* 2014. **27:** p. 102-112; Spiess C. *et al. Mol Immunol* 2015. **67**(2): 95-106.

In addition to bispecific molecules that recognize epitopes on separate molecules, the concept of linking two antibody binding domains that recognize adjacent epitopes on the same protein (biparatopic) has a long history (see Neri D. *J Mol Biol.* 1995. 246(3): p. 367-73). Biparatopic V_{HH} molecules have been disclosed (for example, Jahnichen S. *et al Proc Natl Acad Sci U S A.* 2010. **107**(47): p. 20565-70; Roovers R.C. *et al Int J Cancer*. 2011 **129**(8): p. 2013-24).

However, it has been suggested that, unlike V_{HH}s, VNARs might not be able efficiently to form dimeric fusion molecules (Simmons D.P. *et al. Immunol Methods*. 2006 **315**(1-2): p. 171-84). (See also comments in *Bispecific Antibodies* Konterman R.E. Springer Publishing 2011; 6.6; also see comments in p322/323 of Strohl W.R. and Strohl L.M., *Therapeutic Antibody Engineering*, Woodhead Publishing 2012).

SUMMARY OF INVENTION

The present invention relates to the provision of multi-domain specific binding molecules comprising two or more VNAR domains. More particularly, the invention relates to the provision of bi- and multi-valent VNARs. The current inventors have recently shown that, contrary to the general understanding in the art, in fact dimeric, trimeric and bispecific fusions of VNARs can be formed.

Recently Muller M.R. *et al mAbs* 2012. 4(6): p. 673-685; WO2013/167883) disclosed a bispecific VNAR that comprises a VNAR in which one domain has specificity for human serum albumin (HSA), which allows the bivalent structure to bind in serum to HSA and so extend the biological half-life of the partner domain. Fusion of VNARs at both the N and C terminus of the HSA-binding VNAR was demonstrated with retention of function of the HSA binding domain. More recently, WO/2014/173975 discloses VNARS that can bind to ICOSL (CD275), a cell surface antigen expressed constitutively on antigen presenting cells (APCs) such as B cells, activated monocytes and dendritic cells and is the ligand for the B7 family member, ICOS (CD278) (Yoshinaga.S., K., *et al., Int. Immunol., 2000*. **12(10):** p. 1439-1447). Certain of these ICOSL VNARs can be linked to HSA-binding VNARS and it was shown that both domains retain functionality. Trimeric forms each recognizing different antigens (hICOSL, mICOSL and HSA) could be prepared and each domain shown to retain function.

However it has not been previously shown that bi- or multispecific VNARs could be formed that recognize the same or different epitopes on the same antigen. Additionally, and unexpectedly, bispecific molecules of this form show improved properties over bivalent molecules formed from the constituent monomers, or the monomer forms themselves, or the monomer joined to a VNAR recognizing HSA.

The present invention relates to specific VNAR domain sequences that have the capability of being combined into multivalent or multispecific entities and within which multidomain entity each domain retains binding function.

Therefore, in a first aspect of the present invention there is provided a multi-domain specific binding molecule comprising two or more VNAR domains which bind to the same or different epitopes of one or more specific antigens.

In certain preferred embodiments the VNARs in the multi-domain specific binding molecule of the first aspect of the invention bind the same antigen on a specific antigen.

In further preferred embodiments, the VNARs of multi-domain specific binding molecule bind different epitopes on a specific antigen. Multi-domain specific binding molecules in accordance with these embodiments may be termed bi-paratopic molecules, as further described herein.

In one embodiment specific VNAR binding domain sequences are combined into multivalent or multispecific entities and, within which multidomain entity each domain retains binding function, wherein the binding domains recognize distinct epitopes on a single antigen.

A preferred embodiment of the invention is a bi- or multi-specific binding molecule comprising two (or more) different VNAR domains wherein the binding specificity is for distinct epitopes on a single specific antigen and in which the resultant entity shows improved properties compared to the individual VNAR binding domains. An example of an improved property includes increased agonistic or antagonistic effect compared to the monomer VNARs.

Preferably the VNAR domains of the multi-domain specific binding molecule of the present invention are separated by a spacer sequence. More preferably, the spacer sequence has independent functionality which is exhibited in the binding molecule. In one embodiment, the spacer sequence is a VNAR domain or functional fragment thereof. In a specific example the spacer may be a VNAR or functional fragment thereof that binds serum albumin, including human serum albumin or ICOSL. In certain embodiments the spacer sequence comprises the amino acid sequence of any one of SEQ ID NO: 67, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88, or a functional fragment having at least 60% sequence identity thereto.In a further embodiment the spacer sequence may be the Fc portion of an immunoglobulin, including but not limited to a human immunoglobulin Fc region. The improved

properties may partially or completely derive from the properties of the spacer, for example by passively separating the VNAR domains in space or by the inherent properties of the spacer such as serum albumin binding which may lead to a longer *in vivo* half-life for the resultant entity, or by the recognition of a second therapeutic auto-immune target such as ICOSL or by introduction of a capacity for engagement with cells of the immune system or complement, in the case of immunoglobulin Fc regions.

Embodiments of the multi-domain specific binding molecule of the invention comprising two or more VNAR domains separated by a spacer sequence may be referred to herein as a Quad-X format.

In other preferred embodiments, the multi-domain specific binding molecule may further comprise one or more non-VNAR domains. The one or more non-VNAR domains may be placed in any position relative to the VNAR domains. Typically, and in preferred embodiments, the non-VNAR domain will be C-terminal or N-terminal to the VNAR domains.

Embodiments of the multi-domain specific binding molecule of the invention comprising two or more VNAR domains and a non-VNAR domain that is C-terminal or N-terminal to the VNAR domains may be referred to herein as a Quad-Y format.

Exemplary non-VNAR domains include, but are not limited to, TNF R1 and immunoglobulin Fc.

The specific antigen can be from a group comprising a cytokine, a growth factor, an enzyme, a cell surface associated molecule, a cell-surface membrane component, an intracellular molecule, an extracellular matrix component, a stromal antigen, a serum protein, a skeletal antigen, a microbial antigen or an antigen from a normally immune-privileged location.

A further aspect of the invention is the specific combination of VNAR binding domains that recognize cytokines

Also provided by the present invention are specific domains that recognize human TNF and bind to an epitope that is different from all other well characterized anti-TNF antibody and VHH binders that are currently used to treat disease.

Accordingly, in a second aspect the present invention provides a TNF-alpha specific VNAR binding domain comprising the following CDRs and hyper-variable regions (HV):

CDR1: HCATSS (SEQ ID NO. 68) or NCGLSS (SEQ ID NO. 69) or NCALSS (SEQ ID NO. 70)
HV2: TNEESISKG (SEQ ID NO. 71)
HV4: SGSKS (SEQ ID NO. 72) or EGSKS (SEQ ID NO. 73)

CDR3: ECQYGLAEYDV (SEQ ID NO. 1) or SWWTQNWRCSNSDV (SEQ ID NO. 6) or YIPCIDELVYMISGGTSGPIHDV (SEQ ID NO. 11)

or a functional variant thereof with a sequence identity of at least 60%.

In particularly preferred embodiments, the TNF-alpha specific VNAR binding domain comprising the amino acid sequence of SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

In preferred embodiments the TNF-alpha specific VNAR domain of the invention is modified at one or more amino acid sequence position to reduce the potential for immunogenicity *in vivo*, by for example humanization, deimmunization or similar technologies, while retaining functional binding activity for the specific epitopes on the specific antigen.

One embodiment of the invention is the specific combination of VNAR binding domains into a resultant multidomain binding molecule that recognize TNFα and which, in the forms outlined in this invention, provide improved functional properties over the individual binding domains. It is known that VNARs can be raised that are claimed to recognize TNFα (Camacho-Villegas T, *et al* MAbs. 2013. **5**(1): P. 80-85; Bojalil R, *et al* BMC Immunol. 2013. 14:17; WO2011/056056; US20110129473; US20140044716). These VNARs have not however been linked to form dimeric or bispecific forms. In addition these domains in a monomeric format are 70 to 200 times less potent than the monomeric anti-TNF VNAR domains described here.

Accordingly, the TNF-alpha specific VNAR binding domain of the second aspect of the invention may be used as one or both VNAR domains in the multi-domain specific binding molecule of the first aspect. Therefore, in a preferred embodiment there is provided a multi-domain specific binding molecule of the first aspect, wherein one or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%. In other preferred embodiments, there is provided a multi-domain specific binding molecule of the first aspect, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

Other preferred embodiments of the first aspect of the invention include the multi-domain specific binding molecule of the first aspect comprising one or more of the VNAR domains having an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%. Yet further embodiments of the first aspect include the multi-domain specific binding molecule of the first aspect comprising two or more of the VNAR domains having an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%.

The VNAR domain or domains used in the first aspect of the invention may be modified at one or more amino acid sequence position to reduce the potential for immunogenicity *in vivo*, by for example humanization, deimmunization or similar technologies, while retaining functional binding activity for the specific epitopes on the specific antigen.

The present invention also provides an isolated nucleic acid comprising a polynucleotide sequence that encodes a binding molecule according to any aspect or embodiment described herein. Furthermore, there is provided herein a method for preparing a binding molecule according to the invention, comprising cultivating or maintaining a host cell comprising the polynucleotide under conditions such that said host cell produces the binding molecule, optionally further comprising isolating the binding molecule.

According to a further aspect of the invention, there is provided a pharmaceutical composition of a specific antigen binding molecule and/or the multi-domain specific binding molecule of the previous aspects of the invention.

Pharmaceutical compositions of the invention may comprise any suitable and pharmaceutically acceptable carrier, diluent, adjuvant or buffer solution. The composition may comprise a further pharmaceutically active agent. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, liposomes, water, glycerol, ethanol and combinations thereof.
Such compositions may comprise a further pharmaceutically active agent as indicated. The additional agents may be therapeutic compounds, e.g. anti-inflammatory drugs, cytotoxic agents, cytostatic agents or antibiotics. Such additional agents may be present in a form suitable for administration to patient in need thereof and such administration may be simultaneous, separate or sequential. The components may be prepared in the form of a kit which may comprise instructions as appropriate.

The pharmaceutical compositions may be administered in any effective, convenient manner effective for treating a patient's disease including, for instance, administration by oral, topical, intravenous, intramuscular, intranasal, or intradermal routes among others. In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

For administration to mammals, and particularly humans, it is expected that the daily dosage of the active agent will be from 0.01 mg/kg body weight, typically around 1 mg/kg, 2mg/kg, 10mg/kg or up to 100 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual which will be dependent on factors including the age, weight, sex and response of the individual. The above dosages are exemplary of the average case. There can, of course, be instances where higher or lower dosages are merited, and such are within the scope of this invention. The

present invention also provides a kit comprising a pharmaceutical composition as defined herein with instructions for use.

According to a further aspect of the invention, there is provided a pharmaceutical composition of the previous aspect for use in medicine. Such uses include methods for the treatment of a disease associated with the interaction between the target antigen of the binding domain of the invention and its ligand partner(s) through administration of a therapeutically effective dose of a pharmaceutical composition of the invention as defined above. The composition may comprise at least one specific antigen binding molecule (VNAR domain) or multi-domain specific binding molecule of the invention, or a combination of such molecules and/or a humanized variant thereof.

In accordance with this aspect of the invention, there is provided a composition for use in the manufacture of a medicament for the treatment of a disease associated with the interaction between target antigen of the binding domain of the invention and its ligand partner(s).

Such compositions may comprise a further pharmaceutically active agent as indicated. The additional agents may be therapeutic compounds, e.g. anti-inflammatory drugs, cytotoxic agents, cytostatic agents or antibiotics. Such additional agents may be present in a form suitable for administration to patient in need thereof and such administration may be simultaneous, separate or sequential. The components may be prepared in the form of a kit which may comprise instructions as appropriate.

According to the invention, there is provided an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention for use in medicine. This aspect of the invention therefore extends to the use of such of an antigen specific antigen binding molecule or multi-domain binding molecule of the invention in the manufacture of a medicament for the treatment of a disease in a patient in need thereof. An antigen specific antigen binding molecule of the invention can also be used to prepare a fusion protein comprising such a specific binding molecule or multi-domain binding molecule as defined above in relation to pharmaceutical compositions of the invention. Such uses also embrace methods of treatment of diseases in patients in need of treatment comprising administration to the patient of a therapeutically effective dosage of a pharmaceutical composition as defined herein comprising an antigen specific antigen binding molecule or multi-domain binding molecule of the invention.

As used herein, the term "treatment" includes any regime that can benefit a human or a non-human animal. The treatment of "non-human animals" in veterinary medicine extends to the treatment of domestic animals, including horses and companion animals (e.g. cats and dogs) and farm/agricultural animals including members of the ovine, caprine, porcine, bovine and equine families. The treatment may be a therapeutic treatment in respect of any existing condition or disorder, or may be prophylactic (preventive treatment). The treatment may be of an inherited or an acquired disease. The treatment may be of an acute or chronic condition. The treatment may be of a condition/disorder associated with

inflammation and/or cancer. The antigen specific antigen binding molecules or multi-domain specific binding molecules of the invention may be used in the treatment of a disorder, including, but not limited to osteoarthritis, scleroderma, renal disease, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, atherosclerosis, or any inflammatory disease.

In a further aspect the present invention provides a method for treating a condition mediated by TNFα, the method comprising the administration of a therapeutically effective amount of a composition of the invention that specifically binds to TNFα.

In yet a further aspect the present invention provides a method for treating at least one condition mediated by ICOSL, comprising the administration of an effective amount of a composition of the invention that specifically binds to ICOSL.

A further aspect of the invention is the specific combination of VNAR binding domains that recognize cell-surface molecules. In certain embodiments, the VNARs of the multi-domain binding molecule bind different classes of ligand or target. One non-limiting example contemplated herein is a multi-domain specific binding molecule of the first aspect of the invention in which at least one VNAR domain binds a target associated with auto-immune disease and at least one VNAR domain binds to a target associated with the inflammatory response.

A particularly preferred multi-domain specific antigen binding molecule of the invention includes a TNF-specific VNAR and an ICOSL specific VNAR. Preferably, the TNF-specific VNAR is the VNAR of the second aspect of the invention.

One embodiment of the invention is the specific combination of VNAR binding domains into a resultant multidomain binding molecule that recognize ICOSL and which, in the forms outlined in this invention, provide improved functional properties over the individual binding domains.

In the present application reference is made to a number of drawings in which:

Figure 1 Anti-hTNF-alpha IgNAR titration of immunized nurse shark plasma using anti-Nurse shark IgNAR hybridoma antibody
ELISA titration of serum from immunized animals, preimmunization and after bleed 5
Binding ELISA measurement of anti-rhTNF-α IgNAR titer in immunized nurse shark. Detection was carried out with GA8 monoclonal anti-nurse IgNAR antibody, and anti-mouse IgG-HRP conjugated antibody was used as secondary antibody.

Figure 2 Neutralisation of hTNF-alpha induced cytotoxicity in L929 cells
In this assay, the ability of the anti-TNF domains (D1 and C4) and control anti-human serum albumin domain (BA11) to neutralize the activity of hTNF-α in a cell bio-assay was determined. Both the D1 and C4 domains demonstrated a similar level of concentration

dependent neutralization (for calculated values see Table 2). The BA11 control does not recognize hTNF and so no neutralization was observed even at the highest concentrations. The hTNF-α + Actinomycin-D acted as control demonstrating classical cytotoxicity in the absence of a neutralizing domain.

Figure 3 *In vitro* rhTNFα neutralization assay in L929 fibrosarcoma cell line.
A. Neutralisation of 1x LD80 [0.3 ng/ml rhTNFα], n = 3 with duplicates per experiment, ± SEM;
B. Neutralisation of 10x LD80 [3 ng/ml rhTNFα], n = 2 with duplicates per experiment, ± SD.

TNF30-Fc is a fusion of an anti-rhTNFα V_{HH} nanobody isolated from immunized camelid fused to IgG Fc (Coppieters *et al.*, Arthritis and Rheumatism, 2006, **54** (6): 1856-1866; Riechmann *et al.*, J. Immunol. Methods, 1999. **231**: 25-38)
Alb8-Fc is a V_{HH} domain which recognizes HSA, fused to IgG Fc.
2V is a negative control VNAR which recognizes no known target. 2V-Fc is a fusion of 2V to IgG Fc.

Only those binders that were specific for hTNF (D1, C4 and TNF-30) were able to demonstrate neutralization of the activity of the free hTNF and in a concentration dependent manner. The neutralization potency was enhanced by conversion from a monomer to a bivalent Fc format. The combination of D1 -Fc and C4-Fc together delivered a neutralization potency that was better than D1-Fc alone or C4 –Fc alone. (see Table 2 for all calculated neutralization values). Both the controls, Alb-8 and 2V, were unable to neutralize even in this bivalent Fc format.

Figure 4 Diagram of format of bivalent and bispecific constructs

Figure 5 ELISA binding of dimeric VNARs together with a TNF-30 VHH control (TNF30-TNF30).
All the tested VNAR domains D1, C4, B4 were either paired with themselves (eg D1 –D1, C4-C4 etc) or paired with each other (eg D1 – C4, D1 - B4) in both possible orientations (eg C4 – D1 , B4 – D1). The ELISA ranking placed the D1 – D1 dimer pair as the best (lowest concentration of VNAR required to reach a saturating signal) and B4- C4 and B4 – B4 as the worst performing in this ELSA format. A number of the VNAR pairings were better than the VHH dimer control.

Figure 6 L929 assay to measure TNF neutralization by VNAR dimer pairs
The neutralizing ability of the anti-TNF α VNAR dimer pairs D1 – D1, D1 – C4, D1 – B4 and the positive control anti-TNF VHH dimer (TNF30 – TNF30) were assessed using an

appropriate bioassay. The domain pairing showing the most potent neutralizing activity in this assay format was the VNAR pair D1 – C4 (for calculated values of neutralisation see Table 2). hTNFα + actinomycin-D treated cells without any neutralizing domain provided an appropriate classical uninhibited cytotoxicity control.

Figure 7 Neutralisation of hTNFα induced cytotoxicity in L929 cells using trimeric anti-hTNF-α VNARs

The lead anti-hTNFα VNAR dimers (D1-D1 and D1-C4) were reformatting into multivalent trimeric constructs by incorporating an anti-HSA humanised VNAR (soloMER™ BA11) in the middle of both dimeric constructs to achieve D1-BA11-D1 and D1-BA11-C4 respectively. The ability of these multivalent trimeric constructs and humira (Adalimumab) and TNF30-BA11-TNF30 to neutralize hTNFα was assessed in a classical L929 assay. The D1-BA11-C4 construct demonstrated comparable neutralizing potency as Adalimumab, and significantly improved potency than the VHH trimeric construct, and also the anti-hTNFα dimeric (D1-C4 and D1-D1) VNARs (see Table 2 for calculated ND_{50} values).

Figure 8 Caco2 Epithelial Permeability in Polarized Caco2 cells

Caco-2 cells were treated and incubated for 18 h with 10 ng/mL TNFα, LPS and IFNγ +/- anti-TNFα protein. 5 µl of 10 mg/ml FITC-Dextran [3000-5000 kDa] was added to the apical chamber and transport across the membrane to the basolateral chamber was measured 24 h later.

Treatment with VNAR/VHH monomers and VNAR control proteins was at 50 nM concentration, while Treatment with VNAR dimers (2C and 2D) and Adalimumab was at 25 nM.

BA11 and 2V are non-TNFα binding VNAR control, while B4 is a non-neutralising TNF-binding VNAR

The ability of the anti-hTNFα VNAR constructs (monomers D1, C4, B4; dimers D1-D1, D1-C4; trimers, D1-BA11-D1, D1-BA11-C4), VHH constructs TNF30, TNF30-TNF30, TNF30-BA11-TNF30, and Adalimumab to prevent intestinal barrier dysfunction in cytokine treated Caco-2 cells was assessed using this classical assay. The VNAR domains D1-C4 and D1-BA11-C4 demonstrated comparable efficacy to Adalimumab. Negative controls BA11 and 2V were unable to prevent intestinal barrier dysfunction.

Figure 9 Epithelial Resistance in Polarized Caco-2 cells

Differentiated Caco-2 cells were treated and incubated for 24 h with 10 ng/mL TNFα and IFNγ +/- anti-TNFα. Effect of cytokine treatment on Trans-epithelial resistance was determined using a volt-ohm meter. Resistance was normalised to the surface area under treatment ($ohm.cm^2$).

Treatment with VNAR/VHH monomers and VNAR control proteins was at 50 nM concentration, while Treatment with VNAR dimers (2C and 2D) and Adalimumab was at 25 nM.
BA11 and 2V are non-TNFα binding VNAR control, while B4 is a non-neutralising TNF-binding VNAR
[n=1 ± SD with ≥ 8 replicates per treatment, one-way ANOVA and Dunnett's post-hoc test using GraphPad Prism 5)

The efficacy of the anti-hTNFα VNAR domains to restore epithelial resistance in cytokine treated Caco-2 cells were investigated in comparison with the efficacy of the VHH TNF30 and the clinically available Adalimumab at equimolar dosing range. The anti-hTNFα dimeric and trimeric VNAR domains demonstrated significant capacity in restoring epithelial resistance in a comparable fashion to the effect observed with Adalimumab. The negative controls BA11 and 2V did not restore epithelial resistance.

Figure 10 Format of ICOSL VNAR-Fc fusions

Figure 11 ICOSL ELISA binding data
Binding ELISA of the different anti-ICOSL Quad-X™ constructs to both human and mouse ICOS ligands.

Figure 12 Formats for multivalent and multispecific VNARs of the invention incorporating the TNF R1 domain, ICOSL VNARs and human IgG Fc

Figure 13 Efficacy data for multivalent and multispecific VNARs incorporating the TNF R1 domain, ICOSL. VNARs and human IgG Fc, which provides additional improved functional characteristics.
VNAR-TNFR1 Fc bi-functional constructs demonstrate specific and potent efficacy in cell based neutralisation assays
Format 1: anti-TNFα scFv
Format 2: anti-mICOSL VNAR (CC3)
Format 3: anti-hICOSL scFv
Format 4: anti-hICOSL VNAR (2D4)

Figure 14 hTNF-alpha Binding profile between VNAR T43 horn shark clone and VNAR Nurse shark D1 and C4.
Binding ELISA of the VNAR T43 from the Horn shark and VNAR D1 and C4 to 1 µg/ml hTNFα coated wells. The binding profile of T43 clone could not be determined at experimental concentration used.

Figure 15 hTNF-alpha Neutralisation efficacy in L929 cells of VNAR T43 horn shark clone and VNAR Nurse shark D1 and C4
Comparison of the neutralising efficacy of anti-hTNFα VNAR monomers D1 and C4 compared to the Horn Shark T43 VNAR at equimolar dosing range. The T43 domain did not demonstrate any dose-dependent neutralising effect, and has similar profile as the unprotected cells treated with hTNFα and Actinomycin-D (See table 2)

Figure 16 Binding profile of a successfully humanised anti-hTNF-alpha D1 (also known as D1 soloMER™)
Binding profile of a number of progressively improved framework humanised versions of VNAR D1 domain. D1-v1, D1-v2, D1-v3 and D1-v4 represents varying extents of humanisation, while VNAR D1 (wt) is the parental VNAR D1 domain. Substituting nurse shark framework amino acid residues with those of DPK-9, human germline kappa did not disrupt the ability of the humanised D1 versions to recognise hTNFα.

Figure 17 Neutralisation efficacy in L929 cells of a D1 soloMER™
The capacity to neutralize hTNFα mediated cytotoxicity in L929 cells was assessed in a humanised VNAR D1 variant. The soloMER D1-v2 retained neutralizing potency for hTNFα induced cytotoxicity.

Figure 18 Formats for multivalent and multispecific VNARs of the invention incorporating the human IgG Fc

Figure 19 hTNF-alpha Binding profile of multivalent/multispecific VNAR-Fc constructs
Demonstrating the binding profile of biparatopic/bispecific D1-Fc-C4 (Quad-X™) vs biparatopic VNAR Fc constructs D1-Fc and C4-Fc. The anti-hTNF-α VNAR Quad-X™ D1-Fc-C4 retained binding to hTNFα, with binding profile comparable and slightly improved comparable to D1-Fc and C4-Fc.

Figure 20 Assessing the hTNF-alpha Neutralising activity of the multivalent/multispecific VNAR-Fc constructs in L929
Assessing Neutralising potency of VNAR Quad-X™ D1-Fc-C4 vs Humira (Adalimumab) in an L929 cell based assay of hTNFα mediated cytotoxicity. VNAR Quad-X™ D1-Fc-C4 retained neutralising capacity and demonstrated a superior neutralising activity compared to Humira (see Table 2 for ND_{50} values).

Figure 21 Formats for multivalent, bi-paratopic VNARs of the invention incorporating anti-mouse TNF-alpha VNAR; and anti-HSA soloMER™ BA11 or ICOSL VNAR domain, A5 or mouse IgG2a Fc

Figure 22A Mouse TNF-alpha binding profile of the bi-paratopic anti-mouse TNF-alpha VNAR constructs.
Reformatting a VNAR anti-mouse TNFα S17 domain as multivalent/multispecific trimer incorporating either an anti-ICOS ligand VNAR A5 or anti-HSA humanised VNAR, soloMER™ BA11. Both constructs retained recognition to mouse TNF-alpha.

Figure 22B HSA binding profile of the bi-paratopic anti-mouseTNF-alpha VNAR constructs.
Reformatting a VNAR anti-mouse TNFα S17 domain as multivalent/multispecific trimer incorporating either an anti-ICOS ligand VNAR A5 or anti-HSA humanised VNAR, soloMER™ BA11. S17-BA11-S17 retained binding to HSA, and the negative control, S17-A5-S17 did not recognise HSA.

Figure 22C Mouse ICOS Ligand binding profile of the bi-paratopic dimeric/trimeric anti-ICOSL VNAR construct.
Binding profile of the reformatted S17-A5-S17 and A5-A5 homodimer to mouse ICOS ligand demonstrated that the reformatted trimeric construct incorporating an anti-mouse ICOS ligand VNAR A5 in the middle as S17-A5-S17 retained binding to mouse ICOS Ligand.

Figure 23 A & B Mouse TNF-alpha Neutralisation in L929 profile of the bi-paratopic and IgG2a Fc fusion anti-mouse TNF-alpha VNAR S17 constructs respectively.
The Neutralising efficacy of the anti-mouse TNFα constructs (S17-A5-S17, S17-BA11-S17, S17-Fc) were assessed in a mouse TNFα mediated cytotoxicity L929 assay. Both trimeric S17 and the bi-paratopic S17-Fc constructs demonstrated neutralising activities against mouse TNFα mediated cytotoxicity in L929 cells. The S17-A5-S17 demonstrated the highest potency amongst the three constructs. BA11 was a negative control in the assay, also hTNFα + Actinomycin D represented a classical cytotoxicity effect observed in the absence of an anti-mouse TNFα inhibitor/neutraliser. Cells alone indicate healthy untreated cells.

Figure 24 CHO-based huICOS/recombinant mouse ICOS Ligand-Fc (ICOSL-Fc) Neutralisation (blocking) Assay-ELISA based.
In this blocking assay, the multivalent VNAR constructs demonstrated significant capacity to block the mouse ICOSL-Fc from interacting with its cognate binding partner, ICOS on CHO cells. This leads to reduced/compromised detection of the Fc portion of the mouse ICOSL-Fc using an anti-human Fc-HRP antibody in a cell based ELISA format. A5-A5 dimer is the most potent blocker, followed by the S17-A5-S17, while S17 monomer is a negative control in this assay.

Figure 25 Binding Cross-reactivity differences between the anti-hTNF-alpha VNAR vs VHH TNF30 and Humira®.

This figure illustrates the binding crossreactivity profile of the VNAR D1-C4 compared to the VHH TNF30 and Humira. The VNAR D1-C4 binds to only human, Dog and Cynomolgus TNFα; the VHH TNF30 including binding to human, dog and cynomolgus TNFα, binds weakly to pig TNFα and also human TNFβ. Humira binds to human, dog, cynomolgus and mouse TNFα. Also see tables table 3A and 3B for detailed binding and neutralisation profiles of these anti-TNFα domains.

Figure 26 BIAcore™ T200 epitope binning analysis of anti-hTNF-alpha VNAR heterodimer vs VHH TNF30 dimer.
This epitope binning data demonstrates that VNAR D1-C4 recognizes and interacts with distinct epitope on the hTNFα molecule from those recognised by VHH TNF30 domain. This assay involves reaching available epitope saturation with the first binding domain (in this instance, VNAR D1-C4 using saturating concentration determined as 100 times its KD value), and then followed with the second binding domain (TNF30).

Figure 27 Functional binding to hTNF-alpha by Quad-X™ and Quad-Y™ constructs in an ELISA format.

Figure 28 Assessing the hTNF-alpha Neutralising activity of the multivalent/multispecific VNAR-Fc constructs in L929
Assessing Neutralising potency of VNAR Quad-X™ D1-Fc-C4, Quad-Y™ D1-C4-Fc and C4-D1-Fc vs Humira (Adalimumab) in an L929 cell based assay of hTNFα mediated cytotoxicity. VNAR Quad-Y™ constructs retained neutralising capacity, with D1-C4-Fc construct demonstrating comparable neutralising activity as Quad-X™ in the presence of either 0.3 ng/ml or 3 ng/ml hTNF-alpha (see Table 2 for ND_{50} values).

Figure 29 The effect of D1-Fc-C4 (Quad-X™) and Humira® on the body weight gain of experimental Tg197 mice. By the end of the study (10 weeks of age), the mean body weights of all groups treated twice weekly from week 3 were as follows: G1- Vehicle= 19.3±1.4 g, G4- Humira® 10 mg/kg= 24.4±1.5 g, G2- D1-Fc-C4 3 mg/kg= 24.1±1.5 g, G5- D1-Fc-C4 10 mg/kg= 24.1±1.7 g and G3- D1-Fc-D4 30 mg/kg= 23.4±1.4 g. Control mice at week 3 had a mean body weight of 9.8±0.2 g. Error bars indicate standard error of the mean

Figure 30 The effect of D1-Fc-C4 (Quad-X™) and Humira® on in vivo arthritis scores of experimental Tg197 mice. By the end of the study (10 weeks of age), the mean in vivo disease severity scores of all groups treated twice weekly from week 3, were as follows: G1- Vehicle= 1.36±0.07, G4- Humira® 10 mg/kg= 0.25±0.05, G2- D1-Fc-C4 3 mg/kg= 0.17±0.04, G5- D1-Fc-C4 10 mg/kg= 0.17±0.04 and G3- D1-Fc-D4 30 mg/kg= 0.17±0.04. Control mice at week 3 had an in vivo arthritic score = 0.13±0.05. Error bars indicate standard error of the mean.

Figure 31 The effect of D1-Fc-C4 (Quad-X™) and Humira® on arthritis histopathology scores of experimental Tg197 mice. By the end of the study (10 weeks of age), the mean arthritis histopathology scores of all groups treated twice weekly from week 3, were as follows: G1- Vehicle= 2.94±0.12, G4- Humira® 10 mg/kg= 0.42±0.07, G2- D1-Fc-C4 3 mg/kg= 0.41±0.03, G5- D1-Fc-C4 10 mg/kg= 0.50±0.05 and G3- D1-Fc-D4 30 mg/kg= 0.42±0.07. Control mice at week 3 had a histopathology score= 1.22±0.10. Error bars indicate standard error of the mean.

Figure 32 Comparison of the effect of D1-Fc-C4 (Quad-X™) and Humira® on the in vivo arthritis scores versus the ankle histopathological scores of experimental Tg197 mice. By the end of study (10 weeks of age), the mean disease severity scores of all groups treated twice weekly from week 3, were as follows: G1- Vehicle= 2.94±0.12 (HS) and 1.36±0.07 (AS), G4- Humira® 10 mg/kg= 0.42±0.07 (HS) and 0.25±0.05 (AS), G2- D1-Fc-C4 3 mg/kg= 0.41±0.03 (HS) and 0.17±0.04 (AS), G5- D1-Fc-C4 10 mg/kg= 0.50±0.05 (HS) and 0.17±0.04 (AS) and G3- D1-Fc-D4 30 mg/kg= 0.42±0.07 (HS) and 0.17±0.04 (AS). Error bars indicate standard error of the mean.

Figure 33 Efficacy evaluation of D1-Fc-C4 (Quad-X™) at 0.5, 1 and 3 mg/kg and D1-BA11-C4 at 30 mg/kg vs Humira® at 1 mg/kg and 3 mg/kg in ameliorating arthritis pathology in the Tg197 model of arthritis.

Figure 34 The effect of D1-Fc-C4 (Quad-X™) at 0.5, 1 and 3 mg/kg and D1-BA11-C4 at 30 mg/kg vs Humira® at 1 mg/kg and 3 mg/kg on the mean group weight of Tg197 model of arthritis.

Figure 35 The effect of different Humira® dosing regimen on in vivo arthritic and histology scores. This was performed as a separate experiment but using identical methods to those described for Figures 29-32.

Figure 36 Twelve rats were immunized with Interphotoreceptor Retinal Binding Protein (IRBP) to induce Experimental Auto-Immune Uveitis (EAU). Four animals each were treated via intraperitoneal injections with a (rodent protein specific) anti TNFα VNAR-Fc at 20 mg/kg on day 8, day 10 and day 12; four animals were treated with dexamethasone intraperitoneal on same days and four animals were treated with vehicle identically. The Optical Coherence Tomography (OCT) of both the anterior and posterior segment of the rats' eyes was performed on days 0, 7, 10, 12, 13, and 14. To minimize any scientific bias of the outcomes, OCT images were scored by an "experimentally blinded observer" for total inflammation using a validated scoring system. The experiment also included a vehicle control and a positive control using a standard dose of Dexamethazone steroid.

Figure 37A & B Assessing the hTNF-alpha Neutralizing activity of soloMER VNAR dimer constructs in L929 cells

Figure 38: Assessing the hTNF-alpha Neutralizing activity of S17-Fc vs S17-Fc-S17 (Quad-X™) constructs in L929 cells. The Fc used in the S17 constructs is derived from mouse IgG2a.

Figure 39A, B & C: Cross-reactivity binding profile of S17-Quad-X™ and D1-C4 Quad-X™ against human and mouse TNF-alpha

Various nucleotide and amino acid sequences are provided herein as follows:

SEQ ID NO 1
TNF VNAR D1 CDR3 AMINO ACID SEQUENCE ECQYGLAEYDV

SEQ ID NO 2
TNF VNAR D1 AMINO ACID SEQUENCE (CDR1 and CDR3 single underlined)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN

SEQ ID NO 3
TNF VNAR D1 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVNAAAHHHHHHGAAESKLISEEDL

SEQ ID NO 4
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR D1
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAAT

SEQ ID NO 5
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR D1 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGCGGCC
GCACATCATCATCACCATCACGGCGCCGCAGAATCAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 6
TNF VNAR C4 CDR3 AMINO ACID SEQUENCE SWWTQNWRCSNSDV

SEQ ID NO 7
TNF VNAR C4 AMINO ACID SEQUENCE (CDR1 and CDR3 underlined)
RVDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRI
NDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVN

SEQ ID NO 8
TNF VNAR C4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined)
ARVDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSL
RINDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVNAAAHHHHHHGAAESKLISEEDL

SEQ ID NO 9
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR C4

GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTG
GTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTG
AAT

SEQ ID NO 10
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR D1 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTG
GTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTG
AATGCGGCCGCACATCATCATCACCATCACGGCGCCGCAGAATCAAAACTCATCTCAGAAGAGG
ATCTG

SEQ ID NO 11
TNF VNAR B4 CDR3 AMINO ACID SEQUENCE YIPCIDELVYMISGGTSGPIHDV

SEQ ID NO 12
TNF VNAR B4 AMINO ACID SEQUENCE (CDR1 and CDR3 single underlined)
ARVDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVN

SEQ ID NO 13
TNF VNAR B4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined)
ARVDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVNAAAHHHHHHGAAESKLISE
EDL

SEQ ID NO 14
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR B4
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATAT
ACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTAT
ACGGAGGTGGCACTGTCGTGACTGTGAAT

SEQ ID NO 15
GCTAGGCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCTCGAGTGGACCAA
ACACC

SEQ ID NO 16
CGCGCCGGATCCGCCACCTCCGCTACCGCCACCTCCGCTACCGCCACCTCCGCTACCGCCACC
TCCATTCACAGTCACGACAGTGCC

SEQ ID NO 17
GGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCAAACACCGC

SEQ ID NO 18
GTCCGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCTGCGGCCCC

SEQ ID NO 19
AATTCCCCTCTAGAAGGCGCGCACTCCGCTCGAGTGGACCAAACACCG

SEQ ID NO 20
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR B4 WITH HIS AND MYC TAGS

GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATAT
ACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTAT
ACGGAGGTGGCACTGTCGTGACTGTGAATGCGGCCGCACATCATCATCACCATCACGGCGCCGC
AGAATCAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 21
TNF VNAR DIMER D1-D1 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGGSGAHS*ARVDQTPQTITKE
TGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVEDSGTY
RCASECQYGLAEYDVYGGGTVVTVNAAAHHHHHHGAAESKLISEEDL

SEQ ID NO 22
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR DIMER D1-D1 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGTAGCGGAGGTGGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCAAACACCGCA
AACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGCCACTGTG
CAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAGCATATCGAAA
GGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTA
ACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTGCCAATATGGACTGGCAGAATATG
ATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGCGGCCGCACATCATCATCACCATCACGG
GGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 23
TNF VNAR DIMER C4-C4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSL
RINDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVN*GGGGSGGGGGSGAHS*ARVDQTPQ
TITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVED
SGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEEDL

SEQ ID NO 24
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR DIMER C4-C4 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTG
GTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTG
AACGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCA
AACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATA
GCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAG
CATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTCCTTTTCTTTGAGAAT
TAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTGGTGGACCCAGAACT
GGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTGAACGCGGCCGCACA
TCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 25
TNF VNAR DIMER B4-B4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVN*GGGGSGGGGGSGAHS*AR
VDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRI

NDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEE
DL

SEQ ID NO 26
NUCLEOTIDE SEQUENCE CODING FOR TNF VNAR DIMER B4-B4 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATAT
ACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTAT
ACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCG
GCGCGCACTCCGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACT
GACCATCAACTGTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAA
AATCTGGCTCAACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGC
GGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGC
AAGGTATATATACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGAT
TCATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGCGGCCGCACATCATCATCACCATC
ACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 27
TNF VNAR DIMER D1-C4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGGSGAHS*ARVDQTPQTITKE
TGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVEDSGTYR
CKLSWWTQNWRCSNSDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEEDL

SEQ ID NO 28
NUCLEOTIDE SEQUENCE CODING FOR THE TNF VNAR DIMER D1-C4 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGTAGCGGAGGTGGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCAAACACCGCA
AACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGCAACTGTG
GGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAGCATATCGAA
AGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCT
AACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTGGTGGACCCAGAACTGGAGATGC
TCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTGAACGCGGCCGCACATCATCATCA
CCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 29
TNF VNAR DIMER D1-B4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (double underlining) (CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLPINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGGSGAHS*ARVDQTPQTITKE
TGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVEDSGTY
RCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEEDL.

SEQ ID NO 30
NUCLEOTIDE SEQUENCE CODIGN FOR THE TNF VNAR DIMER D1-B4 NUCLEOTIDE SEQUENCE WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGTAGCGGAGGTGGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCAAACACCGCA

AACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGTAACTGTG
CATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCAACAAACGAGGAGAGCATATCGAAA
GGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTA
ACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATATACCTTGCATCGATGAACTGGTATA
TATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTATACGGAGGTGGCACTGTCGTGACT
GTGAATGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAG
AGGATCTG

SEQ ID NO 31
TNF VNAR DIMER B4-D1 AMINO ACID SEQUENCE (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVN*GGGGSGGGGGSGAHS*AR
VDQTPQTITKETGESLPINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRI
NDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEEDL

SEQ ID NO 32
TNF VNAR DIMER B4-D1 NUCLEOTIDE SEQUENCE
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATAT
ACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTAT
ACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCG
GCGCGCACTCCGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACT
GACCATCAACTGTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAA
AATCGGGCTCAACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGC
GGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGC
GCTTCCGAGTGCCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTG
TGAATGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATCTC
AGAAGAGGATCTG

SEQ ID NO 33
TNF VNAR DIMER C4-B4 AMINO ACID SEQUENCE (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSL
RINDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVN*GGGGSGGGGGSGAHS*ARVDQTPQ
TITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVED
SGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEED

SEQ ID NO 34
TNF VNAR DIMER C4-B4 NUCLEOTIDE SEQUENCE
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTG
GTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTG
AACGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCGGCGCGCACTCCGCTCGAGTGGACCA
AACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATA
GTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCAACAAACGAGGAGAGC
ATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTCCTTTTCTTTGAGAATT
AATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATATACCTTGCATCGATGAA
CTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTATACGGAGGTGGCACTGT
CGTGACTGTGAATGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATC
TCAGAAGAGGATCTG

SEQ ID NO 35
TNF VNAR DIMER B4-C4 AMINO ACID SEQUENCE (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)

ARVDQTPQTITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVN*GGGGSGGGGGSGAHS*AR
VDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRIN
DLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVNAAAHHHHHHGAAEQKLISEED

SEQ ID NO 36
TNF VNAR DIMER B4-C4 NUCLEOTIDE SEQUENCE
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATAT
ACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTAT
ACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCG
GCGCGCACTCCGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACT
GACCATCAACTGTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAA
AATCGGGCTCAACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAA
GGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGC
AAGTTAAGCTGGTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTG
TCGTGACTGTGAACGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAA
ACTCATCTCAGAAGAGGATCTG

SEQ ID NO 37
TNF VNAR D1-BA11-C4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGSGGGGSGGGGSGAHS*TR
VDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTIS
SLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK*GGGGSGGGGSGGGGSGGGGSGAHS*ARVDQTPQ
TITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVED
SGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVNHHHHHHHEQKLISEEDL

SEQ ID NO 38
NUCLEOTIDE SEQUENCE CODING FOR THE TNF VNAR D1-BA11-C4 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGATCCGGGGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGTGGAGCTCA
TTCAACAAGAGTGGACCAAAGTCCAAGCTCTCTGTCCGCCAGTGTGGGCGACCGCGTGACCATC
ACCTGCGTCCTGACTGATACCAGCTATCCTCTGTACAGCACATACTGGTATCGGAAGAATCCCGG
TTCCAGCAACAAGGAGCAGATTTCCATCTCCGGCCGCTATAGTGAATCAGTCAACAAGGGCACTA
AGTCCTTTACCCTGACAATCAGTTCCCTGCAGCCCGAGGACTCCGCCACCTATTACTGCAGAGCT
ATGAGTACAAATATCTGGACCGGGGACGGAGCTGGTACCAAGGTGGAGATCAAGGGAGGTGGC
GGTTCCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGGGCCCATTC
TGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAAC
TGTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTC
AACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGT
CCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCT
GGTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGT
GAATCATCACCATCACCATCACCATGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 39
TNF VNAR D1-BA11-D1 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGSGGGGSGGGGSGAHS*TR
VDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTIS
SLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK*GGGGSGGGGSGGGGSGGGGSGAHS*ARVDQTPQ

TITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVED
SGTYRCASECQYGLAEYDVYGGGTVVTVNHHHHHHHHEQKLISEEDL

SEQ ID NO 40
NUCLEOTIDE SEQUENCE CODING FOR THE TNF VNAR D1-BA11-D1 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGATCCGGGGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGTGGAGCTCA
TTCAACAAGAGTGGACCAAAGTCCAAGCTCTCTGTCCGCCAGTGTGGGCGACCGCGTGACCATC
ACCTGCGTCCTGACTGATACCAGCTATCCTCTGTACAGCACATACTGGTATCGGAAGAATCCCGG
TTCCAGCAACAAGGAGCAGATTTCCATCTCCGGCCGCTATAGTGAATCAGTCAACAAGGGCACTA
AGTCCTTTACCCTGACAATCAGTTCCCTGCAGCCCGAGGACTCCGCCACCTATTACTGCAGAGCT
ATGAGTACAAATATCTGGACCGGGGACGGAGCTGGTACCAAGGTGGAGATCAAGGGAGGTGGC
GGTTCCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGGGCCCATTC
TGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAAC
TGTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTC
AACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGT
CCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGT
GCCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATCATCAC
CATCACCATCACCATGAACAAAAACTCATCTCAGAAGAGGATCTG

SEQ ID NO 41
TNF VNAR D1-BA11-B4 AMINO ACID SEQUENCE WITH HIS AND MYC TAGS (His and Myc Tags - double underlining, CDR1 and CDR3 single underlined, linker shown in italics)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGSGGGGSGGGGSGAHS*TR
VDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTIS
SLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK*GGGGSGGGGSGGGGSGGGGSGAHS*ARVDQTPQ
TITKETGESLTINCVLRDSNCALSSMYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVED
SGTYRCKVYIPCIDELVYMISGGTSGPIHDVYGGGTVVTVNHHHHHHHHEQKLISEEDL

SEQ ID NO 42
NUCLEOTIDE SEQUENCE CODING FOR THE TNF VNAR D1-BA11-B4 WITH HIS AND MYC TAGS
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGATCCGGGGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGTGGAGCTCA
TTCAACAAGAGTGGACCAAAGTCCAAGCTCTCTGTCCGCCAGTGTGGGCGACCGCGTGACCATC
ACCTGCGTCCTGACTGATACCAGCTATCCTCTGTACAGCACATACTGGTATCGGAAGAATCCCGG
TTCCAGCAACAAGGAGCAGATTTCCATCTCCGGCCGCTATAGTGAATCAGTCAACAAGGGCACTA
AGTCCTTTACCCTGACAATCAGTTCCCTGCAGCCCGAGGACTCCGCCACCTATTACTGCAGAGCT
ATGAGTACAAATATCTGGACCGGGGACGGAGCTGGTACCAAGGTGGAGATCAAGGGAGGTGGC
GGTTCCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGAGGTGGCGGTAGCGGGGCCCATTC
TGCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAAC
TGTGTCCTACGAGATAGTAACTGTGCATTGTCCAGCATGTACTGGTATCGCAAAAAATCTGGCTC
AACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGT
CCTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGGTATATA
TACCTTGCATCGATGAACTGGTATATATGATCAGTGGGGGTACCTCTGGCCCGATTCATGATGTA
TACGGAGGTGGCACTGTCGTGACTGTGAATCATCACCATCACCATCACCATGAACAAAAACTCAT
CTCAGAAGAGGATCTG

SEQ ID NO 43
ICOS VNAR 2D4-Fc-2D4 AMINO ACID SEQUENCE (linkers shown in italics, Fc portion underlined)

TRVDQTPRTATKETGESLTINCVLTDTDYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAFTWPWEWPDRWFRPWYDGAGTVLTVN*GGGGSGGGADQ*EPKSSDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK*TAAAATAAAATAAAATAAAA*TRVDQTPRTATKETGESLTINCVLTDT
DYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFSLRIKDLTVADSATYYCKAFTWPWEWP
DRWFRPWYDGAGTVLTVN

SEQ ID NO 44
ICOS VNAR 2D4-Fc-2D4 NUCELOTIDE SEQUENCE

ACACGTGTTGACCAGACACCGCGTACCGCAACCAAAGAAACCGGTGAAAGCCTGACCATT
AATTGTGTTC TGACCGATAC CGATTATGGT TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT
CCGGGTACAA CCGATTGGGA ACGTATGAGC ATTGGTGGTC GTTATGTTGA AAGCGTGAAT
AAAGGTGCCA AAAGCTTTAG CCTGCGCATT AAAGATCTGA CCGTTGCAGA TAGCGCAACC
TATTACTGTA AAGCATTCAC TTGGCCGTGG GAATGGCCGG ACCGTTGGTT CCGTCCGTGG
TATGATGGTG CAGGCACCGT TCTGACCGTT AATGGCGGTG GTGGTTCTGG TGGTGGTGCT
GATCAGGAGC CCAAATCTTC TGACAAAACT CACACATGTC CACCGTGCCC AGCACCTGAA
CTCCTGGGTG GACCGTCAGT CTTCCTCTTC CCCCCAAAAC CCAAGGACAC CCTCATGATC
TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA GCCACGAAGA CCCTGAGGTC
AAGTTCAACT GGTACGTGGA CGGCGTGGAG GTGCATAATG CCAAGACAAA GCCGCGGGAG
GAGCAGTACA ACAGCACGTA CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG
CTGAATGGCA AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG
AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC CCTGCCCCCA
TCCCGGGAGG AGATGACCAA GAACCAGGTC AGCCTGACCT GCCTGGTCAA AGGCTTCTAT
CCCAGCGACA TCGCCGTGGA GTGGGAGAGC AATGGGCAGC CGGAGAACAA CTACAAGACC
ACGCCTCCCG TGCTGGACTC CGACGGCTCC TTCTTCCTCT ATAGCAAGCT CACCGTGGAC
AAGAGCAGGT GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC
AACCACTACA CGCAGAAGAG CCTCTCCCTG TCCCCGGGTA AAACCGCCGC CGCCGCCACC
GCCGCCGCCG CCACCGCCGC CGCCGCCACC GCCGCGGCCG CCACACGTGT TGATCAGACA
CCGCGTACCG CAACCAAAGA AACCGGTGAA AGCCTGACCA TTAATTGTGT TCTGACCGAT
ACCGATTATG GTTTGTTCTC CACCAGCTGG TTTCGTAAAA ATCCGGGTAC AACCGATTGG
GAACGTATGA GCATTGGTGG TCGTTATGTT GAAAGCGTGA ATAAAGGTGC CAAAAGCTTT
AGCCTGCGCA TTAAAGATCT GACCGTTGCA GATAGCGCAA CCTATTACTG TAAAGCATTC
ACTTGGCCGT GGGAATGGCC GGACCGTTGG TTCCGTCCGT GGTATGATGG TGCAGGCACC
GTTCTGACCG TTAAT

SEQ ID NO 45
ICOS VNAR 2D4-Fc-CC3 AMINO ACID SEQUENCE (linkers shown in italics, Fc portion underlined)
TRVDQTPRTATKETGESLTINCVLTDTDYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAFTWPWEWPDRWFRPWYDGAGTVLTVN*GGGGSGGGADQ*EPKSSDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK*TAAAATAAAATAAAATAAAA*TRVDQTPRTATKETGESLTINCVLTDT
EYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFSLRIKDLTVADSATYYCKALGWWPPAF
PHWYDGAGTVLTVN

SEQ ID NO 46
ICOS VNAR 2D4-Fc-CC3 NUCELOTIDE SEQUENCE

ACACGTGTTG ACCAGACACC GCGTACCGCA ACCAAAGAAA CCGGTGAAAG CCTGACCATT
AATTGTGTTC TGACCGATAC CGATTATGGT TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT
CCGGGTACAA CCGATTGGGA ACGTATGAGC ATTGGTGGTC GTTATGTTGA AAGCGTGAAT
AAAGGTGCCA AAAGCTTTAG CCTGCGCATT AAAGATCTGA CCGTTGCAGA TAGCGCAACC
TATTACTGTA AAGCATTCAC TTGGCCGTGG GAATGGCCGG ACCGTTGGTT CCGTCCGTGG
TATGATGGTG CAGGCACCGT TCTGACCGTT AATGGCGGTG GTGGTTCTGG TGGTGGTGCT

GATCAGGAGC CCAAATCTTC TGACAAAACT CACACATGTC CACCGTGCCC AGCACCTGAA CTCCTGGGTG GACCGTCAGT CTTCCTCTTC CCCCCAAAAC CCAAGGACAC CCTCATGATC TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA GCCACGAAGA CCCTGAGGTC AAGTTCAACT GGTACGTGGA CGGCGTGGAG GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG CTGAATGGCA AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC CCTGCCCCCA TCCCGGGAGG AGATGACCAA GAACCAGGTC AGCCTGACCT GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGCTGGACTC CGACGGCTCC TTCTTCCTCT ATAGCAAGCT CACCGTGGAC AAGAGCAGGT GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC AACCACTACA CGCAGAAGAG CCTCTCCCTG TCCCCGGGTA AAACCGCCGC CGCCGCCACC GCCGCCGCCG CCACCGCCGC CGCCGCCACC GCCGCGGCCG CCACACGTGT TGATCAGACA CCGCGTACCG CAACCAAAGA AACCGGTGAA AGCCTGACCA TTAATTGTGT TCTGACCGAT ACCGAGTATG GTTTGTTCTC CACCAGCTGG TTTCGTAAAA ATCCGGGTAC AACCGATTGG GAACGTATGA GCATTGGTGG TCGTTATGTT GAAAGCGTGA ATAAAGGTGC CAAAAGCTTT AGCCTGCGCA TTAAAGATCT GACCGTTGCA GATAGCGCAA CCTATTACTG TAAAGCACTG GGTTGGTGGC CGCCGGCTTT CCCGCATTGG TATGATGGTG CAGGCACCGT TCTGACCGTT AAT

SEQ ID NO 47
ICOS VNAR CC3-Fc-2D4 AMINO ACID SEQUENCE (linkers shown in italics, Fc portion underlined)

TRVDQTPRTATKETGESLTINCVLTDTEYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS LRIKDLTVADSATYYCKALGWWPPAFPHWYDGAGTVLTVN*GGGGSGGGGRT*EPKSSDKTHTCPPC PAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK*TAAAATAAAATAAAATAAAA*TRVDQTPRTATKETGESLTINCVLTDTDYGL FSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFSLRIKDLTVADSATYYCKAFTWPWEWPDRWF RPWYDGAGTVLTVN

SEQ ID NO 48
ICOS VNAR CC3-Fc-2D4 NUCLEOTIDE SEQUENCE

ACACGTGTTG ATCAGACACC GCGTACCGCA ACCAAAGAAA CCGGTGAAAG CCTGACCATT AATTGTGTTC TGACCGATAC CGAGTATGGT TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT CCGGGTACAA CCGATTGGGA ACGTATGAGC ATTGGTGGTC GTTATGTTGA AAGCGTGAAT AAAGGTGCCA AAAGCTTTAG CCTGCGCATT AAAGATCTGA CCGTTGCAGA TAGCGCAACC TATTACTGTA AAGCACTGGG TTGGTGGCCG CCGGCTTTCC CGCATTGGTA TGATGGTGCA GGCACCGTTC TGACCGTTAA TGGCGGTGGT GGTTCTGGTG GTGGTGGTCG TACGGAGCCC AAATCTTCTG ACAAAACTCA CACATGCCCA CCGTGCCCAG CACCTGAAGC CGCTGGGGCA CCGTCAGTCT TCCTCTTCCC CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT GAGGTCACAT GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG TACGTGGACG GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC AGCACGTACC GTGTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG GAGTACAAGT GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA AACCATCTCC AAAGCCAAAG GGCAGCCCCG AGAACCACAG GTGTACACCC TGCCCCCATC CCGGGAGGAG ATGACCAAGA ACCAGGTCAG CCTGACCTGC CTGGTCAAAG GCTTCTATCC CAGCGACATC GCCGTGGAGT GGGAGAGCAA TGGGCAGCCG GAGAACAACT ACAAGACCAC GCCTCCCGTG CTGGACTCCG ACGGCTCCTT CTTCCTCTAT AGCAAGCTCA CCGTGGACAA GAGCAGGTGG CAGCAGGGGA ACGTCTTCTC ATGCTCCGTG ATGCATGAGG CTCTGCACAA CCACTACACG CAGAAGAGCC TCTCCCTGTC CCCGGGTAAA ACCGCCGCCG CCGCCACCGC CGCCGCCGCC ACCGCCGCCG CCGCCACCGC CGCGGCCGCC ACACGTGTTG ATCAGACACC GCGTACCGCA ACCAAAGAAA CCGGTGAAAG CCTGACCATT AATTGTGTTC TGACCGATAC CGATTATGGT TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT CCGGGTACAA CCGATTGGGA ACGTATGAGC ATTGGTGGTC GTTATGTTGA AAGCGTGAAT AAAGGTGCCA AAAGCTTTAG CCTGCGCATT AAAGATCTGA CCGTTGCAGA TAGCGCAACC TATTACTGTA AAGCATTCAC TTGGCCGTGG GAATGGCCGG ACCGTTGGTT CCGTCCGTGG TATGATGGTG CAGGCACCGT TCTGACCGTT AAT

SEQ ID NO 49
ICOS VNAR CC3-Fc-CC3 AMINO ACID SEQUENCE (linkers shown in italics, Fc portion underlined)
TRVDQTPRTATKETGESLTINCVLTDTEYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKALGWWPPAFPHWYDGAGTVLTVN*GGGGSGGGGRT*EPKSSDKTHTCPPC
PAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK*TAAAATAAAATAAAATAAAA*TRVDQTPRTATKETGESLTINCVLTDTEYGL
FSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFSLRIKDLTVADSATYYCKALGWWPPAFPHWY
DGAGTVLTVN

SEQ ID NO 50
ICOS VNAR **CC3-Fc-CC3** NUCLEOTIDE SEQUENCE

ACACGTGTTG ATCAGACACC GCGTACCGCA ACCAAAGAAA CCGGTGAAAG CCTGACCATT
AATTGTGTTC TGACCGATAC CGAGTATGGT TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT
CCGGGTACAA CCGATTGGGA ACGTATGAGC ATTGGTGGTC GTTATGTTGA AAGCGTGAAT
AAAGGTGCCA AAAGCTTTAG CCTGCGCATT AAAGATCTGA CCGTTGCAGA TAGCGCAACC
TATTACTGTA AAGCACTGGG TTGGTGGCCG CCGGCTTTCC CGCATTGGTA TGATGGTGCA
GGCACCGTTC TGACCGTTAA TGGCGGTGGT GGTTCTGGTG GTGGTGGTCG TACGGAGCCC
AAATCTTCTG ACAAAACTCA CACATGCCCA CCGTGCCCAG CACCTGAAGC CGCTGGGGCA
CCGTCAGTCT TCCTCTTCCC CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT
GAGGTCACAT GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG
TACGTGGACG GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC
AGCACGTACC GTGTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG
GAGTACAAGT GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA AACCATCTCC
AAAGCCAAAG GGCAGCCCCG AGAACCACAG GTGTACACCC TGCCCCCATC CCGGGAGGAG
ATGACCAAGA ACCAGGTCAG CCTGACCTGC CTGGTCAAAG GCTTCTATCC CAGCGACATC
GCCGTGGAGT GGGAGAGCAA TGGGCAGCCG GAGAACAACT ACAAGACCAC GCCTCCCGTG
CTGGACTCCG ACGGCTCCTT CTTCCTCTAT AGCAAGCTCA CCGTGGACAA GAGCAGGTGG
CAGCAGGGGA ACGTCTTCTC ATGCTCCGTG ATGCATGAGG CTCTGCACAA CCACTACACG
CAGAAGAGCC TCTCCCTGTC CCCGGGTAAA ACCGCCGCCG CCGCCACCGC CGCCGCCGCC
ACCGCCGCCG CCGCCACCGC CGCGGCCGCC ACACGTGTTG ATCAGACACC GCGTACCGCA
ACCAAAGAAA CCGGTGAAAG CCTGACCATT AATTGTGTTC TGACCGATAC CGAGTATGGT
TTGTTCTCCA CCAGCTGGTT TCGTAAAAAT CCGGGTACAA CCGATTGGGA ACGTATGAGC
ATTGGTGGTC GTTATGTTGA AAGCGTGAAT AAAGGTGCCA AAAGCTTTAG CCTGCGCATT
AAAGATCTGA CCGTTGCAGA TAGCGCAACC TATTACTGTA AAGCACTGGG TTGGTGGCCG
CCGGCTTTCC CGCATTGGTA TGATGGTGCA GGCACCGTTC TGACCGTTAA T

SEQ ID NO 51
SoloMER™ VNAR D1-v1 AMINO ACID SEQUENCE WITH HIS TAG
ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFS
LRINDLTVEDSGTYRCASECQYGLAEYDVYGGGTKVEIKHHHHHH

SEQ ID NO 52
NUCLEOTIDE SEQUENCE CODING FOR THE SoloMER™ VNAR D1-v1 WITH HIS TAG
GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCGCAAGAAG
TCCGGCTCCACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTACGTGGAGACCGTGAACTCC
GGCTCCAAGTCCTTCTCCCTGCGCATCAACGACCTGACCGTGGAGGACTCCGGCACC
TACCGCTGCGCCTCCGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTACGGCGGCGGCACC
AAGGTGGAGATCAAGCACCACCACCACCACCAC

SEQ ID NO 53
SoloMER™ VNAR D1-v2 AMINO ACID SEQUENCE WITH HIS TAG
ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFT
LTISSLQPEDFATYYCASECQYGLAEYDVYGGGTKVEIKHHHHHH

SEQ ID NO 54

NUCLEOTIDE SEQUENCE CODING FOR THE SoloMER™ VNAR D1-v2 WITH HIS TAG
GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCGCAAGAAG
TCCGGCTCCACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTACGTGGAGACCGTGAACTCC
GGCTCCAAGTCCTTCACCCTGACCATCTCCTCCCTGCAGCCCGAGGACTTCGCCACC
TACTACTGCGCCTCCGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTACGGCGGCGGCACC
AAGGTGGAGATCAAGCACCACCACCACCACCAC

SEQ ID NO 55
SoloMER™ VNAR D1-v3 AMINO ACID SEQUENCE WITH HIS TAG
ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYQQKPGKTNEESISKGGRYVETVNSGSKSFT
LTISSLQPEDFATYYCASECQYGLAEYDVYGGGTKVEIKHHHHHH

SEQ ID NO 56
NUCLEOTIDE SEQUENCE CODING FOR THE SoloMER™ VNAR D1-v3 WITH HIS TAG
GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCAGCAGAAG
CCCGGCAAGACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTACGTGGAGACCGTGAACTCC
GGCTCCAAGTCCTTCACCCTGACCATCTCCTCCCTGCAGCCCGAGGACTTCGCCACC
TACTACTGCGCCTCCGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTACGGCGGCGGCACC
AAGGTGGAGATCAAGCACCACCACCACCACCAC

SEQ ID NO 57
SoloMER™ VNAR D1-v4 AMINO ACID SEQUENCE WITH HIS TAG
ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYRKKPGSTNEESISKGGRFSGSGSSGSKSFT
LTISSLQPEDFATYYCASECQYGLAEYDVFGQGTKVEIKHHHHHH

SEQ ID NO 58
NUCLEOTIDE SEQUENCE CODING FOR THE SoloMER™ VNAR D1-v4 WITH HIS TAG
GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCGCAAGAAG
CCCGGCTCCACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTTCTCCGGCTCCGGCTCCTCC
GGCTCCAAGTCCTTCACCCTGACCATCTCCTCCCTGCAGCCCGAGGACTTCGCCACC
TACTACTGCGCCTCCGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTTCGGCCAGGGCACC
AAGGTGGAGATCAAGCACCACCACCACCACCAC

SEQ ID NO 59
Quad-X™ D1-Fc-C4 AMINO ACID SEQUENCE (Fc portion underlined)
ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVNGSGGGSGGGGSGEPKSSDKTHTCPPCP
APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGGSGAHSARVDQTPQTITKETGESLTINCVLRD
SNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVEDSGTYRCKLSWWTQNWR
CSNSDVYGGGTVVTVN

SEQ ID NO 60
NUCLEOTIDE SEQUENCE CODING FOR THE Quad-X™ D1-Fc-C4
GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGATCC
GGTGGTGGGTCCGGAGGAGGTGGCTCAGGAGAGCCCAAATCTAGCGACAAAACTCACACATGC
CCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCA
AGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACG
AAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAA
GCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCGTCCTGCACCA
GGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC

GAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAT
CCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAG
CGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCC
CGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGG
CAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGA
AGAGCCTCTCCCTGTCTCCGGGGAAAGGAGGTGGCGGTTCCGGAGGTGGCGGTAGCGGAGGT
GGCGGTAGCGGAGGTGGCGGTAGCGGGGCCCATTCTGCTCGAGTGGACCAAACACCGCAAACA
ATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGCAACTGTGGGTT
GTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAGCATATCGAAAGGT
GGACGATATGTTGAAACAATTAACGAAGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCTAACA
GTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTGGTGGACCCAGAACTGGAGATGCTCAA
ATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTGAAT

SEQ ID NO 61

Quad-Y-D1C4™ D1-C4-Fc AMINO ACID SEQUENCE (Fc portion underlined)

ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVNGGGGSGGGGGGSGAHSARVDQTPQTITKE
TGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVEDSGTYR
CKLSWWTQNWRCSNSDVYGGGTVVTVNGGGSGGGGSGEPKSSDKTHTCPPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

SEQ ID NO 62

NUCLEOTIDE SEQUENCE CODING FOR THE Quad-Y-D1C4™ AMINO ACID SEQUENCE

GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGTAGCGGAGGTGGTGGCGGATCCGGGGCGCACTCCGCTCGAGTGGACCAAACACCGCA
AACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGCAACTGTG
GGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAGCATATCGAA
AGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCT
AACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTGGTGGACCCAGAACTGGAGATGC
TCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTGAACGGTGGTGGGTCCGGAGGAG
GTGGCTCAGGAGAGCCCAAATCTAGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGA
ACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCC
CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTAC
AACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGG
AGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGC
CAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAA
GAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGG
GAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT
CATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCC
GGGGAAA

SEQ ID NO 63

Quad-Y-C4D1™ C4-D1-Fc AMINO ACID SEQUENCE (Fc portion underlined)

ARVDQTPQTITKETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSL
RINDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTVVTVNGGGGSGGGGGSGAHSARVDQTPQ
TITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSLRINDLTVED
SGTYRCASECQYGLAEYDVYGGGTVVTVNGGGSGGGGSGEPKSSDKTHTCPPCPAPELLGGPSVF
LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

SEQ ID NO 64
NUCLEOTIDE SEQUENCE CODING FOR THE Quad-Y-C4D1™ AMINO ACID SEQUENCE

GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCAACTGTGGGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTG
GTGGACCCAGAACTGGAGATGCTCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTG
AACGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCGGGGCGCACTCCGCTCGAGTGGACCA
AACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATA
GCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAG
CATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTCCTTTTCTTTGAGAAT
TAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTGCCAATATGGACTGG
CAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGTGGTGGGTCCGGAGGAGG
TGGCTCAGGAGAGCCCAAATCTAGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA
CTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC
GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCA
ACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACA
ACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGA
GTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAG
AACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGG
AGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCT
CCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTC
ATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG
GGGAAA

SEQ ID NO 65 - 2D4
TRVDQTPRTATKETGESLTINCVLTDTDYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAFTWPWEWPDRWFRPWYDGAGTVLTVN

SEQ ID NO 66 - CC3
TRVDQTPRTATKETGESLTINCVLTDTEYGLFSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKALGWWPPAFPHWYDGAGTVLTVN

SEQ ID NO 67 - BA11
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK

SEQ ID NO 68 - CDR1
HCATSS

SEQ ID NO 69 – CDR1
NCGLSS

SEQ ID NO 70 – CDR1
NCALSS

SEQ ID NO 71 – HV2
TNEESISKG

SEQ ID NO 72 – HV4
SGSKS

SEQ ID NO 73 – HV4

EGSKS

SEQ ID NO 74 - NARF4For1
ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG ACA GTG CCA CCT C

SEQ ID NO 75 - NARF4For2
ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG GCA GTG CCA TCT C

SEQ ID NO 76 - NARF1Rev
ATA ATA AGG AAT TCC ATG GCT CGA GTG GAC CAA ACA CCG

SEQ ID NO 77 - E06
TRVDQTPRTATRETGESLTINCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYVESVNKGTKSFSL
RIKDLTVADSATYICRAMGTNIWTGDGAGTVLTVN

SEQ ID NO 78 - hE06v1.10
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 79 - AC9
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSSTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 80 - AD4
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISMSGRYSESVNKSTKSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 81 - AG11
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVETK

SEQ ID NO 82 - AH7
TRVDQTPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSSTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK
SEQ ID NO 83 - BB10
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNFWTGDGAGTKVEIK

SEQ ID NO 84 - BB11
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMATNIWTGDGAGTKVEIK

SEQ ID NO 85 - BC3
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSNNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 86 - BD12
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTNSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 87 - BE4
TRVDQSPSSLSASVGDRVTITCVLTDTSYSLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNIWTGDGAGTKVEIK

SEQ ID NO 88 - BH4
TRVDQSPSSLSASVGDRVTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDFATYYCRAMGTNLWTGDGAGTKVEIK

SEQ ID NO 89 - TNF VNAR DIMER D1-C4 with $(Gly_4Ser)_3$ AMINO ACID SEQUENCE WITH HIS TAG
(linker shown in italics and tag double underlined)

ARVDQTPQTITKETGESLTINCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFSL
RINDLTVEDSGTYRCASECQYGLAEYDVYGGGTVVTVN*GGGGSGGGGGSGGGS*ARVDQTPQTITK
ETGESLTINCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVEDSGTY
RCKLSWWTQNWRCSNSDVYGGGTVVTVNAAAHHHHHH

SEQ ID NO 90 - TNF VNAR DIMER D1-C4 with $(Gly_4Ser)_3$ NEUCLEOTIDE SEQUENCE WITH HIS TAG

GCTCGAGTGGACCAAACACCGCAAACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACT
GTGTCCTACGAGATAGCCACTGTGCAACCTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCA
ACAAACGAGGAGAGCATATCGAAAGGTGGACGATATGTTGAAACAGTTAACAGCGGATCAAAGTC
CTTTTCTTTGAGAATTAATGATCTAACAGTTGAAGACAGTGGCACGTATCGATGCGCTTCCGAGTG
CCAATATGGACTGGCAGAATATGATGTATACGGAGGTGGCACTGTCGTGACTGTGAATGGAGGT
GGCGGTAGCGGAGGTGGTGGCGGATCCGGCGGTGGTTCCGCTCGAGTGGACCAAACACCGCA
AACAATAACAAAGGAGACGGGCGAATCACTGACCATCAACTGTGTCCTACGAGATAGCAACTGTG
GGTTGTCCAGCACGTACTGGTATCGCAAAAAATCGGGCTCAACAAACGAGGAGAGCATATCGAA
AGGTGGACGATATGTTGAAACAATTAACGAAGGATCAAAGTCCTTTTCTTTGAGAATTAATGATCT
AACAGTTGAAGACAGTGGCACGTATCGATGCAAGTTAAGCTGGTGGACCCAGAACTGGAGATGC
TCAAATTCCGATGTATACGGAGGTGGCACTGTCGTGACTGTGAACGCGGCCGCACATCATCATCA
CCATCAC

SEQ ID NO 91 - TNF soloMER™ DIMER D1v2-C4v1 with $(Gly_4Ser)_3$ AMINO ACID SEQUENCE WITH HIS TAG (linker shown in italics and tag double underlined)

ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFT
LTISSLQPEDFATYYCASECQYGLAEYDVYGGGTKVEIK*GGGGSGGGGGSGGGS*ARVDQSPSSLSA
SVGDRVTITCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLRINDLTVEDSGT
YRCKLSWWTQNWRCSNSDVYGGGTKVEIKAAAHHHHHH

SEQ ID NO 92 - TNF soloMER™ DIMER D1v2-C4v1 with $(Gly_4Ser)_3$ NUCLEOTIDE SEQUENCE WITH HIS TAG

GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCGCAAGAAGTCCGGCT
CCACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTACGTGGAGACCGTGAACTCCGGCTCCA
AGTCCTTCACCCTGACCATCTCCTCCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCGCCTC
CGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTACGGCGGCGGCACCAAGGTGGAGATCAA
GGGAGGTGGCGGTAGCGGAGGTGGTGGCGGATCCGGCGGTGGTTCCGCCCGCGTGGACCAGT
CCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACCTGCGTGCTGCGCGACT
CCAACTGCGGCCTGTCCTCCACCTACTGGTACCGCAAGAAGTCCGGCTCCACCAACGAGGAGTC
CATCTCCAAGGGCGGCCGCTACGTGGAGACCATCAACGAGGGCTCCAAGTCCTTCTCCCTGCGC
ATCAACGACCTGACCGTGGAGGACTCCGGCACCTACCGCTGCAAGCTGTCCTGGTGGACCCAGA
ACTGGCGCTGCTCCAACTCCGACGTGTACGGCGGCGGCACCAAGGTGGAGATCAAGGCGGCCG
CACATCATCATCACCATCAC

SEQ ID NO 93 - TNF soloMER™ DIMER D1v2-C4v1 with $(Gly_4Ser)_5$ AMINO ACID SEQUENCE WITH HIS TAG (linker shown in italics and tag double underlined)

ARVDQSPSSLSASVGDRVTITCVLRDSHCATSSTYWYRKKSGSTNEESISKGGRYVETVNSGSKSFT
LTISSLQPEDFATYYCASECQYGLAEYDVYGGGTKVEIK*GGGGSGGGGSGGGGSGGGGSGGGGS*A
RVDQSPSSLSASVGDRVTITCVLRDSNCGLSSTYWYRKKSGSTNEESISKGGRYVETINEGSKSFSLR
INDLTVEDSGTYRCKLSWWTQNWRCSNSDVYGGGTKVEIKAAAHHHHHH

SEQ ID NO 94 - TNF soloMER™ DIMER D1v2-C4v1 with $(Gly_4Ser)_5$ NUCLEOTIDE SEQUENCE WITH HIS TAG

GCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACCGCGTGACCATCACC
TGCGTGCTGCGCGACTCCCACTGCGCCACCTCCTCCACCTACTGGTACCGCAAGAAGTCCGGCT
CCACCAACGAGGAGTCCATCTCCAAGGGGGGCCGCTACGTGGAGACCGTGAACTCCGGCTCCA
AGTCCTTCACCCTGACCATCTCCTCCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCGCCTC
CGAGTGCCAGTACGGCCTGGCCGAGTACGACGTGTACGGCGGCGGCACCAAGGTGGAGATCAA
GGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAGGTGGCGGTGGTTCTGGCGGTGGCGGTAGTG
GCGGAGGTGGTAGTGCCCGCGTGGACCAGTCCCCCTCCTCCCTGTCCGCCTCCGTGGGCGACC
GCGTGACCATCACCTGCGTGCTGCGCGACTCCAACTGCGGCCTGTCCTCCACCTACTGGTACCG
CAAGAAGTCCGGCTCCACCAACGAGGAGTCCATCTCCAAGGGCGGCCGCTACGTGGAGACCAT

CAACGAGGGCTCCAAGTCCTTCTCCCTGCGCATCAACGACCTGACCGTGGAGGACTCCGGCACC
TACCGCTGCAAGCTGTCCTGGTGGACCCAGAACTGGCGCTGCTCCAACTCCGACGTGTACGGCG
GCGGCACCAAGGTGGAGATCAAGGCGGCCGCACATCATCATCACCATCAC

SEQ ID NO 95 - TNF S17-Quad-X™ with $(Gly_4Ser)_5$ AMINO ACID SEQUENCE (Fc portion underlined)
ASVNQTPRTATKETGESLTINCVLTDTHAKVFTTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYICRAGGYLSQPRVYWDVYGAGTVLTVNGGGGSGGGGRTEPRGPTIKPCPPCKC
PAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNS
TLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLT
CMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGL
HNHHTTKSFSRTPGKGGGGSGGGGSGGGGSGGGGSGAHSASVNQTPRTATKETGESLTINCVLTD
THAKVFTTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFSLRIKDLTVADSATYICRAGGYLSQPR
VYWDVYGAGTVLTVN

SEQ ID NO 96 - TNF S17-Quad-X™ with $(Gly_4Ser)_5$ NUCLEOTIDE SEQUENCE
GCAAGCGTTAATCAGACACCGCGTACCGCAACCAAAGAAACCGGTGAAAGCCTGACCATTAATTG
TGTTCTGACCGATACCCATGCTAAAGTTTTCACTACCAGCTGGTTTCGTAAAAATCCGGGTACAAC
CGATTGGGAACGTATGAGCATTGGTGGTCGTTATGTTGAAAGCGTGAATAAAGGTGCCAAAAGCT
TTAGCCTGCGCATTAAAGATCTGACCGTTGCAGATAGCGCAACCTATATCTGTCGTGCCGGTGGT
TACCTGTCTCAGCCGCGTGTTTACTGGGATGTTTATGGTGCAGGCACCGTTCTGACCGTTAATGG
CGGTGGTGGTTCTGGTGGTGGTGGTCGTACGGAGCCTCGAGGCCCCACAATCAAGCCCTGTCC
TCCATGCAAATGCCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGA
TCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGA
GGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACA
CAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACC
AGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCAT
CGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCAC
CAGAAGAAGAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAA
GACATTTACGTGGAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGT
CCTGGACTCTGATGGTTCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGG
AAAGAAATAGCTACTCCTGTTCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGC
TTCTCCCGGACTCCGGGTAAAGGAGGTGGCGGTTCCGGAGGTGGCGGTAGCGGAGGTGGCGG
TAGCGGAGGTGGCGGTAGCGGGGCCCATTCTGCAAGCGTTAATCAGACACCGCGTACCGCAAC
CAAAGAAACCGGTGAAAGCCTGACCATTAATTGTGTTCTGACCGATACCCATGCTAAAGTTTTCAC
TACCAGCTGGTTTCGTAAAAATCCGGGTACAACCGATTGGGAACGTATGAGCATTGGTGGTCGTT
ATGTTGAAAGCGTGAATAAAGGTGCCAAAAGCTTTAGCCTGCGCATTAAAGATCTGACCGTTGCA
GATAGCGCAACCTATATCTGTCGTGCCGGTGGTTACCTGTCTCAGCCGCGTGTTTACTGGGATGT
TTATGGTGCAGGCACCGTTCTGACCGTTAAT

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Amino acids are represented herein as either a single letter code or as the three-letter code or both.

The term "affinity purification" means the purification of a molecule based on a specific attraction or binding of the molecule to a chemical or binding partner to form a combination or complex which allows the molecule to be separated from impurities while remaining bound or attracted to the partner moiety.

The term "Complementarity Determining Regions" or CDRs (i.e., CDR1 and CDR3) refers to the amino acid residues of a VNAR domain the presence of which are necessary for antigen binding. Each

VNAR typically has CDR regions identified as CDR1 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" and/or those residues from a "hypervariable loop" (HV). In some instances, a complementarity determining region can include amino acids from both a CDR region and a hypervariable loop. According to the generally accepted nomenclature for VNAR molecules, a CDR2 region is not present.

"Framework regions" (FW) are those VNAR residues other than the CDR residues. Each VNAR typically has five framework regions identified as FW1, FW2, FW3a, FW3b and FW4. VNAR domains therefore typically have the structure FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 in the N- to C-terminal direction.

"Cell", "cell line", and "cell culture" are used interchangeably (unless the context indicates otherwise) and such designations include all progeny of a cell or cell line. Thus, for example, terms like "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

"Control sequences" when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, etc. Eukaryotic cells use control sequences such as promoters, polyadenylation signals, and enhancers.

The term "coat protein" means a protein, at least a portion of which is present on the surface of the virus particle. From a functional perspective, a coat protein is any protein which associates with a virus particle during the viral assembly process in a host cell, and remains associated with the assembled virus until it infects another host cell.

The "detection limit" for a chemical entity in a particular assay is the minimum concentration of that entity which can be detected above the background level for that assay. For example, in the phage ELISA, the "detection limit" for a particular phage displaying a particular antigen binding fragment is the phage concentration at which the particular phage produces an ELISA signal above that produced by a control phage not displaying the antigen binding fragment.

A "fusion protein" and a "fusion polypeptide" refer to a polypeptide having two portions covalently linked together, where each of the portions is a polypeptide having a different property. The property may be a biological property, such as activity in vitro or in vivo. The property may also be a simple chemical or physical property, such as binding to a target antigen, catalysis of a reaction, etc. The two portions may be linked directly by a single peptide bond or through a peptide linker containing one or

more amino acid residues. Generally, the two portions and the linker will be in reading frame with each other. Preferably, the two portions of the polypeptide are obtained from heterologous or different polypeptides.

The term "fusion protein" in this text means, in general terms, one or more proteins joined together by chemical means, including hydrogen bonds or salt bridges, or by peptide bonds through protein synthesis or both.

"Heterologous DNA" is any DNA that is introduced into a host cell. The DNA may be derived from a variety of sources including genomic DNA, cDNA, synthetic DNA and fusions or combinations of these. The DNA may include DNA from the same cell or cell type as the host or recipient cell or DNA from a different cell type, for example, from an allogenic or xenogenic source. The DNA may, optionally, include marker or selection genes, for example, antibiotic resistance genes, temperature resistance genes, etc.

A "highly diverse position" refers to a position of an amino acid located in the variable regions of the light and heavy chains that have a number of different amino acid represented at the position when the amino acid sequences of known and/or naturally occurring antibodies or antigen binding fragments are compared. The highly diverse positions are typically in the CDR regions.

"Identity" describes the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. Identity also means the degree of sequence relatedness (homology) between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. While there exist a number of methods to measure identity between two polypeptide or two polynucleotide sequences, methods commonly employed to determine identity are codified in computer programs. Preferred computer programs to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, *et al., Nucleic Acids Res,* 1984, **12,** 387 BLASTP, BLASTN, and FASTA (Atschul *et al*., *J. Molec. Biol.* (1990) 215, 403).

Preferably, the amino acid sequence of the protein has at least 60% identity, using the default parameters of the BLAST computer program (Atschul *et al., J. Mol. Biol.* 1990 **215**, 403-410) provided by HGMP (Human Genome Mapping Project), at the amino acid level, to the amino acid sequences disclosed herein.

More preferably, the protein sequence may have at least 65%, 66%, 67%, 68%, 69%, 70%, 75%, 80%, 85%, 90% and still more preferably 95% (still more preferably at least 96%, 97%, 98% or 99%) identity, at the nucleic acid or amino acid level, to the amino acid sequences as shown herein.

The protein may also comprise a sequence which has at least 60%, 65%, 66%, 67%, 68%, 69%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity with a sequence disclosed herein, using the default parameters of the BLAST computer program provided by HGMP, thereto.

A "library" refers to a plurality of VNARs or VNAR fragment sequences or the nucleic acids that encode these sequences. The origin of the library can be from non-natural sources or synthetic in nature where diversity has been engineered into a natural or combination of natural frameworks or can be from a natural source as exemplified from VNAR domains isolated from RNA extracted from an immunized animal.

"Ligation" is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol- chloroform extraction and ethanol precipitation or by silica purification. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 \ig of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

A "mutation" is a deletion, insertion, or substitution of a nucleotide(s) relative to a reference nucleotide sequence, such as a wild type sequence.

"Natural" or "naturally occurring" VNARs, refers to VNARs identified from a non-synthetic source, for example, from a tissue source obtained ex vivo, or from the serum of an animal of the Elasmobranchii subclass. These VNARs can include VNARs generated in any type of immune response, either natural or otherwise induced. Natural VNARs include the amino acid sequences, and the nucleotide sequences that constitute or encode these antibodies. As used herein, natural VNARs are different than "synthetic VNARs", synthetic VNARs referring to VNAR sequences that have been changed from a source or template sequence, for example, by the replacement, deletion, or addition, of an amino acid, or more than one amino acid, at a certain position with a different amino acid, the different amino acid providing an antibody sequence different from the source antibody sequence.

The term "nucleic acid construct" generally refers to any length of nucleic acid which may be DNA, cDNA or RNA such as mRNA obtained by cloning or produced by chemical synthesis. The DNA may be single or double stranded. Single stranded DNA may be the coding sense strand, or it may be the

non-coding or anti-sense strand. For therapeutic use, the nucleic acid construct is preferably in a form capable of being expressed in the subject to be treated.

"Operably linked" when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promotor or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contingent and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

"Phage display" is a technique by which variant polypeptides are displayed as fusion proteins to at least a portion of coat protein on the surface of phage, e.g., filamentous phage, particles. Phage display technology allows for the preparation of large libraries of randomized protein variants which can be rapidly and efficiently sorted for those sequences that bind to a target antigen with high affinity. The display of peptide and protein libraries on phage can be used for screening millions of polypeptides for ones with specific binding properties. Polyvalent phage display methods have been used for displaying small random peptides and small proteins through fusions to the genes encoding coat proteins pill, pV III, pVI, pVII or pIX of filamentous phage.

A "phagemid" is a plasmid vector having a bacterial origin of replication, e.g., ColEI, and a copy of an intergenic region of a bacteriophage. The phagemid may be used on any known bacteriophage, including filamentous bacteriophage and lambdoid bacteriophage. The plasmid will also generally contain a selectable marker for antibiotic resistance. Segments of DNA cloned into these vectors can be propagated as plasmids. When cells harboring these vectors are provided with all genes necessary for the production of phage particles, the mode of replication of the plasmid changes to rolling circle replication to generate copies of one strand of the plasmid DNA and package phage particles. The phagemid may form infectious or non-infectious phage particles. This term includes phagemids which contain a phage coat protein gene or fragment thereof linked to a heterologous polypeptide gene as a gene fusion such that the heterologous polypeptide is displayed on the surface of the phage particle. An example of a phagemid display vector is pWRIL-1.

The term "phage vector" means a double stranded replicative form of a bacteriophage containing a heterologous gene and capable of replication. The phage vector has a phage origin of replication allowing phage replication and phage particle formation. The phage is preferably a filamentous bacteriophage, such as an M13, fl, fd, Pf3 phage or a derivative thereof, or a lambdoid phage, such as lambda, 21 , phi80, phi81 , or a derivative thereof.

The term "protein" means, in general terms, a plurality of amino acid residues joined together by peptide bonds. It is used interchangeably and means the same as peptide, oligopeptide, oligomer or polypeptide, and includes glycoproteins and derivatives thereof. The term "protein" is also intended to include fragments, analogues, variants and derivatives of a protein wherein the fragment, analogue, variant or derivative retains essentially the same biological activity or function as a reference protein. Examples of protein analogues and derivatives include peptide nucleic acids, and DARPins (Designed Ankyrin Repeat Proteins). A "polypeptide of the invention" is TNFα specific antigen binding molecule as defined herein.

A fragment, analogue, variant or derivative of the protein may be at least 25 preferably 30 or 40, or up to 50 or 100, or 60 to 120 amino acids long, depending on the length of the original protein sequence from which it is derived. A length of 90 to 120, 100 to 1 10 amino acids may be convenient in some instances.

The fragment, derivative, variant or analogue of the protein may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably, a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or auxiliary sequence which is employed for purification of the polypeptide. Such fragments, derivatives, variants and analogues are deemed to be within the scope of those skilled in the art from the teachings herein.

“Oligonucleotides" are short-length, single-or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid-phase techniques). Further methods include the polymerase chain reaction (PCR) used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides can be purified on polyacrylamide gels or molecular sizing columns or by precipitation. DNA is "purified" when the DNA is separated from non-nucleic acid impurities (which may be polar, non-polar, ionic, etc.).

A "source" or "template" VNAR", as used herein, refers to a VNAR or VNAR antigen binding fragment whose antigen binding sequence serves as the template sequence upon which diversification according to the criteria described herein is performed. An antigen binding sequence generally includes within a VNAR preferably at least one CDR, preferably including framework regions.

A "transcription regulatory element" will contain one or more of the following components: an enhancer element, a promoter, an operator sequence, a repressor gene, and a transcription termination sequence.

"Transformation" means a process whereby a cell takes up DNA and becomes a "transformant". The DNA uptake may be permanent or transient. A "transformant" is a cell which has taken up and maintained DNA as evidenced by the expression of a phenotype associated with the DNA (e.g., antibiotic resistance conferred by a protein encoded by the DNA).

A "variant" or "mutant" of a starting or reference polypeptide (for example, a source VNAR or a CDR thereof), such as a fusion protein (polypeptide) or a heterologous polypeptide (heterologous to a phage), is a polypeptide that (1) has an amino acid sequence different from that of the starting or reference polypeptide and (2) was derived from the starting or reference polypeptide through either natural or artificial mutagenesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. For example, a fusion polypeptide of the invention generated using an oligonucleotide comprising a nonrandom codon set that encodes a sequence with a variant amino acid (with respect to the amino acid found at the corresponding position in a source VNAR or antigen binding fragment) would be a variant polypeptide with respect to a source VNAR or antigen binding fragment. Thus, a variant CDR refers to a CDR comprising a variant sequence with respect to a starting or reference polypeptide sequence (such as that of a source VNAR or antigen binding fragment). A variant amino acid, in this context, refers to an amino acid different from the amino acid at the corresponding position in a starting or reference polypeptide sequence (such as that of a source VNAR or antigen binding fragment). Any combination of deletion, insertion, and substitution may be made to arrive at the final variant or mutant construct, provided that the final construct possesses the desired functional characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites.
A "wild-type" or "reference" sequence or the sequence of a "wild-type" or "reference" protein/polypeptide, such as a coat protein, or a CDR of a source VNAR, may be the reference sequence from which variant polypeptides are derived through the introduction of mutations. In general, the "wild-type" sequence for a given protein is the sequence that is most common in nature.

Similarly, a "wild-type" gene sequence is the sequence for that gene which is most commonly found in nature. Mutations may be introduced into a "wild-type" gene (and thus the protein it encodes) either through natural processes or through man induced means. The products of such processes are "variant" or "mutant" forms of the original "wild-type" protein or gene.

General methods for DNA manipulation, transfection methods and culture methods are well known to those skilled in the art. In this respect, reference is made to Molecular Cloning: A Laboratory Manual *(Fourth Edition)* Cold Spring Harbor Publishing.

Isolation of VNARs

VNAR domains may be obtained from phage-displayed libraries constructed using tissues from target-immunized sharks (Dooley, H., *et al. Mol Immunol*, 2003. **40**(1): p. 25-33; Nuttall, S.D., *et al*, Proteins, 2004. 55(1): p. 187-97; and Dooley, H., *et al.*, *Proc Natl Acad Sci U S A,* 2006. **103**(6): p. 1846-51), WO2003/014161, incorporated by reference describes a useful method for immunizing a shark and obtaining binding domains.

VNAR binding domains may also be obtained from synthetic libraries comprising VNAR sequences. WO2014/173959, incorporated by reference, describes a useful method for developing VNAR libraries and obtaining binding domains.

Additionally it has been shown that libraries with synthetic diversity targeted to CDR3 can be used to obtain binding domains based on VNAR structures (Nuttall, S.D., *et al. Mol Immunol*, 2001. 38(4): p. 313-26; Nuttall, S.D., *et al. Eur J Biochem*, 2003. 270(17): p. 3543-54; Shao, C.Y., *et al. Mol Immunol,* 2007. 44(4): p. 656-65 and Liu, J.L., *et al. BMC Biotechnol*, 2007. 7: p. 78; WO2005/118629.

VNARS of the invention may be further adapted to reduce potential immunogenicity when administered to man (humanization).

Humanization of antibody variable domains is a technique well-known in the art to modify an antibody which has been raised, in a species other than humans, against a therapeutically useful target so that the humanized form may avoid unwanted immunological reaction when administered to a human subject. The methods involved in humanization are summarized in Almagro J.C and William Strohl W. *Antibody Engineering: Humanization, Affinity Maturation, and Selection Techniques* in *Therapeutic Monoclonal Antibodies: From Bench to Clinic*. Edited by An J. 2009 John Wiley & Sons, Inc and in Strohl W.R. and Strohl L.M., *Therapeutic Antibody Engineering*, Woodhead Publishing 2012.

Although IgNARs have distinct origins compared to immunoglobulins and have very little sequence homology compared to immunoglobulin variable domains there are some structural similarities between immunoglobulin and IgNAR variable domains, so that similar processes can be applied to the VNAR domain. For example, WO2013/167883, incorporated by reference, provides a description of the humanization of VNARs, see also Kovalenko O.V., *et al. J Biol Chem*. 2013**. 288(24):** p. 17408-19.

Protein expression

Nucleic acid sequences encoding antigen specific antigen binding molecules or multi-domain specific binding molecules of the invention may be present in a nucleic acid construct. Such nucleic acid constructs may be in the form of a vector, for example, an expression vector, and may include, among

others, chromosomal, episomal and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculo-viruses, papova-viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Generally, any vector suitable to maintain, propagate or express nucleic acid to express a polypeptide in a host, may be used for expression in this regard.

The nucleic acid construct may suitably include a promoter or other regulatory sequence which controls expression of the nucleic acid. Promoters and other regulatory sequences which control expression of a nucleic acid have been identified and are known in the art. The person skilled in the art will note that it may not be necessary to utilise the whole promoter or other regulatory sequence. Only the minimum essential regulatory element may be required and, in fact, such elements can be used to construct chimeric sequences or other promoters. The essential requirement is, of course, to retain the tissue and/or temporal specificity. The promoter may be any suitable known promoter, for example, the human cytomegalovirus (CMV) promoter, the CMV immediate early promoter, the HSV thymidine kinase, the early and late SV40 promoters or the promoters of retroviral LTRs, such as those of the Rous Sarcoma virus (RSV) and metallothionine promoters such as the mouse metallothionine-I promoter. The promoter may comprise the minimum comprised for promoter activity (such as a TATA element, optionally without enhancer element) for example, the minimum sequence of the CMV promoter. Preferably, the promoter is contiguous to the nucleic acid sequence.

As stated herein, the nucleic acid construct may be in the form of a vector. Vectors frequently include one or more expression markers which enable selection of cells transfected (or transformed) with them, and preferably, to enable a selection of cells containing vectors incorporating heterologous DNA. A suitable start and stop signal will generally be present.

The vector may be any suitable expression vector, such as pET. The vector may include such additional control sequences as desired, for example selectable markers (e.g. antibiotic resistance, fluorescence, etc.), transcriptional control sequences and promoters, including initiation and termination sequences.

The promoter may be any suitable promoter for causing expression of the protein encoded by a nucleic acid sequence of the invention, e.g. a CMV promoter, human phosphoglycerate kinase (hPGK) promoter.

Such vectors may be present in a host cell. Representative examples of appropriate host cells for expression of the nucleic acid construct of the invention include virus packaging cells which allow encapsulation of the nucleic acid into a viral vector; bacterial cells, such as *Streptococci, Staphylococci, E. coli, Streptomyces* and *Bacillus subtilis*; single cells, such as yeast cells, for

example, *Saccharomyces cerevisiae*, and *Aspergillus* cells; insect cells such as *Drosophila S2* and *Spodoptera* Sf9 cells, animal cells such as CHO, COS, C127, 3T3, PHK.293, and Bowes Melanoma cells and other suitable human cells; and plant cells e.g. *Arabidopsis thaliana*. Suitably, the host cell is a eukaryotic cell, such as a CHO cell or a HEK293 cell.

Introduction of an expression vector into the host cell can be achieved by calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic – lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Sambrook et al, Molecular Cloning, a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Mature proteins can be expressed in host cells, including mammalian cells such as CHO cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can be employed to produce such proteins using RNAs derived from the nucleic acid construct of the third aspect of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al, Molecular Cloning, a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

The invention also provides a host cell comprising any of the polynucleotides and/or vectors of the invention described herein. According to the invention, there is provided a process for the production of an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention, comprising the step of expressing a nucleic acid sequence encoding said molecule in a suitable host cell as defined herein.

Proteins can be recovered and purified from recombinant cell cultures by standard methods including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, lectin and/or heparin chromatography. For therapy, the nucleic acid construct, e.g. in the form of a recombinant vector, may be purified by techniques known in the art, such as by means of column chromatography as described in Sambrook *et al*, Molecular Cloning, a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

This aspect of the invention therefore extends to processes for preparing a fusion protein of the invention comprising production of the fusion protein recombinantly by expression in a host cell, purification of the expressed fusion protein by means of peptide bond linkage, hydrogen or salt bond or chemical cross linking. In some embodiments of this aspect of the invention, the fusion protein could be prepared using hydrogen or salt bonds where the peptide is capable or multimerisation, for example dimerisation or trimerisation.

The antigen specific antigen binding molecule or multi-domain specific binding molecule may comprise additional N-terminal or C-terminal sequences which are cleaved off prior to use which may assist in purification and/or isolation during processes for the production of the molecule as described herein. For example, $(Ala)_3(His)_6$ at the C-terminal end of the molecule.

Also included within the invention are variants, analogues, derivatives and fragments having the amino acid sequence of the protein in which several e.g. 5 to 10, or 1 to 5, or 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the protein of the present invention. Also especially preferred in this regard are conservative substitutions where the properties of a protein of the present invention are preserved in the variant form compared to the original form. Variants also include fusion proteins comprising an antigen specific antigen binding molecule according to the invention.

As discussed above, an example of a variant of the present invention includes a protein in which there is a substitution of one or more amino acids with one or more other amino acids. The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a substance can often be substituted by one or more other such amino acids without interfering with or eliminating a desired activity of that substance. Such substitutions may be referred to as “non-conservative” amino acid substitutions.

Thus the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains). Substitutions of this nature are often referred to as “conservative” or “semi-conservative” amino acid substitutions.

Amino acid deletions or insertions may also be made relative to the amino acid sequence for the fusion protein referred to above. Thus, for example, amino acids which do not have a substantial effect on the activity of the polypeptide, or at least which do not eliminate such activity, may be deleted. Such deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining activity. This can enable the amount of polypeptide required for a particular purpose to be reduced - for example, dosage levels can be reduced.

Amino acid insertions relative to the sequence of the fusion protein above can also be made. This may be done to alter the properties of a substance of the present invention (e.g. to assist in identification, purification or expression, as explained above in relation to fusion proteins).

Amino acid changes relative to the sequence for the fusion protein of the invention can be made using any suitable technique e.g. by using site-directed mutagenesis.

It should be appreciated that amino acid substitutions or insertions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids. Whether or not natural or synthetic amino acids are used, it is preferred that only L- amino acids are present.

A protein according to the invention may have additional N-terminal and/or C-terminal amino acid sequences. Such sequences can be provided for various reasons, for example, glycosylation.

A fusion protein may comprise an antigen specific antigen binding molecule of the present invention fused to a heterologous peptide or protein sequence providing a structural element to the fusion protein. In other embodiments, the fusion protein may comprise an antigen specific antigen binding molecule of the present invention fused with a molecule having biological activity. The molecule may be a peptide or protein sequence, or another biologically active molecule.

For example, the antigen specific antigen binding molecule may be fused to a heterologous peptide sequence which may be a poly-amino acid sequence, for example a plurality of histidine residues or a plurality of lysine residues (suitably 2, 3, 4, 5, or 6 residues), or an immunoglobulin domain (for example an Fc domain).

References to heterologous peptides sequences include sequences from other mammalian species, such as murine and human and any heterologous peptides sequences originated from other VNAR domains.

Where the fusion protein comprises an antigen specific antigen binding molecule of the present invention fused with a molecule having biological activity, a biologically active moiety may be a peptide or protein having biological activity such as an enzyme, immunoglobulin, cytokine or a fragment thereof. Alternatively, the biologically active molecule may be an antibiotic, an anti-cancer drug, an NSAID, a steroid, an analgesic, a toxin or other pharmaceutically active agent. Anti-cancer drugs may include cytotoxic or cytostatic drugs.

In some embodiments, the fusion protein may comprise an antigen specific antigen binding molecule of the invention fused to another immunoglobulin variable or constant region, or another antigen specific antigen binding molecule of the invention. In other words, fusions of antigen specific antigen binding molecules of the invention of variable length, e.g. dimers, trimers, tetramers, or higher

multimer (i.e. pentamers, hexamers, heptamers octamers, nonamers, or decamers, or greater). In specific embodiments this can be represented as a multimer of monomer VNAR subunits.

In fusion proteins of the present invention, the antigen specific antigen binding molecule may be directly fused or linked via a linker moiety to the other elements of the fusion protein. The linker may be a peptide, peptide nucleic acid, or polyamide linkage. Suitable peptide linkers may include a plurality of amino acid residues, for example, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 25 amino acids., such as $(Gly)_4$, $(Gly)_5$, $(Gly)_4Ser$, $(Gly)_4(Ser)(Gly)_4$, or combinations thereof or a multimer thereof (for example a dimer, a trimer, or a tetramer, or greater). For example, a suitable linker may be $(GGGGS)_3$. Alternative linkers include $(Ala)_3(His)_6$ or multimers thereof. Also included is a sequence which has at least 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, using the default parameters of the BLAST computer program provided by HGMP, thereto.

Vectors constructed as described in accordance with the invention are introduced into a host cell for amplification and/or expression. Vectors can be introduced into host cells using standard transformation methods including electroporation, calcium phosphate precipitation and the like. If the vector is an infectious particle such as a virus, the vector itself provides for entry into the host cell. Transfection of host cells containing a replicable expression vector which encodes the gene fusion and production of phage particles according to standard procedures provides phage particles in which the fusion protein is displayed on the surface of the phage particle.

Replicable expression vectors are introduced into host cells using a variety of methods. In one embodiment, vectors can be introduced into cells using: Cells are grown in culture in standard culture broth, optionally for about 6-48 hours (or to OD600 = 0.6-0.8) at about 37°C, and then the broth is centrifuged and the supernatant removed (e.g. decanted). Initial purification is preferably by resuspending the cell pellet in a buffer solution (e.g. 1.0 mM HEPES pH 7.4) followed by recentrifugation and removal of supernatant. The resulting cell pellet is resuspended in dilute glycerol (e.g. 5-20% v/v) and again recentrifuged to form a cell pellet and the supernatant removed. The final cell concentration is obtained by resuspending the cell pellet in water or dilute glycerol to the desired concentration.

The use of higher DNA concentrations during electroporation (about 10x) increases the transformation efficiency and increases the amount of DNA transformed into the host cells. The use of high cell concentrations also increases the efficiency (about 10x). The larger amount of transferred DNA produces larger libraries having greater diversity and representing a greater number of unique members of a combinatorial library. Transformed cells are generally selected by growth on antibiotic containing medium.

Pharmaceutical compositions and uses

According to the invention, there is provided a pharmaceutical composition of antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention. Such compositions include fusion proteins comprising said antigen specific antigen binding molecules.

The pharmaceutical composition may also comprise an antigen specific antigen binding molecule of the present invention fused to a therapeutic protein, or a fragment thereof. The therapeutic protein may be a hormone, a growth factor (e.g. TGFβ, epidermal growth factor (EGF), platelet derived growth factor (PDGF), nerve growth factor (NGF), colony stimulating factor (CSF), hepatocyte growth factor, insulin-like growth factor, placenta growth factor); a differentiation factor; a blood clotting factor (for example, Factor VIIa, Factor VIII, Factor IX, VonWillebrand Factor or Protein C) or another protein from the blood coagulation cascade (for example, antithrombin); a cytokine e.g. an interleukin, (e.g. IL1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32 or IL-33 or an interferon (e.g. IFN-α, IFN-β and IFN-γ), tumour necrosis factor (TNF), IFN-γ inducing factor (IGIF), a bone morphogenetic protein (BMP, e.g. BMP-1, BMP-2, BMP-3, BMP-4, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP10, BMP-11, BMP-12, BMP-13); an interleukin receptor antagonist (e.g. IL-1ra, IL-1RII); a chemokine (e.g. MIPs (Macrophage Inflammatory Proteins) e.g. MIP1α and MIP1β; MCPs (Monocyte Chemotactic Proteins) e.g. MCP1, 2 or 3; RANTES (regulated upon activation normal T-cell expressed and secreted)); a trophic factor; a cytokine inhibitor; a cytokine receptor; an enzyme, for example a free-radical scavenging enzyme e.g. superoxide dismutase or catalase or a pro-drug converting enzyme (e.g. angiotensin converting enzyme, deaminases, dehydrogenases, reductases, kinases and phosphatases); a peptide mimetic; a protease inhibitor; a tissue inhibitor of metalloproteinases (TIMPs e.g. TIMP1, TIMP2, TIMP3 or TIMP4) or a serpin (inhibitors of serine proteases).

In other embodiments of the invention, the therapeutic protein in the fusion protein may be an antibody, or a engineered fragment thereof, including Fab, Fc, $F(ab')_2$ (including chemically linked $F(ab')_2$ chains), Fab', scFv (including multimer forms thereof, i.e. di-scFv, or tri-scFv), sdAb, or BiTE (bi-specific T-cell engager). Antibody fragments also include variable domains and fragments thereof, as well as other VNAR type fragments (IgNAR molecules).

The pharmaceutical composition may be composed of a number of antigen specific antigen binding molecules of the invention, for example dimers, trimers, or higher order multimers, i.e. 2, 3, 4, 5, 6, 7, or 8-mers, fused to the therapeutic protein.

The fusion of the antigen specific antigen binding molecules of the invention to the therapeutic protein may at any convenient site on the protein and may be N-, C- and/or N-/C-terminal fusion(s). In one embodiment of the invention, the fusion of the antigen specific antigen binding molecules of the invention is to both the N- and C- terminals of a therapeutic protein.

Pharmaceutical compositions of the invention may comprise any suitable and pharmaceutically acceptable carrier, diluent, adjuvant or buffer solution. The composition may comprise a further pharmaceutically active agent. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, liposomes, water, glycerol, ethanol and combinations thereof.

Such compositions may comprise a further pharmaceutically active agent as indicated. The additional agents may be therapeutic compounds, e.g. anti-inflammatory drugs, cytotoxic agents, cytostatic agents or antibiotics. Such additional agents may be present in a form suitable for administration to patient in need thereof and such administration may be simultaneous, separate or sequential. The components may be prepared in the form of a kit which may comprise instructions as appropriate.

The pharmaceutical compositions may be administered in any effective, convenient manner effective for treating a patient's disease including, for instance, administration by oral, topical, intravenous, intramuscular, intranasal, or intradermal routes among others. In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

For administration to mammals, and particularly humans, it is expected that the daily dosage of the active agent will be from 0.01mg/kg body weight, typically around 1mg/kg, 2mg/kg or up to 4mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual which will be dependent on factors including the age, weight, sex and response of the individual. The above dosages are exemplary of the average case. There can, of course, be instances where higher or lower dosages are merited, and such are within the scope of this invention.

According to the invention, there is provided an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention for use in medicine. This aspect of the invention therefore extends to the use of such of an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention in the manufacture of a medicament for the treatment of a disease in a patient in need thereof. An antigen specific antigen binding molecule of the invention can also be used to prepare a fusion protein comprising such a specific binding molecule as defined above in relation to pharmaceutical compositions of the invention.

Such uses also embrace methods of treatment of diseases in patients in need of treatment comprising administration to the patient of a therapeutically effective dosage of a pharmaceutical composition as defined herein comprising an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention.

As used herein, the term "treatment" includes any regime that can benefit a human or a non-human animal. The treatment of "non-human animals" in veterinary medicine extends to the treatment of domestic animals, including horses and companion animals (e.g. cats and dogs) and farm/agricultural

animals including members of the ovine, caprine, porcine, bovine and equine families. The treatment may be a therapeutic treatment in respect of any existing condition or disorder, or may be prophylactic (preventive treatment). The treatment may be of an inherited or an acquired disease. The treatment may be of an acute or chronic condition. The treatment may be of a condition/disorder associated with inflammation and/or cancer. The antigen specific antigen binding molecules or multi-domain specific binding molecules of the invention may be used in the treatment of a disorder, including, but not limited to osteoarthritis, scleroderma, renal disease, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, atherosclerosis, or any inflammatory disease.

The antigen specific antigen binding molecules or multi-domain specific binding molecules of the present invention may also be used to investigate the nature of a disease condition in a patient. The antigen specific antigen binding molecules or multi-domain specific binding molecules may be used to prepare images of sites of disease in the body of a subject using imaging techniques such as X-ray, gamma-ray, or PET scanning, or similar. The invention may therefore extend to a method of imaging a site of disease in a subject, comprising administration of a suitably detectably labeled antigen specific antigen binding molecule or multi-domain specific binding molecules to a subject and scanning the subject's body subsequently. Alternatively, administration of said molecules to a subject may provide for a test result by analysing a sample from the subject following administration of the molecule. Such embodiments may include a method of diagnosis of a disease or medical condition in a subject comprising administration of an antigen specific antigen binding molecule or multi-domain specific binding molecule of the invention. The multi-domain specific binding molecules of the invention may be especially useful with regard to diagnostic sensitivity, in particular when multiple VNARs that target different epitopes on the same antigen are used.

Measurement of binding

Detection and measurement of binding of a VNAR to a target can be measured in a number of ways well known in the art including ELISA and surface plasmon resonance.

Functional activity

VNARs of the invention may function in a number of ways including binding to and neutralizing the biological effects of a molecule such as a cytokine, binding to a receptor preventing ligand binding or causing a biological effect post-binding.

Methods of measuring the functional activity of a binding domain are known in the art.

The present invention is illustrated in further details by the following non-limiting examples.

EXAMPLE 1: Isolation of specific antigen binding VNARs

A. TNF binding VNARs

Immunization and selection

Nurse sharks *[Ginglymostoma cirratum]* were placed in containers containing artificial sea water containing 0.1 % (w/v) tricaine methanesulfonate [MS-222]. Following attainment of desired level of narcosis, they were removed for immunisation or bleeding. hTNFα [250 µg] emulsified in complete Freund's adjuvant [CFA] was injected using a 20 gauge needle into the lateral fin of the shark. Boosts were given at 4 week intervals intravenously into the caudal vein as soluble antigen in Phosphate buffered saline (PBS) [sample 0.45 µM sterile filtered]. Blood samples were collected from the caudal vein into a 30 ml syringe containing 200 µl porcine heparin [1000 U/ml in PBS]. Blood samples were spun at 2000 rpm for 10 min to separate blood cells from plasma. The plasma supernatant fraction was carefully removed into a sterile tube with RNA stabilisation buffer, stored at – 80 °C.

Detection of hTNFα specific IgNAR in shark serum

An ELISA plate was coated with 1 µg/ml rhTNFα, incubated at 37 °C for 1 h followed by blocking in 4 % (w/v) MPBS for 1 h at 37 °C. Shark sera [pre-bleed, bleed 4 and 5] were added to designated wells in a 1:2 dilution series and incubated for 1 h at 37 °C. The plate was incubated with 100 µl/well of purified anti-Nurse shark IgNAR monoclonal antibody [GA8] at a dilution of 1:200 in PBS. Binding signal was generated by the addition of anti-mouse IgG-HRP at a dilution of 1:2000 in 0.1% (v/v) Tween-20 PBS (PBST), incubated at room temperature for 1 h. The plate was washed 3x with PBST after every step, and a further 3x PBS after incubation with anti-mouse IgG-horseradish peroxidase (HRP) conjugated antibody [Sigma]. The plate developed by adding SureBlue TMB Microwell Peroxidase Substrate [Thermo Scientific], the reaction stopped with 1 M H_2SO_4 and absorbance measured at 450 nm wavelength using a microplate reader.

rhTNFα specific IgNAR response following each immunisation boost was measured by binding ELISA using sera obtained after each boost. GA8, a mouse monoclonal anti-Nurse shark IgNAR antibody, diluted as hybridoma tissue culture supernatant in PBS was used as the detection antibody (Haines *et al*., 2005; Müller, *et al*. 2012). Result showed a convincing trend of IgNAR increase over time following immunisation as shown in bleeds 4 and 5, also a background response seen in the pre-bleed sample suggest no significant rhTNFα-specific IgNAR response prior to immunisation [Figure 1].

Total RNA isolation from PBLs and PCR amplification

Peripheral blood lymphocytes [PBLs] were harvested from the plasma of the bleed with the best IgNAR response [Bleed 5] and total RNA prepared. Total RNA from the harvested PBLs was used at

approximately 2 µg/µl as template for cDNA synthesis using Superscript III First strand synthesis supermix [Invitrogen]. cDNA was generated with the framework specific primers NARF4For1 [5'-ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG ACA GTG CCA CCT C-3'] (SEQ ID NO. 74) and NARF4For2 [5'-ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG GCA GTG CCA TCT C-3'] (SEQ ID NO. 75) (see Dooley, H., *et al*, *Mol. Immunol*, 2003. **40**(1): p. 25-33). Following cDNA synthesis, the common framework one specific primer NARF1Rev [5'-ATA ATA AGG AAT TCC ATG GCT CGA GTG GAC CAA ACA CCG-3'] (SEQ ID NO. 76) was introduced and IgNAR V region DNA amplified using a 3-step polymerase chain reaction (PCR) amplification protocol. The resultant PCR product of approximately 400 base pairs was ran on 1.5% agarose gel, and NAR V region cut out and purified [QIAquick purification kit, QIAGEN]. Purified DNA was digested at the primer-encoded restriction sites [underlined] with the restriction enzymes *NcoI* and *NotI* [New England Biolabs], and re-purified.

Library construction

The Phagemid vector pHEN2 was digested with the restriction enzymes *NcoI* and *NotI*, PCR purified [QIAquick PCR purification] and ligated to similarly prepared PCR product. Ligated material was transformed into Electroporation-competent *E. coli* TG1 cells [Lucigen]. Transformed cells were plated on TYE agar plates containing 2 % glucose [w/v], 100 µg/ml ampicillin and grown overnight at 37 °C. Library size was calculated and colonies scraped from plates and aliquots of the library stock stored at – 80 °C.

Phage display selection

A single aliquot of library stock equivalent to OD_{600} of 0.1 was added to 2 x TY growth media containing 2 % glucose [w/v], 100 µg/ml ampicillin, and grown at 37 °C to mid-log phase [OD_{600} of 0.4 to 0.6] prior to infection with M13K07 helper phage [New England Biolabs]. Library expression was conducted overnight in 2 x TY media, 0.2 % glucose, 100 µg/ml ampicillin and 50 µg/ml kanamycin at 30 °C. Phage were precipitated from the culture supernatant with polyethylene glycol (PEG) and used for bio-panning. The library was panned against biotinylated rhTNFα captured on Dynabeads® M-280 streptavidin beads [Dynabeads, Invitrogen]. Library phage and Dynabeads® M-280 streptavidin were separately pre-blocked with block solution [3 % (w/v) milk, 1 % (w/v) BSA in PBS] for 1 h, rotating at room temperature. Biotinylated-rhTNFα [400 nM] was added to blocked beads and incubated for 1 h, rotating at room temperature. In a different tube, library phage was incubated with previously blocked streptavidin beads for 1 h rotating at room temperature. Unbound phage was recovered using the Dynabeads magnetic rack and recovered phage is here-in referred to as deselected phage. Phage were deselected by incubating with blocked beads, 1 h rotating at room temperature. Biotin-rhTNFα decorated beads were incubated with deselected phage for 1 h, rotating at room temperature. Beads were washed 5x PBST and 5x PBS prior to a strict 8 min elution with 400 µl of 100 mM Triethylamine (TEA), and neutralised by adding 200 µl of 1 M Tris-HCl pH 7.5. Mid-log phase *E. coli* TG1 cells [10

ml] were infected with 400 µl eluted phage for 30 min, at 37 °C. Then grown overnight at 37 °C on TYE agar plates containing 2 % glucose (w/v), 100 µg/ml ampicillin. Three further rounds of selection were conducted and stringency was increased in round 3 and 4 by reducing the concentration of biotin-rhTNFα to 200 nM.

Screening and selection of clones

Enrichment of antigen binding monoclonal phage was evaluated using ELISA plates coated with 1 µg/ml rhTNFα, blocked with 4 % [w/v] Milk-PBS. Binding was detected with anti-M13-HRP conjugated monoclonal antibody [GE Healthcare]. Also monoclonal phage was analysed for selectivity and specificity against Streptavidin and HSA coated ELISA plates respectively.

The library was subjected to four iterative rounds of panning against rhTNFα. The biopanning antigen concentration was kept constant for rounds 1 and 2 but was reduced by half for subsequent rounds of panning in a bid to favour high-affinity binders. Enrichment of positive monoclonal phage binders were evaluated at the end of each round of biopanning for rhTNFα binding by ELISA. A steady increase in antigen binding was observed from pre-selected clones through round 2, with a drop in the number of monoclonal phage binders after rounds 3 and 4. rhTNFα monoclonal binders increased from about 6 % [11/184] in round 0 [pre-selected library] to 99.46 % [183/184] following round 2.

A number of unique sequences were identified from the library panning. These include VNARs named D1, C4 and B4.

	FW1	CDR1	FW2	HV2
D1	ARVDQTPQTITKETGESLTINCVLRDS	HCATSS	TYWYRKKSGS	TNEESISKG
C4	ARVDQTPQTITKETGESLTINCVLRDS	NCGLSS	TYWYRKKSGS	TNEESISKG
B4	ARVDQTPQTITKETGESLTINCVLRDS	NCALSS	MYWYRKKSGS	TNEESISKG

	FW3a	HV4	FW3b
D1	GRYVETVN	SGSKS	FSLRINDLTVEDSGTYRCAS
C4	GRYVETIN	EGSKS	FSLRINDLTVEDSGTYRCKL
B4	GRYVETVN	SGSKS	FSLRINDLTVEDSGTYRCKV

	CDR3	FW4	
D1	ECQYGLAEY_____DV	YGGGTVVTVN	SEQ ID NO 2
C4	SWWTQNWRCSNS_____DV	YGGGTVVTVN	SEQ ID NO 7
B4	YIPCIDELVYMISGGTSGPIH_DV	YGGGTVVTVN	SEQ ID NO 12

The Cysteine (C) residues in CDR1 and CDR3 (double underlined) are typical of Type II VNARs and are observed to form a second disulphide bridge in addition to the canonical Immunoglobulin superfamily bridge between the Cysteines in FW1 and FW3b (single underlined).

Expression of VNARs that bind to TNFα

Preparation of soluble VNAR protein in cytoplasm of SHuffle cells

The IgNAR V region inserts of interest identified from the monoclonal phage screening were cloned into the expression vector pET28b (+) (Novagen) *via* the *XbaI* and *EcoRI* restriction enzyme sites. VNAR DNA was prepared from *E. coli* TG1 culture (using QIAprep miniprep kit, QIAGEN) and PCR amplified using in-house designed primer pair XbaI_NARFW1_#127 (SEQ ID 26) and EcoRI_stop_myc_#129 (SEQ ID 29) introducing cloning sites *Xba*I and *EcoR*I respectively, while primer SEQ ID 29 incorporated c-myc, 6x Histidine tags and a stop codon into the VNAR gene sequence. Purified VNAR DNA PCR product and pET28b (+) plasmid DNA were digested at 37 ºC, 2 h with 50 U *Xba*I and 10 U *EcoR*I-HF. Digested samples were purified, ligated and transformed into electrocompetent *E. coli* SHuffle® T7 Express cells [New England Biolabs], and selected on TYE agar plates containing 50 µg/ml kanamycin. The VNAR anti-hTNFα –D1, C4 and B4 fusion proteins were expressed in the cytoplasm of SHuffle® cells upon induction with IPTG at 30 °C. Cells were harvested by centrifugation, and the cell pellet treated with Bugbuster™ protein extraction reagent [Novagen] to lyse cells and release soluble protein. The VNAR soluble protein was purified by immobilised metal affinity chromatography [IMAC] *via* the hexa-histidine tail, and eluted from IMAC resin with 500 mM Imidazole, pH 8. Protein samples were dialysed against PBS, pH 7.4 before use. Protein concentration was determined using Ultraspec 6300 pro UV/Visible spectrophotometer [Amersham, Biosciences]. Total purified protein was visualised on Coomassie blue stained SDS-PAGE. The purified VNAR monomeric protein migrated as a single band of approximately 14 kDa [including hexa-histidine and c-myc tags] with no evidence of protein aggregation. Purity was estimated to be about 90 % based on an SDS-PAGE gel

Determination of protein integrity and purity

Denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE] was used to assess purified protein purity and size. Protein samples were prepared in NuPAGE® LDS sample buffer [Life Technologies] containing 5 % β-mercaptoethanol and heated to 95 °C for 5 min. Denatured protein samples were loaded onto NuPAGE® 4 – 12 % Bis-Tris Gel [Life Technologies] immersed in a MES SDS running buffer, and electrophoresis carried out at 160 volts, for 55 min. A Full Range recombinant protein molecular weight marker [GE Healthcare] was used as molecular weight ladder standard. The gel was washed in distilled water, and stained with Coomassie blue for 1 h followed by an overnight de-staining process in distilled water.

Determination of selectivity and specificity

Specificity and selectivity of binding was determined on ELISA plates coated with either 1µg/ml Biotin-TNF and rhTNFα, or 10 µg/ml HSA, BSA, streptavidin, single stranded DNA, thyroglobulin or lysozyme. ELISA plates were suitably blocked in 4 % [w/v] Milk-PBS, and protein samples loaded at a top concentration of 1 µg/ml and serial dilution performed. Binding was detected with an anti-c-myc-HRP conjugated monoclonal antibody [Roche].

To obtain more accurate binding data certain molecules were also measured using surface plasmon resonance with BIACore T200 or Octet RED96 instruments.

BIACore™ T200 (GE Healthcare)

Amine coupling is a very common approach for immobilising the ligand to the chip surface. The chip surface has a dextran matrix derivatised with carboxyl groups, which after activation with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), form reactive succinimide esters which allows the covalent capturing of the ligand via any available primary amine groups (e.g. Lysine) on the ligand.
TNFα was diluted 1/10 in 10mM Sodium acetate buffer pH 5.5 and injected unto the activated chip. An "aim for" software immobilisation wizard or a specific time period may be used to aim-for 200 RU of immobilised TNFα. In addition the run buffer was changed to PBS in absence of 0.05% Tween20 as it was thought perhaps the detergent may affect ligand activity. A final ligand immobilised level of 202 RU was obtained.
Start-up cycles were composed of a 60s buffer injection at a flow rate of 30µl/min followed by a 30s dissociation period. The anti-TNFα sample cycles included a 120s injection of sample at 30µl/min followed by a regeneration step of a 60s injection of 10mM glycine pH2 at 30µ/min. Finally, a 120s stabilisation period was included at the end of each cycle to allow for baseline equilibration prior to beginning the next cycle.
The concentration series screened and the dissociation periods were variable and were as follows: All samples were assayed at a top start concentration of 100 nM and a 1200s dissociation time, with the exception of all monomeric domains D1, C4, B4, TNF43 and TNF30 where the dissociation time was 600s. B4 and TNF43 VNAR were assayed at top start concentrations of 500nM and 5µM respectively. 5 blank sample cycles were included to be used to generate double referenced datasets.
Binding responses for domain were analysed using the BIACore™ T200 evaluation software and double referenced data was fitted to a 1: 1 Langmuir model to obtain kinetic and affinity characterisation.

OCTET® RED96 [ForteBio™]

Biolayer interferometry (BLI) was used to determine the equilibrium dissociation constant (K_D). Dip and read streptavidin biosensors were rehydrated for at least 30 min in PBS, pH 7.4. Sensors were loaded with 20 µg/ml biotinylated hTNF-α and anti-TNF-α VNAR proteins were serially diluted with top concentration of 100 nM while TNF43 and VNAR negative controls were assayed at top concentration of 1 µM. Binding association was monitored for 10 min followed by a 5 min dissociation time. For all anti-TNF-α VNAR measurements, kinetic data sets were fitted using a two-site model since the curve fit data showed complex multiphasic curves, however for the control anti-TNF-α nanobody, TNF30 a 1:1 Langmuir binding with Mass Transport model was used.

The data obtained is shown in TABLE 1. TABLE 1 indicates that the monomer VNARs tested have at least a 500 fold lower binding affinity for TNFα compared with the TNF 43 VNAR .

Binding molecule	**K_a (M^{-1} S^{-1})**	**K_d (S^{-1})**	**KD [BIAcore T200]**	**KD [Octet ForteBio]**
D1	1.2 E+03	2.06E- 02	**50 nM**	**1.9 nM**
C4	2.8 E+05	3.3 E - 02	**70 nM**	**6.4 nM**
TNF30 VHH	5 E+04	1.6E - 07	**16 nM**	**N/A**
TNF43 VNAR	Very Weak affinity- No binding data obtained. Highest conc tested 500 nM			**> 1000nM**
BB10	Negative control- No binding data obtained			**>1000 nM**
D1-D1	5E+05	3.16E-04	**n/a**	**0.6 nM**
D1-C4	1.8E+05	1.07E-04	**5 nM**	**0.17 nM**
D1-B4	2.7E+05	5E-04	**n/a**	**15.9 nM**
TNF30-TNF30	3E+04	3E-05	**n/a**	**0.4 nM**
D1-BA11-D1	1.9E+06	2E-04	**4 nM**	**0.1 nM**
D1-BA11-C4	2E+05	1.6E-04	**0.6 nM**	**0.13 nM**
D1-BA11-B4	1.7E+06	6E-03	**n/a**	**0.33 nM**
TNF30-BA11-TNF30	9E+04	1.5E-05	**0.4 nM**	**0.38 nM**

TABLE 1 TNF VNAR SPR binding data

In vitro Neutralisation assay

To determine the neutralisation capacity and ND_{50} for the VNAR domains, mouse fibrosarcoma cell line L929 [ATCC, CCL-1] was grown in Dulbecco modified eagle medium [GIBCO] supplemented with 10 % heat inactivated fetal bovine serum [GIBCO] and 1µg/ml actinomycin D [R & D systems]. For each VNAR clone 5,000 cells per well were incubated in a 96 well plate in duplicate for 24 h at 37 °C with 5 % CO_2 and humidity. LD_{50} [1x at 0.25 ng/ml] and 10x LD_{50} [2.5 ng/ml] of rhTNFα was added to wells containing either VNAR proteins serially diluted or cells alone. Plates were then incubated for 24

h at 37 °C with 5 % CO_2 and humidity. Cytotoxicity or cell survival was measured by adding 50 µl of 1:20 dilution WST-1 cell proliferation reagent [Roche], and incubated for 4-8 h at 37 °C with 5 % CO_2 and humidity. Absorbance was read at 450-560 nm.

TNFα in the presence of 1 µg/ml actinomycin D causes cytotoxicity in L929 fibrosarcoma cells, with an LD_{50} between 0.25 – 0.3 ng/ml. We demonstrated that our VNAR protein domains at nanomolar concentrations were capable of neutralising up to ten times the LD_{50} of rhTNFα [Figure 3]. In this experiment the VNARs were joined at their C terminal end by peptide linkers to the IgG Fc domains so as to form bivalent molecules for comparison to the control anti-TNFα antibody MAB210.

When measured as single domains in the neutralization assay the D1 and C4 VNARs did not appear as efficacious as the TNF30 VHH nanobody (Figure 2). This appears to correlate with single site binding affinity. However when combined as a mixture (Figure 3) or in bivalent or bispecific formats (Figures xxxx) they unexpectedly demonstrated improved properties over dimeric TNF30 VHH nanobody.

Paracellular flux assay

Human epithelial colorectal adenocarcinoma cells (Caco-2) were cultured in DMEM supplemented with 10 (% v/v) heat inactivated FBS and 1 % (v/v) Penicillin-Streptomycin (10,000 units/ml and 10,000 µg/ml respectively). Cells were grown to 90 % confluence in T75 flasks before seeding on 24 wells, 0.4 µm semipermeable tissue culture transwell inserts (Corning Inc.). Viable cells number was determined by suspending 10 µl cell suspension in 90 µl of 0.4 % trypan blue exclusion dye (Beckman Coulter), and carefully transferring the mixture onto a haemocytometer with a cover slip attached.

Following viable cell number determination, 1 x 10^5 cells were seeded per transwell inserts in a final DMEM volume of 100 µl, while 600 µl DMEM without cells was transferred into the outer containing wells. Transwell plates were incubated at 37 ºC with 5 % (v/v) CO_2, and spent DMEM + 10 % (v/v) FBS replaced every 48 h. Cell proliferation was monitored under a phase contrast microscope (40x magnification objective) until cells attain 100 % confluence, usually between 5 – 7 days post-seeding. Caco-2 cells were grown for a further 21 days allowing differentiation, with spent medium changed every 48 h until differentiation.

Designated insert wells containing polarised cells (apical side) in 100 µl DMEM with 10 % (v/v) HI-FBS were treated with 10 ng/ml hTNFα, IFNγ, LPS with or without anti-TNFα VNAR proteins. Treated cells were incubated for 18 h at 37 ºC with 5 % (v/v) CO_2. Following incubation for 18 h with cytokines ± anti-TNFα VNARs, phase contrast images of treated cells were captured followed by the addition of 5 µl of 10 mg/ml Fluorescein isothiocyanate-labelled dextran, molecular weight (3 - 5 kDa) to apical side (insert wells) of Caco-2 monolayer. Medium from the basolateral side of the transwell chamber was collected 24 h after addition of FITC-dextran.

Fluorescence intensity was measured using a Synergy HT (BioTek®) microplate reader at 485 nm excitation and 520 nm emission wavelengths.

Epithelial resistance dysfunction assay

Human epithelial colorectal adenocarcinoma cells (Caco-2) were cultured in DMEM supplemented with 10 (% v/v) heat inactivated FBS and 1 % (v/v) Penicillin-Streptomycin (10,000 units/ml and 10,000 µg/ml respectively). Cells were grown to 90 % confluence in T75 flasks before seeding on 12 or 24 wells, 0.4 µm semipermeable tissue culture transwell inserts (Corning Inc.). The protocol described previously was followed until cells achieved full differentiation.

Designated insert wells containing polarised cells (apical side) in 200 µl DMEM with 10 % (v/v) HI-FBS were treated with 10 ng/ml hTNFα, and IFNγ with or without anti-TNFα VNAR proteins. Treated cells were incubated for 24 h at 37 ºC with 5 % (v/v) CO_2, and humidity. Following incubation for 24 h with cytokines ± anti-TNFα VNARs, transepithelial electrical resistance (TEER) was measured in the apical chamber using Millicell® ERS-2 Epithelial (Volt/Ohm) meter and MERSSTX01 probe (Merck Millipore). Measured resistance values were normalised to the surface area under treatment.

It is important to note that 12 well tissue culture transwell inserts were seeded with 5 x 10^6 cells / well containing 500 µl DMEM with outer well (basolateral side) containing 1.5 ml DMEM. Also during TEER measurement, DMEM volume in the insert and outer wells were increased to 500 µl and 1.5 ml respectively to allow volt-ohm meter electrodes to fully submerge in the medium without touching the base of the wells.

B. ICOSL binding VNARS

The isolation and characterization of ICOSL binding VNARS 2D4 and CC3 are disclosed in WO2014/173975 and WO2014/173959.

2. Formation of multivalent and multispecific VNARs.

A TNF binding domains

Figure 3 indicated that the combination of the D1-Fc and C4-Fc molecules showed increased neutralisation capability in the bivalent form. Therefore VNARs D1 and C4 and other combinations were prepared as bivalent or bispecific fusions to demonstrate that when combined together as fusions the same improvement in neutralisation capacity is seen.

<u>Construction of dimers and trimers</u>

Figure 4 provides a diagram of the format of bivalent and bispecific constructs

Two or three separate PCR reactions were set up to amplify the N-terminal, middle terminal [in the case of a trimer], and C-terminal VNAR domains using the oligonucleotide combinations listed below, and each oligonucleotide habouring a specific/unique cloning site, and/or 6x his-tag and c-myc tag for ease of purification and detection respectively.

Dimer construction PCR oligos:

N-terminal fragment oligonucleotide pair:

XbaI_FW1 TNF_#127: SEQ ID 15
GCTAGGCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCTCGAGTGGACCAA
ACACC

GS_BamHI_Rev_#130: SEQ ID 16
CGCGCCGGATCCGCCACCTCCGCTACCGCCACCTCCGCTACCGCCACCTCCGCTACCGCCACC
TCCATTCACAGTCACGACAGTGCC

C-terminal oligonucleotide pair:
GS_BamHI_For_#132: SEQ ID 17
GGTGGCGGatccGGCGCGCACTCCGCTCGAGTGGACCAAACACCGC

EcoRI_stop_myc_#129: SEQ ID 18
GTCCGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCTGCGGCCCC

Trimer construction PCR oligonucleotides: Here we utilized an in-house designed DNA cassette habouring BA11 gene as the middle fragment flanked by Xba1/BamH1 and APA1/EcoR1 cloning sites on its N- and C- terminals respectively. Oligonucleotide pairs listed above can be utilised in the PCR amplification steps, as well as oligonucleotides habouring both Xba1 and BssH11 site in the N-terminal forward oligonucleotide, thus allowing sub-cloning the trimer gene into an in-house eukaryotic expression vector, pEEE2A. Otherwise all clonings are carried out in pET28b (+) expression vector.

Xba1/BssH11-FW1NAR_#197 [Trimer cassette]: SEQ ID 19
AATTCCCCTCTAGAAGGCGCGCACTCCGCTCGAGTGGACCAAACACCG

A PCR reaction of 2 µl VNAR DNA (50 – 100 ng), 2 µl forward and reverse oligonucleotide primers (final concentration 1 µM), 5 µl of 10X Taq polymerase buffer, 0.25 µl of Taq polymerase (final concentration 25 U/ml), 0.5 µl dNTPs (final concentration 0.1mM), and 38.25 µl H_2O with a final reaction volume of 50 µl. A PCR program was started with 5 min at 98 °C. This was followed by a 30 cycle of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 1 min. A final extension at 72 °C for

5 min. Amplicons were checked by agarose gel electrophoresis, and purified using QIAquick PCR purification kit. Eluted DNA digested with appropriate restriction endonuclease.

Expression of dimers and trimers in *E. coli* SHuffle® T7 Express cells

The VNAR regions cloned into the expression vector pET28b (+) *via* the *XbaI* and *EcoRI* restriction enzyme sites, and resulting purified plasmid containing VNAR gene was transformed into electrocompetent *E. coli* SHuffle® T7 Express cells [NEB], and selected on TYE agar plates containing 50 µg/ml kanamycin. The anti-hTNFα VNAR –D1, C4 and B4 fusion proteins were expressed in the cytoplasm of SHuffle cells upon induction with IPTG at 30 °C.

Single colony of transformed *E. coli* SHuffle® T7 Express cells was grown in 5 ml 2x TY-Kanamycin medium until OD_{600} 0.4 – 0.6 usually achieved between 4 – 6 h incubation at 37 ºC, 250 rpm. This log phase culture was used to inoculate 50 ml TB medium containing kanamycin and PO_4 salts, incubated overnight at 30 ºC, 250 rpm until they attain OD_{600} 6.0 – 10.0. Cells were centrifuged at 4000 rpm, 30 ºC for 15 min, then resuspended in fresh TB-kanamycin-PO_4 salt medium and allowed to recover for 1 – 2 h at 30 ºC, 250 rpm. Cytoplasmic protein expression was induced using a final IPTG concentration of 1 mM, cells incubated at 30 ºC, 200 rpm for 12 – 16 h post-induction. Cells were harvested by centrifugation at 6000 rpm, 25 ºC for 10 min, and cell pellet wet weight determined. Cell pellet was resuspended in 5 ml / gram of wet cell paste BugBuster™ protein extraction reagent plus Benzonase® (Novagen, UK), and cell suspension was placed on a shaking platform at 10 – 15 rpm, room temperature for 20 min. Cell suspension was centrifuged at 6000 rpm, 4 ºC for 20 min, and soluble protein collected in the supernatant was ready for affinity purification *via* polyhistidine tag using immobilised metal affinity chromatography (IMAC) resin (nickel-nitrilotriacetic acid, Ni-NTA or Ni-Sepharose). VNAR fusion protein was eluted with 300 – 500 mM imidazole, pH 8.0 and eluate dialysed against PBS (1 L PBS / 1 ml eluted protein), pH 7.4 overnight and then PBS replaced for a further 3 – 4 h dialysis. Protein quality was assessed via SDS-PAGE and quantified using the Ultraspec 6300 pro uv/visible spectrophotometer (Amersham Biosciences, GE Healthcare).

For eukaryotic cell expression, domains cloned into the BA11 trimer cassette were digested using *BssH*II and *EcoR*1 enzymes, and subcloned into pEEE2A eukaryotic expression vector utilising a CMV promoter, and transformed into an E. coli strain for plasmid propagation. Isolated and purified plasmid vector containing the VNAR trimer gene was co-incubated with linear polyethylenimine [PEI] for 20 minutes at room temperature. The mixture of plasmid DNA: PEI was transferred unto a cell culture flask containing HEK293 cells with cell growth density of 90 % confluence. Transfected HEK293 cells were incubated at 37 °C, 5 % v/v CO_2 for 5 – 7 days. Cell culture supernatant was harvested, and expressed protein purified using IMAC resin, and dialysed against PBS.

Binding and TNF neutralization data

Figure 5 shows ELISA binding of dimeric and bispecific constructs to TNFα.

The initial ELISA data indicated that the bispecfic D1-C4 construct had increased avidity (combined binding affinity) compared to the TNF30 nanobody dimeric construct.

A number of these were later measured for binding to immobilized TNF in surface plasmon resonance.

TABLE 1 indicates that of the dimeric molecules measured, the D1-C4 bispecific molecule showed superior binding affinity (avidity) compared to the TNF30 bivalent nanonbody construct.

Figure 6 shows TNF neutralization data for a number of bivalent or bispecific VNAR fusions, compared to the bivalent TNF nanobody. When the binding molecules were tested for TNF neutralisation in the L929 assay, the D1-C4 dimer was equivalent to or superior to the TNF30 nanobody dimeric construct. In this experiment the D1-D1 dimer was inferior to the D1-B4 dimer.

TABLE 1 Shows SPR binding data for the trimeric constructs tested. This indicates that the introduction of the additional domain, acting as a spacer between the TNFα binding domains, appears significantly to improve the relative affinity (avidity) of the molecules for TNFα.

When measured in the TNFα neutralisation assay, the D1-BA11-C4 trimeric construct was equivalent to adalimumab and superior to the TNF30 nanobody construct. In this assay the bivalent molecule comprising the D1 domains was equivalent in efficacy to the TNF30 nanobody construct.

Figure 7 shows the results of an experiment to measure the ability of the various VNAR formats to neutralize TNFα function.

TABLE 2 summarizes the neutralisation data. When the spacer domain is included both the D1-BA11-D1 and D1-BA11-C4 show a ten-fold or better improvement in neutralisation ability, with the D1-BA11-C4 showing approximately equivalent efficacy to adalimumab and MAB210. The TNF30-BA11-TNF30 also shows an improvement over the TNF30-TNF30 dimeric form but not as markedly. The GlySer linker length of D1-C4 construct (SEQ ID NO 27 & 28) was extended from a (Gly4Ser)2 to a (Gly4Ser)3 with a consequent improvement in hTNF-alpha neutralizing potency.

The data from these experiments are shown in table 2. Further comparative data is given in table 3.

Binding Molecule	**ND_{50} (nM)** **[≥n=3 ± SEM; except otherwise stated]**
TNF43 [Horn Shark VNAR]	7100 nM (Publication: Camacho-Villegas, Tanya, et al. *MAbs* 5(1): 2013; U.S. Patent No. 8,496,933. 30 Jul. 2013)

TNF43 [Horn Shark VNAR]	No neutralisation seen *in vitro* at concentrations up to 500 nM (also see Fig.15 at 100 nM)
D1	30 ± 3.5
C4	100 ± 0.1
TNF30 [VHH]	9.2 ± 2.1
D1-Fc	0.9 ± 0.14
C4-Fc	0.52 ± 0.2
TNF30-Fc	0.7 ± 0.07
D1-D1	7.0 ± 2.4
D1-C4	0.76 ± 0.06
D1-C4 $(Gly_4Ser)_3$	0.08 ± 0.02***(n =2 with 3 replicates each)
D1-B4	8.0 ± 2.5
TNF30- TNF30	0.8 ± 0.27
Adalimumab	0.03 ± 0.009
D1-BA11-D1	0.38 ± 0.03
D1-BA11-C4	0.02 ± 0.09
TNF30-BA11- TNF30	0.3 ± 0.14
D1-Fc-C4 (Quad-X™)	0.002 ± 0.0011
D1-C4-Fc (Quad-Y™)	0.005 ± 0.0005 (n=2 ± SD)
C4-D1-Fc (Quad-Y™)	0.012 ± 0.0016 (n=2 ± SD)

TABLE 2 TNF Neutralisation data using 0.3 ng/ml (LD80) of hTNF-alpha (unless otherwise stated)

A. Binding (B) and Neutralisation (N) data obtained by the inventors

	Human (Bind/Neutralise) B/N	Dog (B/N)	Cynomolgus (B/N)	Rat (B/N)	Mouse (B/N)	Rabbit (B/N)	Pig (B/N)	Human TNF-β (B/N)
Lead anti-hTNF-α VNARs (D1 and C4)	+++/+++	+++/+++	+++/+++	-/-	-/-	-/-	-/-	-/-
Nanobody Lead (VHH) TNF 30	+++/+++	+++/+++	+++/+++	-/-	-/-	-/-	+/+	++/-
Adalimumab (Humira)	+++/+++	+++/+++	+++/+++	-/-	++/++	-/-	-/-	-/-

Note: **+++** denotes strong binding/neutralisation activity, **++** moderate; **+** very weak activity and **–** denotes no binding/neutralisation activity observed.

B. Cross-reactivity data of clinically available anti-hTNF-alpha biologics as reported in the literature [Assessment Report for Cimzia, European Medicines Agency (2009). Doc. Ref.: EMEA/664021/2009; Assessment Report for Simponi. European Medicines Agency (2009). Doc Ref.: EMEA/446762/2009; Assessment Report for Enbrel. European Medicines Agency (2008). Procedure No. EMEA/H/C/262/11/94; Scientific Discussion on Remicade, European Medicines Agency (2005) (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000240/WC500050885.pdf) Last assessed on 21st Sept 2017; Scientific Discussion on Humira, European Medicines Agency (2004). (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000481/WC500050867.pdf). Last assessed on 21st Sept 2017]

	Human (Bind/Neutralise) B/N	Dog (B/N)	Cynomolgus (B/N)	Rat (B/N)	Mouse (B/N)	Rabbit (B/N)	Pig (B/N)	Human TNF-β (B/N)
Adalimumab (Humira)	+/+	+/+	+/+	-/-	+/+	-/-	-/-	-/-
Infliximab (Remicade)	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Etanercept (Enbrel)	+/+	-/-	+/+		+/+			+/+
Certolizumab (Cimzia)	+/+	-/-	+/+					
Golimumab (Simponi)	+/+	+/+	+/+	-/-	-/-	+/+		-/-

Note: +/- denotes yes or no binding/neutralisation respectively.

TABLE 3 Cross-reactivity profile of anti-hTNF-alpha VNAR lead construct compared to commercially available anti-hTNF-alpha mAbs and pre-clinical VHH TNF30

Functional activity

Background

Human epithelial colorectal adenocarcinoma cells (Caco-2) develop morphological characteristics of normal enterocytes when grown on suitable platform (e.g., plastic dishes, nitrocellulose filters). More so collagen coated polycarbonate or polyester membrane have been demonstrated to be suitable for

Caco-2 monolayers as an intestinal epithelial transport model systems (Wang, F., et al. *Am. J. Path.* 166.2 (2005): 409-419.; Hidalgo, I.J., *et al Gastroenterology* 1989. **96:** 736-49.).

A principle function of epithelial membrane is the maintenance of a barrier to hydrophilic solutes such as Inulin and Dextran. This barrier is compromised in certain diseases involving the intestinal epithelium, which include but not limited to infectious, immune-mediated and idiopathic diseases (Clayburgh, D.R., *et al Lab Invest.* 2004. **84(3):** 282-291; Wang, F., et al. *Am. J. Path.* 2005. **166(2):** 409-419). Intestinal barrier dysfunctions, measured as increases in paracellular permeability and reduction of intestinal epithelial resistance are closely associated with inflammatory bowel diseases (IBD), such as Crohn's disease (Irvine E.J. and Marshall J.K., *Gastroenterology* 2000. **119.6**: 1740-1744.; Wyatt et al., *The Lancet* 1993. **341**(8858): 1437-1439.). Also there are evidence supporting the reduction of epithelial tight junction proteins in IBD, consequently contributing to the loss of solutes resulting in leak flux diarrhea (Schulzke J.D. *et al., Ann N Y Acad Sci*. 2009 **1165**:294-300; Schmitz H. *et al., J. Cell Sci* 1999. **112(1)**: 137-146). Finally Interferon-γ (IFN-γ), TNFα and Lipopolysaccharide (LPS) have been shown to synergistically induce intestinal epithelial barrier dysfunction in human epithelial cell lines (Wang et al., *J. Cell Science* 1999. 112(1): 137-146; Schuerer-Maly C.C *et al*., *Immunology* 1994. **81(1)**: 85).

Anti-TNFα treatment have been shown to repair the intestinal barrier dysfunction in Crohn's disease (Suenaert P. *et al., Am J Gastroenterol* 2002. **97**(8): 2000-2004) thus we examined our anti-TNF VNAR domains to demonstrate these would repair these dysfunctions induced *in-vitro*. We hypothesized that bi-specific/multivalent VNAR domains would be more effective in the prevention of these dysfunctions when compared to VNAR monomers.

FITC-Dextran paracellular flux across polarised monolayer of Caco-2 cells

Human epithelial colorectal adenocarcinoma cells (Caco-2) were cultured in DMEM supplemented with 10 (% v/v) heat inactivated FBS and 1 % (v/v) Penicillin-Streptomycin (10,000 units/ml and 10,000 µg/ml respectively). Cells were grown to 90 % confluence in T75 flasks before seeding on 24 wells, 0.4 µm semipermeable tissue culture transwell inserts (Corning Inc.). Viable cells number was determined by suspending 10 µl cell suspension in 90 µl of 0.4 % trypan blue exclusion dye (Beckman Coulter), and carefully transferring the mixture onto a haemocytometer with a cover slip attached.

Following viable cell number determination, 1 x 10^5 cells were seeded per transwell inserts in a final DMEM volume of 100 µl, while 600 µl DMEM without cells was transferred into the outer containing wells. Transwell plates were incubated at 37 ºC with 5 % (v/v) CO_2, and humidity, and spent DMEM + 10 % (v/v) FBS replaced every 48 h. Cell proliferation was monitored under a phase contrast microscope (40x magnification objective) until cells attain 100 % confluence, usually between 5 – 7 days post-seeding. Caco-2 cells are grown for a further 21 days allowing differentiation, with spent medium changed every 48 h until differentiation.

Designated insert wells containing polarised cells (apical side) in 100 µl DMEM with 10 % (v/v) HI-FBS were treated with 10 ng/ml hTNFα, IFN-γ and LPS with or without anti-TNFα VNAR proteins. Treated cells were incubated for 18 h at 37 °C with 5 % (v/v) CO_2, and humidity. Following incubation for 18 h with cytokines ± anti-TNFα VNARs, phase contrast images of treated cells were captured followed by the addition of 5 µl of 10 mg/ml Fluorescein isothiocyanate-labelled dextran, molecular weight (3 - 5 kDa) to apical side (insert wells) of Caco-2 monolayer. Medium from the basolateral side of the transwell chamber was collected 24 h after addition of FITC-dextran.

Fluorescence intensity was measured using a Synergy HT (BioTek®) microplate reader at 485 nm excitation and 520 nm emission wavelengths.

Figure 8 shows the permeability data from an experiment measuring paracellular flux across polarised monolayer of Caco-2 cells comparing several of the TNF VNAR multidomain binding molecules. This experiment shows that the various bivalent and bispecifc forms show improved function over the monomer forms as a lower concentration of dimer or trimer delivered an increased level of protection of challenged cells.

Epithelial resistance dysfunction assay in polarised Caco-2 cell monolayer

Human epithelial colorectal adenocarcinoma cells (Caco-2) were cultured in DMEM supplemented with 10 (% v/v) heat inactivated FBS and 1 % (v/v) Penicillin-Streptomycin (10,000 units/ml and 10,000 µg/ml respectively). Cells were grown to 90 % confluence in T75 flasks before seeding on 12 or 24 wells, 0.4 µm semipermeable tissue culture transwell inserts (Corning Inc.). The protocol described previously in section 0 was followed until cells achieved full differentiation.

Designated insert wells containing polarised cells (apical side) in 200 µl DMEM with 10 % (v/v) HI-FBS were treated with 10 ng/ml hTNFα, and IFN-γ with or without anti-TNFα VNAR proteins. Treated cells were incubated for 24 h at 37 °C with 5 % (v/v) CO_2, and humidity. Following incubation for 24 h with cytokines ± anti-TNFα VNARs, transepithelial electrical resistance (TEER) was measured in the apical chamber using Millicell® ERS-2 Epithelial (Volt/Ohm) meter and MERSSTX01 probes (Merck Millipore). Measured resistance values were normalised to the surface area under treatment.
It is important to note that 12 well tissue culture transwell inserts were seeded with 5 x 10^6 cells / well containing 500 µl DMEM with outer well (basolateral side) containing 1.5 ml DMEM. Also during TEER measurement, DMEM volume in the insert and outer wells were increased to 500 µl and 1.5 ml respectively to allow volt-ohm meter electrodes to fully submerge in the medium without touching the base of the wells.

Figure 9 shows an assay measuring epithelial resistance in polarized Caco-2 cells. This experiment shows that the various bivalent and bispecifc forms show improved function over the monomer forms.

B ICOSL binding domains

Construction of multivalent forms and enhanced efficacy data.

2D4 and CC3 Fc fusions

Figure 10 shows formats for multivalent and multispecific VNARs of the invention incorporating ICOSL VNARs (and human IgG Fc, which provides additional improved functional characteristics.

Method

Selected VNAR monomeric domains were PCR amplified and subcloned into a eukaryotic expression vector. This cloning was onto the 5' terminal end of a Human IgG1 Fc encoding DNA fragment (this Human IgG1 Fc fragment also encoded a full length Human IgG1 hinge sequence with the 5 prime most Cys residue which normally disulphide bridges to the light chain mutated to a Serine).

Whilst subject to PCR amplification oligonucleotides were used to introduce amino acid residues inserting a linker sequence between the carboxyl terminal end of the VNAR domain and the N terminal residue of the Human IgG1 hinge region as well as restriction endonuclease sites compatible with mammalian vector expression system. The linker sequences introduced by this process were either GGGGSGGGGRT whereby the nucleic acid sequence encoding the underlined RT amino acid residues introduces a *BsiW1* restriction endonuclease site or GGGGSGGGADQ in which codon usage of the underlined GADQ amino acid residues introduces a *BclI* site. Both of these sites are compatible with cloning sites in different versions of the Fc eukaryotic expression vector. At the 5' end of all amplicons a unique BssHII site is introduced which is compatible with eukaryotic vector construction.

DNA sequences to introduce linker VNAR domain fusions to the carboxyl terminal end of the Fc were designed and synthesis of these intermediate fragments was carried out by GeneArt (Invitrogen). The N-terminus of these fragments utilized a naturally-occurring *Bsr*GI site within the human IgG1-derived CH3 region, and an *EcoR1* site in the vector. These constructs introduced a linker with amino acid sequence TAAAATAAAATAAAATAAAA between the carboxyl terminal end of the Fc domain and the amino terminal end of the VNAR domain. Codon usage at the underlined triple alanine region of the linker allows for the introduction of a *NotI* restriction site which can be utilised in subsequent cloning work to assemble further bispecific VNAR constructs.

Post PEI-mediated transfection and transient expression in suspension HEK 293 cells using serum free media, expression levels of NAR Fc fusion proteins were determined by ELISA. Protein A affinity chromatography to purify the VNAR Fc proteins was performed after an initial 0.2 µm filtration clarification step to remove cell debris. A second chromatographic step to polish affinity purified protein was performed using ion exchange or size exclusion chromatography with buffer exchange as appropriate between steps. Proteins were concentrated using Amicon ultra filtration units and final

protein concentrations determined by UV spectroscopy. Analytical SEC and SDS PAGE was used to determine integrity of final purified proteins.

ICOSL neutralization assay

Ligand-receptor neutralisation assays were conducted as follows: CHO cells expressing human ICOS receptor were grown to confluency in DMEM/F12 + 5% FBS media in 96-well cell culture plates (Greiner, Bio-One). A total of 20 µl at 1µg/ml of rmB7-H2/Fc (158-B7, R&D Systems) was preincubated for 1 h with 40 µl of serially diluted anti-ICOSL-VNAR-Fc in DMEM/F12 + 2% FBS and then added to the cells. Following 1 h incubation at 16°C, cells were gently washed three times with DMEM/F12 + 2% FBS and incubated for 40 min at 16°C with goat anti-human Fc-HRP (SIGMA) diluted 1:10,000 in the same media. Cells were washed and developed with TMB substrate.

Figure 11 shows ELISA binding data, indicating that the ICOSL VNARS bind to their cognate antigens in these formats

Figure 12 shows formats for multivalent and multispecific VNARs of the invention incorporating the TNF R1 domain, ICOSL VNARs and human IgG Fc, which provides additional improved functional characteristics.

Figure 13 shows efficacy data for multivalent and multispecific VNARs of the invention incorporating the TNF R1 domain, ICOSL VNARs and human IgG Fc, which provides additional improved functional characteristics.

The data shows that the fusions are able to bind to TNF via TNFR1, and to mICOSL and hICOSL via the VNAR domains. The constructs are able to inhibit binding of m or hICOSL to their cognate CHO-expressed receptors

These data demonstrate that VNARs combined into multivalent formats are capable of binding to their targets and the molecules are able to show improved properties over the monomer VNARs

In vivo Pre-clinical Studies

Background of the study

The Tg197 murine model of rheumatoid arthritis is a transgenic mouse line carrying and expressing wild type and 3'-modified human tumour necrosis factor (hTNF-α) transgenes. These transgenic mice develop chronic polyarthritis with 100 % incidence at 4-7 weeks of age, dependent on the over expression of bioactive human TNF-α (Keffer et al. 1991, EMBO J., Vol. 10, pp. 4025-4031). The exemplification of the therapeutic efficacy of the first anti-TNF-α therapeutic antibody, Remicade® and other anti-TNF-alpha biologics were established using the Tg197 mouse model (Shealy et al., 2002,

Arthritis Research & Therapy, 4(5), p.R7; Shealy et al., 2010, MAbs (Vol. 2, No. 4, pp. 428-439). Taylor & Francis).

The aim of the study was to evaluate the therapeutic efficacy of anti-TNF-α D1-Fc-C4 (Quad-X™) in comparison to Humira® in preventing arthritic symptoms in the Tg197 transgenic mouse model of arthritis.

Methods

A total of 40 mice were allocated to each of the 5 test groups, G1-G5 (Table 4). For the purpose of this study, transgenic mice were allocated to groups consisting of 8 mice each that received the test compounds or vehicle buffer (Phosphate buffered saline, PBS, pH 7.4), twice weekly subcutaneously starting at the third week of age, prior to the establishment of arthritis, and continuing over 7 weeks, until the 10th week of age. One additional group of transgenic mice (2 male and 2 female) untreated animals were used as 3-week old control mice for histopathological status, and were sacrificed prior to the first dose administration.

Mice were allocated into groups prior to performing the first arthritis scoring. Age and gender balanced study consisted of 8 ((4♂ and 4♀) heterozygous Tg197 for groups G1-G5 mice that were pooled from different litters of synchronized mating upon weaning. The assignment of the mice to the different experimental groups was performed in a fashion that ensured equal distribution of body weight among the different groups at the start of the study. In vivo arthritis scores was evaluated as described in Table 5.

At the 10th week of age, all animals were sacrificed and the blood (serum isolated and stored at -80°C) and the two ankle joints of each animal were collected. Ankle joints of all experimental animals were dissected, calcified and further processed to perform histopathological evaluation of arthritis. Ankle histopathology was assessed by microscopic examination according to the histopathology scoring systems described in Table 6, and only representative images were included in the results section.

Table 4. Experimental groups

Group No.[1]	Test article	Dose (mg/kg)	Dose frequency (weeks)[2]	Duration of administration (weeks)	Dose volume (ml/kg)	Route of administration	Animal number	Age at sacrifice (weeks)[3]
G1	Vehicle	0	2/wk	7	10	s.c.	8(4♂/4♀)	10
G4	Humira®	10	2/wk	7	10	s.c.	8(4♂/4♀)	10
G2	Test article (D1-Fc-C4)	3	2/wk	7	10	s.c.	8(4♂/4♀)	10
G5	Test article (D1-Fc-C4)	10	2/wk	7	10	s.c.	8(4♂/4♀)	10
G3	Test article (D1-Fc-C4)	30	2/wk	7	10	s.c.	8(4♂/4♀)	10
3-wk old control mice	-	-	-	-	-	-	4(2♂/2♀)	3

1. The study was performed in a blinded fashion resulting in the random order of groups described in the table above.

2. Administration was initiated at the age of 3 weeks.
3. All mice of groups 1-5 were sacrificed 48 hours after the last dose administration. 3-week old control animals were sacrificed at the study initiation just before the first dose administration.

Table 5. Evaluation of in vivo arthritis score

ARTHRITIS SCORE[1]	CHARACTERISTICS
0 / no disease	no arthritis (normal appearance, mouse can support its weight clinging to an inverted or tilted surface such as a wire grid or a cage lid for a period of time, whole body flexibility/evasiveness normal, grip strength maximum)
0.5 / mild disease	onset of arthritis (mild joint swelling, all other parameters as above)
1 / mild to moderate disease	mild to moderate (joint distortion by swelling, inflamed paw, all other parameters as above)
1.5 / moderate disease	moderate arthritis (joint-paw swelling, distortion + last finger inward deformation, brief support clinging to an inverted or tilted surface such as a wire grid or a cage lid, whole body flexibility reduced, reduced grip strength)
2 / moderate to severe disease	moderate to severe arthritis (severe joint, paw and finger swelling, joint - leg deformation, no support clinging to an inverted or tilted surface such as a wire grid or a cage lid, no whole-body flexibility, no grip strength, climbing/feeding affected, starts shaking when trying to move, but manages to move forward)
2.5 / severe disease	severe arthritis (as above 2 + finger deformation in front paws, mouse movement impaired, shaking not willing to move)
3 / very severe disease	very severe arthritis (ankylosis detected on flexion and severely impaired movement, mouse moribund, not shaking anymore, cannot turn/flip around readily when tilted to the side).

[1]The addition of an extra 0.25 on the scoring of some assessments signifies a tendency towards the next more severe phenotype, i.e. when one, but not all the criteria from the next scale of severity are present. For example, "1.75" means "1.5" with severe swelling but no joint deformation and some strength on flexion.

In vivo arthritis scores with group average scores are depicted as graph in the results section.

Table 6. Cumulative histopathological criteria for scoring arthritic phenotype in the ankle joints

SCORE[1]	DISEASE	CRITERIA
0	Normal	no detectable pathology
1	Mild	hyperplasia of the synovial membrane and presence of polymorphonuclear infiltrates. Mild tendonitis may be present.
2	Moderate	pannus and fibrous tissue formation and focal subchondrial bone erosion
3	Moderate-Severe	cartilage destruction and bone erosion
4	Severe	extensive cartilage destruction and bone erosion. Bone outline structure is lost

(Adapted from: Pettit, A.R., *et al.*, 2001, The American journal of pathology, 159(5), pp.1689-1699; Mould, A.W., *et al.*, 2003, Arthritis & Rheumatology, 48(9), pp.2660-2669)

[1] Half marks are given when some but not all of the features from the next higher score are present. Hence, a score of "2.5" means pannus and fibrous tissue formation and focal subchondrial bone erosion (score 2), with more bone erosion spread outside and around subchondrial foci, but not as broad and with cartilage destruction, as to justify a score "3".

Histopathological scores with group average scores are depicted as bar graph in the results section and tables in the appendix. Illustrative histopathology images at 25x magnification are also presented in the appendix.

Results

The evaluation of the efficacy of the anti-hTNF-α D1-Fc-C4 (Quad-X™) and Humira® on the Tg197 arthritis model was performed following a prophylactic administration scheme, i.e. starting treatment at the 3rd week of age of the mice when they exhibited mild evidence of in vivo arthritis pathology and early histopathological lesions. By the 10th week of age, the in vivo arthritic score in the vehicle treated control group G1 increased dramatically compared to the 3-week old untreated animals, while at the same age the histopathological lesions observed in the animals of G1 were statistically more severe than that seen in the 3-week control mice group.

- ***Efficacy evaluation of the therapeutic effect of the test article D1-Fc-C4 anti-hTNFα in the in vivo and histopathological as well as body weight arthritis symptoms***
  - The 3, 10 and 30 mg/kg dose regimens of D1-Fc-C4 test article afforded statistical significant robust inhibition of the Tg197 in vivo and histopathological arthritic pathology compared to the vehicle treated mice in G1. More specifically, after 14 doses administered twice weekly for a period of 7 weeks, the dose regimens resulted in:

    ~88 inhibition of the in vivo and ~86% inhibition of arthritis histopathology score following the 3 mg/kg D1-Fc-D4 test article treatment of animals in G2

    ~88% significant inhibition of the in vivo and ~83% inhibition of arthritis histopathology score following the 10 mg/kg D1-Fc-C4 test article treatment of animals in G5

    ~88% significant inhibition of the in vivo and ~86% inhibition of arthritis histopathology score following the 30 mg/kg D1-Fc-C4 test article treatment of animals in G3

  - Similar findings were observed in mean body weight curves of the D1-Fc-C4 test article treated mice which appeared to gain more body weight in all dose levels compared to the vehicle treated mice in G1 although statistical significance was achieved only in the 3 mg/kg and in the 10 mg/kg dose regimens.

- ***D1-Fc-C4 test article dose response efficacy evaluation***
  - Treatment with the 3, 10 and 30mg/Kg doses of D1-Fc-D4 test article did not exhibit a dose-dependent response efficact as shown by the in vivo arthritic evaluations and body weight scores as well as from the histopathological evaluations in which all doses acted similarly and their therapeutic effects were statistically undifferentiated.

- ***Efficacy evaluation of the therapeutic effect of Humira® in the in vivo and histopathological as well as body weight arthritis symptoms***

- The 10 mg/kg dose regimen of Humira® afforded robust statistical significant inhibition of the Tg197 in vivo and histological arthritic pathology compared to the vehicle treated mice in G1. More specifically, after 14 doses administered twice weekly for a period of 7 weeks, we observed:

  ~82% inhibition of the in vivo and ~86% inhibition of the arthritis histopathology score following the 10 mg/kg Humira® treatment of animals in G4

- Similar findings were observed in mean body weight curve of the Humira® treated mice which appeared to gain more body weight compared to the vehicle treated mice in G1.

➢ Dose response comparison between D1-Fc-C4 test article and Humira®

The comparative examination of the inhibitory effects between D1-Fc-C4 test article and Humira® across the 10 mg/kg dose revealed that they were statistically undifferentiated in all parameters evaluated, including body weights, in vivo arthritic scores and histopathological evaluations.

➢ ***Histopathological comparison of the effect of D1-Fc-C4 test article and Humira® to the 3-week old control animals***

The inhibitory effects of the 3, 10 and 30mg/Kg of the D1-Fc-C4 test article as well as the 10mg/Kg Humira resulted in lower histopathology lesions at week 10 and statistically differentiated from the 3-week old control untreated animals.

Table 7 Mean group body weights

Mean group body weight[1] (g)	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
3-week old control mice	9.8±0.2	-	-	-	-	-	-	-
G1-Vehicle	10.0±0.5	12.8±0.8	17.0±0.8	18.4±0.8	18.1±1.0	18.8±1.2	19.6±1.3	19.3±1.4
G4-Humira® 10 mg/kg	10.0±0.3	13.0±0.6	18.3±0.8	20.8±0.7	21.2±1.4	22.6±1.3	23.4±1.4	24.4±1.5
G2-D1-Fc-C4 3 mg/kg	10.0±0.4	13.6±0.8	18.5±0.9	20.4±1.0	21.5±1.4	22.3±1.5	23.0±1.5	24.1±1.5
G5-D1-Fc-C4 10 mg/kg	10.0±0.3	13.4±0.7	18.1±1.0	20.2±0.9	20.7±1.3	21.9±1.5	23.2±1.5	24.1±1.7
G3-D1-Fc-C4 30 mg/kg	10.0±0.4	13.8±0.6	18.3±0.7	20.0±1.0	20.7±1.1	21.7±1.2	22.3±1.3	23.4±1.4

1. Data are presented as mean±SEM

Table 8 Mean group in vivo arthritis scores

Mean group *in vivo* arthritic scores[1]	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
3-week old control mice	0.13 ±0.05	-	-	-	-	-	-	-
G1- Vehicle	0.14 ±0.03	0.34 ±0.03	0.58 ±0.04	0.84 ±0.05	0.94 ±0.04	0.98 ±0.04	1.20 ±0.06	1.36 ±0.07
G4- Humira® 10 mg/kg	0.17 ±0.04	0.28 ±0.04	0.23 ±0.05	0.19 ±0.04	0.33 ±0.04	0.25 ±0.05	0.28 ±0.04	0.25 ±0.05
G2- D1-Fc-C4 3 mg/kg	0.13 ±0.04	0.20 ±0.03	0.17 ±0.04	0.17 ±0.04	0.19 ±0.04	0.17 ±0.03	0.16 ±0.03	0.17 ±0.04
G5- D1-Fc-C4 10 mg/kg	0.19 ±0.04	0.20 ±0.04	0.17 ±0.03	0.28 ±0.03	0.25 ±0.03	0.27 ±0.03	0.25 ±0.02	0.17 ±0.04
G3- D1-Fc-C4 30 mg/kg	0.09 ±0.03	0.17 ±0.04	0.17 ±0.04	0.19 ±0.05	0.22 ±0.04	0.22 ±0.04	0.20 ±0.03	0.17 ±0.04

1. Data are presented as mean±SEM

Table 9 Mean group arthritis histopathology scores

Mean group histopathology scores[1]	Week 3	Week 10
3-week old control mice	1.22±0.10	-
G1- Vehicle	-	2.94±0.12
G4- Humira® 10 mg/kg	-	0.42±0.07
G2- D1-Fc-C4 3 mg/kg	-	0.41±0.03
G5- D1-Fc-C4 10 mg/kg	-	0.50±0.05
G3- D1-Fc-C4 30 mg/kg	-	0.42±0.07

1. Data are presented as mean±SEM

In addition, a second exemplification of the *in vitro* potency enhancement using the VNAR S17 Quad-X™ construct targeting mouse TNF-alpha was conducted.

VNAR S17 is a specific anti-mouse TNF-alpha with no binding or neutralizing activity against human TNF-alpha. VNAR S17-Fc is a potent neutralizer of mouse TNF-alpha with in vitro potency (ND50) of approximately 8 nM. When designed as a Quad-X™ construct (S17-Fc-S17), *in vitro* neutralizing potency improved by ≈ 40-fold to 0.2 nM (Figure 30).

Furthermore, it has been demonstrated that the S17 Quad-X™ and D1-C4 Quad-X™ constructs recognize distinct species of TNF-alpha (Figure 31).

Discussion and conclusion

The results of this study show that the reference Humira® and D1-Fc-C4 (Quad-X™) and D1-BA11-C4 anti-hTNF-α articles inhibited the arthritic phenotype observed in Tg197 animals thus resulting in increased body weight and reduced in vivo and histopathological arthritic pathology as compared to the vehicle treated animals.

The therapeutic effect of the reference Humira® was evaluated at 10 mg/kg dose (Fig 29-32) and resulted in statistically significant inhibition of the in vivo arthritic and ankle histopathological evaluations when compared to the vehicle treated mice. In Figure 33, 1 mg/kg Humira® show significant disease breakthrough at 8 weeks. In a previous Tg197 mice model study using a dosing regimen of 1 mg/kg, 3 mg/kg, 10 mg/kg and 30 mg/kg Humira®, it was shown that there was significant disease breakthrough in the group of mice treated with either 1 mg/kg (Figure 33) or 3 mg/kg Humira® (Figure 35). These groups of mice had time-dependent disease progression similar to the untreated group, and in vivo arthritic (AS) and histopathology scores (HS) significantly higher than the groups treated with either 10 mg/kg or 30 mg/kg Humira® (Figure 33).

The D1-Fc-C4 (Quad-X™) test articles did not exhibit a dose-dependent response as all evaluated doses, i.e. 0.5, 1, 3 mg/kg, 10 mg/kg and 30 mg/kg demonstrated similar and statistically undifferentiated therapeutic effects with complete control of the disease. Furthermore, in vivo arthritic and histopathological evaluations revealed that the therapeutic effect of the 3 mg/kg dose of D1-Fc-C4 test article was statistically comparable to that of 10 mg/kg Humira®. We also did not observe any sign of disease breakthrough in the 3 mg/kg D1-Fc-C4 treated mice at 10 weeks of age, neither did we observe any with 0.5 and 1 mg/kg D1-Fc-C4 at 8 weeks of age.

We have therefore further exemplified that the D1-Fc-C4 anti-hTNF-α domain is more potent than the standard therapy, Humira® in neutralising the effects of TNF-alpha both *in vitro* (L929 and Caco2 data----Figures 2, 3, 6 to 8, 20, 23 and 28) and *in vivo* (Figures 29-35). We have also demonstrated the in vivo efficacy of a non-Fc based tandem multivalent VNAR, D1-BA11-C4 (Figures 33 and 34).

Finally we were able to show that an anti-TNF VNAR (D1 – Fc) was also able to control and treat Uveitis (with a similar potency to Dexamethasone) in a rat model of inflammatory eye disease if administered systemically in an Fc alone format (Figure 36).

CLAIMS

1. A multi-domain specific binding molecule comprising two or more VNAR domains which bind to the same or different epitopes of one or more specific antigens.

2. The multi-domain specific binding molecule of claim 1, wherein one or more of the VNAR domains in the multi-domain specific binding molecule exhibit higher binding affinity for their target compared to the monomeric VNAR.

3. The multi-domain specific binding molecule of claim 1 or 2, further comprising a spacer sequence between the VNAR domains.

4. The multi-domain specific binding molecule of claim 3, wherein the spacer sequence has independent functionality which is exhibited in the binding molecule.

5. The multi-domain specific binding molecule of claim 4, wherein the spacer sequence is a VNAR domain or a functional fragment thereof.

6. The multi-domain specific binding molecule of claim 5, wherein the spacer sequence is a VNAR or functional fragment thereof that binds to serum albumin.

7. The multi-domain specific binding molecule of claim 6, wherein the spacer sequence is a VNAR or functional fragment thereof that binds to human serum albumin.

8. The multi-domain specific binding molecule of claim 7, wherein the spacer sequence has an amino acid sequence comprising the amino acid sequence of any one of SEQ ID NO: 67, SEQ ID NO: 67, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88, or a functional fragment having at least 60% sequence identity thereto.

9. The multi-domain specific binding molecule of claim 4, wherein the spacer sequence is derived from an immunoglobulin Fc region.

10. The multi-domain specific binding molecule of claim 9, wherein the spacer sequence is derived from a human immunoglobulin Fc region.

11. The multi-domain specific binding molecule of claim 1 or 2, further comprising one or more non-VNAR domains.

12. The multi-domain specific binding molecule of claim 11, wherein the non-VNAR domain is C-terminal or N-terminal to the VNAR domains.

13. The multi-domain specific binding molecule of claim 11 or 12, wherein at least one of the non-VNAR domains is selected from the group comprising TNF R1 and immunoglobulin Fc.

14. The multi-domain specific binding molecule of any one of claims 1 to 13, further characterized in that the specific antigen is from a group comprising of a cytokine, a growth factor, an enzyme, a hormone, a cell surface associated molecule, a cell-surface membrane component, an intracellular molecule, an extracellular matrix component, a stromal antigen, a serum protein, a skeletal antigen, a microbial antigen, or an antigen from a normally immune-privileged location.

15. A TNF-alpha specific VNAR binding domain comprising the following CDRs and hyper-variable regions (HV):

CDR1: HCATSS or NCGLSS or NCALSS
HV2: TNEESISKG
HV4: SGSKS or EGSKS
CDR3: ECQYGLAEYDV or SWWTQNWRCSNSDV or YIPCIDELVYMISGGTSGPIHDV

or a functional variant thereof with a sequence identity of at least 60%.

16. The TNF-alpha specific VNAR binding domain of claim 15, wherein the VNAR binding domain comprises the amino acid sequence of SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

17. The TNF-alpha specific VNAR binding domain of claim 15 or 16, wherein the VNAR domain is humanized or de-immunized.

18. The multi-domain specific binding molecule of claim 1, wherein one or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

19. The multi-domain specific binding molecule of claim 17, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

20. The multi-domain specific binding molecule of claim 1, wherein one or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%.

21. The multi-domain specific binding molecule of claim 19, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%.

22. The multi-domain specific binding molecule of claim 8, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

23. The multi-domain specific binding molecule of claim 8, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%.

24. The multi-domain specific binding molecule of claim 10, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 2, 7 or 12, or a functional variant thereof with a sequence identity of at least 60%.

25. The multi-domain specific binding molecule of claim 10, wherein two or more of the VNAR domains have an amino acid sequence selected from the group comprising SEQ ID 65 or 66, or a functional variant thereof with a sequence identity of at least 60%.

26. The binding molecule as claimed in any one of the preceding claims modified at one or more amino acid sequence positions to reduce potential immunogenicity when administered to a human.

27. An isolated nucleic acid comprising a polynucleotide sequence that encodes a binding molecule according to any preceding claim.

28. A method for preparing a binding molecule, comprising cultivating or maintaining a host cell comprising the polynucleotide of claim 26 under conditions such that said host cell produces the binding molecule, optionally further comprising isolating the binding molecule.

29. A pharmaceutical composition comprising the multi-domain specific binding molecule of any one of claims 1 to 24 and optionally at least one pharmaceutically acceptable carrier.

30. The multi-domain specific binding molecule of any one of claims 1 to 24, for use in therapy.

31. The use of a specific antigen binding molecule of any one of claims 1 to 24 in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

32. A method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a pharmaceutical composition of claim 28.

33. A method for treating a condition mediated by TNFα, the method comprising the administration of a therapeutically effective amount of a composition of claim 28 that specifically binds to TNFα.

34. A method for treating at least one condition mediated by ICOSL, comprising the administration of an effective amount of a composition of claim 28 that specifically binds to ICOSL.

Figure 1

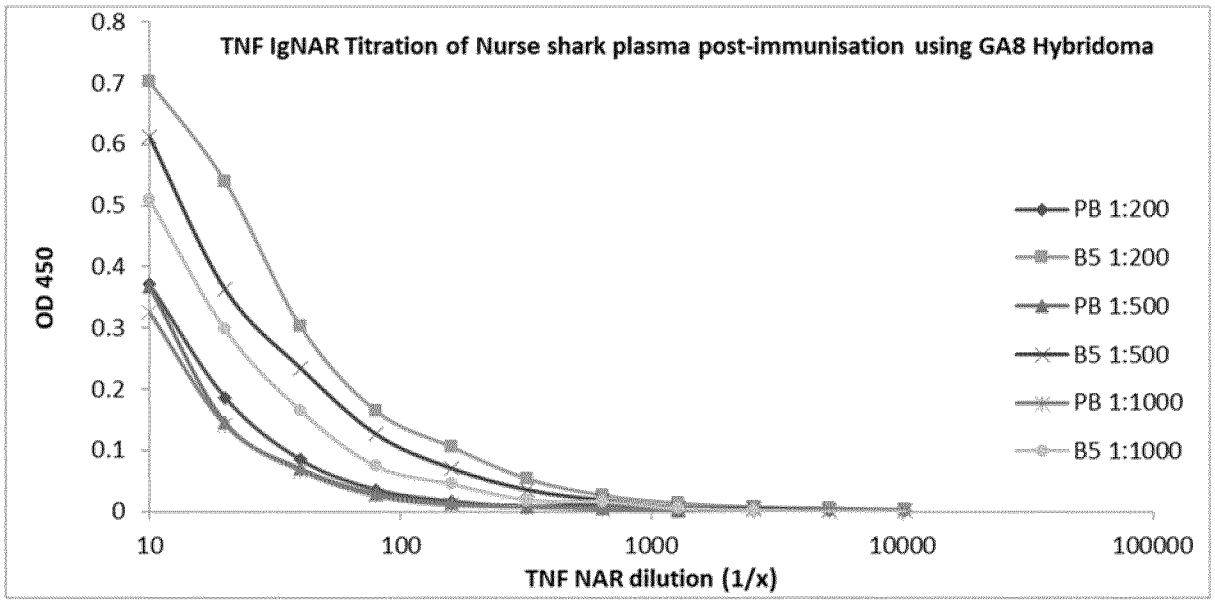

TNF IgNAR Titration of Nurse shark plasma post-immunisation using GA8 Hybridoma
OD 450
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
100
1000
10000
100000
TNF NAR dilution (1/x)
PB 1:200
B5 1:200
PB 1:500
B5 1:500
PB 1:1000
B5 1:1000

Figure 2

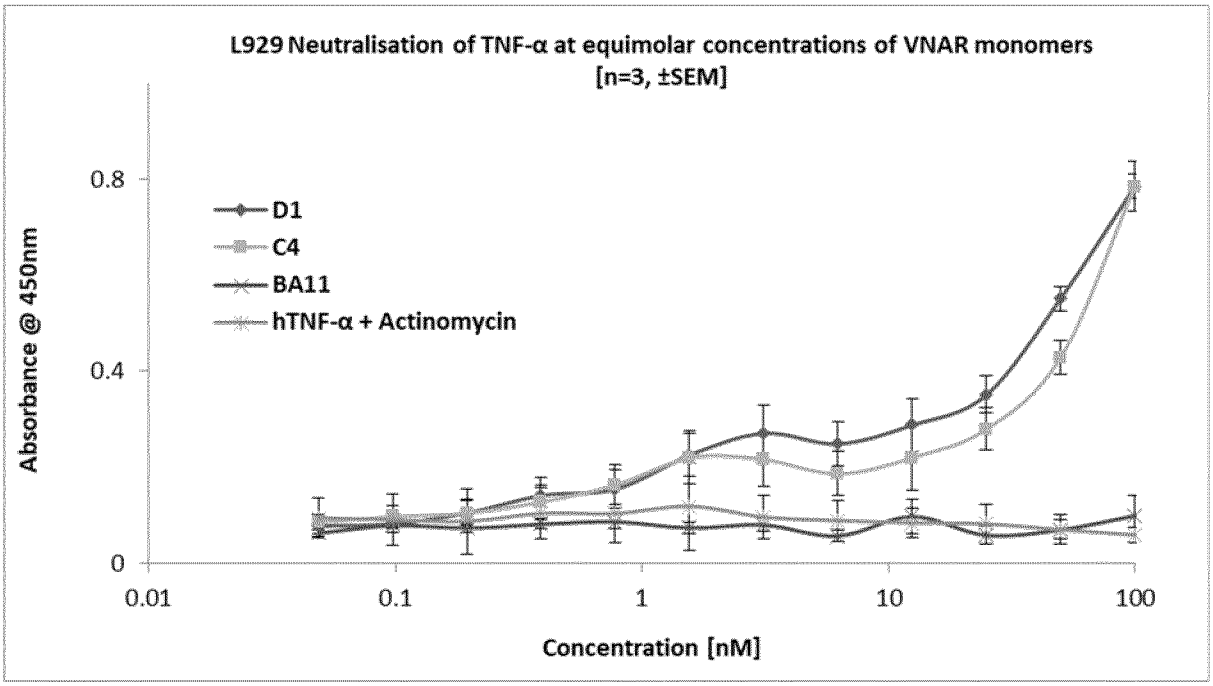

L929 Neutralisation of TNF-α at equimolar concentrations of VNAR monomers
[n=3, ±SEM]
Absorbance @ 450nm
0.8
0.4
0
D1
C4
BA11
hTNF-α + Actinomycin
0.01
0.1
1
10
100
Concentration [nM]

Figure 3

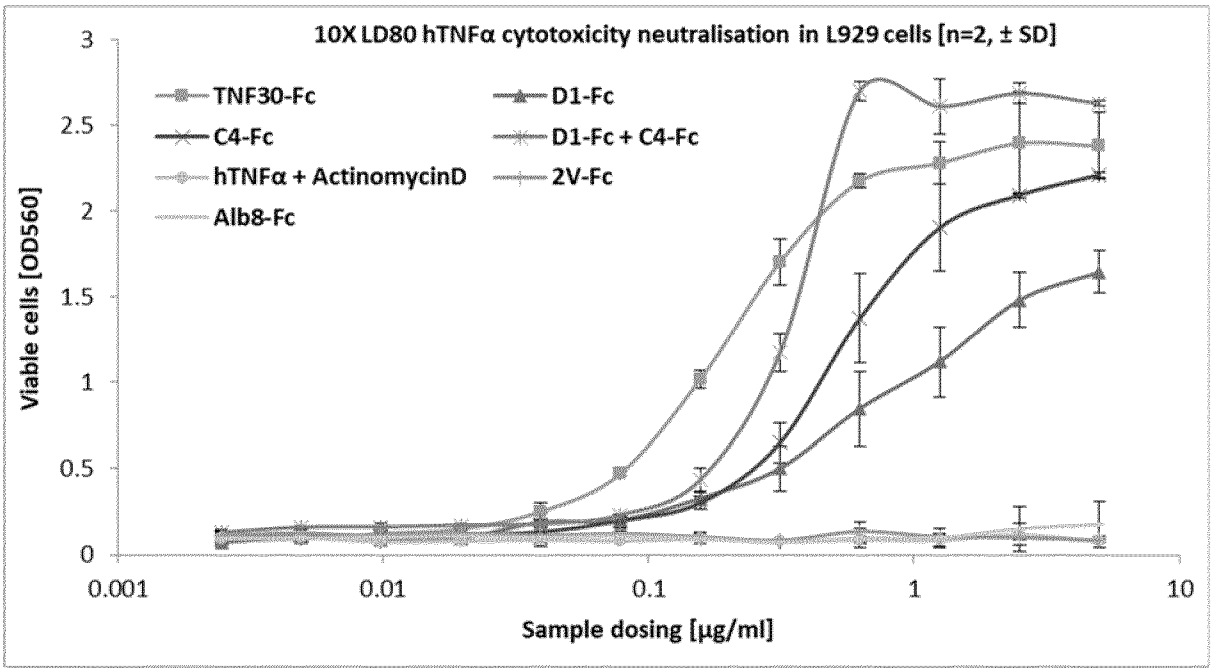

10X LD80 hTNFα cytotoxicity neutralisation in L929 cells [n=2, ± SD]
TNF30-Fc
D1-Fc
C4-Fc
D1-Fc + C4-Fc
hTNFα + ActinomycinD
2V-Fc
Alb8-Fc
Viable cells [OD560]
Sample dosing [µg/ml]
0
0.5
1
1.5
2
2.5
3
0.001
0.01
0.1
1
10

Figure 4

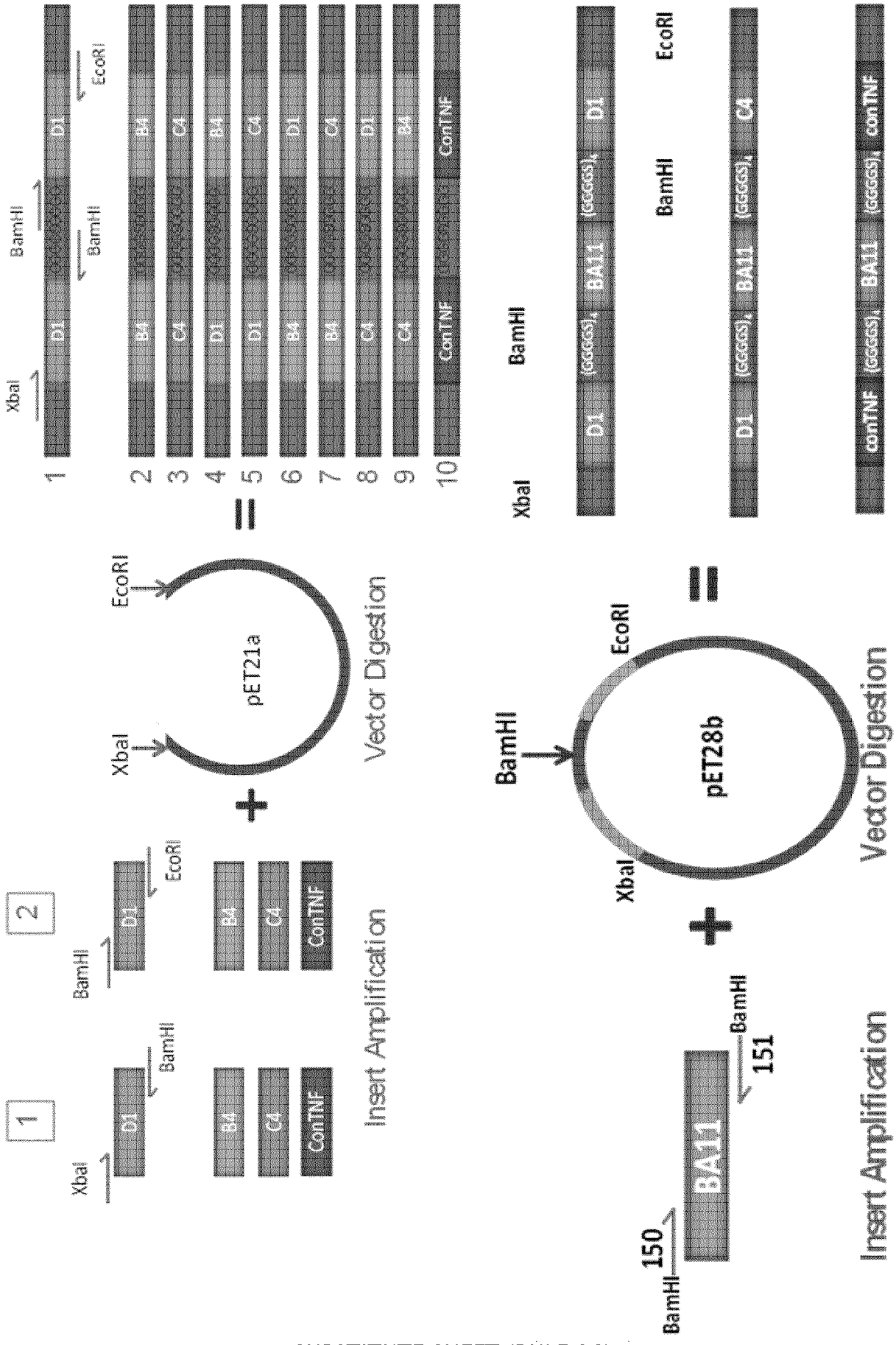

XbaI
BamHI
BamHI
EcoRI
D1
B4
C4
ConTNF
1
2
3
4
5
6
7
8
9
10
=
BamHI
XbaI
(GGGGS)4
BA11
EcoRI
BamHI
pET21a
Vector Digestion
Insert Amplification
pET28b
150
151

Figure 5

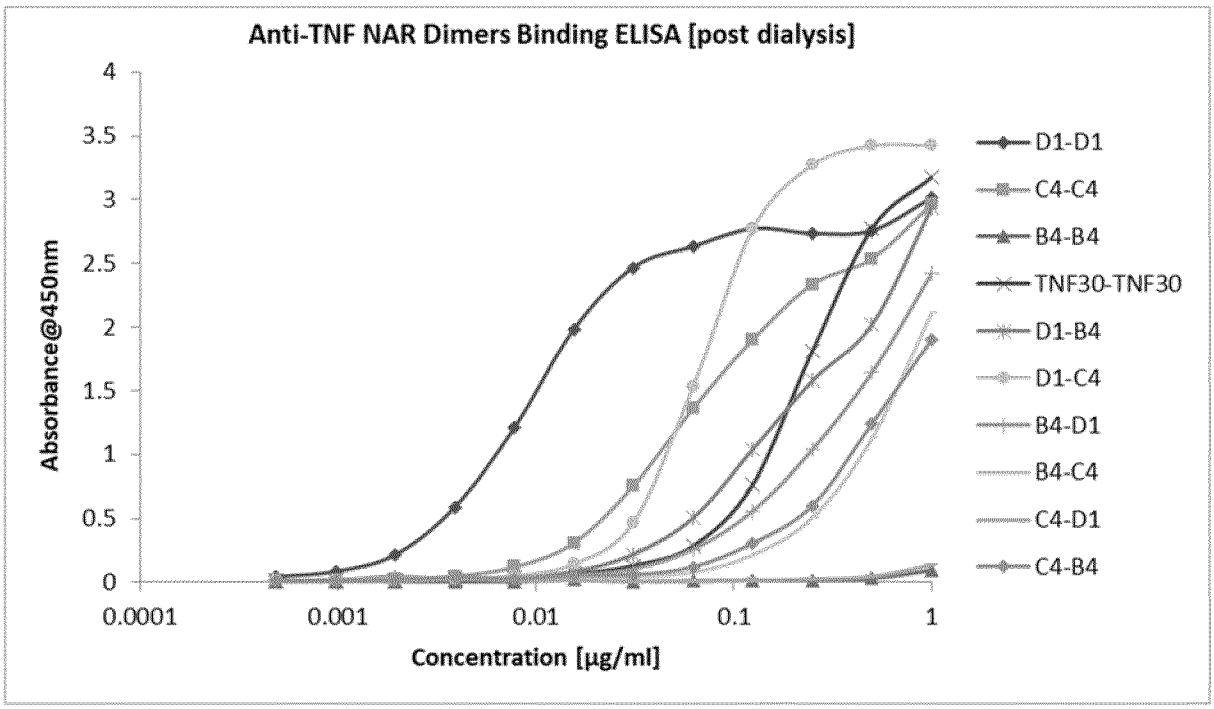

Anti-TNF NAR Dimers Binding ELISA [post dialysis]
Absorbance@450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
0.0001
0.001
0.01
0.1
1
Concentration [µg/ml]
D1-D1
C4-C4
B4-B4
TNF30-TNF30
D1-B4
D1-C4
B4-D1
B4-C4
C4-D1
C4-B4

Figure 6

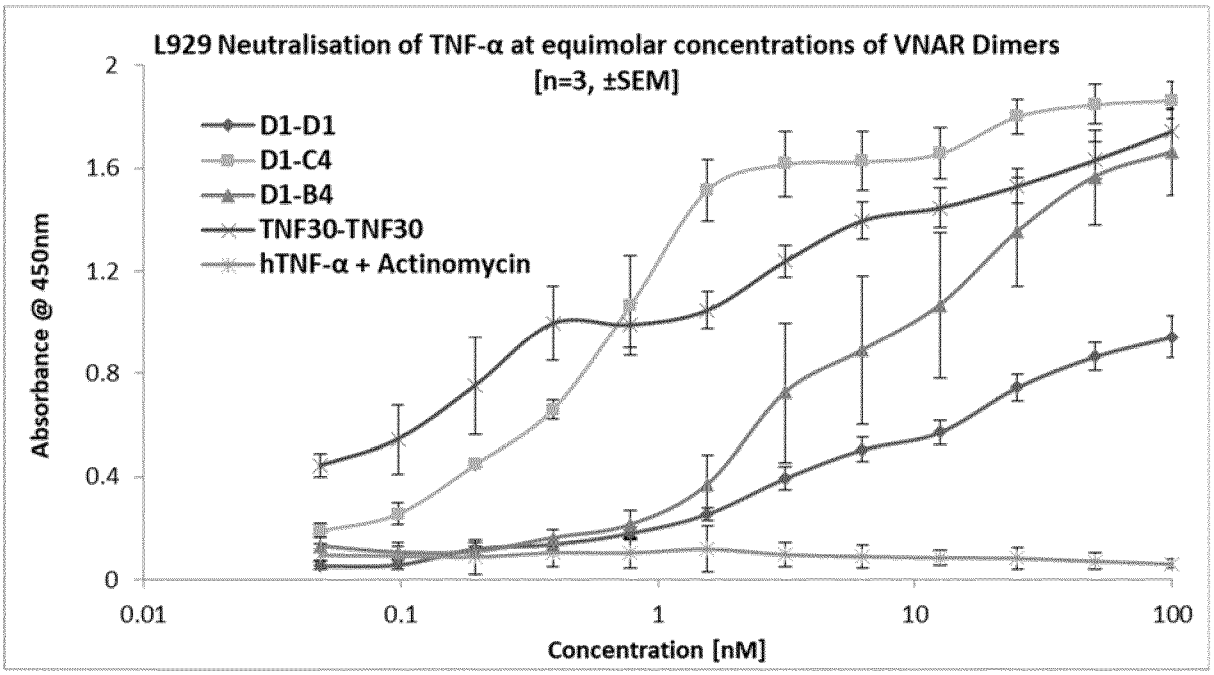

L929 Neutralisation of TNF-α at equimolar concentrations of VNAR Dimers
[n=3, ±SEM]
D1-D1
D1-C4
D1-B4
TNF30-TNF30
hTNF-α + Actinomycin
Absorbance @ 450nm
Concentration [nM]
0
0.4
0.8
1.2
1.6
2
0.01
0.1
1
10
100

Figure 7

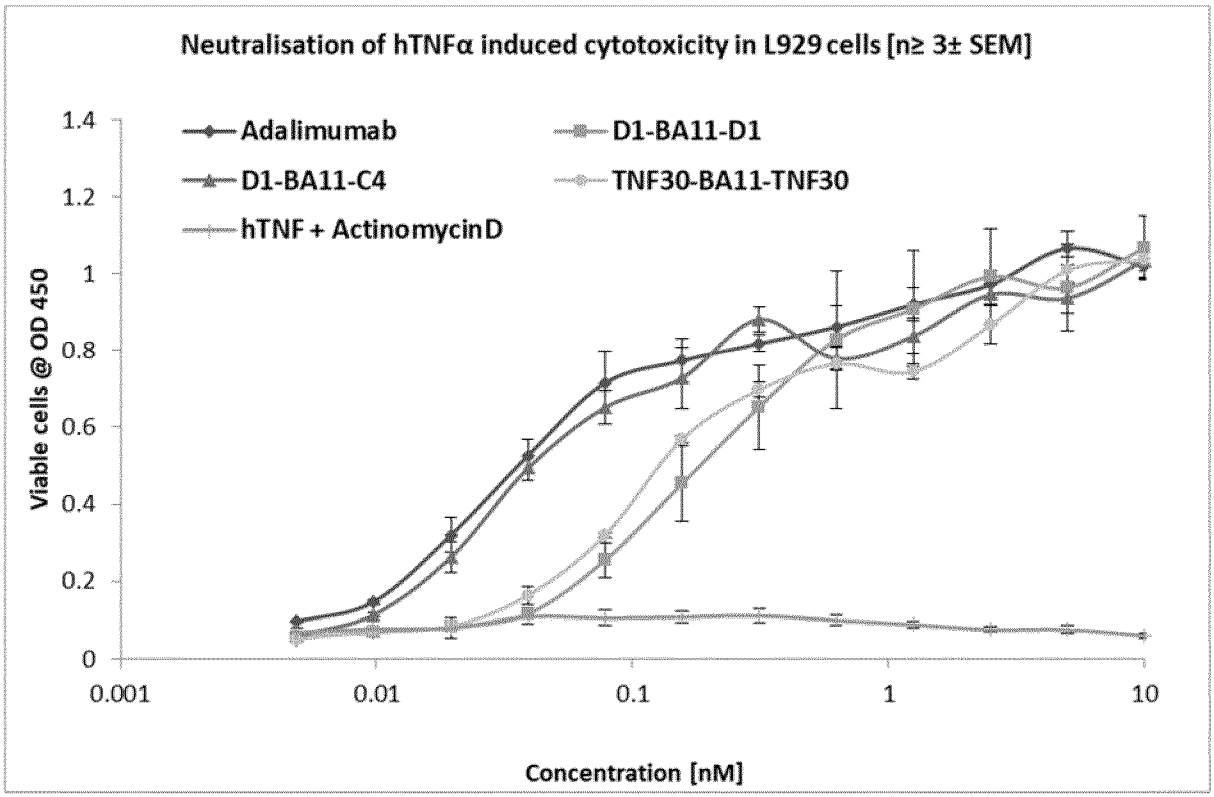

Neutralisation of hTNFα induced cytotoxicity in L929 cells [n≥ 3± SEM]
Adalimumab
D1-BA11-D1
D1-BA11-C4
TNF30-BA11-TNF30
hTNF + ActinomycinD
Viable cells @ OD 450
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
Concentration [nM]

Figure 8

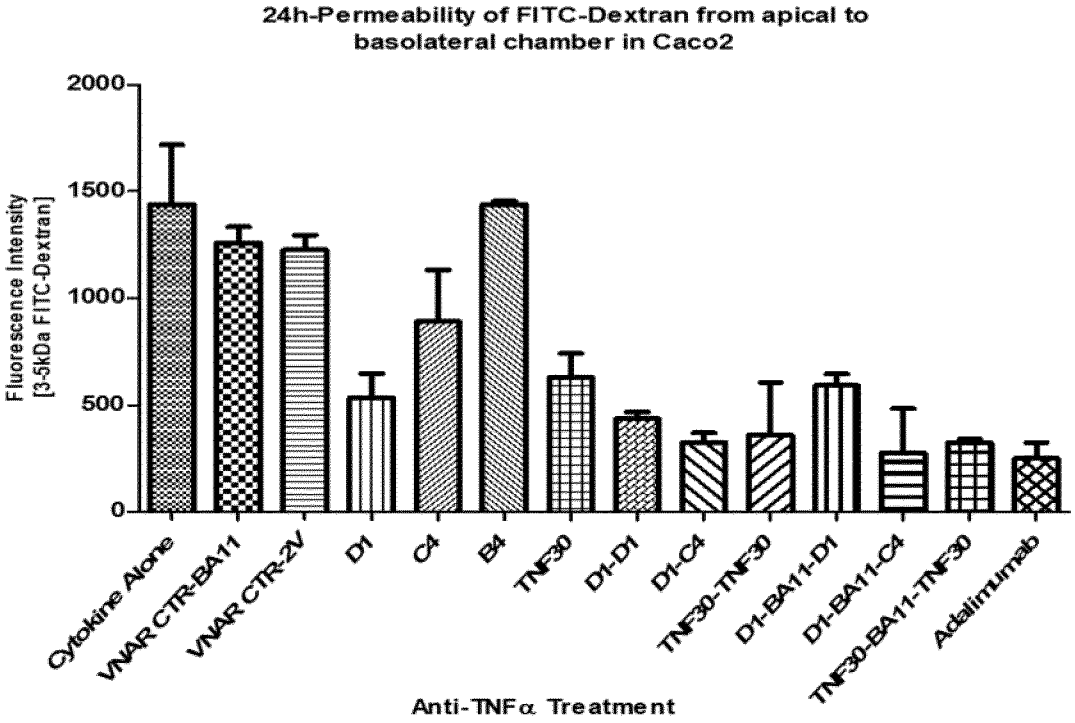

24h-Permeability of FITC-Dextran from apical to basolateral chamber in Caco2
Fluorescence Intensity [3-5kDa FITC-Dextran]
2000
1500
1000
500
0
Cytokine Alone
VNAR CTR-BA11
VNAR CTR-2V
D1
C4
B4
TNF30
D1-D1
D1-C4
TNF30-TNF30
D1-BA11-D1
D1-BA11-C4
TNF30-BA11-TNF30
Adalimumab
Anti-TNFα Treatment

Figure 9

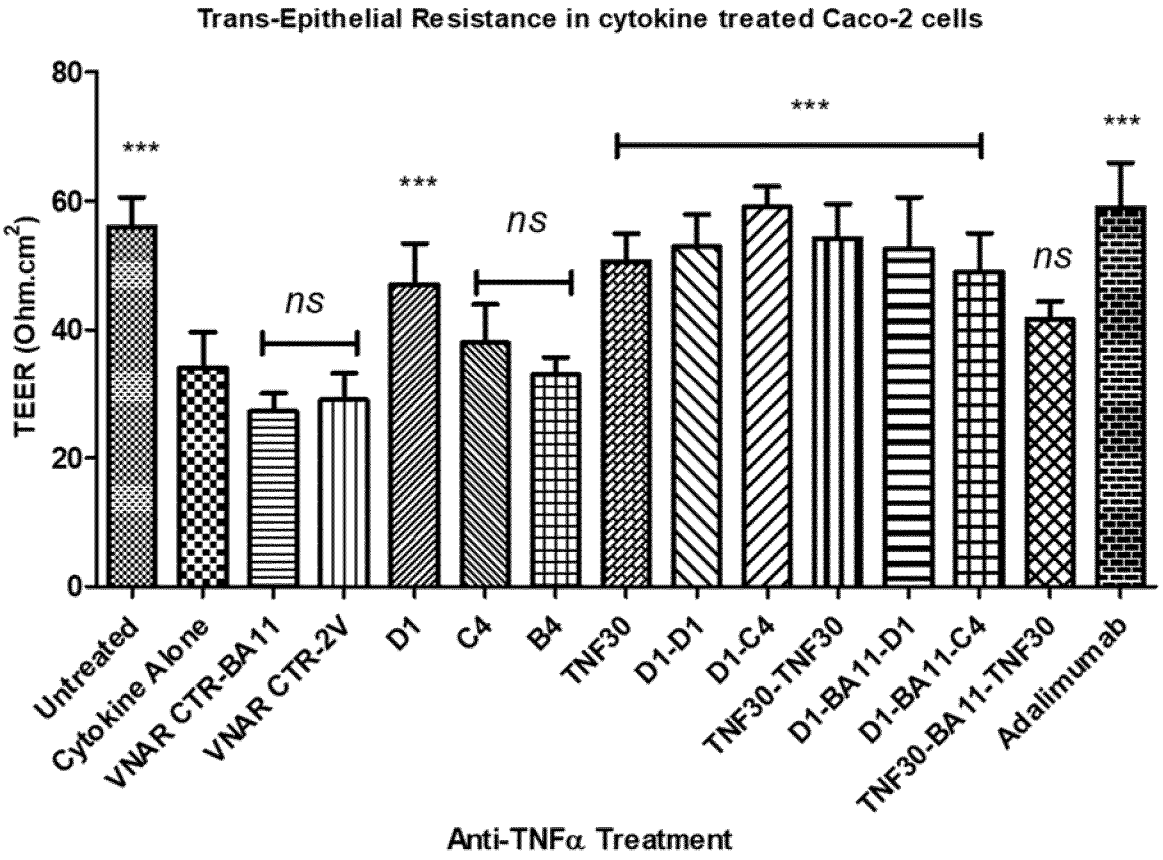

Trans-Epithelial Resistance in cytokine treated Caco-2 cells
TEER (Ohm.cm^2)
80
60
40
20
0
***
***
ns
ns
***
***
Untreated
Cytokine Alone
VNAR CTR-BA11
VNAR CTR-2V
D1
C4
B4
TNF30
D1-D1
D1-C4
TNF30-TNF30
D1-BA11-D1
D1-BA11-C4
TNF30-BA11-TNF30
Adalimumab
Anti-TNFα Treatment

Figure 10

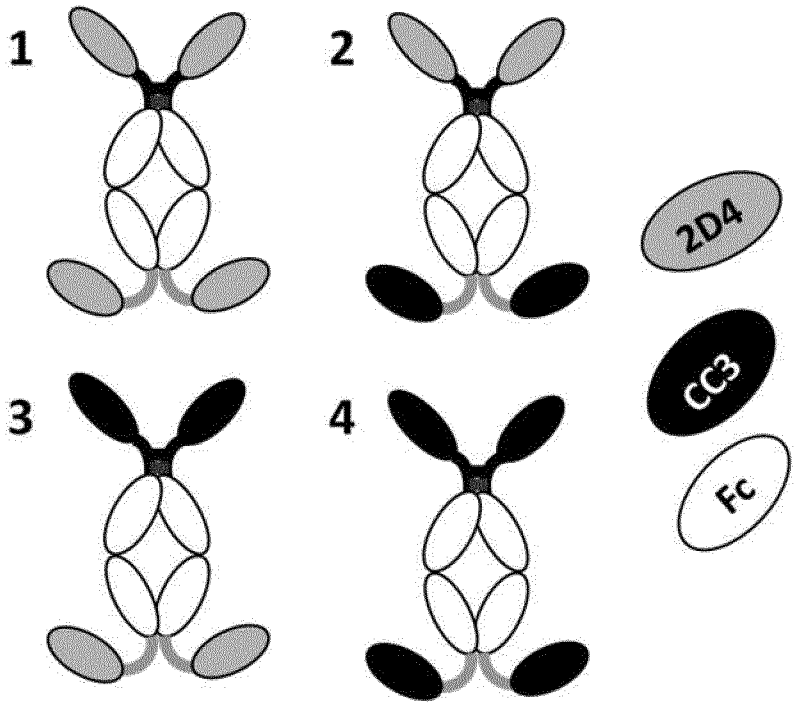
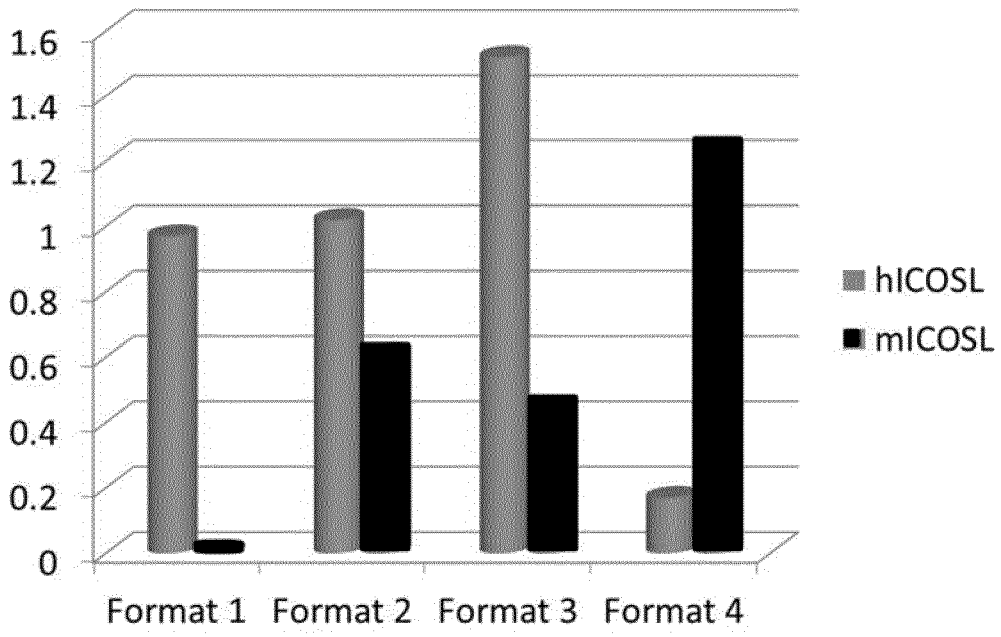


Figure 11



Anti-hICOSL or mICOSL binding ELISA data of four VNAR-Fc combinations, using 1/8 dilution of supernatant from transiently transfected cells

Figure 12

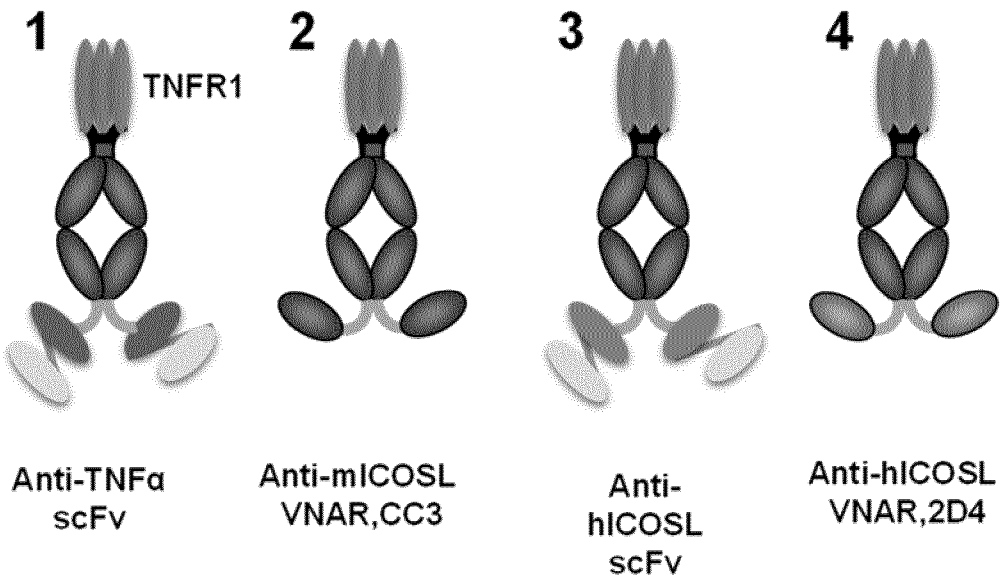
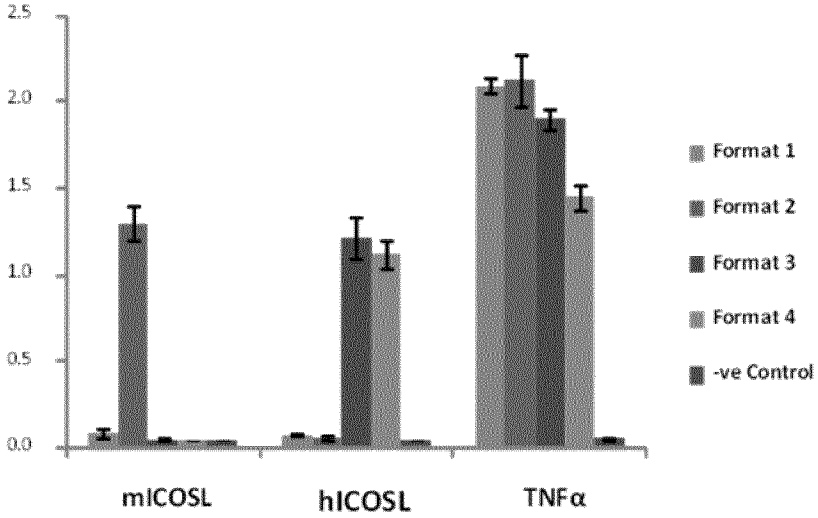
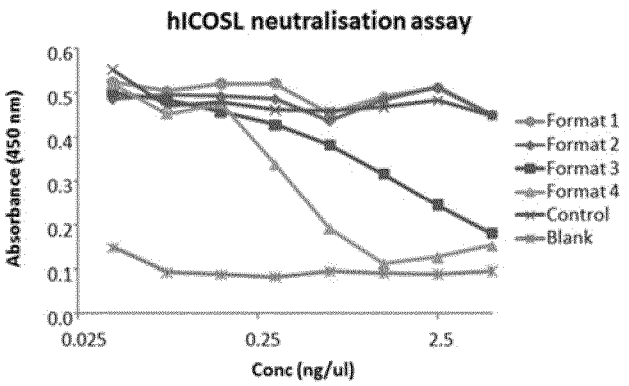

1
TNFR1
2
3
4
Anti-TNFα scFv
Anti-mICOSL VNAR,CC3
Anti-hICOSL scFv
Anti-hICOSL VNAR,2D4

Figure 13


2.5
2.0
1.5
1.0
0.5
0.0
mICOSL
hICOSL
TNFα
Format 1
Format 2
Format 3
Format 4
-ve Control


hICOSL neutralisation assay
Absorbance (450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.025
0.25
2.5
Conc (ng/ul)
Format 1
Format 2
Format 3
Format 4
Control
Blank

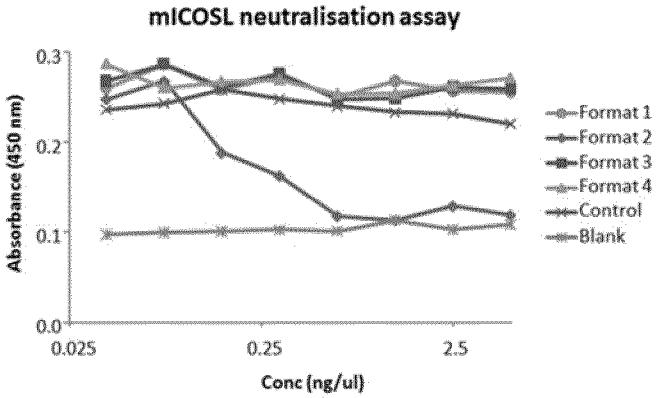

mICOSL neutralisation assay
Absorbance (450 nm)
0.3
0.2
0.1
0.0
0.025
0.25
2.5
Conc (ng/ul)
Format 1
Format 2
Format 3
Format 4
Control
Blank

Figure 14

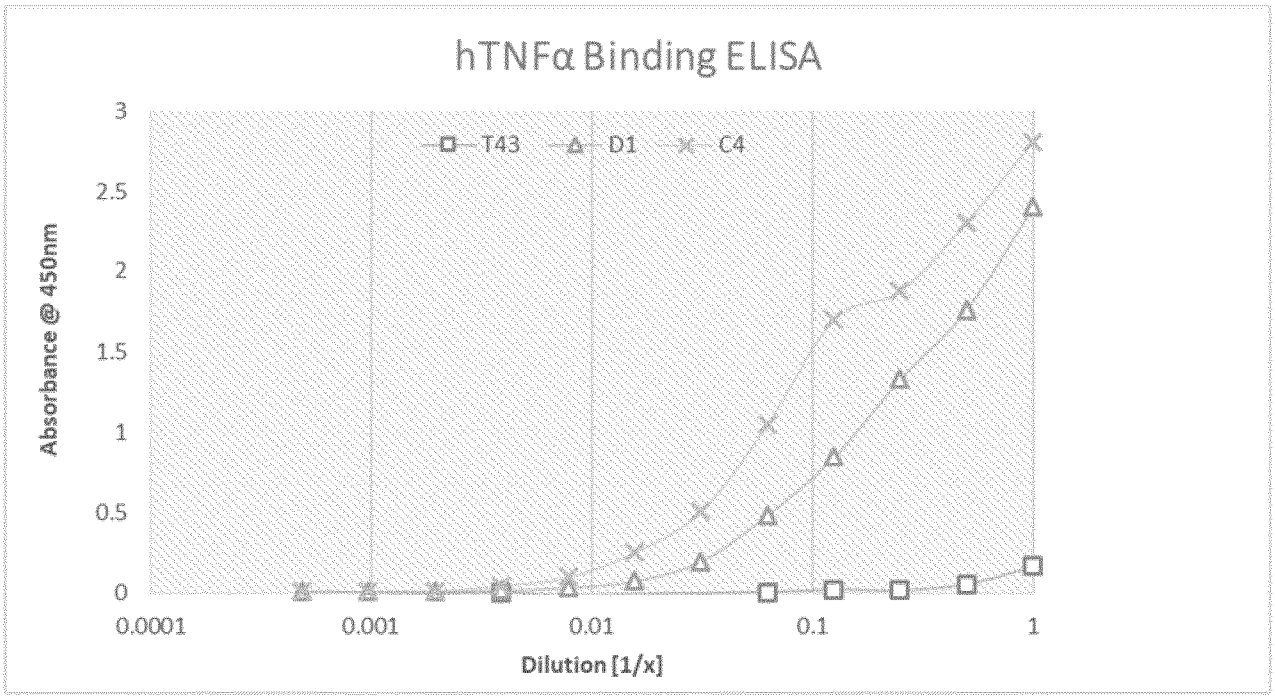

hTNFα Binding ELISA
T43
D1
C4
Absorbance @ 450nm
3
2.5
2
1.5
1
0.5
0
0.0001
0.001
0.01
0.1
1
Dilution [1/x]

Figure 15

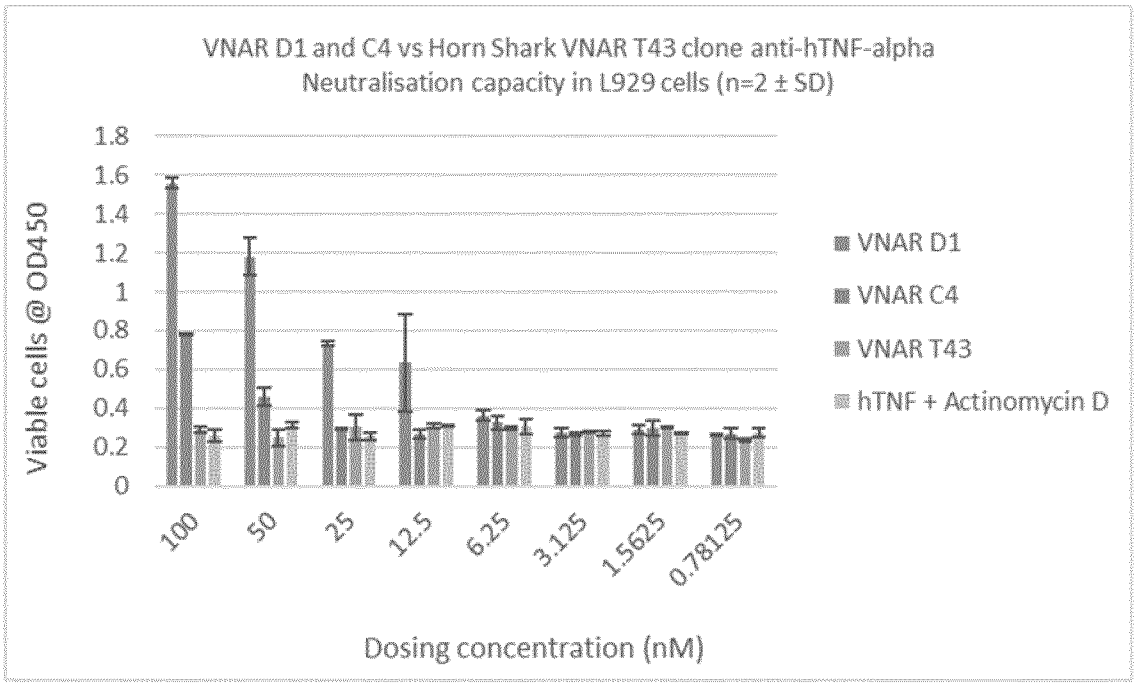

VNAR D1 and C4 vs Horn Shark VNAR T43 clone anti-hTNF-alpha
Neutralisation capacity in L929 cells (n=2 ± SD)
Viable cells @ OD450
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
100
50
25
12.5
6.25
3.125
1.5625
0.78125
Dosing concentration (nM)
VNAR D1
VNAR C4
VNAR T43
hTNF + Actinomycin D

Figure 16

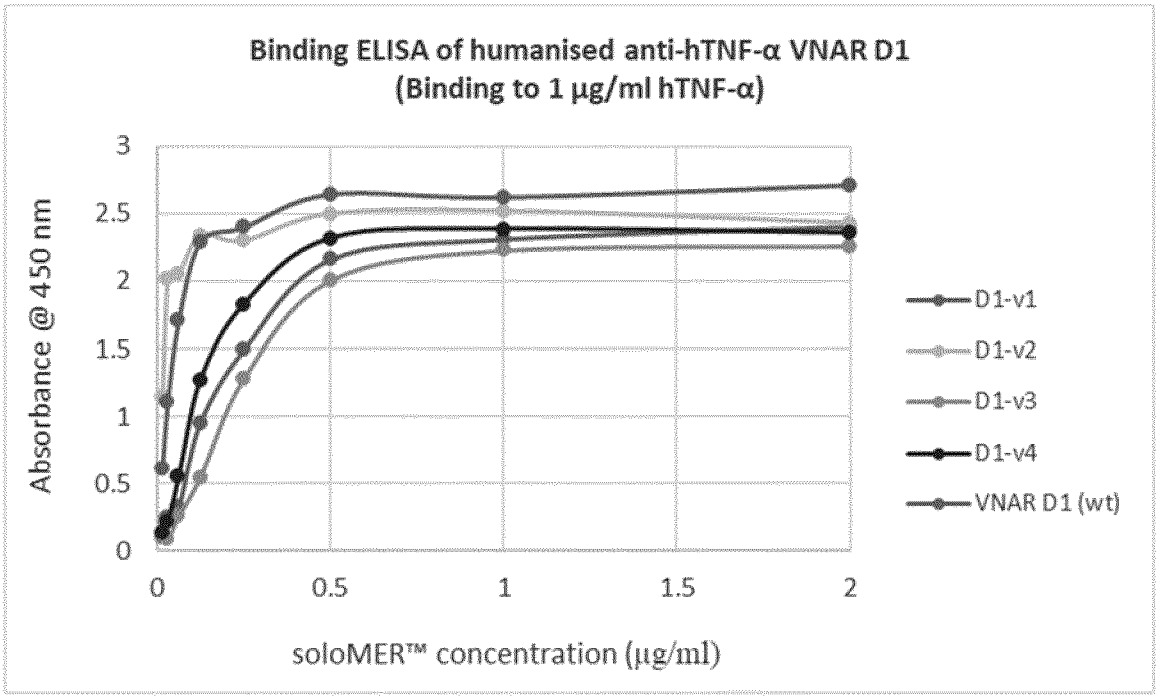

Binding ELISA of humanised anti-hTNF-α VNAR D1
(Binding to 1 µg/ml hTNF-α)
Absorbance @ 450 nm
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
soloMER™ concentration (µg/ml)
D1-v1
D1-v2
D1-v3
D1-v4
VNAR D1 (wt)

Figure 17

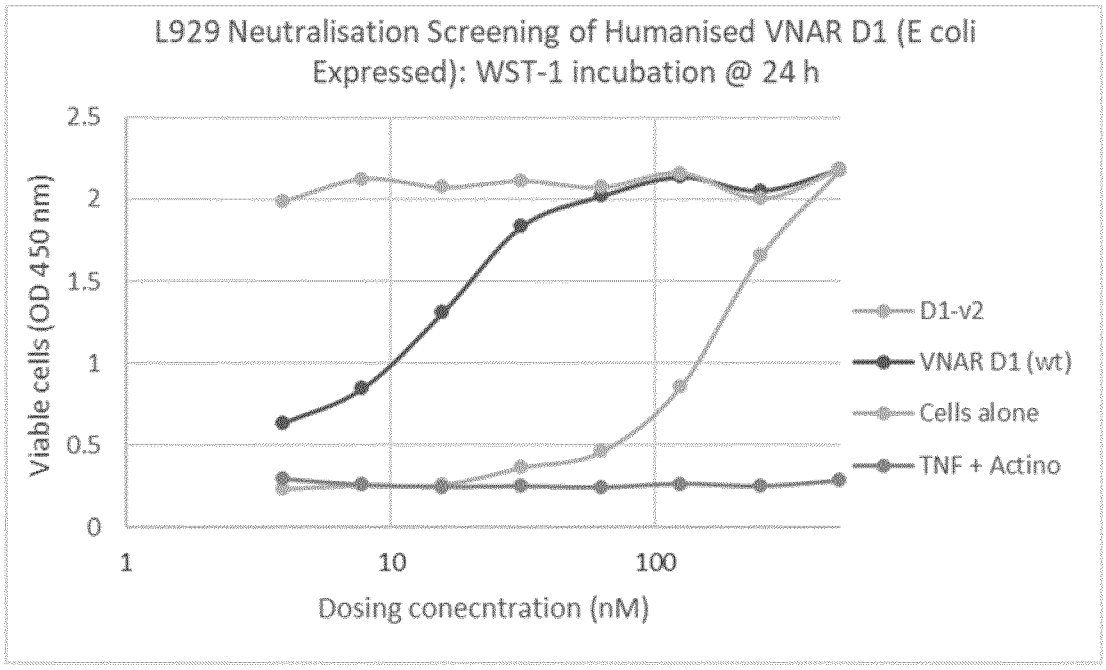

L929 Neutralisation Screening of Humanised VNAR D1 (E coli Expressed): WST-1 incubation @ 24 h
Viable cells (OD 450 nm)
2.5
2
1.5
1
0.5
0
1
10
100
Dosing conecntration (nM)
D1-v2
VNAR D1 (wt)
Cells alone
TNF + Actino

Figure 18

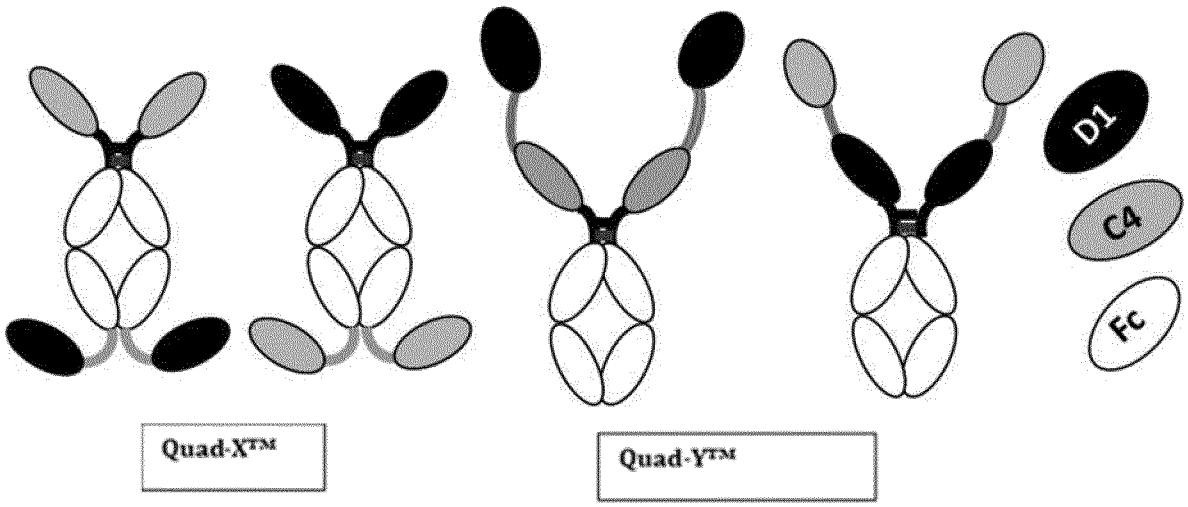


Figure 19

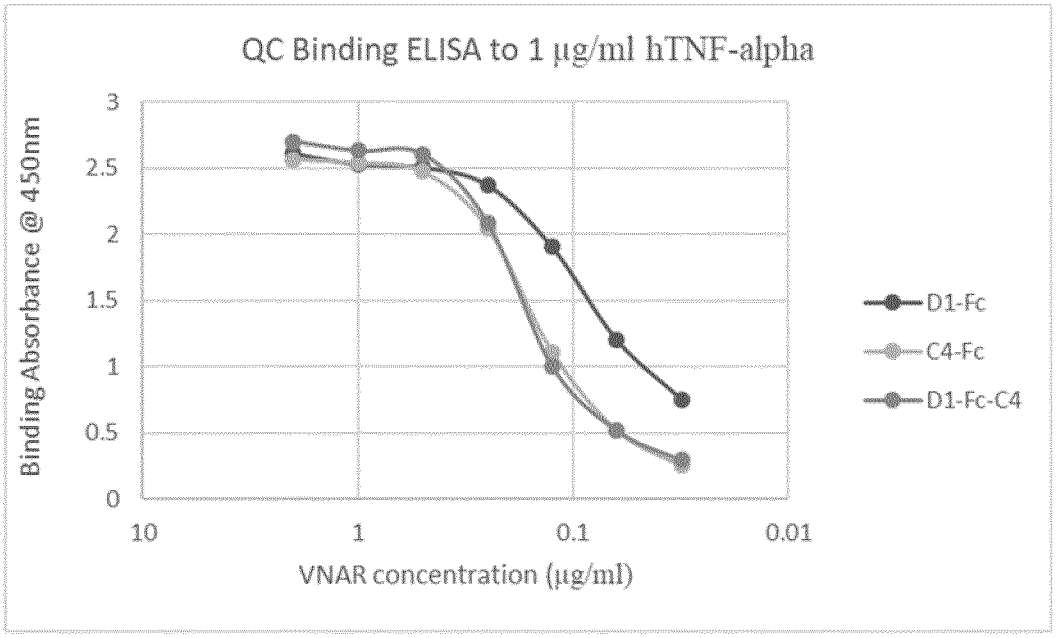

QC Binding ELISA to 1 µg/ml hTNF-alpha
Binding Absorbance @ 450nm
3
2.5
2
1.5
1
0.5
0
10
1
0.1
0.01
VNAR concentration (µg/ml)
D1-Fc
C4-Fc
D1-Fc-C4

Figure 20

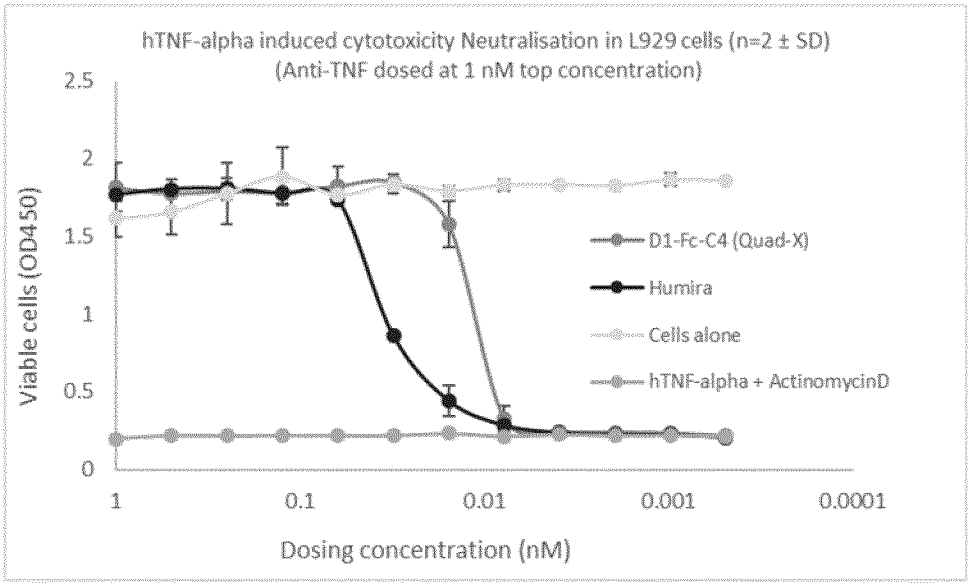

hTNF-alpha induced cytotoxicity Neutralisation in L929 cells (n=2 ± SD)
(Anti-TNF dosed at 1 nM top concentration)
Viable cells (OD450)
2.5
2
1.5
1
0.5
0
1
0.1
0.01
0.001
0.0001
Dosing concentration (nM)
D1-Fc-C4 (Quad-X)
Humira
Cells alone
hTNF-alpha + ActinomycinD

Figure 21

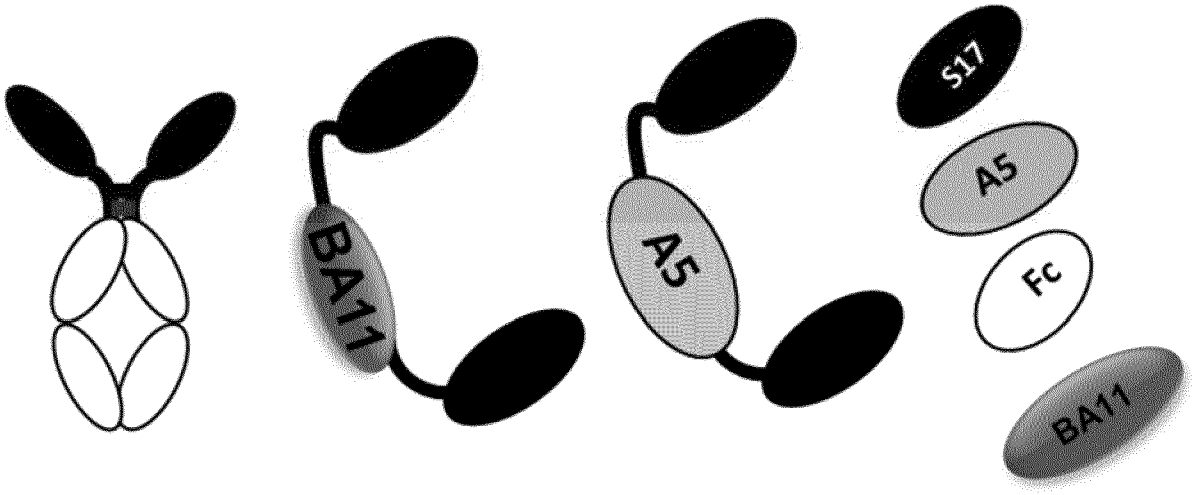
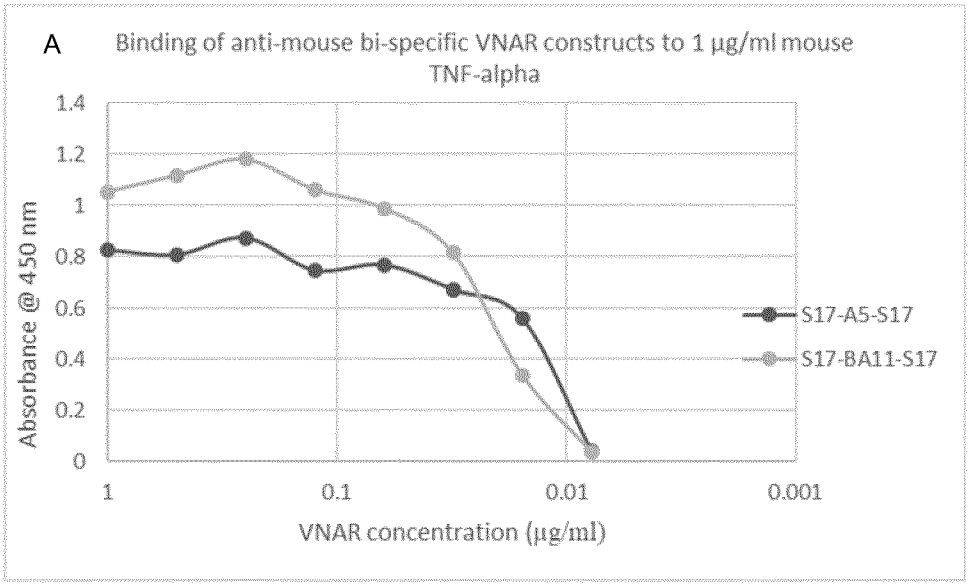
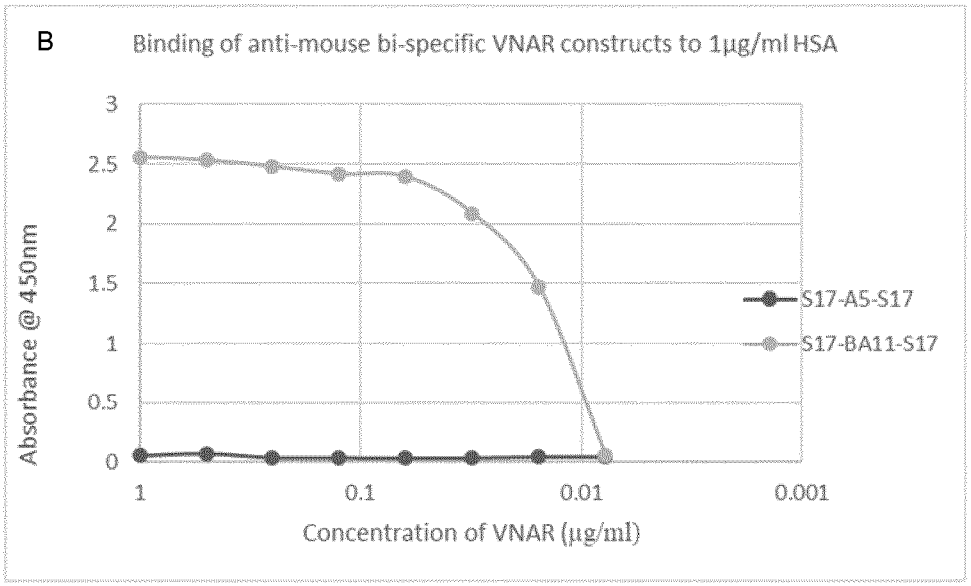


Figure 22


A
Binding of anti-mouse bi-specific VNAR constructs to 1 µg/ml mouse TNF-alpha
Absorbance @ 450 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
0.1
0.01
0.001
VNAR concentration (µg/ml)
S17-A5-S17
S17-BA11-S17


B
Binding of anti-mouse bi-specific VNAR constructs to 1µg/ml HSA
Absorbance @ 450nm
3
2.5
2
1.5
1
0.5
0
1
0.1
0.01
0.001
Concentration of VNAR (µg/ml)
S17-A5-S17
S17-BA11-S17

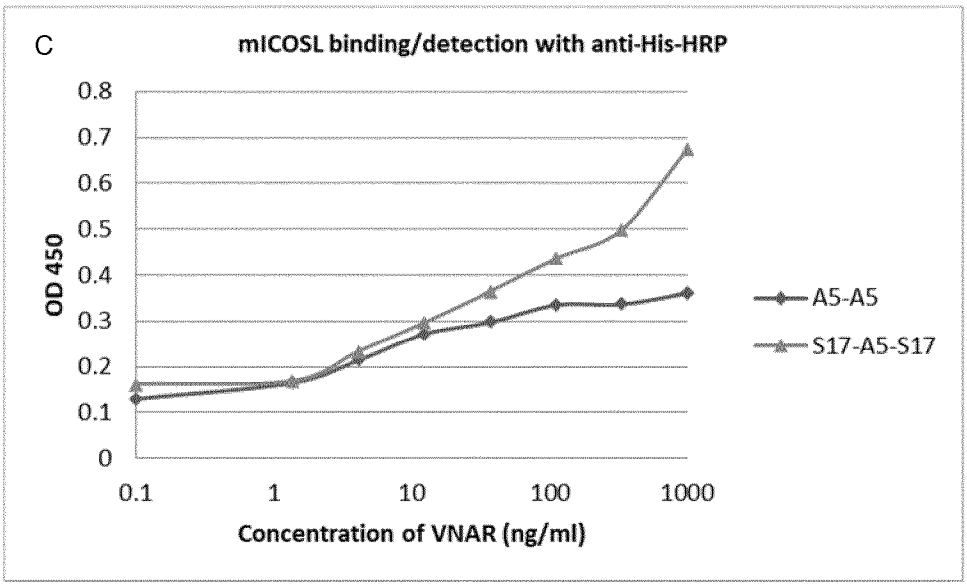
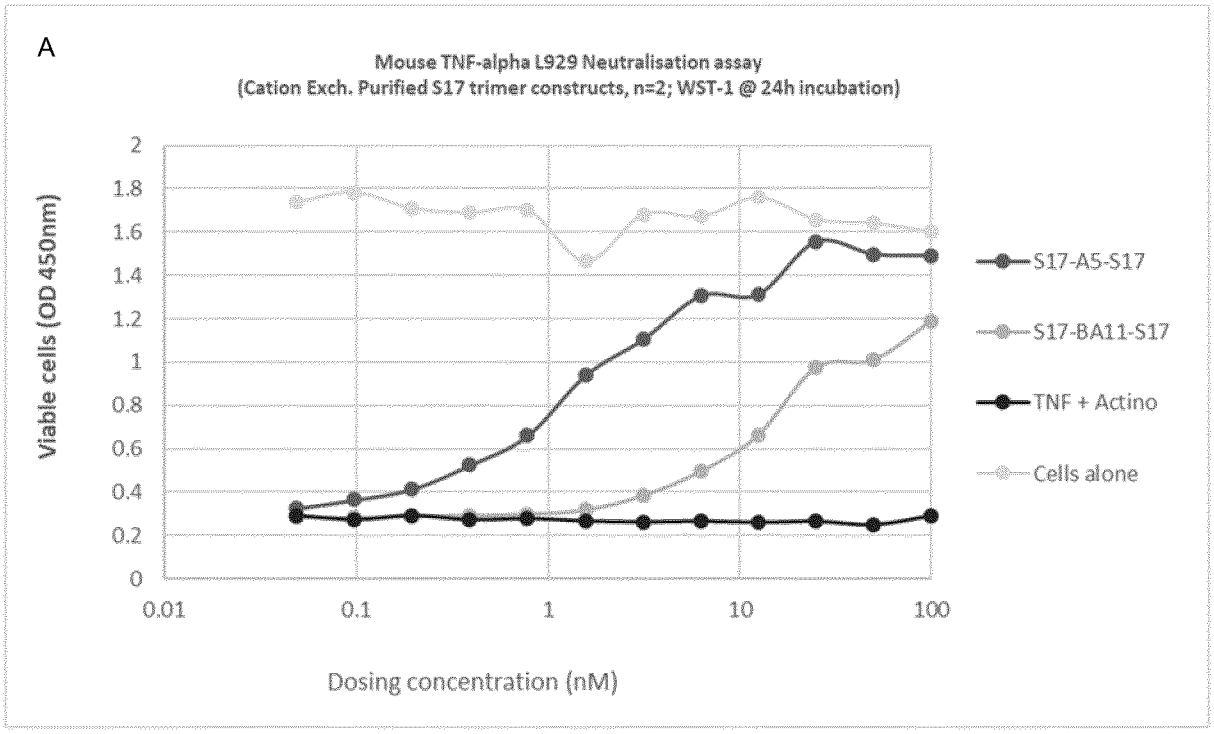

C
mICOSL binding/detection with anti-His-HRP
OD 450
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
1000
Concentration of VNAR (ng/ml)
A5-A5
S17-A5-S17

Figure 23


A
Mouse TNF-alpha L929 Neutralisation assay
(Cation Exch. Purified S17 trimer constructs, n=2; WST-1 @ 24h incubation)
Viable cells (OD 450nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
Dosing concentration (nM)
S17-A5-S17
S17-BA11-S17
TNF + Actino
Cells alone

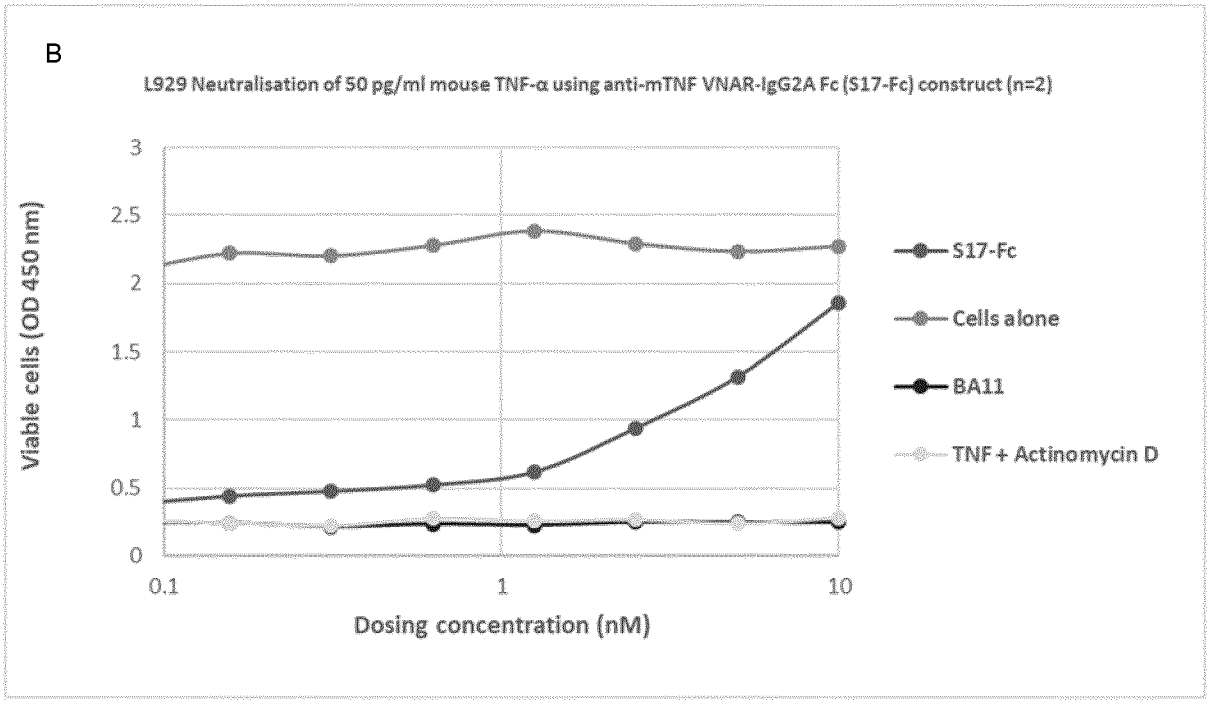

B
L929 Neutralisation of 50 pg/ml mouse TNF-α using anti-mTNF VNAR-IgG2A Fc (S17-Fc) construct (n=2)
Viable cells (OD 450 nm)
3
2.5
2
1.5
1
0.5
0
0.1
1
10
Dosing concentration (nM)
S17-Fc
Cells alone
BA11
TNF + Actinomycin D

Figure 24

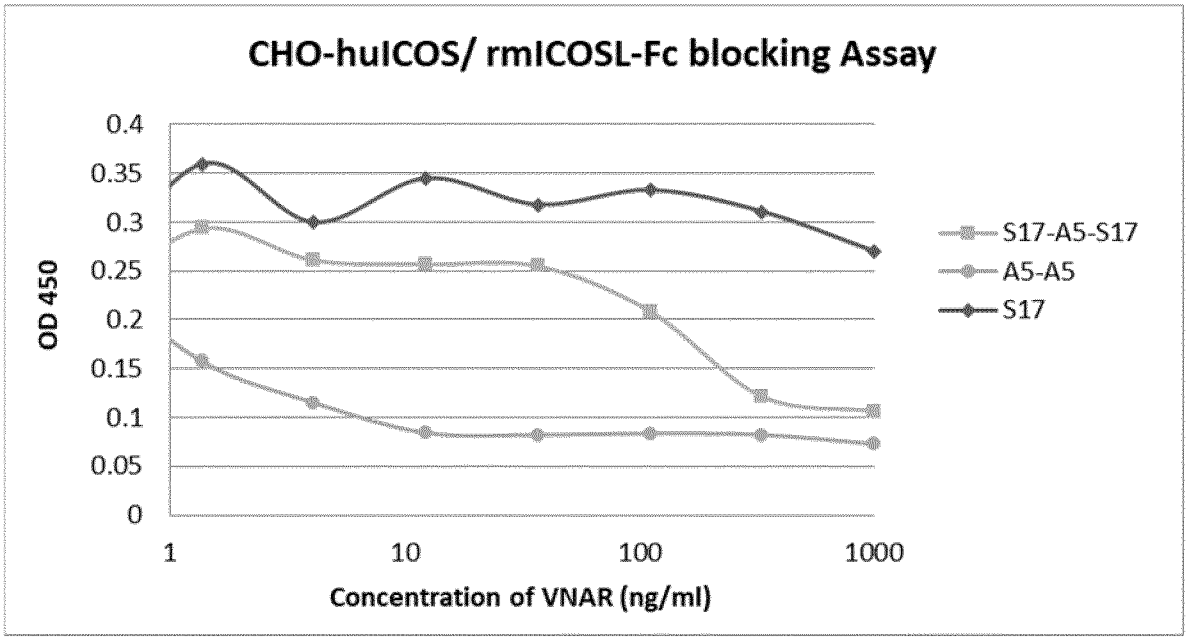

CHO-huICOS/ rmICOSL-Fc blocking Assay
OD 450
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
10
100
1000
Concentration of VNAR (ng/ml)
S17-A5-S17
A5-A5
S17

Figure 25

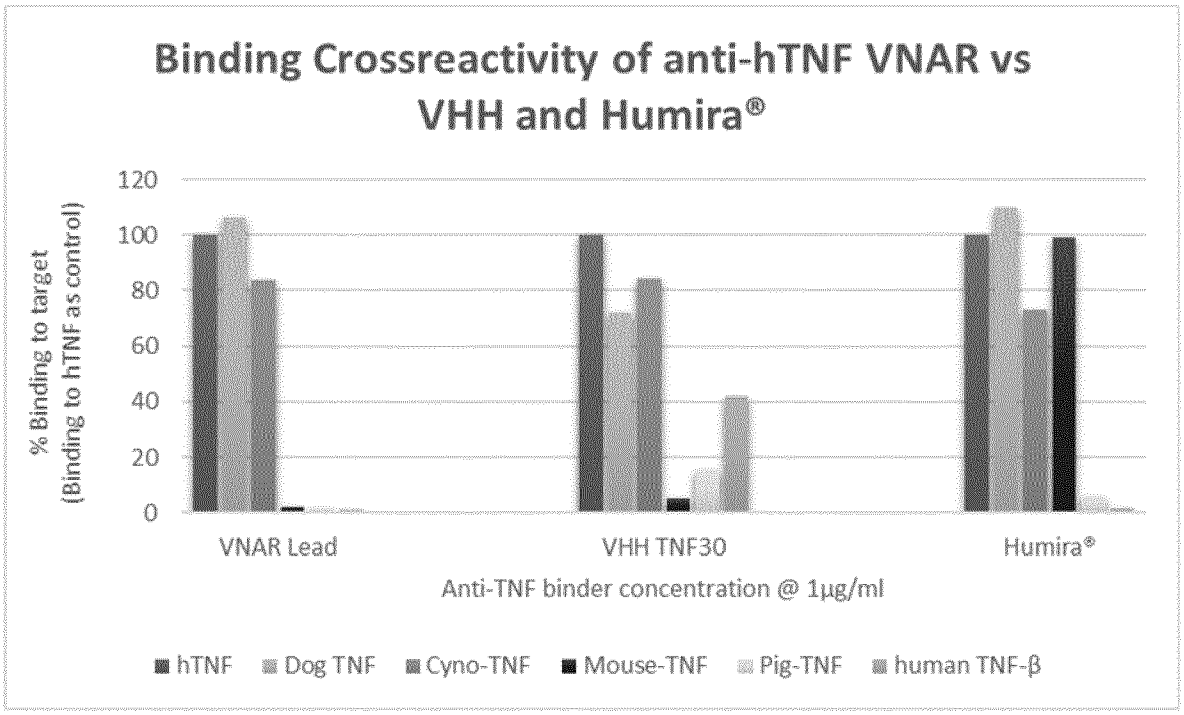

Binding Crossreactivity of anti-hTNF VNAR vs VHH and Humira®
% Binding to target (Binding to hTNF as control)
120
100
80
60
40
20
0
VNAR Lead
VHH TNF30
Humira®
Anti-TNF binder concentration @ 1µg/ml
hTNF
Dog TNF
Cyno-TNF
Mouse-TNF
Pig-TNF
human TNF-β

Figure 26

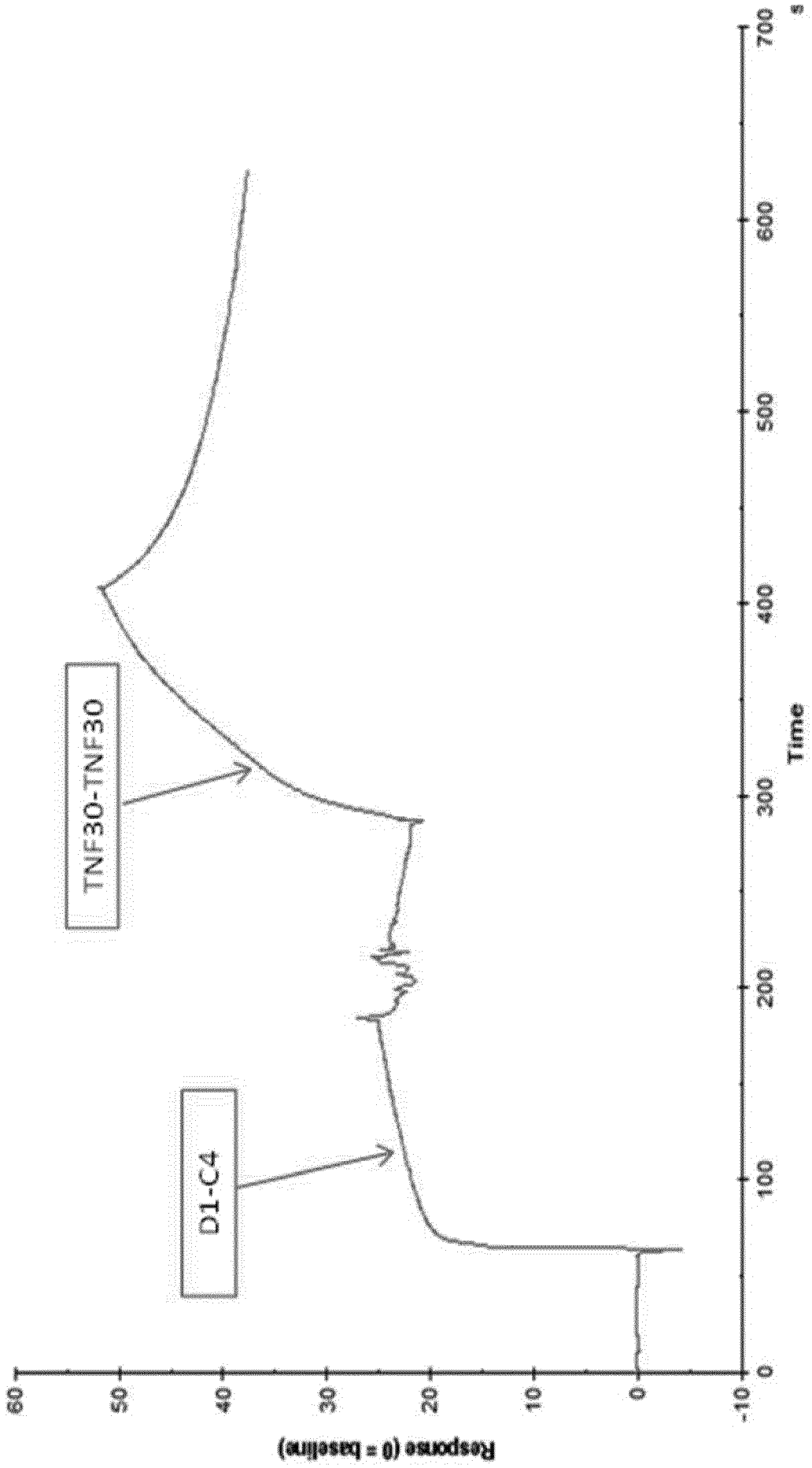

TNF30-TNF30
D1-C4
Response (0 = baseline)
Time
s
60
50
40
30
20
10
0
-10
0
100
200
300
400
500
600
700

Figure 27

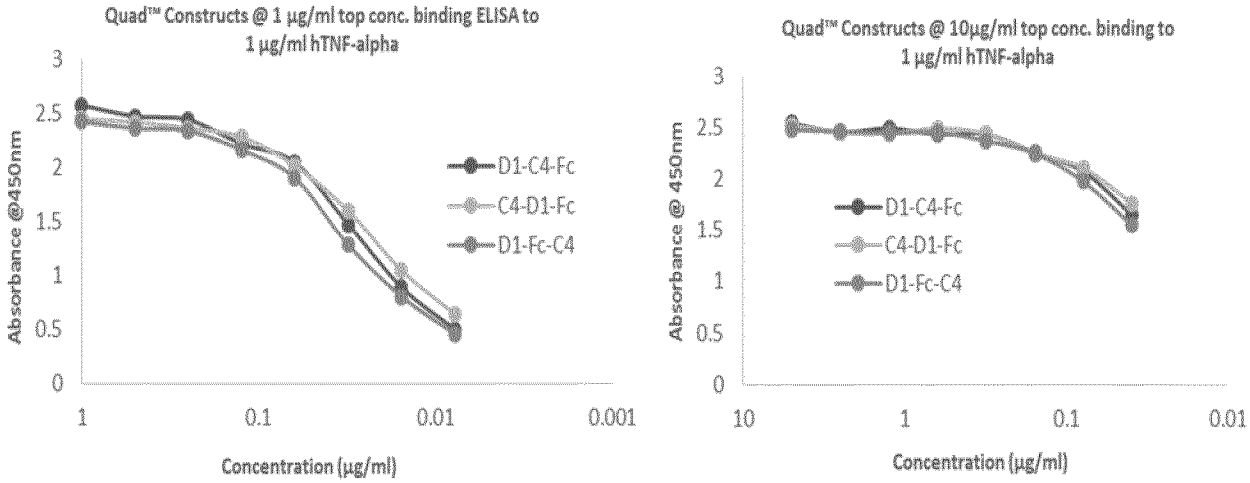

Quad™ Constructs @ 1 µg/ml top conc. binding ELISA to 1 µg/ml hTNF-alpha
Absorbance @450nm
3
2.5
2
1.5
1
0.5
0
1
0.1
0.01
0.001
Concentration (µg/ml)
D1-C4-Fc
C4-D1-Fc
D1-Fc-C4
Quad™ Constructs @ 10µg/ml top conc. binding to 1 µg/ml hTNF-alpha
Absorbance @ 450nm
3
2.5
2
1.5
1
0.5
0
10
1
0.1
0.01
Concentration (µg/ml)
D1-C4-Fc
C4-D1-Fc
D1-Fc-C4

Figure 28

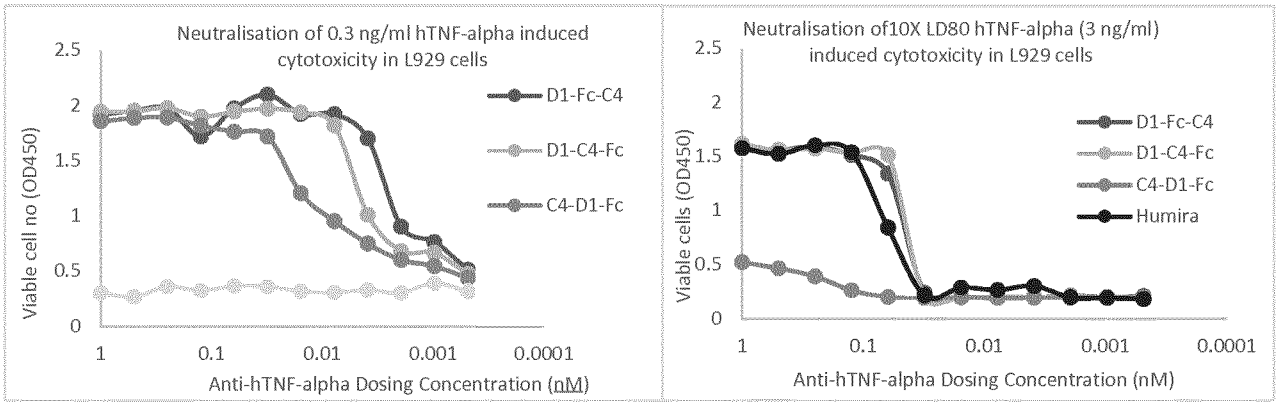

Neutralisation of 0.3 ng/ml hTNF-alpha induced cytotoxicity in L929 cells
Viable cell no (OD450)
2.5
2
1.5
1
0.5
0
1
0.1
0.01
0.001
0.0001
Anti-hTNF-alpha Dosing Concentration (nM)
D1-Fc-C4
D1-C4-Fc
C4-D1-Fc
Neutralisation of10X LD80 hTNF-alpha (3 ng/ml) induced cytotoxicity in L929 cells
Viable cells (OD450)
Anti-hTNF-alpha Dosing Concentration (nM)
Humira

Figure 29

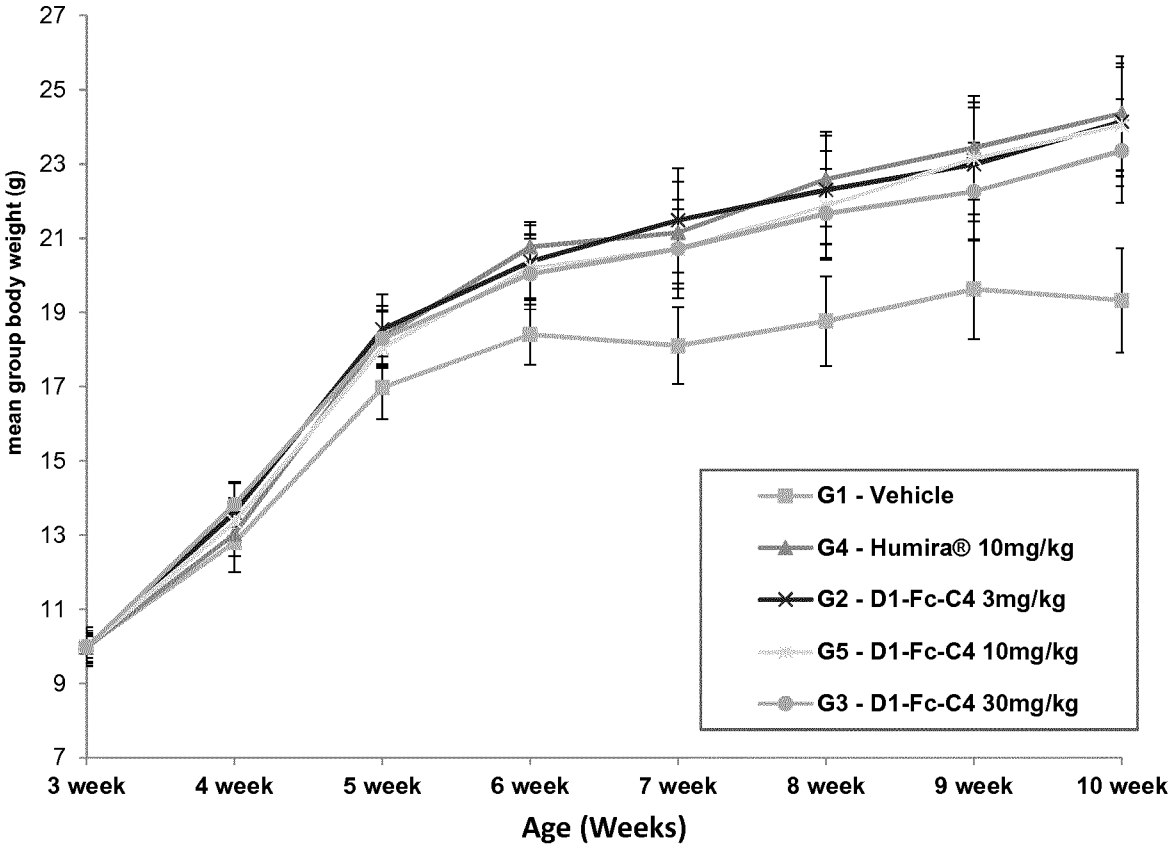

mean group body weight (g)
27
25
23
21
19
17
15
13
11
9
7
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week
Age (Weeks)
G1 - Vehicle
G4 - Humira® 10mg/kg
G2 - D1-Fc-C4 3mg/kg
G5 - D1-Fc-C4 10mg/kg
G3 - D1-Fc-C4 30mg/kg

Figure 30

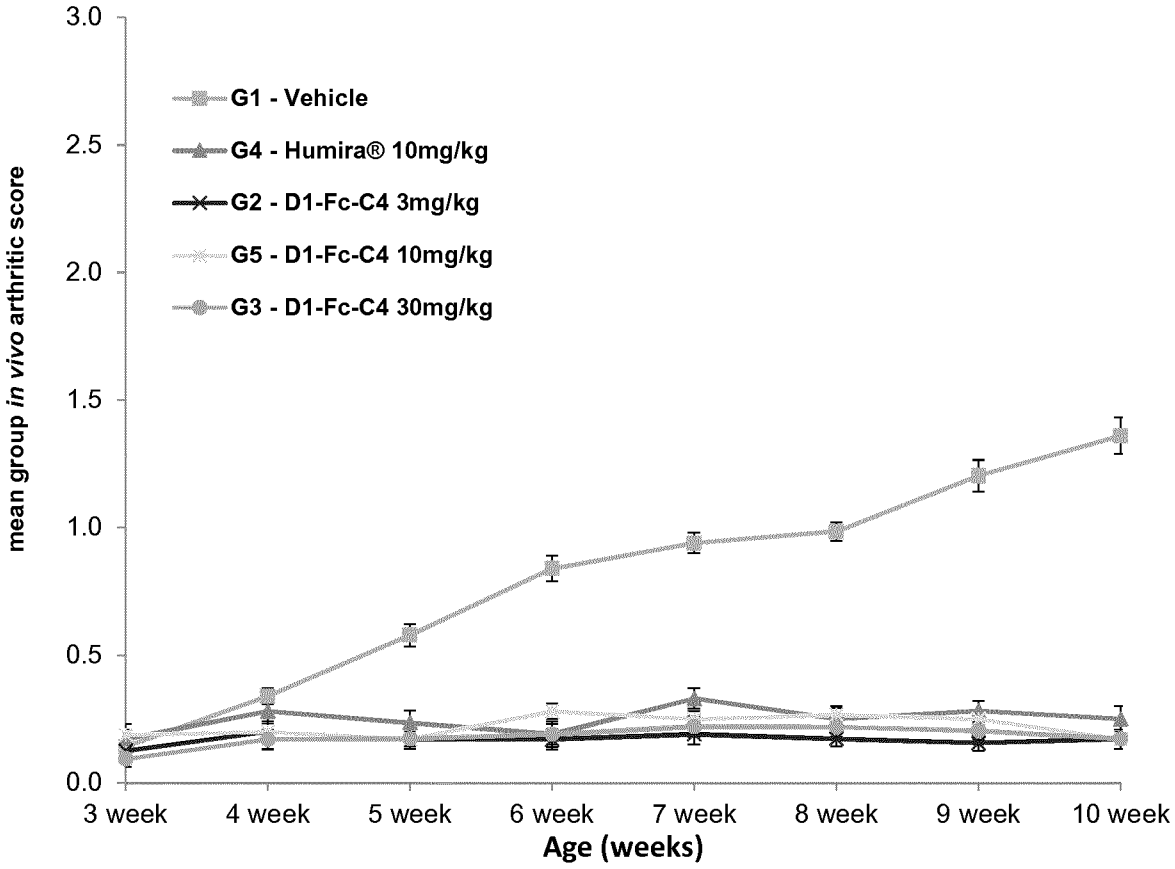

3.0
2.5
2.0
1.5
1.0
0.5
0.0
mean group *in vivo* arthritic score
G1 - Vehicle
G4 - Humira® 10mg/kg
G2 - D1-Fc-C4 3mg/kg
G5 - D1-Fc-C4 10mg/kg
G3 - D1-Fc-C4 30mg/kg
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week
Age (weeks)

Figure 31

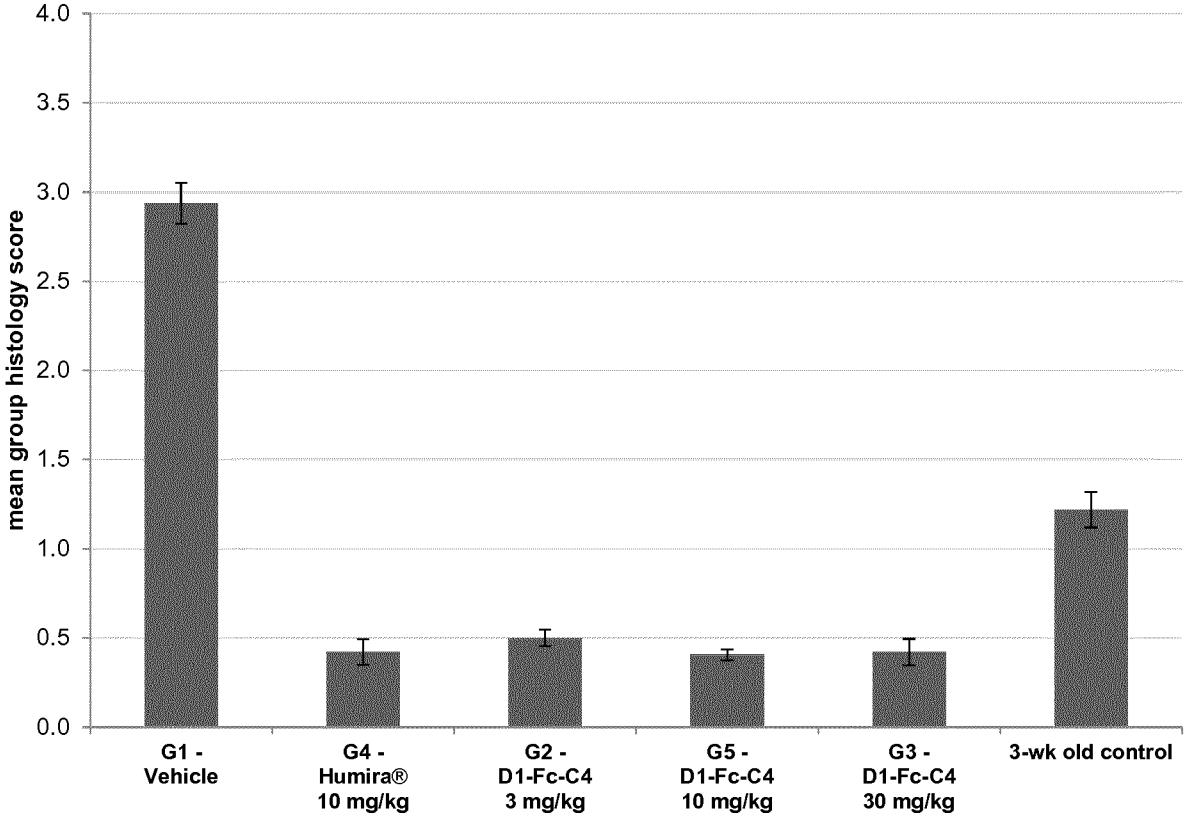

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
mean group histology score
G1 - Vehicle
G4 - Humira® 10 mg/kg
G2 - D1-Fc-C4 3 mg/kg
G5 - D1-Fc-C4 10 mg/kg
G3 - D1-Fc-C4 30 mg/kg
3-wk old control

Figure 32

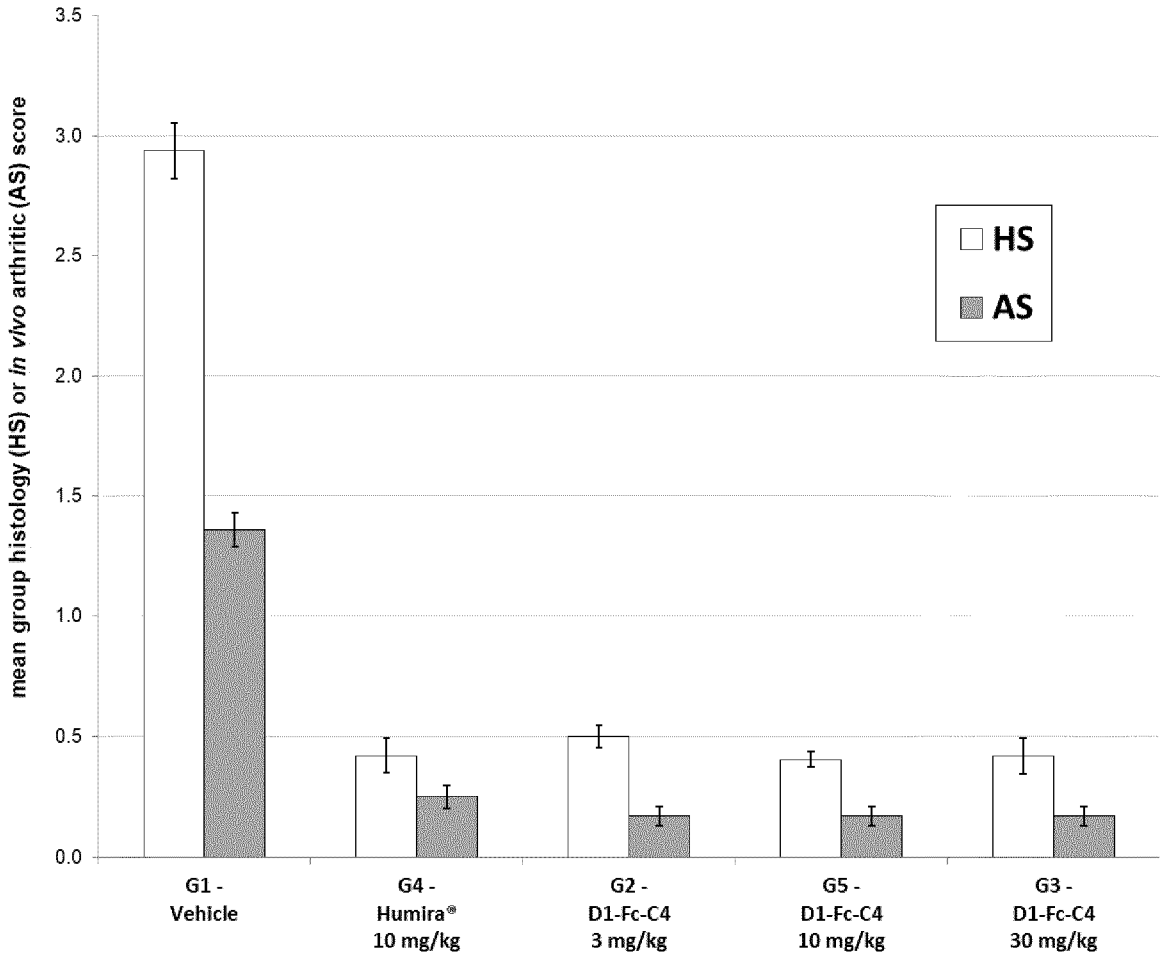

mean group histology (HS) or in vivo arthritic (AS) score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
HS
AS
G1 - Vehicle
G4 - Humira® 10 mg/kg
G2 - D1-Fc-C4 3 mg/kg
G5 - D1-Fc-C4 10 mg/kg
G3 - D1-Fc-C4 30 mg/kg

Figure 33

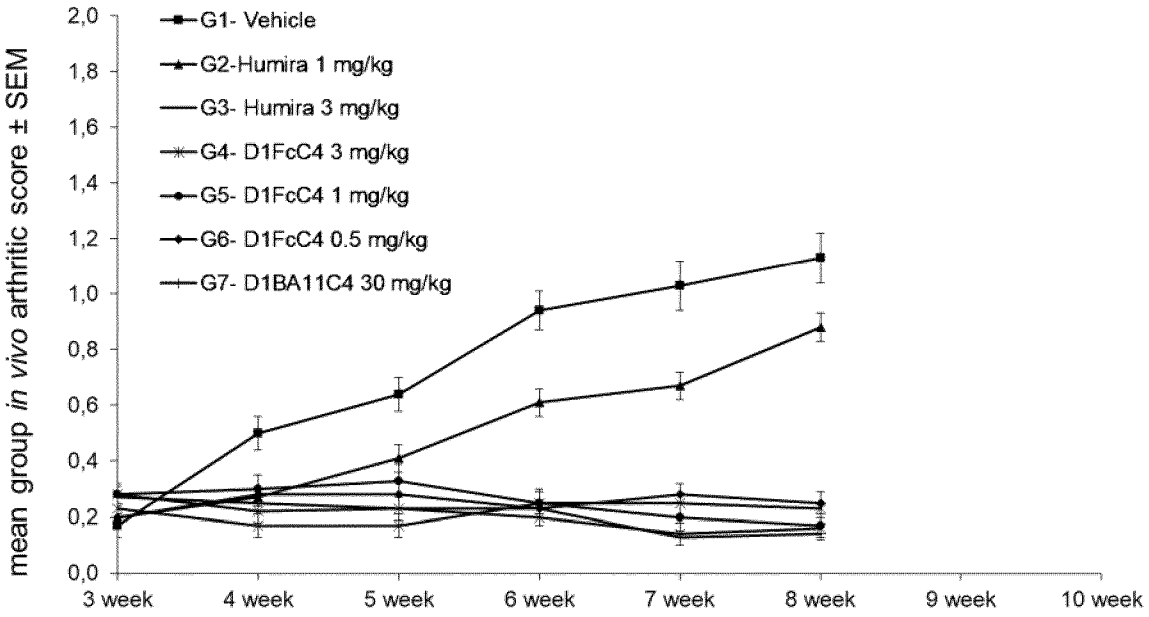

mean group *in vivo* arthritic score ± SEM
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
G1- Vehicle
G2-Humira 1 mg/kg
G3- Humira 3 mg/kg
G4- D1FcC4 3 mg/kg
G5- D1FcC4 1 mg/kg
G6- D1FcC4 0.5 mg/kg
G7- D1BA11C4 30 mg/kg
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week

Figure 34

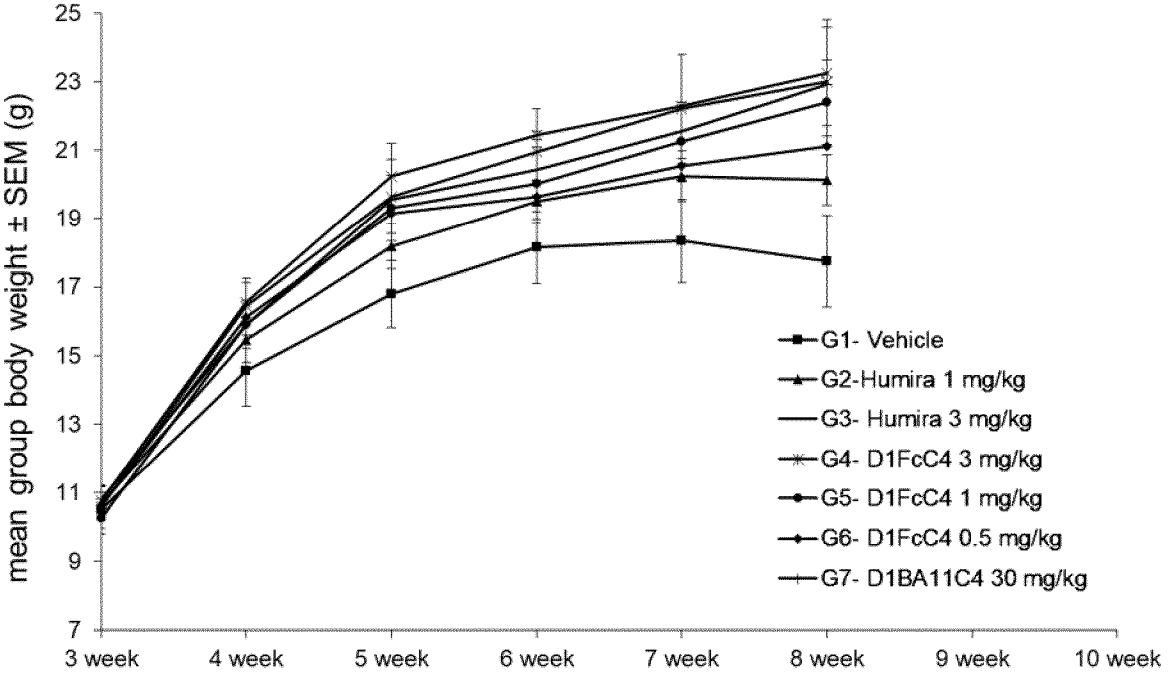
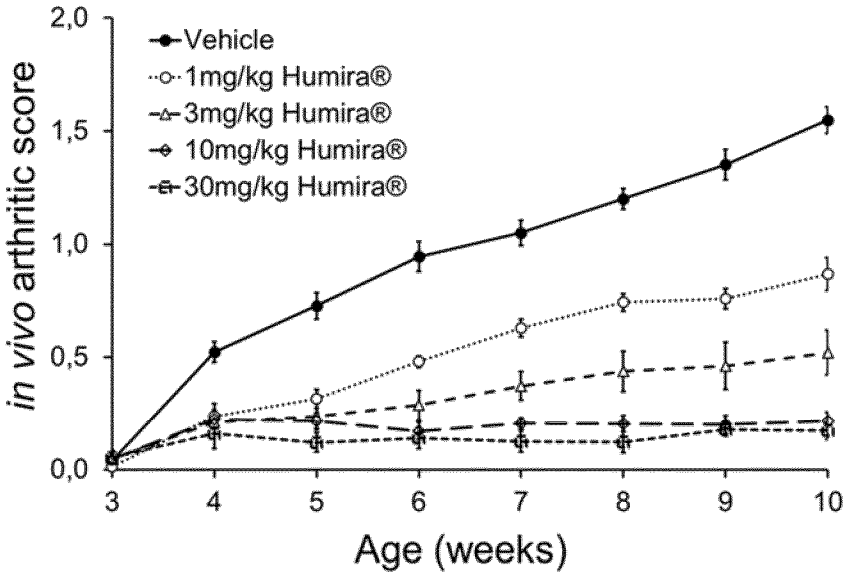

mean group body weight ± SEM (g)
25
23
21
19
17
15
13
11
9
7
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week
G1- Vehicle
G2-Humira 1 mg/kg
G3- Humira 3 mg/kg
G4- D1FcC4 3 mg/kg
G5- D1FcC4 1 mg/kg
G6- D1FcC4 0.5 mg/kg
G7- D1BA11C4 30 mg/kg

Figure 35


2,0
1,5
1,0
0,5
0,0
in vivo arthritic score
Vehicle
1mg/kg Humira®
3mg/kg Humira®
10mg/kg Humira®
30mg/kg Humira®
3
4
5
6
7
8
9
10
Age (weeks)

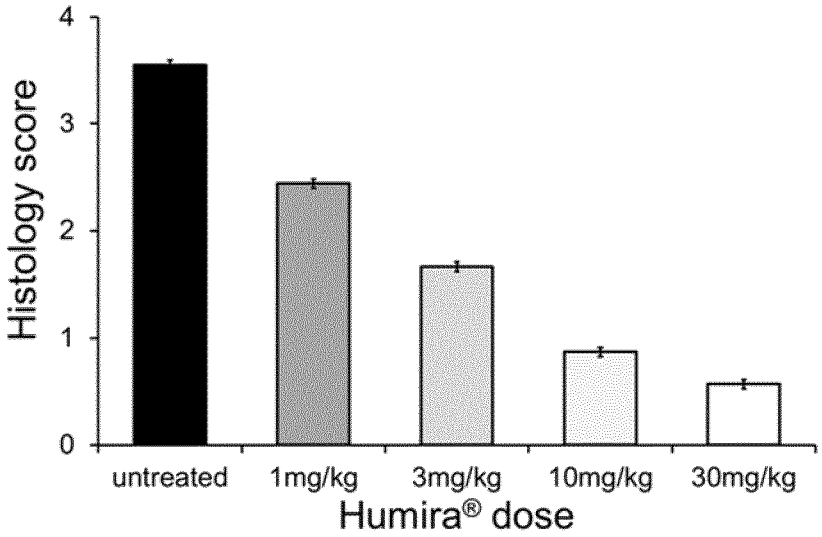

4
3
2
1
0
Histology score
untreated
1mg/kg
3mg/kg
10mg/kg
30mg/kg
Humira® dose

Figure 36

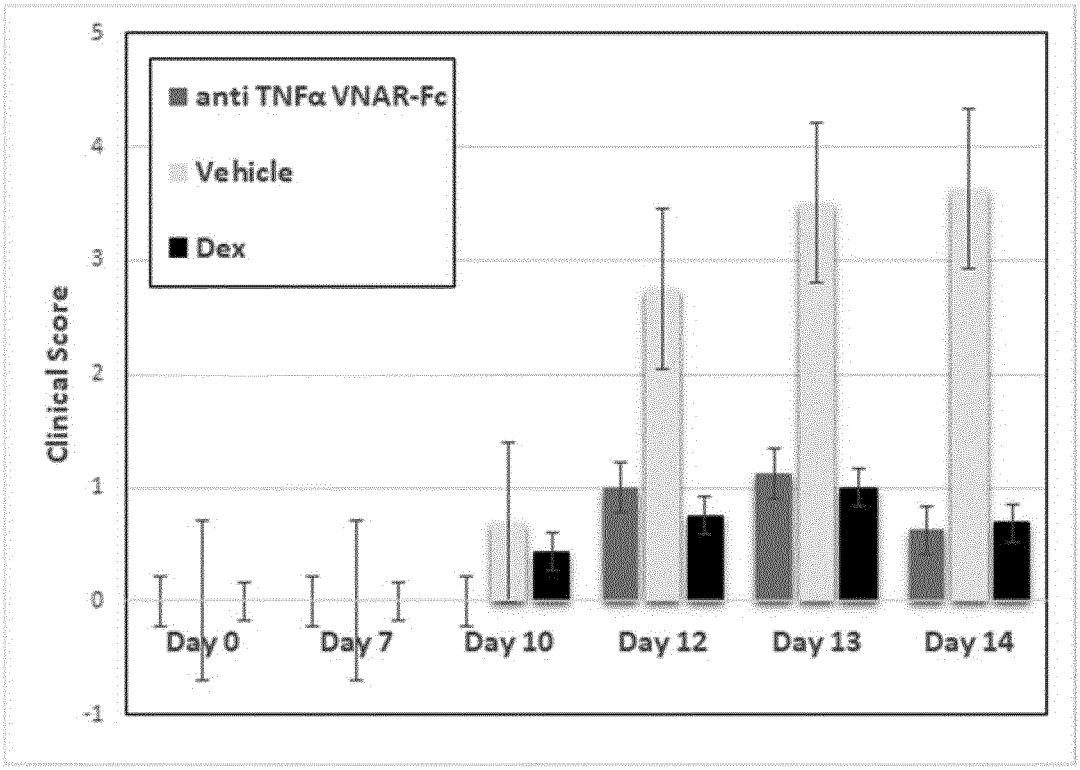

anti TNFα VNAR-Fc
Vehicle
Dex
Clinical Score
5
4
3
2
1
0
-1
Day 0
Day 7
Day 10
Day 12
Day 13
Day 14

Figure 37A

Figure 37A:

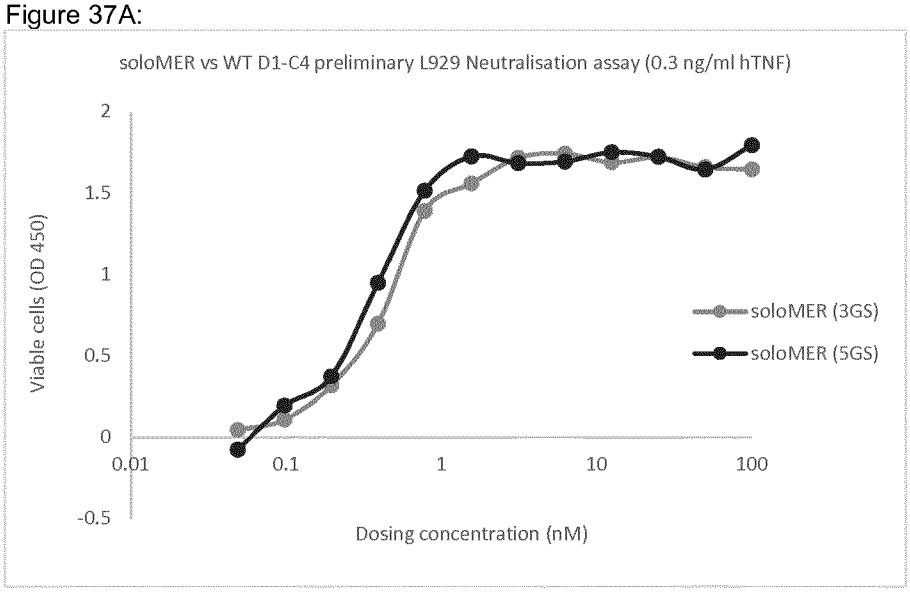


Figure 37B:

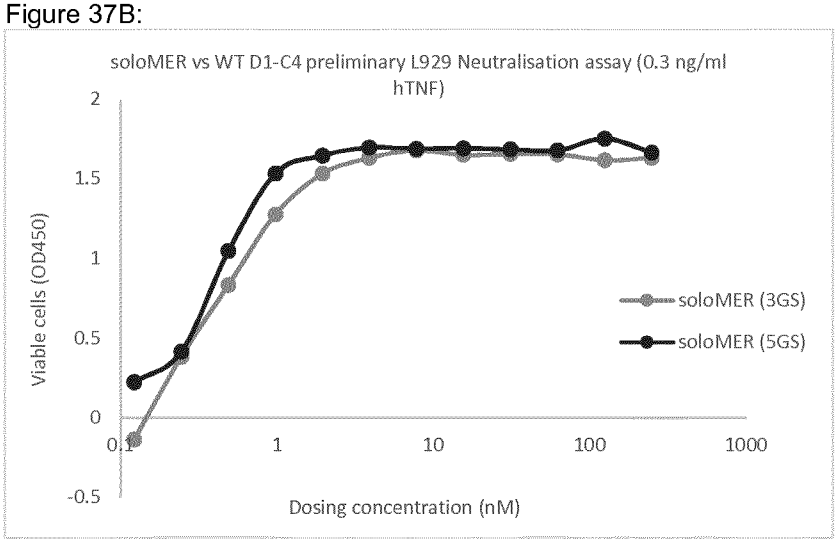


Figure 38

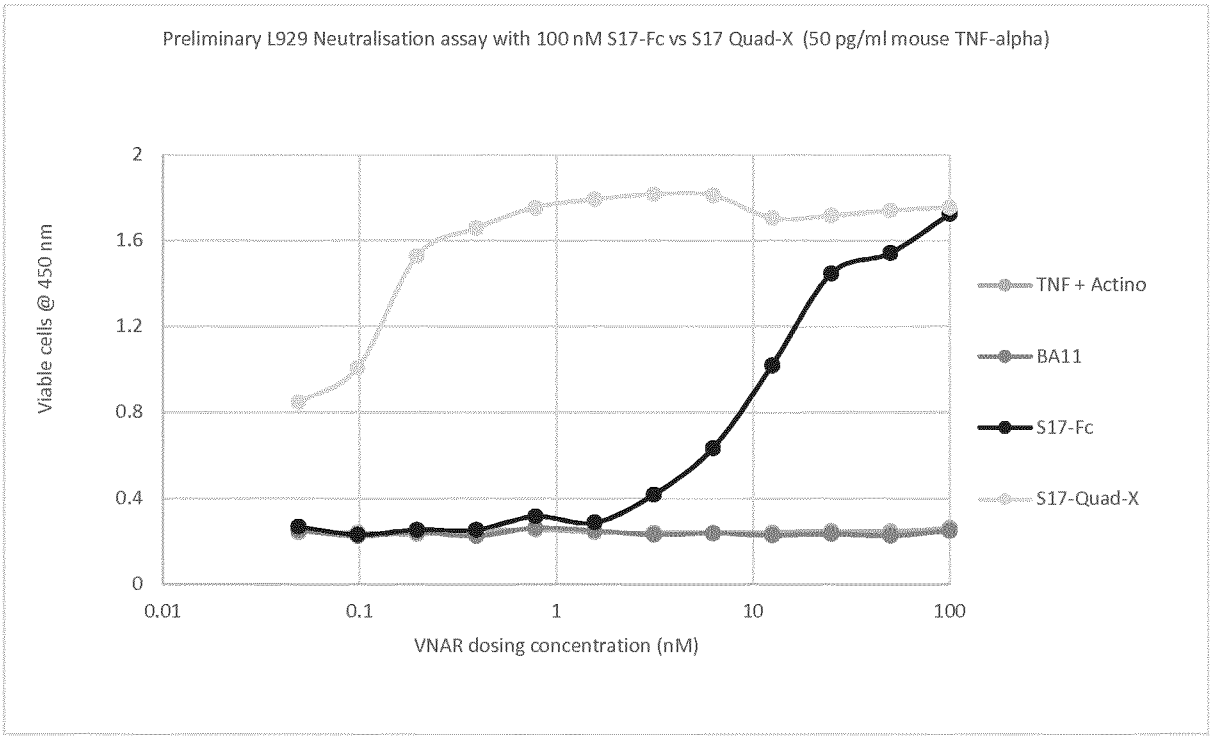

Preliminary L929 Neutralisation assay with 100 nM S17-Fc vs S17 Quad-X (50 pg/ml mouse TNF-alpha)
Viable cells @ 450 nm
2
1.6
1.2
0.8
0.4
0
0.01
0.1
1
10
100
VNAR dosing concentration (nM)
TNF + Actino
BA11
S17-Fc
S17-Quad-X

Figure 39A

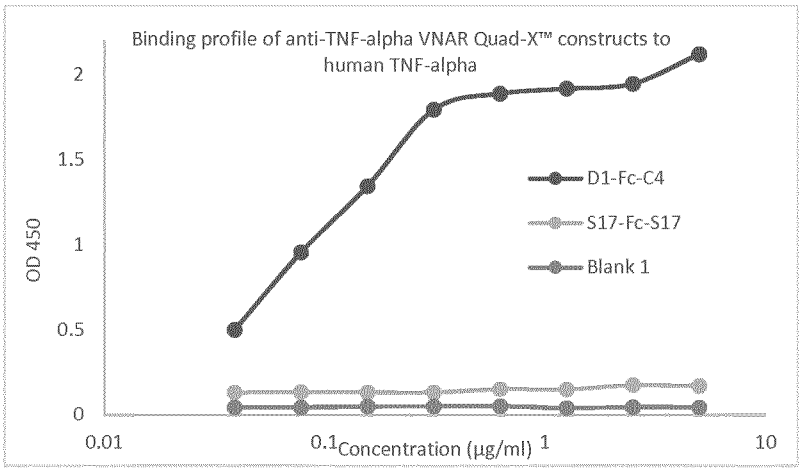


Figure 39B

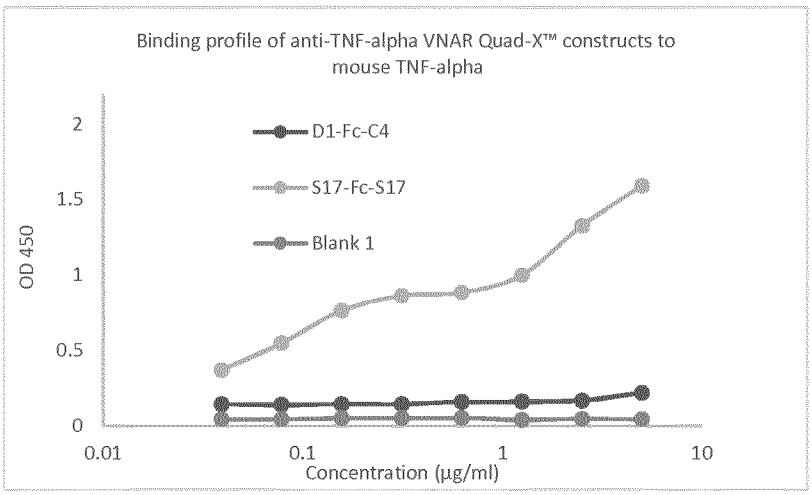


Figure 39C