DUAL VARIABLE REGION ANTIBODY-LIKE BINDING PROTEINS HAVING CROSS-OVER BINDING REGION ORIENTATION

FIELD OF THE INVENTION

The invention relates to antibody-like binding proteins comprising four polypeptide chains that form four antigen binding sites, wherein each pair of polypeptides forming the antibody-like binding protein possesses dual variable domains having a cross-over orientation. The invention also relates to methods for making such antigen-like binding proteins.

BACKGROUND OF THE INVENTION

Naturally occurring IgG antibodies are bivalent and monospecific. Bispecific antibodies having binding specificities for two different antigens can be produced using recombinant technologies and are projected to have broad clinical applications. It is well known that complete IgG antibody molecules are Y-shaped molecules comprising four polypeptide chains: two heavy chains and two light chains. Each light chain consists of two domains, the N-terminal domain being known as the variable or V_L domain (or region) and the C-terminal domain being known as the constant (or C_L) domain (constant kappa (Cκ) or constant lambda (Cλ) domain). Each heavy chain consists of four or five domains, depending on the class of the antibody. The N-terminal domain is known as the variable (or V_H) domain (or region), which is followed by the first constant (or C_{H1}) domain, the hinge region, and then the second and third constant (or C_{H2} and C_{H3}) domains. In an assembled antibody, the V_L and V_H domains associate together to form an antigen binding site. Also, the C_L and C_{H1} domains associate together to keep one heavy chain associated with one light chain. The two heavy-light chain heterodimers associate together by interaction of the C_{H2} and C_{H3} domains and interaction between the hinge regions on the two heavy chains.

It is known that proteolytic digestion of an antibody can lead to the production of antibody fragments (Fab and Fab2). Such fragments of the whole antibody can exhibit antigen binding activity. Antibody fragments can also be produced recombinantly. Fv fragments, consisting only of the variable domains of the heavy and light chains associated with each other may be obtained. These Fv fragments are monovalent for antigen binding. Smaller fragments such as individual variable domains (domain antibodies or dABs; Ward *et al.*, 1989, *Nature* 341(6242): 544-46),

and individual complementarity determining regions or CDRs (Williams *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86(14): 5537-41) have also been shown to retain the binding characteristics of the parent antibody, although most naturally occurring antibodies generally need both a V_H and V_L to retain full binding potency.

Single chain variable fragment (scFv) constructs comprise a V_H and a V_L domain of an antibody contained in a single polypeptide chain wherein the domains are separated by a flexible linker of sufficient length (more than 12 amino acid residues), that forces intramolecular interaction, allowing self-assembly of the two domains into a functional epitope binding site (Bird *et al.*, 1988, *Science* 242(4877): 423-26). These small proteins (MW ~25,000 Da) generally retain specificity and affinity for their antigen in a single polypeptide and can provide a convenient building block for larger, antigen-specific molecules.

An advantage of using antibody fragments rather than whole antibodies in diagnosis and therapy lies in their smaller size. They are likely to be less immunogenic than whole antibodies and more able to penetrate tissues. A disadvantage associated with the use of such fragments is that they have only one antigen binding site, leading to reduced avidity. In addition, due to their small size, they are cleared very fast from the serum, and hence display a short half-life.

It has been of interest to produce bispecific antibodies (BsAbs) that combine the antigen binding sites of two antibodies within a single molecule, and therefore, would be able to bind two different antigens simultaneously. Besides applications for diagnostic purposes, such molecules pave the way for new therapeutic applications, *e.g.*, by redirecting potent effector systems to diseased areas (where cancerous cells often develop mechanisms to suppress normal immune responses triggered by monoclonal antibodies, like antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC)), or by increasing neutralizing or stimulating activities of antibodies. This potential was recognized early on, leading to a number of approaches for obtaining such bispecific antibodies. Initial attempts to couple the binding specificities of two whole antibodies against different target antigens for therapeutic purposes utilized chemically fused heteroconjugate molecules (Staerz *et al.*, 1985, *Nature* 314(6012): 628-31).

Bispecific antibodies were originally made by fusing two hybridomas, each capable of producing a different immunoglobulin (Milstein *et al.*, 1983, *Nature* 305(5934): 537-40), but the complexity of species (up to ten different species)

produced in cell culture made purification difficult and expensive (George *et al.*, 1997, THE ANTIBODIES 4: 99-141 (Capra *et al.*, ed., Harwood Academic Publishers)). Using this format, a mouse IgG2a and a rat IgG2b antibody were produced together in the same cell (*e.g.*, either as a quadroma fusion of two hybridomas, or in engineered CHO cells). Because the light chains of each antibody associate preferentially with the heavy chains of their cognate species, three major species of antibody are assembled: the two parental antibodies, and a heterodimer of the two antibodies comprising one heavy/light chain pair of each, associating via their Fc portions. The desired heterodimer can be purified from this mixture because its binding properties to Protein A are different from those of the parental antibodies: rat IgG2b does not bind to Protein A, whereas the mouse IgG2a does. Consequently, the mouse-rat heterodimer binds to Protein A but elutes at a higher pH than the mouse IgG2a homodimer, and this makes selective purification of the bispecific heterodimer possible (Lindhofer *et al.*, 1995, *J. Immunol.* 155(1): 219-25). The resulting bispecific heterodimer is fully non-human, hence highly immunogenic, which could have deleterious side effects (*e.g.*, "HAMA" or "HARA" reactions), and/or neutralize the therapeutic. There remained a need for engineered bispecifics with superior properties that can be readily produced in high yield from mammalian cell culture.

Despite the promising results obtained using heteroconjugates or bispecific antibodies produced from cell fusions as cited above, several factors made them impractical for large scale therapeutic applications. Such factors include: rapid clearance of heteroconjugates *in vivo*, the laboratory intensive techniques required for generating either type of molecule, the need for extensive purification of heteroconjugates away from homoconjugates or mono-specific antibodies, and the generally low yields obtained.

Genetic engineering has been used with increasing frequency to design, modify, and produce antibodies or antibody derivatives with a desired set of binding properties and effector functions. A variety of recombinant methods have been developed for efficient production of BsAbs, both as antibody fragments (Carter *et al.*, 1995, *J. Hematother.* 4(5): 463-70; Pluckthun *et al.*, 1997, *Immunotechnology* 3(2): 83-105; Todorovska *et al.*, 2001, *J. Immunol. Methods* 248(1-2): 47-66) and full length IgG formats (Carter, 2001, *J. Immunol. Methods* 248(1-2): 7-15).

Combining two different scFvs results in BsAb formats with minimal molecular mass, termed sc-BsAbs or Ta-scFvs (Mack *et al.*, 1995, *Proc. Natl. Acad.*

Sci. U.S.A. 92(15): 7021-25; Mallender *et al.*, 1994, *J. Biol. Chem.* 269(1): 199-206). BsAbs have been constructed by genetically fusing two scFvs to a dimerization functionality such as a leucine zipper (Kostelny *et al.*, 1992, *J. Immunol.* 148(5): 1547-53; de Kruif *et al.*, 1996, *J. Biol. Chem.* 271(13): 7630-34).

Diabodies are small bivalent and bispecific antibody fragments. The fragments comprise a V_H connected to a V_L on the same polypeptide chain, by using a linker that is too short (less than 12 amino acid residues) to allow pairing between the two domains on the same chain. The domains are forced to pair intermolecularly with the complementary domains of another chain and create two antigen-binding sites. These dimeric antibody fragments, or "diabodies," are bivalent and bispecific (Holliger *et al.*, 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90(14): 6444-48). Diabodies are similar in size to a Fab fragment. Polypeptide chains of V_H and V_L domains joined with a linker of between 3 and 12 amino acid residues form predominantly dimers (diabodies), whereas with a linker of between 0 and 2 amino acid residues, trimers (triabodies) and tetramers (tetrabodies) predominate. In addition to the linker length, the exact pattern of oligomerization seems to depend on the composition as well as the orientation of the variable domains (Hudson *et al.*, 1999, *J. Immunol. Methods* 231(1-2): 177-89). The predictability of the final structure of diabody molecules is very poor.

Although sc-BsAb and diabody-based constructs display interesting clinical potential, it was shown that such non-covalently associated molecules are not sufficiently stable under physiological conditions. The overall stability of a scFv fragment depends on the intrinsic stability of the V_L and V_H domains as well as on the stability of the domain interface. Insufficient stability of the V_H-V_L interface of scFv fragments has often been suggested as a main cause of irreversible scFv inactivation, since transient opening of the interface, which would be allowed by the peptide linker, exposes hydrophobic patches that favor aggregation and therefore instability and poor production yield (Wörn *et al.*, 2001, *J. Mol. Biol.* 305(5): 989-1010).

An alternative method of manufacturing bispecific bivalent antigen-binding proteins from V_H and V_L domains is described in U.S. Patent No. 5,989,830. Such double head and dual Fv configurations are obtained by expressing a bicistronic vector, which encodes two polypeptide chains. In the Dual-Fv configuration, the variable domains of two different antibodies are expressed in a tandem orientation on two separate chains (one heavy chain and one light chain), wherein one polypeptide

chain has two times a V_H in series separated by a peptide linker (V_{H1}-linker-V_{H2}) and the other polypeptide chain consists of complementary V_L domains connected in series by a peptide linker (V_{L1}-linker-V_{L2}). In the cross-over double head configuration, the variable domains of two different antibodies are expressed in a tandem orientation on two separate polypeptide chains (one heavy chain and one light chain), wherein one polypeptide chain has two times a V_H in series separated by a peptide linker (V_{H1}-linker-V_{H2}) and the other polypeptide chain consists of complementary V_L domains connected in series by a peptide linker in the opposite orientation (V_{L2}-linker-V_{L1}). Molecular modeling of the constructs suggested the linker size to be long enough to span 30-40 Å (15–20 amino acid residues).

Increasing the valency of an antibody is of interest as it enhances the functional affinity of that antibody due to the avidity effect. Polyvalent protein complexes (PPC) with an increased valency are described in U.S. Patent Application Publication No. US 2005/0003403 A1. PPCs comprise two polypeptide chains generally arranged laterally to one another. Each polypeptide chain typically comprises three or four "v-regions," which comprise amino acid sequences capable of forming an antigen binding site when matched with a corresponding v-region on the opposite polypeptide chain. Up to about six "v-regions" can be used on each polypeptide chain. The v-regions of each polypeptide chain are connected linearly to one another and may be connected by interspersed linking regions. When arranged in the form of the PPC, the v-regions on each polypeptide chain form individual antigen binding sites. The complex may contain one or several binding specificities.

A strategy was proposed by Carter *et al.* (Ridgway *et al.*, 1996, *Protein Eng.* 9(7): 617-21; Carter, 2011, *J. Immunol. Methods* 248(1-2): 7-15) to produce a Fc heterodimer using a set of "knob-into-hole" mutations in the C_{H3} domain of Fc. These mutations lead to the alteration of residue packing complementarity between the C_{H3} domain interface within the structurally conserved hydrophobic core so that formation of the heterodimer is favored as compared with homodimers, which achieves good heterodimer expression from mammalian cell culture. Although the strategy led to higher heterodimer yield, the homodimers were not completely suppressed (Merchant *et al.*, 1998, *Nat. Biotechnol.* 16(7): 677-81.

Gunasekaran *et al.* explored the feasibility of retaining the hydrophobic core integrity while driving the formation of Fc heterodimer by changing the charge complementarity at the C_{H3} domain interface (Gunasekaran *et al.*, 2010, *J. Biol.*

Chem. 285(25): 19637-46). Taking advantage of the electrostatic steering mechanism, these constructs showed efficient promotion of Fc heterodimer formation with minimum contamination of homodimers through mutation of two pairs of peripherally located charged residues. In contrast to the knob-into-hole design, the homodimers were evenly suppressed due to the nature of the electrostatic repulsive mechanism, but not totally avoided.

Davis *et al.* describe an antibody engineering approach to convert Fc homodimers into heterodimers by interdigitating β-strand segments of human IgG and IgA C_{H3} domains, without the introduction of extra interchain disulfide bonds (Davis *et al.*, 2010, *Protein Eng. Des. Sel.* 23(4): 195-202). Expression of SEEDbody (Sb) fusion proteins by mammalian cells yields Sb heterodimers in high yield that are readily purified to eliminate minor by-products.

U.S. Patent Application Publication No. US 2010/331527 A1 describes a bispecific antibody based on heterodimerization of the C_{H3} domain, introducing in one heavy chain the mutations H95R and Y96F within the C_{H3} domain. These amino acid substitutions originate from the C_{H3} domain of the IgG3 subtype and will heterodimerize with an IgG1 backbone. A common light chain prone to pair with every heavy chain is a prerequisite for all formats based on heterodimerization though the C_{H3} domain. A total of three types of antibodies are therefore produced: 50% having a pure IgG1 backbone, one-third having a pure H95R and Y96F mutated backbone, and one-third having two different heavy chains (bispecific). The desired heterodimer can be purified from this mixture because its binding properties to Protein A are different from those of the parental antibodies: IgG3-derived C_{H3} domains do not bind to Protein A, whereas the IgG1 does. Consequently, the heterodimer binds to Protein A, but elutes at a higher pH than the pure IgG1 homodimer, and this makes selective purification of the bispecific heterodimer possible.

U.S. Patent No. 7,612,181 describes a Dual-Variable-Domain IgG (DVD-IgG) bispecific antibody that is based on the Dual-Fv format described in U.S. Patent No. 5,989,830. A similar bispecific format was also described in U.S. Patent Application Publication No. US 2010/0226923 A1. The addition of constant domains to respective chains of the Dual-Fv (C_{H1}-Fc to the heavy chain and kappa or lambda constant domain to the light chain) led to functional bispecific antibodies without any need for additional modifications (*i.e.*, obvious addition of constant domains to

enhance stability). Some of the antibodies expressed in the DVD-Ig/TBTI format show a position effect on the second (or innermost) antigen binding position (Fv2). Depending on the sequence and the nature of the antigen recognized by the Fv2 position, this antibody domain displays a reduced affinity to its antigen (*i.e.*, loss of on-rate in comparison to the parental antibody). One possible explanation for this observation is that the linker between V_{L1} and V_{L2} protrudes into the CDR region of Fv2, making the Fv2 somewhat inaccessible for larger antigens.

The second configuration of a bispecific antibody fragment described in U.S. Patent No. 5,989,830 is the cross-over double head (CODH), having the following orientation of variable domains expressed on two chains:

V_{L1}-linker-V_{L2}, for the light chain, and

V_{H2}-linker-V_{H1}, for the heavy chain

The '830 patent discloses that a bispecific cross-over double-head antibody fragment (construct GOSA.E) retains higher binding activity than a Dual-Fv (*see* page 20, lines 20-50 of the '830 patent), and further discloses that this format is less impacted by the linkers that are used between the variable domains (*see* page 20-21 of the '830 patent).

SUMMARY OF THE INVENTION

The invention provides an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, wherein two polypeptide chains have a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

and two polypeptide chains have a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \qquad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

The invention also provides an antibody-like binding protein comprising two polypeptide chains that form two antigen binding sites, wherein a first polypeptide chain has a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

and a second polypeptide chain has a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the first and second polypeptides form a cross-over light chain-heavy chain pair.

The invention further provides a method of making an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, comprising identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H; assigning either the light chain or the heavy chain as template chain; assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1}; assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \quad [II]$$

determining maximum and minimum lengths for L_1, L_2, L_3, and L_4; generating the polypeptide structures of formulas I and II; selecting polypeptide structures of formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

The invention further provides a method of making an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, comprising identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H; assigning either the light chain or the heavy chain as template chain; assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1}; assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

determining maximum and minimum lengths for L_1, L_2, L_3, and L_4; generating polypeptide structures of formulas I and II; selecting polypeptide structures of formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Specific embodiments of the invention will become evident from the following more detailed description of certain embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic representation of the antigen binding domains Fv1 and Fv2 within the dual V region configuration and arrangement of their respective peptide linkers L_L and L_H in the TBTI format.

Figure 2. Schematic diagram (2D) of the antigen binding domains Fv1 (anti-IL4) and Fv2 (anti-IL13) within the cross-over dual variable (CODV) configuration and the arrangement of their respective peptide linkers.

Figure 3. Schematic representation of the Fv anti-IL4 and Fab anti-IL13 showing one possible spatial arrangement obtained by protein-protein docking of Fv of anti-IL4 and the Fv of anti-IL13.

Figure 4. Assessment of tetravalent and bispecific binding ability of the CODV protein in a BIACORE assay by injecting the two antigens sequentially or simultaneously over a DVD-Ig protein–coated chip. The maximal signal observed by sequential injection can be obtained by co-injection of both antigens, demonstrating saturation of all binding sites.

Figure 5. Schematic diagram (2D) of the antigen binding domains within the CODV configuration and arrangement of their respective peptide linker L_L (L_1 and L_2) and L_H (L_3 and L_4). In panel A, the light chain is kept in a "linear or template" alignment, whereas the heavy chain is in the "cross-over" configuration. In panel B, the heavy chain is kept in a "linear or template" alignment and the light chain is in the "cross-over" configuration.

Figure 6. Schematic representation of CODV-Ig design based on whether the light chain or heavy chain is used as "template."

Figure 7. Comparison of TBTI/DVD-Ig or CODV-Ig molecules incorporating anti-

IL4 and anti-IL13 sequences.

Figure 8. Comparison of CODV-Fab and B-Fab formats in a cytotoxic assay using NALM-6 cells.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides antibody-like binding proteins comprising four polypeptide chains that form four antigen binding sites, wherein each pair of polypeptides forming an antibody-like binding protein possesses dual variable domains having a cross-over orientation. The invention also provides methods for making such antigen-like binding proteins.

Computer modeling predicted that the cross-over double-head (CODH) design of U.S. Patent No. 5,989,830 would yield a complex in which both binding sites face in the opposite direction, without the restraints suggested for the Dual-Fv configuration of U.S. Patent No. 7,612,181. In particular, computer modeling indicated that the length of the amino acid linkers between the variable domains was not critical for the CODH design, but was important for permitting full access to both antigen binding sites in the Dual-Fv design. As with the DVD-Ig/TBTI format, antibody-like binding protein constructs were prepared in which constant domains were attached to the CODH configuration to form antibody-like binding proteins comprising four polypeptide chains that form four antigen binding sites, wherein each pair of polypeptides forming an antibody-like binding protein possesses dual variable domains having a cross-over orientation (*i.e.*, CODH-Ig). CODH-Ig molecules are expected to possess significantly improved stability as compared with CODH molecules (as DVD-Ig/TBTI possessed improved stability over Dual-Fv molecules).

In order to test the above hypothesis, a CODH-Ig molecule was prepared using the anti-IL4 and anti-IL13 antibody sequences described in U.S. Patent Application Publication No. US 2010/0226923 A1. The CODH-Ig molecule differed from the CODH molecule of US 2010/0226923 with respect to the lengths of amino acid linkers separating the variable domains on the respective polypeptide chains. The CODH-Ig molecules were expressed in cells following transient transfection and were then purified by Protein A chromatography. Although their size-exclusion chromatography (SEC) profiles showed aggregation levels of 5-10%, none of the CODH-Ig molecules were functional, and thus none of the CODH-Ig molecules was

able to bind all of its target antigens. The lack of antigen binding activity may have been due to a perturbed dimerization of the Fv-regions of the heavy and light chains due to unsuitable linker lengths compromising correct paratope formation. As a result, a protocol was developed to identify suitable amino acid linkers for insertion between the two variable domains and the second variable domain and constant domain on both the heavy and light polypeptide chains of an antibody-like binding protein. This protocol was based on protein-protein docking of homology and experimental models of the FvIL4 and FvIL13 regions, respectively, inclusion of the Fc1 domain the model, and construction of appropriate linkers between the FvIL4 and FvIL13 regions and between the Fv and constant Fc1 regions.

Standard recombinant DNA methodologies are used to construct the polynucleotides that encode the polypeptides which form the antibody-like binding proteins of the invention, incorporate these polynucleotides into recombinant expression vectors, and introduce such vectors into host cells. *See e.g.*, Sambrook *et al.*, 2001, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Laboratory Press, 3rd ed.). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclature utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Similarly, conventional techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, delivery, and treatment of patients.

1. General Definitions

As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

The term "polynucleotide" as used herein refers to single-stranded or double-stranded nucleic acid polymers of at least 10 nucleotides in length. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides

or deoxyribonucleotides or a modified form of either type of nucleotide. Such modifications include base modifications such as bromuridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single-stranded and double-stranded forms of DNA.

An "isolated polynucleotide" is a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the isolated polynucleotide: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence.

An "isolated polypeptide" is one that: (1) is free of at least some other polypeptides with which it would normally be found, (2) is essentially free of other polypeptides from the same source, *e.g.*, from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is not associated (by covalent or noncovalent interaction) with portions of a polypeptide with which the "isolated polypeptide" is associated in nature, (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (7) does not occur in nature. Such an isolated polypeptide can be encoded by genomic DNA, cDNA, mRNA or other RNA, of synthetic origin, or any combination thereof. Preferably, the isolated polypeptide is substantially free from polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

The term "human antibody" as used herein includes antibodies having variable and constant regions substantially corresponding to human germline immunoglobulin sequences. In some embodiments, human antibodies are produced in non-human mammals, including, but not limited to, rodents, such as mice and rats, and lagomorphs, such as rabbits. In other embodiments, human antibodies are produced in hybridoma cells. In still other embodiments, human antibodies are produced recombinantly.

Naturally occurring antibodies typically comprise a tetramer. Each such tetramer is typically composed of two identical pairs of polypeptide chains, each pair having one full-length "light" chain (typically having a molecular weight of about 25 kDa) and one full-length "heavy" chain (typically having a molecular weight of about 50-70 kDa). The terms "heavy chain" and "light chain" as used herein refer to any immunoglobulin polypeptide having sufficient variable domain sequence to confer specificity for a target antigen. The amino-terminal portion of each light and heavy chain typically includes a variable domain of about 100 to 110 or more amino acids that typically is responsible for antigen recognition. The carboxy-terminal portion of each chain typically defines a constant domain responsible for effector function. Thus, in a naturally occurring antibody, a full-length heavy chain immunoglobulin polypeptide includes a variable domain (V_H) and three constant domains (C_{H1}, C_{H2}, and C_{H3}), wherein the V_H domain is at the amino-terminus of the polypeptide and the C_{H3} domain is at the carboxyl-terminus, and a full-length light chain immunoglobulin polypeptide includes a variable domain (V_L) and a constant domain (C_L), wherein the V_L domain is at the amino-terminus of the polypeptide and the C_L domain is at the carboxyl-terminus.

Human light chains are typically classified as kappa and lambda light chains, and human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subclasses, including, but not limited to, IgG1, IgG2, IgG3, and IgG4. IgM has subclasses including, but not limited to, IgM1 and IgM2. IgA is similarly subdivided into subclasses including, but not limited to, IgA1 and IgA2. Within full-length light and heavy chains, the variable and constant domains typically are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. *See*, *e.g.*, FUNDAMENTAL IMMUNOLOGY (Paul, W., ed., Raven Press, 2nd ed., 1989), which is incorporated by reference in its entirety for all purposes. The variable regions of each light/heavy chain pair typically form an antigen binding site. The variable domains of naturally occurring antibodies typically exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair typically are aligned by the framework regions, which may enable binding to a specific epitope. From the amino-terminus to the carboxyl-terminus, both light

and heavy chain variable domains typically comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4.

The term "native Fc" as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is preferably of human origin and can be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (*i.e.*, disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (*e.g.*, IgG, IgA, and IgE) or subclass (*e.g.*, IgG1, IgG2, IgG3, IgA1, and IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" as used herein refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). Exemplary Fc variants, and their interaction with the salvage receptor, are known in the art. Thus, the term "Fc variant" can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the antibody-like binding proteins of the invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has be modified, that affect or are involved in: (1) disulfide bond formation, (2) incompatibility with a selected host cell, (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC).

The term "Fc domain" as used herein encompasses native Fc and Fc variants and sequences as defined above. As with Fc variants and native Fc molecules, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means.

The term "antibody-like binding protein" as used herein refers to a non-naturally occurring (or recombinant) molecule that specifically binds to at least one target antigen, and which comprises four polypeptide chains that form four antigen binding sites, wherein two polypeptide chains have a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

and two polypeptide chains have a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \qquad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair. The term "antibody-like binding protein" as used herein also refers to a non-naturally occurring (or recombinant) molecule that specifically binds to at least one target antigen, and which comprises two polypeptide chains that form two antigen binding sites, wherein a first polypeptide chain has a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

and a second polypeptide chain has a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the first and second polypeptides form a cross-over light chain-heavy chain pair. A "recombinant" molecule is one that has been prepared, expressed, created, or isolated by recombinant means.

One embodiment of the invention provides antibody-like binding proteins having biological and immunological specificity to between one and four target antigens. Another embodiment of the invention provides nucleic acid molecules comprising nucleotide sequences encoding polypeptide chains that form such antibody-like binding proteins. Another embodiment of the invention provides expression vectors comprising nucleic acid molecules comprising nucleotide sequences encoding polypeptide chains that form such antibody-like binding proteins. Yet another embodiment of the invention provides host cells that express such antibody-like binding proteins (*i.e.*, comprising nucleic acid molecules or vectors encoding polypeptide chains that form such antibody-like binding proteins).

The term "swapability" as used herein refers to the interchangeability of variable domains within the CODV format and with retention of folding and ultimate binding affinity. "Full swapability" refers to the ability to swap the order of both V_{H1} and V_{H2} domains, and therefore the order of V_{L1} and V_{L2} domains, in a CODV-Ig (*i.e.*, to reverse the order) or CODV-Fab while maintaining full functionality of the antibody-like binding protein as evidenced by the retention of binding affinity. Furthermore, it should be noted that the designations V_H and V_L within a particular CODV-Ig or CODV-Fab refer only to the domain's location on a particular protein chain in the final format. For example, V_{H1} and V_{H2} could be derived from V_{L1} and V_{L2} domains in parent antibodies and placed into the V_{H1} and V_{H2} positions in the antibody-like binding protein. Likewise, V_{L1} and V_{L2} could be derived from V_{H1} and V_{H2} domains in parent antibodies and placed in the V_{H1} and V_{H2} positions in the antibody-like binding protein. Thus, the V_H and V_L designations refer to the present location and not the original location in a parent antibody. V_H and V_L domains are therefore "swappable."

An "isolated" antibody-like binding protein is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody-like binding protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In

preferred embodiments, the antibody-like binding protein will be purified: (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody-like binding proteins include the antibody-like binding protein *in situ* within recombinant cells since at least one component of the antibody-like binding protein's natural environment will not be present.

The terms "substantially pure" or "substantially purified" as used herein refer to a compound or species that is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition). In some embodiments, a substantially purified fraction is a composition wherein the species comprises at least about 50% (on a molar basis) of all macromolecular species present. In other embodiments, a substantially pure composition will comprise more than about 80%, 85%, 90%, 95%, or 99% of all macromolar species present in the composition. In still other embodiments, the species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The term "antigen" or "target antigen" as used herein refers to a molecule or a portion of a molecule that is capable of being bound by an antibody-like binding protein, and additionally is capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. A target antigen may have one or more epitopes. With respect to each target antigen recognized by an antibody-like binding protein, the antibody-like binding protein is capable of competing with an intact antibody that recognizes the target antigen. A "bivalent" antibody-like binding protein, other than a "multispecific" or "multifunctional" antibody-like binding protein, is understood to comprise antigen binding sites having identical antigenic specificity.

A bispecific or bifunctional antibody typically is an artificial hybrid antibody having two different heavy chain/light chain pairs and two different binding sites or epitopes. Bispecific antibodies may be produced by a variety of methods including, but not limited to, fusion of hybridomas or linking of F(ab') fragments.

A F(ab) fragment typically includes one light chain and the V_H and C_{H1} domains of one heavy chain, wherein the V_H-C_{H1} heavy chain portion of the F(ab) fragment cannot form a disulfide bond with another heavy chain polypeptide. As used herein, a F(ab) fragment can also include one light chain containing two variable domains separated by an amino acid linker and one heavy chain containing two variable domains separated by an amino acid linker and a C_{H1} domain.

A F(ab') fragment typically includes one light chain and a portion of one heavy chain that contains more of the constant region (between the C_{H1} and C_{H2} domains), such that an interchain disulfide bond can be formed between two heavy chains to form a $F(ab')_2$ molecule.

The phrases "biological property," "biological characteristic," and the term "activity" in reference to an antibody-like binding protein of the invention are used interchangeably herein and include, but are not limited to, epitope affinity and specificity, ability to antagonize the activity of the antigen target (or targeted polypeptide), the *in vivo* stability of the antibody-like binding protein, and the immunogenic properties of the antibody-like binding protein. Other identifiable biological properties or characteristics of an antibody-like binding protein include, for example, cross-reactivity, (*i.e.*, with non-human homologs of the antigen target, or with other antigen targets or tissues, generally), and ability to preserve high expression levels of protein in mammalian cells. The aforementioned properties or characteristics can be observed or measured using art-recognized techniques including, but not limited to ELISA, competitive ELISA, surface plasmon resonance analysis, *in vitro* and *in vivo* neutralization assays, and immunohistochemistry with tissue sections from different sources including human, primate, or any other source as the need may be.

The term "immunologically functional immunoglobulin fragment" as used herein refers to a polypeptide fragment that contains at least the CDRs of the immunoglobulin heavy or light chains from which the polypeptide fragment was derived. An immunologically functional immunoglobulin fragment is capable of binding to a target antigen.

A "neutralizing" antibody-like binding protein as used herein refers to a molecule that is able to block or substantially reduce an effector function of a target antigen to which it binds. As used herein, "substantially reduce" means at least about 60%, preferably at least about 70%, more preferably at least about 75%, even more

preferably at least about 80%, still more preferably at least about 85%, most preferably at least about 90% reduction of an effector function of the target antigen.

The term "epitope" includes any determinant, preferably a polypeptide determinant, capable of specifically binding to an immunoglobulin or T-cell receptor. In certain embodiments, epitope determinants include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics and/or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody or antibody-like binding protein. In certain embodiments, an antibody-like binding protein is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In preferred embodiments, an antibody-like binding protein is said to specifically bind an antigen when the equilibrium dissociation constant is $\leq 10^{-8}$ M, more preferably when the equilibrium dissociation constant is $\leq 10^{-9}$ M, and most preferably when the dissociation constant is $\leq 10^{-10}$ M.

The dissociation constant (K_D) of an antibody-like binding protein can be determined, for example, by surface plasmon resonance. Generally, surface plasmon resonance analysis measures real-time binding interactions between ligand (a target antigen on a biosensor matrix) and analyte (an antibody-like binding protein in solution) by surface plasmon resonance (SPR) using the BIAcore system (Pharmacia Biosensor; Piscataway, NJ). Surface plasmon analysis can also be performed by immobilizing the analyte (antibody-like binding protein on a biosensor matrix) and presenting the ligand (target antigen). The term "K_D," as used herein refers to the dissociation constant of the interaction between a particular antibody-like binding protein and a target antigen.

The term "specifically binds" as used herein refers to the ability of an antibody-like protein or an antigen-binding fragment thereof to bind to an antigen containing an epitope with an Kd of at least about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, or more, and/or to bind to an epitope with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen.

The term "linker" as used herein refers to one or more amino acid residues inserted between immunoglobulin domains to provide sufficient mobility for the domains of the light and heavy chains to fold into cross over dual variable region

immunoglobulins. A linker is inserted at the transition between variable domains or between variable and constant domains, respectively, at the sequence level. The transition between domains can be identified because the approximate size of the immunoglobulin domains are well understood. The precise location of a domain transition can be determined by locating peptide stretches that do not form secondary structural elements such as beta-sheets or alpha-helices as demonstrated by experimental data or as can be assumed by techniques of modeling or secondary structure prediction. The linkers described herein are referred to as L_1, which is located on the light chain between the N-terminal V_{L1} and V_{L2} domains; L_2, which is also on the light chain is located between the V_{L2} and C-terminal C_L domains. The heavy chain linkers are known as L_3, which is located between the N-terminal V_{H2} and V_{H1} domains; and L_4, which is located between the V_{H1} and C_{H1}-Fc domains. The linkers L_1, L_2, L_3, and L_4 are independent, but they may in some cases have the same sequence and/or length.

The term "vector" as used herein refers to any molecule (*e.g.*, nucleic acid, plasmid, or virus) that is used to transfer coding information to a host cell. The term "vector" includes a nucleic acid molecule that is capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double-stranded DNA molecule into which additional DNA segments may be inserted. Another type of vector is a viral vector, wherein additional DNA segments may be inserted into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell and thereby are replicated along with the host genome. In addition, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms "plasmid" and "vector" may be used interchangeably herein, as a plasmid is the most commonly used form of vector. However, the invention is intended to include other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses, and adeno-associated viruses), which serve equivalent functions.

The term "operably linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably linked to a coding sequence may be capable of effecting the replication, transcription, and/or translation of the coding sequence. For example, a coding sequence is operably linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

The phrase "recombinant host cell" (or "host cell") as used herein refers to a cell into which a recombinant expression vector has been introduced. A recombinant host cell or host cell is intended to refer not only to the particular subject cell, but also to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but such cells are still included within the scope of the term "host cell" as used herein. A wide variety of host cell expression systems can be used to express the antibody-like binding proteins of the invention, including bacterial, yeast, baculoviral, and mammalian expression systems (as well as phage display expression systems). An example of a suitable bacterial expression vector is pUC19. To express an antibody-like binding protein recombinantly, a host cell is transformed or transfected with one or more recombinant expression vectors carrying DNA fragments encoding the polypeptide chains of the antibody-like binding protein such that the polypeptide chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibody-like binding protein can be recovered.

The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transformation, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably

transformed when the DNA is replicated with the division of the cell. The term "transfection" as used herein refers to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art. Such techniques can be used to introduce one or more exogenous DNA molecules into suitable host cells.

The term "naturally occurring" as used herein and applied to an object refers to the fact that the object can be found in nature and has not been manipulated by man. For example, a polynucleotide or polypeptide that is present in an organism (including viruses) that can be isolated from a source in nature and that has not been intentionally modified by man is naturally-occurring. Similarly, "non-naturally occurring" as used herein refers to an object that is not found in nature or that has been structurally modified or synthesized by man.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids; unnatural amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for the polypeptide chains of the antibody-like binding proteins of the invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and imino acids (*e.g.*, 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxyl-terminal direction, in accordance with standard usage and convention.

Naturally occurring residues may be divided into classes based on common side chain properties:

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp, Tyr, Pro;

(2) polar hydrophilic: Arg, Asn, Asp, Gln, Glu, His, Lys, Ser, Thr ;

(3) aliphatic: Ala, Gly, Ile, Leu, Val, Pro;

(4) aliphatic hydrophobic: Ala, Ile, Leu, Val, Pro;

(5) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(6) acidic: Asp, Glu;

(7) basic: His, Lys, Arg;

(8) residues that influence chain orientation: Gly, Pro;

(9) aromatic: His, Trp, Tyr, Phe; and

(10) aromatic hydrophobic: Phe, Trp, Tyr.

Conservative amino acid substitutions may involve exchange of a member of one of these classes with another member of the same class. Conservative amino acid substitutions may encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid residues. Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class.

A skilled artisan will be able to determine suitable variants of the polypeptide chains of the antibody-like binding proteins of the invention using well-known techniques. For example, one skilled in the art may identify suitable areas of a polypeptide chain that may be changed without destroying activity by targeting regions not believed to be important for activity. Alternatively, one skilled in the art can identify residues and portions of the molecules that are conserved among similar polypeptides. In addition, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

The term "patient" as used herein includes human and animal subjects.

A "disorder" is any condition that would benefit from treatment using the antibody-like binding proteins of the invention. "Disorder" and "condition" are used interchangeably herein and include chronic and acute disorders or diseases, including those pathological conditions that predispose a patient to the disorder in question.

The terms "treatment" or "treat" as used herein refer to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those having the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

The terms "pharmaceutical composition" or "therapeutic composition" as used herein refer to a compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient.

The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of an antibody-like binding protein.

The terms "effective amount" and "therapeutically effective amount" when used in reference to a pharmaceutical composition comprising one or more antibody-like binding proteins refer to an amount or dosage sufficient to produce a desired therapeutic result. More specifically, a therapeutically effective amount is an amount of an antibody-like binding protein sufficient to inhibit, for some period of time, one or more of the clinically defined pathological processes associated with the condition being treated. The effective amount may vary depending on the specific antibody-like binding protein that is being used, and also depends on a variety of factors and conditions related to the patient being treated and the severity of the disorder. For example, if the antibody-like binding protein is to be administered *in vivo*, factors such as the age, weight, and health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work would be among those factors considered. The determination of an effective amount or therapeutically effective amount of a given pharmaceutical composition is well within the ability of those skilled in the art.

One embodiment of the invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of an antibody-like binding protein.

2. Antibody-like Binding Proteins

In one embodiment of the invention, the antibody-like binding proteins comprise four polypeptide chains that form four antigen binding sites, wherein two polypeptide chains have a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

and two polypeptide chains have a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \qquad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

In another embodiment of the invention, the antibody-like binding proteins comprise two polypeptide chains that form two antigen binding sites, wherein a first polypeptide chain has a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

and a second polypeptide chain has a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the first and second polypeptides form a cross-over light chain-heavy chain pair.

The antibody-like binding proteins of the invention may be prepared using domains or sequences obtained or derived from any human or non-human antibody, including, for example, human, murine, or humanized antibodies.

In some antibody-like binding proteins of the invention, the length of L_3 is at least twice the length of L_1. In other antibody-like binding proteins of the invention, the length of L_4 is at least twice the length of L_2. In some antibody-like binding proteins of the invention, the length of L_1 is at least twice the length of L_3. In other antibody-like binding proteins of the invention, the length of L_2 is at least twice the length of L_4.

In some antibody-like binding proteins of the invention, L_1 is 3 to 12 amino acid residues in length, L_2 is 3 to 14 amino acid residues in length, L_3 is 1 to 8 amino

acid residues in length, and L_4 is 1 to 3 amino acid residues in length. In other antibody-like binding proteins, L_1 is 5 to 10 amino acid residues in length, L_2 is 5 to 8 amino acid residues in length, L_3 is 1 to 5 amino acid residues in length, and L_4 is 1 to 2 amino acid residues in length. In a preferred antibody-like binding protein, L_1 is 7 amino acid residues in length, L_2 is 5 amino acid residues in length, L_3 is 1 amino acid residues in length, and L_4 is 2 amino acid residues in length.

In some antibody-like binding proteins of the invention, L_1 is 1 to 3 amino acid residues in length, L_2 is 1 to 4 amino acid residues in length, L_3 is 2 to 15 amino acid residues in length, and L_4 is 2 to 15 amino acid residues in length. In other antibody-like binding proteins, L_1 is 1 to 2 amino acid residues in length, L_2 is 1 to 2 amino acid residues in length, L_3 is 4 to 12 amino acid residues in length, and L_4 is 2 to 12 amino acid residues in length. In a preferred antibody-like binding protein, L_1 is 1 amino acid residue in length, L_2 is 2 amino acid residues in length, L_3 is 7 amino acid residues in length, and L_4 is 5 amino acid residues in length.

In some antibody-like binding proteins of the invention, L_1, L_3, or L_4 may be equal to zero. However, in antibody-like binding proteins wherein L_1, L_3, or L_4 is equal to zero, the corresponding transition linker between the variable region and constant region or between the dual variable domains on the other chain cannot be zero. In some embodiments, L_1 is equal to zero and L_3 is 2 or more amino acid residues, L_3 is equal to zero and L_1 is equal to 1 or more amino acid residues, or L_4 is equal to 0 and L_2 is 3 or more amino acid residues.

In some antibody-like binding proteins of the invention, at least one of the linkers selected from the group consisting of L_1, L_2, L_3, and L_4 contains at least one cysteine residue.

Examples of suitable linkers include a single glycine (Gly) residue; a diglycine peptide (Gly-Gly); a tripeptide (Gly-Gly-Gly); a peptide with four glycine residues (Gly-Gly-Gly-Gly; SEQ ID NO: 25); a peptide with five glycine residues (Gly-Gly-Gly-Gly-Gly; SEQ ID NO: 26); a peptide with six glycine residues (Gly-Gly-Gly-Gly-Gly-Gly; SEQ ID NO: 27); a peptide with seven glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly; SEQ ID NO: 28); a peptide with eight glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly; SEQ ID NO: 29). Other combinations of amino acid residues may be used such as the peptide Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 30) and the peptide Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 31). Other suitable linkers include a single Ser, and Val residue; the dipeptide Arg-Thr, Gln-Pro,

Ser-Ser, Thr-Lys, and Ser-Leu; Thr-Lys-Gly-Pro-Ser (SEQ ID NO: 52), Thr-Val-Ala-Ala-Pro (SEQ ID NO: 53), Gln-Pro-Lys-Ala-Ala (SEQ ID NO: 54), Gln-Arg-Ile-Glu-Gly (SEQ ID NO: 55); Ala-Ser-Thr-Lys-Gly-Pro-Ser (SEQ ID NO: 48), Arg-Thr-Val-Ala-Ala-Pro-Ser (SEQ ID NO: 49), Gly-Gln-Pro-Lys-Ala-Ala-Pro (SEQ ID NO: 50), and His-Ile-Asp-Ser-Pro-Asn-Lys (SEQ ID NO: 51). The examples listed above are not intended to limit the scope of the invention in any way, and linkers comprising randomly selected amino acids selected from the group consisting of valine, leucine, isoleucine, serine, threonine, lysine, arginine, histidine, aspartate, glutamate, asparagine, glutamine, glycine, and proline have been shown to be suitable in the antibody-like binding proteins of the invention (*see* Example 12).

The identity and sequence of amino acid residues in the linker may vary depending on the type of secondary structural element necessary to achieve in the linker. For example, glycine, serine, and alanine are best for linkers having maximum flexibility. Some combination of glycine, proline, threonine, and serine are useful if a more rigid and extended linker is necessary. Any amino acid residue may be considered as a linker in combination with other amino acid residues to construct larger peptide linkers as necessary depending on the desired properties.

In some antibody-like binding proteins of the invention, V_{L1} comprises the amino acid sequence of SEQ ID NO: 1; V_{L2} comprises the amino acid sequence of SEQ ID NO: 3; V_{H1} comprises the amino acid sequence of SEQ ID NO: 2; and V_{H2} comprises the amino acid sequence of SEQ ID NO: 4.

In some embodiments of the invention, the antibody-like binding protein is capable of specifically binding one or more antigen targets. In preferred embodiments of the invention, the antibody-like binding protein is capable of specifically binding at least one antigen target selected from the group consisting of B7.1, B7.2, BAFF, BlyS, C3, C5, CCL11 (eotaxin), CCL15 (MIP-1d), CCL17 (TARC), CCL19 (MIP-3b), CCL2 (MCP-1), CCL20 (MIP-3a), CCL21 (MIP-2), SLC, CCL24 (MPIF-2/eotaxin-2), CCL25 (TECK), CCL26 (eotaxin-3), CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (mcp-2), CD3, CD19, CD20, CD24, CD40, CD40L, CD80, CD86, CDH1 (E-cadherin), Chitinase, CSF1 (M-CSF), CSF2 (GM-CSF), CSF3 (GCSF), CTLA4, CX3CL1 (SCYD1), CXCL12 (SDF1), CXCL13, EGFR, FCER1A, FCER2, HER2, IGF1R, IL-1, IL-12, IL13, IL15, IL17, IL18, IL1A, IL1B, IL1F10, IL1β, IL2, IL4, IL6, IL7, IL8, IL9, IL12/23, IL22, IL23, IL25, IL27, IL35, ITGB4 (b 4 integrin), LEP (leptin), MHC class II, TLR2,

TLR4, TLR5, TNF, TNF-a, TNFSF4 (OX40 ligand), TNFSF5 (CD40 ligand), Toll-like receptors, TREM1, TSLP, TWEAK, XCR1 (GPR5/CCXCR1), DNGR-1(CLEC91), and HMGB1. In other embodiments of the invention, the antibody-like binding protein is capable of inhibiting the function of one or more of the antigen targets.

In some embodiments of the invention, the antibody-like binding protein is bispecific and capable of binding two different antigen targets or epitopes. In a preferred embodiment of the invention, the antibody-like binding protein is bispecific and each light chain-heavy chain pair is capable of binding two different antigen targets or epitopes. In a more preferred embodiment, the antibody-like binding protein is capable of binding two different antigen targets that are selected from the group consisting of IL4 and IL13, IGF1R and HER2, IGF1R and EGFR, EGFR and HER2, BK and IL13, PDL-1 and CTLA-4, CTLA4 and MHC class II, IL-12 and IL-18, IL-1α and IL-1β, TNFα and IL12/23, TNFα and IL-12p40, TNFα and IL-1β, TNFα and IL-23, and IL17 and IL23. In an even more preferred embodiment, the antibody-like binding protein is capable of binding the antigen targets IL4 and IL13.

In some embodiments of the invention, the antibody-like binding protein specifically binds IL4 with an on-rate of 2.97 E+07 and an off-rate of 3.30 E-04 and specifically binds IL13 with an on-rate of 1.39 E+06 and an off-rate of 1.63 E-04. In other embodiments of the invention, the antibody-like binding protein specifically binds IL4 with an on-rate of 3.16 E+07 and an off-rate of 2.89 E-04 and specifically binds IL13 with an on-rate of 1.20 E+06 and an off-rate of 1.12 E-04.

In one embodiment of the invention, an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites is prepared by identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H; assigning either the light chain or the heavy chain as template chain; assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1}; assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \qquad [II]$$

determining maximum and minimum lengths for L_1, L_2, L_3, and L_4; generating polypeptide structures of formulas I and II; selecting polypeptide structures of

formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

In another embodiment of the invention, an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites is prepared by identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H; assigning either the light chain or the heavy chain as template chain; assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1}; assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

determining maximum and minimum lengths for L_1, L_2, L_3, and L_4; generating polypeptide structures of formulas I and II; selecting polypeptide structures of formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are an amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

In other embodiments of the invention, an antibody-like binding protein in which the first antibody variable domain and the second antibody variable domain are the same is prepared.

One embodiment of the invention provides a method for making an antibody-like binding protein, comprising expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Another embodiment of the invention provides a method for making an antibody-like binding protein, comprising expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptide of formula I and the polypeptide of formula II form a cross-over light chain-heavy chain pair.

3. Uses for Antibody-like Binding Proteins

The antibody-like binding proteins of the invention can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays for the detection and quantitation of one or more target antigens. The antibody-like binding proteins will bind the one or more target antigens with an affinity that is appropriate for the assay method being employed.

For diagnostic applications, in certain embodiments, antibody-like binding proteins can be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, ^{99}Tc, ^{111}In, or ^{67}Ga; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, β-galactosidase, or horseradish peroxidase.

The antibody-like binding proteins of the invention are also useful for *in vivo* imaging. An antibody-like binding protein labeled with a detectable moiety can be administered to an animal, preferably into the bloodstream, and the presence and location of the labeled antibody in the host assayed. The antibody-like binding protein can be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

The invention also relates to a kit comprising an antibody-like binding protein and other reagents useful for detecting target antigen levels in biological samples. Such reagents can include a detectable label, blocking serum, positive and negative control samples, and detection reagents.

4. Antibody-Like Binding Protein Therapeutic Compositions and

Administration Thereof

Therapeutic or pharmaceutical compositions comprising antibody-like binding proteins are within the scope of the invention. Such therapeutic or pharmaceutical compositions can comprise a therapeutically effective amount of an antibody-like binding protein, or antibody-like binding protein-drug conjugate, in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration.

Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The pharmaceutical composition can contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrogen-sulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrins), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or polyethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), tonicity enhancing agents (such as alkali metal halides – preferably sodium or potassium chloride – or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants (*see, e.g.,* REMINGTON'S

PHARMACEUTICAL SCIENCES (18th Ed., A.R. Gennaro, ed., Mack Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The optimal pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage. Such compositions can influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the antibody-like binding protein.

The primary vehicle or carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier for injection can be water, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute. In one embodiment of the invention, antibody-like binding protein compositions can be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents in the form of a lyophilized cake or an aqueous solution. Further, the antibody-like binding protein can be formulated as a lyophilizate using appropriate excipients such as sucrose.

The pharmaceutical compositions of the invention can be selected for parenteral delivery. Alternatively, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

When parenteral administration is contemplated, the therapeutic compositions for use in this invention can be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired antibody-like binding protein in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which an antibody-like binding protein is

formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which can then be delivered via a depot injection. Hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a pharmaceutical composition can be formulated for inhalation. For example, an antibody-like binding protein can be formulated as a dry powder for inhalation. Antibody-like binding protein inhalation solutions can also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions can be nebulized.

It is also contemplated that certain formulations can be administered orally. In one embodiment of the invention, antibody-like binding proteins that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the antibody-like binding protein. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Another pharmaceutical composition can involve an effective quantity of antibody-like binding proteins in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions of the invention will be evident to those skilled in the art, including formulations involving antibody-like binding proteins in sustained- or controlled-delivery formulations. Techniques for

formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides, copolymers of L-glutamic acid and gamma ethyl-L-glutamate, poly(2-hydroxyethyl-methacrylate), ethylene vinyl acetate, or poly-D(-)-3-hydroxybutyric acid. Sustained-release compositions can also include liposomes, which can be prepared by any of several methods known in the art.

Pharmaceutical compositions of the invention to be used for *in vivo* administration typically must be sterile. This can be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method can be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration can be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations can be stored either in a ready-to-use form or in a form (*e.g.*, lyophilized) requiring reconstitution prior to administration.

The invention also encompasses kits for producing a single-dose administration unit. The kits can each contain both a first container having a dried protein and a second container having an aqueous formulation. Also included within the scope of this invention are kits containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes).

The effective amount of an antibody-like binding protein pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the antibody-like binding protein is being used, the route of administration, and the size (body weight, body surface, or organ size) and condition (the age and general health) of the patient. Accordingly, the

clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage can range from about 0.1 μg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In other embodiments, the dosage can range from 0.1 μg/kg up to about 100 mg/kg; or 1 μg/kg up to about 100 mg/kg; or 5 μg/kg, 10 μg/kg, 15 μg/kg, 20 μg/kg, 25 μg/kg, 30 μg/kg, 35 μg/kg, 40 μg/kg, 45 μg/kg, 50 μg/kg, 55 μg/kg, 60 μg/kg, 65 μg/kg, 70 μg/kg, 75 μg/kg, up to about 100 mg/kg.

Dosing frequency will depend upon the pharmacokinetic parameters of the antibody-like binding protein in the formulation being used. Typically, a clinician will administer the composition until a dosage is reached that achieves the desired effect. The composition can therefore be administered as a single dose, as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages can be ascertained through use of appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, *e.g.*, orally; through injection by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, intraportal, or intralesional routes; by sustained release systems; or by implantation devices. Where desired, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device.

The composition can also be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration.

5. Examples

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and should not be construed as limiting the scope of the invention in any way.

Example 1. Design and Engineering of Bispecific Cross-over Dual Variable Region Antibody-like Binding Proteins

The cross-over dual variable region in an Fv format was described in U.S. Patent No. 5,989,830 and was referred to as a cross-over double head (CODH) configuration. Molecular modeling predicted that the cross-over double-head (CODH) design results in a complex with both binding sites facing in opposite directions, without the restraints suggested for the Dual-Fv configuration. The CODH Fv format was examined to determine whether it could be converted into complete antibody-like molecules by adding a C_L domain to the light chain and an Fc region to the heavy chain. A similar conversion was successful for the corresponding dual variable domains (DVD-Ig) and TBTI as described in U.S. Patent No. 7,612,181 and International Publication No. WO 2009/052081. The arrangement of the variable regions in the CODH format is shown in the structures below, which indicate the amino to carboxyl orientation of the peptide chains:

(a) light chain: NH_2-V_{L1}-Linker-V_{L2}-COOH

(b) heavy chain: NH_2-V_{H2}-Linker-V_{H1}-COOH

The amino to carboxyl terminal arrangement of the variable regions in (a) and (b) above can be distinguished from the arrangement in the Dual-Fv configuration shown in (c) and (d) below:

(c) light chain: NH_2-V_{L1}-Linker-V_{L2}-COOH

(d) heavy chain: NH_2-V_{H1}-Linker-V_{H2}-COOH

The main difference to note is the distinct placement of the corresponding light chain and heavy chain variable regions (V_{H1}/V_{L1} and V_{H2}/V_{L2}) with respect to each other in the two dual variable region configurations. The corresponding V_{L1} and V_{H1} domains were both at the N-terminus of the light and heavy chains in dual variable region configuration. In contrast, in the cross-over configuration, one half of one pair of an antibody variable region was separated spatially within the protein chain in the cross-over configuration. In the cross-over configuration, the V_{L1} domain would be at the N-terminus of the protein light chain but the pairing V_{H1} domain is at the C-terminus of the cross-over configuration heavy chain. The spatial relationship between V_{L1} and V_{H1} found in the dual variable region configuration is the arrangement found in natural antibodies.

One potential disadvantage of the dual Fv configuration is that the linker L_L separating the two variable regions protrudes into the antigen binding site of the Fv2

domain (*see* Figure 1). This protrusion may interfere with antigen binding and result in a perturbed accessibility of Antigen 2 to Fv2. This perturbed accessibility or interference may prevent antigen binding. In addition, this interference might be more pronounced where the size of Antigen 2 is larger. Indeed, it has been documented in U.S. Patent No. 7,612,181 that the binding affinity and neutralization ability of a DVD-Ig molecule depends on which antigen specificity is presented at the N-terminus or C-terminus. *See* U.S. Patent No. 7,612,181, Example 2.

Therefore, to create more stable antibody-like binding proteins that are not subject to loss of antigen affinity as compared to the parental antibody, cross-over dual variable region molecules having a C_L domain on the light chain and an Fc region on the heavy chain were designed and constructed. The polypeptides that form these antibody-like proteins have the structures shown below, in which the amino to carboxyl terminal orientation of the polypeptide chains is indicated:

(e) light chain: NH_2-V_{L1}-Linker-V_{L2}-C_L-COOH

(f) heavy chain: NH_2-V_{H2}-Linker-V_{H1}-C_{H1}-Fc-COOH

To evaluate whether this bispecific antibody-like protein design would bind to two different antigens, two previously generated and humanized variable regions from antibodies specific for IL4 (parental humanized anti-IL4) and IL13 (parental humanized anti-IL13) were used to construct the bispecific antibody-like molecules shown in Table 1. Sequencing of the mouse antibodies and the humanization process were described in International Publication No. WO 2009/052081 (TBTI). Briefly, amino acid sequences of the variable heavy and light chains of the murine anti-IL13 clone B-B13 and the murine anti-IL4 clone 8D4-8 were determined by amino acid sequencing. The murine sequences were humanized and then back-translated into nucleotide sequences as described in Example 5 of International Publication No. WO 2009/052081, which is incorporated herein by reference in its entirety. The parental humanized anti-IL4 V_H and V_L, and parental humanized anti-IL13 V_H and V_L sequences were combined and arranged as shown in Table 1. The shorthand codes in column one of Table 1 were created to simplify discussion of these antibody-like binding proteins. The antibody-like binding proteins differ in the size of the linker inserted between the two variable regions as shown in Table 1. DNA molecules encoding the polypeptides shown in Table 1 were generated from the back-translated parental anti-IL4 and anti-IL13 antibodies. C_{H1}, C_L, and Fc domains were obtained from IGHG1 (GenBank Accession No. 569F4) and IGKC (GenBank Accession No.

Q502W4).

Table 1. Cross-over Double Head Immunoglobulins

Shorthand Code for Protein	Protein Description	SEQ ID NO:
Parental anti-IL4 Light Chain	anti-IL4 V_L	1
Parental anti-IL4 Heavy Chain	anti-IL4 V_H	2
Parental anti-IL13 Light Chain	anti-IL13 V_L	3
Parental anti-IL13 Heavy Chain	anti-IL13 V_H	4
Heavy Chain Codes		
IL13(G4S)IL4CH1-Fc	anti-IL13 V_H-(G_4S)-anti-IL4 V_H-C_{H1}-Fc	5
IL13(G4S2)IL4CH1-Fc	anti-IL13 V_H-$(G_4S)_2$-anti-IL4 V_H -C_{H1}-Fc	6
IL4(G4S)IL13CH1-Fc	anti-IL4 V_H-(G_4S)-anti-IL13 V_H -C_{H1}-Fc	7
IL4(G4S2)IL13CH1-Fc	anti-IL4 V_H-$(G_4S)_2$-anti-IL13 V_H -C_{H1}-Fc	8
Light Chain Codes		
IL13(G4S)IL4CL	anti-IL13 V_L-(G_4S)-anti-IL4 V_L-CL	9
IL13(G4S2)IL4CL	anti-IL13 V_L -$(G_4S)_2$-anti-IL4 V_L-CL	10
IL4(G4S)IL13CL	anti-IL4 V_L -(G_4S)-anti-IL13 V_L -CL	11
IL4(G4S2)IL13CL	anti-IL4 V_L -$(G_4S)_2$-anti-IL13 V_L -CL	12

The protein combinations shown in Table 2 were expressed by transient transfection and purified by Protein A chromatography. In each case, size exclusion chromatography revealed less than 12% aggregation, with most having less than 7% aggregation; but none of the cross-over double head immunoglobulins were found to display any ability to bind either IL4 or IL13. However, no functional antibody-like binding could be detected and the reasons for this lack of activity could not be ascertained. It was previously predicted that this arrangement would show superior stability over the dual variable region domain antibodies described in U.S. Patent No. 7,612,181 and International Publication No. WO 2009/052081.

Table 2. Binding of CODH-Ig to IL4 and IL13

Protein Combination	Aggregation	IL4 binding	IL13 binding
anti-IL13 V_H-(G_4S)-anti-IL4 V_H-C_{H1}-Fc anti-IL4 V_L-(G_4S)-anti-IL13 V_L-C_L	5.4%	ND*	ND
anti-IL13 V_H -(G_4S)-anti-IL4 V_H - C_{H1}-Fc anti-IL4 V_L -$(G_4S)_2$-anti-IL13 V_L -CL	6.3%	ND	ND
anti-IL13 V_H -$(G_4S)_2$-anti-IL4 V_H - C_{H1}-Fc anti-IL4 V_L -(G_4S)-anti-IL13 V_L -CL	11.5%	ND	ND
anti-IL13 V_H -$(G_4S)_2$-anti-IL4 V_H - C_{H1}-	10.1%	ND	ND

Fc anti-IL4 V_L -$(G_4S)_2$-anti-IL13 V_L -CL			
anti-IL4 V_H -(G_4S)-anti-IL13 V_H - C_{H1}-Fc anti-IL13 V_L -(G_4S)-anti-IL4 V_L -CL	2.7%	ND	ND
anti-IL4 V_H -(G_4S)-anti-IL13 V_H - C_{H1}-Fc anti-IL13 V_L -$(G_4S)_2$-anti-IL4 V_L -CL	3.6%	ND	ND
anti-IL4 V_H -$(G_4S)_2$-anti-IL13 V_H - C_{H1}-Fc anti-IL13 V_L -(G_4S)-anti-IL4 V_L -CL	2.9%	ND	ND
anti-IL4 V_H -$(G_4S)_2$-anti-IL13 V_H - C_{H1}-Fc anti-IL13 V_L -$(G_4S)_2$-anti-IL4 V_L -CL	10.8%	ND	ND

* ND means none detected

Example 2. Design of CODV-Ig Proteins by Molecular Modeling

To obtain fully functional antibody-like proteins utilizing the cross-over double head configuration that are amendable to incorporation of the Fc and C_{L1} domains, a molecular modeling protocol was developed for the inclusion and evaluation of different linkers between the constant and variable domains and between the dual variable domains on both the heavy and light chains. The question was whether the addition of unique linkers between each constant/variable domain interface and between the two variable/variable domain interfaces on both the heavy and light chains would allow proper protein folding to occur and produce functional antibody-like molecules in the cross-over dual variable region configuration (*see* Figure 2). In other words, a total of four independent and unique linkers were evaluated (*see* Figure 2). This molecular modeling protocol was based on protein-protein docking of homology models and experimental models of the Fv_{IL4} and Fv_{IL13} regions, respectively, in combination with appropriate linkers between the Fv_{IL4} and Fv_{IL13} regions and between the Fv and constant or Fc regions.

The independent linkers were assigned unique names as follows: L_1 refers to the linker between N-terminal V_L and the C-terminal V_L on the light chain; L_2 refers to the linker between the C-terminal V_L and C_L on the light chain; L_3 refers to the linker between N-terminal V_H and the C-terminal V_H on the heavy chain; L_4 refers to the linker between the C-terminal V_H and C_{H1} (and Fc) on the heavy chain. It should be noted that the designations V_H and V_L refer only to the domain's location on a particular protein chain in the final format. For example, V_{H1} and V_{H2} could be derived from V_{L1} and V_{L2} domains in parent antibodies and placed into the V_{H1} and

V_{H2} positions in a CODV-Ig. Likewise, V_{L1} and V_{L2} could be derived from V_{H1} and V_{H2} domains in parent antibodies and placed into the V_{H1} and V_{H2} positions in a CODV-Ig. Thus, V_H and V_L designations refer to present location and not the original location in a parent antibody.

In more detail, a homology model of Fv_{IL4} was constructed on PDB entries 1YLD (light chain) and 1IQW (heavy chain). The Fv_{IL4} dimer was recomposed on an in-house crystal structure of the IL13/anti-IL13 Fab_{IL13} complex and optimized. In order to obtain an estimate of the volume required by IL4 when bound to Fv_{IL4}, the crystal structure of IL4 (1RCB.pdb) was docked to the homology model of Fv_{IL4}. Next, twenty-two putative models of the complex were generated that merited further consideration.

In parallel, the homology model of Fv_{IL4} was docked to Fv_{IL13} extracted from an in-house crystal structure of the IL13/Fab_{IL13} complex. One superior solution was found that permitted construction of relatively short linkers while showing no steric interference for antigen binding and placement of the constant domains as was the case for dual variable region immunoglobulins (*see* Figure 3). In this arrangement Fv_{IL4} (V_{L1}) was placed at the N-terminus of the light chain, followed by Fv_{IL13} (V_{L2}) and Fc (C_{L1}) on the light chain C-terminus. On the heavy chain, Fv_{IL13} (V_{H2}) was placed N-terminally, followed by Fv_{IL4} (V_{H1}) and the constant regions (C_{H1} - C_{H2} - C_{H3}).

As shown in Table 3, the models of the light chain suggested that the linker L_1 between the V_{L1} and V_{L2} domains and the linker L_2 between the V_{L2} and C_{L1} domains should be between one to three and zero to two glycine residues long, respectively. Models of the heavy chain suggested that the linker L_3 between the V_{H2} and V_{H1} domains and the linker L_4 between the V_{H1} and C_{H1} domains should be between two to six and four to seven glycine residues long, respectively (*see* Table 3 and Figure 2). In this example, glycine was used as a prototypical amino acid for the linkers but other amino acid residues may also serve as linkers. The structural stability of the proposed models was verified by optimization of the linker conformations, minimization, and molecular dynamics calculations. Systematic combination between four light chain and six heavy chain constructs resulted in 24 possible cross-over dual variable region bispecific anti-IL4 and anti-IL13 antibody-like binding proteins (*see* Table 4).

Table 3. Proposed Linker Lengths

Linker Between	Maximal Linker Insertion	Minimal Linker Insertion	Linker Name
V_{L1}-V_{L2}	Gly_3	Gly	L_1
V_{L2}-C_L	Gly_2	None	L_2
V_{H2}-V_{H1}	Gly_6	Gly_2	L_3
V_{H1}-C_{H1}	Gly_7	Gly_4	L_4

Table 4. CODV-Ig for Expression

Code*	Heavy Chains (N to C terminal)	SEQ ID NO:
HC1	IL13 V_H -(Gly6)-IL4 V_H -(Gly7)-C_{H1}-Fc	13
HC2	IL13 V_H -(Gly6)-IL4 V_H -(Gly4)- C_{H1}-Fc	14
HC3	IL13 V_H -(Gly2)-IL4 V_H -(Gly7)- C_{H1}-Fc	15
HC4	IL13 V_H -(Gly2)-IL4 V_H -(Gly4)- C_{H1}-Fc	16
HC5	IL13 V_H -(Gly4)-IL4 V_H -(Gly7)- C_{H1}-Fc	17
HC6	IL13 V_H -(Gly4)-IL4 V_H -(Gly4)- C_{H1}-Fc	18
Code*	Light Chains (N to C terminal)	
LC1	IL4 V_L-(Gly3)-IL13 V_L -C_{L1}	19
LC2	IL4 V_L -(Gly)-IL13 V_L - C_{L1}	20
LC3	IL4 V_L -(Gly3)-IL13 V_L -(Gly2)- C_{L1}	21
LC4	IL4 V_L -(Gly)-IL13 V_L -(Gly2)- C_{L1}	22
C_{L1}	C_{L1} human light chain constant domain	23
C_{H1}-Fc	C_{H1} human heavy chain constant domain and Fc region	24
Gly4	peptide linker with 4 glycines (GGGG)	25
Gly5	peptide linker with 5 glycines (GGGGG)	26
Gly6	peptide linker with 6 glycines (GGGGGG)	27
Gly7	peptide linker with 7 glycines (GGGGGGG)	28
Gly8	peptide linker with 8 glycines (GGGG GGGG)	29

*A short hand code was devised to represent the associated structures. Codes beginning with HC represent the adjacent heavy chain and codes beginning with LC represent the adjacent light chains.

In Table 4, the prefix "anti" is not included but it is intended to mean that IL13 refers to anti-IL13 and IL4 refers to anti-IL4.

Example 3. Generation of CODV-Ig Expression Plasmids

Nucleic acid molecules encoding the variable heavy and light chains of the six heavy chains and four light chains described in Table 4 were generated by gene synthesis at Geneart (Regensburg, Germany). The variable light chain domains were

fused to the constant light chain (IGKC, GenBank Accession No. Q502W4) by digestion with the restriction endonucleases ApaLI and BsiWI and subsequently ligated into the ApaLI/BsiWI sites of the episomal expression vector pFF, an analogon of the pTT vector described by Durocher *et al.*, (2002, *Nucl. Acids Res.* 30(2): E9), creating the mammalian expression plasmid for expression of the light chains.

The variable heavy chain domains were fused to the "Ted" variant of the human constant heavy chain (IGHG1, GenBank Accession No. 569F4), or alternatively, to a 6x His tagged C_{H1} domain from the human constant IGHG1 in order to create a bispecific Fab. Next, the V_H domain was digested with the restriction endonucleases ApaLI and ApaI and then fused to the IGHG1 or His tagged C_{H1} domain respectively, by ligation into the ApaLI/ApaI sites of the episomal expression vector pFF, creating the mammalian expression plasmids for expression of the heavy chains (IgG1 or Fab respectively).

Example 4. Expression of CODV-Ig

The expression plasmids encoding the heavy and light chains of the corresponding constructs were propagated in *E. coli* DH5a cells. Plasmids used for transfection were prepared from *E. coli* using the Qiagen EndoFree Plasmid Mega Kit.

HEK 293-FS cells growing in Freestyle Medium (Invitrogen) were transfected with indicated LC and HC plasmids encoding the heavy chains and light chains shown in Table 4 using 293fectin (Invitrogen) transfection reagent as described by the manufacturer. After 7 days, cells were removed by centrifugation and the supernatant was passed over a 0.22 μm filter to remove particles.

CODV-IgG1 constructs were purified by affinity chromatography on Protein A columns (HiTrap Protein A HP Columns, GE Life Sciences). After elution from the column with 100 mM acetate buffer and 100 mM NaCl, pH 3.5, the CODV-IgG1 constructs were desalted using HiPrep 26/10 Desalting Columns, formulated in PBS at a concentration of 1 mg/mL and filtered using a 0.22 μm membrane.

Bispecific CODV Fab constructs were purified by IMAC on HiTrap IMAC HP Columns (GE Life Sciences). After elution from the column with a linear gradient (Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 50 - 500 mM imidazole, pH 7.4), the protein containing fractions were pooled and desalted using

HiPrep 26/10 Desalting Columns, formulated in PBS at a concentration of 1 mg/mL and filtered using a 0.22 μm membrane.

Protein concentration was determined by measurement of absorbance at 280 nm. Each batch was analyzed by SDS-PAGE under reducing and non-reducing conditions to determine the purity and molecular weight of each subunit and of the monomer.

A Nunc F96-MaxiSorp-Immuno plate was coated with goat anti-Human IgG (Fc specific) [NatuTec A80-104A]. The antibody was diluted to 10 μg/ml in carbonate coating buffer (50 mM sodium carbonate, pH 9.6) and dispensed at 50 μL per well. The plate was sealed with adhesive tape, and stored overnight at 4° C. The plate was washed three times with Wash buffer (PBS, pH 7.4 and 0.1% Tween20). 150 μL of blocking solution (1% BSA / PBS) was dispensed into each well to cover the plate. After 1 hour at room temperature, the plate was washed three times with Wash buffer. 100 μL of sample or standards (in a range from 1500 ng/ml to 120 ng/ml) were added and allowed to sit for 1 hour at room temperature. The plate was washed three times with Wash buffer. 100 μL of goat anti-Human IgG-FC – HRP conjugate [NatuTec A80-104P-60] diluted 1:10.000 were added using incubation solution (0.1% BSA, PBS, pH 7.4, and 0.05% Tween20). After 1 hour incubation at room temperature, the plate was washed three times with Wash buffer. 100 μL of ABTS substrate (10 mg ABTS tablet (Pierce 34026) in 0.1 M Na_2HPO_4, 0.05 M citric acid solution, pH 5.0). Addition of 10 μL of 30% H_2O_2 / 10 ml Substrate buffer prior to use) were dispensed to each well, and the color was allowed to develop. After the color developed (approximately 10 to 15 minutes), 50 μL of 1% SDS solution were added to stop the reaction. The plate was read at A_{405}.

Example 5. Characterization of CODV-Ig Variants

To determine whether the CODV-Ig antibody-like protein heavy and light chains were pairing and folding properly, the aggregation level was measured by analytical size-exclusion chromatography (SEC). Analytical SEC was performed on assembled pairs using an ÄKTA explorer 10 (GE Healthcare) equipped with a TSKgel G3000SWXL column (7.8 mm x 30 cm) and TSKgel SWXL guard column (Tosoh Bioscience). The analysis was run at 1 ml/min using 250 mM NaCl, 100 mM Na-phosphate, pH 6.7, with detection at 280 nm. 30 μL of protein sample (at 0.5-1 mg/ml) were applied onto the column. For estimation of the molecular size, the

column was calibrated using a gel filtration standard mixture (MWGF-1000, SIGMA Aldrich). Data evaluation was performed using UNICORN software v5.11.

Table 5 shows the results of the first set of 24 different CODV-Ig molecules made using the anti-IL4 and anti-IL13 variable region combinations described in Table 4. The codes assigned in Table 4 represent the adjacent structures shown in Table 4. For the pairs of light chain and heavy chains where protein was produced, aggregation levels were measured using SEC. The results are shown in Table 5 where LC4 ($L_1 = 1$; $L_2 = 2$) was most successful in pairing with all six heavy chains. LC4 corresponds to the structure IL4 V_L-(Gly)-IL13 V_L-(Gly2)-C_{L1} having linker L_1 equal to 1, where a single amino acid residue separated the two V_L domains of the dual variable region light chain. In addition, LC4 had L_2 equal to 2, which contained a Gly-Gly dipeptide linker between the central V_L and the C-terminal C_{H1}.

Table 5. Levels of Aggregation Among Pairs of Heavy and Light Chains

	HC1	HC2	HC3	HC4	HC5	HC6
LC1	>50%	>50%	ND*	ND	ND	ND
LC2	ND	ND	ND	>50%	ND	ND
LC3	ND	ND	ND	ND	ND	ND
LC4	7.2%	6.8%	6.8%	7.1%	6.3%	5.9%

*ND indicates that no protein was produced

Where CODV-Ig molecules were produced, a single-concentration BIACORE experiment at intermediate IL13 and IL4 concentrations was performed to verify binding to target antigens. CODV-Ig antibody-like molecules corresponding to the LC4:HC4 and LC4:HC6 combinations described in Table 4 were chosen for assessment of a full kinetic analysis using surface plasmon resonance.

As depicted in Table 5, most of the CODV-Ig molecules could not be produced at all or only as aggregates (up to 90%). The heavy chain/light chain combinations giving rise to acceptable aggregation levels (5-10%) after one chromatography step were the ones combined with the light chain IL4 V_L-(Gly)-IL13 V_L-(Gly2)-C_{L1}. The light chain was the most finicky chain within these CODV-Ig variants and served as platform to accept different heavy chains with different linker compositions.

1. **Kinetic Analysis**

Two pairs of heavy and light chains were selected for full kinetic analysis. Recombinant human IL13 and IL4 was purchased from Chemicon (USA). Kinetic characterization of purified antibodies was performed using surface plasmon resonance technology on a BIACORE 3000 (GE Healthcare). A capture assay using a species specific antibody (*e.g.*, human-Fc specific MAB 1302, Chemicon) for capture and orientation of the investigated antibodies was used. The capture antibody was immobilized via primary amine groups (11000 RU) on a research grade CM5 chip (GE Life Sciences) using standard procedures. The analyzed antibody was captured at a flow rate of 10 µL/min with an adjusted RU value that would result in maximal analyte binding of 30 RU. Binding kinetics were measured against recombinant human IL4 and IL13 over a concentration range between 0 to 25 nM in HBS EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005 % Surfactant P20) at a flow rate of 30 µl/min. Chip surfaces were regenerated with 10 mM glycine, pH 2.5. Kinetic parameters were analyzed and calculated in the BIAevaluation program package v4.1 using a flow cell without captured antibody as reference.

Table 6 below shows the comparison of kinetics of the parental BB13 (anti-IL13) and 8D4 (anti-IL4) antibodies (expressed as IgGs) with the respective domains within the CODV-Ig format (Table 4, Codes LC4:HC4 and LC4:HC6). As shown in Table 6, the CODV-Ig constructs did not exhibit decreased binding properties against the corresponding antigens, when compared to the parental anti-IL13 and anti-IL4 antibodies. The loss in the on-rate observed in the DVD-Ig/TBTI format using the same Fv sequences did not occur with the CODV-Ig configuration. The opposite facing binding sites should allow for binding large antigens or bridging different cells with a bispecific antibody-like configuration, and would also be suitable for a wider selection of parental antibodies. A further advantage of the CODV-Ig was that no linker residues protrude into the antigen binding site and reduce accessibility of the antigen.

Table 6. Kinetic Analysis of LC4:HC4 and LC4:HC6

Code		On-rate [1/Ms]	Off-rate [1/s]	KD [M]
	parental anti-IL4 mAb	2.49E+07	1.95E-04	7.83E-12
	parental anti-IL13 mAb	1.59E+06	1.30E-04	8.18E-11

LC4:HC4	CODV-Ig with IL4	3.16E+07	2.89E-04	9.14E-12
LC4:HC4	CODV-IG with IL13	1.20E+06	1.12E-04	9.34E-11
LC4:HC6	CODV-Ig with IL4	2.97E+07	3.30E-04	1.11E-11
LC4:HC6	CODV-IG with IL13	1.39E+06	1.63E-04	1.18E-10

2. Co-injection of IL4 and IL13 for Demonstration of Additive Antigen Binding by CODV-Ig

To investigate additive binding of both antigens, a wizard-driven co-inject method was applied in which one antigen was injected immediately followed by the other antigen after a lag time (IL4 then IL13 and vice versa). The resulting binding level could be compared to that achieved with a 1:1 mixture of both antigens in the same concentration. In order to show additive binding of both the IL4 and IL13 antigens by the CODV-Ig molecules, a BIACORE experiment was performed with a CODV-Ig combination [HC4:LC4] by co-injection of both antigens in three separate analysis cycles (*see* Figure 4). The co-injection was done with 3.125 nM IL4/25 nM IL13 (and vice versa) and with a 1:1 mixture of 3.125 nM IL4 and 25 nM IL13. A co-injection of HBS-EP buffer was done as a reference. At a time point of 800 seconds, an identical binding level of 63 RU was achieved after injection of the antigen mixture or co-injection of the antigens, regardless of the co-injection sequence. When CODV-Ig protein had been saturated by the first antigen (IL4), the second antigen (IL13) was injected and a second binding signal was observed. This observation was reproduced when the antigen injection sequence was reversed. This demonstrates the additive and non-inhibition of binding of both antigens by the CODV-Ig. Therefore, the CODV-Ig construct was able to bind both antigens simultaneously (*i.e.*, exhibit bispecificity) saturating all binding sites (*i.e.*, exhibited tetravalency).

Example 6. Tolerance of Linker Lengths for CODV-Ig

The tolerance for linkers of various lengths was evaluated by constructing CODV-Ig molecules having different combinations of linker lengths for L_1, L_2 on the light chain and L_3 and L_4 on the heavy chain. CODV-Ig constructs were generated with heavy chain linkers L_3 and L_4 varying between 1 through 8 residues for L_3 and either 0 or 1 residues for L_4. The heavy chain contained anti-IL4 as the N-terminal binding domain and anti-IL13 as the C-terminal binding domain followed by C_{H1}-Fc. The light chain linkers L_1 and L_2 were varied from 3 to 12 residues for L_1 and from 3 to 14 residues for L_2. The light chain contained anti-IL13 as the N-terminal binding

domain and anti-IL4 as the C-terminal binding domain followed by C_{L1}.

1. Characterization of CODV-Ig Variants

Determination of aggregation level was by analytical size-exclusion chromatography (SEC). Analytical SEC was performed using an ÄKTA explorer 10 (GE Healthcare) equipped with a TSKgel G3000SWXL column (7.8 mm x 30 cm) and TSKgel SWXL guard column (Tosoh Bioscience). The analysis was run at 1 ml/min using 250 mM NaCl, 100 mM Na-phosphate pH 6.7, with detection at 280 nm. 30 µL of protein sample (at 0.5-1 mg/ml) was applied onto the column. For estimation of the molecular size, the column was calibrated using a gel filtration standard mixture (MWGF-1000, SIGMA Aldrich). Data evaluation was performed using UNICORN software v5.11.

Recombinant human IL13 and IL4 were purchased from Chemicon (USA). Recombinant human TNF-α was purchased from Sigma Aldrich (H8916-10µg), recombinant human IL-1β (201-LB/CF), recombinant human IL-23 (1290-IL/CF), recombinant human EGFR (344 ER), and recombinant human HER2 (1129-ER-50) were purchased from R&D Systems.

Kinetic binding analysis by Biacore was performed as follows. Surface plasmon resonance technology on a Biacore 3000 (GE Healthcare) was used for detailed kinetic characterization of purified antibodies. A capture assay using a species-specific antibody (*e.g.*, human-Fc specific MAB 1302, Chemicon) for capture and orientation of the investigated antibodies was used. For determination of IL4 and IL13 binding kinetics, the corresponding CODV Fabs as in Example 10, Table 12 were captured using the anti-human Fab capture Kit (GE Healthcare). The capture antibody was immobilized via primary amine groups (11000 RU) on a research grade CM5 chip (GE Life Sciences) using standard procedures. The analyzed antibody was captured at a flow rate of 10 µL/min with an adjusted RU value that would result in maximal analyte binding of 30 RU. Binding kinetics were measured against recombinant human IL4 and IL13 over a concentration range between 0 to 25 nM in HBS EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) at a flow rate of 30 µL/min. Chip surfaces were regenerated with 10 mM glycine pH 2.5. Kinetic parameters were analyzed and calculated in the BIAevaluation program package v4.1 using a flow cell without captured antibody as a reference.

Binding affinities of CODV-Ig, CODV-Fab, and TBTI against EGFR and HER2 were measured using a Proteon XPR36 protein interaction array system (Biorad). The antigens were immobilized by amine reactive coupling on GLC sensor chips (Biorad). Dilution series of the bispecific antibody variants in PBSET buffer (Biorad) were analyzed in parallel in one-shot kinetics mode with double referencing. Data were analyzed using Proteon Manager Software v3.0 (Biorad) with either Langmuir 1:1 model with mass transfer or bivalent analyte model.

Table 7 summarizes the results for yield, aggregation (as measured by size exclusion chromatography), and binding affinity for CODV-Ig having different size combinations of linkers. The results revealed that CODV-Ig molecules in which L_2 was zero generally could not be produced, or where protein was produced, there was a high level of aggregation (*see* Batch ID Nos. 101, 102, 106-111, and 132-137 in Table 7). Therefore, in contrast to the molecular modeling prediction from Example 2, where L_2 equal to zero was within the acceptable range, these results indicate that the V_{L2}-C_L transition (or L_2) requires a linker of at least one residue (*see* Table 7).

Table 7. Optimization of Linker Sizes for CODV-Ig

Batch ID	Alignment on LC1*	L_1	L_2	L_3	L_4	Yield[1] [mg/L]	Aggregation [%]	KD (Antigen1) IL4 [pM]	KD (Antigen2) IL13 [pM]
101	IL4 x IL13	0	0	0	0	n.p.	–	–	–
102	IL4 x IL13	0	0	2	4	1.0	66.4	35	125
103	IL4 x IL13	0	1	2	4	2.0	10.8	4	124
104	IL4 x IL13	0	2	2	4	1.0	10.0	3	137
105	IL4 x IL13	0	2	4	4	2.0	8.5	6	94
106	IL4 x IL13	1	0	2	4	4.2	61.0	–	–
107	IL4 x IL13	1	0	2	7	n.p.	–	–	–
108	IL4 x IL13	1	0	4	4	n.p.	–	–	–
109	IL4 x IL13	1	0	4	7	n.p.	–	–	–
110	IL4 x IL13	1	0	6	4	3.0	75.0	–	–
111	IL4 x IL13	1	0	6	7	5.6	91.0	–	–
112	IL4 x IL13	1	1	2	4	5.3	8.8	5	55
113	IL4 x IL13	1	2	0	0	2.0	1.9	6	59
114	IL4 x IL13	1	2	0	2	1.0	7.2	4	75
115	IL4 x IL13	1	2	0	4	2.0	8.0	11	62
116	IL4 x IL13	1	2	1	4	3.0	5.7	5	74
117	IL4 x IL13	1	2	12	12	4.0	9.2	7	75
118	IL4 x IL13	1	2	12	9	7.0	11.8	7	74
119	IL4 x IL13	1	2	15	15	10.0	7.9	6	64
120	IL4 x IL13	1	2	2	2	2.0	5.9	6	70
121	IL4 x IL13	1	2	2	4	7.0	7.1	11	118

122	IL4 x IL13	1	2	2	7	6.6	6.8	1	50
123	IL4 x IL13	1	2	4	4	5.8	5.9	9	93
124	IL4 x IL13	1	2	4	7	5.6	6.3	6	51
125	IL4 x IL13	1	2	6	4	7.6	6.8	8	64
126	IL4 x IL13	1	2	6	7	8.2	7.2	9	56
127	IL4 x IL13	1	2	9	12	4.0	8.7	2	83
128	IL4 x IL13	1	2	9	9	10.2	14.0	12	94
129	IL4 x IL13	1	3	2	4	4.0	4.7	5	70
130	IL4 x IL13	1	4	2	4	7.1	6.0	4	57
131	IL4 x IL13	2	2	2	4	1.0	7.3	5	50
132	IL4 x IL13	3	0	2	4	n.p.	–	–	–
133	IL4 x IL13	3	0	2	7	n.p.	–	–	–
134	IL4 x IL13	3	0	4	4	n.p.	–	–	–
135	IL4 x IL13	3	0	4	7	n.p.	–	–	–
136	IL4 x IL13	3	0	6	4	9.8	93.0	–	–
137	IL4 x IL13	3	0	6	7	5.8	90.0	–	–
138	IL4 x IL13	3	2	2	4	n.p.	–	–	–
139	IL4 x IL13	3	2	2	7	n.p.	–	–	–
140	IL4 x IL13	3	2	4	4	n.p.	–	–	–
141	IL4 x IL13	3	2	4	7	n.p.	–	–	–
142	IL4 x IL13	3	2	6	4	n.p.	–	–	–
143	IL4 x IL13	3	2	6	7	n.p.	–	–	–

* Alignment on the heavy chain must be IL13V_H-L_3-IL4V_H-L_4-C_{H1}-Fc
[1] n.p. means the construct was not producible.

In addition, the CODV-Ig linker lengths described above were found to be more sensitive to increases in 1 amino acid residue than increases in 2 amino acid residues. For example, while Batch ID Nos. 103 and 104 differ by 1 amino acid residue in L_2, Batch ID No. 103 shows 6 fold more aggregation and Batch ID No. 104 displays less aggregation and twice the yield. In contrast, Batch ID Nos. 104 and 105, which differ by two residues in L_2, displayed similar profiles with respect to yield, aggregation, and binding.

Example 7. Heavy Chain as Template Chain for CODV-Ig

In examples 1 through 5, the optimal short linker sizes on the light chain suggested that the light chain was serving as a template by remaining in a linear arrangement and that larger linkers were required on the heavy chain in order for the heavy chain to fold properly into the cross-over configuration to conform to the template light chain (*see* Figure 5, Panel A). Whether the short linkers specifically placed on the heavy chain to maintain a liner arrangement on the heavy chain rendered the heavy chain the "template" chain, and whether the pattern would repeat itself and larger linkers would be required to allow the non-template chain to fold

properly and accommodate the now template heavy chain was evaluated next (*see* Figure 5, Panel B).

Figure 6 illustrates these principles of CODV-Ig design based on having either the light chain or the heavy chain as the "template." To evaluate the generic nature of this concept, CODV-Ig constructs were generated with heavy chain linkers L_3 and L_4 varying between 1 through 8 residues for L_3 and either 0 or 1 residues for L_4. The heavy chain contained anti-IL4 as the N-terminal binding domain and anti-IL13 as the C-terminal binding domain followed by C_{H1}-Fc. The light chain linkers L_1 and L_2 were varied from 3 to 12 residues for L_1 and from 3 to 14 residues for L_2. The light chain contained anti-IL13 as the N-terminal binding domain and anti-IL4 as the C-terminal binding domain followed by C_{L1}.

Table 8 summarizes the results for yield, aggregation (as measured by size exclusion chromatography), and binding affinity for CODV-Ig having different size combinations of linkers and where the heavy chain is maintained in a linear arrangement as the template chain and the light chain is allowed to fold in a cross-over configuration. The results revealed that CODV-Ig molecules in which L_4 was zero generally could not be produced, or where protein was produced, there was a high level of aggregation (similar to molecules in which L_2 was equal to zero) (*see* Batch ID Nos. 207-209, 211-212, 219-224, 231-236, 243-252, and 263-266 in Table 8). One exception was Batch ID No. 210, in which L_1 was 7, L_2 was 5, L_3 was 2, and L_4 was zero. This arrangement produced a sufficient amount of protein and had an acceptable level of aggregation and binding, which suggested that some combination of linker sizes could be found to compensate for a zero length linker at L_4 in some circumstances.

Table 8. Optimization of Linker Sizes with Heavy Chain as Template

Batch ID	Alignment on HC*	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (Antigen1) IL4 [pM]	KD (Antigen2) IL13 [pM]
201	IL4 x IL13	5	3	1	2	6.3	12	4	72
202	IL4 x IL13	5	5	1	2	10.5	7.0	9	54
203	IL4 x IL13	7	3	1	2	19.3	9.4	80	46
204	IL4 x IL13	7	5	1	2	15.3	5.2	3	25
205	IL4 x IL13	10	3	1	2	4.7	4.0	8	58
206	IL4 x IL13	10	5	1	2	9.1	3.9	4	58
207	IL4 x IL13	5	3	2	0	6.7	25.3	3	33

208	IL4 x IL13	5	5	2	0	10.2	18.4	10	77
209	IL4 x IL13	7	3	2	0	16.2	22.2	5	47
210	IL4 x IL13	7	5	2	0	14.7	9.7	4	47
211	IL4 x IL13	10	3	2	0	2.1	12.8	7	53
212	IL4 x IL13	10	5	2	0	7.0	36.3	10	29
213	IL4 x IL13	5	3	2	2	4.0	13.2	5	27
214	IL4 x IL13	5	5	2	2	8.0	7.9	10	53
215	IL4 x IL13	7	3	2	2	14.9	11.5	4	50
216	IL4 x IL13	7	5	2	2	7.5	3.6	11	40
217	IL4 x IL13	10	3	2	2	2.4	4.4	8	79
218	IL4 x IL13	10	5	2	2	4.6	6.6	4	36
219	IL4 x IL13	3	6	3	0	2.1	51.8	8	71
220	IL4 x IL13	3	10	3	0	3.9	59.4	1	42
221	IL4 x IL13	3	14	3	0	1.9	57.6	35	81
222	IL4 x IL13	6	6	3	0	4.0	11.8	7	53
223	IL4 x IL13	6	10	3	0	10.3	16.6	6	23
224	IL4 x IL13	6	14	3	0	5.1	13.5	9	52
225	IL4 x IL13	3	6	3	2	2.8	71.6	6	68
226	IL4 x IL13	3	10	3	2	7.3	65.8	6	64
227	IL4 x IL13	3	14	3	2	1.6	53.6	7	39
228	IL4 x IL13	6	6	3	2	4.0	19.1	7	44
229	IL4 x IL13	6	10	3	2	2.2	15.4	3	14
230	IL4 x IL13	6	14	3	2	4.0	16.2	6	76
231	IL4 x IL13	5	3	5	0	n.p.	–	–	–
232	IL4 x IL13	5	5	5	0	0.6	24.9	8	70
233	IL4 x IL13	7	3	5	0	0.4	15.1	3	113
234	IL4 x IL13	7	5	5	0	1.3	30.7	3	122
235	IL4 x IL13	10	3	5	0	0.1	11.3	2	82
236	IL4 x IL13	10	5	5	0	0.4	18.6	11	112
237	IL4 x IL13	5	3	5	2	2.1	45.3	8.1	101.0
238	IL4 x IL13	5	5	5	2	0.6	45.4	9.3	67.2
239	IL4 x IL13	7	3	5	2	n.p.	–	–	–
240	IL4 x IL13	7	5	5	2	1.6	31.7	4	65
241	IL4 x IL13	10	3	5	2	0.2	14.7	7	119
242	IL4 x IL13	10	5	5	2	1.1	17.6	10	37
243	IL4 x IL13	3	6	6	0	1.6	54.3	5	50
244	IL4 x IL13	3	10	6	0	1.5	63.9	10	10
245	IL4 x IL13	3	14	6	0	1.0	61.5	10	69
246	IL4 x IL13	6	6	6	0	1.1	16.2	6	57
247	IL4 x IL13	6	10	6	0	4.7	27.9	2	41
248	IL4 x IL13	6	14	6	0	0.9	18.1	10	79
249	IL4 x IL13	10	6	6	0	0.3	8.7	3	87
250	IL4 x IL13	10	8	6	0	0.7	21.3	8	53
251	IL4 x IL13	12	6	6	0	1.3	9.7	8	70
252	IL4 x IL13	12	8	6	0	1.3	11.7	7	85
253	IL4 x IL13	3	6	6	2	5.1	66.8	6	66
254	IL4 x IL13	3	10	6	2	2.4	62.4	6	80
255	IL4 x IL13	3	14	6	2	2.0	72.1	2	60
256	IL4 x IL13	6	6	6	2	2.0	32.4	4	81
257	IL4 x IL13	6	10	6	2	1.9	29.8	7	30

258	IL4 x IL13	6	14	6	2	2.5	24.6	5	70
259	IL4 x IL13	10	6	6	2	1.4	16.4	8	71
260	IL4 x IL13	10	8	6	2	0.8	16.6	10	71
261	IL4 x IL13	12	6	6	2	1.2	12.3	5	265
262	IL4 x IL13	12	8	6	2	1.1	13.2	4	111
263	IL4 x IL13	10	6	8	0	2.4	10.8	2	74
264	IL4 x IL13	10	8	8	0	0.8	8.0	7	22
265	IL4 x IL13	12	6	8	0	1.0	9.5	8	66
266	IL4 x IL13	12	8	8	0	2.0	9.3	3	69
267	IL4 x IL13	10	6	8	2	1.4	15.0	9	170
268	IL4 x IL13	10	8	8	2	1.0	12.9	4	52
269	IL4 x IL13	12	6	8	2	1.2	8.8	5	66
270	IL4 x IL13	12	8	8	2	2.4	11.7	3	72

* Alignment on the light chain must be $IL13V_L$-L_1-$IL4V_L$-L_2-C_{L1}

The results from Tables 7 and 8 clearly show that linkers are required between the variable and constant domains to allow optimal folding. Only in rare arrangements was a linker equal to zero tolerated (*see* Batch ID Nos. 103-105, in which L_1 (LC) was zero, and Batch No. 210, in which L_4 was zero). However, in each case, the corresponding transition linker between the variable region and constant region on the other chain could not be zero.

The results above indicated that the combinations L_1=7, L_2=5, L_3=1, and L_4=2 were a good starting point for optimizing a new CODV-Ig in which the heavy chain is the template. The ranges in Table 9 were shown to be reasonable ranges for successfully engineering a new CODV-Ig from two parent antibodies.

Table 9. Ranges of Linker Sizes for Either LC or HC as Template

Linker	LC as Template		HC as Template	
	Max Range	Min Range	Max Range	Min Range
L_1	1-3	1-2	3-12	5-10
L_2	1-4	1-2	3-14	5-8
L_3	2-15	4-12	1-8	1-5
L_4	2-15	2-12	1-3	1-2

Example 8. Universal Applicability of CODV-Ig Format

To evaluate the suitability of the CODV-Ig format for engineering new antibody-like binding proteins the variable regions from numerous existing human and humanized antibodies having specificity for insulin-like growth factor 1 receptor

(IGF1R(1)), a second antibody to insulin-like growth factor 1 receptor (IGF1R(2)), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), tumor necrosis factor - alpha (TNFα), Interleukin 12 and 23 (IL-12/23) and interleukin 1beta (IL-1β) were incorporated into the CODV-Ig format (*see* Table 10).

Table 10. Descriptive Codes for Heavy and Light Chains Used in Bispecific CODV-Ig

Code*	Heavy Chains (N to C terminal)	SEQ ID NO:
HC10	IGF1R(1) V_H -(Gly)-HER2 V_H -(Gly2)-C_{H1}-Fc	32
HC11	HER2 V_H -(Gly)- IGF1R(1) V_H -(Gly2)- C_{H1}-Fc	33
HC12	IGF1R(2) V_H -(Gly)-EGFR V_H -(Gly2)-C_{H1}-Fc	34
HC13	EGFR V_H -(Gly)- IGF1R(2) V_H -(Gly2)- C_{H1}-Fc	35
HC14	TNFα V_H -(Gly)-IL12/23 V_H -(Gly2)- C_{H1}-Fc	36
HC15	IL12/23 V_H -(Gly)-TNFα V_H -(Gly2)- C_{H1}-Fc	37
HC16	TNFα V_H -(Gly)-IL1β V_H -(Gly2)- C_{H1}-Fc	38
HC17	IL1β V_H -(Gly)-TNFα V_H -(Gly2)- C_{H1}-Fc	39
Code*	Light Chains (N to C terminal)	
LC10	HER2 V_L-(Gly7)-IGF1R(1) V_L -(Gly5)-C_{L1}	40
LC11	IGF1R(1) V_L -(Gly7)- HER2 V_L-(Gly5)- C_{L1}	41
LC12	EGFR V_L-(Gly7)-IGF1R(2) V_L -(Gly5)-C_{L1}	42
LC13	IGF1R(2) V_L -(Gly7)- EGFR V_L-(Gly5)- C_{L1}	43
LC14	IL12/23 V_L-(Gly7)- TNFα V_L -(Gly5)-C_{L1}	44
LC15	TNFα V_L -(Gly7)- IL12/23 V_L-(Gly5)- C_{L1}	45
LC16	IL1β V_L-(Gly7)- TNFα V_L -(Gly5)-C_{L1}	46
LC17	TNFα V_L-(Gly7)- IL1β V_L -(Gly5)-C_{L1}	47
C_{L1}	C_{L1} human light chain constant domain	23
C_{H1}-Fc	C_{H1} human heavy chain constant domain and Fc region	24
Gly4	peptide linker with 4 glycines (GGGG)	25
Gly5	peptide linker with 5 glycines (GGGGG)	26
Gly6	peptide linker with 6 glycines (GGGGGG)	27
Gly7	peptide linker with 7 glycines (GGGGGGG)	28
Gly8	peptide linker with 8 glycines (GGGG GGGG)	29

*A short hand code was devised to represent the associated structures. Codes beginning with HC represent the adjacent heavy chain and codes beginning with LC represent the adjacent light chains.

The antibody variable regions from known human and humanized antibodies were used to test the universal applicability of the CODV-Ig format in designing bispecific antibody-like binding proteins. In addition, the possibility of positional effects with regard to the placement of certain antibody variable regions either N-terminal or C-terminal on either the heavy chain or the light chain was examined. Based on the design of CODV-Ig molecules having a linker composition of L_1= 7,

L_2= 5, L_3= 1, and L_4=2, different sequences of antibodies were introduced into the CODV-Ig format.

Activities of bispecific antibodies or derivatives against IL1β and TNFα were determined by using commercially available HEK-Blue TNFα/IL1β reporter cells (InvivoGen). To determine antibody activities against TNFα and IL1β, the cytokines were pre-incubated for 1 hour with different concentrations of the antibodies and added to 50,000 HEK Blue TNFα/IL1β cells. Cytokine mediated induction of SEAP was measured after 24 hours in the culture supernatant with the QUANTI-Blue assay (InvivoGen).

As shown in Table 11, all constructs showed good to excellent protein yield and acceptable levels of aggregation (*see*, in particular, Batch ID Nos. 301 and 302 in Table 11). The measured affinity for each antibody variable domain was within the published or expected affinity. In cases where affinity was assessed, no positional effects were detected. In summary, as shown in the following tables, no positional effects were seen with any of the antibody variable domains used or with the use of these domains on either antibody chain.

Table 11. Universal Use of CODV-Ig Format for Bispecific Antibody-like Binding Proteins

Batch ID DC/LC Codes[1]	Alignment on HC	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (Antigen 1) [pM]	KD (Antigen 2) [pM]	IC50 [pM] TNFα Cellular Assay
301 HC10/LC10	IGF1R(1) x HER2	7	5	1	2	70	5.9	n.m.	153 (HER2)	–
302 HC11/LC11	HER2 x IGF1R(1)	7	5	1	2	60	1.8	163 (HER2)	n.m.	–
303 HC12/LC12	IGF1R(2) x EGFR	7	5	1	2	17	2.7	n.m.	–	–
304 HC13/LC13	EGFR x IGF1R(2)	7	5	1	2	9.5	4.3	–	n.m.	–
305 HC14/LC14	TNFα x IL12/23	7	5	1	2	7.1	7.5	321 (TNFα)	65 (IL23)	95
306 HC15/LC15	IL12/23 x TNFα	7	5	1	2	11.9	7.1	118 (IL23)	543 (TNFα)	138
307 HC16/LC16	TNFα x IL1β	7	5	1	2	6.6	13.6	340 (TNFα)	155 (IL1β)	136
308 HC17/LC17	IL1β x TNFα	7	5	1	2	2.4	5.7	97.5 (IL1β)	358 (TNFα)	138

*n.m.= not measurable by Biacore.

[1] - Heavy chain and light chains corresponding to codes can be found in Table 10.

Example 9. Retention of Parental Antibody Affinity in CODV-Ig Format

The identical antibody sequences for anti-IL4 and anti-IL13 were incorporated into either the TBTI/DVD-Ig or CODV-Ig formats for a direct comparison of these configurations, the positioning of the linkers, and affinities of the resulting molecules. As shown in Figure 7, the parental affinity of each of the antibodies was maintained in the CODV format. As shown in the upper panel of Figure 7, when the variable regions were placed in the TBTI/DVD-Ig format, a drop in affinity of the IL4 antibody positioned at the inner Fv2 position was manifested as a reduction of the on-rate of antibody binding to the antigen. In contrast, there was no loss in affinity for the CODV-Ig format as compared to the parental antibodies (*see* Figure 7, lower panel).

Example 10. Adaptability of CODV-Ig to Fab Format

The ability of the CODV-Ig format to provide fragments such as Fab fragments was evaluated next. Two different variable heavy chains were fused to each other through linker L_3 and elongated C-terminally by linker L_4. This V_H complex was then fused to the C_{H1} domain of IGHG1 (GenBank Accession No. Q569F4) harboring C-terminally the five amino acid sequence DKTHT (SEQ ID NO: 60) from the hinge region followed by six histidine residues. Two different variable light chains were fused to each other in a cross-over configuration to the corresponding heavy chain through linker L_1 and extended C-terminally by linker L_2 and subsequently fused to the constant kappa chain (IGKC, GenBank Accession No. Q502W4).

Fab fragments were expressed by transient transfection as described previously. Seven days post-transfection, cells were removed by centrifugation, 10 % vol/vol 1M Tris-HCl, pH 8,0, was added and the supernatant was passed over a 0.22 μm filter to remove particles. The Fab proteins were captured using HisTrap High Performance columns (GE Healthcare) and eluted via imidazole gradient. The protein containing fractions were pooled and desalted using PD-10 or Sephadex columns. Concentrated and sterile filtered (0.22 μm) protein solutions were adjusted to 1 mg/ml and kept at 4°C until use.

Immediate advantages were observed in that the Fab-like molecules in a CODV orientation showed no tendency to aggregate and retained the affinities of the parental antibodies (*see* Table 12). Binding protein constructs from Batch ID Nos. 401-421 directly compared antibody-like proteins in which antibody variable regions were arranged as in CODV-Ig molecules with the heavy chain as the template (401, 402, 406, and 407), CODV Fab-like fragments (402, 408, 413, 418, and 421), four domain antibody-like molecules in TBTI/DVD-Ig format (404, 409, 414, and 419), and CODV-Ig with no linkers (405, 410, 415, and 420). As shown in Table 12, the results of this comparison indicated that there is more likely to be a loss in affinity as compared to the parent antibodies when the variable region is incorporated into a TBTI or DVD-Ig format. In contrast, both the CODV-Ig and CODV-Ig Fab-like formats were better able to maintain parental affinities. The results further confirmed that CODV-Ig molecules require linkers between the variable regions and between the variable regions and the constant domains (*see* Table 12).

Table 12. Use of CODV-Ig Format for Fab-like Fragments

Batch ID	Sample-ID	Format	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (Antigen 1) [pM]	KD (Antigen 2) [pM]	IC50 [pM] TNFα Cellular Assay
401	TNFα x IL12/23	CODV-Ig	7	5	1	2	7.1	7.5	321 (TNFα)	90 (IL23)	95
402	IL12/23 x TNFα	CODV-Ig	7	5	1	2	11.9	7.1	118 (IL23)	543 (TNFα)	138
403	TNFα x IL12/23	CODV-Fab	7	5	1	2	18.7	1.7	232 (TNFα)	41 (IL23)	785
404	TNFα x IL12/23	TBTI	(G4S)2	0	(G4S)2	0	26.0	8.7	219 (TNFα)	399 (IL23)	–
405	TNFα x IL12/23	CODV-Ig	0	0	0	0	3.5	71	–	–	–
406	IL1β x TNFα	CODV-Ig	7	5	1	2	2.4	5.7	98 (IL1β)	358 (TNFα)	139
407	TNFα x IL1β	CODV-Ig	7	5	1	2	6.6	13.6	340 (TNFα)	155 (IL1β)	122
408	IL1β x TNFα	CODV-Fab	7	5	1	2	8.6	0	179 (IL1β)	–	–
409	IL1β x TNFα	TBTI	(G4S)2	0	(G4S)2	0	1.3	40.5	133 (IL1β)	456 (TNFα)	–
410	TNFα x IL1β	CODV-Ig	0	0	0	0	n.p.	–	–	–	–
411	EGFR x IGF1R(2)	CODV-Ig	7	5	1	2	9.5	4.3	124nM (EGFR)	n.m.	–
412	IGF1R(2) x EGFR	CODV-Ig	7	5	1	2	17	2.7	n.m.	–	–
413	EGFR x IGF1R(2)	CODV-Fab	7	5	1	2	13.3	0	42nM (EGFR)	n.m.	–
414	EGFR x IGF1R(2)	TBTI	(G4S)2	0	(G4S)2	0	2.1	2.9	7nM (EGFR)	n.m.	–
415	EGFR x IGF1R(2)	CODV-Ig	0	0	0	0	4.4	100	–	–	–
416	HER2 x IGF1R(1)	CODV-Ig	7	5	1	2	60.0	1.8	163 (HER2)	n.m.	–
417	IGF1R(1) x HER2	CODV-Ig	7	5	1	2	70	5.9	n.m.	41 (HER2)	–
418	HER2 x IGF1R(1)	CODV-Fab	7	5	1	2	34.4	0	190 (HER2)	n.m.	–
419	HER2 x IGF1R(1)	TBTI	(G4S)2	0	(G4S)2	0	6.3	6.8	56 (HER2)	n.m.	–
420	IGF1R(1) x HER2	CODV-Ig	0	0	0	0	0.35	54.5	–	–	–
421	IL4 x IL13	CODV-Fab	7	5	1	2	8.7	14	12 (IL4)	48 (IL13)	–

Example 11. Substitution of Variable Domains within CODV-Ig and CODV-Fab

To characterize the CODV format in a T-cell engaging approach, bispecific CODV Fab-like binding proteins (CODV-Fab) having a TCR binding site (CD3epsilon) and a CD19 binding site were generated and compared to a bispecific Fab derived from the TBTI/DVD-Ig format (B-Fab). To investigate the importance of the orientation of the binding sites (TCR x CD19 vs. CD19 x TCR), both orientations were evaluated for each of the binding proteins.

The binding proteins were characterized in a cytotoxic assay using NALM-6 (CD19 expressing) cells as target cells and primary human T-cells as effector cells. CD3 positive cells were isolated from freshly prepared human PBMC's. Effector and target cells were mixed at a ratio of 10:1 and incubated for 20 hours with the indicated concentrations of bispecific binding proteins (*see* Figure 8). Apoptotic target cells were determined in a FACS-based assay using 7-Aminoactinomycin staining.

The B-Fab format in the configuration CD3-CD19 (1060) was shown to be active in inducing T-cell mediated cytotoxicity towards NALM-6 cells with an EC50 of 3.7 ng/ml. A similarly high activity was observed for the CD19-CD3 CODV-Fab (1109) with an EC50 of 3.2 ng/ml (*see* Figure 8).

A swap of the configuration of the B-Fab molecule (Fab of the TBTI / DVD-Ig format) to a CD19-CD3 orientation resulted in a significant loss of activity (*see* Figure 8). The swapped B-Fab molecule showed no activity at concentrations that were maximal for both orientations of CODV-Ig Fab and the other orientation of B-Fab. For the CODV-Ig Fabs and one orientation of B-Fab, a maximum response was observed (ranging between 1 and 100 ng/ml). For the CD19-CD3 orientation of B-Fab, even at the maximal concentration (30 μg/ml), the optimal cytotoxic response was not reached. In sharp contrast, a change in the orientation of the domains in the CODV-Fab towards CD3-CD19 (1108), resulted in a molecule with significant activity in this assay (*see* Figure 8). Although domain swapping in the CODV-Fab also reduced the induction of T-cell mediated cytotoxicity (increase of EC50 by factor ~100), this effect was far less pronounced than was seen in the B-Fab format and the molecule was able to induce cytotoxicity up to the maximal level. The data were representative and obtained from three independent experiments.

Example 12. Influence of Amino Acid Sequence Identity on CODV-Ig Linkers

The optimized construct corresponding to Batch ID 204 (*see* Example 7 and Table 8) was chosen to investigate the influence of linker composition on the linkers L_1 to L_4. Linker lengths were set at 7, 5, 1, and 2 residues in length for L_1, L_2, L_3, and L_4, respectively (*see* Table 13). Test sequences were derived from naturally occurring linkers at the transitions between natural antibody V_H and C_{H1} domains or between antibody Fv and C_L domains of kappa or lambda light chains. The candidate sequences were ASTKGPS (SEQ ID NO: 48), which is derived from the V_H and C_{H1} domain transition, RTVAAPS (SEQ ID NO: 49) and GQPKAAP (SEQ ID NO: 50), which were derived from the Fv and C_L domain transitions of kappa and lambda light chains, respectively. Furthermore, one construct was generated with an arbitrary linker composition to show that any sequence can be potentially used in linkers L_1 to L_4. This linker composition was obtained by randomly distributing the amino acids valine, leucine, isoleucine, serine, threonine, lysine, arginine, histidine, aspartate, glutamate, asparagine, glutamine, glycine, and proline at the 15 positions of the four linkers. The aromatic amino acids phenylalanine, tyrosine, and tryptophan, as well as the amino acids methionine and cysteine were deliberately excluded to avoid potential increases in aggregation.

A three-dimensional model of the construct for Batch ID No. 204 was generated to assure suitability or refine the choices of linker composition. Thus, serine was chosen for linker L_3 as positively and negatively charged residues are observed nearby in the three-dimensional model. The residues in linker L_4 were selected to be compatible with solvent exposure of these positions as suggested by the model. Similarly, no problems were anticipated or predicted for the linker compositions of L_1 and L_2. Three-dimensional models of selected proposals for linker composition were constructed.

As shown in Table 12, linker composition may have a dramatic influence on yield. Sequences that were derived from lambda chain on L_1 (comparing Batch ID Nos. 505-507 with Batch ID Nos. 501-503) were more productive protein generators (up to 8 fold increase). Indeed, the linkers based on random generation also produced good yields, as shown in Table 13, Batch ID No. 508. Therefore, linker composition should be one parameter considered during CODV-Ig optimization.

Table 13. Effect of Linker Composition on CODV-Ig

Batch ID	Alignment on HC	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (IL4) [pM]	KD (IL13) [pM]	IC50 Cellular Assay IL4 [nM]	IC50 Cellular Assay IL13 [nM]
501	IL4 x IL13	ASTKGPS (SEQ ID NO: 48)	TKGPS (SEQ ID NO: 52)	S	RT	10.8	1.2	1	55	0.034	4.5
502	IL4 x IL13	ASTKGPS (SEQ ID NO: 48)	TVAAP (SEQ ID NO: 53)	S	QP	15.8	2.9	3	61	0.049	2.4
503	IL4 x IL13	ASTKGPS (SEQ ID NO: 48)	TVAAP (SEQ ID NO: 53)	S	SS	11.6	3.5	3	52	0.047	2.1
504	IL4 x IL13	RTVAAPS (SEQ ID NO: 49)	QPKAA (SEQ ID NO: 54)	S	TK	15	1.9	8	71	0.042	1.4
505	IL4 x IL13	GQPKAAP (SEQ ID NO: 50)	TKGPS (SEQ ID NO: 52)	S	RT	71.7	1.9	6	68	0.033	0.9
506	IL4 x IL13	GQPKAAP (SEQ ID NO: 50)	TVAAP (SEQ ID NO: 53)	S	TK	49.3	1.7	7	55	0.045	1.8
507	IL4 x IL13	GQPKAAP (SEQ ID NO: 50)	QPKAA (SEQ ID NO: 54)	S	RT	62.4	2	1	69	0.040	2.0
508	IL4 x IL13	HIDSPNK (SEQ ID NO: 51)	QRIEG (SEQ ID NO: 55)	V	SL	37.7	2.1	1	44	0.054	1.3

Activities of bispecfic antibodies or derivatives against cytokines IL4 and IL13 were determined in commercially available HEK-Blue IL-4/IL-13 reporter cells (InvivoGen). HEK-Blue IL-4/IL-13 cells are designed to monitor the activation of the STAT6 pathway by IL-4 or IL13. Stimulation of the cells with either cytokine results in production of the reporter gene secreted embryonic alkaline phosphatase (SEAP), which can be measured in the culture supernatant with the QUANTI-Blue assay. To test antibody activities against IL4 or IL13, the cytokines were pre-incubated for 1 hour with different concentrations of the antibodies and added to 50,000 HEK-Blue IL-4/IL-13 cells. Cytokine-mediated induction of SEAP was measured after 24 hours incubation in the cell culture supernatant with the QUANTI-Blue assay (InvivoGen).

Example 13. Introduction of Cysteines into CODV-Ig Linkers

Published data suggest that the stability of antibodies and antibody-derived proteins can be increased by the introduction of non-natural disulfide bridges (*see* Wozniak-Knopp *et al.*, 2012, "Stabilisation of the Fc Fragment of Human IgG1 by Engineered Intradomain Disulfide Bonds," *PLoS ONE* 7(1): e30083). To examine whether the equivalent Fc fragment derived from a human IgG1 antibody and engineered into a CODV-Ig molecule can be stabilized by the introduction of inter- and intra-chain disulfide bridges, the equivalent Fc positions of the CODV-Ig construct Batch ID No. 204 (from Example 7) were mutated to cysteine residues, and the mutant proteins were overproduced, purified, and characterized (*see* Table 14).

As shown in Table 14, each of the mutated CODV-Ig molecules containing additional cysteine residues had melting temperatures that were the same as the melting temperature for the CODV-Ig construct Batch ID No. 204.

In addition, two simultaneous cysteines were introduced at Kabat positions 100 for the light chain and 44 for the heavy chain on each of the variable domains as described in Brinkmann *et al.*, 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90: 7538-42. These positions have been shown to be structurally conserved within antibody folds, and therefore tolerable of cysteine substitution without interfering with the integrity of the individual domains.

As shown in Table 15, CODV and CODV-Ig constructs in which cysteine

residues were introduced at Kabat positions 100 for the light chain and 44 for the heavy chain on each of the variable domains had higher melting temperatures than the CODV and CODV-Ig constructs in which cysteine residues were not introduced at these positions (*see*, *e.g.*, Batch ID Nos. 704 and 706 and Batch ID Nos. 713 and 714).

5

1. Thermostability Measurements of CODV and TBTI Variants

Melting points (Tm) of CODV and TBTI variants were determined using differential scanning fluorimetry (DSF). Samples were diluted in D-PBS buffer (Invitrogen) to a final concentration of 0.2 μg/μl and added to 2μl of a 40x concentrated 10 solution of SYPRO-Orange dye (Invitrogen) in D-PBS in white semi-skirt 96-well plates. All measurements were done in duplicate using a MyiQ2 real time PCR instrument (Biorad). Tm values were extracted from the negative first derivative of the melting curves using iQ5 Software v2.1.

Next the effect of introducing cysteine residues directly into the linkers or within 15 the variable region was examined. In this example, Batch ID No. 204 (from Example 7 and Table 8) was used as the model CODV-Ig binding protein, and cysteine was substituted for glycine in L_1, L_3, or the variable region on the basis of the three-dimensional model. As shown in Table 16 below, the results indicate how the introduction of cysteine pairs would affect yield and aggregation. The envisioned 20 mutations were all modeled to ascertain that disulfide bonds were properly formed and correct geometry was maintained for the linkers and their environment in the models. Nevertheless, Batch ID No. 808 showed good yield and little aggregation, thus suggesting that a proper cysteine bridge could be formed.

25 While the invention has been described in terms of various embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed. In addition, the section headings used herein are for organizational purposes only and are not to be construed as limiting the 30 subject matter described.

Each embodiment herein described may be combined with any other embodiment or embodiments unless clearly indicated to the contrary. In particular, any feature or

embodiment indicated as being preferred or advantageous may be combined with any other feature or features or embodiment or embodiments indicated as being preferred or advantageous, unless clearly indicated to the contrary.

All references cited in this application are expressly incorporated by reference herein.

Table 14. Effect of Disulfide Bridge Stabilization on CODV-Ig

Batch ID	Alignment on HC	Introduced Mutations IGHG1	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (IL4) [pM]	KD (IL13) [pM]	IC50 Cellular Assay IL4 [nM]	IC50 Cellular Assay IL13 [nM]	Tm [°C]
601	IL4 x IL13	P344C & A432C	7	5	1	2	21.6	0.9	n.d.	n.d.	0.032	1.7	64
602	IL4 x IL13	S376C & P397C	7	5	1	2	13.9	0.8	10	100	0.034	1.5	64
603	IL4 x IL13	P446G447 to G446E447C448	7	5	1	2	15.7	1.3	4	64	0.036	1.4	64

Table 15. Effect of Disulfide Bridge Stabilization on CODV-Ig

Batch ID	Alignment on HC	Format	Introduced Mutations LC	Introduced Mutations HC	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (Antigen 1) [pM]	KD (Antigen 2) [pM]	Tm [°C]
704	IL13 x IL4	CODV	–	–	7	5	1	2	15.3	5.2	3	25	64
706	IL4 x IL13	CODV	G100C & G100C	G44C & G44C	7	5	1	2	3.2	17.7	8	61	68
713	IGF1R(1) x HER2	CODV-Ig	–	–	7	5	1	2	60	1.8	163 (HER2)	–	64
714	IGF1R(1) x HER2	CODV-Ig	G100C & Q100C	G44C & G44C	7	5	1	2	3.4	7.9	362 (HER2)	–	68

Table 16A. Effect of Disulfide Bridge Stabilization on CODV-Ig

Batch ID	Alignment on HC	Introduced Mutations LC	Introduced Mutations HC	L_1	L_2	L_3	L_4	Yield [mg/L]	Aggregation [%]	KD (IL4) [pM]	KD (IL13) [pM]	Tm
801	IL4 x IL13			7	5	1	2	15.3	5.2	3	25	64
802	IL4 x IL13	G114C	Q43C	GG**C**GGGG (SEQ ID NO: 56)	GGGGG (SEQ ID NO: 26)	G	GG	3.0	11.3	6	34	64
803	IL4 x IL13	G115C	Q43C	GGG**C**GGG (SEQ ID NO: 57)	GGGGG (SEQ ID NO: 26)	G	GG	4.0	9.4	6	20	63
804	IL4 x IL13	G116C	Q43C	GGGG**C**GG (SEQ ID NO: 58)	GGGGG (SEQ ID NO: 26)	G	GG	11.1	9.6	2	33	64
805	IL4 x IL13	G117C	Q43C	GGGGG**C**G (SEQ ID NO: 59)	GGGGG (SEQ ID NO: 26)	G	GG	5.8	10.6	7	45	64
806	IL4 x IL13	S60C	S122C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	n.p.	–	–	–	–
807	IL4 x IL13	S60C	S123C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	7.5	27.9	2	202	66
808	IL4 x IL13	S60C	G124C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	**C**	GG	1.1	5.8	5	70	64
809	IL4 x IL13	G61C	S122C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	8.7	6.9	6	81	65

Table 16B. Effect of Disulfide Bridge Stabilization on CODV-Ig

Batch ID	Alignment on HC	Introduced Mutations LC	Introduced Mutations HC	L_1	L_2	L_3	L_4	IC50 Cellular Assay IL4 [nM]	IC50 Cellular Assay IL13 [nM]
801	IL4 x IL13			7	5	1	2	0.045	1.8
802	IL4 x IL13	G114C	Q43C	GG**C**GGGG (SEQ ID NO: 56)	GGGGG (SEQ ID NO: 26)	G	GG	0.027	1.2
803	IL4 x IL13	G115C	Q43C	GGG**C**GGG (SEQ ID NO: 57)	GGGGG (SEQ ID NO: 26)	G	GG	0.036	1.5
804	IL4 x IL13	G116C	Q43C	GGGG**C**GG (SEQ ID NO: 58)	GGGGG (SEQ ID NO: 26)	G	GG	0.040	1.8
805	IL4 x IL13	G117C	Q43C	GGGGG**C**G (SEQ ID NO: 59)	GGGGG (SEQ ID NO: 26)	G	GG	0.047	1.9
806	IL4 x IL13	S60C	S122C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	–	–
807	IL4 x IL13	S60C	S123C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	0.048	10.1
808	IL4 x IL13	S60C	G124C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	**C**	GG	0.023	3.8
809	IL4 x IL13	G61C	S122C	GGGGGGG (SEQ ID NO: 28)	GGGGG (SEQ ID NO: 26)	G	GG	0.035	2.8

WHAT IS CLAIMED IS:

Claim 1. An antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, wherein two polypeptide chains have a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

and two polypeptide chains have a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Claim 2. The antibody-like binding protein of claim 1, wherein:

L_1 is 3 to 12 amino acid residues in length;

L_2 is 3 to 14 amino acid residues in length;

L_3 is 1 to 8 amino acid residues in length; and

L_4 is 1 to 3 amino acid residues in length.

Claim 3. The antibody-like binding protein of either claim 1 or 2, wherein:

L_1 is 5 to 10 amino acid residues in length;

L_2 is 5 to 8 amino acid residues in length;

L_3 is 1 to 5 amino acid residues in length; and

L_4 is 1 to 2 amino acid residues in length.

Claim 4. The antibody-like binding protein of any one of claims 1-3, wherein:

L_1 is 7 amino acid residues in length;

L_2 is 5 amino acid residues in length;

L_3 is 1 amino acid residues in length; and

L_4 is 2 amino acid residues in length.

Claim 5. The antibody-like binding protein of claim 1, wherein:

L_1 is 1 to 3 amino acid residues in length;

L_2 is 1 to 4 amino acid residues in length;

L_3 is 2 to 15 amino acid residues in length; and

L_4 is 2 to 15 amino acid residues in length.

Claim 6. The antibody-like binding protein of either claim 1 or 5, wherein:

L_1 is 1 to 2 amino acid residues in length;

L_2 is 1 to 2 amino acid residues in length;

L_3 is 4 to 12 amino acid residues in length; and

L_4 is 2 to 12 amino acid residues in length.

Claim 7. The antibody-like binding protein of any one of claims 1, 5, or 6, wherein:

L_1 is 1 amino acid residue in length;

L_2 is 2 amino acid residues in length;

L_3 is 7 amino acid residues in length; and

L_4 is 5 amino acid residues in length.

Claim 8. The antibody-like binding protein of claim 1, wherein L_1 is 0 amino acid residues in length and L_3 is 2 or more amino acid residues in length.

Claim 9. The antibody-like binding protein of claim 1, wherein L_3 is 0 amino acid residues in length and L_1 is 1 or more amino acid residues in length.

Claim 10. The antibody-like binding protein of claim 1, wherein L_4 is 0 amino acid residues in length and L_2 is 3 or more amino acid residues in length.

Claim 11. The antibody-like binding protein of any one of claims 1-10, wherein the binding protein is capable of specifically binding one or more antigen targets.

Claim 12. The antibody-like binding protein of any one of claims 1-11, wherein the one or more antigen targets is selected from the group consisting of B7.1, B7.2, BAFF, BlyS, C3, C5, CCL11 (eotaxin), CCL15 (MIP-1d), CCL17 (TARC), CCL19 (MIP-3b), CCL2 (MCP-1), CCL20 (MIP-3a), CCL21 (MIP-2), SLC, CCL24 (MPIF-2/eotaxin-2), CCL25 (TECK), CCL26 (eotaxin-3), CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (mcp-2), CD3, CD19, CD20, CD24, CD40, CD40L, CD80, CD86, CDH1 (E-cadherin), Chitinase, CSF1 (M-CSF), CSF2 (GM-CSF), CSF3 (GCSF), CTLA4, CX3CL1 (SCYD1), CXCL12 (SDF1), CXCL13, EGFR, FCER1A, FCER2, HER2, IGF1R, IL-1, IL-12, IL13, IL15, IL17, IL18, IL1A, IL1B, IL1F10, IL1β, IL2, IL4, IL6, IL7, IL8, IL9, IL12/23, IL22, IL23, IL25, IL27, IL35, ITGB4 (b 4 integrin), LEP (leptin), MHC class II, TLR2, TLR4, TLR5, TNF, TNFα, TNFSF4 (OX40 ligand), TNFSF5 (CD40 ligand), Toll-like receptors, TREM1, TSLP, TWEAK, XCR1 (GPR5/CCXCR1), DNGR-1(CLEC91), and HMGB1.

Claim 13. The antibody-like binding protein of any one of claims 1-12, wherein the binding protein is bispecific and capable of binding two different antigen targets.

Claim 14. The antibody-like binding protein of any one of claims 1-13, wherein the two different antigen targets are selected from the group consisting of IL4 and IL13, IGF1R and HER2, IGF1R and EGFR, EGFR and HER2, BK and IL13, PDL-1 and CTLA-4, CTLA4 and MHC class II, IL-12 and IL-18, IL-1α and IL-1β, TNFα and IL12/23, TNFα and IL-12p40, TNFα and IL1β, TNFα and IL-23, and IL17 and IL23.

Claim 15. The antibody-like binding protein of any one of claims 1-14, wherein the binding protein is capable of inhibiting the function of one or more of the antigen targets.

Claim 16. The antibody-like binding protein of any one of claims 1-15, wherein at least one of the linkers selected from the group consisting of L_1, L_2, L_3, and L_4 contains at least one cysteine residue.

Claim 17. An antibody-like binding protein comprising two polypeptide chains that form two antigen binding sites, wherein a first polypeptide chain has a structure represented by the formula:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

and a second polypeptide chain has a structure represented by the formula:

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the first and second polypeptides form a cross-over light chain-heavy chain pair.

Claim 18. The antibody-like binding protein of claim 17, wherein:

L_1 is 3 to 12 amino acid residues in length;

L_2 is 3 to 14 amino acid residues in length;

L_3 is 1 to 8 amino acid residues in length; and

L_4 is 1 to 3 amino acid residues in length.

Claim 19. The antibody-like binding protein of either claim 17 or 18, wherein:

L_1 is 5 to 10 amino acid residues in length;

L_2 is 5 to 8 amino acid residues in length;

L_3 is 1 to 5 amino acid residues in length; and

L_4 is 1 to 2 amino acid residues in length.

Claim 20. The antibody-like binding protein of any one of claims 17-19, wherein:

L_1 is 7 amino acid residues in length;

L_2 is 5 amino acid residues in length;

L_3 is 1 amino acid residues in length; and

L_4 is 2 amino acid residue in length.

Claim 21. The antibody-like binding protein of claim 17, wherein:

L_1 is 1 to 3 amino acid residues in length;

L_2 is 1 to 4 amino acid residues in length;

L_3 is 2 to 15 amino acid residues in length; and

L_4 is 2 to 15 amino acid residues in length.

Claim 22. The antibody-like binding protein of either claim 17 or 21, wherein:

L_1 is 1 to 2 amino acid residues in length;

L_2 is 1 to 2 amino acid residues in length;

L_3 is 4 to 12 amino acid residues in length; and

L_4 is 2 to 12 amino acid residues in length.

Claim 23. The antibody-like binding protein of any one of claims 17, 21, or 22, wherein:

L_1 is 1 amino acid residue in length;

L_2 is 2 amino acid residues in length;

L_3 is 7 amino acid residues in length; and

L_4 is 5 amino acid residues in length.

Claim 24. The antibody-like binding protein of claim 17, wherein L_1 is 0 amino acid residues in length and L_3 is 2 or more amino acid residues in length.

Claim 25. The antibody-like binding protein of claim 17, wherein L_3 is 0 amino acid residues in length and L_1 is 1 or more amino acid residues in length.

Claim 26. The antibody-like binding protein of claim 17, wherein L_4 is 0 amino acid residues in length and L_2 is 3 or more amino acid residues in length.

Claim 27. The antibody-like binding protein of any one of claims 17-26, wherein the binding protein is capable of specifically binding one or more antigen targets.

Claim 28. The antibody-like binding protein of any one of claims 17-27, wherein the one or more antigen targets is selected from the group consisting of B7.1, B7.2, BAFF, BlyS, C3, C5, CCL11 (eotaxin), CCL15 (MIP-1d), CCL17 (TARC), CCL19 (MIP-3b), CCL2 (MCP-1), CCL20 (MIP-3a), CCL21 (MIP-2), SLC, CCL24 (MPIF-2/eotaxin-2), CCL25 (TECK), CCL26 (eotaxin-3), CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (mcp-2), CD3, CD19, CD20, CD24, CD40, CD40L, CD80, CD86, CDH1 (E-cadherin), Chitinase, CSF1 (M-CSF), CSF2 (GM-CSF), CSF3 (GCSF), CTLA4, CX3CL1 (SCYD1), CXCL12 (SDF1), CXCL13, EGFR, FCER1A, FCER2, HER2, IGF1R, IL-1, IL-12, IL13, IL15, IL17, IL18, IL1A, IL1B, IL1F10, IL1β, IL2, IL4, IL6, IL7, IL8, IL9, IL12/23, IL22, IL23, IL25, IL27, IL35, ITGB4 (b 4 integrin), LEP (leptin), MHC class II, TLR2, TLR4, TLR5, TNF, TNFα, TNFSF4 (OX40 ligand), TNFSF5 (CD40 ligand), Toll-like receptors, TREM1, TSLP, TWEAK, XCR1 (GPR5/CCXCR1), DNGR-1(CLEC91), and HMGB1.

Claim 29. The antibody-like binding protein of any one of claims 17-28, wherein the binding protein is bispecific and capable of binding two different antigen targets.

Claim 30. The antibody-like binding protein of any one of claims 17-29, wherein the two different antigen targets are selected from the group consisting of IL4 and IL13,

IGF1R and HER2, IGF1R and EGFR, EGFR and HER2, BK and IL13, PDL-1 and CTLA-4, CTLA4 and MHC class II, IL-12 and IL-18, IL-1α and IL-1β, TNFα and IL12/23, TNFα and IL-12p40, TNFα and IL1β, TNFα and IL-23, and IL17 and IL23.

Claim 31. The antibody-like binding protein of any one of claims 17-30, wherein the binding protein is capable of inhibiting the function of one or more of the antigen targets.

Claim 32. The antibody-like binding protein of any one of claims 17-31, wherein at least one of the linkers selected from the group consisting of L_1, L_2, L_3, and L_4 contains at least one cysteine residue.

Claim 33. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the antibody-like binding protein of any one of claims 1-32.

Claim 34. An expression vector comprising the nucleic acid molecule of claim 33.

Claim 35. An isolated host cell comprising the nucleic acid molecule of claim 33 or the expression vector of claim 34.

Claim 36. The host cell of claim 35, wherein the host cell is a mammalian cell or an insect cell.

Claim 37. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of the antibody-like binding protein of any one of claims 1-32.

Claim 38. A method for making the antibody-like binding protein of any one of claims 1-16, comprising expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \qquad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \qquad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Claim 39. A method for making the antibody-like binding protein of any one of claims 17-32, comprising expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptide of formula I and the polypeptide of formula II form a cross-over light chain-heavy chain pair.

Claim 40. A method of making an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, comprising:

(a) identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H;

(b) assigning either the light chain or the heavy chain as template chain;

(c) assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1};

(d) assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1}\text{-}Fc \quad [II]$$

(e) determining maximum and minimum lengths for L_1, L_2, L_3, and L_4;

(f) generating polypeptide structures of formulas I and II;

(g) selecting polypeptide structures of formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain;

Fc is the immunoglobulin hinge region and C_{H2}, C_{H3} immunoglobulin heavy chain constant domains; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Claim 41. The method of claim 40, wherein the first antibody variable domain and the second antibody variable domain are the same.

Claim 42. A method of making an antibody-like binding protein comprising four polypeptide chains that form four antigen binding sites, comprising:

(a) identifying a first antibody variable domain that binds a first target antigen and a second antibody variable domain that binds a second target antigen, each containing a V_L, and a V_H;

(b) assigning either the light chain or the heavy chain as template chain;

(c) assigning the V_L of the first antibody variable domain or the second antibody variable domain as V_{L1};

(d) assigning a V_{L2}, a V_{H1}, and a V_{H2} according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}L_2\text{-}C_L \quad [I]$$

$$V_{H2}\text{-}L_3\text{-}V_{H1}\text{-}L_4\text{-}C_{H1} \quad [II]$$

(e) determining maximum and minimum lengths for L_1, L_2, L_3, and L_4;

(f) generating polypeptide structures of formulas I and II;

(g) selecting polypeptide structures of formulas I and II that bind the first target antigen and the second target antigen when combined to form the antibody-like binding protein;

wherein:

V_{L1} is a first immunoglobulin light chain variable domain;

V_{L2} is a second immunoglobulin light chain variable domain;

V_{H1} is a first immunoglobulin heavy chain variable domain;

V_{H2} is a second immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is the immunoglobulin C_{H1} heavy chain constant domain; and

L_1, L_2, L_3, and L_4 are amino acid linkers;

and wherein the polypeptides of formula I and the polypeptides of formula II form a cross-over light chain-heavy chain pair.

Claim 43. The method of claim 42, wherein the first antibody variable domain and the second antibody variable domain are the same.