

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
15 July 2004 (15.07.2004)

PCT

(10) International Publication Number
WO 2004/058821 A2

(51) International Patent Classification⁷: **C07K 16/00**

(74) Agent: **MASCHIO, Antonio**; D Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).

(21) International Application Number:
PCT/GB2003/005646

(22) International Filing Date:
24 December 2003 (24.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0230202.4 27 December 2002 (27.12.2002) GB
PCT/GB03/002804 30 June 2003 (30.06.2003) GB
0327706.8 28 November 2003 (28.11.2003) GB

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): **DOMANTIS LIMITED** [GB/GB]; Abington, Granta Park, Cambridge CB1 6GS (GB).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **WINTER, Greg** [GB/GB]; Room H3, Great Court, Trinity College, Trinity Street, Cambridge CB2 1TQ (GB). **TOMLINSON, Ian** [GB/GB]; Domantis Limited, Granta Park, Abington, Cambridge CB1 6GS (GB). **IGNATOVICH, Olga** [BY/GB]; Domantis Limited, Granta Park, Abington, Cambridge CB1 6GS (GB). **WOOLVEN, Ben** [GB/GB]; Domantis Limited, Granta Park, Abington, Cambridge CB1 6GS (GB).

Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LIGAND

(57) Abstract: The invention provides a dual-specific ligand comprising a first immunoglobulin variable domain having a first binding specificity for a target ligand and a complementary or non-complementary immunoglobulin variable domain having a second binding specificity for a receptor of the target ligand.



WO 2004/058821 A2

Ligand

The present invention relates to dual specific ligands. In particular, the invention provides a method for the preparation of dual-specific ligands comprising a first immunoglobulin single variable domain binding to a first antigen or epitope, and a second immunoglobulin single variable domain binding to a second antigen or epitope. More particularly, the invention relates to dual-specific ligands wherein binding to at least one of the first and second antigens or epitopes acts to increase the half-life of the ligand *in vivo*. Open and closed conformation ligands comprising more than one binding specificity are described.

Introduction

The antigen binding domain of an antibody comprises two separate regions: a heavy chain variable domain (V_H) and a light chain variable domain (V_L : which can be either V_K or V_λ). The antigen binding site itself is formed by six polypeptide loops: three from V_H domain (H1, H2 and H3) and three from V_L domain (L1, L2 and L3). A diverse primary repertoire of V genes that encode the V_H and V_L domains is produced by the combinatorial rearrangement of gene segments. The V_H gene is produced by the recombination of three gene segments, V_H , D and J_H . In humans, there are approximately 51 functional V_H segments (Cook and Tomlinson (1995) *Immunol Today*, **16**: 237), 25 functional D segments (Corbett *et al.* (1997) *J. Mol. Biol.*, **268**: 69) and 6 functional J_H segments (Ravetch *et al.* (1981) *Cell*, **27**: 583), depending on the haplotype. The V_H segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_H domain (H1 and H2), whilst the V_H , D and J_H segments combine to form the third antigen binding loop of the V_H domain (H3). The V_L gene is produced by the recombination of only two gene segments, V_L and J_L . In humans, there are approximately 40 functional V_K segments (Schäble and Zachau (1993) *Biol. Chem. Hoppe-Seyler*, **374**: 1001), 31 functional V_λ segments (Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220; Kawasaki *et al.* (1997) *Genome Res.*, **7**: 250), 5 functional J_K segments (Hieter *et al.* (1982) *J. Biol. Chem.*, **257**: 1516) and 4 functional J_λ segments (Vasicek

and Leder (1990) *J. Exp. Med.*, **172**: 609), depending on the haplotype. The V_L segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_L domain (L1 and L2), whilst the V_L and J_L segments combine to form the third antigen binding loop of the V_L domain (L3). Antibodies selected from this primary repertoire are believed to be sufficiently diverse to bind almost all antigens with at least moderate affinity. High affinity antibodies are produced by "affinity maturation" of the rearranged genes, in which point mutations are generated and selected by the immune system on the basis of improved binding.

Analysis of the structures and sequences of antibodies has shown that five of the six antigen binding loops (H1, H2, L1, L2, L3) possess a limited number of main-chain conformations or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, **196**: 901; Chothia *et al.* (1989) *Nature*, **342**: 877). The main-chain conformations are determined by (i) the length of the antigen binding loop, and (ii) particular residues, or types of residue, at certain key position in the antigen binding loop and the antibody framework. Analysis of the loop lengths and key residues has enabled us to predict the main-chain conformations of H1, H2, L1, L2 and L3 encoded by the majority of human antibody sequences (Chothia *et al.* (1992) *J. Mol. Biol.*, **227**: 799; Tomlinson *et al.* (1995) *EMBO J.*, **14**: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, **263**: 800; Shirai *et al.* (1996) *FEBS Letters*, **399**: 1).

Bispecific antibodies comprising complementary pairs of V_H and V_L regions are known in the art. These bispecific antibodies must comprise two pairs of V_H and V_L s, each V_H/V_L pair binding to a single antigen or epitope. Methods described involve hybrid hybridomas (Milstein & Cuello AC, *Nature* 305:537-40), minibodies (Hu *et al.*, (1996) *Cancer Res* 56:3055-3061;), diabodies (Holliger *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 6444-6448; WO 94/13804), chelating recombinant antibodies (CRAbs; (Neri *et al.*, (1995) *J. Mol. Biol.* 246, 367-373), biscFv (e.g. Atwell *et al.*, (1996) *Mol. Immunol.* 33, 1301-1312), "knobs in holes" stabilised antibodies (Carter *et al.*, (1997) *Protein Sci.* 6, 781-

788). In each case each antibody species comprises two antigen-binding sites, each fashioned by a complementary pair of V_H and V_L domains. Each antibody is thereby able to bind to two different antigens or epitopes at the same time, with the binding to EACH antigen or epitope mediated by a V_H and its complementary V_L domain. Each of these techniques presents its particular disadvantages; for instance in the case of hybrid hybridomas, inactive V_H/V_L pairs can greatly reduce the fraction of bispecific IgG. Furthermore, most bispecific approaches rely on the association of the different V_H/V_L pairs or the association of V_H and V_L chains to recreate the two different V_H/V_L binding sites. It is therefore impossible to control the ratio of binding sites to each antigen or epitope in the assembled molecule and thus many of the assembled molecules will bind to one antigen or epitope but not the other. In some cases it has been possible to engineer the heavy or light chains at the sub-unit interfaces (Carter *et al.*, 1997) in order to improve the number of molecules which have binding sites to both antigens or epitopes but this never results in all molecules having binding to both antigens or epitopes.

There is some evidence that two different antibody binding specificities might be incorporated into the same binding site, but these generally represent two or more specificities that correspond to structurally related antigens or epitopes or to antibodies that are broadly cross-reactive.. For example, cross-reactive antibodies have been described, usually where the two antigens are related in sequence and structure, such as hen egg white lysozyme and turkey lysozyme (McCafferty *et al.*, WO 92/01047) or to free hapten and to hapten conjugated to carrier (Griffiths AD *et al.* *EMBO J* 1994 13:14 3245-60). In a further example, WO 02/02773 (Abbott Laboratories) describes antibody molecules with "dual specificity". The antibody molecules referred to are antibodies raised or selected against multiple antigens, such that their specificity spans more than a single antigen. Each complementary V_H/V_L pair in the antibodies of WO 02/02773 specifies a single binding specificity for two or more structurally related antigens; the V_H and V_L domains in such complementary pairs do not each possess a separate specificity. The antibodies thus have a broad single specificity which encompasses two antigens, which are structurally related. Furthermore natural autoantibodies have been described that are polyreactive (Casali & Notkins, *Ann. Rev. Immunol.* 7, 515-531), reacting with at least two (usually more) different antigens or epitopes that are not structurally related. It

has also been shown that selections of random peptide repertoires using phage display technology on a monoclonal antibody will identify a range of peptide sequences that fit the antigen binding site. Some of the sequences are highly related, fitting a consensus sequence, whereas others are very different and have been termed mimotopes (Lane & Stephen, Current Opinion in Immunology, 1993, 5, 268-271). It is therefore clear that a natural four-chain antibody, comprising associated and complementary V_H and V_L domains, has the potential to bind to many different antigens from a large universe of known antigens. It is less clear how to create a binding site to two given antigens in the same antibody, particularly those which are not necessarily structurally related.

10

Protein engineering methods have been suggested that may have a bearing on this. For example it has also been proposed that a catalytic antibody could be created with a binding activity to a metal ion through one variable domain, and to a hapten (substrate) through contacts with the metal ion and a complementary variable domain (Barbas et al., 1993 Proc. Natl. Acad. Sci USA 90, 6385-6389). However in this case, the binding and catalysis of the substrate (first antigen) is proposed to require the binding of the metal ion (second antigen). Thus the binding to the V_H/V_L pairing relates to a single but multi-component antigen.

Methods have been described for the creation of bispecific antibodies from camel antibody heavy chain single domains in which binding contacts for one antigen are created in one variable domain, and for a second antigen in a second variable domain. However the variable domains were not complementary. Thus a first heavy chain variable domain is selected against a first antigen, and a second heavy chain variable domain against a second antigen, and then both domains are linked together on the same chain to give a bispecific antibody fragment (Conrath et al., J. Biol. Chem. 270, 27589-27594). However the camel heavy chain single domains are unusual in that they are derived from natural camel antibodies which have no light chains, and indeed the heavy chain single domains are unable to associate with camel light chains to form complementary V_H and V_L pairs.

30

Single heavy chain variable domains have also been described, derived from natural antibodies which are normally associated with light chains (from monoclonal antibodies

or from repertoires of domains; see EP-A-0368684). These heavy chain variable domains have been shown to interact specifically with one or more related antigens but have not been combined with other heavy or light chain variable domains to create a ligand with a specificity for two or more different antigens. Furthermore, these single domains have been shown to have a very short *in vivo* half-life. Therefore such domains are of limited therapeutic value.

It has been suggested to make bispecific antibody fragments by linking heavy chain variable domains of different specificity together (as described above). The disadvantage with this approach is that isolated antibody variable domains may have a hydrophobic interface that normally makes interactions with the light chain and is exposed to solvent and may be "sticky" allowing the single domain to bind to hydrophobic surfaces. Furthermore, in the absence of a partner light chain the combination of two or more different heavy chain variable domains and their association, possibly via their hydrophobic interfaces, may prevent them from binding to one in not both of the ligands they are able to bind in isolation. Moreover, in this case the heavy chain variable domains would not be associated with complementary light chain variable domains and thus may be less stable and readily unfold (Worn & Pluckthun, 1998 Biochemistry 37, 13120-7).

20

Summary of the invention

The inventors have described, in their copending international patent application WO 03/002609 as well as copending unpublished UK patent application 0230203.2, dual specific immunoglobulin ligands which comprise immunoglobulin single variable domains which each have different specificities. The domains may act in competition with each other or independently to bind antigens or epitopes on target molecules.

In a first configuration, the present invention provides a further improvement in dual specific ligands as developed by the present inventors, in which one specificity of the ligand is directed towards a protein or polypeptide target, and another specificity is directed to a receptor for the target.

Therefore, in a first aspect, the invention provides a dual specific ligand comprising a first dAb specific for a target ligand, and a second dAb specific for a receptor for the target ligand.

- 5 Preferably, the dual specific ligand is an open conformation ligand and can bind both the target ligand and the target ligand receptor simultaneously.

Preferred dual specific ligands comprise at least one specificity for TNF alpha and at least one specificity for TNF Receptor 1 (p55). Advantageously, the specificities are provided
10 by one or more dAbs arranged in Fab, F(ab')₂ or IgG formats. Preferred dAbs are TAR1-5-19 V_K and TAR2h-10-27 V_H as set forth below.

The invention may also comprise further modifications and configurations of the dual specific ligands as set forth in the accompanying claims and detailed herein.

15 Accordingly, in a further aspect, there is provided a dual-specific ligand comprising a first immunoglobulin single variable domain having a binding specificity to a first antigen or epitope and a second complementary immunoglobulin single variable domain having a binding activity to a second antigen or epitope, wherein one or both of said antigens or
20 epitopes acts to increase the half-life of the ligand *in vivo* and wherein said first and second domains lack mutually complementary domains which share the same specificity, provided that said dual specific ligand does not consist of an anti-HSA V_H domain and an anti-β galactosidase V_K domain. Preferably, neither of the first or second variable domains binds to human serum albumin (HSA).

25 Antigens or epitopes which increase the half-life of a ligand as described herein are advantageously present on proteins or polypeptides found in an organism *in vivo*. Examples include extracellular matrix proteins, blood proteins, and proteins present in various tissues in the organism. The proteins act to reduce the rate of ligand clearance
30 from the blood, for example by acting as bulking agents, or by anchoring the ligand to a desired site of action. Examples of antigens/epitopes which increase half-life *in vivo* are given in Annex 1 below.

Increased half-life is useful in *in vivo* applications of immunoglobulins, especially antibodies and most especially antibody fragments of small size. Such fragments (Fvs, disulphide bonded Fvs, Fabs, scFvs, dAbs) suffer from rapid clearance from the body; thus, whilst they are able to reach most parts of the body rapidly, and are quick to produce and easier to handle, their *in vivo* applications have been limited by their only brief persistence *in vivo*. The invention solves this problem by providing increased half-life of the ligands *in vivo* and consequently longer persistence times in the body of the functional activity of the ligand.

Methods for pharmacokinetic analysis and determination of ligand half-life will be familiar to those skilled in the art. Details may be found in *Kenneth, A et al: Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists* and in *Peters et al, Pharmacokinetic analysis: A Practical Approach* (1996). Reference is also made to "Pharmacokinetics", M Gibaldi & D Perron, published by Marcel Dekker, 2nd Rev. ex edition (1982), which describes pharmacokinetic parameters such as t alpha and t beta half lives and area under the curve (AUC).

Half lives ($t_{1/2}$ alpha and $t_{1/2}$ beta) and AUC can be determined from a curve of serum concentration of ligand against time. The WinNonlin analysis package (available from Pharsight Corp., Mountain View, CA94040, USA) can be used, for example, to model the curve. In a first phase (the alpha phase) the ligand is undergoing mainly distribution in the patient, with some elimination. A second phase (beta phase) is the terminal phase when the ligand has been distributed and the serum concentration is decreasing as the ligand is cleared from the patient. The t alpha half life is the half life of the first phase and the t beta half life is the half life of the second phase. Thus, advantageously, the present invention provides a ligand or a composition comprising a ligand according to the invention having a t_{α} half-life in the range of 15 minutes or more. In one embodiment, the lower end of the range is 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 10 hours, 11 hours or 12 hours. In addition, or alternatively, a ligand or composition according to the invention will have a t_{α} half life in the range of up to and including 12 hours. In one embodiment, the upper end of the range is 11, 10, 9, 8, 7, 6 or 5 hours. An example of a suitable range is 1 to 6 hours, 2 to 5 hours or 3 to 4 hours.

Advantageously, the present invention provides a ligand or a composition comprising a ligand according to the invention having a $t_{1/2}$ half-life in the range of 2.5 hours or more. In one embodiment, the lower end of the range is 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 10 hours, 11 hours, or 12 hours. In addition, or alternatively, a ligand or composition according to the invention has a $t_{1/2}$ half-life in the range of up to and including 21 days. In one embodiment, the upper end of the range is 12 hours, 24 hours, 2 days, 3 days, 5 days, 10 days, 15 days or 20 days. Advantageously a ligand or composition according to the invention will have a $t_{1/2}$ half life in the range 12 to 60 hours. In a further embodiment, it will be in the range 12 to 48 hours. In a further embodiment still, it will be in the range 12 to 26 hours.

In addition, or alternatively to the above criteria, the present invention provides a ligand or a composition comprising a ligand according to the invention having an AUC value (area under the curve) in the range of 1 mg.min/ml or more. In one embodiment, the lower end of the range is 5, 10, 15, 20, 30, 100, 200 or 300mg.min/ml. In addition, or alternatively, a ligand or composition according to the invention has an AUC in the range of up to 600 mg.min/ml. In one embodiment, the upper end of the range is 500, 400, 300, 200, 150, 100, 75 or 50 mg.min/ml. Advantageously a ligand according to the invention will have a AUC in the range selected from the group consisting of the following: 15 to 150mg.min/ml, 15 to 100 mg.min/ml, 15 to 75 mg.min/ml, and 15 to 50mg.min/ml.

In a first embodiment, the dual specific ligand comprises two complementary variable domains, i.e. two variable domains that, in their natural environment, are capable of operating together as a cognate pair or group even if in the context of the present invention they bind separately to their cognate epitopes. For example, the complementary variable domains may be immunoglobulin heavy chain and light chain variable domains (V_H and V_L). V_H and V_L domains are advantageously provided by scFv or Fab antibody fragments. Variable domains may be linked together to form multivalent ligands by, for example: provision of a hinge region at the C-terminus of each V domain and disulphide bonding between cysteines in the hinge regions; or provision of dAbs each with a cysteine at the C-terminus of the domain, the cysteines being disulphide bonded together; or production of V-CH & V-CL to produce a Fab format; or use of peptide linkers (for

example Gly₄Ser linkers discussed hereinbelow) to produce dimers, trimers and further multimers.

5 The inventors have found that the use of complementary variable domains allows the two domain surfaces to pack together and be sequestered from the solvent. Furthermore the complementary domains are able to stabilise each other. In addition, it allows the creation of dual-specific IgG antibodies without the disadvantages of hybrid hybridomas as used in the prior art, or the need to engineer heavy or light chains at the sub-unit interfaces.

10 The dual-specific ligands of the first aspect of the present invention have at least one V_H/V_L pair. A bispecific IgG according to this invention will therefore comprise two such pairs, one pair on each arm of the Y-shaped molecule. Unlike conventional bispecific antibodies or diabodies, therefore, where the ratio of chains used is determinative in the success of the preparation thereof and leads to practical difficulties,

15 the dual specific ligands of the invention are free from issues of chain balance. Chain imbalance in conventional bi-specific antibodies results from the association of two different V_L chains with two different V_H chains, where V_L chain 1 together with V_H chain 1 is able to bind to antigen or epitope 1 and V_L chain 2 together with V_H chain 2 is able to bind to antigen or epitope 2 and the two correct pairings are in some way linked to

20 one another. Thus, only when V_L chain 1 is paired with V_H chain 1 and V_L chain 2 is paired with V_H chain 2 in a single molecule is bi-specificity created. Such bi-specific molecules can be created in two different ways. Firstly, they can be created by association of two existing V_H/V_L pairings that each bind to a different antigen or epitope (for example, in a bi-specific IgG). In this case the V_H/V_L pairings must come all together in a

25 1:1 ratio in order to create a population of molecules all of which are bi-specific. This never occurs (even when complementary CH domain is enhanced by "knobs into holes" engineering) leading to a mixture of bi-specific molecules and molecules that are only able to bind to one antigen or epitope but not the other. The second way of creating a bi-specific antibody is by the simultaneous association of two different V_H chain with two

30 different V_L chains (for example in a bi-specific diabody). In this case, although there tends to be a preference for V_L chain 1 to pair with V_H chain 1 and V_L chain 2 to pair with V_H chain 2 (which can be enhanced by "knobs into holes" engineering of the V_L and V_H domains), this pairing is never achieved in all molecules, leading to a mixed formulation whereby incorrect pairings occur that are unable to bind to either antigen or epitope.

Bi-specific antibodies constructed according to the dual-specific ligand approach according to the first aspect of the present invention overcome all of these problems because the binding to antigen or epitope 1 resides within the V_H or V_L domain and the binding to antigen or epitope 2 resides with the complementary V_L or V_H domain, respectively. Since V_H and V_L domains pair on a 1:1 basis all V_H/V_L pairings will be bi-specific and thus all formats constructed using these V_H/V_L pairings (Fv, scFvs, Fabs, minibodies, IgGs etc) will have 100% bi-specific activity.

10 In the context of the present invention, first and second "epitopes" are understood to be epitopes which are not the same and are not bound by a single monospecific ligand. In the first configuration of the invention, they are advantageously on different antigens, one of which acts to increase the half-life of the ligand *in vivo*. Likewise, the first and second antigens are advantageously not the same.

15

The dual specific ligands of the invention do not include ligands as described in WO 02/02773. Thus, the ligands of the present invention do not comprise complementary V_H/V_L pairs which bind any one or more antigens or epitopes co-operatively. Instead, the ligands according to the first aspect of the invention comprise a V_H/V_L complementary pair, wherein the V domains have different specificities.

20

Moreover, the ligands according to the first aspect of the invention comprise V_H/V_L complementary pairs having different specificities for non-structurally related epitopes or antigens. Structurally related epitopes or antigens are epitopes or antigens which possess sufficient structural similarity to be bound by a conventional V_H/V_L complementary pair which acts in a co-operative manner to bind an antigen or epitope; in the case of structurally related epitopes, the epitopes are sufficiently similar in structure that they "fit" into the same binding pocket formed at the antigen binding site of the V_H/V_L dimer.

25

30 In a further aspect, the present invention provides a ligand comprising a first immunoglobulin variable domain having a first antigen or epitope binding specificity and a second immunoglobulin variable domain having a second antigen or epitope binding specificity wherein one or both of said first and second variable domains bind to an

antigen which increases the half-life of the ligand *in vivo*, and the variable domains are not complementary to one another.

5 In one embodiment, binding to one variable domain modulates the binding of the ligand to the second variable domain.

In this embodiment, the variable domains may be, for example, pairs of V_H domains or pairs of V_L domains. Binding of antigen at the first site may modulate, such as enhance or inhibit, binding of an antigen at the second site. For example, binding at the first site at
10 least partially inhibits binding of an antigen at a second site. In such an embodiment, the ligand may for example be maintained in the body of a subject organism *in vivo* through binding to a protein which increases the half-life of the ligand until such a time as it becomes bound to the second target antigen and dissociates from the half-life increasing protein.

15 Modulation of binding in the above context is achieved as a consequence of the structural proximity of the antigen binding sites relative to one another. Such structural proximity can be achieved by the nature of the structural components linking the two or more antigen binding sites, eg by the provision of a ligand with a relatively rigid structure that
20 holds the antigen binding sites in close proximity. Advantageously, the two or more antigen binding sites are in physically close proximity to one another such that one site modulates the binding of antigen at another site by a process which involves steric hindrance and/or conformational changes within the immunoglobulin molecule.

25 The first and the second antigen binding domains may be associated either covalently or non-covalently. In the case that the domains are covalently associated, then the association may be mediated for example by disulphide bonds or by a polypeptide linker such as (Gly₄Ser)_n, where n = from 1 to 8, eg, 2, 3, 4, 5 or 7.

30 Ligands according to the invention may be combined into non-immunoglobulin multi-ligand structures to form multivalent complexes, which bind target molecules with the same antigen, thereby providing superior avidity, while at least one variable domain binds an antigen to increase the half life of the multimer. For example natural bacterial

receptors such as SpA have been used as scaffolds for the grafting of CDRs to generate ligands which bind specifically to one or more epitopes. Details of this procedure are described in US 5,831,012. Other suitable scaffolds include those based on fibronectin and affibodies. Details of suitable procedures are described in WO 98/58965. Other
5 suitable scaffolds include lipocallin and CTLA4, as described in van den Beuken *et al.*, J. Mol. Biol. (2001) 310, 591-601, and scaffolds such as those described in WO0069907 (Medical Research Council), which are based for example on the ring structure of bacterial GroEL or other chaperone polypeptides.

10 Protein scaffolds may be combined; for example, CDRs may be grafted on to a CTLA4 scaffold and used together with immunoglobulin V_H or V_L domains to form a ligand. Likewise, fibronectin, lipocallin and other scaffolds may be combined.

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

20 Where V-gene repertoires are used variation in polypeptide sequence is preferably located within the structural loops of the variable domains. The polypeptide sequences of either variable domain may be altered by DNA shuffling or by mutation in order to enhance the interaction of each variable domain with its complementary pair.

25 In a preferred embodiment of the invention the 'dual-specific ligand' is a single chain Fv fragment. In an alternative embodiment of the invention, the 'dual-specific ligand' consists of a Fab region of an antibody. The term "Fab region" includes a Fab-like region where two V_H or two V_L domains are used.

30 The variable regions may be derived from antibodies directed against target antigens or epitopes. Alternatively they may be derived from a repertoire of single antibody domains

such as those expressed on the surface of filamentous bacteriophage. Selection may be performed as described below.

In a further aspect, the invention provides a method for producing a ligand comprising a first immunoglobulin single variable domain having a first binding specificity and a second single immunoglobulin single variable domain having a second (different) binding specificity, one or both of the binding specificities being specific for an antigen which increases the half-life of the ligand *in vivo*, the method comprising the steps of:

- (a) selecting a first variable domain by its ability to bind to a first epitope,
- 10 (b) selecting a second variable region by its ability to bind to a second epitope,
- (c) combining the variable domains; and
- (d) selecting the ligand by its ability to bind to said first epitope and to said second epitope.

15 The ligand can bind to the first and second epitopes either simultaneously or, where there is competition between the binding domains for epitope binding, the binding of one domain may preclude the binding of another domain to its cognate epitope. In one embodiment, therefore, step (d) above requires simultaneous binding to both first and second (and possibly further) epitopes; in another embodiment, the binding to the first and second epitopes is not simultaneous.

The epitopes are preferably on separate antigens.

Ligands advantageously comprise V_H/V_L combinations, or V_H/V_H or V_L/V_L combinations of immunoglobulin variable domains, as described above. The ligands may moreover comprise camelid V_{HH} domains, provided that the V_{HH} domain which is specific for an antigen which increases the half-life of the ligand *in vivo* does not bind Hen egg white lysozyme (HEL), porcine pancreatic alpha-amylase or NmC-A; hcg, BSA-linked RR6 azo dye or *S. mutans* HG982 cells, as described in Conrath *et al.*, (2001) JBC 276:7346-7350 and WO99/23221, neither of which describe the use of a specificity for an antigen which increases half-life to increase the half life of the ligand *in vivo*.

In one embodiment, said first variable domain is selected for binding to said first epitope in absence of a complementary variable domain. In a further embodiment, said first variable domain is selected for binding to said first epitope/antigen in the presence of a third variable domain in which said third variable domain is different from said second variable domain and is complementary to the first domain. Similarly, the second domain may be selected in the absence or presence of a complementary variable domain.

The antigens or epitopes targeted by the ligands of the invention, in addition to the half-life enhancing protein, may be any antigen or epitope but advantageously is an antigen or epitope that is targeted with therapeutic benefit. The invention provides ligands, including open conformation, closed conformation and isolated dAb monomer ligands, specific for any such target, particularly those targets further identified herein. Such targets may be, or be part of, polypeptides, proteins or nucleic acids, which may be naturally occurring or synthetic. In this respect, the ligand of the invention may bind the epitope or antigen and act as an antagonist or agonist (eg, EPO receptor agonist). One skilled in the art will appreciate that the choice is large and varied. They may be for instance human or animal proteins, cytokines, cytokine receptors, enzymes co-factors for enzymes or DNA binding proteins. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, EGF receptor, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, EpoR, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF- β 1, insulin, IFN- γ , IGF-I, IGF-II, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin α , Inhibin β , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotoxin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 α , SDF1 β , SCF, SCGF, stem cell factor (SCF), TARC, TGF- α , TGF- β , TGF- β 2, TGF- β 3, tumour necrosis factor (TNF), TNF- α , TNF- β , TNF receptor I, TNF receptor II, TNIL-1, TPO, VEGF, VEGF receptor 1, VEGF receptor 2, VEGF receptor 3, GCP-2, GRO/MGSA, GRO- β , GRO- γ , HCC1, 1-309, HER 1, HER 2, HER 3 and HER 4.

Cytokine receptors include receptors for the foregoing cytokines. It will be appreciated that this list is by no means exhaustive.

In one embodiment of the invention, the variable domains are derived from a respective
5 antibody directed against the antigen or epitope. In a preferred embodiment the variable domains are derived from a repertoire of single variable antibody domains.

In a further aspect, the present invention provides one or more nucleic acid molecules encoding at least a dual-specific ligand as herein defined. The dual specific ligand may
10 be encoded on a single nucleic acid molecule; alternatively, each domain may be encoded by a separate nucleic acid molecule. Where the ligand is encoded by a single nucleic acid molecule, the domains may be expressed as a fusion polypeptide, in the manner of a scFv molecule, or may be separately expressed and subsequently linked together, for example using chemical linking agents. Ligands expressed from separate nucleic acids will be
15 linked together by appropriate means.

The nucleic acid may further encode a signal sequence for export of the polypeptides from a host cell upon expression and may be fused with a surface component of a filamentous bacteriophage particle (or other component of a selection display system)
20 upon expression.

In a further aspect the present invention provides a vector comprising nucleic acid encoding a dual specific ligand according to the present invention.

25 In a yet further aspect, the present invention provides a host cell transfected with a vector encoding a dual specific ligand according to the present invention.

Expression from such a vector may be configured to produce, for example on the surface of a bacteriophage particle, variable domains for selection. This allows selection of
30 displayed variable regions and thus selection of 'dual-specific ligands' using the method of the present invention.

The present invention further provides a kit comprising at least a dual-specific ligand according to the present invention.

Dual-Specific ligands according to the present invention preferably comprise combinations of heavy and light chain domains. For example, the dual specific ligand may comprise a V_H domain and a V_L domain, which may be linked together in the form of an scFv. In addition, the ligands may comprise one or more C_H or C_L domains. For example, the ligands may comprise a C_{H1} domain, C_{H2} or C_{H3} domain, and/or a C_L domain, $C_{\mu 1}$, $C_{\mu 2}$, $C_{\mu 3}$ or $C_{\mu 4}$ domains, or any combination thereof. A hinge region domain may also be included. Such combinations of domains may, for example, mimic natural antibodies, such as IgG or IgM, or fragments thereof, such as Fv, scFv, Fab or $F(ab')_2$ molecules. Other structures, such as a single arm of an IgG molecule comprising V_H , V_L , C_{H1} and C_L domains, are envisaged.

In a preferred embodiment of the invention, the variable regions are selected from single domain V gene repertoires. Generally the repertoire of single antibody domains is displayed on the surface of filamentous bacteriophage. In a preferred embodiment each single antibody domain is selected by binding of a phage repertoire to antigen.

In a preferred embodiment of the invention each single variable domain may be selected for binding to its target antigen or epitope in the absence of a complementary variable region. In an alternative embodiment, the single variable domains may be selected for binding to its target antigen or epitope in the presence of a complementary variable region. Thus the first single variable domain may be selected in the presence of a third complementary variable domain, and the second variable domain may be selected in the presence of a fourth complementary variable domain. The complementary third or fourth variable domain may be the natural cognate variable domain having the same specificity as the single domain being tested, or a non-cognate complementary domain – such as a “dummy” variable domain.

30

Preferably, the dual specific ligand of the invention comprises only two variable domains although several such ligands may be incorporated together into the same protein, for example two such ligands can be incorporated into an IgG or a multimeric

immunoglobulin, such as IgM. Alternatively, in another embodiment a plurality of dual specific ligands are combined to form a multimer. For example, two different dual specific ligands are combined to create a tetra-specific molecule.

5 It will be appreciated by one skilled in the art that the light and heavy variable regions of a dual-specific ligand produced according to the method of the present invention may be on the same polypeptide chain, or alternatively, on different polypeptide chains. In the case that the variable regions are on different polypeptide chains, then they may be linked via a linker, generally a flexible linker (such as a polypeptide chain), a chemical linking
10 group, or any other method known in the art.

In a further aspect, the present invention provides a composition comprising a dual-specific ligand, obtainable by a method of the present invention, and a pharmaceutically acceptable carrier, diluent or excipient.

15

Moreover, the present invention provides a method for the treatment and/or prevention of disease using a 'dual-specific ligand' or a composition according to the present invention.

In a second configuration, the present invention provides multispecific ligands which
20 comprise at least two non-complementary variable domains. For example, the ligands may comprise a pair of V_H domains or a pair of V_L domains. Advantageously, the domains are of non-camelid origin; preferably they are human domains or comprise human framework regions (FWs) and one or more heterologous CDRs. CDRs and framework regions are those regions of an immunoglobulin variable domain as defined in
25 the Kabat database of Sequences of Proteins of Immunological Interest.

Preferred human framework regions are those encoded by germline gene segments DP47 and DPK9. Advantageously, FW1, FW2 and FW3 of a V_H or V_L domain have the sequence of FW1, FW2 or FW3 from DP47 or DPK9. The human frameworks may
30 optionally contain mutations, for example up to about 5 amino acid changes or up to about 10 amino acid changes collectively in the human frameworks used in the ligands of the invention.

The variable domains in the multispecific ligands according to the second configuration of the invention may be arranged in an open or a closed conformation; that is, they may be arranged such that the variable domains can bind their cognate ligands independently and simultaneously, or such that only one of the variable domains may bind its cognate
5 ligand at any one time.

The inventors have realised that under certain structural conditions, non-complementary variable domains (for example two light chain variable domains or two heavy chain variable domains) may be present in a ligand such that binding of a first epitope to a first
10 variable domain inhibits the binding of a second epitope to a second variable domain, even though such non-complementary domains do not operate together as a cognate pair.

Advantageously, the ligand comprises two or more pairs of variable domains; that is, it comprises at least four variable domains. Advantageously, the four variable domains
15 comprise frameworks of human origin.

In a preferred embodiment, the human frameworks are identical to those of human germline sequences.

20 The present inventors consider that such antibodies will be of particular use in ligand binding assays for therapeutic and other uses.

Thus, in a first aspect of the second configuration, the present invention provides a method for producing a multispecific ligand comprising the steps of:

- 25 a) selecting a first epitope binding domain by its ability to bind to a first epitope,
- b) selecting a second epitope binding domain by its ability to bind to a second epitope,
- c) combining the epitope binding domains; and
- d) selecting the closed conformation multispecific ligand by its ability to bind to said
30 first second epitope and said second epitope.

In a further aspect of the second configuration, the invention provides method for preparing a closed conformation multi-specific ligand comprising a first epitope binding

domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity, wherein the first and second binding specificities compete for epitope binding such that the closed conformation multi-specific ligand may not bind both epitopes simultaneously, said
5 method comprising the steps of:

- a) selecting a first epitope binding domain by its ability to bind to a first epitope,
- b) selecting a second epitope binding domain by its ability to bind to a second epitope,
- 10 c) combining the epitope binding domains such that the domains are in a closed conformation; and
- d) selecting the closed conformation multispecific ligand by its ability to bind to said first second epitope and said second epitope, but not to both said first and second epitopes simultaneously.

15

Moreover, the invention provides a closed conformation multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity, wherein the first and second binding specificities compete for epitope binding
20 such that the closed conformation multi-specific ligand may not bind both epitopes simultaneously.

An alternative embodiment of the above aspect of the of the second configuration of the invention optionally comprises a further step (b1) comprising selecting a third or further
25 epitope binding domain. In this way the multi-specific ligand produced, whether of open or closed conformation, comprises more than two epitope binding specificities. In a preferred aspect of the second configuration of the invention, where the multi-specific ligand comprises more than two epitope binding domains, at least two of said domains are in a closed conformation and compete for binding; other domains may compete for
30 binding or may be free to associate independently with their cognate epitope(s).

According to the present invention the term 'multi-specific ligand' refers to a ligand which possesses more than one epitope binding specificity as herein defined.

As herein defined the term 'closed conformation' (multi-specific ligand) means that the epitope binding domains of the ligand are attached to or associated with each other, optionally by means of a protein skeleton, such that epitope binding by one epitope binding domain competes with epitope binding by another epitope binding domain. That is, cognate epitopes may be bound by each epitope binding domain individually but not simultaneously. The closed conformation of the ligand can be achieved using methods herein described.

“Open conformation” means that the epitope binding domains of the ligand are attached to or associated with each other, optionally by means of a protein skeleton, such that epitope binding by one epitope binding domain does not compete with epitope binding by another epitope binding domain.

As referred to herein, the term 'competes' means that the binding of a first epitope to its cognate epitope binding domain is inhibited when a second epitope is bound to its cognate epitope binding domain. For example, binding may be inhibited sterically, for example by physical blocking of a binding domain or by alteration of the structure or environment of a binding domain such that its affinity or avidity for an epitope is reduced.

In a further embodiment of the second configuration of the invention, the epitopes may displace each other on binding. For example, a first epitope may be present on an antigen which, on binding to its cognate first binding domain, causes steric hindrance of a second binding domain, or a conformational change therein, which displaces the epitope bound to the second binding domain.

Advantageously, binding is reduced by 25% or more, advantageously 40%, 50%, 60%, 70%, 80%, 90% or more, and preferably up to 100% or nearly so, such that binding is completely inhibited. Binding of epitopes can be measured by conventional antigen binding assays, such as ELISA, by fluorescence based techniques, including FRET, or by techniques such as surface plasmon resonance which measure the mass of molecules.

According to the method of the present invention, advantageously, each epitope binding domain is of a different epitope binding specificity.

In the context of the present invention, first and second “epitopes” are understood to be epitopes which are not the same and are not bound by a single monospecific ligand. They may be on different antigens or on the same antigen, but separated by a sufficient distance that they do not form a single entity that could be bound by a single mono-specific V_H/V_L binding pair of a conventional antibody. Experimentally, if both of the individual variable domains in single chain antibody form (domain antibodies or dAbs) are separately competed by a monospecific V_H/V_L ligand against two epitopes then those two epitopes are not sufficiently far apart to be considered separate epitopes according to the present invention.

The closed conformation multispecific ligands of the invention do not include ligands as described in WO 02/02773. Thus, the ligands of the present invention do not comprise complementary V_H/V_L pairs which bind any one or more antigens or epitopes co-operatively. Instead, the ligands according to the invention preferably comprise non-complementary V_H-V_H or V_L-V_L pairs. Advantageously, each V_H or V_L domain in each V_H-V_H or V_L-V_L pair has a different epitope binding specificity, and the epitope binding sites are so arranged that the binding of an epitope at one site competes with the binding of an epitope at another site.

According to the present invention, advantageously, each epitope binding domain comprises an immunoglobulin variable domain. More advantageously, each immunoglobulin variable domain will be either a variable light chain domain (V_L) or a variable heavy chain domain V_H . In the second configuration of the present invention, the immunoglobulin domains when present on a ligand according to the present invention are non-complementary, that is they do not associate to form a V_H/V_L antigen binding site. Thus, multi-specific ligands as defined in the second configuration of the invention comprise immunoglobulin domains of the same sub-type, that is either variable light chain domains (V_L) or variable heavy chain domains (V_H). Moreover, where the ligand according to the invention is in the closed conformation, the immunoglobulin domains may be of the camelid V_{HH} type.

In an alternative embodiment, the ligand(s) according to the invention do not comprise a camelid V_{HH} domain. More particularly, the ligand(s) of the invention do not comprise one or more amino acid residues that are specific to camelid V_{HH} domains as compared to
5 human V_H domains.

Advantageously, the single variable domains are derived from antibodies selected for binding activity against different antigens or epitopes. For example, the variable domains may be isolated at least in part by human immunisation. Alternative methods are known
10 in the art, including isolation from human antibody libraries and synthesis of artificial antibody genes.

The variable domains advantageously bind superantigens, such as protein A or protein L. Binding to superantigens is a property of correctly folded antibody variable domains, and
15 allows such domains to be isolated from, for example, libraries of recombinant or mutant domains.

Epitope binding domains according to the present invention comprise a protein scaffold and epitope interaction sites (which are advantageously on the surface of the protein
20 scaffold).

Epitope binding domains may also be based on protein scaffolds or skeletons other than immunoglobulin domains. For example natural bacterial receptors such as SpA have been used as scaffolds for the grafting of CDRs to generate ligands which bind specifically to
25 one or more epitopes. Details of this procedure are described in US 5,831,012. Other suitable scaffolds include those based on fibronectin and affibodies. Details of suitable procedures are described in WO 98/58965. Other suitable scaffolds include lipocallin and CTLA4, as described in van den Beuken *et al.*, J. Mol. Biol. (2001) 310, 591-601, and scaffolds such as those described in WO0069907 (Medical Research Council), which are
30 based for example on the ring structure of bacterial GroEL or other chaperone polypeptides.

Protein scaffolds may be combined; for example, CDRs may be grafted on to a CTLA4 scaffold and used together with immunoglobulin V_H or V_L domains to form a multivalent ligand. Likewise, fibronectin, lipocallin and other scaffolds may be combined.

- 5 It will be appreciated by one skilled in the art that the epitope binding domains of a closed conformation multispecific ligand produced according to the method of the present invention may be on the same polypeptide chain, or alternatively, on different polypeptide chains. In the case that the variable regions are on different polypeptide chains, then they may be linked via a linker, advantageously a flexible linker (such as a polypeptide chain),
10 a chemical linking group, or any other method known in the art.

The first and the second epitope binding domains may be associated either covalently or non-covalently. In the case that the domains are covalently associated, then the association may be mediated for example by disulphide bonds.

15

- In the second configuration of the invention, the first and the second epitopes are preferably different. They may be, or be part of, polypeptides, proteins or nucleic acids, which may be naturally occurring or synthetic. In this respect, the ligand of the invention may bind an epitope or antigen and act as an antagonist or agonist (eg, EPO receptor
20 agonist). The epitope binding domains of the ligand in one embodiment have the same epitope specificity, and may for example simultaneously bind their epitope when multiple copies of the epitope are present on the same antigen. In another embodiment, these epitopes are provided on different antigens such that the ligand can bind the epitopes and bridge the antigens. One skilled in the art will appreciate that the choice of epitopes and
25 antigens is large and varied. They may be for instance human or animal proteins, cytokines, cytokine receptors, enzymes co-factors for enzymes or DNA binding proteins. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, EGF receptor, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, EpoR, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine
30 (CX3C), GDNF, G-CSF, GM-CSF, GF- β 1, insulin, IFN- γ , IGF-I, IGF-II, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGF), Inhibin α , Inhibin β , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotoxin, Mullerian inhibitory substance,

monocyte colony inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β -NGF, NT-3, NT-4, 5 Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 α , SDF1 β , SCF, SCGF, stem cell factor (SCF), TARC, TGF- α , TGF- β , TGF- β 2, TGF- β 3, tumour necrosis factor (TNF), TNF- α , TNF- β , TNF receptor I, TNF receptor II, TNIL-1, TPO, VEGF, VEGF receptor 1, VEGF receptor 2, VEGF receptor 3, GCP-2, GRO/MGSA, GRO- β , GRO- γ , HCC1, 1-309, HER 1, HER 2, HER 3, HER 4, TACE recognition site, TNF BP-I 10 and TNF BP-II, as well as any target disclosed in Annex 2 or Annex 3 hereto, whether in combination as set forth in the Annexes, in a different combination or individually. Cytokine receptors include receptors for the foregoing cytokines, e.g. IL-1 R1; IL-6R; IL-10R; IL-18R, as well as receptors for cytokines set forth in Annex 2 or Annex 3 and also receptors disclosed in Annex 2 and 3. It will be appreciated that this list is by no means 15 exhaustive. Where the multispecific ligand binds to two epitopes (on the same or different antigens), the antigen(s) may be selected from this list.

Advantageously, dual specific ligands may be used to target cytokines and other molecules which cooperate synergistically in therapeutic situations in the body of an 20 organism. The invention therefore provides a method for synergising the activity of two or more cytokines, comprising administering a dual specific ligand capable of binding to said two or more cytokines. In this aspect of the invention, the dual specific ligand may be any dual specific ligand, including a ligand composed of complementary and/or non-complementary domains, a ligand in an open conformation, and a ligand in a closed 25 conformation. For example, this aspect of the invention relates to combinations of V_H domains and V_L domains, V_H domains only and V_L domains only.

Synergy in a therapeutic context may be achieved in a number of ways. For example, target combinations may be therapeutically active only if both targets are targeted by the 30 ligand, whereas targeting one target alone is not therapeutically effective. In another embodiment, one target alone may provide some low or minimal therapeutic effect, but together with a second target the combination provides a synergistic increase in therapeutic effect.

Preferably, the cytokines bound by the dual specific ligands of this aspect of the invention are selected from the list shown in Annex 2.

- 5 Moreover, dual specific ligands may be used in oncology applications, where one specificity targets CD89, which is expressed by cytotoxic cells, and the other is tumour specific. Examples of tumour antigens which may be targeted are given in Annex 3.

10 In one embodiment of the second configuration of the invention, the variable domains are derived from an antibody directed against the first and/or second antigen or epitope. In a preferred embodiment the variable domains are derived from a repertoire of single variable antibody domains. In one example, the repertoire is a repertoire that is not created in an animal or a synthetic repertoire. In another example, the single variable domains are not isolated (at least in part) by animal immunisation. Thus, the single
15 domains can be isolated from a naïve library.

The second configuration of the invention, in another aspect, provides a multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope
20 binding specificity. The first and second binding specificities may be the same or different.

In a further aspect, the present invention provides a closed conformation multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope
25 binding specificity wherein the first and second binding specificities are capable of competing for epitope binding such that the closed conformation multi-specific ligand cannot bind both epitopes simultaneously.

30 In a still further aspect, the invention provides open conformation ligands comprising non-complementary binding domains, wherein the domains are specific for a different epitope on the same target. Such ligands bind to targets with increased avidity. Similarly, the invention provides multivalent ligands comprising non-complementary

binding domains specific for the same epitope and directed to targets which comprise multiple copies of said epitope, such as IL-5, PDGF-AA, PDGF-BB, TGF beta, TGF beta2, TGF beta3 and TNF α , for example human TNF Receptor 1 and human TNF α .

- 5 In a similar aspect, ligands according to the invention can be configured to bind individual epitopes with low affinity, such that binding to individual epitopes is not therapeutically significant; but the increased avidity resulting from binding to two epitopes provides a therapeutic benefit. In a particular example, epitopes may be targeted which are present individually on normal cell types, but present together only on
10 abnormal or diseased cells, such as tumour cells. In such a situation, only the abnormal or diseased cells are effectively targeted by the bispecific ligands according to the invention.

Ligand specific for multiple copies of the same epitope, or adjacent epitopes, on the same
15 target (known as chelating dAbs) may also be trimeric or polymeric (tetrameric or more) ligands comprising three, four or more non-complementary binding domains. For example, ligands may be constructed comprising three or four V_H domains or V_L domains.

- 20 Moreover, ligands are provided which bind to multisubunit targets, wherein each binding domain is specific for a subunit of said target. The ligand may be dimeric, trimeric or polymeric.

Preferably, the multi-specific ligands according to the above aspects of the invention are
25 obtainable by the method of the first aspect of the invention.

According to the above aspect of the second configuration of the invention, advantageously the first epitope binding domain and the second epitope binding domains are non-complementary immunoglobulin variable domains, as herein defined. That is
30 either V_H-V_H or V_L-V_L variable domains.

Chelating dAbs in particular may be prepared according to a preferred aspect of the invention, namely the use of anchor dAbs, in which a library of dimeric, trimeric or

multimeric dAbs is constructed using a vector which comprises a constant dAb upstream or downstream of a linker sequence, with a repertoire of second, third and further dAbs being inserted on the other side of the linker. For example, the anchor or guiding dAb may be TAR1-5 (V κ), TAR1-27(V κ), TAR2h-5(VH) or TAR2h-6(V κ).

5

In alternative methodologies, the use of linkers may be avoided, for example by the use of non-covalent bonding or natural affinity between binding domains such as V_H and V κ . The invention accordingly provides a method for preparing a chelating multimeric ligand comprising the steps of:

- 10 (a) providing a vector comprising a nucleic acid sequence encoding a single binding domain specific for a first epitope on a target;
- (b) providing a vector encoding a repertoire comprising second binding domains specific for a second epitope on said target, which epitope can be the same or different to the first epitope, said second epitope being adjacent to said first epitope; and
- 15 (c) expressing said first and second binding domains; and
- (d) isolating those combinations of first and second binding domains which combine together to produce a target-binding dimer.

The first and second epitopes are adjacent such that a multimeric ligand is capable of
20 binding to both epitopes simultaneously. This provides the ligand with the advantages of increased avidity if binding. Where the epitopes are the same, the increased avidity is obtained by the presence of multiple copies of the epitope on the target, allowing at least two copies to be simultaneously bound in order to obtain the increased avidity effect.

25 The binding domains may be associated by several methods, as well as the use of linkers. For example, the binding domains may comprise cys residues, avidin and streptavidin groups or other means for non-covalent attachment post-synthesis; those combinations which bind to the target efficiently will be isolated. Alternatively, a linker may be present between the first and second binding domains, which are expressed as a single
30 polypeptide from a single vector, which comprises the first binding domain, the linker and a repertoire of second binding domains, for instance as described above.

In a preferred aspect, the first and second binding domains associate naturally when bound to antigen; for example, V_H and V_K domains, when bound to adjacent epitopes, will naturally associate in a three-way interaction to form a stable dimer. Such associated proteins can be isolated in a target binding assay. An advantage of this procedure is that
5 only binding domains which bind to closely adjacent epitopes, in the correct conformation, will associate and thus be isolated as a result of their increased avidity for the target.

In an alternative embodiment of the above aspect of the second configuration of the
10 invention, at least one epitope binding domain comprises a non-immunoglobulin 'protein scaffold' or 'protein skeleton' as herein defined. Suitable non-immunoglobulin protein scaffolds include but are not limited to any of those selected from the group consisting of: SpA, fibronectin, GroEL and other chaperones, lipocalin, CTLA4 and affibodies, as set forth above.

15 According to the above aspect of the second configuration of the invention, advantageously, the epitope binding domains are attached to a 'protein skeleton'. Advantageously, a protein skeleton according to the invention is an immunoglobulin skeleton.

20 According to the present invention, the term 'immunoglobulin skeleton' refers to a protein which comprises at least one immunoglobulin fold and which acts as a nucleus for one or more epitope binding domains, as defined herein.

25 Preferred immunoglobulin skeletons as herein defined includes any one or more of those selected from the following: an immunoglobulin molecule comprising at least (i) the CL (kappa or lambda subclass) domain of an antibody; or (ii) the CH1 domain of an antibody heavy chain; an immunoglobulin molecule comprising the CH1 and CH2 domains of an antibody heavy chain; an immunoglobulin molecule comprising the CH1, CH2 and CH3
30 domains of an antibody heavy chain; or any of the subset (ii) in conjunction with the CL (kappa or lambda subclass) domain of an antibody. A hinge region domain may also be included. Such combinations of domains may, for example, mimic natural antibodies,

such as IgG or IgM, or fragments thereof, such as Fv, scFv, Fab or F(ab')₂ molecules. Those skilled in the art will be aware that this list is not intended to be exhaustive.

5 Linking of the skeleton to the epitope binding domains, as herein defined may be achieved at the polypeptide level, that is after expression of the nucleic acid encoding the skeleton and/or the epitope binding domains. Alternatively, the linking step may be performed at the nucleic acid level. Methods of linking a protein skeleton according to the present invention, to the one or more epitope binding domains include the use of protein chemistry and/or molecular biology techniques which will be familiar to those skilled in
10 the art and are described herein.

Advantageously, the closed conformation multispecific ligand may comprise a first domain capable of binding a target molecule, and a second domain capable of binding a molecule or group which extends the half-life of the ligand. For example, the molecule or
15 group may be a bulky agent, such as HSA or a cell matrix protein. As used herein, the phrase "molecule or group which extends the half-life of a ligand" refers to a molecule or chemical group which, when bound by a dual-specific ligand as described herein increases the in vivo half-life of such dual specific ligand when administered to an animal, relative to a ligand that does not bind that molecule or group. Examples of
20 molecules or groups that extend the half-life of a ligand are described hereinbelow. In a preferred embodiment, the closed conformation multispecific ligand may be capable of binding the target molecule only on displacement of the half-life enhancing molecule or group. Thus, for example, a closed conformation multispecific ligand is maintained in circulation in the bloodstream of a subject by a bulky molecule such as HSA. When a
25 target molecule is encountered, competition between the binding domains of the closed conformation multispecific ligand results in displacement of the HSA and binding of the target.

Ligands according to any aspect of the present invention, as well as dAb monomers
30 useful in constructing such ligands, may advantageously dissociate from their cognate target(s) with a K_d of 300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12} M), preferably 50nM to 20pM, or 5nM to 200pM or 1nM to 100pM, 1×10^{-7} M or less, 1×10^{-8} M or less, 1×10^{-9} M or less, 1×10^{-10} M or less, 1×10^{-11} M or less; and/or a K_{off} rate constant of 5 x

10^{-1} to $1 \times 10^{-7} \text{ S}^{-1}$, preferably 1×10^{-2} to $1 \times 10^{-6} \text{ S}^{-1}$, or 5×10^{-3} to $1 \times 10^{-5} \text{ S}^{-1}$, or $5 \times 10^{-1} \text{ S}^{-1}$ or less, or $1 \times 10^{-2} \text{ S}^{-1}$ or less, or $1 \times 10^{-3} \text{ S}^{-1}$ or less, or $1 \times 10^{-4} \text{ S}^{-1}$ or less, or $1 \times 10^{-5} \text{ S}^{-1}$ or less, or $1 \times 10^{-6} \text{ S}^{-1}$ or less as determined by surface plasmon resonance. The K_d rate constant is defined as $K_{\text{off}}/K_{\text{on}}$.

5

In particular the invention provides an anti-TNF α dAb monomer (or dual specific ligand comprising such a dAb), homodimer, heterodimer or homotrimer ligand, wherein each dAb binds TNF α . The ligand binds to TNF α with a K_d of 300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12} M), preferably 50nM to 20pM, more preferably 5nM to 200pM and most preferably 1nM to 100pM; expressed in an alternative manner, the K_d is $1 \times 10^{-7} \text{ M}$ or less, preferably $1 \times 10^{-8} \text{ M}$ or less, more preferably $1 \times 10^{-9} \text{ M}$ or less, advantageously $1 \times 10^{-10} \text{ M}$ or less and most preferably $1 \times 10^{-11} \text{ M}$ or less; and/or a K_{off} rate constant of 5×10^{-1} to $1 \times 10^{-7} \text{ S}^{-1}$, preferably 1×10^{-2} to $1 \times 10^{-6} \text{ S}^{-1}$, more preferably 5×10^{-3} to $1 \times 10^{-5} \text{ S}^{-1}$, for example $5 \times 10^{-1} \text{ S}^{-1}$ or less, preferably $1 \times 10^{-2} \text{ S}^{-1}$ or less, more preferably $1 \times 10^{-3} \text{ S}^{-1}$ or less, advantageously $1 \times 10^{-4} \text{ S}^{-1}$ or less, further advantageously $1 \times 10^{-5} \text{ S}^{-1}$ or less, and most preferably $1 \times 10^{-6} \text{ S}^{-1}$ or less, as determined by surface plasmon resonance.

15

Preferably, the ligand neutralises TNF α in a standard L929 assay with an ND50 of 500nM to 50pM, preferably or 100nM to 50pM, advantageously 10nM to 100pM, more preferably 1nM to 100pM; for example 50nM or less, preferably 5nM or less, advantageously 500pM or less, more preferably 200pM or less and most preferably 100pM or less.

20

Preferably, the ligand inhibits binding of TNF alpha to TNF alpha Receptor I (p55 receptor) with an IC50 of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less. Preferably, the TNF α is Human TNF α .

25

Furthermore, the invention provides a an anti-TNF Receptor I dAb monomer, or dual specific ligand comprising such a dAb, that binds to TNF Receptor I with a K_d of 300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12} M), preferably 50nM to 20pM, more preferably 5nM to 200pM and most preferably 1nM to 100pM, for example $1 \times 10^{-7} \text{ M}$ or less, preferably 1

30

$\times 10^{-8}$ M or less, more preferably 1×10^{-9} M or less, advantageously 1×10^{-10} M or less and most preferably 1×10^{-11} M or less; and/or a K_{off} rate constant of 5×10^{-1} to 1×10^{-7} S^{-1} , preferably 1×10^{-2} to 1×10^{-6} S^{-1} , more preferably 5×10^{-3} to 1×10^{-5} S^{-1} , for example 5×10^{-1} S^{-1} or less, preferably 1×10^{-2} S^{-1} or less, advantageously 1×10^{-3} S^{-1} or less, more preferably 1×10^{-4} S^{-1} or less, still more preferably 1×10^{-5} S^{-1} or less, and most preferably 1×10^{-6} S^{-1} or less as determined by surface plasmon resonance.

Preferably, the dAb monomer or ligand neutralises $\text{TNF}\alpha$ in a standard assay (eg, the L929 or HeLa assays described herein) with an ND_{50} of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less.

Preferably, the dAb monomer or ligand inhibits binding of TNF alpha to TNF alpha Receptor I (p55 receptor) with an IC_{50} of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less. Preferably, the TNF Receptor I target is Human $\text{TNF}\alpha$.

Furthermore, the invention provides a dAb monomer (or dual specific ligand comprising such a dAb) that binds to serum albumin (SA) with a K_d of 1nM to 500 μM (ie, $\times 10^{-9}$ to 5×10^{-4}), preferably 100nM to 10 μM . Preferably, for a dual specific ligand comprising a first anti-SA dAb and a second dAb to another target, the affinity (eg K_d and/or K_{off} as measured by surface plasmon resonance, eg using BiaCore) of the second dAb for its target is from 1 to 100000 times (preferably 100 to 100000, more preferably 1000 to 100000, or 10000 to 100000 times) the affinity of the first dAb for SA. For example, the first dAb binds SA with an affinity of approximately 10 μM , while the second dAb binds its target with an affinity of 100pM. Preferably, the serum albumin is human serum albumin (HSA).

In one embodiment, the first dAb (or a dAb monomer) binds SA (eg, HSA) with a K_d of approximately 50, preferably 70, and more preferably 100, 150 or 200 nM.

The invention moreover provides dimers, trimers and polymers of the aforementioned dAb monomers, in accordance with the foregoing aspect of the present invention.

5 Ligands according to the invention, including dAb monomers, dimers and trimers, can be linked to an antibody Fc region, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region. For example, vectors encoding ligands linked as a single nucleotide sequence to an Fc region may be used to prepare such polypeptides.

10 In a further aspect of the second configuration of the invention, the present invention provides one or more nucleic acid molecules encoding at least a multispecific ligand as herein defined. In one embodiment, the ligand is a closed conformation ligand. In another embodiment, it is an open conformation ligand. The multispecific ligand may be encoded on a single nucleic acid molecule; alternatively, each epitope binding domain
15 may be encoded by a separate nucleic acid molecule. Where the ligand is encoded by a single nucleic acid molecule, the domains may be expressed as a fusion polypeptide, or may be separately expressed and subsequently linked together, for example using chemical linking agents. Ligands expressed from separate nucleic acids will be linked together by appropriate means.

20

The nucleic acid may further encode a signal sequence for export of the polypeptides from a host cell upon expression and may be fused with a surface component of a filamentous bacteriophage particle (or other component of a selection display system) upon expression. Leader sequences, which may be used in bacterial expression and/or
25 phage or phagemid display, include pelB, stII, ompA, phoA, bla and pelA.

In a further aspect of the second configuration of the invention the present invention provides a vector comprising nucleic acid according to the present invention.

30 In a yet further aspect, the present invention provides a host cell transfected with a vector according to the present invention.

Expression from such a vector may be configured to produce, for example on the surface of a bacteriophage particle, epitope binding domains for selection. This allows selection of displayed domains and thus selection of 'multispecific ligands' using the method of the present invention.

5

In a preferred embodiment of the second configuration of the invention, the epitope binding domains are immunoglobulin variable regions and are selected from single domain V gene repertoires. Generally the repertoire of single antibody domains is displayed on the surface of filamentous bacteriophage. In a preferred embodiment each
10 single antibody domain is selected by binding of a phage repertoire to antigen.

The present invention further provides a kit comprising at least a multispecific ligand according to the present invention, which may be an open conformation or closed conformation ligand. Kits according to the invention may be, for example, diagnostic
15 kits, therapeutic kits, kits for the detection of chemical or biological species, and the like.

In a further aspect still of the second configuration of the invention, the present invention provides a homogenous immunoassay using a ligand according to the present invention.

20 In a further aspect still of the second configuration of the invention, the present invention provides a composition comprising a closed conformation multispecific ligand, obtainable by a method of the present invention, and a pharmaceutically acceptable carrier, diluent or excipient.

25 Moreover, the present invention provides a method for the treatment of disease using a 'closed conformation multispecific ligand' or a composition according to the present invention.

In a preferred embodiment of the invention the disease is cancer or an inflammatory
30 disease, eg rheumatoid arthritis, asthma or Crohn's disease.

In a further aspect of the second configuration of the invention, the present invention provides a method for the diagnosis, including diagnosis of disease using a closed

conformation multispecific ligand, or a composition according to the present invention. Thus in general the binding of an analyte to a closed conformation multispecific ligand may be exploited to displace an agent, which leads to the generation of a signal on displacement. For example, binding of analyte (second antigen) could displace an enzyme (first antigen) bound to the antibody providing the basis for an immunoassay, especially if the enzyme were held to the antibody through its active site.

Thus in a final aspect of the second configuration, the present invention provides a method for detecting the presence of a target molecule, comprising:

- (a) providing a closed conformation multispecific ligand bound to an agent, said ligand being specific for the target molecule and the agent, wherein the agent which is bound by the ligand leads to the generation of a detectable signal on displacement from the ligand;
- (b) exposing the closed conformation multispecific ligand to the target molecule; and
- (c) detecting the signal generated as a result of the displacement of the agent.

15

According to the above aspect of the second configuration of the invention, advantageously, the agent is an enzyme, which is inactive when bound by the closed conformation multi-specific ligand. Alternatively, the agent may be any one or more selected from the group consisting of the following: the substrate for an enzyme, and a fluorescent, luminescent or chromogenic molecule which is inactive or quenched when bound by the ligand.

20

Sequences similar or homologous (e.g., at least about 70% sequence identity) to the sequences disclosed herein are also part of the invention. In some embodiments, the sequence identity at the amino acid level can be about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. At the nucleic acid level, the sequence identity can be about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. Alternatively, substantial identity exists when the nucleic acid segments will hybridize under selective hybridization conditions (e.g., very high stringency hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

30

Calculations of "homology" or "sequence identity" or "similarity" between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

Advantageously, the BLAST algorithm (version 2.0) is employed for sequence alignment, with parameters set to default values. The BLAST algorithm is described in detail at the world wide web site ("www") of the National Center for Biotechnology Information ("ncbi") of the National Institutes of Health ("nih") of the U.S. government ("gov"), in the "/Blast/" directory, in the "blast_help.html" file. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87(6):2264-8 (see the "blast_help.html" file, as described above) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. The programs are not generally useful for motif-style searching. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (1994).

The five BLAST programs available at the National Center for Biotechnology Information web site perform the following tasks:

- “blastp” compares an amino acid query sequence against a protein sequence database;
- 5 “blastn” compares a nucleotide query sequence against a nucleotide sequence database;
- “blastx” compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database;
- “tblastn” compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).
- 10 “tblastx” compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

- 15 DESCRIPTIONS Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page). See also EXPECT and CUTOFF.

- ALIGNMENTS Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database
20 sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

- EXPECT The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely
25 by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

- 30 CUTOFF Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF

values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

MATRIX Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and
5 TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992, Proc. Natl. Acad. Sci. USA 89(22):10915-9). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

10 STRAND Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993)
15 Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States, 1993, Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see the world wide web site of the NCBI). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g.,
20 hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in
25 nucleotide sequence (e.g., "N" repeated 13 times) and the letter "X" in protein sequences (e.g., "X" repeated 9 times).

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.
30 It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the

statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi Causes NCBI gi identifiers to be shown in the output, in addition to the
5 accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at the NCBI world wide web site described above, in the "/BLAST" directory.

10

According to a further aspect the present invention provides a dual specific ligand comprising a first single immunoglobulin variable domain having a binding specificity to a first antigen or epitope and a second immunoglobulin single variable domain having a binding activity to a second antigen or epitope wherein said first and second domains lack
15 mutually complementary domains which share the same specificity.

According to the above aspect of the invention, preferably a dual-specific ligand has an IgG format which comprises two complementary pairs of mammalian dAbs wherein each Dab comprising each complementary pair has a different target binding specificity.
20 Advantageously a dual specific molecule according to this embodiment of the invention comprises one or more Dabs which exhibits an epitope binding specificity of of 50nM or more.

According to the above aspect of the invention the two different dAbs may be both VH
25 domains, both VL domains or at least one VH and a VL domain.

According to the above aspect of the invention, preferably the dual-specific ligand comprises at least one pair of Dabs which are complementary to one another.

30 Preferably a dual-specific ligand having an IgG format as described binds to its respective targets in a non-competitive manner.

In an alternative embodiment of the above aspect of the invention, a dual-specific ligand having an IgG format as described above binds to its respective targets in a competitive manner.

- 5 Advantageously a dual-specific ligand according to the above aspect of the invention has an IgG format and comprises one pair of identical dAbs which can bind simultaneously to two copies of the corresponding target.

10 More advantageously, a dual-specific ligand according to the above aspect of the invention comprise two pairs of identical dAbs wherein both pairs of identical dAbs can bind simultaneously to two copies of the corresponding targets

Advantageously, a dual-specific ligand according to the above aspect of the invention comprises 4 identical dAbs, preferably mammalian Dabs.

15

In an alternative embodiment of the above aspect of the invention, a dual-specific molecule according to the invention has a Fab format.

20 Advantageously, a dual-specific ligand having a Fab format as herein described binds to its respective targets in a non-competitive manner.

In an alternative embodiment of the above aspect of the invention, a dual-specific ligand having a Fab format as described above binds to its respective targets in a competitive manner.

25

Suitable targets for the dual-specific ligands according to the aspect of the invention described above include any one or more of those in the list consisting of the following: . One skilled in the art will appreciate that the choice of epitopes and antigens is large and varied. They may be for instance human or animal proteins, cytokines, cytokine receptors, 30 enzymes co-factors for enzymes or DNA binding proteins. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, EGF receptor, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, EpoR, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF,

GM-CSF, GF- β 1, insulin, IFN- γ , IGF-I, IGF-II, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin α , Inhibin β , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotactin, Mullerian inhibitory substance, monocyte colony
 5 inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 α , SDF1 β , SCF, SCGF, stem cell factor
 10 (SCF), TARC, TGF- α , TGF- β , TGF- β 2, TGF- β 3, tumour necrosis factor (TNF), TNF- α , TNF- β , TNF receptor I, TNF receptor II, TNIL-1, TPO, VEGF, VEGF receptor 1, VEGF receptor 2, VEGF receptor 3, GCP-2, GRO/MGSA, GRO- β , GRO- γ , HCC1, 1-309, HER 1, HER 2, HER 3, HER 4, TACE recognition site, TNF BP-I and TNF BP-II, as well as any target disclosed in Annex 2 or Annex 3 hereto, whether in combination as set forth in
 15 the Annexes, in a different combination or individually.

Advantageously, according to the final aspect of the invention, preferably the dual-specific ligands exhibit the ability to neutralise *in vitro* or in cell based assays

20 Ligands according to any aspect of the present invention, as well as dAb monomers useful in constructing such ligands, may advantageously dissociate from their cognate target(s) with a K_d of 300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12} M), preferably 50nM to 20pM, or 5nM to 200pM or 1nM to 100pM, 1×10^{-7} M or less, 1×10^{-8} M or less, 1×10^{-9} M or less, 1×10^{-10} M or less, 1×10^{-11} M or less; and/or a K_{off} rate constant of 5×10^{-1} to 1×10^{-7} S $^{-1}$, preferably 1×10^{-2} to 1×10^{-6} S $^{-1}$, or 5×10^{-3} to 1×10^{-5} S $^{-1}$, or 5×10^{-1}
 25 S $^{-1}$ or less, or 1×10^{-2} S $^{-1}$ or less, or 1×10^{-3} S $^{-1}$ or less, or 1×10^{-4} S $^{-1}$ or less, or 1×10^{-5} S $^{-1}$ or less, or 1×10^{-6} S $^{-1}$ or less as determined by surface plasmon resonance. The K_d rate constant is defined as K_{off}/K_{on} .

30 In particular the invention provides a dual-specific ligand wherein the affinity of binding to target with a K_d of 300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12} M), preferably 50nM to 20pM, more preferably 5nM to 200pM and most preferably 1nM to 100pM; expressed in an alternative manner, the K_d is 1×10^{-7} M or less, preferably 1×10^{-8} M or less, more

preferably 1×10^{-9} M or less, advantageously 1×10^{-10} M or less and most preferably 1×10^{-11} M or less; and/or a K_{off} rate constant of 5×10^{-1} to $1 \times 10^{-7} \text{ s}^{-1}$, preferably 1×10^{-2} to $1 \times 10^{-6} \text{ s}^{-1}$, more preferably 5×10^{-3} to $1 \times 10^{-5} \text{ s}^{-1}$, for example $5 \times 10^{-1} \text{ s}^{-1}$ or less, preferably $1 \times 10^{-2} \text{ s}^{-1}$ or less, more preferably $1 \times 10^{-3} \text{ s}^{-1}$ or less, advantageously $1 \times 10^{-4} \text{ s}^{-1}$ or less, further advantageously $1 \times 10^{-5} \text{ s}^{-1}$ or less, and most preferably $1 \times 10^{-6} \text{ s}^{-1}$ or less, as determined by surface plasmon resonance.

Preferably, the ligand neutralises $\text{TNF}\alpha$ in a standard L929 assay with an ND_{50} of 500nM to 50pM, preferably 100nM to 50pM, advantageously 10nM to 100pM, more preferably 1nM to 100pM; for example 50nM or less, preferably 5nM or less, advantageously 500pM or less, more preferably 200pM or less and most preferably 100pM or less.

Preferably, the ligand inhibits binding of TNF alpha to TNF alpha Receptor I (p55 receptor) with an IC_{50} of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less. Preferably, the $\text{TNF}\alpha$ is Human $\text{TNF}\alpha$.

According to the above aspect of the invention dual-specific ligands preferably exhibit a binding affinity of at least 50nM.

In a preferred aspect, the invention relates to a dual specific ligand which binds to a target ligand and a receptor for the target ligand. For example, the ligand may be $\text{TNF}\alpha$ and the target ligand receptor may be TNF Receptor 1. Advantageously, the dual specific ligand according to the invention is able to bind both the target ligand and the target ligand receptor simultaneously, i.e. is in an open configuration.

According to the present invention, advantageously a dual-specific ligand as described herein is a TAR1/TAR2 dual specific Fab, F(ab')_2 or IgG as herein described and is specific for human TNF alpha and the human TNFR1 (p55 receptor). Preferably, each arm comprises a complementary VH/VL pair. More preferably, the VL of each pair is V_{K} . More preferably still the V_{K} has TNF as target and the VH of each pair has the p55

receptor as a target. According to these Fab or IgG formats the dAbs advantageously bind their targets simultaneously, that is with no significant competition.

Most advantageously a TAR1/TAR2 IgG or Fab format dual-specific ligand is as
5 described herein in the Examples.

Those skilled in the art will appreciate that the vectors/constructs provided in the Examples and used for the generation of TAR1/TAR2 dual-specific ligands and the dAbs comprising them represent a mere sample of suitable vectors/constructs for use according
10 to the above aspect of the invention. Vectors/constructs suitable for use include the following:

(a) Eukaryotic leader- V_H or V_L - C_H1 -hinge- C_H2 - C_H3 . In this embodiment the leader may be mammalian, for example a CD33 or IgG K leader or functional variant/fragment of
15 these, or at least 80% homologous with any of these leaders.

According to the present invention there is also provided an expression vector, preferably yeast or mammalian in nature comprising a construct as described above in (a).

20 According to a further aspect still, there is provided a host, preferably mammalian cells such as Cos cells comprising a vector as described above.

In a final aspect of the invention there is provided a V_H dAb monomer designated TAR2h-10-27 dAb having the amino acid sequence given below (a) and which binds to
25 the human TNF receptor1 (p55 receptor):

EVQLLESGGGLVQPGGSLRLSCAASGFTFEWYWMGWVRQAPGKGLEWVSAISG
SGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDAAVYYCAKVKLG GGP N
FGYRGQGTLVTVSSAA

30

TAR2h-10-27 nucleic acid coding sequence

GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCC
 CTGCGTCTCTCCTGTGCAGCCTCCGGATTACCTTTGAGTGGTATTGGATGGG
 TTGGGTCCGCCAGGCTCCAGGGAAGGGTCTAGAGTGGGTCTCAGCTATCAGT
 GGTAAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCA
 5 TCTCCCGCGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGCGT
 GCCGAGGACGCCGCGGTATATTACTGTGCGAAAGTTAAGTTGGGGGGGGGGGC
 CTAATTTTGGCTACCGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGCGGC
 CGC

10 Advantageously, this dAb is comprised within a dual-specific ligand. Dual specific
 ligands include scFv, Fab and Ig molecules, and may be in open or closed conformations.
 Particularly preferred are dual specific Fab and IgG formats, comprising complementary
 TAR1-5-19 V_K and TAR2h-10-27 V_H domains. Advantageously, the polypeptide is in an
 open conformation.

15

Brief Description of the Figures

20 Figure 1 shows the diversification of V_H/HSA at positions H50, H52, H52a, H53,
 H55, H56, H58, H95, H96, H97, H98 (DVT or NNK encoded
 respectively) which are in the antigen binding site of V_H HSA. The
 sequence of V_K is diversified at positions L50, L53.

25 Figure 2 shows Library 1: Germline V_K/DVT V_H,
 Library 2: Germline V_K/NNK V_H,
 Library 3: Germline V_H/DVT V_K
 Library 4: Germline V_H/NNK V_K
 In phage display/ScFv format. These libraries were pre-selected for
 binding to generic ligands protein A and protein L so that the majority of
 30 the clones and selected libraries are functional. Libraries were selected on
 HSA (first round) and β -gal (second round) or HSA β -gal selection or on
 β -gal (first round) and HSA (second round) β -gal HSA selection. Soluble

scFv from these clones of PCR are amplified in the sequence. One clone encoding a dual specific antibody K8 was chosen for further work.

Figure 3 shows an alignment of V_H chains and V_K chains.

Figure 4 shows the characterisation of the binding properties of the K8 antibody, the binding properties of the K8 antibody characterised by monoclonal phage ELISA, the dual specific K8 antibody was found to bind HSA and β -gal and displayed on the surface of the phage with absorbant signals greater than 1.0. No cross reactivity with other proteins was detected.

Figure 5 shows soluble scFv ELISA performed using known concentrations of the K8 antibody fragment. A 96-well plate was coated with 100 μ g of HSA, BSA and β -gal at 10 μ g/ml and 100 μ g/ml of Protein A at 1 μ g/ml concentration. 50 μ g of the serial dilutions of the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirm the dual specific nature of the K8 antibody.

Figure 6 shows the binding characteristics of the clone K8 V_K /dummy V_H analysed using soluble scFv ELISA. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al*, Methods Enzymol. 1996;267:83-109 and the supernatant containing scFv assayed directly. Soluble scFv ELISA is performed as described in example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind β -gal, whereas binding BSA was abolished.

Figure 7 shows the sequence of variable domain vectors 1 and 2.

Figure 8 is a map of the C_H vector used to construct a V_H1/V_H2 multispecific ligand.

Figure 9 is a map of the V_K vector used to construct a V_K1/V_K2 multispecific ligand.

- Figure 10 TNF receptor assay comparing TAR1-5 dimer 4, TAR1-5-19 dimer 4 and TAR1-5-19 monomer.
- 5 Figure 11 TNF receptor assay comparing TAR1-5 dimers 1-6. All dimers have been FPLC purified and the results for the optimal dimeric species are shown.
- 10 Figure 12 TNF receptor assay of TAR1-5 19 homodimers in different formats: dAb-linker-dAb format with 3U, 5U or 7U linker, Fab format and cysteine hinge linker format.
- 15 Figure 13 Dummy VH sequence for library 1. The sequence of the VH framework based on germline sequence DP47 – JH4b. Positions where NNK randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 1 are indicated in bold underlined text.
- 20 Figure 14 Dummy VH sequence for library 2. The sequence of the VH framework based on germline sequence DP47 – JH4b. Positions where NNK randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 2 are indicated in bold underlined text.
- 25 Figure 15 Dummy V_K sequence for library 3. The sequence of the V_K framework based on germline sequence DP_K9 - J_K1. Positions where NNK randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 3 are indicated in bold underlined text.
- 30 Figure 16 Nucleotide and amino acid sequence of anti MSA dAbs MSA 16 and MSA 26.
- 30 Figure 17 Inhibition biacore of MSA 16 and 26. Purified dAbs MSA16 and MSA26 were analysed by inhibition biacore to determine K_d. Briefly, the dAbs were tested to determine the concentration of dAb required to achieve 200RUs of response on a biacore CM5 chip coated with a high density of MSA. Once the required concentrations of dAb had been determined,

MSA antigen at a range of concentrations around the expected K_d was premixed with the dAb and incubated overnight. Binding to the MSA coated biacore chip of dAb in each of the premixes was then measured at a high flow-rate of 30 μ l/minute.

5

Figure 18

Serum levels of MSA16 following injection. Serum half life of the dAb MSA16 was determined in mouse. MSA16 was dosed as single i.v. injections at approx 1.5mg/kg into CD1 mice. Modelling with a 2 compartment model showed MSA16 had a $t_{1/2\alpha}$ of 0.98hr, a $t_{1/2\beta}$ of 36.5hr and an AUC of 913hr.mg/ml. MSA16 had a considerably lengthened half life compared with HEL4 (an anti-hen egg white lysozyme dAb) which had a $t_{1/2\alpha}$ of 0.06hr and a $t_{1/2\beta}$ of 0.34hr.

10

Figure 19

ELISA (a) and TNF receptor assay (c) showing inhibition of TNF binding with a Fab-like fragment comprising MSA26Ck and TAR1-5-19CH. Addition of MSA with the Fab-like fragment reduces the level of inhibition. An ELISA plate coated with 1 μ g/ml TNF α was probed with dual specific V κ C $_H$ and V κ C κ Fab like fragment and also with a control TNF α binding dAb at a concentration calculated to give a similar signal on the ELISA. Both the dual specific and control dAb were used to probe the ELISA plate in the presence and in the absence of 2mg/ml MSA. The signal in the dual specific well was reduced by more than 50% but the signal in the dAb well was not reduced at all (see figure 19a). The same dual specific protein was also put into the receptor assay with and without MSA and competition by MSA was also shown (see figure 19c). This demonstrates that binding of MSA to the dual specific is competitive with binding to TNF α .

15

20

25

Figure 20

TNF receptor assay showing inhibition of TNF binding with a disulphide bonded heterodimer of TAR1-5-19 dAb and MSA16 dAb. Addition of MSA with the dimer reduces the level of inhibition in a dose dependant manner. The TNF receptor assay (figure 19 (b)) was conducted in the presence of a constant concentration of heterodimer (18nM) and a dilution

30

series of MSA and HSA. The presence of HSA at a range of concentrations (up to 2 mg/ml) did not cause a reduction in the ability of the dimer to inhibit TNF α . However, the addition of MSA caused a dose dependant reduction in the ability of the dimer to inhibit TNF α (figure 19a). This demonstrates that MSA and TNF α compete for binding to the cys bonded TAR1-5-19, MSA16 dimer. MSA and HSA alone did not have an effect on the TNF binding level in the assay.

Figure 21: Shows the vectors used for Fab construction according to the invention.

Figure 22: Shows the binding of Fab comprising TAR1/TAR2 Dabs to TNF and TNFR1 via an ELISA assay.

Figure 23: Shows the results of sandwich ELISA to test the ability of TAR1/TAR2 Fab to bind to both TNF and TNFR antigens simultaneously, that is to test whether the Fab is of open or closed conformation.

Figure 24: Shows the results of competition ELISA to test the ability of TAR1/TAR2 Fab to bind to both antigens simultaneously, that is to test whether the Fab is of open or closed conformation.

Figure 25: Shows the results of cell based assays using Fab dual specific ligands according to the invention:

(a) to test human TNF cytotoxicity on murine cells

(b) shows a murine TNF cytotoxicity assay on murine cells with human soluble TAR2.

(c) Shows murine TNF induced IL-8 secretion on human cells.

(d) Shows human TNF induced IL-8 secretion on human cells.

Figure 26: Shows murine TNF cytotoxicity on murine cells with soluble human TNFR1 and increasing concentrations of mutant TNF (competition on cells).

Figure 27: shows the construction of IgG vectors which express IgG1 heavy chain constant region and light chain kappa constant region respectively.

Figure 28 shows the binding of TAR1/TAR2 IgG to TNF and TNFR1 in ELISA assay.

5

Figure 29: Shows the analysis of TAR1/TAR2 IgG properties in cell assays.

- (a) Human TNF cytotoxicity on murine cells.
- (b) Murine TNF cytotoxicity assay on murine cells with human soluble TNF receptor.
- (c) Human TNF induced IL-8 release from human cells.
- 10 (d) Murine TNF induced IL-8 secretion from human cells.

Figure 30: Shows Human TNF induced IL-8 secretion on human cells

Figure 31: Shows the amino acid sequence of the Dab designated TAR2 which binds to
15 human TNFR1 (p55 receptor).

Detailed Description of the Invention

Definitions

5 **Complementary** Two immunoglobulin domains are “complementary” where they belong to families of structures which form cognate pairs or groups or are derived from such families and retain this feature. For example, a V_H domain and a V_L domain of an antibody are complementary; two V_H domains are not complementary, and two V_L domains are not complementary. Complementary domains may be found in other
10 members of the immunoglobulin superfamily, such as the V_α and V_β (or γ and δ) domains of the T-cell receptor. In the context of the second configuration of the present invention, non-complementary domains do not bind a target molecule cooperatively, but act independently on different target epitopes which may be on the same or different molecules. Domains which are artificial, such as domains based on protein scaffolds
15 which do not bind epitopes unless engineered to do so, are non-complementary. Likewise, two domains based on (for example) an immunoglobulin domain and a fibronectin domain are not complementary.

Immunoglobulin This refers to a family of polypeptides which retain the
20 immunoglobulin fold characteristic of antibody molecules, which contains two β sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*, including widespread roles in the immune system (for example, antibodies, T-cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules)
25 and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which possess binding domains. Preferably, the present invention relates to antibodies.

Combining Variable domains according to the invention are combined to form a group
30 of domains; for example, complementary domains may be combined, such as V_L domains being combined with V_H domains. Non-complementary domains may also be combined. Domains may be combined in a number of ways, involving linkage of the domains by covalent or non-covalent means.

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

Repertoire A collection of diverse variants, for example polypeptide variants which differ in their primary sequence. A library used in the present invention will encompass a repertoire of polypeptides comprising at least 1000 members.

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

A '**closed conformation multi-specific ligand**' describes a multi-specific ligand as herein defined comprising at least two epitope binding domains as herein defined. The term 'closed conformation' (multi-specific ligand) means that the epitope binding domains of the ligand are arranged such that epitope binding by one epitope binding domain competes with epitope binding by another epitope binding domain. That is, cognate epitopes may be bound by each epitope binding domain individually but not simultaneously. The closed conformation of the ligand can be achieved using methods herein described.

10 **Antibody** An antibody (for example IgG, IgM, IgA, IgD or IgE) or fragment (such as a Fab, F(ab')₂, Fv, disulphide linked Fv, scFv, closed conformation multispecific antibody, disulphide-linked scFv, diabody) whether derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

15

Dual-specific ligand A ligand comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein the variable regions are capable of binding to two different antigens or two epitopes on the same antigen which are not normally bound by a monospecific immunoglobulin. For example, the two epitopes may be on the same hapten, but are not the same epitope or sufficiently adjacent to be bound by a monospecific ligand. The dual specific ligands according to the invention are composed of variable domains which have different specificities, and do not contain mutually complementary variable domain pairs which have the same specificity.

20

Antigen A molecule that is bound by a ligand according to the present invention. Typically, antigens are bound by antibody ligands and are capable of raising an antibody response *in vivo*. It may be a polypeptide, protein, nucleic acid or other molecule. Generally, the dual specific ligands according to the invention are selected for target specificity against a particular antigen. In the case of conventional antibodies and fragments thereof, the antibody binding site defined by the variable loops (L1, L2, L3 and H1, H2, H3) is capable of binding to the antigen.

25

30

Epitope A unit of structure conventionally bound by an immunoglobulin V_H/V_L pair. Epitopes define the minimum binding site for an antibody, and thus represent the target of specificity of an antibody. In the case of a single domain antibody, an epitope represents the unit of structure bound by a variable domain in isolation.

5

Generic ligand A ligand that binds to all members of a repertoire. Generally, not bound through the antigen binding site as defined above. Non-limiting examples include protein A, protein L and protein G.

10 **Selecting** Derived by screening, or derived by a Darwinian selection process, in which binding interactions are made between a domain and the antigen or epitope or between an antibody and an antigen or epitope. Thus a first variable domain may be selected for binding to an antigen or epitope in the presence or in the absence of a complementary variable domain.

15

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

25 **Half-life** The time taken for the serum concentration of the ligand to reduce by 50%, *in vivo*, for example due to degradation of the ligand and/or clearance or sequestration of the ligand by natural mechanisms. The ligands of the invention are stabilised *in vivo* and their half-life increased by binding to molecules which resist degradation and/or clearance or sequestration. Typically, such molecules are naturally occurring proteins which
30 themselves have a long half-life *in vivo*. The half-life of a ligand is increased if its functional activity persists, *in vivo*, for a longer period than a similar ligand which is not specific for the half-life increasing molecule. Thus, a ligand specific for HSA and a target molecule is compared with the same ligand wherein the specificity for HSA is not

present, that it does not bind HSA but binds another molecule. For example, it may bind a second epitope on the target molecule. Typically, the half life is increased by 10%, 20%, 30%, 40%, 50% or more. Increases in the range of 2x, 3x, 4x, 5x, 10x, 20x, 30x, 40x, 50x or more of the half life are possible. Alternatively, or in addition, increases in the range of up to 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 150x of the half life are possible.

Homogeneous immunoassay An immunoassay in which analyte is detected without need for a step of separating bound and un-bound reagents.

10

Substantially identical (or “substantially homologous”) A first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain, *e.g.*, conserved amino acid substitutions) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities. In the case of antibodies, the second antibody has the same binding specificity and has at least 50% of the affinity of the same.

As used herein, the terms “**low stringency**,” “**medium stringency**,” “**high stringency**,” or “**very high stringency conditions**” describe conditions for nucleic acid hybridization and washing. Guidance for performing hybridization reactions can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, which is incorporated herein by reference in its entirety. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: (1) low stringency hybridization conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for low stringency conditions); (2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; (3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and preferably (4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one

or more washes at 0.2X SSC, 1% SDS at 65°C. Very high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified.

TAR1-5-19 Dab- is a single domain antibody (Dab) specific for human TNF α .

5

TAR2h-10-27 Dab- is a single domain antibody (Dab) specific for human TNF receptor 1 (p55 receptor).

TAR1/TAR2 Fab, F(ab')₂ or IgG are Fab, F(ab')₂ or IgG formatted dual specific
10 antibodies comprising TAR1-5-19 and TAR2h-10-27 Dabs as herein described.

Detailed Description of the Invention

15 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold
20 Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods.

Preparation of immunoglobulin based multi-specific ligands

25

Dual specific ligands according to the invention, whether open or closed in conformation according to the desired configuration of the invention, may be prepared according to previously established techniques, used in the field of antibody engineering, for the preparation of scFv, "phage" antibodies and other engineered antibody molecules.
30 Techniques for the preparation of antibodies, and in particular bispecific antibodies, are for example described in the following reviews and the references cited therein: Winter & Milstein, (1991) Nature 349:293-299; Plueckthun (1992) Immunological Reviews 130:151-188; Wright *et al.*, (1992) Crit. Rev. Immunol.12:125-168; Holliger, P. &

- Winter, G. (1993) *Curr. Op. Biotechn.* 4, 446-449; Carter, *et al.* (1995) *J. Hematother.* 4, 463-470; Chester, K.A. & Hawkins, R.E. (1995) *Trends Biotechn.* 13, 294-300; Hoogenboom, H.R. (1997) *Nature Biotechnol.* 15, 125-126; Fearon, D. (1997) *Nature Biotechnol.* 15, 618-619; Plückthun, A. & Pack, P. (1997) *Immunotechnology* 3, 83-105;
- 5 Carter, P. & Merchant, A.M. (1997) *Curr. Opin. Biotechnol.* 8, 449-454; Holliger, P. & Winter, G. (1997) *Cancer Immunol. Immunother.* 45, 128-130.

The invention provides for the selection of variable domains against two different antigens or epitopes, and subsequent combination of the variable domains.

10

The techniques employed for selection of the variable domains employ libraries and selection procedures which are known in the art. Natural libraries (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581; Vaughan *et al.* (1996) *Nature Biotech.*, 14: 309) which use rearranged V genes harvested from human B cells are well known to those skilled in the

15 art. Synthetic libraries (Hoogenboom & Winter (1992) *J. Mol. Biol.*, 227: 381; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89: 4457; Nissim *et al.* (1994) *EMBO J.*, 13: 692; Griffiths *et al.* (1994) *EMBO J.*, 13: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, 248: 97) are prepared by cloning immunoglobulin V genes, usually using PCR. Errors in the PCR process can lead to a high degree of randomisation. V_H and/or V_L libraries may be

20 selected against target antigens or epitopes separately, in which case single domain binding is directly selected for, or together.

A preferred method for making a dual specific ligand according to the present invention comprises using a selection system in which a repertoire of variable domains is selected

25 for binding to a first antigen or epitope and a repertoire of variable domains is selected for binding to a second antigen or epitope. The selected variable first and second variable domains are then combined and the dual-specific ligand selected for binding to both first and second antigen or epitope. Closed conformation ligands are selected for binding both first and second antigen or epitope in isolation but not simultaneously.

30

A. Library vector systems

A variety of selection systems are known in the art which are suitable for use in the present invention. Examples of such systems are described below.

5

Bacteriophage lambda expression systems may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989) *Science*, **246**: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**: 8095; Persson *et al.* (1991) *Proc.*
10 *Natl. Acad. Sci. U.S.A.*, **88**: 2432) and are of use in the invention. Whilst such expression systems can be used to screen up to 10^6 different members of a library, they are not really suited to screening of larger numbers (greater than 10^6 members).

Of particular use in the construction of libraries are selection display systems, which
15 enable a nucleic acid to be linked to the polypeptide it expresses. As used herein, a selection display system is a system that permits the selection, by suitable display means, of the individual members of the library by binding the generic and/or target ligands.

Selection protocols for isolating desired members of large libraries are known in the art,
20 as typified by phage display techniques. Such systems, in which diverse peptide sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith (1990) *Science*, **249**: 386), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen (McCafferty *et al.*,
25 WO 92/01047). The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids
30 (phagebodies). An advantage of phage-based display systems is that, because they are biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage

or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

Methods for the construction of bacteriophage antibody display libraries and lambda
5 phage expression libraries are well known in the art (McCafferty *et al.* (1990) *Nature*,
348: 552; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 4363; Clackson *et al.*
(1991) *Nature*, **352**: 624; Lowman *et al.* (1991) *Biochemistry*, **30**: 10832; Burton *et al.*
(1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 10134; Hoogenboom *et al.* (1991) *Nucleic Acids*
Res., **19**: 4133; Chang *et al.* (1991) *J. Immunol.*, **147**: 3610; Breitling *et al.* (1991) *Gene*,
10 **104**: 147; Marks *et al.* (1991) *supra*; Barbas *et al.* (1992) *supra*; Hawkins and Winter
(1992) *J. Immunol.*, **22**: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, **267**: 16007; Lerner *et al.*
(1992) *Science*, **258**: 1313, incorporated herein by reference).

One particularly advantageous approach has been the use of scFv phage-libraries (Huston
15 *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.*, **85**: 5879-5883; Chaudhary *et al.* (1990) *Proc.*
Natl. Acad. Sci. U.S.A., **87**: 1066-1070; McCafferty *et al.* (1990) *supra*; Clackson *et al.*
(1991) *Nature*, **352**: 624; Marks *et al.* (1991) *J. Mol. Biol.*, **222**: 581; Chiswell *et al.*
(1992) *Trends Biotech.*, **10**: 80; Marks *et al.* (1992) *J. Biol. Chem.*, **267**). Various
embodiments of scFv libraries displayed on bacteriophage coat proteins have been
20 described. Refinements of phage display approaches are also known, for example as
described in WO96/06213 and WO92/01047 (Medical Research Council *et al.*) and
WO97/08320 (Morphosys), which are incorporated herein by reference.

Other systems for generating libraries of polypeptides involve the use of cell-free
25 enzymatic machinery for the *in vitro* synthesis of the library members. In one method,
RNA molecules are selected by alternate rounds of selection against a target ligand and
PCR amplification (Tuerk and Gold (1990) *Science*, **249**: 505; Ellington and Szostak
(1990) *Nature*, **346**: 818). A similar technique may be used to identify DNA sequences
which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic*
30 *Acids Res.*, **18**: 3203; Beaudry and Joyce (1992) *Science*, **257**: 635; WO92/05258 and
WO92/14843). In a similar way, *in vitro* translation can be used to synthesise
polypeptides as a method for generating large libraries. These methods which generally
comprise stabilised polysome complexes, are described further in WO88/08453,

WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection.

5

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

15

B. Library Construction.

Libraries intended for selection, may be constructed using techniques known in the art, for example as set forth above, or may be purchased from commercial sources. Libraries which are useful in the present invention are described, for example, in WO99/20749. Once a vector system is chosen and one or more nucleic acid sequences encoding polypeptides of interest are cloned into the library vector, one may generate diversity within the cloned molecules by undertaking mutagenesis prior to expression; alternatively, the encoded proteins may be expressed and selected, as described above, before mutagenesis and additional rounds of selection are performed. Mutagenesis of nucleic acid sequences encoding structurally optimised polypeptides is carried out by standard molecular methods. Of particular use is the polymerase chain reaction, or PCR, (Mullis and Faloona (1987) *Methods Enzymol.*, **155**: 335, herein incorporated by reference). PCR, which uses multiple cycles of DNA replication catalysed by a thermostable, DNA-dependent DNA polymerase to amplify the target sequence of interest, is well known in the art. The construction of various antibody libraries has been

25

30

discussed in Winter *et al.* (1994) Ann. Rev. Immunology 12, 433-55, and references cited therein.

PCR is performed using template DNA (at least 1fg; more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers; it may be advantageous to use a larger amount of primer when the primer pool is heavily heterogeneous, as each sequence is represented by only a small fraction of the molecules of the pool, and amounts become limiting in the later amplification cycles. A typical reaction mixture includes: 2µl of DNA, 25 pmol of oligonucleotide primer, 2.5 µl of 10X PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 µl of 1.25 µM dNTP, 0.15 µl (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 µl. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler. The length and temperature of each step of a PCR cycle, as well as the number of cycles, is adjusted in accordance to the stringency requirements in effect. Annealing temperature and timing are determined both by the efficiency with which a primer is expected to anneal to a template and the degree of mismatch that is to be tolerated; obviously, when nucleic acid molecules are simultaneously amplified and mutagenised, mismatch is required, at least in the first round of synthesis. The ability to optimise the stringency of primer annealing conditions is well within the knowledge of one of moderate skill in the art. An annealing temperature of between 30 °C and 72 °C is used. Initial denaturation of the template molecules normally occurs at between 92°C and 99°C for 4 minutes, followed by 20-40 cycles consisting of denaturation (94-99°C for 15 seconds to 1 minute), annealing (temperature determined as discussed above; 1-2 minutes), and extension (72°C for 1-5 minutes, depending on the length of the amplified product). Final extension is generally for 4 minutes at 72°C, and may be followed by an indefinite (0-24 hour) step at 4°C.

C. Combining single variable domains

Domains useful in the invention, once selected, may be combined by a variety of methods known in the art, including covalent and non-covalent methods.

Preferred methods include the use of polypeptide linkers, as described, for example, in connection with scFv molecules (Bird *et al.*, (1988) Science 242:423-426). Discussion of

suitable linkers is provided in *Bird et al.* Science 242, 423-426; *Hudson et al.*, Journal Immunol Methods 231 (1999) 177-189; *Hudson et al.*, Proc Nat Acad Sci USA 85, 5879-5883. Linkers are preferably flexible, allowing the two single domains to interact. One linker example is a (Gly₄ Ser)_n linker, where n=1 to 8, eg, 2, 3, 4, 5 or 7. The linkers used
5 in diabodies, which are less flexible, may also be employed (*Holliger et al.*, (1993) PNAS (USA) 90:6444-6448).

In one embodiment, the linker employed is not an immunoglobulin hinge region.

10 Variable domains may be combined using methods other than linkers. For example, the use of disulphide bridges, provided through naturally-occurring or engineered cysteine residues, may be exploited to stabilise V_H-V_H, V_L-V_L or V_H-V_L dimers (*Reiter et al.*, (1994) Protein Eng. 7:697-704) or by remodelling the interface between the variable domains to improve the "fit" and thus the stability of interaction (*Ridgeway et al.*, (1996)
15 Protein Eng. 7:617-621; *Zhu et al.*, (1997) Protein Science 6:781-788).

Other techniques for joining or stabilising variable domains of immunoglobulins, and in particular antibody V_H domains, may be employed as appropriate.

20 In accordance with the present invention, dual specific ligands can be in "closed" conformations in solution. A "closed" configuration is that in which the two domains (for example V_H and V_L) are present in associated form, such as that of an associated V_H-V_L pair which forms an antibody binding site. For example, scFv may be in a closed conformation, depending on the arrangement of the linker used to link the V_H and V_L
25 domains. If this is sufficiently flexible to allow the domains to associate, or rigidly holds them in the associated position, it is likely that the domains will adopt a closed conformation.

Similarly, V_H domain pairs and V_L domain pairs may exist in a closed conformation.
30 Generally, this will be a function of close association of the domains, such as by a rigid linker, in the ligand molecule. Ligands in a closed conformation will be unable to bind both the molecule which increases the half-life of the ligand and a second target molecule.

Thus, the ligand will typically only bind the second target molecule on dissociation from the molecule which increases the half-life of the ligand.

Moreover, the construction of V_H/V_H , V_L/V_L or V_H/V_L dimers without linkers provides
5 for competition between the domains.

Ligands according to the invention may moreover be in an open conformation. In such a conformation, the ligands will be able to simultaneously bind both the molecule which increases the half-life of the ligand and the second target molecule. Typically, variable
10 domains in an open configuration are (in the case of V_H-V_L pairs) held far enough apart for the domains not to interact and form an antibody binding site and not to compete for binding to their respective epitopes. In the case of V_H/V_H or V_L/V_L dimers, the domains are not forced together by rigid linkers. Naturally, such domain pairings will not compete for antigen binding or form an antibody binding site.

15

Fab fragments and whole antibodies will exist primarily in the closed conformation, although it will be appreciated that open and closed dual specific ligands are likely to exist in a variety of equilibria under different circumstances. Binding of the ligand to a target is likely to shift the balance of the equilibrium towards the open configuration.
20 Thus, certain ligands according to the invention can exist in two conformations in solution, one of which (the open form) can bind two antigens or epitopes independently, whilst the alternative conformation (the closed form) can only bind one antigen or epitope; antigens or epitopes thus compete for binding to the ligand in this conformation.

25 Although the open form of the dual specific ligand may thus exist in equilibrium with the closed form in solution, it is envisaged that the equilibrium will favour the closed form; moreover, the open form can be sequestered by target binding into a closed conformation. Preferably, therefore, certain dual specific ligands of the invention are present in an equilibrium between two (open and closed) conformations.

30

Dual specific ligands according to the invention may be modified in order to favour an open or closed conformation. For example, stabilisation of V_H-V_L interactions with disulphide bonds stabilises the closed conformation. Moreover, linkers used to join the

domains, including V_H domain and V_L domain pairs, may be constructed such that the open from is favoured; for example, the linkers may sterically hinder the association of the domains, such as by incorporation of large amino acid residues in opportune locations, or the designing of a suitable rigid structure which will keep the domains physically spaced apart.

D. Characterisation of the dual-specific ligand.

The binding of the dual-specific ligand to its specific antigens or epitopes can be tested by methods which will be familiar to those skilled in the art and include ELISA. In a preferred embodiment of the invention binding is tested using monoclonal phage ELISA.

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

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

The diversity of the selected phage monoclonal antibodies may also be assessed by gel electrophoresis of PCR products (Marks *et al.* 1991, *supra*; Nissim *et al.* 1994 *supra*), probing (Tomlinson *et al.*, 1992) J. Mol. Biol. 227, 776) or by sequencing of the vector DNA.

E. Structure of 'Dual-specific ligands'.

As described above, an antibody is herein defined as an antibody (for example IgG, IgM, IgA, IgA, IgE) or fragment (Fab, Fv, disulphide linked Fv, scFv, diabody) which comprises at least one heavy and a light chain variable domain, at least two heavy chain

variable domains or at least two light chain variable domains. It may be at least partly derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

5

In a preferred embodiment of the invention the dual-specific ligand comprises at least one single heavy chain variable domain of an antibody and one single light chain variable domain of an antibody, or two single heavy or light chain variable domains. For example, the ligand may comprise a V_H/V_L pair, a pair of V_H domains or a pair of V_L domains.

10

The first and the second variable domains of such a ligand may be on the same polypeptide chain. Alternatively they may be on separate polypeptide chains. In the case that they are on the same polypeptide chain they may be linked by a linker, which is preferentially a peptide sequence, as described above.

15

The first and second variable domains may be covalently or non-covalently associated. In the case that they are covalently associated, the covalent bonds may be disulphide bonds.

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

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

30

In a preferred embodiment of the invention the 'dual-specific ligand' is a single chain Fv fragment. In an alternative embodiment of the invention, the 'dual-specific ligand' consists of a Fab format.

- 5 In a further aspect, the present invention provides nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

One skilled in the art will appreciate that, depending on the aspect of the invention, both antigens or epitopes may bind simultaneously to the same antibody molecule.
10 Alternatively, they may compete for binding to the same antibody molecule. For example, where both epitopes are bound simultaneously, both variable domains of a dual specific ligand are able to independently bind their target epitopes. Where the domains compete, the one variable domain is capable of binding its target, but not at the same time as the other variable domain binds its cognate target; or the first variable domain is
15 capable of binding its target, but not at the same time as the second variable domain binds its cognate target.

The variable regions may be derived from antibodies directed against target antigens or epitopes. Alternatively they may be derived from a repertoire of single antibody domains
20 such as those expressed on the surface of filamentous bacteriophage. Selection may be performed as described below.

In general, the nucleic acid molecules and vector constructs required for the performance of the present invention may be constructed and manipulated as set forth in standard
25 laboratory manuals, such as Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, USA.

The manipulation of nucleic acids useful in the present invention is typically carried out in recombinant vectors.

30

Thus in a further aspect, the present invention provides a vector comprising nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

As used herein, vector refers to a discrete element that is used to introduce heterologous DNA into cells for the expression and/or replication thereof. Methods by which to select or construct and, subsequently, use such vectors are well known to one of ordinary skill in the art. Numerous vectors are publicly available, including bacterial plasmids, bacteriophage, artificial chromosomes and episomal vectors. Such vectors may be used for simple cloning and mutagenesis; alternatively gene expression vector is employed. A vector of use according to the invention may be selected to accommodate a polypeptide coding sequence of a desired size, typically from 0.25 kilobase (kb) to 40 kb or more in length. A suitable host cell is transformed with the vector after *in vitro* cloning manipulations. Each vector contains various functional components, which generally include a cloning (or "polylinker") site, an origin of replication and at least one selectable marker gene. If given vector is an expression vector, it additionally possesses one or more of the following: enhancer element, promoter, transcription termination and signal sequences, each positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding a ligand according to the invention.

Both cloning and expression vectors generally contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors unless these are used in mammalian cells able to replicate high levels of DNA, such as COS cells.

Advantageously, a cloning or expression vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline,

complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

Since the replication of vectors encoding a ligand according to the present invention is most conveniently performed in *E. coli*, an *E. coli*-selectable marker, for example, the β -lactamase gene that confers resistance to the antibiotic ampicillin, is of use. These can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or pUC19.

Expression vectors usually contain a promoter that is recognised by the host organism and is operably linked to the coding sequence of interest. Such a promoter may be inducible or constitutive. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the β -lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (*trp*) promoter system and hybrid promoters such as the *tac* promoter. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the coding sequence.

The preferred vectors are expression vectors that enables the expression of a nucleotide sequence corresponding to a polypeptide library member. Thus, selection with the first and/or second antigen or epitope can be performed by separate propagation and expression of a single clone expressing the polypeptide library member or by use of any selection display system. As described above, the preferred selection display system is bacteriophage display. Thus, phage or phagemid vectors may be used, eg pIT1 or pIT2.

Leader sequences useful in the invention include *pelB*, *stII*, *ompA*, *phoA*, *bla* and *pelA*. One example are phagemid vectors which have an *E. coli* origin of replication (for double stranded replication) and also a phage origin of replication (for production of single-stranded DNA). The manipulation and expression of such vectors is well known in

the art (Hoogenboom and Winter (1992) *supra*; Nissim *et al.* (1994) *supra*). Briefly, the vector contains a β -lactamase gene to confer selectivity on the phagemid and a lac promoter upstream of a expression cassette that consists (N to C terminal) of a pelB leader sequence (which directs the expressed polypeptide to the periplasmic space), a multiple cloning site (for cloning the nucleotide version of the library member), optionally, one or more peptide tag (for detection), optionally, one or more TAG stop codon and the phage protein pIII. Thus, using various suppressor and non-suppressor strains of *E. coli* and with the addition of glucose, iso-propyl thio- β -D-galactoside (IPTG) or a helper phage, such as VCS M13, the vector is able to replicate as a plasmid with no expression, produce large quantities of the polypeptide library member only or produce phage, some of which contain at least one copy of the polypeptide-pIII fusion on their surface.

Construction of vectors encoding ligands according to the invention employs conventional ligation techniques. Isolated vectors or DNA fragments are cleaved, tailored, and religated in the form desired to generate the required vector. If desired, analysis to confirm that the correct sequences are present in the constructed vector can be performed in a known fashion. Suitable methods for constructing expression vectors, preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses for assessing expression and function are known to those skilled in the art. The presence of a gene sequence in a sample is detected, or its amplification and/or expression quantified by conventional methods, such as Southern or Northern analysis, Western blotting, dot blotting of DNA, RNA or protein, *in situ* hybridisation, immunocytochemistry or sequence analysis of nucleic acid or protein molecules. Those skilled in the art will readily envisage how these methods may be modified, if desired.

Structure of closed conformation multispecific ligands

According to one aspect of the second configuration of the invention present invention, the two or more non-complementary epitope binding domains are linked so that they are in a closed conformation as herein defined. Advantageously, they may be further attached to a skeleton which may, as a alternative, or on addition to a linker described

herein, facilitate the formation and/or maintenance of the closed conformation of the epitope binding sites with respect to one another.

(I) Skeletons

5 Skeletons may be based on immunoglobulin molecules or may be non-immunoglobulin in origin as set forth above. Preferred immunoglobulin skeletons as herein defined includes any one or more of those selected from the following: an immunoglobulin molecule comprising at least (i) the CL (kappa or lambda subclass) domain of an antibody; or (ii) the CH1 domain of an antibody heavy chain; an immunoglobulin molecule comprising the
10 CH1 and CH2 domains of an antibody heavy chain; an immunoglobulin molecule comprising the CH1, CH2 and CH3 domains of an antibody heavy chain; or any of the subset (ii) in conjunction with the CL (kappa or lambda subclass) domain of an antibody. A hinge region domain may also be included.. Such combinations of domains may, for example, mimic natural antibodies, such as IgG or IgM, or fragments thereof, such as Fv,
15 scFv, Fab or F(ab')₂ molecules. Those skilled in the art will be aware that this list is not intended to be exhaustive.

(II) Protein scaffolds

Each epitope binding domain comprises a protein scaffold and one or more CDRs which
20 are involved in the specific interaction of the domain with one or more epitopes. Advantageously, an epitope binding domain according to the present invention comprises three CDRs. Suitable protein scaffolds include any of those selected from the group consisting of the following: those based on immunoglobulin domains, those based on fibronectin, those based on affibodies, those based on CTLA4, those based on chaperones
25 such as GroEL, those based on lipocalin and those based on the bacterial Fc receptors SpA and SpD. Those skilled in the art will appreciate that this list is not intended to be exhaustive.

F: Scaffolds for use in Constructing Dual Specific Ligands

30

i. Selection of the main-chain conformation

The members of the immunoglobulin superfamily all share a similar fold for their polypeptide chain. For example, although antibodies are highly diverse in terms of their

- primary sequence, comparison of sequences and crystallographic structures has revealed that, contrary to expectation, five of the six antigen binding loops of antibodies (H1, H2, L1, L2, L3) adopt a limited number of main-chain conformations, or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, **196**: 901; Chothia *et al.* (1989) *Nature*, **342**: 877).
- 5 Analysis of loop lengths and key residues has therefore enabled prediction of the main-chain conformations of H1, H2, L1, L2 and L3 found in the majority of human antibodies (Chothia *et al.* (1992) *J. Mol. Biol.*, **227**: 799; Tomlinson *et al.* (1995) *EMBO J.*, **14**: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it
- 10 also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, **263**: 800; Shirai *et al.* (1996) *FEBS Letters*, **399**: 1).
- 15 The dual specific ligands of the present invention are advantageously assembled from libraries of domains, such as libraries of V_H domains and/or libraries of V_L domains. Moreover, the dual specific ligands of the invention may themselves be provided in the form of libraries. In one aspect of the present invention, libraries of dual specific ligands and/or domains are designed in which certain loop lengths and key residues have been
- 20 chosen to ensure that the main-chain conformation of the members is known. Advantageously, these are real conformations of immunoglobulin superfamily molecules found in nature, to minimise the chances that they are non-functional, as discussed above. Germline V gene segments serve as one suitable basic framework for constructing antibody or T-cell receptor libraries; other sequences are also of use. Variations may
- 25 occur at a low frequency, such that a small number of functional members may possess an altered main-chain conformation, which does not affect its function.

Canonical structure theory is also of use to assess the number of different main-chain conformations encoded by ligands, to predict the main-chain conformation based on

30 ligand sequences and to chose residues for diversification which do not affect the canonical structure. It is known that, in the human V_K domain, the L1 loop can adopt one of four canonical structures, the L2 loop has a single canonical structure and that 90% of human V_K domains adopt one of four or five canonical structures for the L3 loop

(Tomlinson *et al.* (1995) *supra*); thus, in the V_{κ} domain alone, different canonical structures can combine to create a range of different main-chain conformations. Given that the V_{λ} domain encodes a different range of canonical structures for the L1, L2 and L3 loops and that V_{κ} and V_{λ} domains can pair with any V_H domain which can encode several canonical structures for the H1 and H2 loops, the number of canonical structure combinations observed for these five loops is very large. This implies that the generation of diversity in the main-chain conformation may be essential for the production of a wide range of binding specificities. However, by constructing an antibody library based on a single known main-chain conformation it has been found, contrary to expectation, that diversity in the main-chain conformation is not required to generate sufficient diversity to target substantially all antigens. Even more surprisingly, the single main-chain conformation need not be a consensus structure - a single naturally occurring conformation can be used as the basis for an entire library. Thus, in a preferred aspect, the dual-specific ligands of the invention possess a single known main-chain conformation.

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

In designing dual specific ligands or libraries thereof the incidence of the different main-chain conformations for each of the six antigen binding loops may be considered separately. For H1, H2, L1, L2 and L3, a given conformation that is adopted by between 20% and 100% of the antigen binding loops of naturally occurring molecules is chosen.

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

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

canonical structures is determined and then combined with the most popular conformation for the H3 loop, as a basis for choosing the single main-chain conformation.

ii. Diversification of the canonical sequence

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

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

20

Various methods have been reported for introducing such diversity. Error-prone PCR (Hawkins *et al.* (1992) *J. Mol. Biol.*, 226: 889), chemical mutagenesis (Deng *et al.* (1994) *J. Biol. Chem.*, **269**: 9533) or bacterial mutator strains (Low *et al.* (1996) *J. Mol. Biol.*, **260**: 359) can be used to introduce random mutations into the genes that encode the
25 molecule. Methods for mutating selected positions are also well known in the art and include the use of mismatched oligonucleotides or degenerate oligonucleotides, with or without the use of PCR. For example, several synthetic antibody libraries have been created by targeting mutations to the antigen binding loops. The H3 region of a human tetanus toxoid-binding Fab has been randomised to create a range of new binding
30 specificities (Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, **89**: 4457). Random or semi-random H3 and L3 regions have been appended to germline V gene segments to produce large libraries with unmutated framework regions (Hoogenboom & Winter (1992) *J. Mol. Biol.*, **227**: 381; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, **89**: 4457;

Nissim *et al.* (1994) *EMBO J.*, **13**: 692; Griffiths *et al.* (1994) *EMBO J.*, **13**: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, **248**: 97). Such diversification has been extended to include some or all of the other antigen binding loops (Crameri *et al.* (1996) *Nature Med.*, **2**: 100; Riechmann *et al.* (1995) *Bio/Technology*, **13**: 475; Morphosys, WO97/08320, supra).

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

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

Diversification of the canonical sequence as it applies to antibody domains

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

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

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

The invention provides two different naive repertoires of binding domains for the construction of dual specific ligands, or a naïve library of dual specific ligands, in which some or all of the residues in the antigen binding site are varied. The "primary" library mimics the natural primary repertoire, with diversity restricted to residues at the centre of the antigen binding site that are diverse in the germline V gene segments (germline diversity) or diversified during the recombination process (junctional diversity). Those residues which are diversified include, but are not limited to, H50, H52, H52a, H53, H55,

H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96. In the "somatic" library, diversity is restricted to residues that are diversified during the recombination process (junctional diversity) or are highly somatically mutated). Those residues which are diversified include, but are not limited to: H31, H33, H35, H95, H96, H97, H98, L30, L31, L32, L34 and L96. All the residues listed above as suitable for diversification in these libraries are known to make contacts in one or more antibody-antigen complexes. Since in both libraries, not all of the residues in the antigen binding site are varied, additional diversity is incorporated during selection by varying the remaining residues, if it is desired to do so. It shall be apparent to one skilled in the art that any subset of any of these residues (or additional residues which comprise the antigen binding site) can be used for the initial and/or subsequent diversification of the antigen binding site.

In the construction of libraries for use in the invention, diversification of chosen positions is typically achieved at the nucleic acid level, by altering the coding sequence which specifies the sequence of the polypeptide such that a number of possible amino acids (all 20 or a subset thereof) can be incorporated at that position. Using the IUPAC nomenclature, the most versatile codon is NNK, which encodes all amino acids as well as the TAG stop codon. The NNK codon is preferably used in order to introduce the required diversity. Other codons which achieve the same ends are also of use, including the NNN codon, which leads to the production of the additional stop codons TGA and TAA.

A feature of side-chain diversity in the antigen binding site of human antibodies is a pronounced bias which favours certain amino acid residues. If the amino acid composition of the ten most diverse positions in each of the V_H , V_K and V_L regions are summed, more than 76% of the side-chain diversity comes from only seven different residues, these being, serine (24%), tyrosine (14%), asparagine (11%), glycine (9%), alanine (7%), aspartate (6%) and threonine (6%). This bias towards hydrophilic residues and small residues which can provide main-chain flexibility probably reflects the evolution of surfaces which are predisposed to binding a wide range of antigens or epitopes and may help to explain the required promiscuity of antibodies in the primary repertoire.

Since it is preferable to mimic this distribution of amino acids, the distribution of amino acids at the positions to be varied preferably mimics that seen in the antigen binding site of antibodies. Such bias in the substitution of amino acids that permits selection of certain polypeptides (not just antibody polypeptides) against a range of target antigens is easily applied to any polypeptide repertoire. There are various methods for biasing the amino acid distribution at the position to be varied (including the use of tri-nucleotide mutagenesis, see WO97/08320), of which the preferred method, due to ease of synthesis, is the use of conventional degenerate codons. By comparing the amino acid profile encoded by all combinations of degenerate codons (with single, double, triple and quadruple degeneracy in equal ratios at each position) with the natural amino acid use it is possible to calculate the most representative codon. The codons (AGT)(AGC)T, (AGT)(AGC)C and (AGT)(AGC)(CT) - that is, DVT, DVC and DVY, respectively using IUPAC nomenclature - are those closest to the desired amino acid profile: they encode 22% serine and 11% tyrosine, asparagine, glycine, alanine, aspartate, threonine and cysteine. Preferably, therefore, libraries are constructed using either the DVT, DVC or DVY codon at each of the diversified positions.

G: Antigens capable of increasing ligand half-life

20

The dual specific ligands according to the invention, in one configuration thereof, are capable of binding to one or more molecules which can increase the half-life of the ligand *in vivo*. Typically, such molecules are polypeptides which occur naturally *in vivo* and which resist degradation or removal by endogenous mechanisms which remove unwanted material from the organism. For example, the molecule which increases the half-life of the organism may be selected from the following:

25

Proteins from the extracellular matrix; for example collagen, laminins, integrins and fibronectin. Collagens are the major proteins of the extracellular matrix. About 15 types of collagen molecules are currently known, found in different parts of the body, eg type I collagen (accounting for 90% of body collagen) found in bone, skin, tendon, ligaments, cornea, internal organs or type II collagen found in cartilage, intervertebral disc, notochord, vitreous humour of the eye.

Proteins found in blood, including:

10

Plasma proteins such as fibrin, α -2 macroglobulin, serum albumin, fibrinogen A, fibrinogen B, serum amyloid protein A, heptaglobin, profilin, ubiquitin, uteroglobulin and β -2-microglobulin;

15 Enzymes and inhibitors such as plasminogen, lysozyme, cystatin C, alpha-1-antitrypsin and pancreatic trypsin inhibitor. Plasminogen is the inactive precursor of the trypsin-like serine protease plasmin. It is normally found circulating through the blood stream. When plasminogen becomes activated and is converted to plasmin, it unfolds a potent enzymatic domain that dissolves the fibrinogen fibers that entangle the blood cells in a blood clot.
20 This is called fibrinolysis.

Immune system proteins, such as IgE, IgG, IgM.

Transport proteins such as retinol binding protein, α -1 microglobulin.

25

Defensins such as beta-defensin 1, Neutrophil defensins 1,2 and 3.

Proteins found at the blood brain barrier or in neural tissues, such as melanocortin receptor, myelin, ascorbate transporter.

30

Transferrin receptor specific ligand-neuropharmaceutical agent fusion proteins (see US5977307);

brain capillary endothelial cell receptor, transferrin, transferrin receptor, insulin, insulin-like growth factor 1 (IGF 1) receptor, insulin-like growth factor 2 (IGF 2) receptor, insulin receptor.

- 5 Proteins localised to the kidney, such as polycystin, type IV collagen, organic anion transporter K1, Heymann's antigen.

Proteins localised to the liver, for example alcohol dehydrogenase, G250.

- 10 Blood coagulation factor X
 α 1 antitrypsin
HNF 1 α

Proteins localised to the lung, such as secretory component (binds IgA).

15

Proteins localised to the Heart, for example HSP 27. This is associated with dilated cardiomyopathy.

Proteins localised to the skin, for example keratin.

20

Bone specific proteins, such as bone morphogenic proteins (BMPs), which are a subset of the transforming growth factor β superfamily that demonstrate osteogenic activity. Examples include BMP-2, -4, -5, -6, -7 (also referred to as osteogenic protein (OP-1) and -8 (OP-2).

25

Tumour specific proteins, including human trophoblast antigen, herceptin receptor, oestrogen receptor, cathepsins eg cathepsin B (found in liver and spleen).

- 30 Disease-specific proteins, such as antigens expressed only on activated T-cells: including LAG-3 (lymphocyte activation gene), osteoprotegerin ligand (OPGL) see *Nature* **402**, 304–309; 1999, OX40 (a member of the TNF receptor family, expressed on activated T cells and the only costimulatory T cell molecule known to be specifically up-regulated in human T cell leukaemia virus type-I (HTLV-I)-producing cells.) See *J Immunol.* 2000

Jul 1;165(1):263-70; Metalloproteases (associated with arthritis/cancers), including CG6512 *Drosophila*, human paraplegin, human FtsH, human AFG3L2, murine ftsH; angiogenic growth factors, including acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), Vascular endothelial growth factor / vascular permeability factor (VEGF/VPF), transforming growth factor- α (TGF α), tumor necrosis factor- α (TNF- α), angiogenin, interleukin-3 (IL-3), interleukin-8 (IL-8), platelet-derived endothelial growth factor (PD-ECGF), placental growth factor (PlGF), midkine platelet-derived growth factor-BB (PDGF), fractalkine.

10 Stress proteins (heat shock proteins)

HSPs are normally found intracellularly. When they are found extracellularly, it is an indicator that a cell has died and spilled out its contents. This unprogrammed cell death (necrosis) only occurs when as a result of trauma, disease or injury and therefore *in vivo*, extracellular HSPs trigger a response from the immune system that will fight infection and disease. A dual specific which binds to extracellular HSP can be localised to a disease site.

Proteins involved in Fc transport

Brambell receptor (also known as FcRB)

20 This Fc receptor has two functions, both of which are potentially useful for delivery
The functions are

- (1) The transport of IgG from mother to child across the placenta
- (2) the protection of IgG from degradation thereby prolonging its serum half life of IgG. It is thought that the receptor recycles IgG from endosome.

25

See Holliger *et al*, Nat Biotechnol 1997 Jul;15(7):632-6.

Ligands according to the invention may designed to be specific for the above targets without requiring any increase in or increasing half life *in vivo*. For example, ligands according to the invention can be specific for targets selected from the foregoing which are tissue-specific, thereby enabling tissue-specific targeting of the dual specific ligand, or a dAb monomer that binds a tissue-specific therapeutically relevant target, irrespective of any increase in half-life, although this may result. Moreover, where the ligand or dAb

monomer targets kidney or liver, this may redirect the ligand or dAb monomer to an alternative clearance pathway *in vivo* (for example, the ligand may be directed away from liver clearance to kidney clearance).

5 H: Use of multispecific ligands according to the second configuration of the invention

Multispecific ligands according to the method of the second configuration of the present invention may be employed in *in vivo* therapeutic and prophylactic applications, *in vitro*
10 and *in vivo* diagnostic applications, *in vitro* assay and reagent applications, and the like. For example antibody molecules may be used in antibody based assay techniques, such as ELISA techniques, according to methods known to those skilled in the art.

As alluded to above, the multispecific ligands according to the invention are of use in
15 diagnostic, prophylactic and therapeutic procedures. Multispecific antibodies according to the invention are of use diagnostically in Western analysis and *in situ* protein detection by standard immunohistochemical procedures; for use in these applications, the ligands may be labelled in accordance with techniques known to the art. In addition, such antibody polypeptides may be used preparatively in affinity chromatography procedures, when
20 complexed to a chromatographic support, such as a resin. All such techniques are well known to one of skill in the art.

Diagnostic uses of the closed conformation multispecific ligands according to the invention include homogenous assays for analytes which exploit the ability of closed
25 conformation multispecific ligands to bind two targets in competition, such that two targets cannot bind simultaneously (a closed conformation), or alternatively their ability to bind two targets simultaneously (an open conformation).

A true homogenous immunoassay format has been avidly sought by manufacturers of
30 diagnostics and research assay systems used in drug discovery and development. The main diagnostics markets include human testing in hospitals, doctor's offices and clinics, commercial reference laboratories, blood banks, and the home, non-human diagnostics (for example food testing, water testing, environmental testing, bio-defence, and

veterinary testing), and finally research (including drug development; basic research and academic research).

At present all these markets utilise immunoassay systems that are built around
5 chemiluminescent, ELISA, fluorescence or in rare cases radio-immunoassay
technologies. Each of these assay formats requires a separation step (separating bound
from un-bound reagents). In some cases, several separation steps are required. Adding
these additional steps adds reagents and automation, takes time, and affects the ultimate
outcome of the assays. In human diagnostics, the separation step may be automated,
10 which masks the problem, but does not remove it. The robotics, additional reagents,
additional incubation times, and the like add considerable cost and complexity. In drug
development, such as high throughput screening, where literally millions of samples are
tested at once, with very low levels of test molecule, adding additional separation steps
can eliminate the ability to perform a screen. However, avoiding the separation creates
15 too much noise in the read out. Thus, there is a need for a true homogenous format that
provides sensitivities at the range obtainable from present assay formats. Advantageously,
an assay possesses fully quantitative read-outs with high sensitivity and a large dynamic
range. Sensitivity is an important requirement, as is reducing the amount of sample
required. Both of these features are features that a homogenous system offers. This is
20 very important in point of care testing, and in drug development where samples are
precious. Heterogenous systems, as currently available in the art, require large quantities
of sample and expensive reagents

Applications for homogenous assays include cancer testing, where the biggest assay is
25 that for Prostate Specific Antigen, used in screening men for prostate cancer. Other
applications include fertility testing, which provides a series of tests for women
attempting to conceive including beta-hcg for pregnancy. Tests for infectious diseases,
including hepatitis, HIV, rubella, and other viruses and microorganisms and sexually
transmitted diseases. Tests are used by blood banks, especially tests for HIV, hepatitis A,
30 B, C, non A non B. Therapeutic drug monitoring tests include monitoring levels of
prescribed drugs in patients for efficacy and to avoid toxicity, for example digoxin for
arrhythmia, and phenobarbital levels in psychotic cases; theophylline for asthma.
Diagnostic tests are moreover useful in abused drug testing, such as testing for cocaine,

marijuana and the like. Metabolic tests are used for measuring thyroid function, anaemia and other physiological disorders and functions.

5 The homogenous immunoassay format is moreover useful in the manufacture of standard clinical chemistry assays. The inclusion of immunoassays and chemistry assays on the same instrument is highly advantageous in diagnostic testing. Suitable chemical assays include tests for glucose, cholesterol, potassium, and the like.

10 A further major application for homogenous immunoassays is drug discovery and development: high throughput screening includes testing combinatorial chemistry libraries versus targets in ultra high volume. Signal is detected, and positive groups then split into smaller groups, and eventually tested in cells and then animals. Homogenous assays may be used in all these types of test. In drug development, especially animal studies and clinical trials heavy use of immunoassays is made. Homogenous assays
15 greatly accelerate and simplify these procedures. Other Applications include food and beverage testing: testing meat and other foods for E. coli, salmonella, etc; water testing, including testing at water plants for all types of contaminants including E. coli; and veterinary testing.

20 In a broad embodiment, the invention provides a binding assay comprising a detectable agent which is bound to a closed conformation multispecific ligand according to the invention, and whose detectable properties are altered by the binding of an analyte to said closed conformation multispecific ligand. Such an assay may be configured in several different ways, each exploiting the above properties of closed conformation multispecific
25 ligands.

The assay relies on the direct or indirect displacement of an agent by the analyte, resulting in a change in the detectable properties of the agent. For example, where the agent is an enzyme which is capable of catalysing a reaction which has a detectable end-point, said
30 enzyme can be bound by the ligand such as to obstruct its active site, thereby inactivating the enzyme. The analyte, which is also bound by the closed conformation multispecific ligand, displaces the enzyme, rendering it active through freeing of the active site. The enzyme is then able to react with a substrate, to give rise to a detectable event. In an

alternative embodiment, the ligand may bind the enzyme outside of the active site, influencing the conformation of the enzyme and thus altering its activity. For example, the structure of the active site may be constrained by the binding of the ligand, or the binding of cofactors necessary for activity may be prevented.

5

The physical implementation of the assay may take any form known in the art. For example, the closed conformation multispecific ligand/enzyme complex may be provided on a test strip; the substrate may be provided in a different region of the test strip, and a solvent containing the analyte allowed to migrate through the ligand/enzyme complex, displacing the enzyme, and carrying it to the substrate region to produce a signal. Alternatively, the ligand/enzyme complex may be provided on a test stick or other solid phase, and dipped into an analyte/substrate solution, releasing enzyme into the solution in response to the presence of analyte.

10

15 Since each molecule of analyte potentially releases one enzyme molecule, the assay is quantitative, with the strength of the signal generated in a given time being dependent on the concentration of analyte in the solution.

Further configurations using the analyte in a closed conformation are possible. For example, the closed conformation multispecific ligand may be configured to bind an enzyme in an allosteric site, thereby activating the enzyme. In such an embodiment, the enzyme is active in the absence of analyte. Addition of the analyte displaces the enzyme and removes allosteric activation, thus inactivating the enzyme.

20

25 In the context of the above embodiments which employ enzyme activity as a measure of the analyte concentration, activation or inactivation of the enzyme refers to an increase or decrease in the activity of the enzyme, measured as the ability of the enzyme to catalyse a signal-generating reaction. For example, the enzyme may catalyse the conversion of an undetectable substrate to a detectable form thereof. For example, horseradish peroxidase is widely used in the art together with chromogenic or chemiluminescent substrates, which are available commercially. The level of increase or decrease of the activity of the enzyme may be between 10% and 100%, such as 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%; in the case of an increase in activity, the increase may be more than 100%, i.e.

30

200%, 300%, 500% or more, or may not be measurable as a percentage if the baseline activity of the inhibited enzyme is undetectable.

In a further configuration, the closed conformation multispecific ligand may bind the substrate of an enzyme/substrate pair, rather than the enzyme. The substrate is therefore unavailable to the enzyme until released from the closed conformation multispecific ligand through binding of the analyte. The implementations for this configuration are as for the configurations which bind enzyme.

Moreover, the assay may be configured to bind a fluorescent molecule, such as a fluorescein or another fluorophore, in a conformation such that the fluorescence is quenched on binding to the ligand. In this case, binding of the analyte to the ligand will displace the fluorescent molecule, thus producing a signal. Alternatives to fluorescent molecules which are useful in the present invention include luminescent agents, such as luciferin/luciferase, and chromogenic agents, including agents commonly used in immunoassays such as HRP.

Therapeutic and prophylactic uses of multispecific ligands prepared according to the invention involve the administration of ligands according to the invention to a recipient mammal, such as a human. Multi-specificity can allow antibodies to bind to multimeric antigen with great avidity. Multispecific ligands can allow the cross-linking of two antigens, for example in recruiting cytotoxic T-cells to mediate the killing of tumour cell lines.

Substantially pure ligands or binding proteins thereof, for example dAb monomers, of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the ligands may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like (Lefkovite and Pernis, (1979 and 1981) Immunological Methods, Volumes I and II, Academic Press, NY).

The ligands or binding proteins thereof, for example dAb monomers, of the present invention will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection, and autoimmune disorders (which include, but are not limited to, Type I diabetes, asthma, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and myasthenia gravis).

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the antibodies or binding proteins thereof in protecting against or treating the disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight *et al.* (1978) *J. Exp. Med.*, **147**: 1653; Reinersten *et al.* (1978) *New Eng. J. Med.*, **299**: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom *et al.* (1988) *Adv. Immunol.*, **42**: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart *et al.* (1984) *Ann. Rev. Immunol.*, **42**: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden *et al.* (1988) *Nature*, **331**: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron *et al.* (1980) *J. Exp. Med.*, **152**: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa *et al.* (1984) *Diabetologia*, **27**: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer *et al.*, eds., Grune and Stratton, New York, pp. 179-213; McFarlin *et al.* (1973) *Science*, **179**: 478; and Satoh *et al.* (1987) *J. Immunol.*, **138**: 179).

Generally, the present ligands will be utilised in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

The ligands of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the ligands of the present invention, or even combinations of ligands according to the present invention having different specificities, such as ligands selected using different target antigens or epitopes, whether or not they are pooled prior to administration.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the selected ligands thereof of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, *via* the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The ligands of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that
5 lyophilisation and reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted upward to compensate.

The compositions containing the present ligands or a cocktail thereof can be administered
10 for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system,
15 but generally range from 0.005 to 5.0 mg of ligand, e.g. antibody, receptor (e.g. a T-cell receptor) or binding protein thereof *per* kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

20

Treatment performed using the compositions described herein is considered "effective" if one or more symptoms is reduced (e.g., by at least 10% or at least one point on a clinical assessment scale), relative to such symptoms present before treatment, or relative to such symptoms in an individual (human or model animal) not treated with such composition.
25 Symptoms will obviously vary depending upon the disease or disorder targeted, but can be measured by an ordinarily skilled clinician or technician. Such symptoms can be measured, for example, by monitoring the level of one or more biochemical indicators of the disease or disorder (e.g., levels of an enzyme or metabolite correlated with the disease, affected cell numbers, etc.), by monitoring physical manifestations (e.g.,
30 inflammation, tumor size, etc.), or by an accepted clinical assessment scale, for example, the Expanded Disability Status Scale (for multiple sclerosis), the Irvine Inflammatory Bowel Disease Questionnaire (32 point assessment evaluates quality of life with respect to bowel function, systemic symptoms, social function and emotional status - score ranges

from 32 to 224, with higher scores indicating a better quality of life), the Quality of Life Rheumatoid Arthritis Scale, or other accepted clinical assessment scale as known in the field. A sustained (e.g., one day or more, preferably longer) reduction in disease or disorder symptoms by at least 10% or by one or more points on a given clinical scale is indicative of "effective" treatment. Similarly, prophylaxis performed using a composition as described herein is "effective" if the onset or severity of one or more symptoms is delayed, reduced or abolished relative to such symptoms in a similar individual (human or animal model) not treated with the composition.

10 A composition containing a ligand or cocktail thereof according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined
15 extracorporeally with the ligands, e.g. antibodies, cell-surface receptors or binding proteins thereof whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

20

I: Use of half-life enhanced dual-specific ligands according to the invention

Dual-specific ligands according to the method of the present invention may be employed in *in vivo* therapeutic and prophylactic applications, *in vivo* diagnostic applications and
25 the like.

Therapeutic and prophylactic uses of dual-specific ligands prepared according to the invention involve the administration of ligands according to the invention to a recipient mammal, such as a human. Dual specific antibodies according to the invention comprise
30 at least one specificity for a half-life enhancing molecule; one or more further specificities may be directed against target molecules. For example, a dual-specific IgG may be specific for four epitopes, one of which is on a half-life enhancing molecule. Dual-specificity can allow antibodies to bind to multimeric antigen with great avidity. Dual-

specific antibodies can allow the cross-linking of two antigens, for example in recruiting cytotoxic T-cells to mediate the killing of tumour cell lines.

Substantially pure ligands or binding proteins thereof, such as dAb monomers, of at least
5 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the ligands may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like
10 (Lefkovite and Pernis, (1979 and 1981) Immunological Methods, Volumes I and II, Academic Press, NY).

The ligands of the present invention will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection,
15 and autoimmune disorders (which include, but are not limited to, Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and myasthenia gravis).

In the instant application, the term "prevention" involves administration of the protective
20 composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

25 Animal model systems which can be used to screen the effectiveness of the dual specific ligands in protecting against or treating the disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight *et al.* (1978) *J. Exp. Med.*, **147**: 1653; Reinersten *et al.* (1978) *New Eng. J. Med.*, **299**: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease
30 with soluble AchR protein from another species (Lindstrom *et al.* (1988) *Adv. Immunol.*, **42**: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart *et al.* (1984) *Ann. Rev. Immunol.*, **42**: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has

been described (Van Eden *et al.* (1988) *Nature*, **331**: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron *et al.* (1980) *J. Exp. Med.*, **152**: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa *et al.* (1984) *Diabetologia*,
5 **27**: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer *et al.*, eds., Grune and Stratton, New York, pp. 179-213; McFarlin *et al.* (1973) *Science*, **179**: 478: and Satoh *et al.* (1987) *J. Immunol.*, **138**: 179).

10

Dual specific ligands according to the invention and dAb monomers able to bind to extracellular targets involved in endocytosis (e.g. Clathrin) enable dual specific ligands to be endocytosed, enabling another specificity able to bind to an intracellular target to be delivered to an intracellular environment. This strategy requires a dual specific ligand
15 with physical properties that enable it to remain functional inside the cell. Alternatively, if the final destination intracellular compartment is oxidising, a well folding ligand may not need to be disulphide free.

Generally, the present dual specific ligands will be utilised in purified form together with
20 pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from
25 thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack
30 (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

The ligands of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs,

such as cyclosporine, methotrexate, adriamycin or cisplatin, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the ligands of the present invention.

5 The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the ligands of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly,
10 intraperitoneally, transdermally, *via* the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

15 The ligands of the invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional
20 immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted upward to compensate.

The compositions containing the present ligands or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an
25 adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of ligand *per* kilogram of body weight, with
30 doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing a ligand according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal.

5 In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the ligands, e.g. antibodies, cell- surface receptors or binding proteins thereof whereby the undesired cells are killed or otherwise
10 removed from the blood for return to the mammal in accordance with standard techniques.

The invention is further described, for the purposes of illustration only, in the following examples. As used herein, for the purposes of dAb nomenclature, human TNF α is
15 referred to as TAR1 and human TNF α receptor 1 (p55 receptor) is referred to as TAR2.

Example 1. Selection of a dual specific scFv antibody (K8) directed against human serum albumin (HSA) and β -galactosidase (β -gal)

20

This example explains a method for making a dual specific antibody directed against β -gal and HSA in which a repertoire of V $_{\kappa}$ variable domains linked to a germline (dummy) V $_H$ domain is selected for binding to β -gal and a repertoire of V $_H$ variable domains linked to a germline (dummy) V $_{\kappa}$ domain is selected for binding to HSA. The selected variable
25 V $_H$ HSA and V $_{\kappa}$ β -gal domains are then combined and the antibodies selected for binding to β -gal and HSA. HSA is a half-life increasing protein found in human blood.

Four human phage antibody libraries were used in this experiment.

30	Library 1	Germline V $_{\kappa}$ /DVT V $_H$	8.46×10^7
	Library 2	Germline V $_{\kappa}$ /NNK V $_H$	9.64×10^7
	Library 3	Germline V $_H$ /DVT V $_{\kappa}$	1.47×10^8

Library 4 Germline V_H/NNK V_K 1.45 x 10⁸

All libraries are based on a single human framework for V_H (V3-23/DP47 and J_H4b) and V_K (O12/O2/DPK9 and J_K1) with side chain diversity incorporated in complementarity
5 determining regions (CDR2 and CDR3).

Library 1 and Library 2 contain a dummy V_K sequence, whereas the sequence of V_H is diversified at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97 and H98 (DVT or NNK encoded, respectively) (Figure 1). Library 3 and Library 4 contain a
10 dummy V_H sequence, whereas the sequence of V_K is diversified at positions L50, L53, L91, L92, L93, L94 and L96 (DVT or NNK encoded, respectively) (Figure 1). The libraries are in phagemid pIT2/ScFv format (Figure 2) and have been preselected for binding to generic ligands, Protein A and Protein L, so that the majority of clones in the unselected libraries are functional. The sizes of the libraries shown above correspond to
15 the sizes after preselection. Library 1 and Library 2 were mixed prior to selections on antigen to yield a single V_H/dummy V_K library and Library 3 and Library 4 were mixed to form a single V_K/dummy V_H library.

Three rounds of selections were performed on β-gal using V_K/dummy V_H library and
20 three rounds of selections were performed on HSA using V_H/dummy V_K library. In the case of β-gal the phage titres went up from 1.1 x 10⁶ in the first round to 2.0 x 10⁸ in the third round. In the case of HSA the phage titres went up from 2 x 10⁴ in the first round to 1.4 x 10⁹ in the third round. The selections were performed as described by Griffith *et al.*, (1993), except that KM13 helper phage (which contains a pIII protein with a protease
25 cleavage site between the D2 and D3 domains) was used and phage were eluted with 1 mg/ml trypsin in PBS. The addition of trypsin cleaves the pIII proteins derived from the helper phage (but not those from the phagemid) and elutes bound scFv-phage fusions by cleavage in the c-myc tag (Figure 2), thereby providing a further enrichment for phages expressing functional scFvs and a corresponding reduction in background (Kristensen &
30 Winter, *Folding & Design* 3: 321-328, Jul 9, 1998). Selections were performed using immunotubes coated with either HSA or β-gal at 100μg/ml concentration.

To check for binding, 24 colonies from the third round of each selection were screened by monoclonal phage ELISA. Phage particles were produced as described by Harrison *et al.*, Methods Enzymol. 1996;267:83-109. 96-well ELISA plates were coated with 100µl of
5 HSA or β-gal at 10µg/ml concentration in PBS overnight at 4°C. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. A selection of clones gave ELISA signals of greater than 1.0 with 50µl supernatant.

10 Next, DNA preps were made from V_H/dummy V_K library selected on HSA and from V_K/dummy V_H library selected on β-gal using the QIAprep Spin Miniprep kit (Qiagen). To access most of the diversity, DNA preps were made from each of the three rounds of selections and then pulled together for each of the antigens. DNA preps were then digested with SalI/NotI overnight at 37°C. Following gel purification of the fragments,
15 V_K chains from the V_K/dummy V_H library selected on β-gal were ligated in place of a dummy V_K chain of the V_H/dummy V_K library selected on HSA creating a library of 3.3 x 10⁹ clones.

This library was then either selected on HSA (first round) and β-gal (second round),
20 HSA/β-gal selection, or on β-gal (first round) and HSA (second round), β-gal/HSA selection. Selections were performed as described above. In each case after the second round 48 clones were tested for binding to HSA and β-gal by the monoclonal phage ELISA (as described above) and by ELISA of the soluble scFv fragments. Soluble antibody fragments were produced as described by Harrison *et al.*, (1996), and standard
25 ELISA protocol was followed Hoogenboom *et al.* (1991) Nucleic Acids Res., 19: 4133, except that 2% Tween/PBS was used as a blocking buffer and bound scFvs were detected with Protein L-HRP. Three clones (E4, E5 and E8) from the HSA/β-gal selection and two clones (K8 and K10) from the β-gal/HSA selection were able to bind both antigens. scFvs from these clones were PCR amplified and sequenced as described by Ignatovich *et al.*,
30 (1999) J Mol Biol 1999 Nov 26;294(2):457-65, using the primers LMB3 and pHENseq. Sequence analysis revealed that all clones were identical. Therefore, only one clone encoding a dual specific antibody (K8) was chosen for further work (Figure 3).

Example 2. Characterisation of the binding properties of the K8 antibody.

5 Firstly, the binding properties of the K8 antibody were characterised by the monoclonal phage ELISA. A 96-well plate was coated with 100µl of HSA and β-gal alongside with alkaline phosphatase (APS), bovine serum albumin (BSA), peanut agglutinin, lysozyme and cytochrome c (to check for cross-reactivity) at 10µg/ml concentration in PBS overnight at 4°C. The phagemid from K8 clone was rescued with KM13 as described by
10 Harrison *et al.*, (1996) and the supernatant (50µl) containing phage assayed directly. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. The dual specific K8 antibody was found to bind to HSA and β-gal when displayed on the surface of the phage with absorbance signals greater than 1.0 (Figure 4). Strong binding to BSA was also observed (Figure 4).
15 Since HSA and BSA are 76% homologous on the amino acid level, it is not surprising that K8 antibody recognised both of these structurally related proteins. No cross-reactivity with other proteins was detected (Figure 4).

Secondly, the binding properties of the K8 antibody were tested in a soluble scFv ELISA.
20 Production of the soluble scFv fragment was induced by IPTG as described by Harrison *et al.*, (1996). To determine the expression levels of K8 scFv, the soluble antibody fragments were purified from the supernatant of 50ml inductions using Protein A-Sepharose columns as described by Harlow and Lane, *Antibodies: a Laboratory Manual*, (1988) Cold Spring Harbor. OD₂₈₀ was then measured and the protein concentration
25 calculated as described by Sambrook *et al.*, (1989). K8 scFv was produced in supernatant at 19mg/l.

A soluble scFv ELISA was then performed using known concentrations of the K8 antibody fragment. A 96-well plate was coated with 100µl of HSA, BSA and β-gal at
30 10µg/ml and 100µl of Protein A at 1µg/ml concentration. 50µl of the serial dilutions of the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirmed the dual specific nature of the K8 antibody (Figure 5).

To confirm that binding to β -gal is determined by the V_K domain and binding to HSA/BSA by the V_H domain of the K8 scFv antibody, the V_K domain was cut out from K8 scFv DNA by *SalI/NotI* digestion and ligated into a *SalI/NotI* digested pIT2 vector containing dummy V_H chain (Figures 1 and 2). Binding characteristics of the resulting clone K8 V_K /dummy V_H were analysed by soluble scFv ELISA. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50 μ) containing scFvs assayed directly. Soluble scFv ELISA was performed as described in Example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind β -gal, whereas binding to BSA was abolished (Figure 6).

Example 3. Selection of single V_H domain antibodies against antigens A and B and single V_K domain antibodies directed against antigens C and D.

This example describes a method for making single V_H domain antibodies directed against antigens A and B and single V_K domain antibodies directed against antigens C and D by selecting repertoires of virgin single antibody variable domains for binding to these antigens in the absence of the complementary variable domains.

Selections and characterisation of the binding clones is performed as described previously (see Example 5, PCT/GB 02/003014). Four clones are chosen for further work:

VH1 – Anti A V_H

VH2 – Anti B V_H

VK1 – Anti C V_K

VK2 – Anti D V_K

The procedures described above in Examples 1-3 may be used, in a similar manner as that described, to produce dimer molecules comprising combinations of V_H domains (i.e., V_H - V_H ligands) and combinations of V_L domains (V_L - V_L ligands).

Example 4. Creation and characterisation of the dual specific ScFv antibodies (VH1/VH2 directed against antigens A and B and VK1/VK2 directed against antigens C and D).

5 This example demonstrates that dual specific ScFv antibodies (VH1/VH2 directed against antigens A and B and VK1/VK2 directed against antigens C and D) could be created by combining V_K and V_H single domains selected against respective antigens in a ScFv vector.

10 To create dual specific antibody VH1/VH2, VH1 single domain is excised from variable domain vector 1 (Figure 7) by *NcoI/XhoI* digestion and ligated into *NcoI/XhoI* digested variable domain vector 2 (Figure 7) to create VH1/ variable domain vector 2. VH2 single domain is PCR amplified from variable domain vector 1 using primers to introduce *SalI* restriction site to the 5' end and *NotI* restriction site to the 3' end. The PCR product is
15 then digested with *SalI/NotI* and ligated into *SalI/NotI* digested VH1/ variable domain vector 2 to create VH1/VH2/ variable domain vector 2.

VK1/VK2/ variable domain vector 2 is created in a similar way. The dual specific nature of the produced VH1/VH2 ScFv and VK1/VK2 ScFv is tested in a soluble ScFv ELISA
20 as described previously (see Example 6, PCT/GB 02/003014). Competition ELISA is performed as described previously (see Example 8, PCT/GB 02/003014).

Possible outcomes:

- VH1/VH2 ScFv is able to bind antigens A and B simultaneously
- 25 -VK1/VK2 ScFv is able to bind antigens C and D simultaneously
- VH1/VH2 ScFv binding is competitive (when bound to antigen A, VH1/VH2 ScFv cannot bind to antigen B)
- VK1/VK2 ScFv binding is competitive (when bound to antigen C, VK1/VK2 ScFv cannot bind to antigen D)

30

Example 5. Construction of dual specific VH1/VH2 Fab and VK1/VK2 Fab and analysis of their binding properties.

To create VH1/VH2 Fab, VH1 single domain is ligated into *NcoI/XhoI* digested CH vector (Figure 8) to create VH1/CH and VH2 single domain is ligated into *SalI/NotI* digested CK vector (Figure 9) to create VH2/CK. Plasmid DNA from VH1/CH and VH2/CK is used to co-transform competent *E. coli* cells as described previously (see Example 8, PCT/GB02/003014).

The clone containing VH1/CH and VH2/CK plasmids is then induced by IPTG to produce soluble VH1/VH2 Fab as described previously (see Example 8, PCT/GB 02/003014).

10

VK1/VK2 Fab is produced in a similar way.

Binding properties of the produced Fabs are tested by competition ELISA as described previously (see Example 8, PCT/GB 02/003014).

15

Possible outcomes:

- VH1/VH2 Fab is able to bind antigens A and B simultaneously
- VK1/VK2 Fab is able to bind antigens C and D simultaneously
- VH1/VH2 Fab binding is competitive (when bound to antigen A, VH1/VH2 Fab cannot bind to antigen B)
- VK1/VK2 Fab binding is competitive (when bound to antigen C, VK1/VK2 Fab cannot bind to antigen D)

20

Example 6

25

Chelating dAb Dimers

Summary

VH and VK homo-dimers are created in a dAb-linker-dAb format using flexible polypeptide linkers. Vectors were created in the dAb linker-dAb format containing glycine-serine linkers of different lengths 3U:(Gly₄Ser)₃, 5U:(Gly₄Ser)₅, 7U:(Gly₄Ser)₇. Dimer libraries were created using guiding dAbs upstream of the linker: TAR1-5 (VK), TAR1-27(VK), TAR2-5(VH) or TAR2-6(VK) and a library of corresponding second

30

dAbs after the linker. Using this method, novel dimeric dAbs were selected. The effect of dimerisation on antigen binding was determined by ELISA and BIAcore studies and in cell neutralisation and receptor binding assays. Dimerisation of both TAR1-5 and TAR1-27 resulted in significant improvement in binding affinity and neutralisation levels.

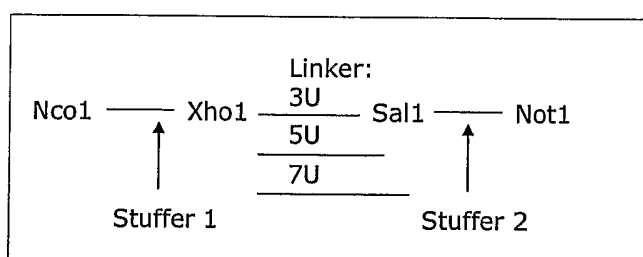
5

1.0 Methods

1.1 Library generation

1.1.1 Vectors

pEDA3U, pEDA5U and pEDA7U vectors were designed to introduce different linker lengths compatible with the dAb-linker-dAb format. For pEDA3U, sense and anti-sense 73-base pair oligo linkers were annealed using a slow annealing program (95°C-5mins, 80°C-10mins, 70°C-15mins, 56°C-15mins, 42°C until use) in buffer containing 0.1MNaCl, 10mM Tris-HCl pH7.4 and cloned using the *Xho1* and *Not1* restriction sites. The linkers encompassed 3 (Gly₄Ser) units and a stuffer region housed between *Sall* and *Not1* cloning sites (scheme 1). In order to reduce the possibility of monomeric dAbs being selected for by phage display, the stuffer region was designed to include 3 stop codons, a *Sac1* restriction site and a frame shift mutation to put the region out of frame when no second dAb was present. For pEDA5U and 7U due to the length of the linkers required, overlapping oligo-linkers were designed for each vector, annealed and elongated using Klenow. The fragment was then purified and digested using the appropriate enzymes before cloning using the *Xho1* and *Not1* restriction sites.



Scheme 1

25

1.1.2 Library preparation

The N-terminal V gene corresponding to the guiding dAb was cloned upstream of the linker using *Nco1* and *Xho1* restriction sites. VH genes have existing compatible sites, however cloning VK genes required the introduction of suitable restriction sites. This was

achieved by using modifying PCR primers (VK-DLIBF: 5' cggccatggcgtaacggacat; VKXho1R: 5' atgtgcgctcgagcggttgattt 3') in 30 cycles of PCR amplification using a 2:1 mixture of SuperTaq (HTBiotechnology Ltd) and *pfu* turbo (Stratagene). This maintained the *NcoI* site at the 5' end while destroying the adjacent *SalI* site and introduced the *XhoI* site at the 3' end. 5 guiding dAbs were cloned into each of the 3 dimer vectors: TAR1-5 (VK), TAR1-27(VK), TAR2-5(VH), TAR2-6(VK) and TAR2-7(VK). All constructs were verified by sequence analysis.

Having cloned the guiding dAbs upstream of the linker in each of the vectors (pEDA3U, 5U and 7U): TAR1-5 (VK), TAR1-27(VK), TAR2-5(VH) or TAR2-6(VK) a library of corresponding second dAbs were cloned after the linker. To achieve this, the complimentary dAb libraries were PCR amplified from phage recovered from round 1 selections of either a VK library against Human TNF α (at approximately 1×10^6 diversity after round 1) when TAR1-5 or TAR1-27 are the guiding dAbs, or a VH or VK library against human p55 TNF receptor (both at approximately 1×10^5 diversity after round 1) when TAR2-5 or TAR2-6 respectively are the guiding dAbs. For VK libraries PCR amplification was conducted using primers in 30 cycles of PCR amplification using a 2:1 mixture of SuperTaq and *pfu* turbo. VH libraries were PCR amplified using primers in order to introduce a *SalI* restriction site at the 5' end of the gene. The dAb library PCRs were digested with the appropriate restriction enzymes, ligated into the corresponding vectors down stream of the linker, using *SalI/NotI* restriction sites and electroporated into freshly prepared competent TG1 cells.

The titres achieved for each library are as follows:

TAR1-5: pEDA3U = 4×10^8 , pEDA5U = 8×10^7 , pEDA7U = 1×10^8
 TAR1-27: pEDA3U = 6.2×10^8 , pEDA5U = 1×10^8 , pEDA7U = 1×10^9
 TAR2h-5: pEDA3U = 4×10^7 , pEDA5U = 2×10^8 , pEDA7U = 8×10^7
 TAR2h-6: pEDA3U = 7.4×10^8 , pEDA5U = 1.2×10^8 , pEDA7U = 2.2×10^8

1.2 Selections

1.2.1 TNF α

Selections were conducted using human TNF α passively coated on immunotubes. Briefly, Immunotubes are coated overnight with 1-4mls of the required antigen. The

immunotubes were then washed 3 times with PBS and blocked with 2% milk powder in PBS for 1-2hrs and washed a further 3 times with PBS. The phage solution is diluted in 2% milk powder in PBS and incubated at room temperature for 2hrs. The tubes are then washed with PBS and the phage eluted with 1mg/ml trypsin-PBS. Three selection strategies were investigated for the TAR1-5 dimer libraries. The first round selections were carried out in immunotubes using human TNF α coated at 1 μ g/ml or 20 μ g/ml with 20 washes in PBS 0.1% Tween. TG1 cells are infected with the eluted phage and the titres are determined (eg, Marks et al J Mol Biol. 1991 Dec 5;222(3):581-97, Richmann et al Biochemistry. 1993 Aug 31;32(34):8848-55).

The titres recovered were:

pEDA3U = 2.8×10^7 (1 μ g/ml TNF) 1.5×10^8 (20 μ g/ml TNF),

pEDA5U = 1.8×10^7 (1 μ g/ml TNF), 1.6×10^8 (20 μ g/ml TNF)

pEDA7U = 8×10^6 (1 μ g/ml TNF), 7×10^7 (20 μ g/ml TNF).

The second round selections were carried out using 3 different methods.

1. In immunotubes, 20 washes with overnight incubation followed by a further 10 washes.
2. In immunotubes, 20 washes followed by 1hr incubation at RT in wash buffer with (1 μ g/ml TNF α) and 10 further washes.
3. Selection on streptavidin beads using 33 pmoles biotinylated human TNF α (Henderikx *et al.*, 2002, *Selection of antibodies against biotinylated antigens*. Antibody Phage Display : Methods and protocols, Ed. O'Brien and Atkin, Humana Press). Single clones from round 2 selections were picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

	Round 1 Human TNF α immuno tube coating concentration	Round 2 selection method 1	Round 2 selection method 2	Round 2 selection method 3
pEDA3U	1 μ g/ml	1×10^9	1.8×10^9	2.4×10^{10}
pEDA3U	20 μ g/ml	6×10^9	1.8×10^{10}	8.5×10^{10}
pEDA5U	1 μ g/ml	9×10^8	1.4×10^9	2.8×10^{10}
pEDA5U	20 μ g/ml	9.5×10^9	8.5×10^9	2.8×10^{10}

pEDA7U	1µg/ml	7.8×10^8	1.6×10^8	4×10^{10}
pEDA7U	20µg/ml	1×10^{10}	8×10^9	1.5×10^{10}

For TAR1-27, selections were carried out as described previously with the following modifications. The first round selections were carried out in immunotubes using human TNF α coated at 1µg/ml or 20µg/ml with 20 washes in PBS 0.1%Tween. The second
5 round selections were carried out in immunotubes using 20 washes with overnight incubation followed by a further 20 washes. Single clones from round 2 selections were picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

10 TAR1-27 titres are as follows:

	Human TNF α immunotube coating conc	Round 1	Round 2
pEDA3U	1µg/ml	4×10^9	6×10^9
pEDA3U	20µg/ml	5×10^9	4.4×10^{10}
pEDA5U	1µg/ml	1.5×10^9	1.9×10^{10}
pEDA5U	20µg/ml	3.4×10^9	3.5×10^{10}
pEDA7U	1µg/ml	2.6×10^9	5×10^9
pEDA7U	20µg/ml	7×10^9	1.4×10^{10}

1.2.2 TNF RECEPTOR 1 (p55 RECEPTOR; TAR2)

Selections were conducted as described previously for the TAR2h-5 libraries only. 3
15 rounds of selections were carried out in immunotubes using either 1µg/ml human p55 TNF receptor or 10µg/ml human p55 TNF receptor with 20 washes in PBS 0.1%Tween with overnight incubation followed by a further 20 washes. Single clones from round 2 and 3 selections were picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

20

TAR2h-5 titres are as follows:

	Round 1 human p55 TNF receptor immunotube coating concentration	Round 1	Round 2	Round 3
pEDA3U	1µg/ml	2.4×10^6	1.2×10^7	1.9×10^9
pEDA3U	10µg/ml	3.1×10^7	7×10^7	1×10^9
pEDA5U	1µg/ml	2.5×10^6	1.1×10^7	5.7×10^8
pEDA5U	10µg/ml	3.7×10^7	2.3×10^8	2.9×10^9
pEDA7U	1µg/ml	1.3×10^6	1.3×10^7	1.4×10^9
pEDA7U	10µg/ml	1.6×10^7	1.9×10^7	3×10^{10}

1.3 Screening

Single clones from round 2 or 3 selections were picked from each of the 3U, 5U and 7U libraries from the different selections methods, where appropriate. Clones were grown in 2xTY with 100µg/ml ampicillin and 1% glucose overnight at 37°C. A 1/100 dilution of this culture was inoculated into 2mls of 2xTY with 100µg/ml ampicillin and 0.1% glucose in 2ml, 96 well plate format and grown at 37°C shaking until OD600 was approximately 0.9. The culture was then induced with 1mM IPTG overnight at 30°C. The supernatants were clarified by centrifugation at 4000rpm for 15 mins in a sorval plate centrifuge. The supernatant preps the used for initial screening.

1.3.1 ELISA

Binding activity of dimeric recombinant proteins was compared to monomer by Protein A/L ELISA or by antigen ELISA. Briefly, a 96 well plate is coated with antigen or Protein A/L overnight at 4°C. The plate washed with 0.05% Tween-PBS, blocked for 2hrs with 2% Tween-PBS. The sample is added to the plate incubated for 1 hr at room temperature. The plate is washed and incubated with the secondary reagent for 1hr at room temperature. The plate is washed and developed with TMB substrate. Protein A/L-HRP or India-HRP was used as a secondary reagent. For antigen ELISAs, the antigen concentrations used were 1µg/ml in PBS for Human TNFα and human THF receptor 1. Due to the presence of the guiding dAb in most cases dimers gave a positive ELISA signal therefore off rate determination was examined by BIAcore.

1.3.2 BIAcore

BIAcore analysys was conducted for TAR1-5 and TAR2h-5 clones. For screening, Human TNF α was coupled to a CM5 chip at high density (approximately 10000 RUs). 50 μ l of Human TNF α (50 μ g/ml) was coupled to the chip at 5 μ l/min in acetate buffer - pH5.5. Regeneration of the chip following analysis using the standard methods is not possible due to the instability of Human TNF α , therefore after each sample was analysed, the chip was washed for 10mins with buffer.

For TAR1-5, clones supernatants from the round 2 selection were screened by BIAcore. 48 clones were screened from each of the 3U, 5U and 7U libraries obtained using the following selection methods:

R1: 1 μ g/ml human TNF α immunotube, R2 1 μ g/ml human TNF α immunotube, overnight wash.

R1: 20 μ g/ml human TNF α immunotube, R2 20 μ g/ml human TNF α immunotube, overnight wash.

R1: 1 μ g/ml human TNF α immunotube, R2 33 pmoles biotinylated human TNF α on beads.

R1: 20 μ g/ml human TNF α immunotube, R2 33 pmoles biotinylated human TNF α beads.

For screening, human p55 TNF receptor was coupled to a CM5 chip at high density (approximately 4000 RUs). 100 μ l of human p55 TNF receptor (10 μ g/ml) was coupled to the chip at 5 μ l/min in acetate buffer - pH5.5. Standard regeneration conditions were examined (glycine pH2 or pH3) but in each case antigen was removed from the surface of the chip therefore as with TNF α , therefore after each sample was analysed, the chip was washed for 10mins with buffer.

For TAR2-5, clones supernatants from the round 2 selection were screened. 48 clones were screened from each of the 3U, 5U and 7U libraries, using the following selection methods:

R1: 1 μ g/ml human p55 TNF receptor immunotube, R2 1 μ g/ml human p55 TNF receptor immunotube, overnight wash.

R1: 10 μ g/ml human p55 TNF receptor immunotube, R2 10 μ g/ml human p55 TNF receptor immunotube, overnight wash.

1.3.3 Receptor and Cell Assays

The ability of the dimers to neutralise in the receptor assay was conducted as follows:

Receptor binding

5 Anti-TNF dAbs were tested for the ability to inhibit the binding of TNF to recombinant TNF receptor 1 (p55). Briefly, Maxisorp plates were incubated overnight with 30mg/ml anti-human Fc mouse monoclonal antibody (Zymed, San Francisco, USA). The wells were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 and then blocked with 1% BSA in PBS before being incubated with 100ng/ml TNF receptor 1 Fc
10 fusion protein (R&D Systems, Minneapolis, USA). Anti-TNF dAb was mixed with TNF which was added to the washed wells at a final concentration of 10ng/ml. TNF binding was detected with 0.2mg/ml biotinylated anti-TNF antibody (HyCult biotechnology, Uben, Netherlands) followed by 1 in 500 dilution of horse radish peroxidase labelled streptavidin (Amersham Biosciences, UK) and then incubation with TMB substrate (KPL,
15 Gaithersburg, USA). The reaction was stopped by the addition of HCl and the absorbance was read at 450nm. Anti-TNF dAb activity lead to a decrease in TNF binding and therefore a decrease in absorbance compared with the TNF only control.

L929 Cytotoxicity Assay

20 Anti-TNF dAbs were also tested for the ability to neutralise the cytotoxic activity of TNF on mouse L929 fibroblasts (Evans, T. (2000) Molecular Biotechnology 15, 243-248). Briefly, L929 cells plated in microtitre plates were incubated overnight with anti-TNF dAb, 100pg/ml TNF and 1mg/ml actinomycin D (Sigma, Poole, UK). Cell viability was measured by reading absorbance at 490nm following an incubation with [3-(4,5-
25 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, USA). Anti-TNF dAb activity lead to a decrease in TNF cytotoxicity and therefore an increase in absorbance compared with the TNF only control.

In the initial screen, supernatants prepared for BIAcore analysis, described above, were
30 also used in the receptor assay. Further analysis of selected dimers was also conducted in the receptor and cell assays using purified proteins.

HeLa IL-8 assay

Anti-TNFR1 or anti-TNF alpha dAbs were tested for the ability to neutralise the induction of IL-8 secretion by TNF in HeLa cells (method adapted from that of Akesson, L. et al (1996) Journal of Biological Chemistry 271, 30517-30523, describing the induction of IL-8 by IL-1 in HUVEC; here we look at induction by human TNF alpha and we use HeLa cells instead of the HUVEC cell line). Briefly, HeLa cells plated in microtitre plates were incubated overnight with dAb and 300pg/ml TNF. Post incubation the supernatant was aspirated off the cells and IL-8 concentration measured via a sandwich ELISA (R&D Systems). Anti-TNFR1 dAb activity lead to a decrease in IL-8 secretion into the supernatant compared with the TNF only control.

10

The L929 assay is used throughout the following experiments; however, the use of the HeLa IL-8 assay is preferred to measure anti-TNF receptor 1 (p55) ligands; the presence of mouse p55 in the L929 assay poses certain limitations in its use.

15 **1.4 Sequence analysis**

Dimers that proved to have interesting properties in the BIAcore and the receptor assay screens were sequenced. Sequences are detailed in the sequence listing.

1.5 Formatting

20 **1.5.1 TAR1-5-19 dimers**

The TAR1-5 dimers that were shown to have good neutralisation properties were re-formatted and analysed in the cell and receptor assays. The TAR1-5 guiding dab was substituted with the affinity matured clone TAR1-5-19. To achieve this TAR1-5 was cloned out of the individual dimer pair and substituted with TAR1-5-19 that had been amplified by PCR. In addition, TAR1-5-19 homodimers were also constructed in the 3U, 5U and 7U vectors. The N terminal copy of the gene was amplified by PCR and cloned as described above and the C-terminal gene fragment was cloned using existing *Sall* and *NotI* restriction sites.

30 **1.5.2 Mutagenesis**

The amber stop codon present in dAb2, one of the C-terminal dAbs in the TAR1-5 dimer pairs was mutated to a glutamine by site-directed mutagenesis.

1.5.3 Fabs

The dimers containing TAR1-5 or TAR1-5-19 were re-formatted into Fab expression vectors. dAbs were cloned into expression vectors containing either the CK or CH genes using *SfiI* and *NotI* restriction sites and verified by sequence analysis. The CK vector is
5 derived from a pUC based ampicillin resistant vector and the CH vector is derived from a pACYC chloramphenicol resistant vector. For Fab expression the dAb-CH and dAb-CK constructs were co-transformed into HB2151 cells and grown in 2xTY containing 0.1% glucose, 100µg/ml ampicillin and 10µg/ml chloramphenicol.

10 1.5.3 Hinge dimerisation

Dimerisation of dAbs via cystine bond formation was examined. A short sequence of amino acids EPKSGDKTHTCPPCP a modified form of the human IgGC1 hinge was engineered at the C terminal region on the dAb. An oligo linker encoding for this sequence was synthesised and annealed, as described previously. The linker was cloned
15 into the pEDA vector containing TAR1-5-19 using *XhoI* and *NotI* restriction sites. Dimerisation occurs *in situ* in the periplasm.

1.6 Expression and purification

1.6.1 Expression

20 Supernatants were prepared in the 2ml, 96-well plate format for the initial screening as described previously. Following the initial screening process selected dimers were analysed further. Dimer constructs were expressed in TOP10F' or HB2151 cells as supernatants. Briefly, an individual colony from a freshly streaked plate was grown overnight at 37°C in 2xTY with 100µg/ml ampicillin and 1% glucose. A 1/100 dilution
25 of this culture was inoculated into 2xTY with 100µg/ml ampicillin and 0.1% glucose and grown at 37°C shaking until OD600 was approximately 0.9. The culture was then induced with 1mM IPTG overnight at 30°C. The cells were removed by centrifugation and the supernatant purified with protein A or L agarose.

Fab and cysteine hinge dimers were expressed as periplasmic proteins in HB2152 cells.
30 A 1/100 dilution of an overnight culture was inoculated into 2xTY with 0.1% glucose and the appropriate antibiotics and grown at 30°C shaking until OD600 was approximately 0.9. The culture was then induced with 1mM IPTG for 3-4 hours at 25°C. The cells were harvested by centrifugation and the pellet resuspended in periplasmic

preparation buffer (30mM Tris-HCl pH8.0, 1mM EDTA, 20% sucrose). Following centrifugation the supernatant was retained and the pellet resuspended in 5mM MgSO₄. The supernatant was harvested again by centrifugation, pooled and purified.

5 1.6.2 Protein A/L purification

Optimisation of the purification of dimer proteins from Protein L agarose (Affitech, Norway) or Protein A agarose (Sigma, UK) was examined. Protein was eluted by batch or by column elution using a peristaltic pump. Three buffers were examined 0.1M Phosphate-citrate buffer pH2.6, 0.2M Glycine pH2.5 and 0.1M Glycine pH2.5. The
10 optimal condition was determined to be under peristaltic pump conditions using 0.1M Glycine pH2.5 over 10 column volumes. Purification from protein A was conducted peristaltic pump conditions using 0.1M Glycine pH2.5.

1.6.3 FPLC purification

15 Further purification was carried out by FPLC analysis on the AKTA Explorer 100 system (Amersham Biosciences Ltd). TAR1-5 and TAR1-5-19 dimers were fractionated by cation exchange chromatography (1ml Resource S – Amersham Biosciences Ltd) eluted with a 0-1M NaCl gradient in 50mM acetate buffer pH4. Hinge dimers were purified by ion exchange (1ml Resource Q Amersham Biosciences Ltd) eluted with a 0-1M NaCl
20 gradient in 25mM Tris HCl pH 8.0. Fabs were purified by size exclusion chromatography using a superose 12 (Amersham Biosciences Ltd) column run at a flow rate of 0.5ml/min in PBS with 0.05% tween. Following purification samples were concentrated using vivaspin 5K cut off concentrators (Vivascience Ltd).

25 2.0 Results

2.1 TAR1-5 dimers

6 x 96 clones were picked from the round 2 selection encompassing all the libraries and selection conditions. Supernatant preps were made and assayed by antigen and Protein L ELISA, BIAcore and in the receptor assays. In ELISAs, positive binding clones were
30 identified from each selection method and were distributed between 3U, 5U and 7U libraries. However, as the guiding dAb is always present it was not possible to discriminate between high and low affinity binders by this method therefore BIAcore analysis was conducted.

BIAcore analysis was conducted using the 2ml supernatants. BIAcore analysis revealed that the dimer Koff rates were vastly improved compared to monomeric TAR1-5. Monomer Koff rate was in the range of 10^{-1} M compared with dimer Koff rates which
 5 were in the range of 10^{-3} - 10^{-4} M. 16 clones that appeared to have very slow off rates were selected, these came from the 3U, 5U and 7U libraries and were sequenced. In addition the supernatants were analysed for the ability to neutralise human TNF α in the receptor assay.

10 6 lead clones (d1-d6 below) that neutralised in these assays and have been sequenced. The results shows that out of the 6 clones obtained there are only 3 different second dAbs (dAb1, dAb2 and dAb3) however where the second dAb is found more than once they are linked with different length linkers.

- 15 **TAR1-5d1:** 3U linker 2nd dAb=dAb1 - 1 μ g/ml Ag immunotube overnight wash
- TAR1-5d2:** 3U linker 2nd dAb=dAb2 - 1 μ g/ml Ag immunotube overnight wash
- TAR1-5d3:** 5U linker 2nd dAb=dAb2 - 1 μ g/ml Ag immunotube overnight wash
- TAR1-5d4:** 5U linker 2nd dAb=dAb3 - 20 μ g/ml Ag immunotube overnight wash
- TAR1-5d5:** 5U linker 2nd dAb=dAb1 - 20 μ g/ml Ag immunotube overnight wash
- 20 **TAR1-5d6:** 7U linker 2nd dAb=dAb1- R1:1 μ g/ml Ag immunotube overnight wash, R2:beads

The 6 lead clones were examined further. Protein was produced from the periplasm and supernatant, purified with protein L agarose and examined in the cell and receptor assays.
 25 The levels of neutralisation were variable (Table 1). The optimal conditions for protein preparation were determined. Protein produced from HB2151 cells as supernatants gave the highest yield (approximately 10mgs/L of culture). The supernatants were incubated with protein L agarose for 2hrs at room temperature or overnight at 4°C. The beads were washed with PBS/NaCl and packed onto an FPLC column using a peristaltic pump. The
 30 beads were washed with 10 column volumes of PBS/NaCl and eluted with 0.1M glycine pH2.5. In general, dimeric protein is eluted after the monomer.

TAR1-5d1-6 dimers were purified by FPLC. Three species were obtained, by FPLC purification and were identified by SDS PAGE. One species corresponds to monomer and the other two species corresponds to dimers of different sizes. The larger of the two species is possibly due to the presence of C terminal tags. These proteins were examined
5 in the receptor assay. The data presented in table 1 represents the optimum results obtained from the two dimeric species (Figure 11)

The three second dAbs from the dimer pairs (ie, dAb1, dAb2 and dAb3) were cloned as monomers and examined by ELISA and in the cell and receptor assay. All three dAbs
10 bind specifically to TNF by antigen ELISA and do not cross react with plastic or BSA. As monomers, none of the dAbs neutralise in the cell or receptor assays.

2.1.2 TAR1-5-19 dimers

TAR1-5-19 was substituted for TAR1-5 in the 6 lead clones. Analysis of all TAR1-5-19
15 dimers in the cell and receptor assays was conducted using total protein (protein L purified only) unless otherwise stated (Table 2). TAR1-5-19d4 and TAR1-5-19d3 have the best ND₅₀ (~5nM) in the cell assay, this is consistent with the receptor assay results and is an improvement over TAR1-5-19 monomer (ND₅₀~30nM). Although purified TAR1-5 dimers give variable results in the receptor and cell assays TAR1-5-19 dimers
20 were more consistent. Variability was shown when using different elution buffers during the protein purification. Elution using 0.1M Phosphate-citrate buffer pH2.6 or 0.2M Glycine pH2.5 although removing all protein from the protein L agarose in most cases rendered it less functional.

TAR1-5-19d4 was expressed in the fermenter and purified on cation exchange FPLC to yield a completely pure dimer. As with TAR1-5d4 three species were obtained, by FPLC purification corresponding to monomer and two dimer species. This dimer was amino acid sequenced. TAR1-5-19 monomer and TAR1-5-19d4 were then examined in the receptor assay and the resulting IC₅₀ for monomer was 30nM and for dimer was 8nM.
25
30 The results of the receptor assay comparing TAR1-5-19 monomer, TAR1-5-19d4 and TAR1-5d4 is shown in figure 10.

TAR1-5-19 homodimers were made in the 3U, 5U and 7U vectors, expressed and purified on Protein L. The proteins were examined in the cell and receptor assays and the resulting IC₅₀s (for receptor assay) and ND₅₀s (for cell assay) were determined (table 3, figure 12).

5

2.2 Fabs

TAR1-5 and TAR1-5-19 dimers were also cloned into Fab format, expressed and purified on protein L agarose. Fabs were assessed in the receptor assays (Table 4). The results showed that for both TAR1-5-19 and TAR1-5 dimers the neutralisation levels were similar to the original Gly₄Ser linker dimers from which they were derived. A TAR1-5-19 Fab where TAR1-5-19 was displayed on both CH and CK was expressed, protein L purified and assessed in the receptor assay. The resulting IC₅₀ was approximately 1nM.

15 2.3 TAR1-27 dimers

3 x 96 clones were picked from the round 2 selection encompassing all the libraries and selection conditions. 2ml supernatant preps were made for analysis in ELISA and bioassays. Antigen ELISA gave 71 positive clones. The receptor assay of crude supernatants yielded 42 clones with inhibitory properties (TNF binding 0-60%). In the majority of cases inhibitory properties correlated with a strong ELISA signal. 42 clones were sequenced, 39 of these have unique second dAb sequences. The 12 dimers that gave the best inhibitory properties were analysed further.

The 12 neutralising clones were expressed as 200ml supernatant preps and purified on protein L. These were assessed by protein L and antigen ELISA, BIAcore and in the receptor assay. Strong positive ELISA signals were obtained in all cases. BIAcore analysis revealed all clones to have fast on and off rates. The off rates were improved compared to monomeric TAR1-27, however the off rate of TAR1-27 dimers was faster (K_{off} is approximately in the range of 10⁻¹ and 10⁻²M) than the TAR1-5 dimers examined previously (K_{off} is approximately in the range of 10⁻³ - 10⁻⁴M). The stability of the purified dimers was questioned and therefore in order to improve stability, the addition of 5% glycerol, 0.5% Triton X100 or 0.5% NP40 (Sigma) was included in the purification of 2 TAR1-27 dimers (d2 and d16). Addition of NP40 or Triton X100™ improved the yield

30

of purified product approximately 2 fold. Both dimers were assessed in the receptor assay. TAR1-27d2 gave IC₅₀ of ~30nM under all purification conditions. TAR1-27d16 showed no neutralisation effect when purified without the use of stabilising agents but gave an IC₅₀ of ~50nM when purified under stabilising conditions. No further analysis was conducted.

2.4 TAR2-5 dimers

3 x 96 clones were picked from the second round selections encompassing all the libraries and selection conditions. 2ml supernatant preps were made for analysis. Protein A and antigen ELISAs were conducted for each plate. 30 interesting clones were identified as having good off-rates by BIAcore (K_{off} ranges between 10⁻² – 10⁻³M). The clones were sequenced and 13 unique dimers were identified by sequence analysis.

Table 1: TAR1-5 dimers

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Receptor/ Cell assay
TAR1-5d1	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~30nM
TAR1-5d2	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~50nM
TAR1-5d3	HB2151	Protein L + FPLC	large dimeric species	0.1M glycine pH2.5	RA~300nM
TAR1-5d4	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~3nM
TAR1-5d5	HB2151	Protein L + FPLC	large dimeric species	0.1M glycine pH2.5	RA~200nM
TAR1-5d6	HB2151	Protein L + FPLC	Large dimeric species	0.1M glycine pH2.5	RA~100nM

*note dimer 2 and dimer 3 have the same second dAb (called dAb2), however have different linker lengths (d2 = (Gly₄Ser)₃, d3 = (Gly₄Ser)₃). dAb1 is the partner dAb to dimers 1, 5 and 6. dAb3 is the partner dAb to dimer4. None of the partner dAbs neutralise alone. FPLC purification is by cation exchange unless otherwise stated. The optimal dimeric species for each dimer obtained by FPLC was determined in these assays.

Table 2: TAR1-5-19 dimers

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Receptor/ Cell assay
TAR1-5-19 d1	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0	RA~15 nM
TAR1-5-19 d2 (no stop codon)	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0 + 0.05%NP40	RA~2nM
TAR1-5-19d3 (no stop codon)	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.5 + 0.05%NP40	RA~8nM
TAR1-5-19d4	TOP10F'	Protein L + FPLC	FPLC purified fraction	0.1M glycine pH2.0	RA~2-5nM CA~12 nM
TAR1-5-19d5	TOP10F'	Protein L	Total protein	0.1M glycine pH2.0 + NP40	RA~8nM CA~10 nM
TAR1-5-19 d6	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0	RA~10 nM

5

Table 3: TAR1-5-19 homodimers

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Receptor/ Cell assay
TAR1-5-19 3U homodimer	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~20 nM CA~30 nM
TAR1-5-19 5U homodimer	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~2nM CA~3nM
TAR1-5-19 7U homodimer	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~10 nM CA~15 nM
TAR1-5-19 cys hinge	HB2151	Protein L + FPLC	FPLC purified dimer fraction	0.1M glycine pH2.5	RA~2nM
TAR1-5-19CH/ TAR1-5-19 CK	HB2151	Protein	Total protein	0.1M glycine pH2.5	RA~1nM

Table 4: TAR1-5/TAR1-5-19 Fabs

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Receptor/ Cell
-------	-----------	--------------	------------------	--------------------	----------------

10

					assay
TAR1-5CH/ dAb1 CK	HB2151	Protein L	Total protein	0.1M citrate pH2.6	RA~90 nM
TAR1-5CH/ dAb2 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~30 nM CA~60 nM
dAb3CH/ TAR1-5CK	HB2151	Protein L	Total protein	0.1M citrate pH2.6	RA~10 0nM
TAR1-5-19CH/ dAb1 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~6n M
dAb1 CH/ TAR1-5-19CK	HB2151	Protein L	0.1M glycine pH2.0	Myc/flag	RA~6n M
TAR1-5-19CH/ dAb2 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~8n M CA~12 nM
TAR1-5-19CH/ dAb3CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~3n M

Example 7

5 dAb dimerisation by terminal cysteine linkage

Summary

For dAb dimerisation, a free cysteine has been engineered at the C-terminus of the protein. When expressed the protein forms a dimer which can be purified by a two step
10 purification method.

PCR construction of TAR1-5-19CYS dimer

See example 8 describing the dAb trimer. The trimer protocol gives rise to a mixture of monomer, dimer and trimer.

15

Expression and purification of TAR1-5-19CYS dimer

The dimer was purified from the supernatant of the culture by capture on Protein L agarose as outlined in the example 8.

20 Separation of TAR1-5-19CYS monomer from the TAR1-5-19CYS dimer

Prior to cation exchange separation, the mixed monomer/dimer sample was buffer exchanged into 50 mM sodium acetate buffer pH 4.0 using a PD-10 column (Amersham

Pharmacia), following the manufacturer's guidelines. The sample was then applied to a 1mL Resource S cation exchange column (Amersham Pharmacia), which had been pre-equilibrated with 50 mM sodium acetate pH 4.0. The monomer and dimer were separated using the following salt gradient in 50 mM sodium acetate pH 4.0:

5

150 to 200 mM sodium chloride over 15 column volumes

200 to 450 mM sodium chloride over 10 column volumes

450 to 1000 mM sodium chloride over 15 column volumes

- 10 Fractions containing dimer only were identified using SDS-PAGE and then pooled and the pH increased to 8 by the addition of 1/5 volume of 1M Tris pH 8.0.

***In vitro* functional binding assay: TNF receptor assay and cell assay**

- 15 The affinity of the dimer for human TNF α was determined using the TNF receptor and cell assay. IC₅₀ in the receptor assay was approximately 0.3-0.8 nM; ND₅₀ in the cell assay was approximately 3-8 nM.

Other possible TAR1-5-19CYS dimer formats

- 20 *PEG dimers and custom synthetic maleimide dimers*

- Nektar (Shearwater) offer a range of bi-maleimide PEGs [mPEG2-(MAL)₂ or mPEG-(MAL)₂] which would allow the monomer to be formatted as a dimer, with a small linker separating the dAbs and both being linked to a PEG ranging in size from 5 to 40 kDa. It has been shown that the 5kDa mPEG-(MAL)₂ (ie, [TAR1-5-19]-Cys-maleimide-PEG x
25 2, wherein the maleimides are linked together in the dimer) has an affinity in the TNF receptor assay of ~ 1-3 nM. Also the dimer can also be produced using TMEA (Tris[2-maleimidoethyl]amine) (Pierce Biotechnology) or other bi-functional linkers.

- It is also possible to produce the disulphide dimer using a chemical coupling procedure
30 using 2,2'-dithiodipyridine (Sigma Aldrich) and the reduced monomer.

Addition of a polypeptide linker or hinge to the C-terminus of the dAb.

A small linker, either (Gly₄Ser)_n where n=1 to 10, eg, 1, 2, 3, 4, 5, 6 or 7, an immunoglobulin (eg, IgG hinge region or random peptide sequence (eg, selected from a library of random peptide sequences) can be engineered between the dAb and the terminal cysteine residue. This can then be used to make dimers as outlined above.

5

Example 8

dAb trimerisation

10

Summary

For dAb trimerisation, a free cysteine is required at the C-terminus of the protein. The cysteine residue, once reduced to give the free thiol, can then be used to specifically couple the protein to a trimeric maleimide molecule, for example TMEA (Tris[2-maleimidoethyl]amine).

15

PCR construction of TAR1-5-19CYS

The following oligonucleotides were used to specifically PCR TAR1-5-19 with a *SalI* and *BamHI* sites for cloning and also to introduce a C-terminal cysteine residue:

20

```

                SalI
                ~~~~~
25      1      Trp Ser Ala Ser Thr Asp* Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
          TGG AGC GCG TCG ACG GAC ATC CAG ATG ACC CAG TCT CCA TCC TCT CTG TCT GCA TCT GTA
          ACC TCG CGC AGC TGC CTG TAG GTC TAC TGG GTC AGA GGT AGG AGA GAC AGA CGT AGA CAT

          Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Asp Ser Tyr Leu His Trp
30      61      GGA GAC CGT GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC ATT GAT AGT TAT TTA CAT TGG
          CCT CTG GCA CAG TGG TAG TGA ACG GCC CGT TCA GTC TCG TAA CTA TCA ATA AAT GTA ACC

          Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Glu Leu Gln
35      121     TAC CAG CAG AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC TAT AGT GCA TCC GAG TTG CAA
          ATG GTC GTC TTT GGT CCC TTT CGG GGA TTC GAG GAC TAG ATA TCA CGT AGG CTC AAC GTT

          Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
40      181     AGT GGG GTC CCA TCA CGT TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC
          TCA CCC CAG GGT AGT GCA AAG TCA CCG TCA CCT AGA CCC TGT CTA AAG TGA GAG TGG TAG

          Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Val Val Trp Arg Pro
241     AGC AGT CTG CAA CCT GAA GAT TTT GCT ACG TAC TAC TGT CAA CAG GTT GTG TGG CGT CCT
          TCG TCA GAC GTT GGA CTT CTA AAA CGA TGC ATG ATG ACA GTT GTC CAA CAC ACC GCA GGA

                                     BamHI
                                     ~~~~~

```

Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Cys *** ** Gly Ser Gly
 301 TTT ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGG TGC TAA TAA GGA TCC GGC
 AAA TGC AAG CCG GTT CCC TGG TTC CAC CTT TAG TTT GCC ACG ATT ATT CCT AGG CCG

5 (* start of TAR1-5-19CYS sequence)

Forward primer

5'-TGGAGCGCGTCGACGGACATCCAGATGACCCAGTCTCCA-3'

10

Reverse primer

5'-TTAGCAGCCGGATCCTTATTAGCACCGTTTGATTTCAC-3'

15 The PCR reaction (50µL volume) was set up as follows: 200µM dNTPs, 0.4µM of each primer, 5 µL of 10x *Pfu*Turbo buffer (Stratagene), 100 ng of template plasmid (encoding TAR1-5-19), 1µL of *Pfu*Turbo enzyme (Stratagene) and the volume adjusted to 50µL using sterile water. The following PCR conditions were used: initial denaturing step 94 °C for 2 mins, then 25 cycles of 94 °C for 30 secs, 64 °C for 30 sec and 72 °C for 30 sec.
 20 A final extension step was also included of 72 °C for 5 mins. The PCR product was purified and digested with *Sal*I and *Bam*HI and ligated into the vector which had also been cut with the same restriction enzymes. Correct clones were verified by DNA sequencing.

25 Expression and purification of TAR1-5-19CYS

TAR1-5-19CYS vector was transformed into BL21 (DE3) pLysS chemically competent cells (Novagen) following the manufacturer's protocol. Cells carrying the dAb plasmid were selected for using 100µg/mL carbenicillin and 37 µg/mL chloramphenicol. Cultures were set up in 2L baffled flasks containing 500 mL of terrific broth (Sigma-Aldrich),
 30 100µg/mL carbenicillin and 37 µg/mL chloramphenicol. The cultures were grown at 30 °C at 200rpm to an O.D.600 of 1-1.5 and then induced with 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside, from Melford Laboratories). The expression of the dAb was allowed to continue for 12-16 hrs at 30 °C. It was found that most of the dAb was present in the culture media. Therefore, the cells were separated from the media by centrifugation

(8,000xg for 30 mins), and the supernatant used to purify the dAb. Per litre of supernatant, 30 mL of Protein L agarose (Affitech) was added and the dAb allowed to batch bind with stirring for 2 hours. The resin was then allowed to settle under gravity for a further hour before the supernatant was siphoned off. The agarose was then packed into
5 a XK 50 column (Amersham Pharmacia) and was washed with 10 column volumes of PBS. The bound dAb was eluted with 100 mM glycine pH 2.0 and protein containing fractions were then neutralized by the addition of 1/5 volume of 1 M Tris pH 8.0. Per litre of culture supernatant 20 mg of pure protein was isolated, which contained a 50:50 ratio of monomer to dimer.

10

Trimerisation of TAR1-5-19CYS

2.5 ml of 100 μ M TAR1-5-19CYS was reduce with 5 mM dithiothreitol and left at room temperature for 20 minutes. The sample was then buffer exchanged using a PD-10 column (Amersham Pharmacia). The column had been pre-equilibrated with 5 mM
15 EDTA, 50 mM sodium phosphate pH 6.5, and the sample applied and eluted following the manufactures guidelines. The sample was placed on ice until required. TMEA (Tris[2-maleimidoethyl]amine) was purchased from Pierce Biotechnology. A 20 mM stock solution of TMEA was made in 100% DMSO (dimethyl sulphoxide). It was found that a concentration of TMEA greater than 3:1 (molar ratio of dAb:TMEA) caused the rapid
20 precipitation and cross-linking of the protein. Also the rate of precipitation and cross-linking was greater as the pH increased. Therefore using 100 μ M reduced TAR1-5-19CYS, 25 μ M TMEA was added to trimerise the protein and the reaction allowed to proceed at room temperature for two hours. It was found that the addition of additives such as glycerol or ethylene glycol to 20% (v/v), significantly reduced the precipitation of
25 the trimer as the coupling reaction proceeded. After coupling, SDS-PAGE analysis showed the presence of monomer, dimer and trimer in solution.

Purification of the trimeric TAR1-5-19CYS

40 μ L of 40% glacial acetic acid was added per mL of the TMEA-TAR1-5-19cys reaction
30 to reduce the pH to ~4. The sample was then applied to a 1mL Resource S cation exchange column (Amersham Pharmacia), which had been pre-equilibrated with 50 mM sodium acetate pH 4.0. The dimer and trimer were partially separated using a salt gradient of 340 to 450 mM Sodium chloride, 50 mM sodium acetate pH 4.0 over 30 column

volumes. Fractions containing trimer only were identified using SDS-PAGE and then pooled and the pH increased to 8 by the addition of 1/5 volume of 1M Tris pH 8.0. To prevent precipitation of the trimer during concentration steps (using 5K cut off Viva spin concentrators; Vivascience), 10% glycerol was added to the sample.

5

***In vitro* functional binding assay: TNF receptor assay and cell assay**

The affinity of the trimer for human TNF α was determined using the TNF receptor and cell assay. IC₅₀ in the receptor assay was 0.3nM; ND₅₀ in the cell assay was in the range of 3 to 10nM (eg, 3nM).

10

Other possible TAR1-5-19CYS trimer formats

TAR1-5-19CYS may also be formatted into a trimer using the following reagents:

PEG trimers and custom synthetic maleimide trimers

15 Nektar (Shearwater) offer a range of multi arm PEGs, which can be chemically modified at the terminal end of the PEG. Therefore using a PEG trimer with a maleimide functional group at the end of each arm would allow the trimerisation of the dAb in a manner similar to that outlined above using TMEA. The PEG may also have the advantage in increasing the solubility of the trimer thus preventing the problem of aggregation. Thus, one could
20 produce a dAb trimer in which each dAb has a C-terminal cysteine that is linked to a maleimide functional group, the maleimide functional groups being linked to a PEG trimer.

Addition of a polypeptide linker or hinge to the C-terminus of the dAb

25 A small linker, either (Gly₄Ser)_n where n= 1 to 10, eg, 1, 2, 3, 4, 5, 6 or 7, an immunoglobulin (eg, IgG hinge region or random peptide sequence (eg, selected from a library of random peptide sequences) could be engineered between the dAb and the terminal cysteine residue. When used to make multimers (eg, dimers or trimers), this again would introduce a greater degree of flexibility and distance between the individual
30 monomers, which may improve the binding characteristics to the target, eg a multisubunit target such as human TNF α .

Example 9.**Selection of a collection of single domain antibodies (dAbs) directed against human serum albumin (HSA) and mouse serum albumin (MSA).**

5

This example explains a method for making a single domain antibody (dAb) directed against serum albumin. Selection of dAbs against both mouse serum albumin (MSA) and human serum albumin (HSA) is described. Three human phage display antibody libraries were used in this experiment, each based on a single human framework for V_H (see Figure 13: sequence of dummy V_H based on V3-23/DP47 and JH4b) or V_K (see Figure 15: sequence of dummy V_K based on o12/o2/DPK9 and Jk1) with side chain diversity encoded by NNK codons incorporated in complementarity determining regions (CDR1, CDR2 and CDR3).

10

15 Library 1 (V_H):

Diversity at positions: H30, H31, H33, H35, H50, H52, H52a, H53, H55, H56, H58, H95, H97, H98.

Library size: 6.2×10^9

20 Library 2 (V_H):

Diversity at positions: H30, H31, H33, H35, H50, H52, H52a, H53, H55, H56, H58, H95, H97, H98, H99, H100, H100a, H100b.

Library size: 4.3×10^9

25 Library 3 (V_K):

Diversity at positions: L30, L31, L32, L34, L50, L53, L91, L92, L93, L94, L96

Library size: 2×10^9

30

The V_H and V_K libraries have been preselected for binding to generic ligands protein A and protein L respectively so that the majority of clones in the unselected libraries are functional. The sizes of the libraries shown above correspond to the sizes after preselection.

Two rounds of selection were performed on serum albumin using each of the libraries separately. For each selection, antigen was coated on immunotube (nunc) in 4ml of PBS at a concentration of 100 μ g/ml. In the first round of selection, each of the three libraries was panned separately against HSA (Sigma) and MSA (Sigma). In the second round of selection, phage from each of the six first round selections was panned against (i) the same antigen again (eg 1st round MSA, 2nd round MSA) and (ii) against the reciprocal antigen (eg 1st round MSA, 2nd round HSA) resulting in a total of twelve 2nd round selections. In each case, after the second round of selection 48 clones were tested for binding to HSA and MSA. Soluble dAb fragments were produced as described for scFv fragments by Harrison *et al*, Methods Enzymol. 1996;267:83-109 and standard ELISA protocol was followed (Hoogenboom *et al*. (1991) Nucleic Acids Res., 19: 4133) except that 2% tween PBS was used as a blocking buffer and bound dAbs were detected with either protein L-HRP (Sigma) (for the V_Ks) and protein A -HRP (Amersham Pharmacia Biotech) (for the V_Hs).

dAbs that gave a signal above background indicating binding to MSA, HSA or both were tested in ELISA insoluble form for binding to plastic alone but all were specific for serum albumin. Clones were then sequenced (see table below) revealing that 21 unique dAb sequences had been identified. The minimum similarity (at the amino acid level) between the V_K dAb clones selected was 86.25% ((69/80)x100; the result when all the diversified residues are different, eg clones 24 and 34). The minimum similarity between the V_H dAb clones selected was 94 % ((127/136)x100).

Next, the serum albumin binding dAbs were tested for their ability to capture biotinylated antigen from solution. ELISA protocol (as above) was followed except that ELISA plate was coated with 1 μ g/ml protein L (for the V_K clones) and 1 μ g/ml protein A (for the V_H clones). Soluble dAb was captured from solution as in the protocol and detection was with biotinylated MSA or HSA and streptavidin HRP. The biotinylated MSA and HSA had been prepared according to the manufacturer's instructions, with the aim of achieving an average of 2 biotins per serum albumin molecule. Twenty four clones were identified that captured biotinylated MSA from solution in the ELISA. Two of these (clones 2 and 38 below) also captured biotinylated HSA. Next, the dAbs were tested for their ability to

bind MSA coated on a CM5 biacore chip. Eight clones were found that bound MSA on the biacore.

dAb capture biotinylated MSA) V _K library 3 template (dummy)	(all H or κ CDR1	CDR2	CDR3	Binds MSA in biacore?	Captures biotinylated HSA?
2, 4, 7, 41, 38, 54	κ SSYLN	RASPLQS	QQTYSVPT		✓ all 4 bind
46, 47, 52, 56	κ SSYLN	RASPLQS	QQTYRIPPT		✓ both bind
13, 15	κ FKSLK	NASYLQS	QQVVYWPVT		
30, 35	κ YYHLK	KASTLQS	QQVRKVPRT		
19,	κ RRYLK	QASVLQS	QQGLYPPT		
22,	κ YNWLK	RASSLQS	QQNVVIPRT		
23,	κ LWHLR	HASLLQS	QOSAVYPKT		
24,	κ FRYLA	HASHLQS	QQRLLYPKT		
31,	κ FYHLA	PASKLQS	QQRARWPRT		
33,	κ IWHLN	RASRLQS	QQVARVPRT		
	κ YRYLR	KASSLQS	QQYVGYPRT		
34,	κ LKYLK	NASHLQS	QQTYYYPIT		
53,	κ LRYLR	KASWLQS	QQVLYYPQT		
11,	κ LRSLK	AASRLQS	QQVVYWPAT	✓	
12,	κ FRHLK	AASRLQS	QQVALYPKT	✓	
17,	κ RKYLR	TASSLQS	QQNLFWPRT	✓	
18,	κ RRYLN	AASSLQS	QQMLFYPKT	✓	
16, 21	κ IKHLK	GASRLQS	QQGARWPQT	✓	
25, 26	κ YYHLK	KASTLQS	QQVRKVPRT	✓	
27,	κ YKHLK	NASHLQS	QQVGRYPKT	✓	
55,	κ FKSLK	NASYLQS	QQVVYWPVT	✓	
V _H library 1 (and 2) template (dummy)	H	XXYXXX	XIXXXGXXTXYADSVKG	XXXX (XXXX) FDY	
8, 10	H	WVYQMD	SISAFGAKTLYADSVKG	LSGKFDY	
36,	H	WSYQMT	SISFSGSSTLYADSVKG	GRDHNYSLFDY	

5

In all cases the frameworks were identical to the frameworks in the corresponding dummy sequence, with diversity in the CDRs as indicated in the table above.

Of the eight clones that bound MSA on the biacore, two clones that are highly expressed in *E. coli* (clones MSA16 and MSA26) were chosen for further study (see example 10). Full nucleotide and amino acid sequences for MSA16 and 26 are given in figure 16.

Example 10.

Determination of affinity and serum half-life in mouse of MSA binding dAbs MSA16 and MSA26.

dAbs MSA16 and MSA26 were expressed in the periplasm of *E. coli* and purified using
5 batch absorption to protein L-agarose affinity resin (Affitech, Norway) followed by
elution with glycine at pH 2.2. The purified dAbs were then analysed by inhibition
biacore to determine K_d . Briefly, purified MSA16 and MSA26 were tested to determine
the concentration of dAb required to achieve 200RUs of response on a biacore CM5 chip
coated with a high density of MSA. Once the required concentrations of dAb had been
10 determined, MSA antigen at a range of concentrations around the expected K_d was
premixed with the dAb and incubated overnight. Binding to the MSA coated biacore chip
of dAb in each of the premixes was then measured at a high flow-rate of 30 μ l/minute.
The resulting curves were used to create Klotz plots, which gave an estimated K_d of
200nM for MSA16 and 70nM for MSA 26 (Figure 17 A & B).

15
Next, clones MSA16 and MSA26 were cloned into an expression vector with the HA tag
(nucleic acid sequence: TATCCTTATGATGTTCTGATTATGCA and amino acid
sequence: YPYDVPDYA) and 2-10 mg quantities were expressed in *E. coli* and purified
from the supernatant with protein L-agarose affinity resin (Affitech, Norway) and eluted
20 with glycine at pH2.2. Serum half life of the dAbs was determined in mouse. MSA26
and MSA16 were dosed as single i.v. injections at approx 1.5mg/kg into CD1 mice.
Analysis of serum levels was by goat anti-HA (Abcam, UK) capture and protein L-HRP
(Invitrogen) detection ELISA which was blocked with 4% Marvel. Washing was with
0.05% tween PBS. Standard curves of known concentrations of dAb were set up in the
25 presence of 1xmouse serum to ensure comparability with the test samples. Modelling
with a 2 compartment model showed MSA-26 had a $t_{1/2\alpha}$ of 0.16hr, a $t_{1/2\beta}$ of 14.5hr and
an area under the curve (AUC) of 465hr.mg/ml (data not shown) and MSA-16 had a $t_{1/2\alpha}$
of 0.98hr, a $t_{1/2\beta}$ of 36.5hr and an AUC of 913hr.mg/ml (figure 18). Both anti-MSA
clones had considerably lengthened half life compared with HEL4 (an anti-hen egg white
30 lysozyme dAb) which had a $t_{1/2\alpha}$ of 0.06hr, and a $t_{1/2\beta}$ of 0.34hr.

Example 11.**Creation of V_H - V_H and V_K - V_K dual specific Fab like fragments**

- 5 This example describes a method for making V_H - V_H and V_K - V_K dual specifics as Fab like fragments. Before constructing each of the Fab like fragments described, dAbs that bind to targets of choice were first selected from dAb libraries similar to those described in example 9. A V_H dAb, HEL4, that binds to hen egg lysozyme (Sigma) was isolated and a second V_H dAb (TAR2h-5) that binds to TNF α receptor (R and D systems) was also
- 10 isolated. The sequences of these are given in the sequence listing. A V_K dAb that binds TNF α (TAR1-5-19) was isolated by selection and affinity maturation and the sequence is also set forth in the sequence listing. A second V_K dAb (MSA 26) described in example 9 whose sequence is in figure 17B was also used in these experiments.
- 15 DNA from expression vectors containing the four dAbs described above was digested with enzymes SalI and NotI to excise the DNA coding for the dAb. A band of the expected size (300-400bp) was purified by running the digest on an agarose gel and excising the band, followed by gel purification using the Qiagen gel purification kit (Qiagen, UK). The DNA coding for the dAbs was then inserted into either the C_H or C_K
- 20 vectors (Figs 8 and 9) as indicated in the table below.

dAb	Target antigen	dAb V_H or dAb V_K	Inserted into vector	tag (C terminal)	Antibiotic resistance
HEL4	Hen egg lysozyme	V_H	C_H	myc	Chloramphenicol
TAR2-5	TNF receptor	V_H	C_K	flag	Ampicillin
TAR1-5-19	TNF α	V_K	C_H	myc	Chloramphenicol
MSA 26	Mouse serum albumin	V_K	C_K	flag	Ampicillin

- The V_H C_H and V_H C_K constructs were cotransformed into HB2151 cells. Separately, the V_K C_H and V_K C_K constructs were cotransformed into HB2151 cells. Cultures of each of
- 25 the cotransformed cell lines were grown overnight (in 2xTy containing 5% glucose, 10 μ g/ml chloramphenicol and 100 μ g/ml ampicillin to maintain antibiotic selection for both C_H and C_K plasmids). The overnight cultures were used to inoculate fresh media (2xTy, 10 μ g/ml chloramphenicol and 100 μ g/ml ampicillin) and grown to OD 0.7-0.9 before induction by the addition of IPTG to express their C_H and C_K constructs.

Expressed Fab like fragment was then purified from the periplasm by protein A purification (for the contrtransformed $V_H C_H$ and $V_H C_K$) and MSA affinity resin purification (for the contrtransformed $V_K C_H$ and $V_K C_K$).

5 V_H - V_H dual specific

Expression of the $V_H C_H$ and $V_H C_K$ dual specific was tested by running the protein on a gel. The gel was blotted and a band the expected size for the Fab fragment could be detected on the Western blot via both the myc tag and the flag tag, indicating that both the $V_H C_H$ and $V_H C_K$ parts of the Fab like fragment were present. Next, in order to
10 determine whether the two halves of the dual specific were present in the same Fab-like fragment, an ELISA plate was coated overnight at 4°C with 100 μ l per well of hen egg lysozyme (HEL) at 3 mg/ml in sodium bicarbonate buffer. The plate was then blocked (as described in example 1) with 2% tween PBS followed by incubation with the $V_H C_H$ / $V_H C_K$ dual specific Fab like fragment. Detection of binding of the dual specific to the
15 HEL was via the non cognate chain using 9e10 (a monoclonal antibody that binds the myc tag, Roche) and anti mouse IgG-HRP (Amersham Pharmacia Biotech). The signal for the $V_H C_H$ / $V_H C_K$ dual specific Fab like fragment was 0.154 compared to a background signal of 0.069 for the $V_H C_K$ chain expressed alone. This demonstrates that the Fab like fragment has binding specificity for target antigen.

20

V_K - V_K dual specific

After purifying the contrtransformed $V_K C_H$ and $V_K C_K$ dual specific Fab like fragment on an MSA affinity resin, the resulting protein was used to probe an ELISA plate coated with 1 μ g/ml TNF α and an ELISA plate coated with 10 μ g/ml MSA. As predicted, there was
25 signal above background when detected with protein L-HRP on bot ELISA plates (data not shown). This indicated that the fraction of protein able to bind to MSA (and therefore purified on the MSA affinity column) was also able to bind TNF α in a subsequent ELISA, confirming the dual specificity of the antibody fragment. This fraction of protein was then used for two subsequent experiments. Firstly, an ELISA plate coated with
30 1 μ g/ml TNF α was probed with dual specific $V_K C_H$ and $V_K C_K$ Fab like fragment and also with a control TNF α binding dAb at a concentration calculated to give a similar signal on the ELISA. Both the dual specific and control dAb were used to probe the ELISA plate in the presence and in the absence of 2mg/ml MSA. The signal in the dual

specific well was reduced by more than 50% but the signal in the dAb well was not reduced at all (see figure 19a). The same protein was also put into the receptor assay with and without MSA and competition by MSA was also shown (see figure 19c). This demonstrates that binding of MSA to the dual specific is competitive with binding to

5 TNF α .

Example 12.

10 **Creation of a V κ - V κ dual specific cys bonded dual specific with specificity for mouse serum albumin and TNF α**

This example describes a method for making a dual specific antibody fragment specific for both mouse serum albumin and TNF α by chemical coupling via a disulphide bond.

15 Both MSA16 (from example 1) and TAR1-5-19 dAbs were recloned into a pET based vector with a C terminal cysteine and no tags. The two dAbs were expressed at 4-10 mg levels and purified from the supernatant using protein L-agarose affinity resin (Affittech, Norway). The cysteine tagged dAbs were then reduced with dithiothreitol. The TAR1-5-19 dAb was then coupled with dithiodipyridine to block reformation of disulphide bonds

20 resulting in the formation of PEP 1-5-19 homodimers. The two different dAbs were then mixed at pH 6.5 to promote disulphide bond formation and the generation of TAR1-5-19, MSA16 cys bonded heterodimers. This method for producing conjugates of two unlike proteins was originally described by King *et al.* (King TP, Li Y Kochoumian L Biochemistry. 1978 vol17:1499-506 Preparation of protein conjugates via intermolecular

25 disulfide bond formation.) Heterodimers were separated from monomeric species by cation exchange. Separation was confirmed by the presence of a band of the expected size on a SDS gel. The resulting heterodimeric species was tested in the TNF receptor assay and found to have an IC₅₀ for neutralising TNF of approximately 18 nM. Next, the receptor assay was repeated with a constant concentration of heterodimer (18nM) and

30 a dilution series of MSA and HSA. The presence of HSA at a range of concentrations (up to 2 mg/ml) did not cause a reduction in the ability of the dimer to inhibit TNF α .

... However, the addition of MSA caused a dose dependant reduction in the ability of the

dimer to inhibit TNF α (figure 20). This demonstrates that MSA and TNF α compete for binding to the cys bonded TAR1-5-19, MSA16 dimer.

Example 13. Cloning and expression of the TAR1/TAR2 dual specific Fab

5

TAR1-5-19 V κ dAb (specific to human TNF alpha) was cloned into pDOM3 CK Amp vector (Figure 21) as a SalI/NotI fragment. TAR2h-10-27 VH dAb (specific to human TNFR1) was cloned into pDOM3 CH Chlor vector (Figure 21) as a SalI/NotI fragment.

- 10 The two vectors with cloned in dAbs were used to co-transform competent HB2151 cells. Amp/Chlor resistant clones (containing both plasmids) were used to make a large scale (10l) fermentor prep of the Fab.

The produced Fab was isolated from the culture supernatant (after 3 hours induction at 15 25C) using sequential Protein A/Protein L purification. The yield of the Fab was 1.5mg.

Example 14. Analysis of Fab properties in ELISA

20 a) Binding of the Fab to TAR1 and TAR2

Binding of the TAR1/TAR2 Fab to TNF and TNFR1 was tested in ELISA. A 96 well plate was coated with 100ul of TNF and TNFR1 at 1ug/ml concentration in PBS overnight at 4C. 50ul (3uM) of Fab was then added to the wells and bound Fab was 25 detected via non-cognate chain, ie using Protein A-HRP on TNF coated wells and Protein L-HRP on TNFR1 coated wells. ELISA demonstrated the ability of the Fab to bind both antigens (Figure 22).

b) Sandwich ELISA

30

To test the ability of the TAR1/TAR2 Fab to bind both antigens simultaneously (open/closed conformation?) a sandwich ELISA was performed. Here a 96 well plate was coated with mutant TNF (that does not bind to TNFR1, but does bind to PEP1-5-19, data

no shown; mutant TNF contains a single point mutation (N141Y) which renders it incapable of binding to TNFRI (Yamadishi et al., 1990, Protein Eng., 3, 713-9)) at 1ug/ml concentration in PBS overnight at 4C. 50ul of Fab (0.5uM) was then added. This was followed by addition of TNFRI-Fc fusion protein (R&D Systems) and detection with
5 Anti-Fc-HRP. The same sandwich ELISA was performed using a control Fab containing TAR1/Ck chain and an irrelevant VH fused to the CH chain. ELISA results demonstrated the ability of the Fab to engage both antigens (TNF and TNFRI) simultaneously, suggesting an open conformation of the molecule (Figure 23).

10

c) Competition ELISA

To test the ability of the TAR1/TAR2 Fab to bind both antigens simultaneously two competition ELISAs were performed.

15

A 96 well plate was coated with 100ul of TNFRI at 1ug/ml concentration in PBS overnight at 4C. A dilution of Fab was chosen such that OD450 of 0.3 was achieved upon detection with Protein L-HRP. This concentration was 6nM. The Fab was pre-incubated for an hour at room temperature with increasing concentrations of mutant TNF (up to
20 160x molar excess). As a negative control Fab was subjected to the same incubation with BSA. Following these incubations the mixtures were then put onto TNFRI coated ELISA plate and incubated for another hour. Bound TAR1/TAR2 Fab was detected using ProteinL-HRP. ELISA demonstrated that TAR1/TAR2 Fab binding to TNFRI was not affected by competing antigen (Figure 24).

25

A 96 well plate was coated with 100ul of mutant TNF at 1ug/ml concentration in PBS overnight at 4C. A dilution of Fab was chosen such that OD450 of 0.3 was achieved upon detection with 9E10 (Sigma) followed by anti mo-HRP (Sigma). This concentration was 25nM. The Fab was pre-incubated for an hour at room temperature with increasing
30 concentrations of soluble TNFRI (up to 10x molar excess). As a negative control Fab was subjected to the same incubation with BSA. Following these incubations the mixtures were then put onto mutant TNF coated ELISA plate and incubated for another hour. Bound TAR1/TAR2 Fab was detected using 9E10 followed by anti mo-HRP. ELISA

demonstrated that TAR1/TAR2 Fab binding to mutant TNF was not affected by competing antigen (Figure 24).

5 **Example 15. Analysis of Fab properties in cell assays.**

To check the degree of functionality of each dAb in a TAR1/TAR2 Fab, the performance of the dual specific molecule was tested in the following cell assays:

10

Human TNF cytotoxicity on murine cells.

This assay tests the activity of TAR1 Dab, as TAR2 Dab cannot bind to murine TNF receptor expressed on the surface of the cells. TAR1-5-19 Dab and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that TAR1-5-19 Dab in a Fab behaves as well as a monomeric TAR1-5-19 dAb (Figure 25).

20 **Murine TNF cytotoxicity assay on murine cells with human soluble TNF receptor.**

20

This assay tests the activity of TAR2h-10-27 (in this assay binding to soluble human TNFRI). TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that TAR2h-10-27 in a Fab behaves as well as a monomeric TAR2h-10-27 dAb (Figure 25).

25

Murine TNF induced IL-8 secretion on human cells.

This assay tests the activity of TAR2h-10-27 Dab (in this assay binding to membrane bound human TNFRI). TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1 + TAR2 dAb mixture were used as controls in this assay. The results demonstrate that TAR2h-10-27 in a Fab behaves as well as a monomeric TAR2h-10-27 dAb (Figure 25).

30

Human TNF induced IL-8 secretion on human cells.

This assay tests the activity of both TAR1-5-19 and TAR2h-10-27 Dabs. TAR1-5-19 Dab and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used
5 as controls in this assay. The results demonstrate that Fab has a similar effect to the TAR2h-10-27 dAb and TAR1-5-19 + TAR2h-10-27 dAb mixture (Figure 25).

Murine TNF cytotoxicity on murine cells with soluble human TNFRI and increasing concentrations of mutant TNF (competition on cells).

10

This assay was performed to test whether increasing concentration of mutant TNF (binding to TAR 1-5-19 Dab) will compromise binding of TAR2h-10-27 Dab to TNFRI in solution. The results of the assay indicate that that is not the case, thus the Fab is able to engage two antigens simultaneously (Figure 26).

15

The assays described above demonstrate that each dAb in a Fab molecule functions as well as a monomeric dAb.

20

Example 16. Construction of IgG vectors.

pcDNA3.1(+) and pcDNA3.1/Zeo(+) backbones (Invitrogen) were used for cloning IgG1
25 heavy chain constant region and light chain kappa constant region, respectively. The overview of the vectors is shown in Figure 27.

Leaders:

30 Two alternative types of leaders were used to facilitate secretion of the expressed protein:
CD33 leader
IgG K- chain leader

The leaders were assembled by the annealing of the two complementary oligos (Table 5) and were cloned into pcDNA3.1(+) and pcDNA3.1/Zeo(+) as NheI/HindIII fragments (Figure 27).

5 **IgG1 heavy chain cloning:**

CH1 domain was PCR amplified from the CH vector (as described in WO 03/002609) using primers shown in Table 5.

10 Hinge region, CH2 and CH3 domains were PCR amplified from pIgplus vector (Novagen) using primers shown in Table 5.

The two products were then PCR assembled to create an IgG1 heavy chain constant region which was cloned into pcDNA3.1(+) as a NotI/XhoI fragment (Figure 27).

15

Kappa light chain cloning:

CK domain was PCR amplified from the CK vector (see see WO 03/002609) using
20 primers shown in Table 5.

It was then cloned into pcDNA3.1/Zeo (+) as a NotI/XhoI fragment (Figure 27).

25 **Example 17. Cloning of TAR1-5-19 and TAR2h-10-27 dAbs into IgG vectors and production of IgG:**

TAR1-5-19 VK dAb (specific to human TNF alpha) was cloned into IgG kappa vectors (with CD33 and IgK leaders) as a HindIII/NotI fragment (Figure 27).

30

TAR2h-10-27 VH dAb (specific to human TNFR1) was cloned into IgG heavy chain vectors (with CD33 and IgK leaders) as a HindIII/NotI fragment (Figure 27).

Heavy and light chain plasmids were then co-transfected into COS7 cells and IgG was expressed transiently for five days. The produced IgG was purified using streamline Protein A. Expression level – 250ng/ml. CD33 and IgG K leaders gave the same level of expression.

5

Purified IgG was checked on a reducing and non-reducing SDS gel (produced bands of expected size) (data not shown).

10 **Example 18. Analysis of IgG properties in ELISA**

a) Binding of the IgG to TNF and TNFRI

Binding of the TAR1/TAR2 IgG to TNF and TNFRI was tested in ELISA. A 96 well
15 plate was coated with 100ul of TNF and TNFRI at 1ug/ml concentration in PBS overnight at 4C. 50ul (200nM) of IgG was then added to the wells and bound IgG was detected via anti-Fc-HRP. ELISA demonstrated the ability of the IgG to bind both antigens (**Figure 28**).

20

Example 19. Analysis of IgG properties in cell assays.

To check the degree of functionality of each dAb in a TAR1/TAR2 IgG, the performance
25 of the dual specific molecule was tested in the following cell assays:

Human TNF cytotoxicity on murine cells.

This assay tests the activity of TAR1-5-19 Dab, as TAR2h-10-27 Dab cannot bind to
30 murine TNF receptor expressed on the surface of the cells. TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that TAR1-5-19 in the IgG behaves better than

monomeric TAR1-5-19 dAb, which indicates that IgG is able to simultaneously engage two molecules of TNF (ND50 of the dimeric molecule) (**Figure 29**).

Murine TNF cytotoxicity assay on murine cells with human soluble TNF receptor.

5

This assay tests the activity of TAR2h-10-27 (in this assay binding to soluble human TNFRI). TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that TAR2h-10-27 in IgG behaves as well as a monomeric TAR2h-10-27 dAb (**Figure 29**).

10

Murine TNF induced IL-8 secretion on human cells.

This assay tests the activity of TAR2h-10-27 (in this assay binding to membrane bound human TNFRI). TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that IgG is able to engage two molecules of TNFR1 on the surface of the cell (agonistic activity) (**Figure 29**). This assay was also repeated with no human TNF present. The results demonstrate that the IgG induces IL-8 release on human cells up to a concentration of 30nM after which the agonistic activity goes down (**Figure 30**).

20

Human TNF induced IL-8 secretion on human cells.

This assay tests the activity of both TAR1-5-19 and TAR2h-10-27. TAR1-5-19 and TAR2h-10-27 dAbs as well as TAR1-5-19 + TAR2h-10-27 dAb mixture were used as controls in this assay. The results demonstrate that IgG has a similar effect to the TARh-10-27 dAb and TAR1-5-19 + TAR2h-10-27 dAb mixture (**Figure 29**). This assay was also repeated with no murine TNF present. The results demonstrate that the IgG induces IL-8 release on human cells up to a concentration of 30nM after which the agonistic activity goes down (**Figure 30**).

30

Table 5. Primers/Oligos

CkbckNot

5' AAGGAAAAAAGCGGCCGCAACTGTGGCTGCACCATC 3'

5

CkforXho

5' CCGCTCGAGTCAACACTCTCCCCTGTTGAAGCTCTTTGTG 3'

Ch1bckNot

10 5' AAGGAAAAAAGCGGCCGCCTCCACCAAGGGCCCATCGGTC 3'

Ch1for

5' GTGAGGTTTGTACAAGATTGTTGGGCTCAACTTTCTTGTCCACC 3'

15 **Fcbck**

5' CCCAAATCTTGTGACAAACCTCAC 3'

FcforXho

20 5' CCGCTCGAGTCATTTACCCGGAGACAGGGAG 3'

LEADER CD33:25 **Leacd1**

5'P CTAGCCACCATGCCGCTGCTGCTACTGCTGCTGCTGTGGGCAGGAGCACTGGCTATGGATA 3'

Leacd230 5'P AGCTTATCCATAGCCAGTGCTCCTGCCCACAGCAGTGGCAGCAGTAGCAGCAGCGGCATGGTGG
3'**LEADER IGGK:**

35

Leak15'P
CTAGCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGT
GACA 3'

40

Leak25'P
AGCTTGTCAACAGTGGAACCTGGAACCCAGAGCAGCAGTACCCATAGCAGGAGTGTGTCTGTCTCCAT
GGTGG 3'

45

SEQBACK

5' TAATACGACTCACTATAGGG 3'

50

SEQFOR

5' TAGAAGGCACAGTCGAGG 3'

55

Data Summary

A summary of data obtained in the experiments set forth in the foregoing examples is set forth in Annex 4.

All publications mentioned in the present specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Annex 1; polypeptides which enhance half-life *in vivo*.

- Alpha-1 Glycoprotein (Orosomucoid) (AAG)
Alpha-1 Antichyromotrypsin (ACT)
5 Alpha-1 Antitrypsin (AAT)
Alpha-1 Microglobulin (Protein HC) (AIM)
Alpha-2 Macroglobulin (A2M)
Antithrombin III (AT III)
Apolipoprotein A-1 (Apo A-1)
10 Apolipoprotein B (Apo B)
Beta-2-microglobulin (B2M)
Ceruloplasmin (Cp)
Complement Component (C3)
Complement Component (C4)
15 C1 Esterase Inhibitor (C1 INH)
C-Reactive Protein (CRP)
Cystatin C (Cys C)
Ferritin (FER)
Fibrinogen (FIB)
20 Fibronectin (FN)
Haptoglobin (Hp)
Hemopexin (HPX)
Immunoglobulin A (IgA)
Immunoglobulin D (IgD)
25 Immunoglobulin E (IgE)
Immunoglobulin G (IgG)
Immunoglobulin M (IgM)
Immunoglobulin Light Chains (kappa/lambda)
Lipoprotein(a) [Lp(a)]
30 Mannose-binding protein (MBP)
Myoglobin (Myo)
Plasminogen (PSM)
Prealbumin (Transthyretin) (PAL)

- Retinol-binding protein (RBP)
- Rheumatoid Factor (RF)
- Serum Amyloid A (SAA)
- Soluble Transferrin Receptor (sTfR)
- 5 Transferrin (Tf)

Annex 2

Pairing	Therapeutic relevant references.
TNF ALPHA/TGF- β	TGF-β and TNF when injected into the ankle joint of collagen induced arthritis model significantly enhanced joint inflammation. In non-collagen challenged mice there was no effect.
TNF ALPHA/IL-1	- TNF and IL-1 synergize in the pathology of uveitis. - TNF and IL-1 synergize in the pathology of malaria (hypoglycaemia, NO). - TNF and IL-1 synergize in the induction of polymorphonuclear (PMN) cells migration in inflammation. - IL-1 and TNF synergize to induce PMN infiltration into the peritoneum. - IL-1 and TNF synergize to induce the secretion of IL-1 by endothelial cells. Important in inflammation. - IL-1 or TNF alone induced some cellular infiltration into knee synovium. IL-1 induced PMNs, TNF – monocytes. Together they induced a more severe infiltration due to increased PMNs. - Circulating myocardial depressant substance (present in sepsis) is low levels of IL-1 and TNF acting synergistically.
TNF ALPHA/IL-2	Most relating to synergistic activation of killer T-cells.
TNF ALPHA/IL-3	- Synergy of interleukin 3 and tumor necrosis factor alpha in stimulating clonal growth of acute myelogenous leukemia blasts is the result of induction of secondary hematopoietic cytokines by tumor necrosis factor alpha. - Cancer Res. 1992 Apr 15;52(8):2197-201.
TNF ALPHA/IL-4	- IL-4 and TNF synergize to induce VCAM expression on endothelial cells. Implied to have a role in asthma. Same for synovium – implicated in RA. - TNF and IL-4 synergize to induce IL-6 expression in keratinocytes. - Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-alpha in combination with either IL-4 or IL-13 through increased mRNA stability. <i>Am J Pathol.</i> 1999 Apr;154(4):1149-58
TNF ALPHA/IL-5	Relationship between the tumor necrosis factor system and the serum interleukin-4, interleukin-5, interleukin-8, eosinophil cationic protein, and immunoglobulin E levels in the bronchial hyperreactivity of adults and their children. <i>Allergy Asthma Proc.</i> 2003 Mar-Apr;24(2):111-8.
TNF ALPHA/IL-6	TNF and IL-6 are potent growth factors for OH-2, a novel human myeloma cell line. <i>Eur J Haematol.</i> 1994 Jul;53(1):31-7.
TNF ALPHA/IL-8	- TNF and IL-8 synergized with PMNs to activate platelets. Implicated in Acute Respiratory Distress Syndrome. - See IL-5/TNF (asthma). Synergism between interleukin-8 and tumor necrosis factor-alpha for neutrophil-mediated platelet activation. <i>Eur Cytokine Netw.</i> 1994 Sep-Oct;5(5):455-60. (adult respiratory distress syndrome (ARDS))
TNF ALPHA/IL-9	
TNF ALPHA/IL-10	IL-10 induces and synergizes with TNF in the induction of HIV expression in chronically infected T-cells.
TNF ALPHA/IL-11	Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. <i>Am J Physiol Cell Physiol.</i> 2002 Sep;283(3):C679-87. (Bone loss)
TNF ALPHA/IL-12	

TNF ALPHA/IL-13	- Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-alpha in combination with either IL-4 or IL-13 through increased mRNA stability. <i>Am J Pathol.</i> 1999 Apr;154(4):1149-58. - Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. <i>Clin Exp Allergy.</i> 2000 Mar;30(3):348-55. - Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. <i>Clin Exp Allergy.</i> 2000 Mar;30(3):348-55 (allergic inflammation) - Implications of serum TNF-beta and IL-13 in the treatment response of childhood nephrotic syndrome. <i>Cytokine.</i> 2003 Feb 7;21(3):155-9.
TNF ALPHA/IL-14	Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. <i>Thorax.</i> 2002 Sep;57(9):774-8.
TNF ALPHA/IL-15	Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. <i>Thorax.</i> 2002 Sep;57(9):774-8.
TNF ALPHA/IL-16	- Tumor necrosis factor-alpha-induced synthesis of interleukin-16 in airway epithelial cells: priming for serotonin stimulation. <i>Am J Respir Cell Mol Biol.</i> 2003 Mar;28(3):354-62. (airway inflammation) - Correlation of circulating interleukin 16 with proinflammatory cytokines in patients with rheumatoid arthritis. <i>Rheumatology (Oxford).</i> 2001 Apr;40(4):474-5. No abstract available. - Interleukin 16 is up-regulated in Crohn's disease and participates in TNBS colitis in mice. <i>Gastroenterology.</i> 2000 Oct;119(4):972-82.
TNF ALPHA/IL-17	- Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with <i>Borrelia burgdorferi</i>. <i>Infect Immun.</i> 2003 Jun;71(6):3437-42. - Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction <i>in vitro</i>. <i>Ann Rheum Dis.</i> 2002 Oct;61(10):870-6. - A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. <i>Eur Respir J.</i> 2003 Mar;21(3):387-93. (Airway inflammation) - Abstract Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. <i>Arthritis Rheum.</i> 2001 Sep;44(9):2078-83.
TNF ALPHA/IL-18	- Association of interleukin-18 expression with enhanced levels of both interleukin-1beta and tumor necrosis factor alpha in knee synovial tissue of patients with rheumatoid arthritis. <i>Arthritis Rheum.</i> 2003 Feb;48(2):339-47. - Abstract Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy. <i>Metabolism.</i> 2003 May;52(5):605-8.
TNF ALPHA/IL-19	Abstract IL-19 induces production of IL-6 and TNF-alpha and results in cell apoptosis through TNF-alpha. <i>J Immunol.</i> 2002 Oct 15;169(8):4288-97.
TNF ALPHA/IL-20	Abstract Cytokines: IL-20 - a new effector in skin inflammation. <i>Curr Biol.</i> 2001 Jul 10;11(13):R531-4
TNF ALPHA/Complement	Inflammation and coagulation: implications for the septic patient. <i>Clin Infect Dis.</i> 2003 May 15;36(10):1259-65. Epub 2003 May 08. Review.
TNF ALPHA/IFN- γ	- MHC induction in the brain. - Synergize in anti-viral response/IFN-β induction. - Neutrophil activation/ respiratory burst. - Endothelial cell activation - Toxicities noted when patients treated with TNF/IFN-γ as anti-viral therapy

	• Fractalkine expression by human astrocytes. • Many papers on inflammatory responses – i.e. LPS, also macrophage activation. • Anti-TNF and anti-IFN-γ synergize to protect mice from lethal endotoxemia.
TGF- β /IL-1	• Prostaglandin synthesis by osteoblasts • IL-6 production by intestinal epithelial cells (inflammation model) • Stimulates IL-11 and IL-6 in lung fibroblasts (inflammation model) • IL-6 and IL-8 production in the retina
TGF- β /IL-6	• Chondrocarcoma proliferation
IL-1/IL-2	• B-cell activation • LAK cell activation • T-cell activation • IL-1 synergy with IL-2 in the generation of lymphokine activated killer cells is mediated by TNF-alpha and beta (lymphotoxin). <i>Cytokine</i>. 1992 Nov;4(6):479-87.
IL-1/IL-3	
IL-1/IL-4	• B-cell activation • IL-4 induces IL-1 expression in endothelial cell activation.
IL-1/IL-5	
IL-1/IL-6	• B cell activation • T cell activation (can replace accessory cells) • IL-1 induces IL-6 expression • C3 and serum amyloid expression (acute phase response) • HIV expression • Cartilage collagen breakdown.
IL-1/IL-7	• IL-7 is requisite for IL-1-induced thymocyte proliferation. Involvement of IL-7 in the synergistic effects of granulocyte-macrophage colony-stimulating factor or tumor necrosis factor with IL-1. <i>J Immunol</i>. 1992 Jan 1;148(1):99-105.
IL-1/IL-8	
IL-1/IL-10	
IL-1/IL-11	• Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. <i>Am J Physiol Cell Physiol</i>. 2002 Sep;283(3):C679-87. (Bone loss)
IL-1/IL-16	• Correlation of circulating interleukin 16 with proinflammatory cytokines in patients with rheumatoid arthritis. <i>Rheumatology</i> (Oxford). 2001 Apr;40(4):474-5. No abstract available.
IL-1/IL-17	• Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with <i>Borrelia burgdorferi</i>. <i>Infect Immun</i>. 2003 Jun;71(6):3437-42. • Contribution of interleukin 17 to human cartilage degradation and synovial inflammation in osteoarthritis. <i>Osteoarthritis Cartilage</i>. 2002 Oct;10(10):799-807. • Abstract Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. <i>Arthritis Rheum</i>. 2001 Sep;44(9):2078-83.
IL-1/IL-18	• Association of interleukin-18 expression with enhanced levels of both interleukin-1beta and tumor necrosis factor alpha in knee synovial tissue of patients with rheumatoid arthritis. <i>Arthritis Rheum</i>. 2003 Feb;48(2):339-47.
IL-1/IFN-g	
IL-2/IL-3	• T-cell proliferation • B cell proliferation

IL-2/IL-4	• B-cell proliferation • T-cell proliferation • (selectively inducing activation of CD8 and NK lymphocytes)IL-2R beta agonist P1-30 acts in synergy with IL-2, IL-4, IL-9, and IL-15: biological and molecular effects. <i>J Immunol.</i> 2000 Oct 15;165(8): 4312-8.
IL-2/IL-5	• B-cell proliferation/ Ig secretion • IL-5 induces IL-2 receptors on B-cells
IL-2/IL-6	• Development of cytotoxic T-cells
IL-2/IL-7	
IL-2/IL-9	• See IL-2/IL-4 (NK-cells)
IL-2/IL-10	• B-cell activation
IL-2/IL-12	• IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. <i>Cell Immunol.</i> 1995 Oct 1;165(1):33-43. (T-cell activation)
IL-2/IL-15	• See IL-2/IL-4 (NK cells) • (T cell activation and proliferation) IL-15 and IL-2: a matter of life and death for T cells <i>in vivo.</i> <i>Nat Med.</i> 2001 Jan;7(1):114-8.
IL-2/IL-16	• Synergistic activation of CD4+ T cells by IL-16 and IL-2. <i>J Immunol.</i> 1998 Mar 1;160(5):2115-20.
IL-2/IL-17	• Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. <i>J Pathol.</i> 2002 Jul;197(3):322-32.
IL-2/IL-18	• Interleukin 18 (IL-18) in synergy with IL-2 induces lethal lung injury in mice: a potential role for cytokines, chemokines, and natural killer cells in the pathogenesis of interstitial pneumonia. <i>Blood.</i> 2002 Feb 15;99(4):1289-98.
IL-2/TGF- β	• Control of CD4 effector fate: transforming growth factor beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. <i>J Exp Med.</i> 1995 Sep 1;182(3):699-709.
IL-2/IFN- γ	• Ig secretion by B-cells • IL-2 induces IFN-γ expression by T-cells
IL-2/IFN- α/β	• None
IL-3/IL-4	• Synergize in mast cell growth • Synergistic effects of IL-4 and either GM-CSF or IL-3 on the induction of CD23 expression by human monocytes: regulatory effects of IFN-alpha and IFN-gamma. <i>Cytokine.</i> 1994 Jul;6(4):407-13.
IL-3/IL-5	
IL-3/IL-6	
IL-3/IFN- γ	• IL-4 and IFN-gamma synergistically increase total polymeric IgA receptor levels in human intestinal epithelial cells. Role of protein tyrosine kinases. <i>J Immunol.</i> 1996 Jun 15;156(12):4807-14.
IL-3/GM-CSF	• Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (allergic inflammation)
IL-4/IL-2	• IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-γ expression in murine NK cells. <i>Blood.</i> 2003 Mar 13 [Epub ahead of print]
IL-4/IL-5	• Enhanced mast cell histamine etc. secretion in response to IgE • A Th2-like cytokine response is involved in bullous pemphigoid. the role of IL-4 and IL-5 in the pathogenesis of the disease. <i>Int J Immunopathol Pharmacol.</i> 1999 May-Aug;12(2):55-61.
IL-4/IL-6	
IL-4/IL-10	
IL-4/IL-11	• Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. <i>Blood.</i>

	1991 Sep 15;78(6):1448-51.
IL-4/IL-12	• Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-gamma production by dendritic cells. <i>J Immunol.</i> 2000 Jan 1;164(1):64-71. (increase Th1/Th2 differentiation) • IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-γ expression in murine NK cells. <i>Blood.</i> 2003 Mar 13 [Epub ahead of print]
IL-4/IL-13	• Abstract Interleukin-4 and interleukin-13 signaling connections maps. <i>Science.</i> 2003 Jun 6;300(5625):1527-8. (allergy, asthma) • Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting established allergy in a mouse model for allergic asthma. <i>J Allergy Clin Immunol.</i> 2003 Jun;111(6):1361-1369.
IL-4/IL-16	• (asthma) Interleukin (IL)-4/IL-9 and exogenous IL-16 induce IL-16 production by BEAS-2B cells, a bronchial epithelial cell line. <i>Cell Immunol.</i> 2001 Feb 1;207(2):75-80
IL-4/IL-17	• Interleukin (IL)-4 and IL-17 synergistically stimulate IL-6 secretion in human colonic myofibroblasts. <i>Int J Mol Med.</i> 2002 Nov;10(5):631-4. (Gut inflammation)
IL-4/IL-24	• IL-24 is expressed by rat and human macrophages. <i>Immunobiology.</i> 2002 Jul;205(3):321-34.
IL-4/IL-25	• Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol.</i> 2002 Jul 1;169(1):443-53. (allergic inflammation) • Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood.</i> 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)
IL-4/IFN- γ	• Abstract Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-gamma. <i>Eur J Immunol.</i> 1991 Jan;21(1):97-101.
IL-4/SCF	• Regulation of human intestinal mast cells by stem cell factor and IL-4. <i>Immunol Rev.</i> 2001 Feb;179:57-60. Review.
IL-5/IL-3	• Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (Allergic inflammation see abstract)
IL-5/IL-6	
IL-5/IL-13	• Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: role of eosinophils, IL-5, eotaxin, and IL-13. <i>J Allergy Clin Immunol.</i> 2003 May;111(5):1049-61.
IL-5/IL-17	• Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. <i>Am J Respir Cell Mol Biol.</i> 2003 Jan;28(1):42-50.
IL-5/IL-25	• Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol.</i> 2002 Jul 1;169(1):443-53. (allergic inflammation) • Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood.</i> 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)
IL-5/IFN- γ	
IL-5/GM-CSF	• Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (Allergic inflammation)
IL-6/IL-10	
IL-6/IL-11	
IL-6/IL-16	• Interleukin-16 stimulates the expression and production of pro-

	inflammatory cytokines by human monocytes. <i>Immunology</i> . 2000 May;100(1):63-9.
IL-6/IL-17	Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. <i>J Biol Chem</i>. 2003 May 9;278(19):17036-43. Epub 2003 Mar 06. (airway inflammation, asthma)
IL-6/IL-19	Abstract IL-19 induces production of IL-6 and TNF-alpha and results in cell apoptosis through TNF-alpha. <i>J Immunol</i>. 2002 Oct 15;169(8):4288-97.
IL-6/IFN-g	
IL-7/IL-2	Interleukin 7 worsens graft-versus-host disease. <i>Blood</i>. 2002 Oct 1;100(7):2642-9.
IL-7/IL-12	Synergistic effects of IL-7 and IL-12 on human T cell activation. <i>J Immunol</i>. 1995 May 15;154(10):5093-102.
IL-7/IL-15	Interleukin-7 and interleukin-15 regulate the expression of the bcl-2 and c-myc genes in cutaneous T-cell lymphoma cells. <i>Blood</i>. 2001 Nov 1;98(9):2778-83. (growth factor)
IL-8/IL-11	Abnormal production of interleukin (IL)-11 and IL-8 in polycythaemia vera. <i>Cytokine</i>. 2002 Nov 21;20(4):178-83.
IL-8/IL-17	- The Role of IL-17 in Joint Destruction. <i>Drug News Perspect</i>. 2002 Jan;15(1):17-23. (arthritis) - Abstract Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. <i>Am J Respir Cell Mol Biol</i>. 2002 Jun;26(6):748-53. (airway inflammation)
IL-8/GSF	Interleukin-8: an autocrine/paracrine growth factor for human hematopoietic progenitors acting in synergy with colony stimulating factor-1 to promote monocyte-macrophage growth and differentiation. <i>Exp Hematol</i>. 1999 Jan;27(1):28-36.
IL-8/VEGF	Intracavitary VEGF, bFGF, IL-8, IL-12 levels in primary and recurrent malignant glioma. <i>J Neurooncol</i>. 2003 May;62(3):297-303.
IL-9/IL-4	Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. <i>Am J Respir Crit Care Med</i>. 2002 Aug 1;166(3):409-16.
IL-9/IL-5	- Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. <i>J Clin Invest</i>. 2002 Jan;109(1):29-39. - Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. <i>Respir Res</i>. 2001;2(2):80-4. Epub 2001 Feb 15. Review. - Abstract Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. <i>Blood</i>. 2000 Sep 15;96(6):2163-71 (asthma)
IL-9/IL-13	- Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. <i>Am J Respir Crit Care Med</i>. 2002 Aug 1;166(3):409-16. - Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. <i>Nat Med</i>. 2002 Aug;8(8):885-9.
IL-9/IL-16	See IL-4/IL-16
IL-10/IL-2	The interplay of interleukin-10 (IL-10) and interleukin-2 (IL-2) in humoral immune responses: IL-10 synergizes with IL-2 to enhance responses of human B lymphocytes in a mechanism which is different from upregulation of CD25 expression. <i>Cell Immunol</i>. 1994 Sep;157(2):478-88.
IL-10/IL-12	
IL-10/TGF-beta	IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. <i>Eur J Immunol</i>. 2003 May;33(5):1205-14.
IL-10/IFN-gamma	

IL-11/IL-6	Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. <i>Bone</i>. 2003 Jan;32(1):1-7. (bone resorption in inflammation)
IL-11/IL-17	- Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis) - IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. <i>J Immunol</i>. 2003 Mar 1;170(5):2655-62.
IL-11/TGF- β	Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis)
IL-12/IL-13	Relationship of Interleukin-12 and Interleukin-13 imbalance with class-specific rheumatoid factors and anticardiolipin antibodies in systemic lupus erythematosus. <i>Clin Rheumatol</i>. 2003 May;22(2):107-11.
IL-12/IL-17	Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. <i>Scand J Gastroenterol</i>. 2003 Feb;38(2):180-5.
IL-12/IL-18	- Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. <i>Cytokine</i>. 1999 Nov;11(11):822-30. - Inflammatory Liver Steatosis Caused by IL-12 and IL-18. <i>J Interferon Cytokine Res</i>. 2003 Mar;23(3):155-62.
IL-12/IL-23	- Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. <i>Nature</i>. 2003 Feb 13;421(6924):744-8. - Abstract A unique role for IL-23 in promoting cellular immunity. <i>J Leukoc Biol</i>. 2003 Jan;73(1):49-56. Review.
IL-12/IL-27	Abstract IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. <i>Immunity</i>. 2002 Jun;16(6):779-90.
IL-12/IFN- γ	IL-12 induces IFN-γ expression by B and T-cells as part of immune stimulation.
IL-13/IL-5	See IL-5/IL-13
IL-13/IL-25	- Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol</i>. 2002 Jul 1;169(1):443-53. (allergic inflammation) - Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood</i>. 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)
IL-15/IL-13	Differential expression of interleukins (IL)-13 and IL-15 in ectopic and eutopic endometrium of women with endometriosis and normal fertile women. <i>Am J Reprod Immunol</i>. 2003 Feb;49(2):75-83.
IL-15/IL-16	IL-15 and IL-16 overexpression in cutaneous T-cell lymphomas: stage-dependent increase in mycosis fungoides progression. <i>Exp Dermatol</i>. 2000 Aug;9(4):248-51.
IL-15/IL-17	Abstract IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. <i>J Immunol</i>. 2003 Feb 15;170(4):2106-12. (airway inflammation)
IL-15/IL-21	IL-21 in Synergy with IL-15 or IL-18 Enhances IFN-gamma Production in Human NK and T Cells. <i>J Immunol</i>. 2003 Jun 1;170(11):5464-9.
IL-17/IL-23	Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. <i>J Biol Chem</i>. 2003 Jan 17;278(3):1910-4. Epub 2002 Nov 03
IL-17/TGF- β	Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis)
IL-18/IL-12	Synergistic proliferation and activation of natural killer cells by interleukin

	12 and interleukin 18. <i>Cytokine</i>. 1999 Nov;11(11):822-30. • Abstract Inhibition of in vitro immunoglobulin production by IL-12 in murine chronic graft-vs.-host disease: synergism with IL-18. <i>Eur J Immunol</i>. 1998 Jun;28(6):2017-24.
IL-18/IL-21	• IL-21 in Synergy with IL-15 or IL-18 Enhances IFN-gamma Production in Human NK and T Cells. <i>J Immunol</i>. 2003 Jun 1;170(11):5464-9.
IL-18/TGF- β	• Interleukin 18 and transforming growth factor beta1 in the serum of patients with Graves' ophthalmopathy treated with corticosteroids. <i>Int Immunopharmacol</i>. 2003 Apr;3(4):549-52.
IL-18/IFN- γ	
Anti-TNF ALPHA/anti-CD4	• Synergistic therapeutic effect in DBA/1 arthritic mice.

Annex 3: Oncology combinations

Target	Disease	Pair with
CD89*	Use as cytotoxic cell recruiter	all
CD19	B cell lymphomas	HLA-DR CD5
HLA-DR	B cell lymphomas	CD89 CD19 CD5
CD38	Multiple myeloma	CD138 CD56 HLA-DR
CD138	Multiple myeloma	CD38 CD56 HLA-DR
CD138	Lung cancer	CD56 CEA
CD33	Acute myelod lymphoma	CD34 HLA-DR
CD56	Lung cancer	CD138 CEA
CEA	Pan carcinoma	MET receptor
VEGF	Pan carcinoma	MET receptor
VEGF receptor	Pan carcinoma	MET receptor
IL-13	Asthma/pulmonary inflammation	IL-4 IL-5 Eotaxin(s) MDC TARC TNF α IL-9 EGFR CD40L IL-25 MCP-1 TGF β
IL-4	Asthma	IL-13 IL-5 Eotaxin(s) MDC TARC TNF α IL-9 EGFR CD40L IL-25 MCP-1 TGF β
Eotaxin	Asthma	IL-5 Eotaxin-2 Eotaxin-3
EGFR	cancer	HER2/neu HER3 HER4
HER2	cancer	HER3

		HER4
TNFR1	RA/Crohn's disease	IL-1R IL-6R IL-18R
TNF α	RA/Crohn's disease	IL-1 α/β IL-6 IL-18 ICAM-1 IL-15 IL-17
IL-1R	RA/Crohn's disease	IL-6R IL-18R
IL-18R	RA/Crohn's disease	IL-6R

Annex 4Data Summary

TARGET	dAb	Equilibrium dissociation constant ($K_d = K_{off}/K_{on}$)	K _{off}	IC ₅₀ for ligand assay	ND50 for cell based neutralisation assay
TAR1	TAR1 monomers	300nM to 5pM (ie, 3×10^{-7} to 5×10^{-12}), preferably 50nM to 20pM	5×10^{-1} to 1×10^{-7}	500nM to 100pM	500nM to 50pM
	TAR1 dimers	As TAR1 monomer	As TAR1 monomer	As TAR1 monomer	As TAR1 monomer
	TAR1 trimers	As TAR1 monomer	As TAR1 monomer	As TAR1 monomer	As TAR1 monomer
	TAR1-5				
	TAR1-27				
	TAR1-5-19 monomer	30nM			
	TAR1-5-19 homodimer			With (Gly ₄ Ser) ₃ linker = 20nm With (Gly ₄ Ser) ₅ linker = 2nm With (Gly ₄ Ser) ₇ linker = 10nm In Fab format = 1nM	=30nM =3nM =15nM
	TAR1-5-19 heterodimers			With (Gly ₄ Ser) _n linker TAR1-5-19 d2 = 2nM TAR1-5-19 d3 = 8nM TAR1-5-19 d4 = 2-5nM TAR1-5-19 d5 = 8nM In Fab format TAR1-5-19CH d1CK = 6nM TAR1-5-19CH d1CH = 6nM TAR1-5-19CH d2CK = 8nM TAR1-5-19CH d3CK = 3nM	= 12nM = 10nM = 12nM

149

TAR1-5 heterodimers			With (Gly ₄ Ser) _n linker TAR1-5d1 = 30nM TAR1-5d2 = 50nM TAR1-5d3 = 300nM TAR1-5d4 = 3nM TAR1-5d5 = 200nM TAR1-5d6 = 100nM In Fab format TAR1-5CH d2CK = 30nM TAR1-5CK d3CH = 100nM = 60nM	
TAR1-5-19 homotrimer			0.3nM	3-10nM (eg, 3nM)
TAR2 monomers	As TAR1 monomer	As TAR1 monomer	500nM to 100pM	500nM to 50pM
TAR2-10				
TAR2-5				
Serum Albumin	Anti-SA monomers	In Dual Specific format, target affinity is 1 to 100,000 x affinity of SA dAb affinity, eg 100pM (target) and 10 μ M SA affinity.	In Dual Specific format, target affinity is 1 to 100,000 x affinity of SA dAb affinity, eg 100pM (target) and 10 μ M SA affinity.	
	MSA-16	200nM		
	MSA-26	70nM		

Claims

1. A dual specific ligand comprising a first dAb specific for a target ligand, and a second dAb specific for a receptor for the target ligand.
5
2. A dual specific ligand according to claim 1, which is an open conformation ligand and can bind both the target ligand and the target ligand receptor simultaneously.
3. A dual specific ligand according to claim 1 or claim 2, wherein the target ligand is
10 TNF α .
4. A dual specific ligand according to claim 3, wherein the dAb specific for TNF α dissociates from human TNF α with a dissociation constant (K_d) of 50nM to 20pM, and a K_{off} rate constant of 5×10^{-1} to $1 \times 10^{-7} \text{ s}^{-1}$, as determined by surface plasmon resonance.
15
5. A dual specific ligand according to claim 3 or claim 4, wherein the dAb specific for TNF α is a V κ .
6. A dual specific ligand according to claim 5, wherein the dAb specific for
20 TNF α comprises the amino acid sequence of TAR1-5-19 or a sequence that is at least 80% homologous thereto.
7. A dual specific ligand according to claim 5, wherein the dAb specific for TNF α comprises the amino acid sequence of TAR1-5 or a sequence that is at least 80%
25 homologous thereto.
8. A dual specific ligand according to claim 5, wherein the dAb specific for TNF α comprises the amino acid sequence of TAR1-27 or a sequence that is at least 80% homologous thereto.
30
9. A dual specific ligand according to any one of claims 1 to 3, wherein the target ligand receptor is TNF receptor 1 (p55).

10. A dual specific ligand according to claim 9, wherein the dAb specific for TNF receptor 1 (p55) dissociates from human TNF receptor 1 with a dissociation constant (K_d) of 50nM to 20pM, and a K_{off} rate constant of 5×10^{-1} to $1 \times 10^{-7} \text{ s}^{-1}$, as determined by surface plasmon resonance.

5

11. A dual specific ligand according to claim 10, wherein the dAb specific for TNF receptor 1 (p55) neutralises human TNF α or TNF receptor 1 in a standard cell assay with an ND50 of 500nM to 50pM.

10 12. A dual specific ligand according to claim 10 or claim 11, wherein the dAb specific for TNF receptor 1 (p55) antagonises the activity of the TNF receptor 1 in a standard cell assay with an ND₅₀ of $\leq 100\text{nM}$, and at a concentration of $\leq 10\mu\text{M}$ the dAb agonises the activity of the TNF receptor 1 by $\leq 5\%$ in the assay.

15 13. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-10 or a sequence that is at least 80% homologous thereto.

14. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb
20 specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-10 or a sequence that is at least 90% homologous thereto.

15. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb
25 specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-5 or a sequence that is at least 80% homologous thereto.

16. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb
specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-5 or a
sequence that is at least 90% homologous thereto.

30

17. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb
specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-10-27 or
a sequence that is at least 80% homologous thereto.

18. A dual specific ligand according to any one of claims 10 to 12, wherein the dAb specific for TNF receptor 1 (p55) comprises the amino acid sequence of TAR2h-10-27 or a sequence that is at least 90% homologous thereto.
- 5 19. A dual specific ligand according to any one of claims 3 to 18, wherein the TNF α and/or TNF receptor 1 (p55) is in human form.
20. A dual specific ligand according to any preceding claim, wherein one or more
10 dAbs further comprises a terminal Cys residue.
21. A dual specific ligand according to claim 1 or claim 2, wherein the target ligand is TNF α and the target ligand receptor is TNF Receptor 1 (p55).
- 15 22. A dual specific ligand according to claim 21, comprising a TAR1-5-19 V_K domain or a sequence that is at least 80% homologous thereto and a TAR2h-10-27 V_H domain or a sequence that is at least 80% homologous thereto.
23. A dual specific ligand according to claim 22, wherein the TAR1-5-19 V_K domain
20 and a TAR2h-10-27 V_H domain, or the homologues thereof, are arranged as complementary domains in a binding site.
24. A dual specific ligand according to any preceding claim, which is a dimer.
- 25 25. A dual specific ligand according to any one of claims 1 to 23, which is a trimer.
26. A dual specific ligand according to claim 23, comprising two ligands specific for TNF α and one ligand specific for TNF Receptor 1 (p55).
- 30 27. A dual specific ligand according to any preceding claim, said dual specific ligand being selected from the group consisting of a Fab, a F(ab')₂ and an IgG immunoglobulin.

28. A dual specific ligand selected from the group consisting of a Fab, a F(ab')₂ and an IgG immunoglobulin, wherein said dual specific ligand is in an open conformation and can bind two or more targets simultaneously, and said dual specific ligand comprises at least one dAb specific for TNF α which is TAR1-5-19 or a sequence at least 80% homologous thereto.

29. A dual specific ligand according to claim 28, which comprises at least one further dAb, which further dAb is a V_H domain.

30. A dual specific ligand selected from the group consisting of a Fab, a F(ab')₂ and an IgG immunoglobulin, wherein said dual specific ligand is in an open conformation and can bind two or more targets simultaneously, and said dual specific ligand comprises at least one dAb specific for TNF Receptor 1 (p55) which is TAR2h-10-27 or a sequence at least 80% homologous thereto.

31. A dual specific ligand according to claim 28, which comprises at least one further dAb, which further dAb is a V_K domain.

32. A dual specific ligand according to claim 29 or claim 31, wherein the further dAb binds serum albumin (SA).

33. A dual specific ligand according to claim 32, wherein the dAb specific for serum albumin (SA) dissociates from SA with a dissociation constant (K_d) of 1nM to 500 μ M, as determined by surface plasmon resonance.

34. A dual specific ligand according to claim 32 or claim 33, wherein the dAb specific for serum albumin (SA) binds SA in a standard ligand binding assay with an IC₅₀ of 1nM to 500 μ M.

35. A dual specific ligand according to any one of claims 32 to 34, wherein the dAb specific for serum albumin (SA) comprises the amino acid sequence of MSA-16 or a sequence that is at least 80% homologous thereto.

36. A dual specific ligand according to any one of claims 32 to 34, wherein the dAb specific for serum albumin (SA) comprises the amino acid sequence of MSA-26 or a sequence that is at least 80% homologous thereto.
- 5 37. A dual specific selected from the group consisting of a Fab, a F(ab')₂ and an IgG immunoglobulin, wherein said dual specific ligand is in an open conformation and can bind two or more targets simultaneously, and said dual specific ligand comprises at least one dAb specific for TNF Receptor 1 (p55) which is TAR2h-10-27 or a sequence at least 80% homologous thereto, and at least one dAb specific for TNF α which is TAR1-5-19 or
10 a sequence at least 80% homologous thereto.
38. A dual specific ligand according to any one of claims 27 to 37, which comprises a universal framework.
- 15 39. A dual specific ligand according to claim 38, wherein the universal framework comprises a V_H framework selected from the group consisting of DP47, DP45 and DP38; and/or the V_L framework is DPK9.
40. A dual specific ligand according to any one of claims 27 to 39, which comprises a
20 binding site for a generic ligand.
41. A dual specific ligand according to claim 40, wherein the generic ligand binding site is selected from the group consisting of protein A, protein L and protein G.
- 25 42. A dual specific ligand according to any one of claims 27 to 41, wherein the ligand comprises a variable domain having one or more framework regions comprising an amino acid sequence that is the same as the amino acid sequence of a corresponding framework region encoded by a human germline antibody gene segment, or the amino acid sequences of one or more of said framework regions collectively comprises up to 5 amino acid
30 differences relative to the amino acid sequence of said corresponding framework region encoded by a human germline antibody gene segment.

43. A dual specific ligand according to any one of claims 27 to 41, wherein the ligand comprises a variable domain, wherein the amino acid sequences of FW1, FW2, FW3 and FW4 are the same as the amino acid sequences of corresponding framework regions encoded by a human germline antibody gene segment, or the amino acid sequences of FW1, FW2, FW3 and FW4 collectively contain up to 10 amino acid differences relative to the amino acid sequences of corresponding framework regions encoded by said human germline antibody gene segment.

44. A dual specific ligand according to claim 42 or claim 43, which comprises an antibody variable domain comprising FW1, FW2 and FW3 regions, and the amino acid sequence of said FW1, FW2 and FW3 are the same as the amino acid sequences of corresponding framework regions encoded by human germline antibody gene segments.

45. A dual specific ligand according to any one of claims 42 to 44, wherein said human germline antibody gene segment is selected from the group consisting of DP47, DP45, DP48 and DPK9.

46. A dual specific ligand according to any preceding claim, comprising a V_H domain that is not a Camelid immunoglobulin variable domain.

47. The ligand of claim 46, comprising a V_H domain that does not contain one or more amino acids that are specific to Camelid immunoglobulin variable domains as compared to human V_H domains.

48. A dAb monomer dAb specific for TNF Receptor 1 (p55) which is TAR2h-10-27 or a sequence at least 80% homologous thereto.

49. A nucleic acid encoding a dAb monomer according to claim 48.

50. A nucleic acid vector comprising a nucleic acid according to claim 49.

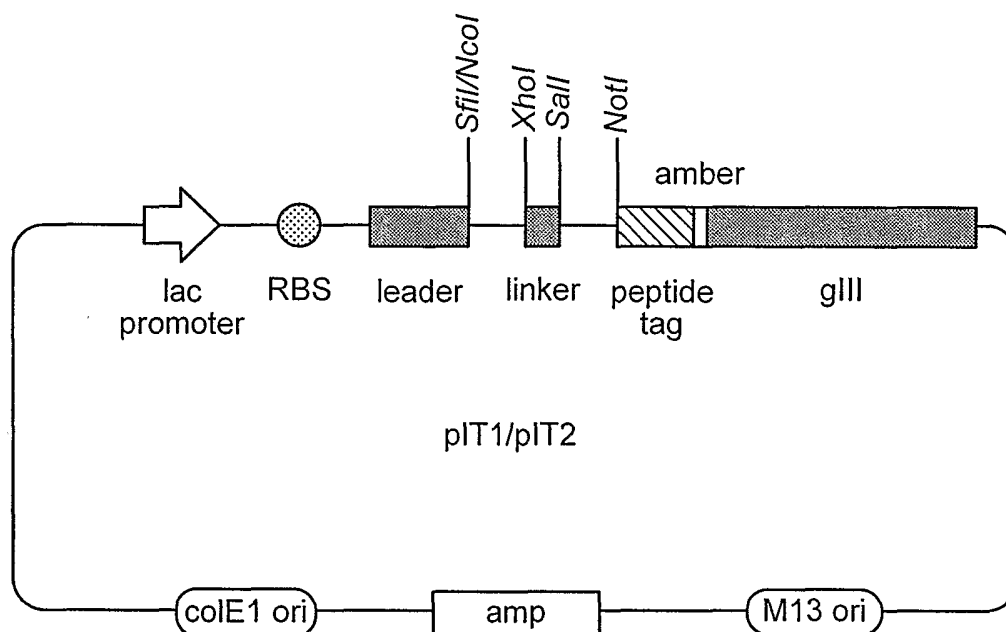
1 / 28

FIG. 1

H1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	F	T	F	S	H30
	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG	TCC	CTG	AGA	CTC	TCC	TGT	GCA	GCC	TCT	GGA	TTC	ACC	TTT	AGC	
	H10										H20										H30										
	H40										H50										H60										
	S	Y	A	M	S	W	V	R	Q	A	P	G	K	G	L	E	W	V	S	A	I	S	G	S	G	S	T	Y	Y		
	AGC	TAT	GCC	ATG	ATG	AGC	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGG	CTG	GAG	TGG	GTC	TCA	GCT	ATT	AGT	GGT	AGT	GGT	AGC	ACA	TAC	TAC	
	H70										H80										H90										
	A	D	S	V	K	G	R	F	T	I	S	R	D	N	S	K	N	T	L	Y	L	Q	M	N	S	L	R	A	E	D	
	GCA	GAC	TCC	GTG	AAG	GGC	CGG	TTC	ACC	ATC	TCC	AGA	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT	CTG	CAA	ATG	AAC	AGC	CTG	AGA	GCC	GAG	GAC	
	H100										H110										H120										
	T	A	V	Y	Y	C	A	K	S	Y	G	A	F	D	Y	W	G	Q	G	T	L	V	T	V	S	S	G	G	G	G	
	ACG	GCC	GTA	TAT	TAC	TGT	CGC	AAA	AGT	TAT	GGT	GCT	TTT	GAC	TAC	TGG	GGC	CAG	GGA	ACC	CTG	GTC	ACC	GTC	TCG	AGC	GGT	GGA	GGC	GGT	
	H130										H140										H150										
	S	G	G	G	G	S	G	G	G	G	S	T	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	
	TCA	GGC	GGA	GGT	GGC	AGC	AGC	GGC	GGT	GGC	TCG	ACG	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT	GTA	GGA	GAC	AGA	
	L1										L2										L3										
	V	T	I	T	C	R	A	S	Q	S	I	S	S	Y	L	N	W	Y	Q	Q	K	P	G	K	A	P	K	L	L	I	
	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC	ATT	AGC	AGC	TAT	TTA	AAT	TGG	TAT	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT	AAG	CTC	CTG	ATC	
	L4										L5										L6										
	Y	A	A	S	S	L	Q	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	
	TAT	GCT	GCA	TCC	AGT	TTG	CAA	AGT	GGG	GTC	CCA	TCA	AGG	TTC	AGT	GGC	AGT	GGG	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	
	L7										L8										L9										
	Q	P	E	D	F	A	T	Y	Y	C	Q	Q	S	Y	S	T	P	N	T	F	G	Q	G	T	K	V	E	I	K	R	
	CAA	CCT	GAA	GAT	TTT	GCA	ACT	TAC	TAC	TGT	CAA	CAG	AGT	TAC	AGT	AGC	CCT	AAAT	ACG	TTC	GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG	
	L10										L11										L12										

2 / 28

FIG. 2



RBS

CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
 -----> M K Y L

LMB3

SfiI NcoI

TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT
 L P T A A A G L L L L A A Q P A M A E V F

XhoI linker

GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT TCA GGC GGA GGT
 D Y W G Q G T L V T V S S G G G G S G G G

Sall NotI

GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG GCG GCC GCA GAA CAA AAA CTC
 G S G G G S T D I Q M T Q A A A E Q K L

<-----
 link seq new

HIS-tag

CAT CAT CAT CAC CAT CAC GGG GCC GCA
 H H H H H H G A A
 (insertion in pIT2 only)

myc-tag Gene III

ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT CAT
 I S E E D L N G A A * T V E S C L A K P H

<-----
 PHEN seq

3 / 28

FIG. 3

V_H chains

FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
1	2	3	4	5	6	7
123456789012345678901234567890	12345	67890123456789	012a3456789012345	67890123456789012345	9	10
						11
						234567890123

V_H dummy

K8	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGSGTYYADSVKVG	RFTISRDNKNTLYIQMNSLRAEDTAVYCAK	SYGAFDY	WGQGTLLVTVSS
VH2	-----	-----	-----	H-SPY-AN-R-----	-----	GLRA---	-----
VH4	-----	-----	-----	D-GAT-SK-G-----P	-----	KVLT---	-----
VHC11	-----	-----	-----	R-NGP-*A-G-----	-----I-----	HGAP---	-----
VHA10sd	-----	-----N-----	-----	S-PAS-LH-R-----	-----	PGLG---	-----
VHA1sd	-----	-----	-----	D-ERT-Y*-R-----	-----	KVLV---	-----
VHA5sd	-----	-----	-----	E-SAN-SK-Q-----	-----L-----	KVLQ---	-----
VHC5sd	-----	-----	-----	T-PAN-*V-R-----	-----	SLLQ---	-----
VHC11sd	-----S-----	-----	-----	D-AAT-SA-S-----	-----	KILK---	-----
				T-SSV-QS-R-----	-----	NLMS---	-----

V_K chains

FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
1	2	3	4	5	6	7
12345678901234567890123	45678901234	567890123456789	0123456	78901234567890123456789012345678	901234567	89012345678

V_K dummyDIQMTQSPSSLSASVGRVTITC RASQSISSYLN WYQKPGKAPKLLIY AASSLQS GVPSPFSGSGGTDFTLTITSSLPQEDFATYYC QQSSTPNT FGQGTKVEIKR

K8	-----	-----	-----	R--H---	-----	--PWRG--
E5sd	-----	-----V-----	-----	L--R---	-----	--NWL-P--
C3	-----	-----	-----	*--L---	-----	--RVYD-L--

4 / 28

FIG. 4

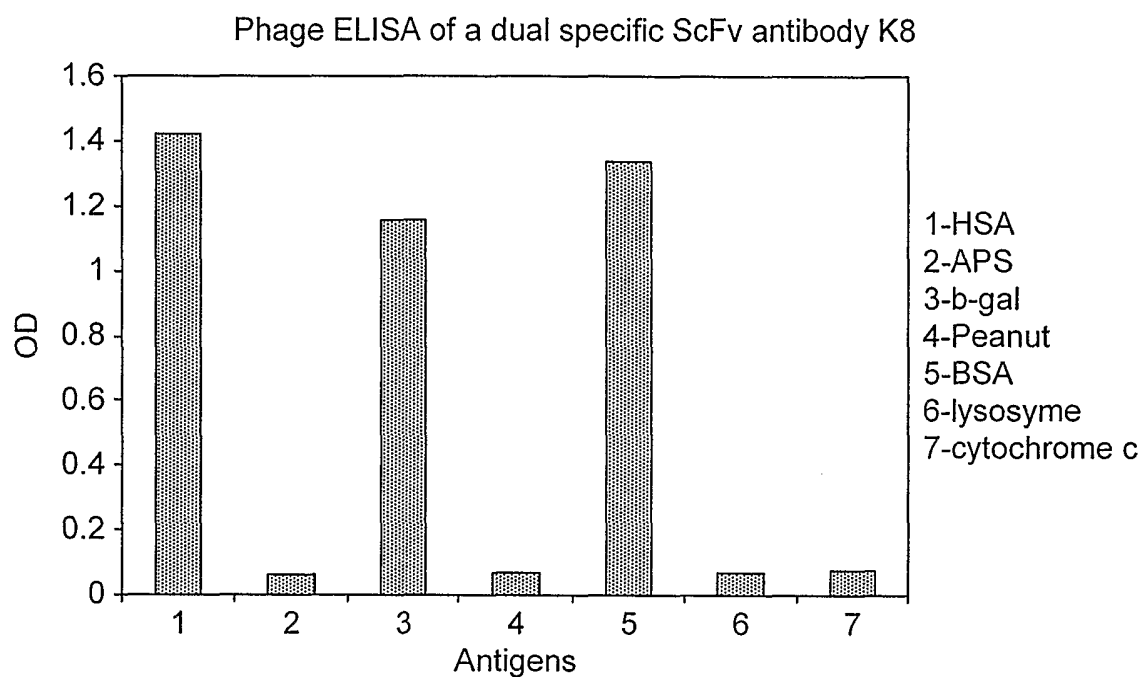
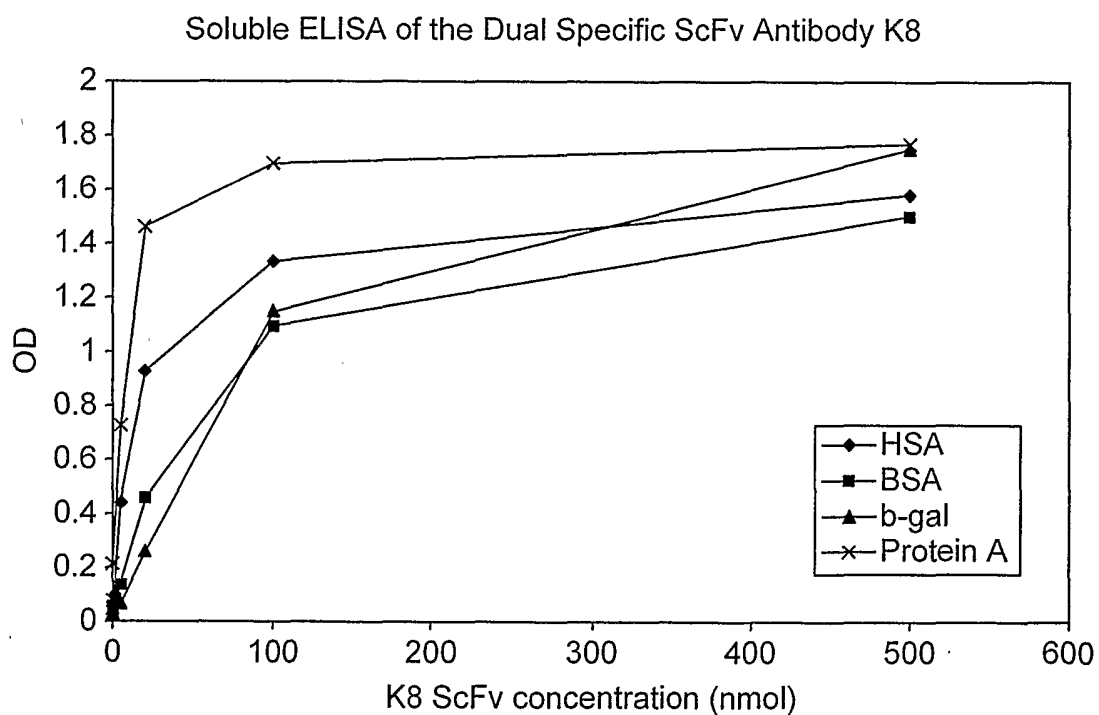


FIG. 5



5 / 28

FIG. 6

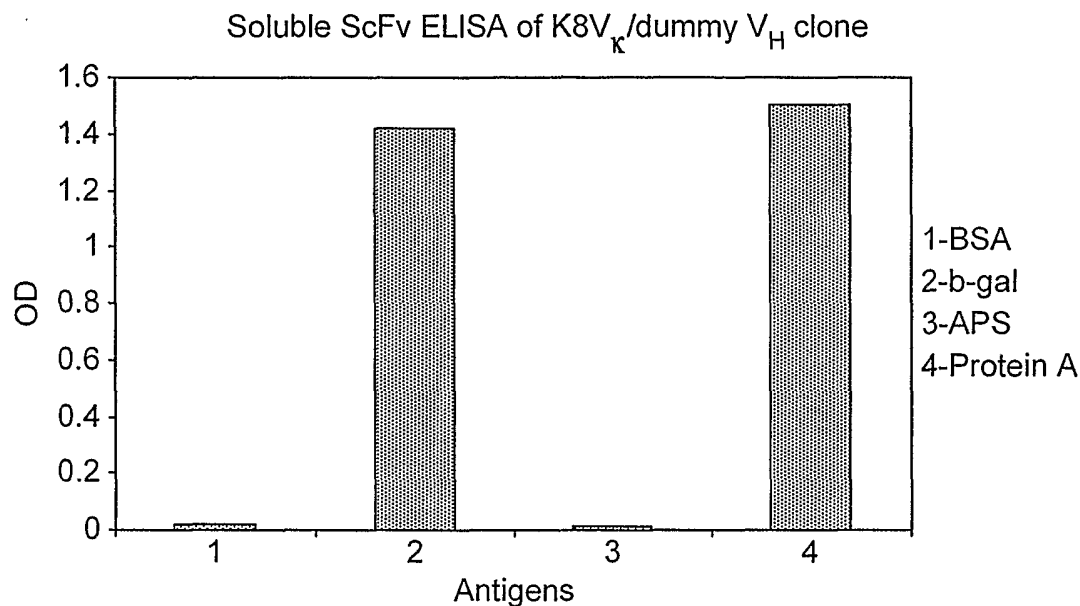


FIG. 7

RBS
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
-----> M K Y L

LMB3

SfiI NcoI
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT
L P T A A A G L L L L A A Q P A M A E V F

XhoI linker
GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT TCA GGC GGA GGT
D Y W G Q G T L V T V S S G G G G S G G G

Sali NotI
GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG GCG GCC GCA GAA CAA AAA CTC
G S G G G G S T D I Q M T Q A A A E Q K L

link seq new

HIS-tag
CAT CAT CAT CAC CAT CAC GGG GCC GCA
H H H H H H G A A
(insertion in V domain vector 2 only)

myc-tag Gene III
ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT CAT
I S E E D L N G A A * T V E S C L A K P H

----->
pHEN seq

6 / 28

FIG. 8

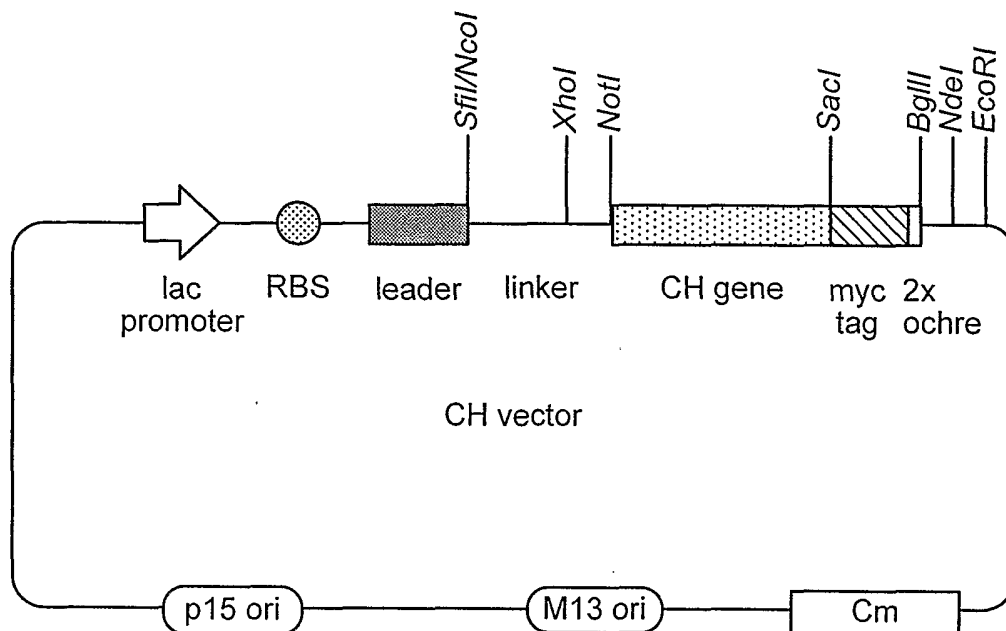
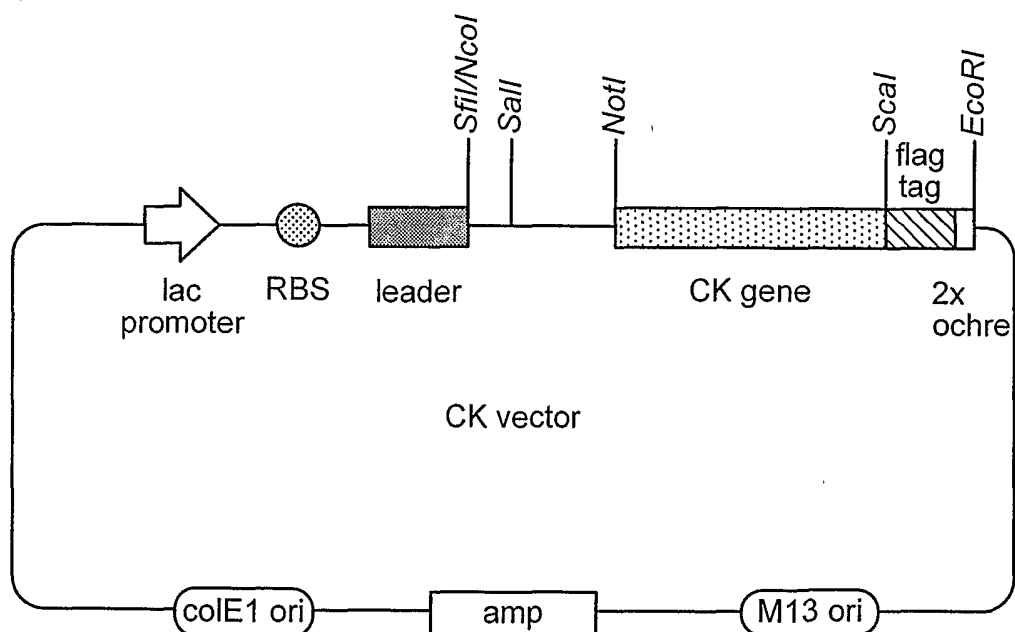


FIG. 9



7 / 28

FIG. 10

TNF receptor assay

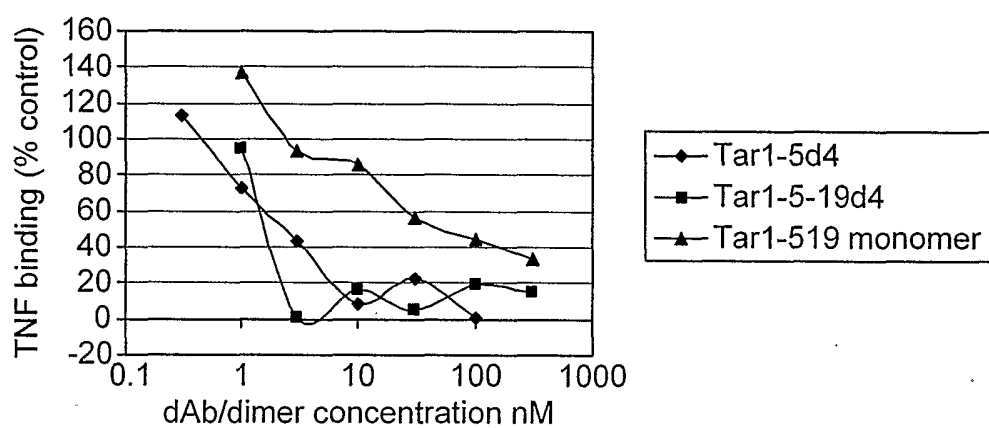
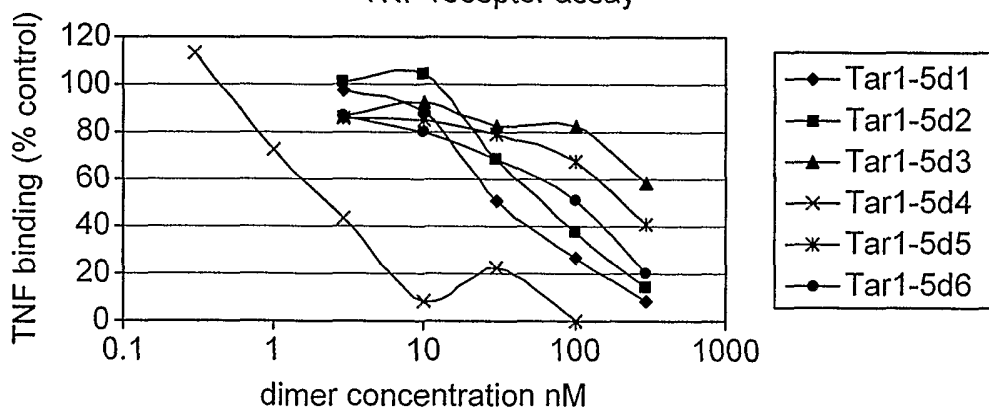


FIG. 11

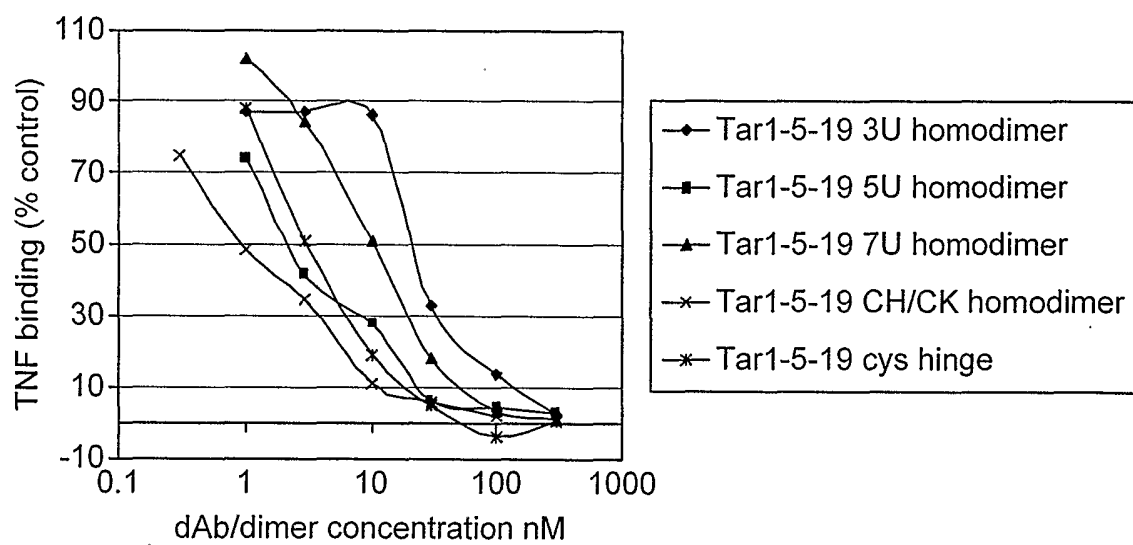
TNF receptor assay



8 / 28

FIG. 12

TNF Receptor assay



9 / 28

FIG. 13

Dummy V_H sequence for library 1

1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G
	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG
	CTC	CAC	GTC	GAC	AAC	CTC	AGA	CCC	CCT	CCG	AAC	CAT	GTC	GGA	CCC	CCC
49	S	L	R	L	S	C	A	A	S	G	F	T	F	<u>S</u>	<u>S</u>	Y
	TCC	CTG	CGT	CTC	TCC	TGT	GCA	GCC	TCC	GGA	TTC	ACC	TTT	<u>AGC</u>	<u>AGC</u>	TAT
	AGG	GAC	GCA	GAG	AGG	ACA	CGT	CGG	AGG	CCT	AAG	TGG	AAA	<u>TCG</u>	<u>TCG</u>	ATA
97	<u>A</u>	M	<u>S</u>	W	V	R	Q	A	P	G	K	G	L	E	W	V
	<u>GCC</u>	ATG	<u>AGC</u>	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGT	CTA	GAG	TGG	GTC
	<u>CGG</u>	TAC	<u>TCG</u>	ACC	CAG	GCG	GTC	CGA	GGT	CCC	TTC	CCA	GAT	CTC	ACC	CAG
145	S	<u>A</u>	I	<u>S</u>	<u>G</u>	<u>S</u>	G	<u>G</u>	<u>S</u>	T	<u>Y</u>	Y	A	D	S	V
	TCA	<u>GCT</u>	ATT	<u>AGT</u>	<u>GGT</u>	<u>AGT</u>	GGT	<u>GGT</u>	<u>AGC</u>	ACA	<u>TAC</u>	TAC	GCA	GAC	TCC	GTG
	AGT	<u>CGA</u>	TAA	<u>TCA</u>	<u>CCA</u>	<u>TCA</u>	CCA	<u>CCA</u>	<u>TCG</u>	TGT	<u>ATG</u>	ATG	CGT	CTG	AGG	CAC
193	K	G	R	F	T	I	S	R	D	N	S	K	N	T	L	Y
	AAG	GGC	CGG	TTC	ACC	ATC	TCC	CGT	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT
	TTC	CCG	GCC	AAG	TGG	TAG	AGG	GCA	CTG	TTA	AGG	TTC	TTG	TGC	GAC	ATA
241	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C
	CTG	CAA	ATG	AAC	AGC	CTG	CGT	GCC	GAG	GAC	ACC	GCG	GTA	TAT	TAC	TGT
	GAC	GTT	TAC	TTG	TCG	GAC	GCA	CGG	CTC	CTG	TGG	CGC	CAT	ATA	ATG	ACA
289	A	K	<u>S</u>	<u>Y</u>	<u>G</u>	<u>A</u>	F	D	Y	W	G	Q	G	T	L	V
	GCG	AAA	<u>AGT</u>	<u>TAT</u>	<u>GGT</u>	<u>GCT</u>	TTT	GAC	TAC	TGG	GGC	CAG	GGA	ACC	CTG	GTC
	CGC	TTT	<u>TCA</u>	<u>ATA</u>	<u>CCA</u>	<u>CGA</u>	AAA	CTG	ATG	ACC	CCG	GTC	CCT	TGG	GAC	CAG
337	T	V	S	S												
	ACC	GTC	TCG	AGC												
	TGG	CAG	AGC	TCG												

10 / 28

FIG. 14

Dummy V_H sequence for library 2

1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G
	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG
	CTC	CAC	GTC	GAC	AAC	CTC	AGA	CCC	CCT	CCG	AAC	CAT	GTC	GGA	CCC	CCC
49	S	L	R	L	S	C	A	A	S	G	F	T	F	<u>S</u>	<u>S</u>	Y
	TCC	CTG	CGT	CTC	TCC	TGT	GCA	GCC	TCC	GGA	TTC	ACC	TTT	<u>AGC</u>	<u>AGC</u>	TAT
	AGG	GAC	GCA	GAG	AGG	ACA	CGT	CGG	AGG	CCT	AAG	TGG	AAA	<u>TCG</u>	<u>TCG</u>	ATA
97	<u>A</u>	M	<u>S</u>	W	V	R	Q	A	P	G	K	G	L	E	W	V
	<u>GCC</u>	ATG	<u>AGC</u>	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGT	CTA	GAG	TGG	GTC
	<u>CGG</u>	TAC	<u>TCG</u>	ACC	CAG	GCG	GTC	CGA	GGT	CCC	TTC	CCA	GAT	CTC	ACC	CAG
145	S	<u>A</u>	I	<u>S</u>	<u>G</u>	<u>S</u>	G	<u>G</u>	<u>S</u>	T	<u>Y</u>	Y	A	D	S	V
	TCA	<u>GCT</u>	ATT	<u>AGT</u>	<u>GGT</u>	<u>AGT</u>	GGT	<u>GGT</u>	<u>AGC</u>	ACA	<u>TAC</u>	TAC	GCA	GAC	TCC	GTG
	AGT	<u>CGA</u>	TAA	<u>TCA</u>	<u>CCA</u>	<u>TCA</u>	CCA	<u>CCA</u>	<u>TCG</u>	TGT	<u>ATG</u>	ATG	CGT	CTG	AGG	CAC
193	K	G	R	F	T	I	S	R	D	N	S	K	N	T	L	Y
	AAG	GGC	CGG	TTC	ACC	ATC	TCC	CGT	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT
	TTC	CCG	GCC	AAG	TGG	TAG	AGG	GCA	CTG	TTA	AGG	TTC	TTG	TGC	GAC	ATA
241	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C
	CTG	CAA	ATG	AAC	AGC	CTG	CGT	GCC	GAG	GAC	ACC	GCG	GTA	TAT	TAC	TGT
	GAC	GTT	TAC	TTG	TCG	GAC	GCA	CGG	CTC	CTG	TGG	CGC	CAT	ATA	ATG	ACA
289	A	K	<u>S</u>	<u>Y</u>	<u>G</u>	<u>A</u>	<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>	F	D	Y	W	G	Q
	GCG	AAA	<u>AGT</u>	<u>TAT</u>	<u>GGT</u>	<u>GCT</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	TTT	GAC	TAC	TGG	GGC	CAG
	CGC	TTT	<u>TCA</u>	<u>ATA</u>	<u>CCA</u>	<u>CGA</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	AAA	CTG	ATG	ACC	CCG	GTC
337	G	T	L	V	T	V	S	S								
	GGA	ACC	CTG	GTC	ACC	GTC	TCG	AGC								
	CCT	TGG	GAC	CAG	TGG	CAG	AGC	TCG								

11 / 28

FIG. 15

Dummy V_k sequence for library 3

1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G
	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT	GTA	GGA
	CTG	TAG	GTC	TAC	TGG	GTC	AGA	GGT	AGG	AGG	GAC	AGA	CGT	AGA	CAT	CCT
49	D	R	V	T	I	T	C	R	A	S	Q	S	I	<u>S</u>	<u>S</u>	<u>Y</u>
	GAC	CGT	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC	ATT	<u>AGC</u>	<u>AGC</u>	<u>TAT</u>
	CTG	GCA	CAG	TGG	TAG	TGA	ACG	GCC	CGT	TCA	GTC	TCG	TAA	<u>TCG</u>	<u>TCG</u>	<u>ATA</u>
97	L	<u>N</u>	W	Y	Q	Q	K	P	G	K	A	P	K	L	L	I
	TTA	<u>AAT</u>	TGG	TAC	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT	AAG	CTC	CTG	ATC
	AAT	<u>TTA</u>	ACC	ATG	GTC	GTC	TTT	GGT	CCC	TTT	CGG	GGA	TTC	GAG	GAC	TAG
145	Y	<u>A</u>	A	S	<u>S</u>	L	Q	S	G	V	P	S	R	F	S	G
	TAT	<u>GCT</u>	GCA	TCC	<u>AGT</u>	TTG	CAA	AGT	GGG	GTC	CCA	TCA	CGT	TTC	AGT	GGC
	ATA	<u>CGA</u>	CGT	AGG	<u>TCA</u>	AAC	GTT	TCA	CCC	CAG	GGT	AGT	GCA	AAG	TCA	CCG
193	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
	AGT	GGA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA	CCT
	TCA	CCT	AGA	CCC	TGT	CTA	AAG	TGA	GAG	TGG	TAG	TCG	TCA	GAC	GTT	GGA
241	E	D	F	A	T	Y	Y	C	Q	Q	<u>S</u>	<u>Y</u>	<u>S</u>	<u>T</u>	P	<u>N</u>
	GAA	GAT	TTT	GCT	ACG	TAC	TAC	TGT	CAA	CAG	<u>AGT</u>	<u>TAC</u>	<u>AGT</u>	<u>ACC</u>	CCT	<u>AAT</u>
	CTT	CTA	AAA	CGA	TGC	ATG	ATG	ACA	GTT	GTC	<u>TCA</u>	<u>ATG</u>	<u>TCA</u>	<u>TGG</u>	GGA	<u>TTA</u>
289	T	F	G	Q	G	T	K	V	E	I	K	R				
	ACG	TTC	GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG				
	TGC	AAG	CCG	GTT	CCC	TGG	TTC	CAC	CTT	TAG	TTT	GCC				

12 / 28

FIG. 16

Nucleotide and amino acid sequence of anti MSA dAbs MSA 16 and MSA 26

A: MSA 16

GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT
D	I	Q	M	T	Q	S	P	S	S	L	S	A	S
GTA	GGA	GAC	CGT	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC
V	G	D	R	V	T	I	T	C	R	A	S	Q	S
ATT	ATT	AAG	CAT	TTA	AAG	TGG	TAC	CAG	CAG	AAA	CCA	GGG	AAA
I	I	K	H	L	K	W	Y	Q	Q	K	P	G	K
GCC	CCT	AAG	CTC	CTG	ATC	TAT	GGT	GCA	TCC	CGG	TTG	CAA	AGT
A	P	K	L	L	I	Y	G	A	S	R	L	Q	S
GGG	GTC	CCA	TCA	CGT	TTC	AGT	GGC	AGT	GGA	TCT	GGG	ACA	GAT
G	V	P	S	R	F	S	G	S	G	S	G	T	D
TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA	CCT	GAA	GAT	TTT	GCT
F	T	L	T	I	S	S	L	Q	P	E	D	F	A
ACG	TAC	TAC	TGT	CAA	CAG	GGG	GCT	CGG	TGG	CCT	CAG	ACG	TTC
T	Y	Y	C	Q	Q	G	A	R	W	P	Q	T	F
GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG				
G	Q	G	T	K	V	E	I	K	R				

B: MSA 26

GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT
D	I	Q	M	T	Q	S	P	S	S	L	S	A	S
GTA	GGA	GAC	CGT	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC
V	G	D	R	V	T	I	T	C	R	A	S	Q	S
ATT	TAT	TAT	CAT	TTA	AAG	TGG	TAC	CAG	CAG	AAA	CCA	GGG	AAA
I	Y	Y	H	L	K	W	Y	Q	Q	K	P	G	K
GCC	CCT	AAG	CTC	CTG	ATC	TAT	AAG	GCA	TCC	ACG	TTG	CAA	AGT
A	P	K	L	L	I	Y	K	A	S	T	L	Q	S
GGG	GTC	CCA	TCA	CGT	TTC	AGT	GGC	AGT	GGA	TCT	GGG	ACA	GAT
G	V	P	S	R	F	S	G	S	G	S	G	T	D
TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA	CCT	GAA	GAT	TTT	GCT
F	T	L	T	I	S	S	L	Q	P	E	D	F	A
ACG	TAC	TAC	TGT	CAA	CAG	GTT	CGG	AAG	GTG	CCT	CGG	ACG	TTC
T	Y	Y	C	Q	Q	V	R	K	V	P	R	T	F
GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG				
G	Q	G	T	K	V	E	I	K	R				

FIG. 17

Inhibition biacore of MSA16

(a)

0	MSA competition 19. Fc=4-3 - 10	10nM	MSA competition 19. Fc=4-3 - 11	50nM	MSA competition 19. Fc=4-3 - 12
100nM	MSA competition 19. Fc=4-3 - 13	200nM	MSA competition 19. Fc=4-3 - 14	300nM	MSA competition 19. Fc=4-3 - 15
400nM	MSA competition 19. Fc=4-3 - 16	600nM	MSA competition 19. Fc=4-3 - 17	800nM	MSA competition 19. Fc=4-3 - 18

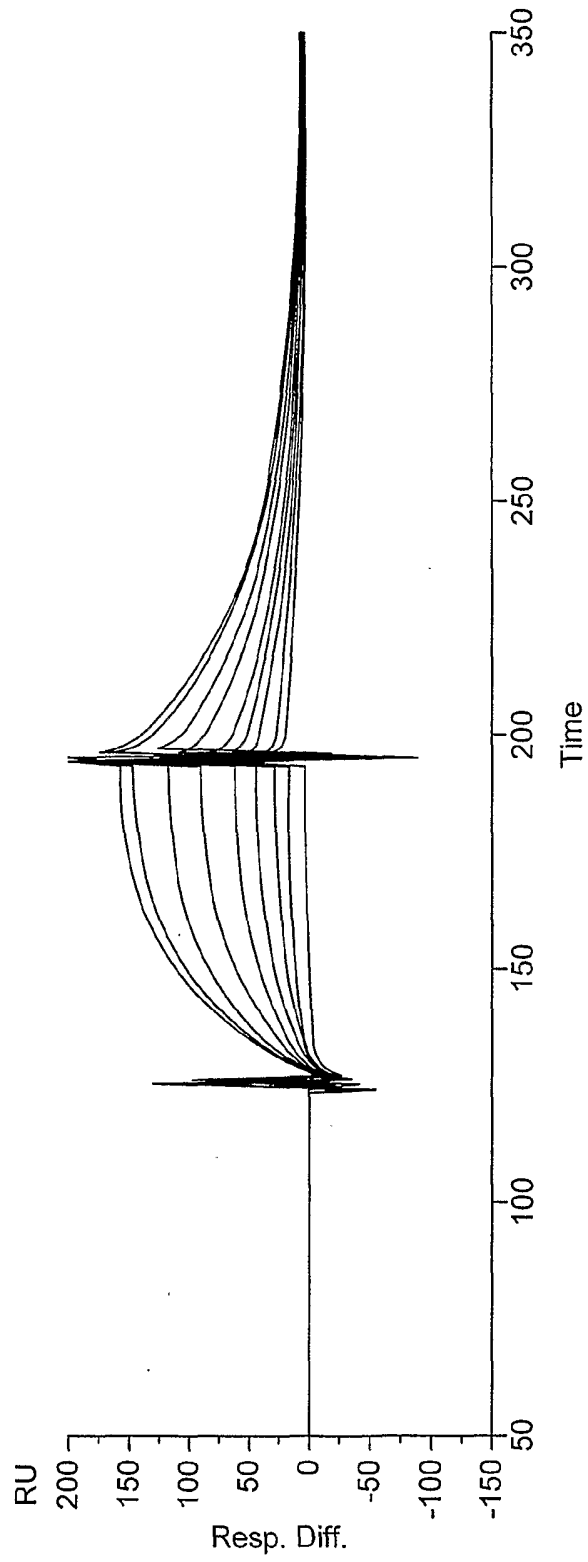


FIG. 17 CONT'D

Inhibition biacore of MSA26

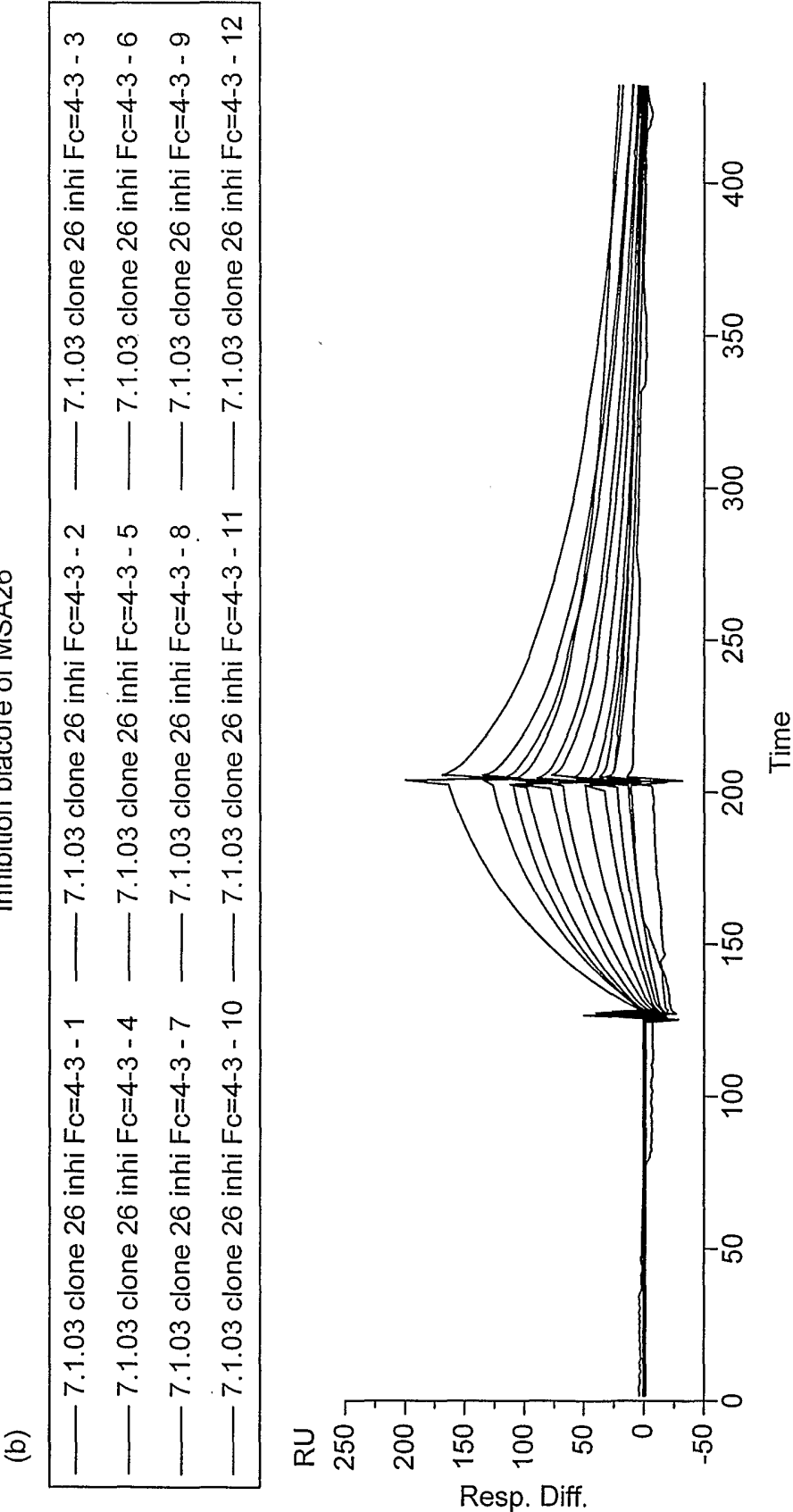
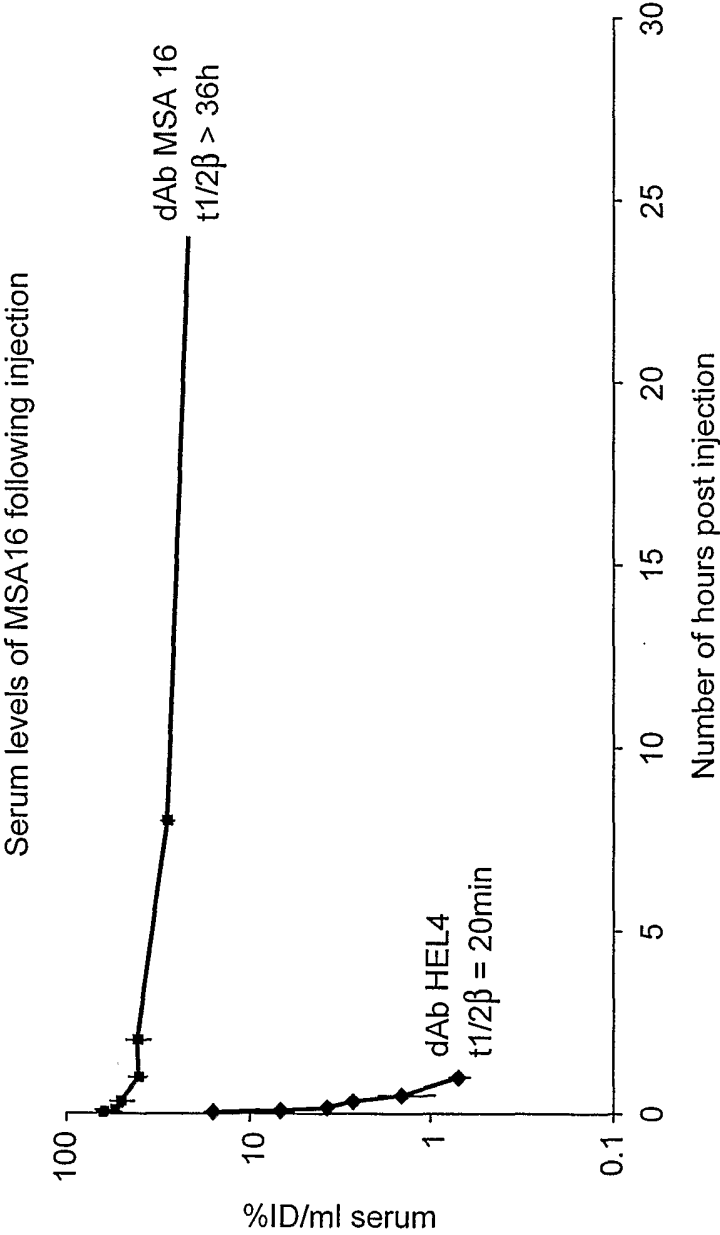
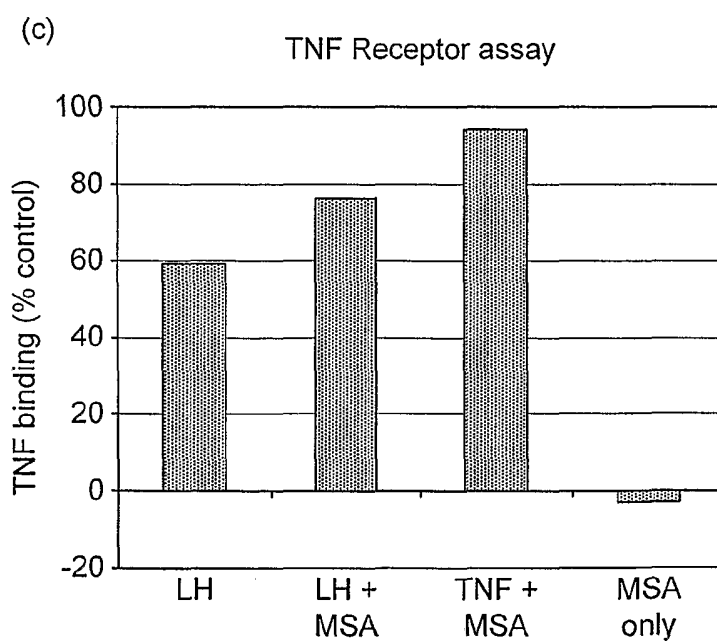
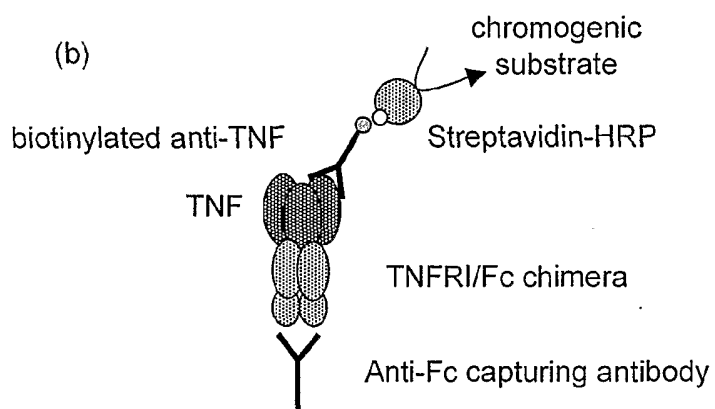
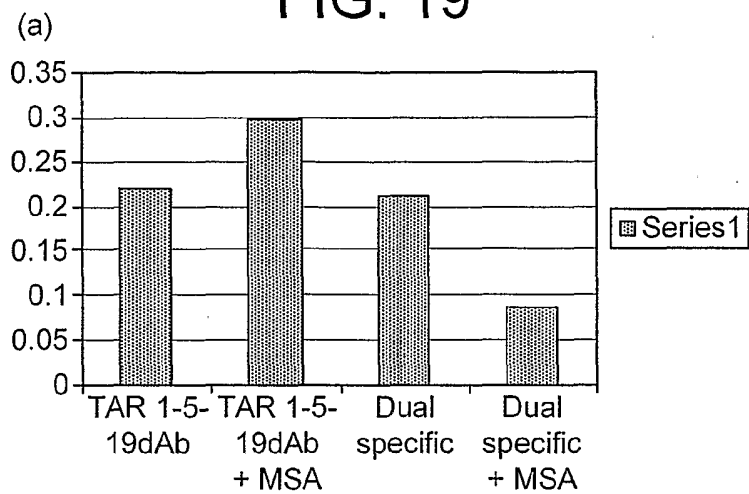


FIG. 18



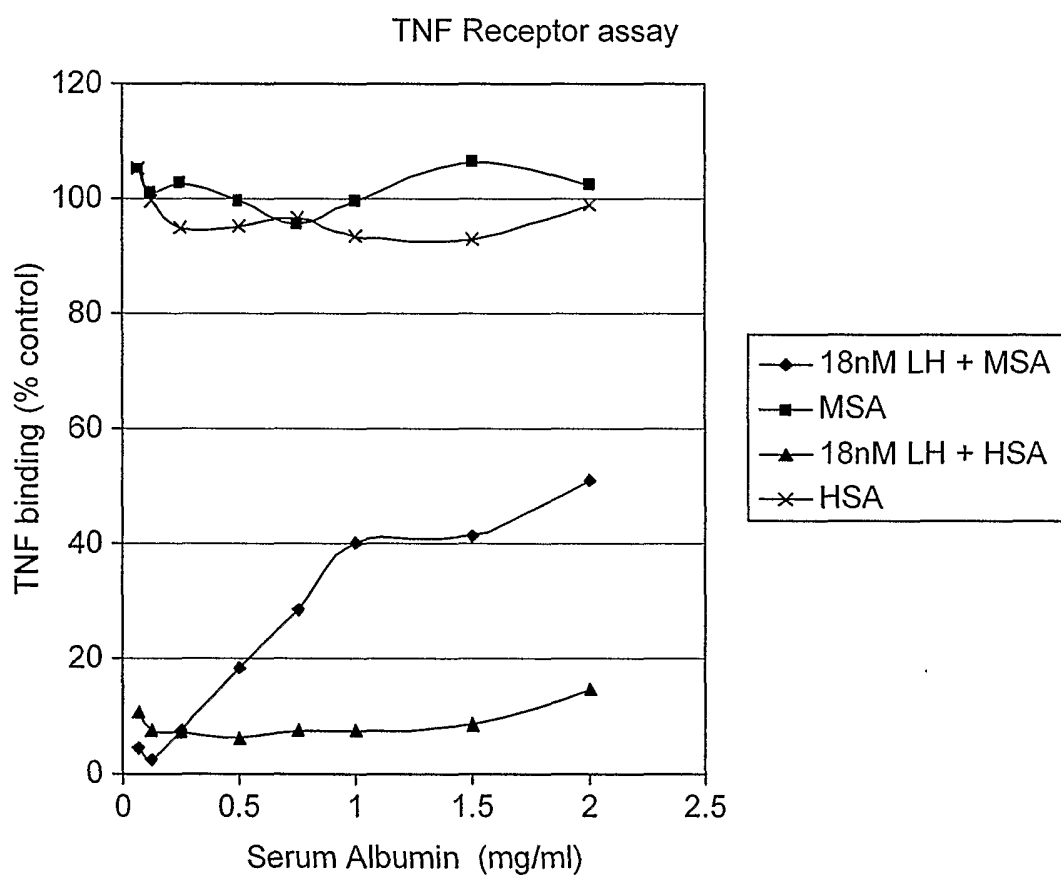
16 / 28

FIG. 19



17 / 28

FIG. 20



18 / 28

FIG. 21

Fab vectors

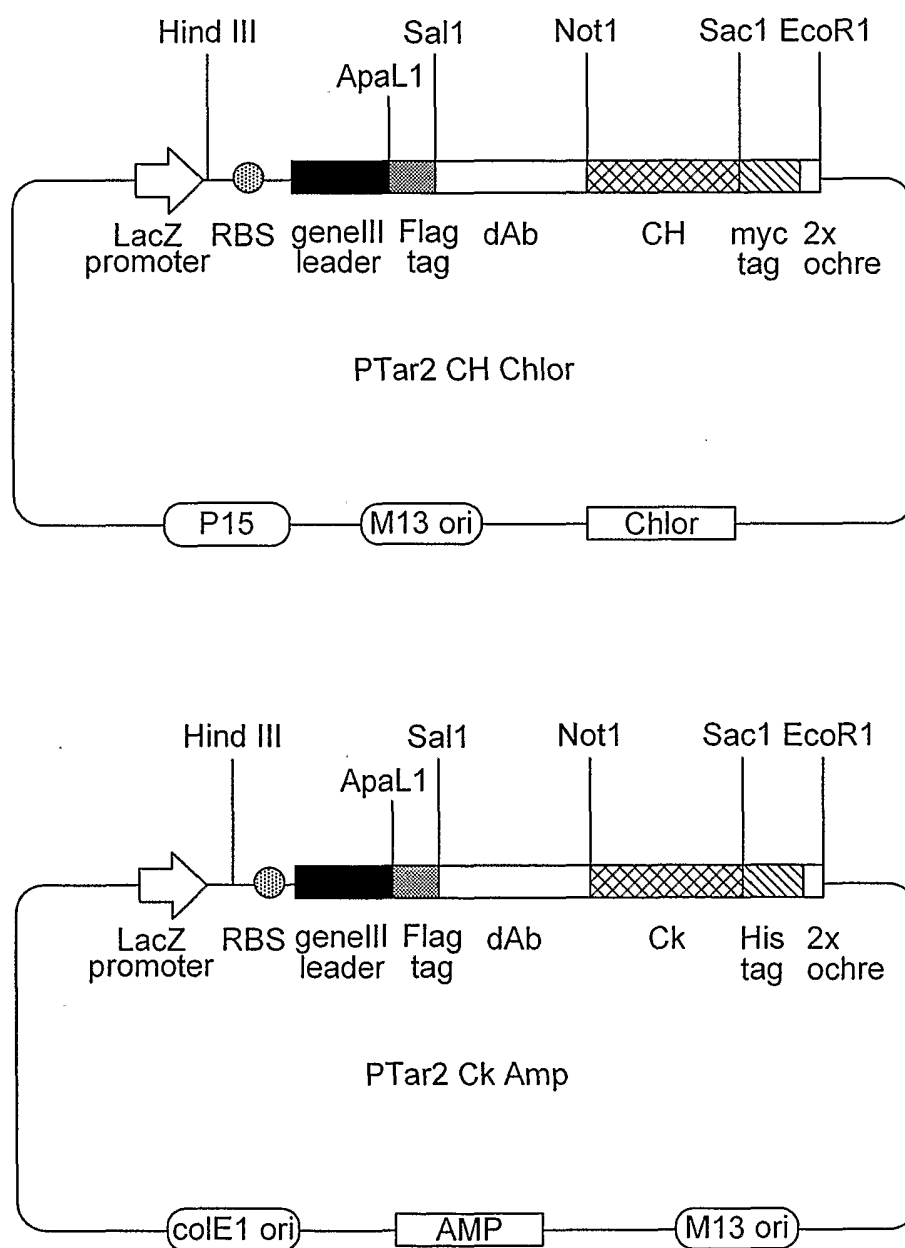


FIG. 22

Binding of the Fab to TNF and TNFRI

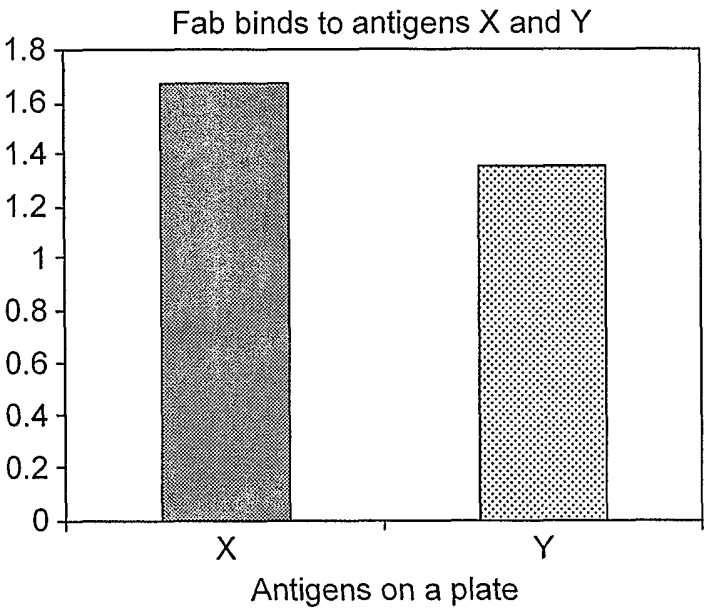


FIG. 23

Sandwich ELISA

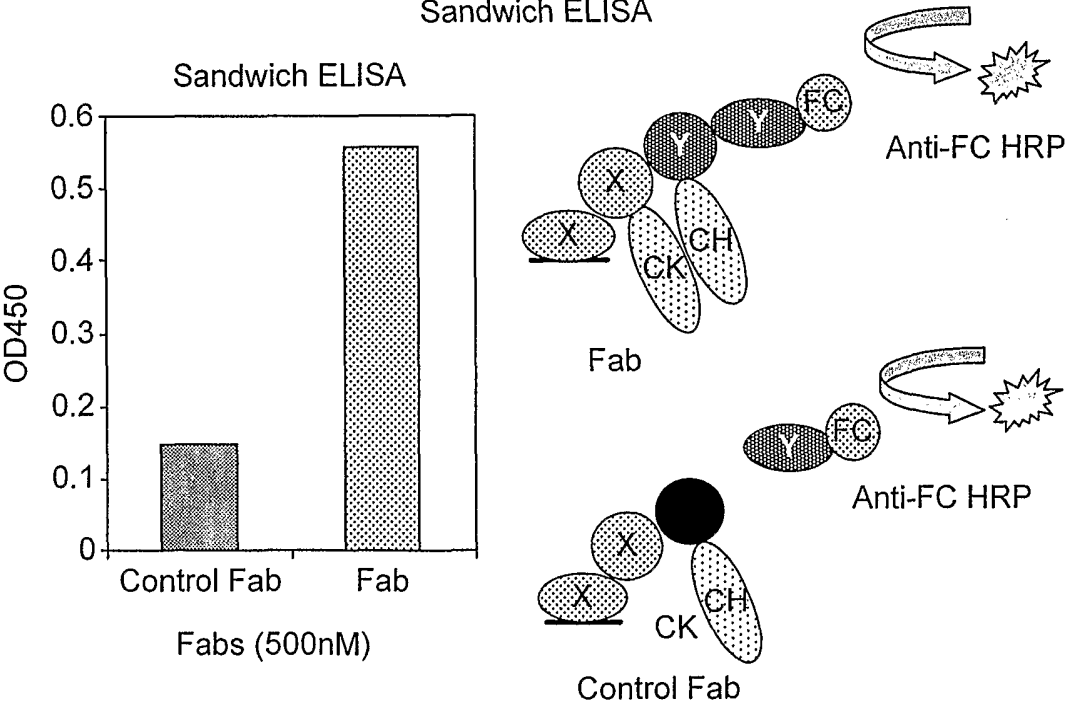


FIG. 24
Competition ELISA

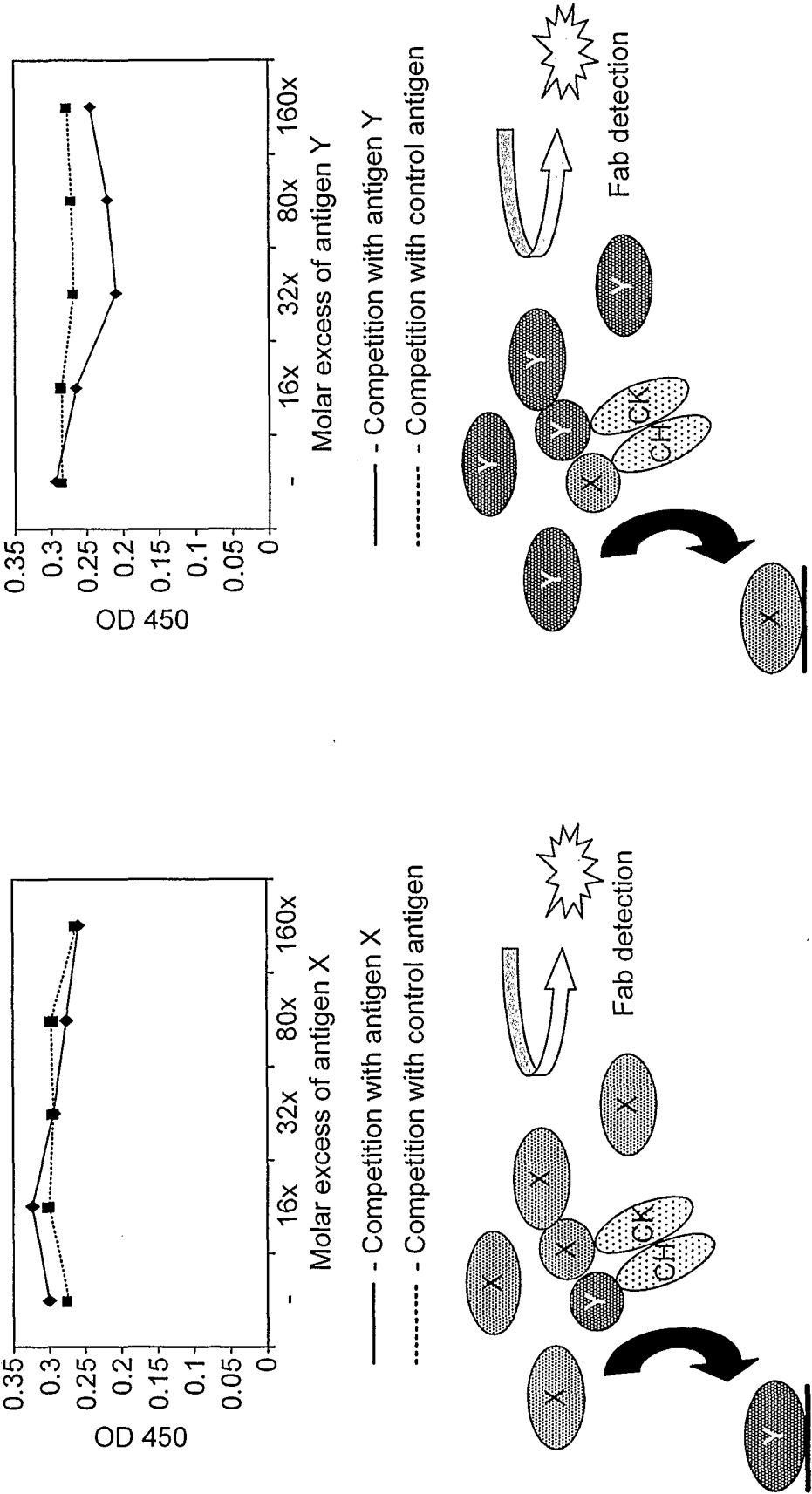


FIG. 25

Cell Assays using Fab

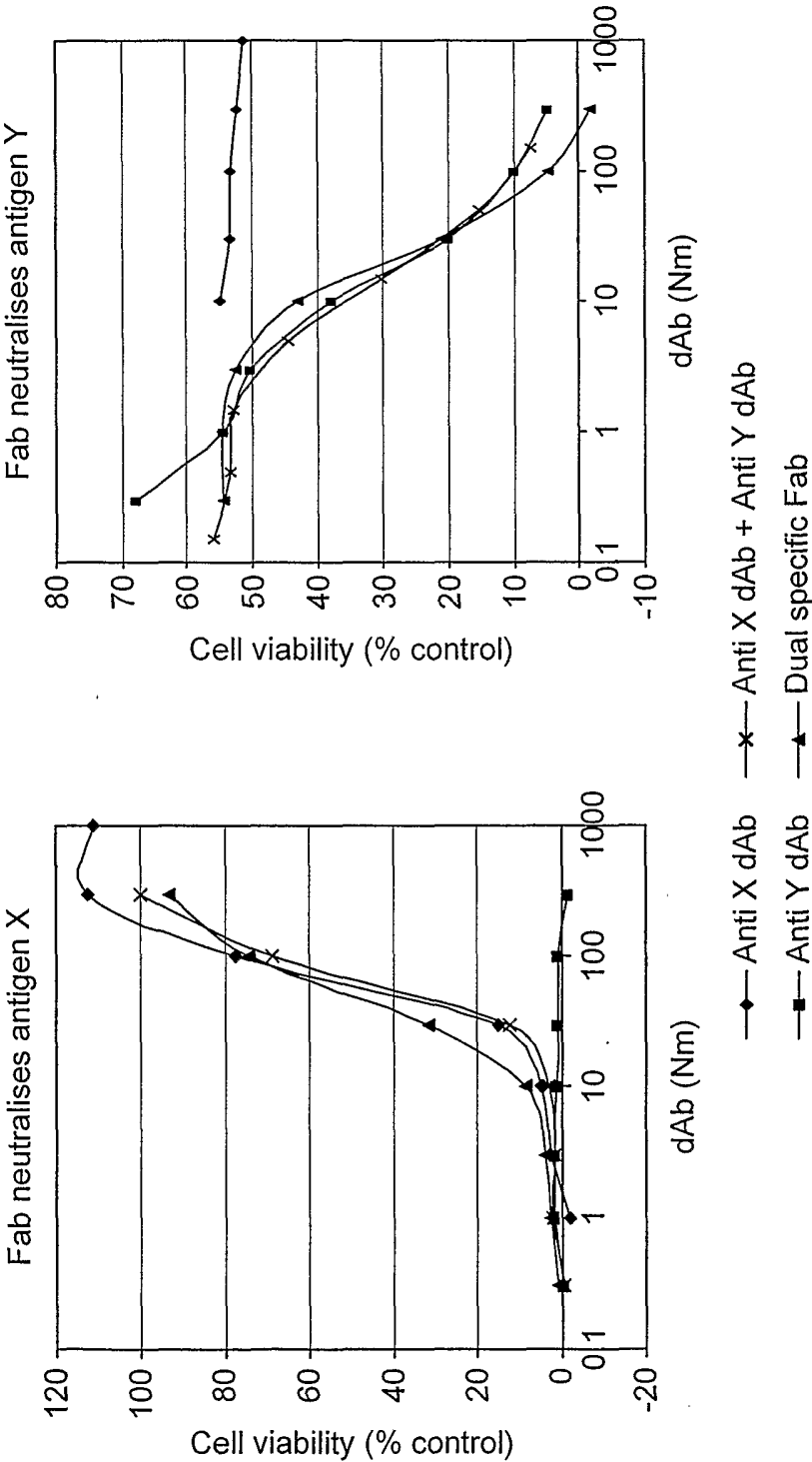


FIG. 26

Competition on cells

murine Antigen X cytotoxicity on murine cells with soluble
human Antigen Y and increasing mutant Antigen X

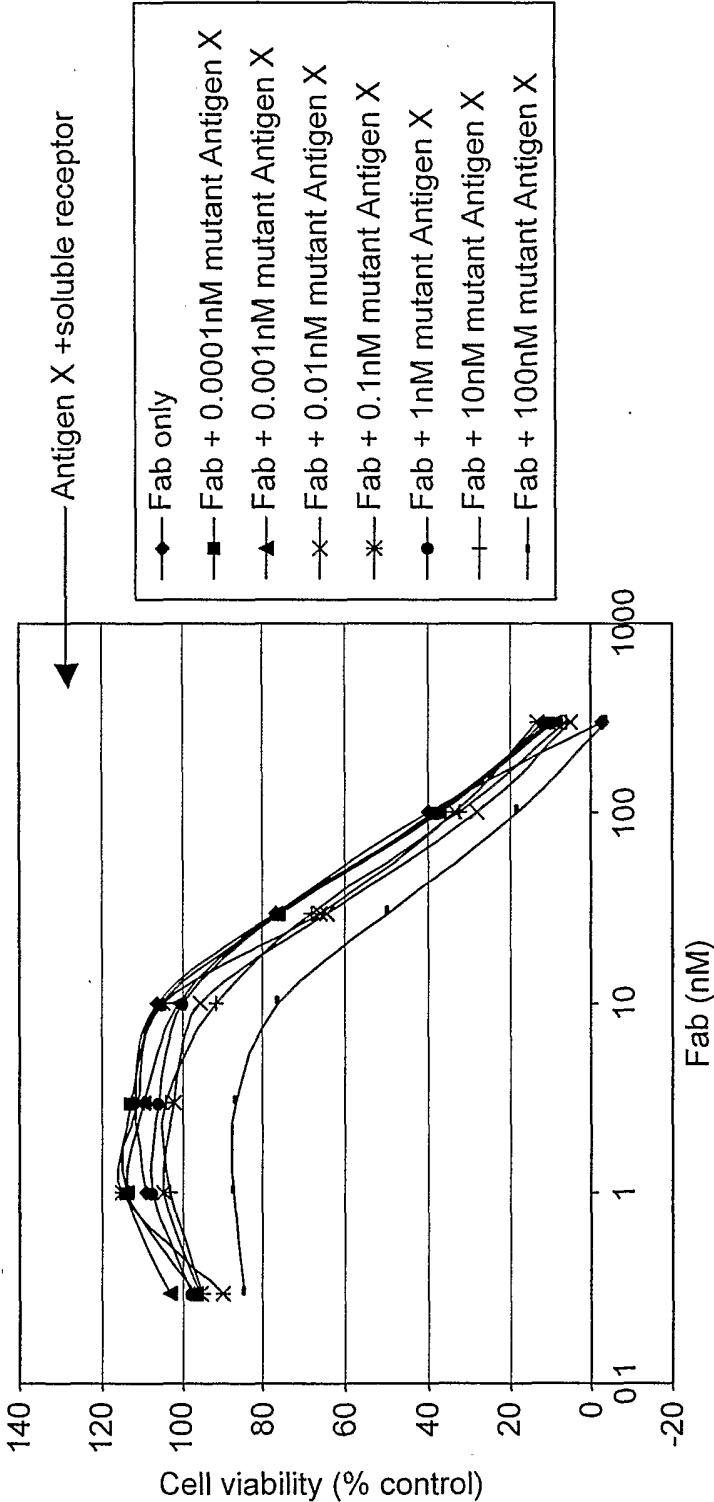
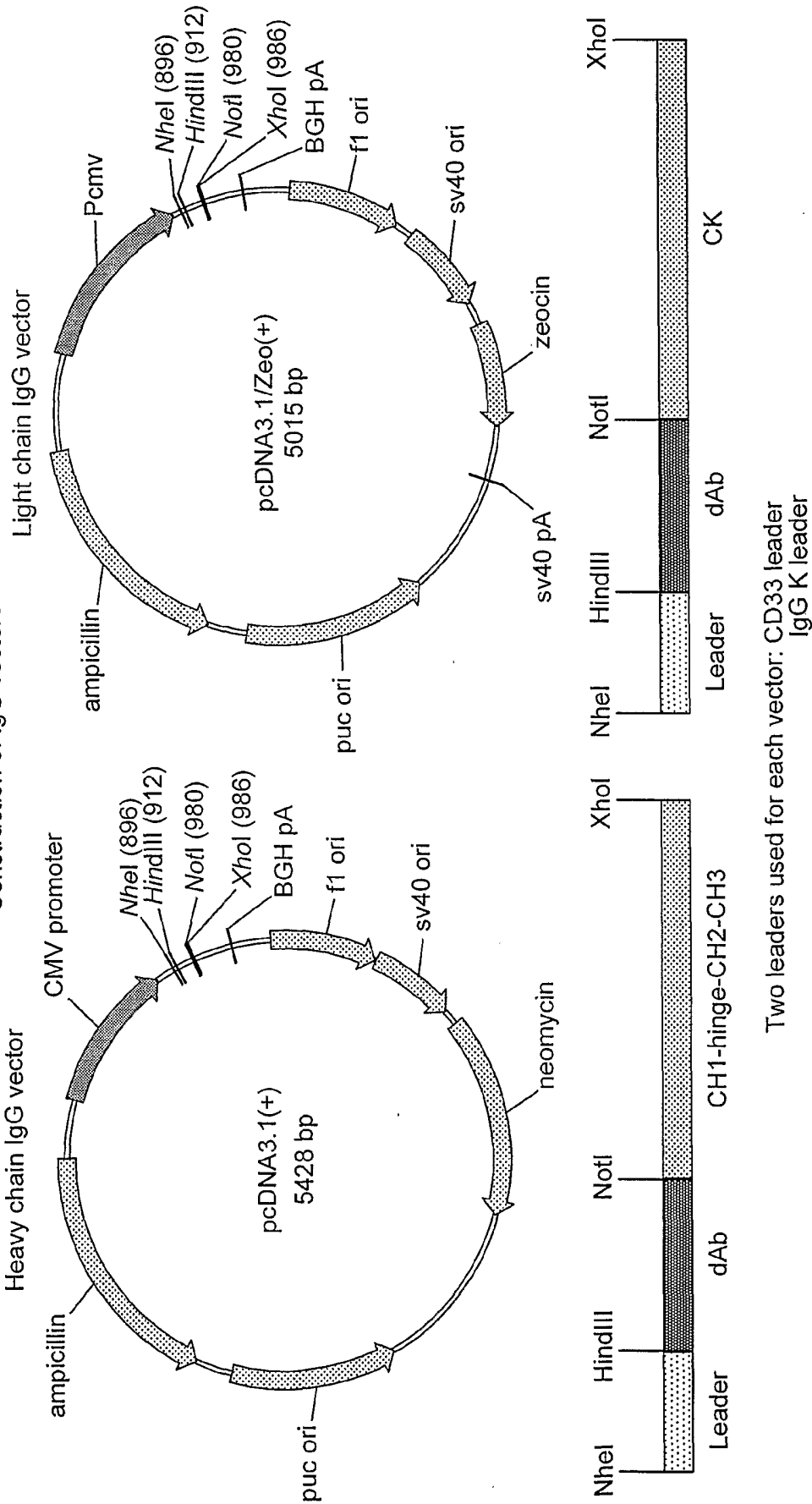


FIG. 27

Construction of IgG vectors



24 / 28

FIG. 28

Binding of the IgG to Antigen X and Antigen Y

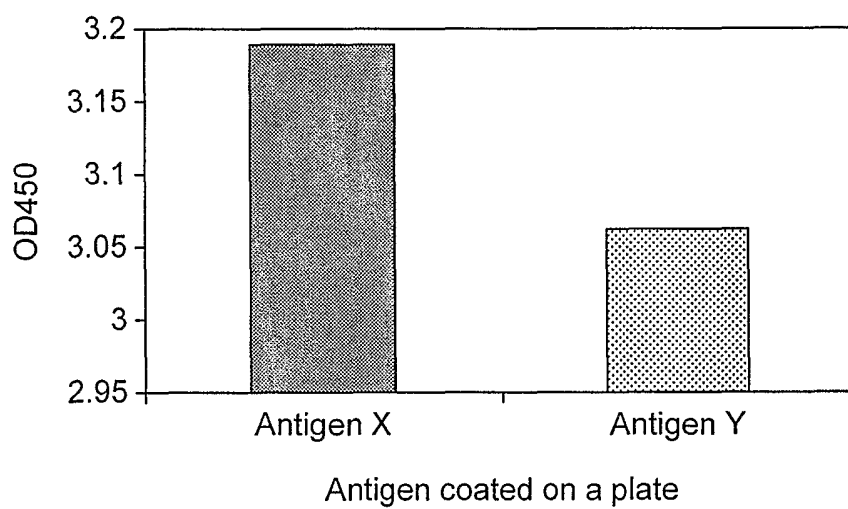


FIG. 29
Cell assays using IgG

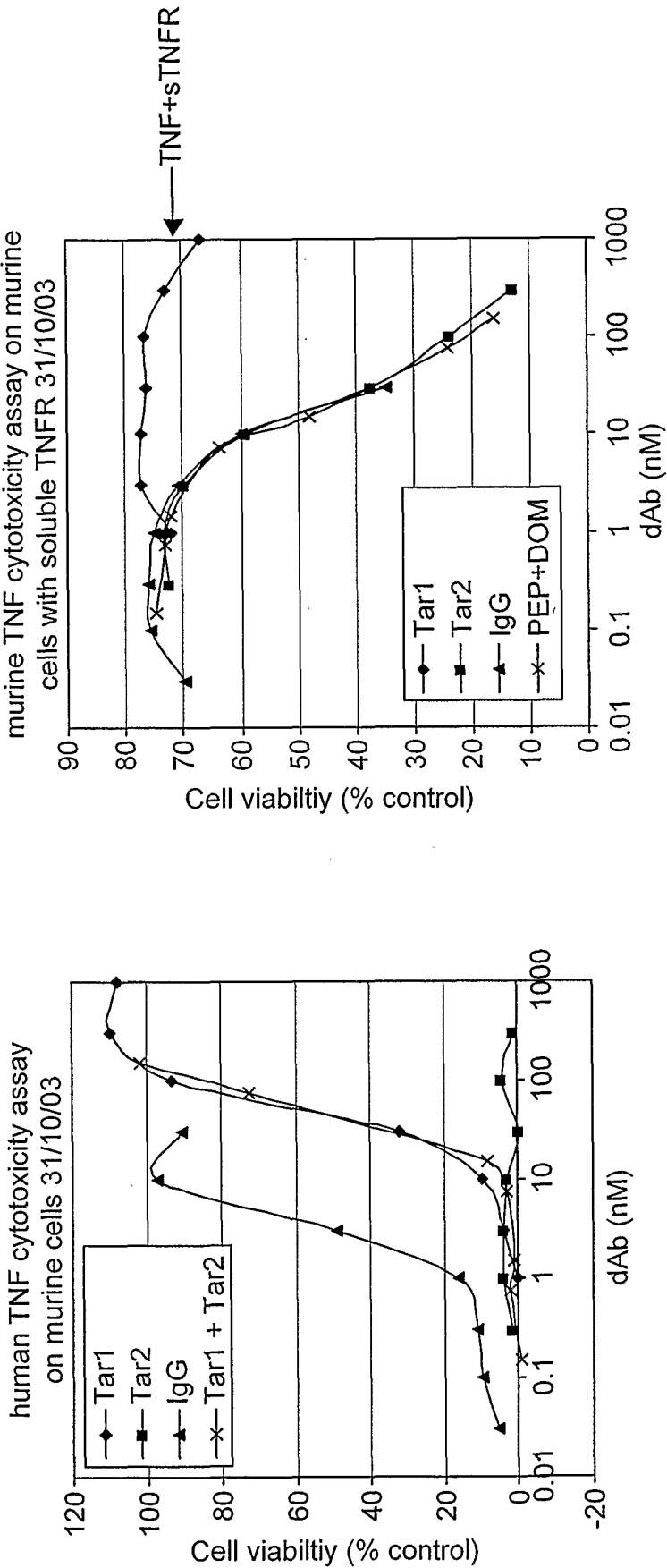


FIG. 29 CONT'D

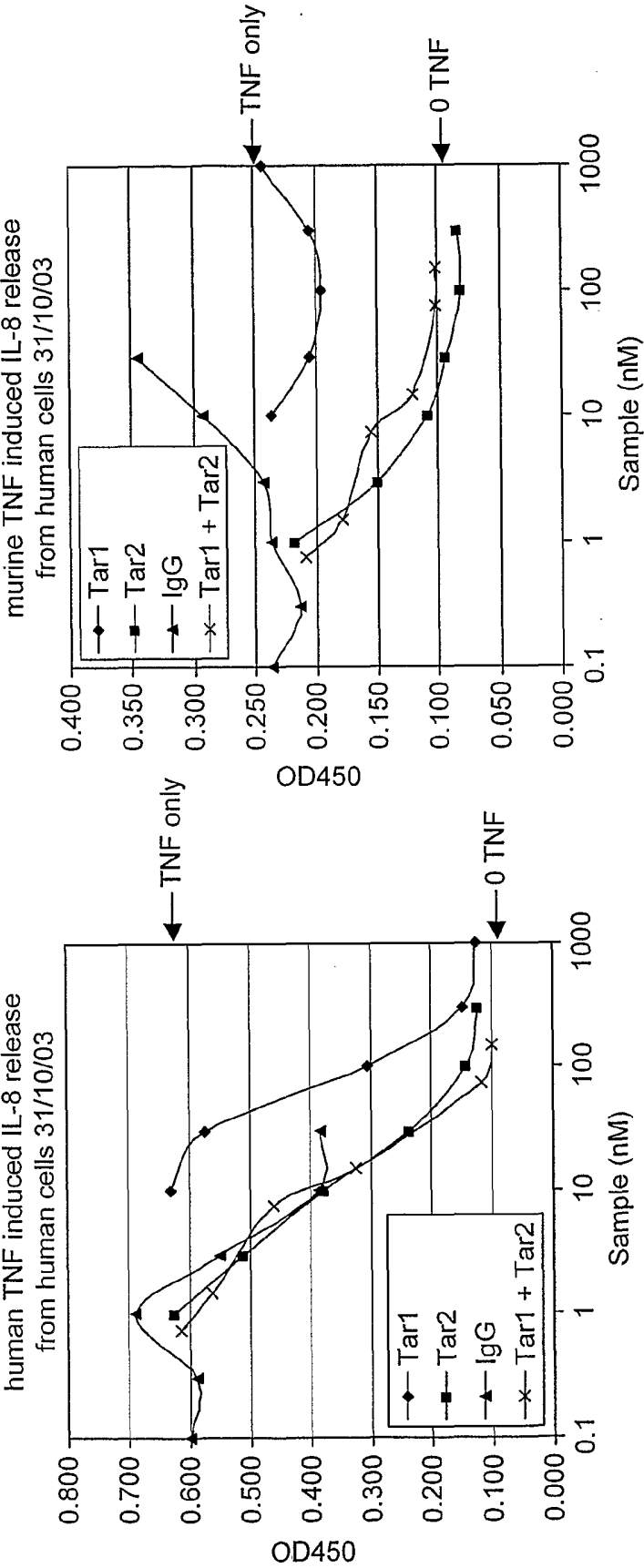


FIG. 30

Cell assays using IgG

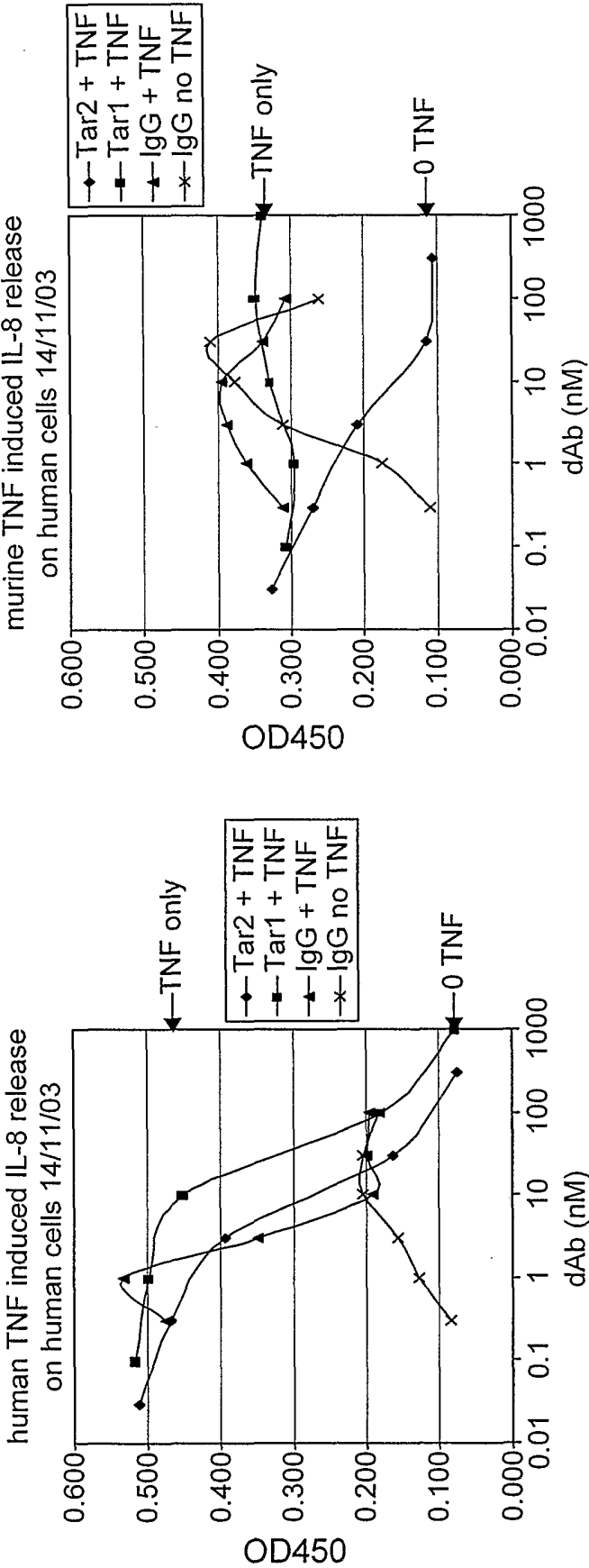


FIG. 31

TAR2h-10-27 Dab amino acid sequence (a) and nucleic acid sequence

TAR2h-10-27 amino acid sequence.

(a) EVQLLES GGLVQPGSLRLSCAASGFTFEWYWMGWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISR
DNSKNTLYLQMNSLRAEDAAVYYCAKVKLG GGNFGRGQGLTVTVSSAA.

(b) GAGGTGCAGCTGTTGGAGTCTGGGGGAGGGCTTGGTACAGCCCTGGGGGGTCCCCTGCGTCTCTCCTGTGCAGCC
TCCGGATTACCTTTGAGTGGTATTGGATGGGTCCGCCAGGCTCCAGGGAAGGTCTAGAGTGGGTC
TCAGCTATCAGTGGTAGTGGTAGCACATACTACGCAGACTCCGTGAAGGCCGGTTCACCATCTCCCGC
GACAA TTC AAGAACACGCTGTATCTGCAAAATGAACAGCCTGCGTGCCGAGGACGCCCGGTATATTACTGT
GCGAAAGTTAAGTTGGGGGGGGGCCTAATTTGGCTACCGGGGCCAGGGAACCCCTGGTCACCGTCTCGAGC
GCGGCCCGC

SEQUENCE LISTING

HEL4

5 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F R I S D E D M .
 GAGTGCAGC TGTTGGAGTC TGGGGGAGGC TTGGTACAGC CTGGGGGTC CCTGCGTCTC TCCTGTGCAG CCTCCGGATT TAGGATTAGC GATGAGGATA
 . G W V R Q A P G K G L E W V S S I Y G P S G S T Y Y A D S V K G R .
 TGGGCTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTATCAAGC ATTATGCCC CTAGCGGTAG CACATACAC GCAGACTCCG TGAAGGGCCG
 10 . F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A S A L
 GTTCACCATC TCCCGTGACA ATTCCAAGAA CACGCTGTAT CTGCAATGA ACAGCTCGG TGCCGAGGAC ACCGCGGTAT ATTATTGCGC GAGTGCTTTG
 E P L S E P L G F W G Q G T L V T V S S
 GAGCCGCTTT CGAGCCCTT GGCCTTTTGG GGTACGGAA CCTGGTCAAC CGTCTCGAGC

15

TAR2-5

20 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F D L Y N M .
 GAGGTGCAGC TGTTGGAGTC TGGGGGAGGC TTGGTACAGC CTGGGGGTC CCTGCGTCTC TCCTGTGCAG CCTCCGGATT CACTTTTGAT CTTTATAATA
 . F W V R Q A P G K G L E W V S F I S Q T G R L T W Y A D S V K G R .
 TGTTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATTT ATTAGTCAGA CTGGTAGGCT TACATGGTAC GCAGACTCCG TGAAGGGCCG
 . F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K T L
 GTTCACCATC TCCCGTGACA ATTCCAAGAA CACGCTGTAT CTGCAATGA ACAGCTCGG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAAACGCTG
 E D F D Y W G Q G T L V T V S S
 GAGGATTTG ACTACTGGGG CCAGGGAACC CTGGTCACCG TCTCGAGC

30

TAR1-5-19

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S V K E F L W .
 GACATCCAGA TGACCAGTC TCCATCCTCC CTGCTGTGCAT CTGTAGGAGA CCGGTGCACC ATCAGCTGCC GGGCAAGTCA GAGCGTTAAG GAGTTTTTAT
 . W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S .
 GGTGGTACCA GCAGAACCA GGGAAAGCC CTAAGTCTCT GATCTATATG GCATCCAAT TGCAAAGTGG GGTCCCATCA CGTTTCACTG GCAGTGGATC
 . G T D F T L T I S L Q P E D F A T Y Y C Q Q K F K L P R T F G Q
 TGGGACAGAT TTCCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTG CTACGTACTA CTGTCAACAG AAGTTTAAGC TGCCTCGTAC GTTCGGCCAA
 G T K V E I K R
 GGGACCAAGG TGAATACAA ACGG

40

TAR2-10

45 GAGGTGCAGCTGTGGAGTC TGGGGGAGGCTTGTTGACAGCCTGGGGGTCCTCGTCTCTCTGTGCAGCCTCCGGATTCACCTTTAGTGGTATTGGATTGGGTGGTCC
 GCCAGGCTCCAGGGAAGGTC TAGAGTGGTCTCAGCTATTAGTGGTAGTGGTAGCACA TACTACGAGAC CTCCTGGAAGGCCGTTTCAACCATCTCCCGGACAATTC
 CAAGAACACGCTGTATCTGCAAAATGAACAGCCTGCGTGGCCGAGACACCGCGGTATATTACTGTGCGAAAGTTAAGTTGGGGGGGGCCCTTAATTTTGACTACTTGGGGCCAG

GGAACCTGGTCAACCGTCTCGAGCGCEVQLLESGGGLVQPGGSLRLSCAASGFTFEWYMWGVRQAPGKGLEWVSAISGSGGTYADSVKGRFTISRDNKNTLYLQMNS
LRAEDTAVYYCAKVKLGGPNFDYWGQTLVTSS

5 E V Q L L E S G G G L V Q P G G S .
1 GAGGTGCAGC TGTGGAGTC TGGGGGAGGC TTGTACAGC CTGGGGGGTC
51 . L R L S C A A S G F T F E W Y W M .
CCTGCGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTGAG TGGTATTGGA
101 . G W V R Q A P G K G L E W V S A
TGGGTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCAGCT
10 I S G S G G S T Y Y A D S V K G R .
151 ATTAGTGGTA GTGGTGGTAG CACATACTAC GCAGACTCCG TGAAGGGCCG
F T I S R D N S K N T L Y L Q M N .
201 GTTCACCATC TCCCGCGACA ATTCCAAGAA CACGCTGTAT CTGCAAAATGA
S L R A E D T A V Y Y C A K V K
251 ACAGCCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAAGTTAAG
L G G G P N F D Y W G Q G T L V T .
301 TTGGGGGGGG GGCCCTAATT TGACTACTGG GGCCAGGGAA CCCTGGTTCAC
Aval

351 . V S S
CGTCTCGAGC

TAR-1 dAb DNA and amino acid sequences.
dAbs 1, 2 and 3 are the partner sequences from TAR1-5 dimers 1-6. dAb1 is the partner dAb to dimers 1, 5 and 6
the second dAbs are the same but the linker lengths are different, likewise, dAb2 is the partner dAb to dimers
2 and 3, dAb3 is the partner dAb to dimer 4. * indicates the presence of an amber stop codon. Sequence homology
between, TAR1-5, TAR1-5-19 and the unique second dAbs is 88%

TAR1-5-19
D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I D S Y L H
GACATCCAGA TGACCCAGTC TCCATCCTCT CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCATTGCC GGGCAAGTCA GAGCATTGAT AGTATTATAC
CTGTAGGTCT ACTGGTCCAG AGGTAGGAGA GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTCACT CTCTAACTA TCAATAAATG
W Y Q Q K P G K A P K L L I Y S A S E L Q S G V P S R F S G S G S
ATTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGT GCACCAAGTG GTCCCATCA CGTTTCAGT GCAGTGCATC
TAACCATGGT CGTCTTTGGT CCCTTTCGGG GATTCAGGA CTAGATATCA CGTAGGCTCA ACCTTCACC CCAGGTAGT GCAAAGTCAC CGTCACTAG
G T D F T L T I S L Q P E D F A T Y Y C Q Q V V W R P F T F G Q
TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTGCTACTA CTGTCAACAG GTTGTGTGGC GTCTTTTAC GTTCGGCCAA
ACCGTGCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTAAAC GATGCATGAT GACAGTTGTC CAACACACCG CAGGAAATG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGC

CCCTGGTTCC ACCTTAGTT TGCG

TAR1-5

5 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I F M N L L
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCCTGTACC ATCACTTACC GGGCAAGTCA GAGCATTTAT ANGAATTTAT
 CTGTAGTCT ACTGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCCGTTCACT CTCGTAAAA TACTTAAATA
 W Y Q Q K P G K A P K L L I Y N A S V L Q S G V P S R F S G S
 10 TGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCTT GATCTATAAT GCATCCGTTG TGCAAAAGTG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 ACACCATGGT CGTCTTTGGT CCCCTTCGGG GATTCGAGGA CTAGATATTA CGTAGGCACA ACGTTTCACC CCAGGTAGT GCAAAGTCAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V V W R P F T F G Q
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGCTACTA CTGTCAACAG GTTGTGTGGC GTCTTTTAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTAAAAAC GATGCATGAT GACAGTTGTC CAACACACCG CAGGAAAAATG CAAGCCCGTT
 G T K V E I K R
 15 GGGACCAAGG TGGAAATCAA ACGG
 CCCTGGTTCC ACCTTAGTT TGCC

Monomer - dAb1: Partner dAb in TAR1-5d1 (3U linker), d5 (5U linker), d6 (7U linker)

20 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I Y D A L E
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCCTGTACC ATCACTTACC GGGCAAGTCA GAGCATTTAT GATCGTTAG
 CTGTAGTCT ACTGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCCGTTCACT CTCGTAAAA TACTTAAATA
 W Y Q Q K P G K A P K L L I Y N A S R L Q S G V P S R F S G S
 25 AGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCTT GATCTATACT GATCTATCTT GCATCCCGT TGCAAAAGTG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 TCACCATGGT CGTCTTTGGT CCCCTTCGGG GATTCGAGGA CTAGATATGA CGTAGGGCCA ACGTTTCACC CCAGGTAGT GCAAAGTCAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V M Q R P V T F G Q
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGCTACTA CTGTCAACAG GTTATGCAGC GTCTGTGTAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTAAAAAC GATGCATGAT GACAGTTGTC CAATACGTCG CAGGACAAATG CAAGCCCGTT
 G T K V E I K R
 30 GGGACCAAGG TGGAAATCAA ACGG
 CCCTGGTTCC ACCTTAGTT TGCC

Monomer - dAb2: Partner dAb in TAR1-5d2 (3U linker), d3 (5U linker)

35 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I Y D A L *
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCCTGTACC ATCACTTACC GGGCAAGTCA GAGCATTTAT GATCGTTTAC
 CTGTAGTCT ACTGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCCGTTCACT CTCGTAAAA TACTTAAATA
 W Y Q Q K P G K A P K L L I Y N A S R L Q S G V P S R F S G S
 40 AGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCTT GATCTATACT GATCTATCTT GCATCCCGT TGCAAAAGTG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 TCACCATGGT CGTCTTTGGT CCCCTTCGGG GATTCGAGGA CTAGATATGA CGTAGGGCCA ACGTTTCACC CCAGGTAGT GCAAAGTCAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V M Q R P V T F G Q
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGCTACTA CTGTCAACAG GTTATGCAGC GTCTGTGTAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTAAAAAC GATGCATGAT GACAGTTGTC CAATACGTCG CAGGACAAATG CAAGCCCGTT
 G T K V E I K R
 45 GGGACCAAGG TGGAAATCAA ACGG
 CCCTGGTTCC ACCTTAGTT TGCC

Monomer - dAb3: Partner dAb in TAR1-5d4 (5U linker)

50 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S V K E F L W

GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAATCA GAGCGTTAAG GAGTTTITAT
 CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT CTGCAAGCG CCGTTCAGT CTCGCAATTC CTCAAAATA
 W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S
 GGTGTACCA GCAGAAACCA GGAAGGCC CTAAGCTCCT GATCTATATG GCATCCAAAT TGCAAGTGG GGTCCCAICA CGTTTCAGT GCATGGATC
 CCACCATGGT CGTCTTTGGT CCCTTTCGG GATTCGAGGA CTAGATATAC CBTAGGTAA AGTTTTCACC CCAGGTAGT GCAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q K F K L P R T F G Q
 TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG AAGTTTAAAG TGCTCTGATC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCAATGAT GACAGTTGTC TTCAAAATTCG ACGGAGCATG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAGG TGGAATCAA ACGG
 CCCTGGTCC ACCTTAGTT TGCC

15 TARI-27 and related partner dab amino acid and DNA sequences (* indicates the presences of an amber stop codon).
 Sequence homology between TARI-27 and the unique second dabs is 90.4%.

TARI-27

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I W T K L H
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTTGG ACGAAGTTAC
 CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT CTGCAAGCGG TAGTGAACGG CCCGTTCACT CTCGTAAACC TGCTTCAATG
 W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S
 ATGGTACCA GCAGAAACCA GGAAGGCC CTAAGCTCCT GATCTATATG GCATCCAAAT TGCAAGTGG GGTCCCAICA CGTTTCAGT GCATGGATC
 TAACCATGGT CGTCTTTGGT CCCTTTCGG GATTCGAGGA CTAGATATAC CBTAGGTAA AGTTTTCACC CCAGGTAGT GCAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q W F S N P S T F G Q
 TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG AAGTTTAAAG TTCTGTGATC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCAATGAT GACAGTTGTC ACCAATCAT TAGGATCATG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAGG TGGAATCAA ACGG
 CCCTGGTCC ACCTTAGTT TGCC

Partner dab monomer in TARI-27d1 (3U linker)

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I * P I L C
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTTAG CCGATTTTAT
 CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCTCT CTGCAAGCGG TAGTGAACGG CCCGTTCACT CTCGTAAACC GGTAAAATA
 W Y Q Q K P G K A P K L L I Y A A S S L Q S G V P S R F S G S
 GTTGGTACCA GCAGAAACCA GGAAGGCC CTAAGCTCCT GATCTATATG GCATCCAAAT TGCAAGTGG GGTCCCAICA CGTTTCAGT GCATGGATC
 CAACCATGGT CGTCTTTGGT CCCTTTCGG GATTCGAGGA CTAGATATAC CBTAGGTAA AGTTTTCACC CCAGGTAGT GCAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q I O H I P V T F G Q
 TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG ATTACAGATA TTCTGTGATC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCAATGAT GACAGTTGTC TAAGTCGTAT AAGGACACTG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAGG TGGAATCAA ACGG
 CCCTGGTCC ACCTTAGTT TGCC

Partner dab monomer in TARI-27d2 (3U linker)

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I G * D L H
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTTGG TAGGATTTAC

CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGAGCTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAACCC ATCCTAAATG
W Y Q Q K P G K A P K L L I Y T A S L L Q S G V P S R F S G S G S
ATTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGTCTCT GATCTATAG GCATCCCTTT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
TAAACATGGT CGTCTTTGGT CCCTTTCGGG GATTGAGGA CTAGATATGC CGTAGGGAAA ACGTTTCACC CAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q Q S A F P N T L G Q
TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTG CTAGTACTA CTGTCAACAG CAGAGTGCCT TTCTTAATAC GCTCGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTTAAAC GATGCAATGAT GACAGTTGTC GTCTCAGAA AAGGATTATG CGAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

10

Partner dab monomer in TAR1-27d7 (3Ulinker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I T K N L L
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CCGTAGGAGA CCGTGTACCC AGGCAAGTCA GAGCATTAACG AAGAATTTAC
CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GGCATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTATTGC TTCTTAAATG
W Y Q Q K P G K A P K L L I Y * A S S L Q S G V P S R F S G S G S
TTTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGTCTCT GATCTATTAG GCATCCTCTT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
AAACCATGGT CGTCTTTGGT CCCTTTCGGG GATTGAGGA CTAGATAATC CGTAGGAGAA ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q L R H K P P T F G Q
TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTG CTAGTACTA CTGTCAACAG CTTCTGTATA AGCTCCGAC GTTCGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTTAAAC GATGCAATGAT GACAGTTGTC TCGGAGGCTG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

25

Partner dab monomer in TAR1-27d8 (3Ulinker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I * K S L R
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC AGGCAAGTCA GAGCATTTAG AAGTCTTTAA
CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAAATC TTCAGAAAT
W Y Q Q K P G K A P K L L I Y H A S D L Q S G V P S R F S G S G S
GGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGTCTCT GATCTATCAT GCATCCGAT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
CCACCATGGT CGTCTTTGGT CCCTTTCGGG GATTGAGGA CTAGATAGTA CGTAGGCTAA ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q M V N S P V T F G Q
TGGGACAGAT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTG CTAGTACTA CTGTCAACAG ATGGTTAATA GTCTGTATAC GTTCGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTTAAAC GATGCAATGAT GACAGTTGTC TACCAATAT CAGGACAATG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

40

Partner dab monomer in TAR1-27d12 (3Ulinker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I * T A L H
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC AGGCAAGTCA GAGCATTTAG ACGGCGGTAC
CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAAATC TGCCGCAATG
W Y Q Q K P G K A P K L L I Y S A S L Q S G V P S R F S G S G S
ATTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGTCTCT GATCTATCT GCATCCAGTT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
TAAACATGGT CGTCTTTGGT CCCTTTCGGG GATTGAGGA CTAGATAAGA CGTAGGTGTC ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG

50

G T D F T L T I S S L Q P E D F A T Y Y C Q Q S S F L P F T F G Q
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGT CTAGTACTA CTGTCAACAG TCGAGTTTTT TGCCTTTTAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTCTAAAC GATGCATGAT GACAGTTGTC AGCTCAAAA ACAGAAAATG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAG TGAATCAAC ACGG
 CCTGGTTCC ACCTTAGTT TGCC

5

10 Partner **dab** monomer in TAR1-27d16 (3U linker)
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I G P N L E
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTTCC GGGCAAGTCA GAGCATTTGG CCGAATTAG
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCACT CTCGTAACCC GGCTTAATC
 W Y Q Q K P G K A P K L L I Y A A S S L Q S G V P S R F S G S G S
 AGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATGCT GCATCCAGTT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 TCACCATGCT CGTCTTTGGT CCCTTTCGGG GATTCGAGGA CTAGATACGA CGTAGGTCAA ACCTTTTACC CCAGGGTAGT GCAAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q Q M G R P R T F G Q
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAAGTTTGG CTACGTACTA CTGTCAACAG CAGATGGGC GTCTTCGGAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTCTAAAC GATGCATGAT GACAGTTGTC GTCTACCCCG CAGGAGCCTG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAG TGAATCAAC ACGG
 CCTGGTTCC ACCTTAGTT TGCC

20

25 Partner **dab** monomer in TAR1-27d23 (5U linker)
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I K H * L A
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTTCC GGGCAAGTCA GAGCATTTAG CATTAGTTAG
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCACT CTCGTAATTC GATATCAATC
 W Y Q Q K P G K A P K L L I Y K A S V L Q S G V P S R F S G S G S
 CTTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAAG GCATCCGTGT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 GAACCATGCT CGTCTTTGGT CCCTTTCGGG GATTCGAGGA CTAGATATTC GGTAGGACA ACCTTTTACC CCAGGGTAGT GCAAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q L R R R P T T F G Q
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGCT CTAGTACTA CTGTCAACAG CTTAGGCGTC GTCTTACTAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTCTAAAC GATGCATGAT GACAGTTGTC GAATCCGCGC CAGGATGATG CAAGCCGGTT
 G T K V E I K R
 GGGACCAAG TGAATCAAC ACGG
 CCTGGTTCC ACCTTAGTT TGCC

30

35

40 Partner **dab** monomer in TAR1-27d30 (7U linker)
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S V K A * L T
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTTCC GGGCAAGTCA GAGCATTTAG GCTTAGTTAA
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCACT CTCGTAATTC CGAATCAATT
 W Y Q Q K P G K A P K L L I Y K A S T L Q S G V P S R F S G S G S
 CTTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAAG GCATCCACTT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 GAACCATGCT CGTCTTTGGT CCCTTTCGGG GATTCGAGGA CTAGATATTC GGTAGTGAAC ACCTTTTACC CCAGGGTAGT GCAAAGTCAAC CGTCACCTAG
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q H S S R P Y T F G Q
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGCT CTAGTACTA CTGTCAACAG CATAGTTCTA GGCCTTATAC GTTCGGCCAA
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTTGA CTCTCTAAAC GATGCATGAT GACAGTTGTC GTATCAAGAT CCGGAATATG CAAGCCGGTT
 G T K V E I K R

45

50

GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

5

Partner dAb monomer in TAR1-27d31 (7U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I E N R L G
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGCATTTAG AATCGGTTAG
CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAACTC TTAGCCCAATC
W Y Q Q K P K A P K L L I Y * A S L L Q S G V P S R F S G S G S
GTTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAG CGTCCCTTGT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
CAACCATGGT CGTCTTTGGT CCCCTTCGGG GATTGAGGA CTAGATAATC CGCAGGAACA ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q D S Y F P R T F G Q
TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGT CTACGTACTA CTGTCAACAG GATTCGTATT TTCTCTGTAC GTTCGGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC CTAGCATATA AGGAGCATG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

10

15

20

Partner dAb monomer in TAR1-27d36 (7U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I M D K L K
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGCATTTAG GATAAGTTAA
CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAACTC CTATTCATTT
W Y Q Q K P K A P K L L I Y * A S I L Q S G V P S R F S G S G S
AGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAG GCATCCATTT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
TCACCATGGT CGTCTTTGGT CCCCTTCGGG GATTGAGGA CTAGATAATC CGTAGTAAA ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q D S G P N T F G Q
TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGT CTACGTACTA CTGTCAACAG GATAGTGGGG GTCCCTAATAC GTTCGGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC CTATCACCCC CAGGATTATG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

25

30

35

Partner dAb monomer in TAR1-27d37 (7U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I G R N L E
GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGCATTTGG AGGAATTTAG
CTGTAGGTCT ACTGGGTTCAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCCGTTTCAGT CTCGTAACTC TCCTTAAATC
W Y Q Q K P K A P K L L I Y D A S H L Q S G V P S R F S G S G S
AGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAG GCATCCATTT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
TCACCATGGT CGTCTTTGGT CCCCTTCGGG GATTGAGGA CTAGATAATC CGTAGGTAAA ACGTTTCACC CCAGGGTAGT GCAAAGTCAC CGTCACCTAG
G T D F T L T I S S L Q P E D F A T Y Y C Q Q S R W L P R T F G Q
TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGT CTACGTACTA CTGTCAACAG TCGCTTTGGC TTCTCTGTAC GTTCGGGCCAA
ACCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC AGCGCAACCG AAGGAGCATG CAAGCCGGTT
G T K V E I K R
GGGACCAAGG TGGAAATCAA ACGG
CCCTGGTTCC ACCTTTAGTT TGCC

40

45

50

Partner dab monomer in TAR1-27d39 (7U linker)
 5 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I R K M L V
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTCTCTGAT CTGTAGAGA CCGTGTACC ATCATTGCC GGGCAAGTCA GAGCATTAGG AAGATGTTAG
 CTGTAGTCT ACTGGTTCAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGACACGTGG TAGTGAACGG CCCGTTGAGT CTGCTAATCC TTCTACAAATC
 W Y Q Q K P G K A P K L L I Y R A S Y L Q S G V P S R F S G S
 TTTGGTACCA GCAGAAACCA GGAAGGCC CTAAGTCCCT GATCTATCG GCATCTATT TGCAGATGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC
 AAACCATGCT CGTCTTTGGT CCCTTTCCGG GATTCCGAGA CTAGATAGCC CGTAGGATAA ACCTTTCCAC CCAGGTAGT GCAAAGTCAC CGTCACCTAG
 10 G T D F T L T I S L Q P E D F A T Y Y C Q Q A F R R P R T F G Q
 TGGACACAGT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAAGATTCTG CTAGTACTA CTGTCAACAG GCTTTTCGGC GGCCTAGGAC GTTCGGCCAA
 ACCCTGTCTA AAGTAGAGT GGTAGTCGTC AGACGTTGGA CTCTAAAC GATGCATGAT GACAGTTGTC CGGATCCCTG CAGCCCGGTT
 G T K V E I K R
 GGGACCAAGG TGGAAATCAA ACGG
 CCCTGGTTCC ACCTTTAGTT TGCC

TAR2 dab amino acid and DNA sequences (* indicates the presences of an amber stop codon) sequence homology
 between TAR2h-5 and the unique second dabs is 83.5%

TAR2h-5
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F D L Y N M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACACG CTGGGGGTC CCTGGTCTC TCCTGTGAG CCTCCGATT CACCTTTGAT CTTTATATA
 CTCACGTCG ACAACCTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GGACGACAG AGGACACGTC GGAGGCTAA GTGGAACCTA GAAATATAT
 F W V R Q A P G K G L E W V S F I S Q T G R L T W Y A D S V K G R
 TGTTTTGGT CGCCAGGCT CCAGGAAGG GTCTAGAGTG GTCTCATTT ATTAGTCAG CTGTAGGCT TACATGGTAC GCAGACTCCG TGAAGGGCCG
 ACAAACCCA GGGGTCCGA GGTCCCTCC CAGATCTCAC CCAGATPAA TAATCAGTCT GACATCCGA ATGTACCATG CGTGTAGGC ACTTCCCGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K T L
 GTTCACCATC TCCCGCAGCA ATTCAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGGCGAGGAC ACCCGGTAT ATTACTGTG GAAACGCTG
 CAAATGGTAG AGGCGCTGT TAAGTTCTT GTGGACATA GACGTTTACT TGTGAGACG ACGCTCCTG TGGCGCCATA TAATGACAG CTTTGGCAG
 E D F D Y W G Q G T L V T V S
 GAGGATTGT ACTACTGGG CCAGGGAACC CTGCTACCG TCTCG
 CTCCTAAAC TGATGACCC GGTCCCTTG GACCAGTGCG AGAGC

Partner dab monomer in TAR2h-5d1 (3U linker)
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F P V Y M M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACACG CTGGGGGTC CCTGGTCTC TCCTGTGAG CCTCCGATT CACCTTTCCG GTTATATGA
 CTCACGTCG ACAACCTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GGACGACAG AGGACACGTC GGAGGCTAA GTGGAAGGC CAAATATAT
 G W V R Q A P G K G L E W V S S I D A L G G R T G Y A D S V K G R
 TGGTTTGGT CGCCAGGCT CCAGGAAGG GTCTAGAGTG GTCTCATTC ATTGATGTC TTGTTGGCG GACAGTTAC GCAGACTCCG TGAAGGGCCG
 ACCCAACCCA GGGGTCCGA GGTCCCTCC CAGATCTCAC CCAGAGTAGC TAACATACAG AACCAACCGC CTGTCCAATG CGTCTGAGC ACTTCCCGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K T M
 GTTCACCATC TCCCGCAGCA ATTCAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGGCGAGGAC ACCCGGTAT ATTACTGTG GAAACCTATG
 CAAATGGTAG AGGCGCTGT TAAGTTCTT GTGGACATA GAGGTTTACT TGTGAGACG ACGCTCCTG TGGCGCCATA TAATGACAG CTTTGGATG
 S N K T H T F D Y W G Q G T L V T V S
 TCGATATAGA CGCATACGTT TGACTACTGG GGCAGGGGA CCCTGGTCC CAGCTCG
 AGCTTATCT GCGATATCAA ACTGATGACC CCGTCCCTT GGGACCAAGT GCAGAGC

Partner dAb monomer in TAR2h-5d2 (3U linker)
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F V A Y N M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTG GCTTATATA
 CTCCACGTCG ACAACCTCAG ACCCTCTCG AACCATGTCG GACCCCCAG GAGCCAGAG AGACACGTC GGAGCCCTAA GTGGAACAC CGAATATAT
 T W V R Q A P G K G L E W V S S I N T F G N * T R Y A D S V K G R
 TGACTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGT GGTCTCAGT ATTAATCTT TTGTAAATTA GACAAGTAC GCAGACTCCG TGAAGGCCG
 ACTGAACCA GCGGTCCGA GTTCCCTTCC CAGATCTAC CACAGTTCA TAATTAAGA AACCATTAAT CTGTCCATG CGTCTGAGC ACTTCCCGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K G S
 GTTCACATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAAAATG ACAGCCCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAGGTAGT
 CAAGTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACCTTACT TGTCCGAGC ACGGCTCTG TGGCGCCATA TAATGACAG CTTTCCATCA
 R P F D Y W G Q G T L V T V S
 AGGCTTTTG ACTACTGGG CCAGGGAACC CTGTCACCG TCTCG
 TCCGGAAC TGATACCCC GTTCCCTTGG GACCATGGC AGAGC

Partner dAb monomer in TAR2h-5d3 (3U linker)
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F * G Y R M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTAG GGGTATCGTA
 CTCCACGTCG ACAACCTCAG ACCCTCTCG AACCATGTCG GACCCCCAG GAGCCAGAG AGACACGTC GGAGCCCTAA GTGGAACAC CCTATAGCAT
 G W V R Q A P G K G L E W V S S I N T F G N * T R Y A D S V K G R
 TGGTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGT GGTCTCAGT ATTACGCTA CTGGTGGAC GACACAGTAC GCAGACTCCG TGAAGGCCG
 ACCCAACCA GCGGTCCGA GTTCCCTTCC CAGATCTAC CACAGTACC TAATGCGAT GACCACCTG CTGTGTCTG CGTCTGAGC ACTTCCCGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K P A
 GTTCACATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAAAATG ACAGCCCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAGCCGCG
 CAAGTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACCTTACT TGTCCGAGC ACGGCTCTG TGGCGCCATA TAATGACAG CTTTGGCCG
 K L V G V G F D Y W G Q G T L V T V S
 AAGCTTTTG GGTGGGT TGAATCTG GGCAGGAA CCTTGTCTAC CGTCTCG
 TTGGAACAC CCCAACCAA ACTGATGACC CCGTCCCTT GGCACCATG GCAGAGC

Partner dAb monomer in TAR2h-5d4 (3U linker)
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R K Y * M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTG GAGTATAGA
 CTCCACGTCG ACAACCTCAG ACCCTCTCG AACCATGTCG GACCCCCAG GAGCCAGAG AGACACGTC GGAGCCCTAA GTGGAACAC TTCATATCT
 G W V R Q A P G K G L E W V S S I N T F G N * T R Y A D S V K G R
 TGGGTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGT GGTCTCAGC ATTGTGCGA AGGTCTAGTAC TACAGATTAC GCAGACTCCG TGAAGGCCG
 ACCCAACCA GCGGTCCGA GTTCCCTTCC CAGATCTAC CACAGTACC TAACACGCT TCCAGTACG ATGTCTAATG CGTCTGAGC ACTTCCCGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K K K
 GTTCACATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAAAATG ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAGGAAG
 CAAGTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACCTTACT TGTCCGAGC ACGGCTCTG TGGCGCCATA TAATGACAG CTTTCTTCT
 R G E N Y F F D Y W G Q G T L V T V S
 AGGGGGAGA ATTAATTTT TGAATCTG GGCAGGAA CCTTGTCTAC CGTCTCG
 TCCCTCTCT TAATAAAAA ACTGATGACC CCGTCCCTT GGCACCATG GCAGAGC

Partner dAb monomer in TAR2h-5d5 (3U linker)
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R R Y S M
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTG CGTATAGTA

CTCCACGTCG ACAACCTCAG ACCCCCTCCG AACCATGTGC GACCCCCAG GAGCGCAGAG AGGACACGTC GGAGGCCCTAA GTGGAAGCC GCCATATCAT
S W V R Q A P G K L E W V S D I S R S G R Y T H Y A D S V K G R
TGTCGTGGGT CCGCAGGCT CCAGGGAAGG GTCTAGATG GGTCTCAGAT ATTCTCGTT CTGGTCGGTA TACACATTAC GCAGACTCCG TGAAGGCCG
ACAGCACCA GCGGTCCGA GGTCCCTTCC CAGATCTAC CAGAGTCTA TAAAGACAA GACCAGCAT ATGTGTAAT CGTCTGAGC ACTTCCCGG
F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K R I
GTTACCATC TCCCGCACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAACGTATT
CAGTGTGTAG AGGCGCTGT TAAGTCTT GTGCGACATA GACGTTTACT TGTGCGACGC ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTGCAATA
D S S Q N G F D Y W G Q G T L V T V S
GATCTCTC AGAATGGGT TGACTACTGG GGCAGGAA CCCTGGTCAC CGTCTCG
CTAAGAGAG TCTTACCAA ACTGATGACC CCGTCCCTT GGGACCATG GCAGAGC

Partner dAb monomer in TAR2h-5d6 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F * G Y K M
GAGTGCAGC TGTGTAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCAG CCTCCGATT CACCTTTAG GGTATAAGA
CTCCACGTCG ACAACCTCAG ACCCCCTCCG AACCATGTGC GACCCCCAG GAGCGCAGAG AGGACACGTC GGAGGCCCTAA GTGGAAGCC CCATATCT
F W V R Q A P G K L E W V S A I S G S T Y Y A D S V K G R
TGTTTGGGT CCGCAGGCT CCAGGAAGG GTCTAGATG GGTCTCAGCT ATTAGTGTGTA GTGGTGTAG CACATACTAC GCAGACTCCG TGAAGGCCG
ACAAACCCA GCGGTCCGA GGTCCCTTCC CAGATCTAC CAGAGTCTA TAATCACCAT CACCACCATC GTGTATGAT CGTCTGAGC ACTTCCCGG
F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K Q K
GTTACCATC TCCCGCACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAACAGAG
CAATGTGTAG AGGCGCTGT TAAGTCTT GTGCGACATA GACGTTTACT TGTGCGACGC ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTGCTCTC
E N F D Y W G Q G T L V T V S
GAGATTTG ACTACTGGG CCAGGAACC CTGGTCACG TCTCG
CTCTTAAAC TGATGACCC CCGTCCCTTGG GACAGTGGC AGAGC

Partner dAb monomer in TAR2h-5d7 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F G D Y A M
GAGTGCAGC TGTGTAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCAG CCTCCGATT CACCTTTAG GGTATAAGA
CTCCACGTCG ACAACCTCAG ACCCCCTCCG AACCATGTGC GACCCCCAG GAGCGCAGAG AGGACACGTC GGAGGCCCTAA GTGGAAGCC CTAATAGAT
W W V R Q A P G K L E W V S V I S S N G G S T F Y A D S V K G R
TGTTTGGGT CCGCAGGCT CCAGGAAGG GTCTAGATG GGTCTCAGT ATTAGTGTGTA GTGGTGTAG TACATTTTAC GCAGACTCCG TGAAGGCCG
ACACACCCA GCGGTCCGA GGTCCCTTCC CAGATCTAC CAGAGTCTA TAATCAGCT TACCACCTC ATGTAAATG CGTCTGAGC ACTTCCCGG
F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K R V
GTTACCATC TCCCGCACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAACGTATT
CAATGTGTAG AGGCGCTGT TAAGTCTT GTGCGACATA GACGTTTACT TGTGCGACGC ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTGCAACA
K K R T P E F D Y W G Q G T L V T V S
CGTAAGAGGA CTCCTGAGTT TGACTACTGG GGCAGGAA CCCTGGTCAC CGTCTCG
GCATTCTCT GAGGACTCAA ACTGATGACC CCGTCCCTT GGGACCATG GCAGAGC

Partner dAb monomer in TAR2h-5d8 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R R Y K M
GAGTGCAGC TGTGTAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCAG CCTCCGATT CACCTTTAG AGGTATAAGA
CTCCACGTCG ACAACCTCAG ACCCCCTCCG AACCATGTGC GACCCCCAG GAGCGCAGAG AGGACACGTC GGAGGCCCTAA GTGGAAGCC TCCATATCT
G W V R Q A P G K L E W V S A I G R N G T K T N Y A D S V K G R
TGTTTGGGT CCGCAGGCT CCAGGAAGG GTCTAGATG GGTCTCAGC ATTTGAGGA ATGTACGAA GACAAATAC GCAGACTCCG TGAAGGCCG

ACCCAACCCA GGCCTGCCGA GGTCCCTTCC CAGATCTCAC CCAGAGTCGC TAACCTCTCT TACCATGCTT CTGTTTAATG CGTCTGAGGC ACTTCCCGGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K I Y
 GTTCACCATC TCCGCGGACA ATTCCAAGAA CACGCTGTAT CTGCAAAATGA ACAGCCTCGG TGCCGAGGAC ACCCGGGTAT ATTACTGTGC GAAAAATTAT
 CAACTGGTAG AGGGCGCTGT TAAGTTCTT GTCCGACATA GACGTTTACT TGTCGGAGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTAAATA
 T G K P A A F D Y W G Q G T L V T V S
 ACGGGGAGC CTGCTGCGTT TGACTACTGG GGCAGGGAA CCCTGGTCAC CGTCTCG
 TGCCCCCTTCG GACGACGCAA ACTGATGACC CCGTCCCTT GGGACCAATG GCAGAGC

10 Partner dAb monomer in TAR2h-5d9 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F K K Y * M
 GAGGTGCAGC TGTGGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTCTGCTCTC TCCTGTGTCAG CCTCGGAT CACCTTTAAG AAGTATAGA
 CTCCACGTCG ACAACTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GAGACACGTC AGGACACGTA GTGGAATTC TTCATATCT
 S W V R Q A P G K G L E W V S A I S G S G G S T Y Y A D S V K G R
 TGCTTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCAGCT ATTAGTGTGA GTGGTGTAG CACACTACTAC GCAGACTCCG TGAAGGGCCG
 ACAGAACCCA GCGGTCCGA GGTCCCTTCC CAGATCTCAC CCAGATCGA TAATCACCAT CACCACCATC GTGTATGATG CGTCTGAGGC ACTTCCCGGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K M L
 GTTCACCATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTCGG TGCCGAGGAC ACCCGGTAT ATTACTGTGC GAAAAATGCTG
 CAACTGGTAG AGGGCGCTGT TAAGTTCTT TTGCGACATA GACGTTTACT TGTCGACGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTACGAC
 R T K N K V F D Y W G Q G T L V T V S
 AGGACTAAGA ATAAGTGT TTGACTACTGG GGCAGGGAA CCCTGGTCAC CGTCTCG
 TCCTGATCT TATTCACAA ACTGATGACC CCGTCCCTT GGGACCAATG GCAGAGC

25 Partner dAb monomer in TAR2h-5d10 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R R Y K M
 GAGGTGCAGC TGTGGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTCTGCTCTC TCCTGTGTCAG CCTCGGAT CACCTTTAAG AAGTATAAGA
 CTCCACGTCG ACAACTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GAGCGACAG AGGACACGTC GGAGCCTTA GTGGAATTC TCCATATCT
 G W V R Q A P G K G L E W V S A I G R N G T K T N Y A D S V K G R
 TGGTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCAGCG ATTGGGAGA ATGGTACGAA GACAAATTA GCAGACTCCG TGAAGGGCCG
 ACCCAACCCA GCGGTCCGA GGTCCCTTCC CAGATCTCAC CCAGAGTCGC TAACCTCTCT TACCATGCTT CTGTTTAATG CGTCTGAGGC ACTTCCCGGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K I Y
 GTTCACCATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTCGG TGCCGAGGAC ACCCGGTAT ATTACTGTGC GAAAAATTAT
 CAACTGGTAG AGGGCGCTGT TAAGTTCTT TTGCGACATA GACGTTTACT TGTCGACGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTAAATA
 T G K P A A F D Y W G Q G T L V T V S
 ACGGGGAGC CTGCTGCGTT TGACTACTGG GGCAGGGAA CCCTGGTCAC CGTCTCG
 TGCCCCCTTCG GACGACGCAA ACTGATGACC CCGTCCCTT GGGACCAATG GCAGAGC

40 Partner dAb monomer in TAR2h-5d11 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F * S Y R M
 GAGGTGCAGC TGTGGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTCTGCTCTC TCCTGTGTCAG CCTCGGAT CACCTTTAAG AAGTATCGGA
 CTCCACGTCG ACAACTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GAGCGACAG AGGACACGTC GGAGCCTTA GTGGAATTC TCAATAGCCT
 G W V R Q A P G K G L E W V S A I S R G R H T S Y A D S V K G R
 TGGTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCAAGT ATTTCGTGGA GGGTAGGCA TACATCTTAC GCAGACTCCG TGAAGGGCCG
 ACCCAACCCA GCGGTCCGA GGTCCCTTCC CAGATCTCAC CCAGATTCGA TAAAGCAGCT CCCCCTCCG ATGAGAATG CGTCTGAGGC ACTTCCCGGC
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K R V
 GTTCACCATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTCGG TGCCGAGGAC ACCCGGTAT ATTACTGTGC GAAAAAGGTT

CAAGTGGTAG AGGCGCTGT TAAGTTCTT GTGACATA GACGTTTACT TGTGGAGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTCCCAA
P G R G R S F D Y W G Q G T L V T V S
CCGGTCGGG GCGTCTCTT TGACTACTGG GGCAGGAA CCTGTGTAC CGTCTCG
GGCCCCAGCC CCGCAAGAA ACTGATGACC CCGTCCCTT GGGACCATG GCAGAGC

5

Partner dAb monomer in TAR2h-5d12 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F P F R R Y R M
GAGGTGCAGC TGTGTGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTCTGTCTC CCTCGGAT CCCTTTCTG CGGTATCGGA
CTCCACGTCG ACAACTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GGAGCCAGG AGGACAGTC GGAGCCCTAA GGGGAAAGCA GCCATAGCCT
R W V R Q A P G K G L E W V S G I S P G G K H T Y A D S V K G R
TGAGGTGGGT CCGCCAGGCT CCAGGAAGG GTCTAGATG GGTCTCAGT ATTCTCCG GTGTTAGCA TACAGCTAC GCAGACTCG TGAAGGGCCG
ACTCCACCA GCGGTCCGA GGTCCCTTCC CAGATCTCAC CAGAGTCCA TAAAGAGCC CACCATCTG ATGTGCTG CGTCTGAGC ACTTCCCGC
F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K G E
GTTACCATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCCTCG TGCCGAGGAC ACCGCGTAT ATTACTGTG GAAAGTGAG
CAAGTGGTAG AGGGCGCTGT TAAGTTCTT GTGCGACATA GACGTTTACT TGTCCGAGC AGGCTCCTG TGGCGCCATA TAATGACACG CTTTCCACTC
G A S S A F D Y W G Q G T L V T V S
GGGGGGCGA GTTCTCGTT TGACTACTGG GGCAGGAA CCCTGGTCAC CGTCTCG
CCCCCGCT CAAGACGCA ACTGATGACC CCGTCCCTT GGGACCATG GCAGAGC

10

15

20

Partner dAb monomer in TAR2h-5d13 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F * R Y G M
GAGGTGCAGC TGTGTGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CCTGTCTC TCTGTGAG CCTCGGAT CACCTTTAG CGGTATGGGA
CTCCACGTCG ACAACTCAG ACCCCTCCG AACCATGTCG GACCCCCAG GGAGCCAGG AGGACAGTC GGAGCCCTAA GTGGAATC GCCATAGCCT
V W V R Q A P G K G L E W V S A I S G S G S T Y Y A D S V K G R
TGTCTGGGT CCGCCAGGCT CCAGGAAGG GTCTAGATG GGTCTCAGT ATTACTGTA GTGTTGAG CACACTAC GCAGACTCG TGAAGGGCCG
ACCAACCCA GCGGTCCGA GGTCCCTTCC CAGATCTCAC CAGAGTCGA TAATACCAT CACCACATC GTGTATGATG CGTCTGAGC ACTTCCCGC
F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K R H
GTTACCATC TCCCGGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTCG GTGCCGAGG ACCGCGTAT ATTACTGTG GAAACGCA
CAAGTGGTAG AGGGCGCTGT TAAGTTCTT GTGCGACATA GACGTTTACT TGTCCGAGC AGGCTCCTG TGGCGCCATA TAATGACACG CTTTCCCGTA
S S E A R Q F D Y W G Q G T L V T V S
AGTCTGAGG CTAGGCAGT TGACTACTGG GGCAGGAA CCCTGGTCAC CGTCTCG
TCAAGACTCC GATCCGTCAA ACTGATGACC CCGTCCCTT GGGACCATG GCAGAGC

25

30

35