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(71) Applicant: **IGM BIOSCIENCES, INC.** [US/US]; 320 Logue Avenue, Mountain View, CA 94043 (US).

(72) Inventors: **KEYT, Bruce**; 1180 Lakeview Drive, Hillsborough, CA 94010 (US). **ZHANG, Fen**; 2014 10th Avenue, San Francisco, CA 94116 (US). **PRESTA, Leonard, George**; 1900 Gough Street, Apt. 206, San Francisco, CA 94109 (US). **YOSHIMURA, Rency**; 4462 Billings Circle, Santa Clara, CA 95054 (US).

(74) Agents: **DREGER, Ginger, R.** et al.; Arnold & Porter, LLP, 1801 Page Mill Road, Suite 110, Palo Alto, CA 94304-1216 (US).

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(54) Title: CONSTANT CHAIN MODIFIED BISPECIFIC, PENTA- AND HEXAVALENT IG-M ANTIBODIES

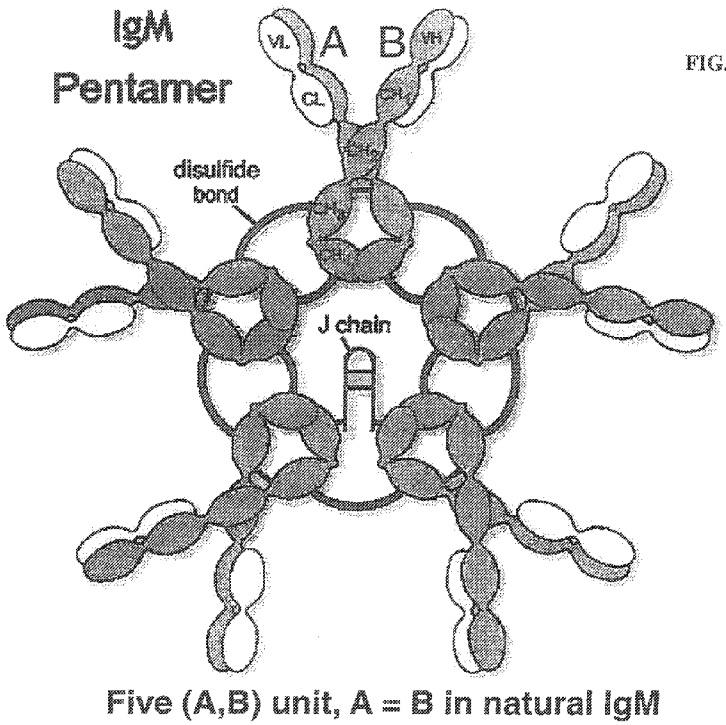

FIG. 1

(57) Abstract: The present invention concerns binding molecules having a penta- or hexameric ring structure, such as, for example, isolated IgM antibodies with five or six bispecific binding units, and methods and means for making and using the same. The invention further concerns multi-specific binding molecules having a penta- or hexameric ring structure, such as, for example, isolated IgM antibodies with five or six bispecific binding units, and methods and means for making and using the same.

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CONSTANT CHAIN MODIFIED BISPECIFIC, PENTA- AND HEXAVALENT IG-M ANTIBODIES

Field of the Invention

The present invention concerns binding molecules with penta- or hexameric structure.

In particular, the invention relates to binding molecules having a penta- or hexameric ring structure comprising five or six bispecific binding units. In the binding molecules of the present invention each of the bispecific binding units binds two different binding targets or different binding regions (e.g. epitopes) on the same binding target, and each of the five or six bispecific binding units have the same binding specificities (bind to the same two binding targets). In a particular embodiment, the invention concerns bispecific antibodies with penta- or hexameric structure, comprising five or six bispecific binding units.

In a different aspect, the invention includes binding molecules comprising five or six monospecific binding units, where (i) each of the monospecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of the monospecific binding units bind to different binding target. The invention further includes binding molecules comprising five or six bispecific binding units, where (i) each of the bispecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, and (ii) at least two of the bispecific binding units bind to different binding targets. In a particular embodiment, the binding molecules are multi-specific IgM antibodies.

Background of the Invention

Since the advent of humanized antibodies, the therapeutic use of antibodies such as Rituxan® (rituximab), Herceptin® (trastuzumab) and Avastin® (bevacizumab), has revolutionized the fields of medicine, including oncology, the treatment of inflammatory disorders, such as rheumatoid arthritis, and many other indications. In the United States, more than 30 human or humanized antibodies have been approved for clinical use, and more than 600 new antibodies or antibody-like molecules are in various stages of development. Some antibodies have antagonistic function on soluble target molecules such as vascular endothelial growth factor (VEGF) or tumor necrosis factor (TNF), whose actions are part of the pathologic process of a disease. Alternatively, antibodies can bind, block and/or induce

destruction of pathologic cells in certain diseases, such as cancer. The main functions of these therapeutic antibodies are binding through the Fab region, and recruitment of effector function via the Fc domain (which also mediates the long circulating half-life of antibodies). One of the major advantages of antibodies compared to small molecule drugs, can be their exquisite specificity. Antibodies can very accurately target selected protein antigens, such as oncogenes, to the exclusion of very similar homologs, allowing for benign safety profiles. Hence, antibodies are well characterized for specific single targeting function.

As the field has progressed, antibody function has been enhanced through creative means of protein engineering, such as to provide higher affinity, longer half-life, and/or better tissue distribution, as well as combination of small and large molecule technologies for increased focus of cell destruction via toxic payload delivery (e.g. antibody-drug conjugates). Another approach to improving antibody function takes advantage of the bivalent binding of the immunoglobulin G (IgG) structure which allows one IgG molecule to bind two antigens. Indeed, in certain applications, there exists good potential for asymmetric antibodies to exert useful functions by simultaneously binding two different target antigens. To address this need, a variety of constructs have been produced to yield a single molecule that can bind two different antigens, allowing for functions never before seen in nature. An example of this bi-specific approach is "blinatumumab" (MT103) which binds the CD3 and CD19 receptors, on T- and B-cells, respectively. This tethering of a cytotoxic T cell to a cancerous B-cell, allows for effective treatment of B-cell leukemia.

However, there remain significant technical difficulties in construction, expression and production of bispecific antibodies. Although bispecific antibodies are regarded as promising therapeutic agents due to their ability to simultaneously bind two different antigens, their utility is limited due to problems with stability and manufacturing complexity.

Various forms of protein engineering have been used to match heterologous heavy chains, plus appropriate pairwise matching of heavy and light chains to efficiently yield a bi-specific IgG. In addition, various bispecific antibody formats, including quadromas, chemical heteroconjugates, recombinant constructs using selected heterodimerization domains and recombinant constructs of minimal size consisting of two minimal antigen-binding sites.

However, all of these efforts have been fraught with difficulty.

Thus, despite efforts directed toward the development of bispecific therapeutic antibodies, there remains a great need for developing more efficient platforms that can lead to more efficient and flexible production of bi- and multispecific antibodies, thereby shortening

the timeline between discovery and clinical introduction of such therapeutics and enabling the design and production of new types of antibody formats with multiple specificities and/or valencies.

Summary of the Invention

The present invention concerns binding molecules having a penta- or hexameric ring structure, such as, for example, isolated IgM antibodies with five or six bispecific binding units, and methods and means for making and using the same.

In one aspect, the invention concerns a binding molecule having a penta- or hexameric ring structure comprising five or six bispecific binding units, wherein each of the bispecific binding units has the same two binding specificities and comprises a first chain comprising at least a Cμ4 domain of an IgM heavy chain constant region conjugated to a first binding region to a first binding target, and a second chain comprising at least a Cμ4 domain of an IgM heavy chain constant region and a second binding region to a second binding target, wherein the first and second binding targets are different, and wherein the first and second chains are assembled to create a bispecific binding unit as a result of an asymmetric interface created between their respective IgM heavy chain constant regions.

In one embodiment, the bispecific binding units are identical.

In another embodiment, the binding molecule further comprises an IgM J chain.

In yet another embodiment, the binding molecule has a pentameric ring structure.

In a further embodiment, the binding molecule has a hexameric ring structure.

In a still further embodiment, in the binding molecule the first and the second chains further comprise a Cμ3 domain of an IgM heavy chain constant region.

In another embodiment, the first and second chains further comprise a Cμ2 domain of an IgM heavy chain constant region.

In other embodiments, the first and second binding targets are selected from peptides, polypeptides, glycoproteins, nucleic acid molecules, and organic and non-organic small molecules, including, without limitation, soluble polypeptides, cell surface receptors, ligands, molecular transporters, enzymes and substrates of enzymes.

In a still further embodiment, the first and second binding targets are two sites on the same soluble target, two sites on the same cell surface receptor target, two different soluble targets, two cell surface receptor targets, one soluble target and one cell surface receptor

target, one soluble or cell surface receptor target and one long residence time target, one soluble target and one matrix protein or substrate target, one soluble or receptor target and one molecular transporter target, or two different cell types.

The conjugation of the binding regions to the rest of the molecule may take place by fusion. Thus, for example, the first and second binding regions may be fused to the N-termini of the first and second IgM heavy chain constant regions, respectively.

In a particular embodiment, the first and second binding regions are variable regions of an antibody.

In another embodiment, the first and second binding targets are two different antigens.

In yet another embodiment, the first and second binding targets are different epitopes on the same antigen.

In further embodiments, the first and second binding regions may be two different antibody heavy chain variable regions, binding to two binding targets, or to different epitopes on the same binding target.

In the binding molecules of the present invention the antibody heavy chain variable regions may be from an IgG, IgA, IgE, and/or IgM antibody, preferably from an IgM antibody. Preferably, the binding molecules herein are bispecific IgM molecules, which may, but are not required to, further comprise at least one IgM light chain variable region sequence associated with one of two different IgM heavy chain variable regions.

In a particular embodiment, in the binding molecules of the present invention at least some of the asymmetric interfaces between the IgM heavy chain constant regions of the two chains of a binding unit are created by a salt bridge formed by pair-wise switches between oppositely charged amino acid residues in at least one of the Cμ2, Cμ3 and/or Cμ4 domains of the two chains of said binding unit.

Thus, a salt bridge may be formed between at least one of the Cμ2-Cμ2, Cμ4-Cμ4, and Cμ2-Cμ3-Cμ4 domains of the two chains of a binding unit.

In one embodiment, the pair-wise switches are selected from the group consisting of E→K, K→E; R→E, E→R; D→K, K→D; and R→D, D→R.

In another embodiment, the binding molecule may comprise at least one pair-wise charged amino acid residue switch in the Cμ4-Cμ4 domains, where the switch may, for

example, be selected from the group consisting of R328E,D↔E339R,K; R344E,D↔S330R,K; K376E,D↔E385R,K; R427E,D↔E339R,K; and T354E,D↔I397R,K.

In a further embodiment, at least one pair-wise charged amino acid switch between the Cμ2-Cμ2 domains, and may, for example, be selected from the group consisting of E167R,K↔K177E,D and K169E,D↔E170R,K.

In a still further embodiment, at least one pair-wise charged amino acid residue switch is in the Cμ2-Cμ3-Cμ4 domains, and may, for example, be selected from the group consisting of D121K,R↔K315D,E; K150E,D↔E385K,R; and K185D,E↔D360K,R.

In a further embodiment, in the binding molecules of the present invention at least some of the asymmetric interfaces between the IgM heavy chain constant regions of the two chains of a binding unit are created through knobs-into-holes connections, which may, for example, be created by mutations selected from the group consisting of knobs: T350→Y,F,W; and H395→Y,F; and holes: L352→ G,A,V,I,M,S,T; H395→A,V,I,L,M,F,Y; F393→W,Y; I397→ A,V,S,T; T350→S,A,V; and T348→S.

In a specific embodiment, in the binding molecules of the present invention the light chain variable region sequences, if present, are coupled to their matching heavy chain variable region by creating an asymmetric interface between the light and heavy chains.

In other embodiments, the asymmetric interface is created by CrossMab technique, knobs-into-holes coupling and/or salt bridges coupling.

The binding molecules of the present invention might comprise a common light chain and/or might be conjugated to a toxin or a chemotherapeutic agent. Preferably, conjugation is by fusion, but conjugation by a chemical linker is also included within the scope of the invention.

The binding molecules herein might be bispecific antibodies with a penta- or hexameric structure, which might be chimeric or humanized.

In a different aspect, the invention concerns a composition comprising at least about 70%, or at least 80%, or at least 90% or at least 95%, of at least 98%, or at last 99% of the binding molecule as hereinabove defined.

In a particular embodiment, the composition is a pharmaceutical composition.

The present invention further concerns a multi-specific binding molecule having a penta- or hexameric ring structure comprising five or six monospecific binding units, wherein (i) each of the monospecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of the monospecific binding units bind to different binding targets, and (iii) an external asymmetric interface is created between the heavy chain constant regions of the neighboring monospecific binding units that bind to different binding targets.

In one embodiment, at least three of the monospecific binding units bind to different binding targets.

In another embodiment, at least four of said monospecific binding units bind to different binding targets.

In yet another embodiment, the binding molecule has a pentameric ring structure and all five monospecific binding units bind to different targets.

In a further embodiment, the binding molecule has a hexameric ring structure and at least five of said monospecific binding units bind to different targets.

In a still further embodiment, all six of the monospecific binding units bind to different targets.

In another aspect, the invention concerns a multi-specific binding molecule having a penta- or hexameric ring structure comprising five or six bispecific binding units, where (i) each of the bispecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of the bispecific binding units bind to different binding targets, (iii) an internal asymmetric interface is created between two IgM heavy chain constant regions of each bispecific binding unit, and (iv) an external asymmetric interface is created between the heavy chain constant regions of the neighboring bispecific binding units binding to different targets.

In one embodiment, at least three of the bispecific binding units bind to different binding targets.

In another embodiment, at least four of the bispecific binding units bind to different binding targets.

In yet another embodiment, the binding molecule has a pentameric ring structure and all five bispecific binding units bind to different targets.

In a further embodiment, the binding molecule has a hexameric ring structure and at least five of the bispecific binding units bind to different targets.

In a still further embodiment, all six bispecific binding units bind to different targets.

In a different embodiment, the multi-specific binding molecule further comprises an IgM J chain.

In various embodiments, the multi-specific binding molecule may have a pentameric or hexameric ring structure.

Regardless of the number and nature of the binding specificities of the multi-specific binding molecules of the present invention, the following specific embodiments apply:

In one embodiment, in at least one of the binding units the IgM heavy chain constant regions further comprise a Cμ2 domain. In yet another embodiment, in all of the binding units the IgM heavy chain constant regions further comprise a Cμ2 domain. In various embodiments, the multi-specific binding molecules of the present invention may bind to binding targets selected from peptides, polypeptides, glycoproteins, nucleic acid molecules, and organic and non-organic small molecules.

In other embodiments, the multi-specific binding molecules of the present invention bind to binding targets selected from soluble polypeptides, cell surface receptors, ligands, molecular transporters, enzymes and substrates of enzymes.

In further embodiments, the multi-specific binding molecules of the present invention binding to different targets are selected from the group consisting of binding units binding to sites on the same soluble target; sites on the same cell surface receptor target; different soluble targets; different cell surface receptor targets; soluble and cell surface receptor targets; soluble or cell surface receptor and long residence time targets; soluble and matrix protein or substrate targets; soluble or receptor and molecular transporter targets, and different cell types.

In a particular embodiment, in the binding units within the binding molecules of the present invention the conjugation between the IgM heavy chain constant regions and the binding region to a binding target is by fusion. Thus, for example, the binding regions may be fused to the N-termini of the IgM heavy chain constant regions.

In one embodiment, at least one of the binding regions is a variable region of an antibody.

In another embodiment, all of the binding regions are antibody heavy chain variable regions.

In yet another embodiment, at least two binding targets are different antigens.

In a further embodiment, at least two binding targets are different epitopes on the same antigen.

In all aspects and embodiments, the antibody heavy chain variable regions may be from an IgG, IgA, IgE, or IgM antibody, preferably from an IgM antibody.

In a preferred embodiment, the multi-specific binding molecule of the present invention is a multi-specific IgM antibody.

In one embodiment, the multi-specific IgM antibody of the present invention further comprises at least one IgM light chain variable region sequence associated with an IgM heavy chain variable region in at least one of the binding units.

In another embodiment, the multi-specific IgM antibody further comprises an IgM light chain variable region sequence associated with each of the IgM heavy chain variable regions.

In all aspects and embodiments, the external asymmetric interface is created by alteration(s) within the Cμ3 domain. In one embodiment, the alteration is created by a salt bridge formed by pair-wise switches between oppositely charged amino acid residues in the Cμ3 domain.

In various embodiments, the salt bridge providing the external asymmetric interface is formed by at least one pair-wise charged amino acid residue switch in the Cμ3-Cμ3 domains, which may, for example be K238 ↔ D293 or K268 ↔ D294 in the neighboring μ chains.

In all aspects and embodiments, in the multi-specific binding molecules, e.g. multi-specific IgM antibodies, of the present invention the internal asymmetric interfaces are created by a salt bridge formed by pair-wise switches between oppositely charged amino acid residues in at least one of the Cμ2, Cμ3 and/or Cμ4 domains.

In one embodiment, a salt bridge is formed between at least one of the Cμ2-Cμ2, Cμ4-Cμ4, and Cμ2-Cμ3-Cμ4 domains of the two chains of said binding unit.

In another embodiment, the pair-wise switches are selected from the group consisting of E→K, K→E; R→E, E→R; D→K, K→D; and R→D, D→R.

In a further embodiment, the multi-specific binding molecule, e.g. multi-specific IgM antibody, comprises at least one pair-wise charged amino acid residue switch in the Cμ4-Cμ4 domains, which may, for example be selected from the group consisting of R328E,D↔E339R,K; R344E,D↔S330R,K; K376E,D↔E385R,K; R427E,D↔E339R,K; and T354E,D↔I397R,K.

In a still further embodiment, the multi-specific binding molecule, e.g. multi-specific IgM antibody, comprises at least one pair-wise charged amino acid switch between the Cμ2-Cμ2 domains, which may, for example, be selected from the group consisting of E167R,K↔K177E,D and K169E,D↔E170R,K.

In another embodiment, the multi-specific binding molecule, e.g. multi-specific IgM antibody, comprises at least one pair-wise charged amino acid residue switch in the Cμ2-Cμ3-Cμ4 domains, which may, for example, be selected from the group consisting of D121K,R↔K315D,E; K150E,D↔E385K,R; and K185D,E↔D360K,R.

In all aspects and embodiments, at least some of the external and/or internal asymmetric interfaces between the IgM heavy chain constant regions may be created through knobs-into-holes connections. For example, at least one knobs-into-hole connection may be created by mutations selected from the group consisting of knobs: T350→Y,F,W; and H395→Y,F; and holes: L352→ G,A,V,I,M,S,T; H395→A,V,I,L,M,F,Y; F393→W,Y; I397→ A,V,S,T; T350→S,A,V; and T348→S.

In the multi-specific IgM antibodies comprising a light chain variable region sequence, such light chain variable region sequences may be coupled to their matching heavy chain variable regions by creating an asymmetric interface between the light and heavy chains. In various embodiments, the asymmetric interface may be created by CrossMab technique, knobs-into-holes coupling and/or salt bridges coupling. In a further embodiment, the binding units of the multi-specific binding molecule comprise a common light chain.

In all aspects and embodiments, the multi-specific binding molecule may be conjugated to a toxin or a chemotherapeutic agent, where the conjugation may, for example, be by fusion and/or through a chemical linker.

The multi-specific IgM antibodies of the present invention may be chimeric or humanized.

In a further aspect, the invention concerns a composition comprising at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 98%,

or at least about 99% of a multi-specific binding molecule herein. The composition may, for example, be a pharmaceutical composition, comprising at least one pharmaceutically acceptable ingredient.

Brief Description of the Drawings

FIG. 1 illustrates the structure of an IgM pentamer, comprising a J chain, wherein chains A and B are identical in native IgM.

FIG. 2A illustrates a five-membered IgM molecule with two binding specificities, where the heavy (μ) chains designated as A and B are different.

FIG.2B illustrates a multi-specific IgM antibody comprising five or six monospecific binding units, where (i) each of the monospecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of the monospecific binding units bind to different binding target.

FIG. 2C illustrates a multi-specific IgM antibody comprising five or six bispecific binding units, where (i) each of the bispecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ4 domain conjugated to a binding region to a binding target, and (ii) at least two of the bispecific binding units bind to different binding targets.

FIG. 3 is a structural model of the A and B heavy chains of an IgM molecule as published in Czajkowsky D.M, Shao Z, PNAS 2009; 106:14960-14965.

FIG. 4A shows the alignment of the CG1, CE1 and CM1constant domains of human IgG1, IgE and IgM, respectively.

FIG. 4B shows the alignment of the CE2 and CM2 constant domains of human IgE and IgM, respectively.

FIG. 4C shows the alignment of the CG2, CE3 and CM3 constant domains of human IgG1, IgE and IgM, respectively.

Fig. 4D shows the alignment of the CG3, CE4 and CM4 constant domains of human IgG, IgE and IgM, respectively.

In FIGs 4A-4D:

human IgE sequence is from GenBank J00222.1; residue numbering from PDB 2WQR; helix (h) and sheet (s) assignments from PDB 2WQR;

human IgG1 sequence is from GenBank J00228.1; residue numbering from PDB 1OQO; helix (h) and sheet (s) assignments from PDB 1OQO;

human IgM sequence is from GenBank X14940.1; residue numbering is sequential from start of CM1 domain; reported variance in human IgM sequences noted below IgM sequence for GenBank CAB37838.1, CAC20458.1, AFM37312.1, X57331.1 and J00260.1

FIG. 5 shows the structure of hetero-monomers prepared in Example 1.

FIG. 6 shows a non-reduced SDS-PAGE gel of wild-type and engineered IgM Fc pairs 2a and 2b.

Lane 1: wild-type Rtx:Fc.

Lane 2: a mixture of Rtx2a:Fc2b, where Rtx2a is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human μ chain with C291S, T350Y, T354E, and I397E mutations and tail piece deletion; and Fc2b is human μ chain CH2 to CH4 and with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion.

Lane 3: a mixture of Rtx2b:Fc2a, where Rtx2b is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human mu chain with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion; and Fc2a consists of a human μ chain CH2 to CH4 region with C291S, T350Y, T354E, and I397E mutations and tail piece deletion. Arrow indicates heterodimer.

FIG. 7 shows a reduced SDS-PAGE gel of wild-type and engineered IgM Fc pairs 1a and 2b, where the designations are the same as in FIG. 6.

FIG. 8 shows a non-reduced SDS-PAGE gel of wild-type and engineered IgM Fc pairs:

Lane 1: wild-type Okt:Fc. Okt, composed of OKT3 (anti-CD3 antibody) scFv fused with CM2 to CM4 of human μ chain.

Lane 2: a mixture of Okt2a:Fc2b, where Okt2a is composed of OKT3 (anti-CD3 antibody) scFv fused with CM2 to CM4 of human μ chain with C291S, T350Y, T354E, and I397E mutations and tail piece deletion;

Lane 3: a mixture of Okt2b:Fc2a, where Okt2b is composed of OKT3 (anti-CD3 antibody) scFv fused with CM2 to CM4 of human μ chain with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion. Arrow indicates heterodimer.

Lanes 4-6: Wild-type Okt:Rtx combination; engineered Okt2a:Rtx2b combination; and Okt2b:Rtx2a combination, where Rtx2a is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human μ chain with C291S, T350Y, T354E, and I397E mutations and tail piece deletion, and Rtx2b is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human mu chain with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion. Arrow indicates the heterodimer.

FIG. 9 shows reduced samples on SDS-PAGE gel of 293F cell transfectants of the same constructs as shown in FIG. 8.

FIG. 10 illustrates how four salt bridges in the Cμ3 region stabilize two neighboring (A.B) μ chains around a disulfide bridge in a multi-specific binding molecule of the present invention.

Table A lists human IgM CM4 domain interface residues in knobs-holes positions and for potential charge introductions.

Table B lists human IgM CM4 domain interface residues for potential charge swaps.

Table C lists human IgM CM2 domain interface residues for potential charge introductions.

Table D lists human IgM CM2 domain interface residues in knobs-holes positions.

Table E lists human IgM CM2 domain interface residues for potential charge swaps.

Table F lists human IgM CM2, CM3 and CM4 domain interface residues for charge exchanges.

Detailed Description of the Invention

I. Definitions

The term "antibody" includes monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), single-chain molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv). The term "immunoglobulin" (Ig) is used

interchangeably with "antibody" herein. The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains.

In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (V_H) followed by three constant domains (C_H) for each of the α and γ chains and four C_H domains for μ and ε isotypes. Each L chain has at the N-terminus, a variable domain (V_L) followed by a constant domain at its other end. The V_L is aligned with the V_H and the C_L is aligned with the first constant domain of the heavy chain (C_{H1}). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a V_H and V_L together forms a single antigen-binding site.

IgM forms polymers where multiple immunoglobulins are covalently linked together with disufide bonds. IgM mostly exists as a pentamer but also as a hexamer and therefore contains 10 or 12 antigen binding sites. The pentameric form optionally contains an additional polypeptide, called the J chain, but can also be made in the absence of J chain. The pentameric IgM molecule has a molecular weight of approximately 970 kDa. Due to its polymeric nature, IgM possesses high avidity and is particularly effective in complement activation. Unlike in IgG, the heavy chain in IgM monomers is composed of one variable and four constant domains. The IgM constant domains are designated herein as CM1 or Cμ1, CM2 or Cμ2, CM3 or Cμ3, and CM4 or Cμ4, wherein the "CM" and Cμ" designations are used interchangeably.

IgA antibodies exist in a monomeric form but can also polymerize. In their secretory form IgA comprise from 2-5 of the basic 4-chain units linked by a J chain and a secretory component.

IgE exists in monomeric form, and has four constant domains, which are referred to as CE1, CE2, CE3 and CE4 in the present application.

The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Some types of antibodies can further be divided into various sub-classes: IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2.

For further details of the structure and properties of the different classes of antibodies, see e.g., Basic and Clinical Immunology, 8th Edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6.

Unless stated otherwise, the term "antibody" specifically includes native human and non-human IgG1, IgG2, IgG3, IgG4, IgE, IgA, IgD and IgM antibodies, including naturally occurring variants. Thus, for example, the human IgM sequence is available under GenBank Accession Number X14940.1, while variants have been reported as GenBank CAB37838.1, CAC20458.1, AFM37312.1, X57331.1, and J00260.1.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al. (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al. (1991) *Nature* 352:624-628 and Marks et al. (1991) *J. Mol. Biol.* 222:581-597, for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6851-6855).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are also replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al. (1986) *Nature* 321:522-525; Riechmann et al. (1988) *Nature* 332:323-329; and Presta (1992) *Curr. Op. Struct. Biol.* 2:593-596.

A "species-dependent antibody" is one which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a second mammalian species. Normally, the species-dependent antibody "binds specifically" to a human antigen (*i.e.* has a binding affinity (K_d) value of no more than about 1×10^{-7} M, preferably no more than about 1×10^{-8} M and most preferably no more than about 1×10^{-9} M) but has a binding affinity for a homologue of the antigen from a second nonhuman mammalian species which is at least about 50 fold, or at least about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be any of the various types of antibodies as defined above, but preferably is a humanized or human antibody.

As used herein, "antibody mutant" or "antibody variant" refers to an amino acid sequence variant of a reference antibody wherein one or more of the amino acid residues of the reference antibody have been modified. The reference antibody can, for example, be a native antibody but also a known variant of a native antibody. Such mutants necessarily have less than 100% sequence identity or similarity with the reference antibody. In a preferred embodiment, the antibody mutant will have an amino acid sequence having at least 75%

amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (*i.e* same residue) or similar (*i.e.* amino acid residue from the same group based on common side-chain properties) with the reference antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence outside of the variable domain shall be construed as affecting sequence identity or similarity.

An "isolated" bispecific or multi-specific binding molecule, such as bispecific or multi-specific antibody, herein is one which has been identified and separated and/or recovered from a component of its natural environment in a recombinant host cell. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the molecule, e.g. antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes, as well as undesired byproducts of the production, such as, for example, monospecific binding units (AA and/or BB in the case of a bispecific molecule comprising AB binding units), or molecules, with less than five bispecific binding units. In preferred embodiments, the bispecific binding molecule, such as antibody, will be purified (1) to greater than 95% by weight, or greater than 98% by weight, or greater than 99% by weight, as determined by SDS-PAGE or SEC-HPLC methods, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a amino acid sequencer, or (3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomassie blue or, preferably, silver stain. Ordinarily, an isolated multi-specific, e.g. bispecific binding molecule, e.g. antibody, will be prepared by at least one purification step.

The term "specific binding" or "specifically binds to" or is "specific for" refers to the binding of a binding molecule, such as an antibody, to a target molecule, e.g., a particular polypeptide or an epitope on a particular polypeptide, peptide, or other target (e.g. a glycoprotein target), and means binding that is measurably different from a non-specific interaction (e.g., a non-specific interaction may be binding to bovine serum albumin or casein). Specific binding can be measured, for example, by determining binding of antibody to a target molecule compared to binding of antibody to a control molecule. For example,

specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. The term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a Kd for the target of at least about 200 nM, alternatively at least about 150 nM, alternatively at least about 100 nM, alternatively at least about 60 nM, alternatively at least about 50 nM, alternatively at least about 40 nM, alternatively at least about 30 nM, alternatively at least about 20 nM, alternatively at least about 10 nM, alternatively at least about 8 nM, alternatively at least about 6 nM, alternatively at least about 4 nM, alternatively at least about 2 nM, alternatively at least about 1 nM, or greater. In certain instances, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

"Binding affinity" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). For example, the Kd can be about 200 nM, 150 nM, 100 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 8 nM, 6 nM, 4 nM, 2 nM, 1 nM, or stronger. Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art.

As used herein, the "Kd" or "Kd value" refers to a dissociation constant measured by a technique appropriate for the antibody and target pair, for example using surface plasmon resonance assays, for example, using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, N.J.) at 25°C. with immobilized antigen CM5 chips at about 10 response units (RU).

The term "bispecific binding unit" is used herein to refer to a molecule comprising a pair of IgM heavy chain constant region polypeptides each comprising at least a CM4 domain, and each conjugated to a binding region to a different binding target. Preferably, the

conjugation is by fusion, preferably to the N-terminus of the IgM heavy chain constant region polypeptide sequence. The term "bispecific binding unit" specifically encompasses, but is not limited to, a "bispecific IgM antibody binding unit," as hereinafter defined. The binding molecules of the present invention have a penta- or hexameric ring structure and comprise five or six bispecific binding units.

The terms "conjugate," "conjugated," and "conjugation" refer to any and all forms of covalent or non-covalent linkage, and include, without limitation, direct genetic or chemical fusion, coupling through a linker or a cross-linking agent, and non-covalent association.

The term "bispecific IgM antibody binding unit" is used in the broadest sense and specifically covers a pair of IgM antibody heavy chain constant region polypeptides, comprising at least a CM4 constant domain, fused to a variable domain sequence (V_H), each variable domain sequence binding to a different target, with or without associated antibody light chain variable domain (V_L) sequences. In one embodiment, the bispecific IgM antibody comprises two V_HV_L antigen binding regions, each capable of binding to a different epitope on one antigen or epitopes on two different antigens. The bispecific IgM antibody binding units can be full length from a single species, or be chimerized or humanized. The bispecific IgM antibodies of the present invention have a penta- or hexameric ring structure comprising five or six bispecific IgM binding units.

A "full length IgM antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody heavy chain variable domain (VH), an antibody constant heavy chain constant domain 1 (CM1 or Cμ1), an antibody heavy chain constant domain 2 (CM2 or Cμ2), an antibody heavy chain constant domain 3 (CM3 or Cμ3), and an antibody heavy chain constant domain 4 (CM4 or Cμ4). The bispecific full length IgM antibodies according to the invention comprise five or six monomers (binding units), each with two antigen binding sites, which specifically bind to two different binding targets (epitopes). The C-terminus of the heavy or light chain of the full length antibody denotes the last amino acid at the C-terminus of the heavy or light chain. The N-terminus of the heavy or light chain of the full length antibody denotes the first amino acid at the N-terminus of the heavy or light chain.

The term "valent" as used herein denotes the presence of a specified number of binding sites in an antibody. As such, the terms "bivalent", "tetravalent", and "hexavalent" denote the presence of two binding sites, four binding sites, and six binding sites, respectively. In the bispecific IgM antibodies according to the invention each binding unit is bivalent.

Accordingly, the bispecific IgM antibodies herein have 10 or 12 valencies. The definition similarly applies to binding molecules that are non-antibodies.

The term "epitope" includes any molecular determinant capable of specific binding to an antibody. In certain embodiments, epitope determinant include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, 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. A "binding region" is a region on a target bound by a binding molecule.

"Polyepitopic specificity" refers to the ability to specifically bind to two or more different epitopes on the same or different target(s). "Monospecific" refers to the ability to bind only one epitope. According to one embodiment the bispecific IgM antibody binds to each epitope with an affinity of at least 10^{-7}M, or 10^{-8} M or better.

The term "target" is used in the broadest sense and specifically includes polypeptides, nucleic acids, carbohydrates, lipids, and other molecules with biological function as they exist in nature. The "target" may, for example, be a cell, wherein the bispecific binding units target two different cell types, different subpopulations of the same cell type (e.g. different B-cell populations) or two different entities on a single cell.

An "antigen-binding site" or "antigen-binding region" of an antibody of the present invention typically contains six complementarity determining regions (CDRs) which contribute in varying degrees to the affinity of the binding site for antigen. There are three heavy chain variable domain CDRs (CDRH1, CDRH2 and CDRH3) and three light chain variable domain CDRs (CDRL1, CDRL2 and CDRL3). The extent of CDR and framework regions (FRs) is determined by comparison to a compiled database of amino acid sequences in which those regions have been defined according to variability among the sequences and/or structural information from antibody/antigen complexes. Also included within the scope of the invention are functional antigen binding sites comprised of fewer CDRs (i.e., where binding specificity is determined by three, four or five CDRs). Less than a complete set of 6 CDRs may be sufficient for binding to some binding targets. Thus, in some instances, the CDRs of a VH or a VL domain alone will be sufficient. Furthermore, certain antibodies might have non-CDR-associated binding sites for an antigen. Such binding sites are specifically included within the present definition.

The term "interface", as used herein, is used to refer to a region, which comprises those "contact" amino acid residues (or other non-amino acid groups such as, for example,

carbohydrate groups,) in a first IgM heavy chain constant region which interact with one or more "contact" amino acid residues (or other non-amino acid groups) in a second IgM heavy chain constant region.

The term "asymmetric interface" is used to refer to an interface (as hereinabove defined) formed between two antibody chains, such as a first and a second IgM heavy chain constant region and/or between an IgM heavy chain constant region and its matching light chain, wherein the contact residues in the first and the second chains are different by design, comprising complementary contact residues. The asymmetric interface can be created by knobs/holes interactions and/or salt bridges coupling (charge swaps) and/or other techniques known in the art, such as for example, by the CrossMab approach for coupling a μ heavy chain to its matching light chain.

A "cavity" or "hole" refers to at least one amino acid side chain which is recessed from the interface of the second polypeptide and therefore accommodates a corresponding protuberance ("knob") on the adjacent interface of the first polypeptide. The cavity (hole) may exist in the original interface or may be introduced synthetically (e.g. by altering nucleic acid encoding the interface). Normally, nucleic acid encoding the interface of the second polypeptide is altered to encode the cavity. To achieve this, the nucleic acid encoding at least one "original" amino acid residue in the interface of the second polypeptide is replaced with DNA encoding at least one "import" amino acid residue which has a smaller side chain volume than the original amino acid residue. It will be appreciated that there can be more than one original and corresponding import residue. The upper limit for the number of original residues which are replaced is the total number of residues in the interface of the second polypeptide. The preferred import residues for the formation of a cavity are usually naturally occurring amino acid residues and are preferably selected from alanine (A), serine (S), threonine (T), valine (V) and glycine (G). Most preferred amino acid residues are serine, alanine or threonine, most preferably alanine. In the preferred embodiment, the original residue for the formation of the protuberance has a large side chain volume, such as tyrosine (Y), arginine (R), phenylalanine (F) or tryptophan (W).

An "original" amino acid residue is one which is replaced by an "import" residue which can have a smaller or larger side chain volume than the original residue. The import amino acid residue can be a naturally occurring or non-naturally occurring amino acid residue, but preferably is the former.

By "non-naturally occurring" amino acid residue is meant a residue which is not encoded by the genetic code, but which is able to covalently bind adjacent amino acid residue(s) in the polypeptide chain. Examples of non-naturally occurring amino acid residues are norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman et al., Meth. Enzym. 202:301-336 (1991), for example. To generate such non-naturally occurring amino acid residues, the procedures of Noren et al. Science 244: 182 (1989) and Ellman et al., supra can be used. Briefly, this involves chemically activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by in vitro transcription and translation of the RNA. The methods of the current invention, in certain embodiments, involve replacing at least one original amino acid residue in an IgM heavy chain, but more than one original residue can be replaced. Normally, no more than the total residues in the interface of the first or second polypeptide will comprise original amino acid residues which are replaced. The preferred original residues for replacement are "buried". By "buried" is meant that the residue is essentially inaccessible to solvent. The preferred import residue is not cysteine to prevent possible oxidation or mispairing of disulfide bonds.

The protuberance is "positionable" in the cavity which means that the spatial location of the protuberance and cavity on the interface of the first polypeptide and second polypeptide respectively and the sizes of the protuberance and cavity are such that the protuberance can be located in the cavity without significantly perturbing the normal association of the first and second polypeptides at the interface. Since protuberances such as Tyr, Phe and Trp do not typically extend perpendicularly from the axis of the interface and have preferred conformations, the alignment of a protuberance with a corresponding cavity relies on modeling the protuberance/cavity pair based upon a three-dimensional structure such as that obtained by X-ray crystallography or nuclear magnetic resonance (NMR). This can be achieved using widely accepted techniques in the art, including techniques of molecular modeling.

By "original nucleic acid" is meant the nucleic acid encoding a polypeptide of interest which can be "altered" (i.e. genetically engineered or mutated) to encode a protuberance or cavity. The original or starting nucleic acid may be a naturally occurring nucleic acid or may comprise a nucleic acid which has been subjected to prior alteration (e.g. a humanized antibody fragment). By "altering" the nucleic acid is meant that the original nucleic acid is mutated by inserting, deleting or replacing at least one codon encoding an amino acid residue

of interest. Normally, a codon encoding an original residue is replaced by a codon encoding an import residue. Techniques for genetically modifying a DNA in this manner have been reviewed in Mutagenesis: a Practical Approach, M. J. McPherson, Ed., (IRL Press, Oxford, UK. (1991), and include site-directed mutagenesis, cassette mutagenesis and polymerase chain reaction (PCR) mutagenesis, for example.

The protuberance or cavity can be "introduced" into the interface of the first or second polypeptide by synthetic means, e.g. by recombinant techniques, in vitro peptide synthesis, those techniques for introducing non-naturally occurring amino acid residues previously described, by enzymatic or chemical coupling of peptides or some combination of these techniques. According, the protuberance or cavity which is "introduced" is "non-naturally occurring" or "non-native", which means that it does not exist in nature or in the original polypeptide (e.g. a humanized monoclonal antibody).

Preferably the import amino acid residue for forming the protuberance has a relatively small number of "rotamers" (e.g. about 3-6). A "rotamer" is an energetically favorable conformation of an amino acid side chain. The number of rotamers for the various amino acid residues are reviewed in Ponders and Richards, J. Mol. Biol. 193: 775-791 (1987).

The term "host cell" as used in the current application denotes any kind of cellular system which can be engineered to generate the antibodies according to the current invention. In one embodiment Chinese hamster ovary (CHO) cells are used as host cells.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

A nucleic acid is "operably linked" when it is placed in a functional relationship with another nucleic acid sequence. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case

of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Detailed Description

IgM is the first immunoglobulin produced by B cells in response to stimulation by antigen, and is present at around 1.5 mg/ml in serum with a half-life of 5 days. IgM is a pentameric or hexameric molecule. Just as IgG, IgM monomers consist of two light and two heavy chains. However, while IgG contains three heavy chain constant domains (CH1, CH2 and CH3), the heavy (μ) chain of IgM additionally contains a fourth constant domain (CH4), similarly to the ε heavy chains in IgE. This extra constant domain is located in place of the IgG and IgA proline-rich hinge region that is responsible for the rotational flexibility of the antigen-binding Fab domains relative to the Fc domain of IgG and IgA antibodies.

Five IgM monomers form a complex with an additional small polypeptide chain (the J chain) to form a native IgM molecule. The J chain is considered to facilitate polymerization of μ chains before IgM is secreted from antibody-producing cells. While crystallization of IgM has proved to be notoriously challenging, Czajkowsky and Shao (PNAS 106(35):14960-14965, 2009) recently published a homology-based structural model of IgM, based on the structure of the IgE Fc domain and the known disulfide pairings. The authors report that the human IgM pentamer is a mushroom-shaped molecule with a flexural bias.

In a natural penta- or hexameric IgM antibody molecule all heavy (μ) chains are identical and the light chains are identical as well. The present invention allows the production of IgM molecules in which two μ chains are different from each other.

In one aspect, the present invention concerns bispecific binding molecules with binding specificities to two different binding regions, having a penta- or hexameric structure, formed by five or six bispecific binding units, wherein each of such bispecific binding units has the same two binding specificities and comprises a first chain comprising at least a CM4 domain of an IgM heavy chain constant region conjugated to a first binding region to a first binding target, and a second chain comprising at least a CM4 domain of an IgM heavy chain constant region and a second binding region to a second binding target, wherein the first and second binding targets are different, and wherein the first and second chains are assembled to create a bispecific binding unit as a result of an asymmetric interface created between their respective IgM heavy chain constant regions.

In various embodiments, the IgM heavy chain constant regions additionally comprise one or both of the CM2 and CM3 domains or fragments thereof, and potentially other IgM heavy chain constant domain sequences. In one embodiment, the binding molecules of the present invention contain a complete IgM heavy (μ) chain constant domain, with one or more modifications to create an asymmetric interface between two heavy chains.

In order to generate an IgM molecule with two different μ heavy chains (chains A and B), a solution must be found for coupling the two matching μ heavy chains (A and B) with two different binding specificities to each other. In addition, if a light chain is needed to form a binding region, a solution must be found to couple each heavy chain with its matching light chain to provide the desired binding specificity.

The coupling can be achieved by salt bridge pairs charge switching (also referred to as charge swaps or charge reversals) between certain residues and/or by creating knobs-holes interactions between the two chains. The heavy chains can also be paired with their matching light chains by using the CrossMab technique. The different approaches can also be combined in order to achieve an optimal result.

In another aspect, the present invention concerns multi-specific binding molecules with binding specificities to two or more different binding targets, having a penta- or hexameric structure. The invention includes binding molecules comprising five or six monospecific binding units, where (i) each of the monospecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of the monospecific binding units bind to different binding target. The invention further includes binding molecules comprising five or six bispecific binding units, where (i) each of the bispecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, and (ii) at least two of the bispecific binding units bind to different binding targets. In a particular embodiment, the binding molecules are multi-specific IgM antibodies.

In various embodiments, the IgM heavy chain constant regions additionally comprise a Cμ2 domain or a fragment thereof, and potentially other IgM heavy chain constant domain sequences. In one embodiment, the binding molecules of the present invention contain a complete IgM heavy (μ) chain constant domain, with one or more modifications to create an asymmetric interface between two heavy chains.

In the multi-specific binding molecules of the present invention which contain at least one bispecific binding unit, in order to generate an IgM molecule with two different μ heavy chains (chains A and B), a solution must be found for coupling the two matching μ heavy chains (A and B) with two different binding specificities to each other via an internal asymmetric interface. In addition, if a light chain is needed to form a binding region, a solution must be found to couple each heavy chain with its matching light chain to provide the desired binding specificity.

In addition, a solution must be found to create an external asymmetric interface between the heavy chain constant regions of the neighboring monospecific binding units that bind to different binding targets.

Techniques for creating internal and external asymmetric interfaces include, without limitation, salt bridge pairs charge switching (also referred to as charge swaps or charge reversals) between certain residues and creation of knobs-holes interactions between two chains. The heavy chains can also be paired with their matching light chains by using the CrossMab technique. The different approaches can also be combined in order to achieve an optimal result.

1. Knobs-into-Holes Technique

To improve the yields of the penta- or hexameric bispecific or multi-specific binding molecules of the present invention, the IgM heavy chain constant regions, e.g. the CM4, CM2 and/or CM3 domains, can be altered by the "knob-into-holes" technique which is described in detail with several examples in e.g. WO 96/027011, Ridgway, J., B., et al., Protein Eng 9 (1996) 617-621; and Merchant, A. M., et al., Nat Biotechnol 16 (1998) 677-681. In this method the interaction surfaces of two IgM heavy chain constant domains are altered to increase the heterodimerization of two heavy chains with different binding specificities and/or between a heavy chain and its matching light chain. Each of the two heavy chain domains, e.g. CM4-CM4, CM2-CM2 and/or CM2-CM3-CM4/CM2-CM3-CM4 can be the "knob", while the other is the "hole". The introduction of a disulfide bridge stabilizes the heterodimers (Merchant, A. M., et al., Nature Biotech 16 (1998) 677-681; Atwell, S., et al., J. Mol. Biol. 270 (1997) 26-35) and increases the yield. Similarly, the matching heavy and light chains can be coupled to each other by this technique Zhu, Z.; Presta, L.G.; Zapata, G.; Carter, P. *Remodeling domain interfaces to enhance heterodimer formation.* Prot. Sci. 6:781-788 (1997).

Following this approach, in the case of bispecific IgM binding molecules within the original interface of the CH4, CH2 and/or CH3 domains of one heavy chain that meets the original interface of the corresponding domain of the other heavy chain within the bispecific IgM binding molecule (e.g. antibody), an amino acid residue may be replaced with an amino acid residue having a larger side chain volume, thereby creating a protuberance within the interface, which is positionable in a cavity within the interface of the corresponding domain in the other IgM heaving chain constant region. Similarly, the second IgM heavy chain may be altered, by replacing an amino acid residue within the interface with a corresponding domain in the constant region of the first IgM heavy chain, with an amino acid residue having a smaller side chain volume, thereby creating a hole (cavity) within the interface between the two heavy chain regions.

Human IgM CM4 and CM2 domain interface residues in knobs-holes positions are shown in Tables A and D. The Tables identify the native residue at the indicated positions of the CM4 sequence shown in FIG. 4D and the CM2 sequence shown in FIG. 4B, respectively, following the numbering shown in those Figures, as well as the potential mutations that can be used to create knobs-holes pairs. Thus, for example, in the CM4 domain the native threonine (T) residue in position 350 may be mutated into tyrosine (Y) to create a knob, which can be combined with any combinations of the potential mutations listed for residues 352, 393 and 395 of the native CM4 sequence (Set #1). Additional mutations at positions 254 and 397, that can be optionally combined with Set #1 are shown in Set #2 and Set #3). Similarly, Set #4 exemplifies knobs mutations at positions 350 and 395 in combination with hole mutations at one or more of positions 352, 393, 395, and 397. Additional mutations for combination with Set#4 are listed in Set #5 and Set #6. The rest of Table A can be read in a similar way. Some of the sets also include charge introductions, i.e. changes from a non-charged residue to a charged residue (similarly to Table C discussed below).

It is emphasized that the listed knobs-holes mutations in Sets #1-30 can be used in various combinations as set forth in Table A. Furthermore, the listed mutations can be combined with other knobs-holes and/or charge swap and/or charge introduction mutations listed in the rest of the Tables. Thus, one or more of the knobs-holes mutations set forth in Table A can be combined with one or more of the knobs-holes mutations shown in Table D, in any combination and/or with one or more of the charge swap/charge introduction mutations listed in Tables B, C, E and F, as discussed hereinbelow. Thus, one can select any set from

Table A and mix it with any set from Table B, mixed with any set from Table C, etc., in any order or combination.

2. Salt bridge pairs charge switching (charge swapping)

Opposite charges attract and similar charges repel each other. The charge of an amino acid molecule is pH dependent and can be characterized by the pK values, which are determined for the alpha amino group (N), the alpha carboxy group (C) and the side chain for free amino acids. The local environment can alter the pK_a of a side chain when the amino acid is part of a protein or peptide.

The charge properties of an amino acid molecule can also be characterized by the isoelectric point (pI), which is the pH at which the overall charge of the molecule is neutral. Since amino acids differ from each other in their side chains, the pI reflects differences in the pKs of the side chains.

Most amino acids (15/20) have a pI close to 6 so they are regarded as having neutral overall charge. Asp and Glu are negatively charged, and His, Lys, Arg are positively charged.

In the interface between two binding units in the mushroom-shaped IgM complex there are four salt bridges, above and below the disulfide bridge connecting the monomers. The residues involved in these interactions (Lys-238, Lys-268, Asp-293 and Asp294) are the same in the two monomers, but their relative disposition in this interface is different, due to the asymmetry of the CM3 domains in the IgM Fc structure.

Positions and amino acid residues for charge swapping or charge introduction mutations are listed in Tables A, B, D, E, and F. As discussed above, or more of these mutations, or sets of mutations, can be combined with one or more sets of knobs-holes mutations to provide a desired asymmetric interface between two different IgM heavy chains and/or between an IgM heavy chain and its matching light chain.

Preferably, the asymmetric interface between two different IgM heavy chain constant regions is created by up to 8, such as, for example, 1-8, or 1-7, or 1-6, or 1-5, or 1-4, or 1-3, or 1-2 mutations in one IgM heavy chain, or 2-10, or 2-9, or 2-8, or 2-7, or 2-6, or 2-5, or 2-4, or 2-3 combined mutations in the two IgM heavy chains.

For multi-specific binding molecules herein, the external asymmetric interface is created by an alteration in the Cμ3 domain. In particular, to create an external asymmetric interface, a salt bridge is formed by pair-wise switches between oppositely charged amino acid residues in the Cμ3 domain. In various embodiments, the salt bridge providing the external

asymmetric interface is formed by at least one pair-wise charged amino acid residue switch in the Cμ3-Cμ3 domains, which may, for example be K238 ↔ D293 or K268 ↔ D294 in the neighboring μ chains.

3. CrossMab technique

As discussed above, the knobs-into-holes technology or charge swapping enables heterodimerization of the antibody heavy chains. Correct association of the light chains and their cognate heavy chains can be achieved by exchange of heavy-chain and light-chain domains within the antigen binding fragment (Fab) of one half of the bispecific antibody binding unit. Crossover can occur as a crossover of the complete VH-CM and VL-CL domains, crossover of only the VH and VL domains. or the CM and CL domains within the one half of the bispecific binding unit of an IgM antibody. This "crossover" retains the antigen-binding affinity but makes the two arms so different that light-chain mispairing can no longer occur. For further details, in the context of IgG antibodies, see, for example, Schaeffer et al., (2011) *Proc Natl Acad Sci USA* 108(27): 11187-11192.

4. Production of bispecific and multi-specific IgM binding molecules

The coding sequences of the heavy chains of the bispecific IgM antibody binding units, with the desired mutations (following the knobs-into-holes, charge swap and/or Cross-Mab technique) may be produced by introducing appropriate nucleotide changes into the antibody DNA, or by nucleotide synthesis. The antibodies can then be produced by recombinant means.

Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity. For the expression of the antibodies in a host cell, nucleic acids encoding the respective modified heavy chains, and optionally light chains, are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6 cells, yeast, or *E. coli* cells, and the antibody is recovered from the cells (supernatant or cells after lysis). General methods for recombinant production of antibodies are described, for example, in the review articles of Makrides, S. C., *Protein Expr. Purif.* 17 (1999) 183-202; Geisse, S., et al., *Protein Expr. Purif.* 8 (1996) 271-282; Kaufman, R. *J., Mol. Biotechnol.* 16 (2000) 151-161; Werner, R. G., *Drug Res.* 48 (1998) 870-880.

The bispecific and multi-specific antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-SEPHAROSE®, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Although due to its complex structure, the large scale production of recombinant IgM has been difficult, several recombinant production systems for IgM using non-lymphoid cells have been reported, including co-expression of the IgM heavy (H) and light (L) chains in C6 glioma cells, CHO cells, and HeLa cells. While the co-expression successfully resulted in the formation of polymer, the yields were typically low (see, e.g.W089/01975 and Wood et al., J. Immunol. 145, 3011-3016 (1990) for expression in CHO cells), and the exact polymeric structure of the penta- or hexameric molecules could not be readily determined. Production of IgM in an immortalized human retina cell line expressing E1A and E1B proteins of an adenovirus is described in U. S. Application Publication No. 20060063234. Further details of the production of the bispecific IgM antibodies of the present invention are provided in the Example below.

The methods of the present invention will result in a composition comprising a bispecific or multi-specific IgM binding molecule, such as a bispecific or multi-specific IgM antibody, as the main component, in combination with various by-products of the manufacturing process, such as monospecific antibodies, antibody fragments, monomers, dimers, trimers, and/or tetramers of the bispecific binding unit, instead of the desired pentameric or hexameric structure. The compositions produced will generally contain at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 92%, or at least about 95%, of the desired penta- or hexameric bispecific binding molecule, e.g. antibody, which will be further purified by methods known in the art to yield a product with a purity of at least about 90%, or at least about 95%, or at least about 98%, or at least about 99%, or at least about 99.5%, or at least about 99.9%.

5. Applications of the bispecific and multi-specific IgM binding molecules

The bispecific and multi-specific IgM binding molecules, e.g. antibodies, of the present invention have widespread therapeutic and diagnostic applications.

In one embodiment, the bispecific binding molecules herein bind to two sites on the same soluble target, such as, for example, VEGF, TNFα, or IL6. The purpose may, for example, be antagonizing multiple sites on the protein and/or increasing the avidity to a given target.

In another embodiment, the bispecific or multi-specific binding molecules herein bind two or more sites on the same cell surface (receptor) target, such as EGFR or HER2 (ErbB2). Thus, for example, a bispecific or multi-specific binding molecule might target both the 4D5 and the 2C4 epitopes on a HER2 molecule. This approach may increase bio-potency and/or avidity to a given target.

In yet another embodiment, the bispecific or multi-specific binding molecules of the present invention bind two or more different soluble targets (globular proteins or peptides), e.g. TNFα and IL6, VEGFα and Ang2, or two cytokines. This approach might result in more complete blocking a specific pathway; blocking of the so called "cytokine storm," or coordinate an enzyme and its substrate, e.g. Factor IXa and Factor X.

In a further embodiment, the bispecific or multi-specific binding molecules herein may bind a soluble target and a cell surface receptor target, such as an angiogenic factor and neo-vascular specific receptor. The purpose of this approach may also be increased delivery and blockade at specific sites or tissues.

In a still further embodiment, the bispecific binding molecules herein are designed to bind two different cell surface receptor targets, such as, for example, HER2 (ErbB2) and HER3 (ErbB3). Similarly, the multi-specific binding molecules herein can be designed to bind two or more different cell surface receptor targets, such as, for example, HER1, HER2 (ErbB2) and HER3 (ErbB3). This may result in enhancing specificity and selectivity and/or in more complete blocking of a given pathway.

Bispecific and multi-specific binding molecules, such as antibodies, of the present invention may also be designed to bind one soluble target or cell surface receptor target and a long residence time target, such as, for example, TNFα and serum albumin, or VEGF and serum albumin. These molecules are expected to have longer circulating half-life than binding molecules without the albumin specificity.

In a further embodiment, the bispecific binding molecules herein may bind one soluble target and a matrix protein or a substrate, such as, for example, VEGFα and hyaluronic acid. Similarly, the multi-specific binding molecules herein may bind one or more soluble targets and one or more matrix proteins and/or substrates, such as, for example, VEGFα and hyaluronic acid. The resultant bi- or multi-specific binding molecules may find utility, for example, in anti-angiogenic therapy of ocular conditions, such as age-related macular degeneration (AMD), due to their increased residence time in the intraocular space.

Bispecific molecules, e.g. antibodies binding one soluble or receptor target, plus a transporter receptor (ie transferrin receptor), e.g. anti-EGFRvIII (mutant form with exon III deleted) found glioblastoma combined with anti-transferrin specificity, can find utility in antibody delivery across blood brain barrier.

Similarly, multi-specific molecules, e.g. antibodies binding one or more soluble or receptor targets, plus one or more transporter receptors (ie transferrin receptor), e.g. anti-EGFRvIII (mutant form with exon III deleted) found glioblastoma combined with anti-transferrin specificity, can find utility in antibody delivery across blood brain barrier.

6. Compositions, pharmaceutical compositions, and methods of treatment

In one aspect, the invention concerns compositions comprising purified bispecific or multi-specific IgM binding molecules, such as bispecific or multi-specific IgM antibodies herein. The compositions generally will contain at least about 80%, or at least about 85%, or at least about 90%, or at least about 92%, or at least about 95%, or at least about 98%, or at least about 99% of the desired bispecific or multi-specific IgM binding molecule, e.g. antibody. The composition may be a pharmaceutical composition, where the bispecific or multi-specific binding molecule, e.g. antibody, is in admixture with at least one pharmaceutically acceptable carrier.

A pharmaceutical composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the target disease or condition and the desired results. To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and/or dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like

into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier preferably is an isotonic buffered saline solution.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

All patent and scientific references cited throughout this disclosure are hereby expressly incorporated by reference herein.

Example 1

1. Generation of DNA constructs with designed mutations

Materials and Methods

a. DNA construct synthesis

All DNA constructs with designed mutations were synthesized by commercial vendors (Genewiz, Inc.), with compatible restriction sites at both ends for subcloning into respective expression vectors, using methods well known in the art.

b. Construction of expression vectors

The synthesized DNA constructs were re-suspended in Tris-EDTA buffer at 1 μg/ml. DNA (1 μg) was subjected to enzyme digestion and the synthesized gene was separated from the carrier plasmid DNA by electrophoresis. The digested DNA was ligated to pre-digested plasmid DNA (pFUSEss-CHIg-hM*03 for μ chain; pFUSE2ss-CLIg-hk for kappa chain, InvivoGen) by standard molecular biology techniques. The ligated DNA was transformed into competent bacteria and plated on LB plates with multiple selective antibiotics. Several bacterial colonies were picked and DNA preparations were made by standard molecular biology techniques. The prepared DNA was verified by sequencing. Only the bacterial clones with 100% match of DNA sequence with the designed DNA sequence were used for plasmid DNA preparation and subsequently for cell transfection.

c. μ Chains of different size

In order to demonstrate that two different μ chains with or without CM4 interaction interface mutation (A and B) were able to couple together, two sets of different sized μ chains were constructed with distinct molecular weights and ligand specificities.

i. The **Rtx** chain is composed of a μ chain for the chimeric anti-CD20 antibody Rituxan (Rituximab) Vh region fused with the CM1 region of human IgM antibody μ chain with a C291S mutation and tail piece deletion:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE
WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVY
YCARSTYYGGDWYFNVWGAGTTVTVSSGSASAPTLFPLVSCENSPSDT
SSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQ
VLLPSKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVP
PRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQA
EAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMC
VPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQNG
EAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTCTVTHTDLPSPL
KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFV
QWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETY
TCVVAHEALPNRVTERTVD

(SEQ ID NO: 1)

The **Rtx** chain has a calculated molecular weight about 60kD (without glycosylation) and 66kD (with 4 N-glycosylation sites) and is able to bind to CD20 positive B cells, such as Raji cells.

ii. The **Fc** chain comprises human IgM μ chain CM2 to CM4 regions, carrying a cMyc tag and having its tail piece replaced by 6His tag and having a C291S mutation:

GSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVG
SGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGL
TFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS
VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTC
TVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLV
TGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVS
EEEWNTGETYTCVVAHEALPNRVTERTVDKSTGKGGGSEQKLISEEDL
NSAVDHHHHHH

(SEQ ID NO: 2)

The **Fc** chain has a molecular weight about 39kD (without glycosylation) and 43kD (with 3 N-glycosylation sites) and is able to bind to anti-myc monoclonal antibody 9E4 or other anti-myc antibodies.

iii. The **Okt** chain is composed of a single chain Fv version of OKT3 (anti-CD3) fused with CM2 of human mu chain with C291S and tail piece deletion:

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLE
WIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVY
YCARYYDDHYSLDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQS
PAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKL
EIKGSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGK
QVGSGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDH
RGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTT
YDSVTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGER
FTCTVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATIT
CLVTGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSIL
TVSEEEWNTGETYTCVVAHEALPNRVTERTVD

(SEQ ID NO: 3)

The **Okt** chain has a calculated molecular weight about 61kD without glycosylation and 67kD including 4 N-glycosylation sites, and is able to bind to CD3 positive T cells.

d. Light chain coupling

i. Native chimeric Rituxan kappa (κ) chain

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYAT
SNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGG
GTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT
HQGLSSPVTKSFNRGEC

(SEQ ID NO: 4)

The kappa chain has a calculated molecular weight about 23kD and is able to link to Rituxan IgM heavy chain.

e. Interface mutations

Knobs and holes, electrostatic charge coupling were asymmetrically introduced into the CM3 interaction interface to maximize hetero-dimerization of two μ chains. Two pairs of CM3 interaction interface mutants were generated.

i. **Fc1a** is a human μ chain CH2 to CH4 region with C291S and T350Y mutations and tail piece deletion:

GSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVG
SGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGL
TFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS
VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTC
TVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATIXCLV
TGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVS
EEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 5)

The **Fc1a** chain has a calculated molecular weight about 36kD without glycosylation and 41kD if 3 N-glycosylation sites are included.

ii. **Fc1b** is human μ chain CH2 to CH4 and with C291S, L352S and H395V mutations and tail piece deletion

GSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVG
SGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGL
TFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS
VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTC

TVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCSV
TGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAVSILTVS
EEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 6)

The **Fc1b** chain has a calculated molecular weight about 36kD without glycosylation and 41kD including 3 N-glycosylation sites.

iii. **Fc2a** consists of a human μ chain CH2 to CH4 region with C291S, T350Y, T354E, and I397E mutations and tail piece deletion.

GSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVG
SGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGL
TFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS
VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTC
TVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATIYCLV
EGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSELTVS
EEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 7)

The **Fc2a** chain has a calculated molecular weight of about 36kD without glycosylation and 41kD including 3 N-glycosylation sites.

iv. **Fc2b** is human μ chain CH2 to CH4 and with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion.

GSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVG
SGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGL
TFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS
VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTC
TVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCSV
KGFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAVSKLTVS
EEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 8)

The **Fc2b** chain has a calculated molecular weight of about 36kD without glycosylation and 41kD including 3 N-glycosylation sites.

f. Interface mutations

Fc2a chain and **Fc2b** chain with knobs, holes, and electrostatic charge coupling were further linked to both Rituxan and the OKT3 (anti-CD3 antibody) scFv by molecular cloning for asymmetrically hetero-dimerization of two μ chains.

i. **Rtx2a** is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human μ chain with C291S, T350Y, T354E, and I397E mutations and tail piece deletion.

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE
WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVY
YCARSTYYGGDWYFNVWGAGTTVTVSSGSASAPTLFPLVSCENSPSDT
SSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQ
VLLPSKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVP
PRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQA
EAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMC
VPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQNG
EAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTCTVTHTDLPSPL
KQTISRPKGVALHRPDVYLLPPAREQLNLRESATIYCLVEGFSPADVFV
QWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSELTVSEEEWNTGET
YTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 9)

The **Rtx2a** chain has a calculated molecular weight of about 61kD without glycosylation and 67kD with 4 N-glycosylation sites.

ii. **Rtx2b** is composed of a μ chain for chimeric Rituxan (anti-CD20) Vh region fused with CM1 to CM4 of human mu chain with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion.

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE
WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVY
YCARSTYYGGDWYFNVWGAGTTVTVSSGSASAPTLFPLVSCENSPSDT
SSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQ
VLLPSKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVP
PRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQA
EAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMC
VPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQNG
EAVKTHTNISESHPNATFSAVGEASISEDDWNSGERFTCTVTHTDLPSPL
KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCSVKGFSPADVFV
QWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAVSKLTVSEEEWNTGET
YTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 10)

The **Rtx2b** chain has a calculated molecular weight of about 61kD without glycosylation and 67kD including 4 N-glycosylation sites.

iii. **Okt2a** is composed of OKT3 (anti-CD3 antibody) scFv fused with CM2 to CM4 of human μ chain with C291S, T350Y, T354E, and I397E mutations and tail piece deletion.

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLE
WIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVY
YCARYYDDHYSLDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQS
PAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKL
EIKGSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGK
QVGSGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDH
RGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTT
YDSVTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGER
FTCTVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATI**Y**
CLV***E***GFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHS***E***
LTVSEEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 11)

The **Okt2a** chain has a calculated molecular weight about 62kD without glycosylation and 68kD including 4 N-glycosylation sites.

iv. **Okt2b** is composed of OKT3 (anti-CD3 antibody) scFv fused with CM2 to CM4 of human μ chain with C291S, L352S, T354K, H395V, and I397K mutations and tail piece deletion.

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLE
WIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVY
YCARYYDDHYSLDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQS
PAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKL
EIKGSGSKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGK
QVGSGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDH
RGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTT
YDSVTISWTRQNGEAVKTHTNISESHPNATFSAVGEASISEDDWNSGER
FTCTVTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATIT
C**S**V***K***GFSPADVFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFA**V**S***K***L
TVSEEEWNTGETYTCVVAHEALPNRVTERTVDKSTGK

(SEQ ID NO: 12)

The **Okt2b** chain has a calculated molecular weight about 62kD without glycosylation and 68kD including 4 N-glycosylation sites.

2. Protein expression, purification and characterization

a. Transfection

IgM was made by co-transfection of several different expression vectors at equal molar ratios into mammalian cells, such as 293F cells (Invitrogen). 10μg of mixed DNA for expression vectors were mixed with 20 μl of 293Fectin (Invitrogen) for 30 minutes at room temperature in 2ml of Opti-MEM (Invitrogen) and then added to 10^7 293F cells. Transfections with 293F cells were incubated for 72 hours post-transfection before harvesting the supernatant.

b. Protein purification by immunoprecipitation.

i. Capture Select IgM (Catalog 2890.05 , BAC, Thermo Fisher)

Transfected supernatant were harvested by centrifugation at 2,000G for 10 minutes. IgM proteins were purified by immunoprecipitation with affinity Capture Select IgM affinity matrix. 100μl of Capture Select IgM slurry were added to 15ml of harvested supernatant. The supernatant and affinity matrix mixtures were incubated at room temperature for 2 hours on a rocker. The affinity matrices were then centrifuged at 300g for 2 minutes, decanting the solution. The affinity matrixes were further washed with PBS plus 0.05% Tween for 3 times. Finally, the purified IgM proteins were washed off from affinity matrices by incubating 20μl of 4x LSD sample loading buffer (Invitrogen) at room temperature for 5 minutes, followed by centrifuging at 10,000g. The affinity matrixes were further washed with 60μl of PBS and the supernatant were pooled for analysis by gel electrophoresis.

c. Gel electrophoresis

i. Non-reducing SDS PAGE

Non-reducing SDS PAGE was used to separate various mutant IgM proteins of different molecular weights. Novex 4-12% Bis-Tris Gel (Life Technologies) was used with Novex MES SDS Running Buffer (Life Technologies).

ii. Reducing SDS-PAGE

NuPage LDS sample buffer (Life Technologies) and NuPage reducing agent dithiothreitol (Life Technologies) were added to IgM protein samples and heated to 80°C for 10 minutes before loading on NuPage Novex 4-12% Bis-Tris Gel (Life Technologies, cat# NP0322). NuPage MES SDS Running Buffer (Life Technologies, cat# NP0002) was used for gel electrophoresis. After electrophoresis is complete, the gel was removed from the apparatus and stained using Colloidal Blue Staining (Life Technologies, manual #LC6025.

iii. Gel band quantitation

Protein gels are dried, then digitized using image scanner. The gel images are processed with Image J program and the amount of protein in a specific band is determined using the gel quantitation function.

iv. Analysis of SDS-PAGE gels

Rtx:Fc including wild-type and engineered IgM Fc pair 2a and 2b SDS-PAGE gels. Lanes 1, 2, and 3 on the non-reduced SDS-PGE gel (FIG. 6) show an upper band for homodimeric Rtx (H2L2, expected MW 168-180 kDa) and a lower band for half-antibody (HL, expected MW 84-90 kDa) for Rtx2a alone, Rtx2b alone and wild-type Rtx. A band for unassociated Fc (expected MW 36-41 kDA) is present in all three lanes; associated Fc (expected MW 72-82 kDa) may also be a component of the 80-90 kDa band. Lane 2 shows the mixture of Rtx2a:Fc2b and lane 3 shows the mixture of Rtx2b:Fc2a. In both lanes heterodimer (expected MW 120-131 kDa) is indicated with an arrow. The engineered Rtx2a:Fc2b and Rtx2b:Fc2a combinations both show the presence of significant heterodimer whereas the wild-type Rtx:Fc combination shows only a small amount of heterodimer.

The Fc is indeed present as seen in lanes 1-3 of the reduced SDS-PAGE shown in FIG. 7: top band is Rtx heavy chain (expected MW 61-67 kDa), middle band is Fc (expected MW 36-41 kDa), and bottom band is Rtx light chain (expected MW 23 kDa).

Okt:Fc including wild-type and engineered IgM Fc pair 2a and 2b , SDS-PAGE, FIG. 8, lanes 1-3

Wild-type Okt:Fc combination (SDS-PAGE, FIG. 8, lane 1) shows an upper band of Okt:Fc heterodimer (expected MW 98-107 kDa), a bottom band for unassociated Fc (expected MW 36-41) and a large middle band representing associated Fc (expected MW 72-82). In contrast, for the Okt2a:Fc2b and Okt2b:Fc2a combinations, the SDS-PAGE gel shown in FIG. 8, lane 2 shows a prominent band for the heterodimer and very light bands for associated Fc2b and the Okt2a homodimer above and congruent with the Okt2a:Fc2b heterodimer. The arrow indicates the heterodimer.

Both the Okt2a and Fc2b are present in the reduced gel (SDS-PAGE gel shown in FIG. 9, lane 2). Similar results are seen for the Okt2b:Fc2a pair on gels shown in FIGs. 8 and 9.

Okt:Rtx including wild-type and engineered IgM Fc pair 2a and 2b SDS-PAGE gels shown in FIGs. 8 and 9, lanes 4-6

Wild-type Okt:Rtx combination (SDS-PAGE gel shown in FIG. 8, lane 4) shows a band of wt Rtx homodimer (H2L2, expected MW 168-180 kDa), a band of wt Rtx half-antibody (HL, expected MW 84-90 kDa) and a light band that may be Okt homodimer (expected MW 124-133 kDa). In contrast, the engineered Okt2a:Rtx2b combination (SDS-PAGE gel shown in FIG. 8, lane 5) shows the presence of significant heterodimer (expected MW 146-157) as well as Rtx2b homodimer (expected MW 168-180 kDa) and half-antibody (expected MW 84-90 kDa). When reduced (SDS-PAGE gel shown in FIG. 9, lane 5), the Rtx2b light chain shows a band at MW 23 kDa; the heavy band between 60-80 kDA is likely comprised of Rtx2b heavy chain (expected MW 61-67 kDa) and

Okt2a heavy chain (expected MW 62-67 kDa). Similar results are seen for the Okt2b:Rtx2a pair.

Conclusions:

For all three systems tested -- Okt:Rtx, Okt:Fc, Rtx:Fc -- the engineered IgM Fc variants showed substantially increased heterodimer formation compared to native (non-engineered) IgM Fc. A single pair of sequences (i.e., Fcs 2a and 2b) were tested and additional variants of the engineered Fc interface can be evaluated to further reduce homodimer formation and optimize heterodimer formation.

3. Bispecific functional analysis

a. ELISA analysis for two ligands

IgM with OKT3 (chain A) and cMyc peptide (chain B) is assayed by ELISA analysis with soluble CD3 epsilon protein capture and anti-cMyc (9E10) detection. Soluble CD3e protein is coated on ELISA plate at 2 mg/ml in 150 mM of $NaHCO_3$ followed by blocking with 3% BSA in PBS. Supernatant (100 µl) containing transfected IgM-OKT3-cMyc is added to blocked ELISA plate for 4 hours at 25 C. After washing with PBS, the 9E10 antibody is added to the ELISA plate for 2 hours at room temperature. Anti-mouse IgG-HRP is added following washes with PBS. The existence of bi-specific IgM is detected by reading with OD 450 after adding HRP substrate.

IgM with Okt3 (chain A) and Rituxan (chain B) is assayed by ELISA analysis with soluble CD3 epsilon protein capture and Protein-L-HRP detection. Soluble CD3e protein is coated on ELISA plate at 2 mg/ml in 150 mM of $NaHCO_3$ following by blocking with 3% BSA in PBS. Supernatant (100 µl) containing transfected IgM-Okta:Rtxb or Oktb:Rtxa is added to blocked ELISA plate for 4 hours at 25 C. After washing with PBS, the Protein-L-HRP is added to the ELISA plate for 2 hours at room temperature. The existence of bi-specific IgM is detected by reading with OD 450 after adding HRP substrate.

b. FACS analysis of target binding

IgM-OKT3-cMyc binding to T cell is confirmed by binding of antibody to T cell line (Peer, positive cell line) and B cell line (Daudi, negative control cell line). After washing,

rhodamine labeled 9E10 is added to the cell suspension. The cell target binding is detected by MFI of both positive and negative controlled cells with or without CD20 antigen.

c. Fluorescent microscopy assay for bispecific binding

Verify bispecific binding of the designed IgM by its ability to bring together, two populations of CD3 positive cells and CD20 positive cells, which have been pre-labeled by two different vital dyes on each cell type. For example:

i. Green Fluorescent cytosolic vital dye (CellTrace™ Calcein Green AM) labeling for CD3 positive cell line (Peer)

ii. Red Fluorescent cytosolic vital dye (CellTrace™ Calcein Red-Orange, AM) labeled CD20 positive B-cell cell line (Daudi)

Example 2

1. Generation of DNA constructs with designed mutations

DNA construct synthesis and construction of expression vectors are performed as in Example 1.

a. μ Chains of different size

The A chain is composed of a full length μ chain for chimeric OKT3 (anti-CD3) Vh region fused with CM1 of human mu chain:

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIG
YINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCAR
YYDDHYCLDYWGQGTTLTVSSGSASAPTLFPLVSCENSPSDTSSVAVGC
LAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQVLLPSKD
VMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVPPRDGFFGN
PRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQAEAKESGPTT
YKVTSTLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMCVPDQDTAIRV
FAIPPSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQNGEAVKTHTNI
SESHPNATFSAVGEASICEDDWNSGERFTCTVTHTDLPSPLKQTISRPK
GVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQP
LSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEAL
PNRVTERTVDKSTGKPTLYNVSLVMSDTAGTCY

(SEQ ID NO: 13)

The A chain has a molecular weight about 63kD and is able to bind to soluble epsilon chain of CD3 (10977-H08H, Sino Biological), or T cells.

The B chain has a cMyc tag fused with CH2 of human μ chain:

QVQLGGPEQKLISEEDLNSAVLPVIAELPPKVSVFVPPRDGFFGNPRKS
KLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQAEAKESGPTTYKVT
STLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMCVPDQDTAIRVFAIP
PSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQNGEAVKTHTNISESH
PNATFSAVGEASICEDDWNSGERFTCTVTHTDLPSPLKQTISRPKGVAL
HRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSPE
KYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRV
TERTVDKSTGKPTLYNVSLVMSDTAGTCY

(SEQ ID NO: 14)

The B chain has a molecular weight about 41kD and is able to bind to anti-myc monoclonal antibody 9E4 or other anti-myc antibodies.

The alternative B chain has a full length μ chain for CrossMab$^{M\text{-}CL}$ (V_H+C_L) Rituximab (anti-CD20) fused with CH2 of human mu chain:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIG
AIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCAR
STYYGGDWYFNVWGAGTTVTVSASVAAPSVFIFPPSDEQLKSGTASVVC
LLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK
ADYEKHKVYACEVTHQGLSSPVTKSFNRGECDKTHLPVIAELPPKVSVF
VPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQ
AEAKESGPTTYKVTSTLTIKESDWLSQSMFTCRVDHRGLTFQQNASSMC
VPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDSVTISWTRQN
GEAVKTHTNISESHPNATFSAVGEASICEDDWNSGERFTCTVTHTDLPS
PLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADV
FVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGET
YTCVVAHEALPNRVTERTVDKSTGKPTLYNVSLVMSDTAGTCY

(SEQ ID NO: 15)

The B chain has a molecular weight about 64kD and is able to bind to CD20 positive B cells.

b. Different light chain coupling

Native chimeric OKT3 kappa chain

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYD
TSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFG
SGTKLEINRAVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW
KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT
HQGLSSPVTKSFNRGEC

(SEQ ID NO: 16)

CrossMab^{CH1-CL} for Rituximab

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYA
TSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFG
GGTKLEIKSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKRVEPKSC

(SEQ ID NO: 17)

c. Different selection markers for different expression vectors

Different selection markers are used on different expression vectors used for co-transfection. Multiple drugs are used for selection of cells in order to accommodate all necessary expression vectors relevant for IgM production. Standard molecular biology techniques are used for cloning specific DNAs into these vectors.

i. Mu chain utilizes Zeocin selection (ant-zn-1, Invivogen). Zeocin is used at a concentration of 100 μg/ml. After transfection with a plasmid containing the Sh ble gene, then the cells are incubated in Opti-CHO medium containing Zeocin at 100 μg/ml to select for stable transfectants.

ii. Kappa chain utilizes Blasticidin S selection (ant-bl-1, Invivogen). Blasticidin S is used at a concentration of 10 μg/ml. After transfection with a plasmid containing the bsr gene, then the cells are incubated in Opti-CHO medium containing Blasticidin S at 10 μg/ml to select for stable transfectants.

d. Protein expression, purification and characterization

i. Transfection

IgM is made by co-transfection of several different expression vectors at equal molar ratios or variable molar ratio (5 to 10 fold difference) into mammalian

cells, such as 293 cells or CHO cells. DNA for expression vectors are mixed with PEI and then added to CHO-S cells. PEI transfection with CHO-S cells is conducted according to established techniques (see "Biotechnology and Bioengineering, Vol 87, 553-545").

ii. Protein purification

- Capture Select IgM (Catalog 2890.05 , BAC, Thermo Fisher)

IgM proteins from transfected CHO-S cell supernatants are purified by affinity Capture Select IgM affinity matrix according to manufacturers' protocol.

- Capto-L (Catalog 17-5478-01, GE Healthcare)

Transfected IgM protein, containing kappa chain, in CHO-S cell supernatant is purified by Capto-L affinity matrix according to manufacturers' protocol

iii. Gel electrophoresis

- Non-reducing SDS PAGE

Non-reducing SDS PAGE separates native IgM and its mutant forms according to size. Pentamic IgM, composed of homodimeric heavy chains (AA), produces a protein band of approximately 1,000,000 molecular weight. Pentameric IgM composed of a shorter version of homodimeric heavy chains (BB) produces a protein band of significantly lower molecular weight. Pentameric IgM composed of heterodimeric heavy chains (chimeric AB) produce multiple proteins with molecular weights greater than BB and less than AA.

NuPage LDS Sample Buffer (Life Technologies) is added to IgM protein samples at 25 C for 30 minutes before loading onto the gel. NativePage Novex 3-12% Bis-Tris Gel (Life Technologies) is used with Novex Tris-Acetate SDS Running Buffer (Life Technologies). Run gel until the dye front reaches the bottom of the gel.

- Reducing SDS-PAGE

NuPage LDS sample buffer (Life Technologies) and NuPage reducing agent dithiothreitol (Life Technologies) are added to IgM protein samples and heated to 80°C for 10 minutes before loading on NuPage Novex 4-12% Bis-Tris Gel

(Life Technologies, cat# NP0322). NuPage MES SDS Running Buffer (Life Technologies, cat# NP0002) is used for gel electrophoresis. Gels are run until the dye front reaches the bottom of the gel.

After electrophoresis is complete, remove gel from apparatus and stain the gel using Colloidal Blue Staining (Life Technologies, manual #LC6025)

- Gel band quantitation

Protein gels are dried, then digitized using image scanner. The gel image is processed with Image J program and the amount of protein in a specific band can be determined using the gel quantitation function

iv. Mass spectrometric analysis to identify/quantify the various mAbs in the bi-specific preparation.

v. Stability analysis using differential scanning calorimetry (DSC)

e. Bi-specific functional analysis

i. ELISA analysis for two ligands

IgM with OKT3 (chain A) and cMyc peptide (chain B) is assayed by ELISA analysis with soluble CD3 epsilon protein capture and anti-cMyc (9E10) detection. Soluble CD3e protein is coated on ELISA plate at 2 mg/ml in 150 mM of $NaHCO_3$ followed by blocking with 3% BSA in PBS. Supernatant (100 µl) containing transfected IgM-OKT3-cMyc is added to blocked ELISA plate for 4 hours at 25 C. After washing with PBS, the 9E10 antibody is added to the ELISA plate for 2 hours at room temperature. Anti-mouse IgG-HRP is added following washes with PBS. The existence of bi-specific IgM is detected by reading with OD 450 after adding HRP substrate.

ii. FACS analysis of target binding

IgM-OKT3-cMyc binding to T cell is confirmed by binding of antibody to T cell line (Peer, positive cell line) and B cell line (Daudi, negative control cell line). After washing, rhodamine labeled 9E10 is added to the cell suspension.

The cell target binding is detected by MFI of both positive and negative controlled cells with or without CD20 antigen.

iii. Fluorescent microscopy assay for bi-specific binding

Verify bi-specific binding of the designed IgM by its ability to bring together, two populations of CD3 positive cells and CD20 positive cells, which have been pre-labeled by two different vital dyes on each cell type. For example:

- Green Fluorescent cytosolic vital dye (CellTrace™ Calcein Green AM) labeling for CD3 positive cell line (Peer)
- Red Fluorescent cytosolic vital dye (CellTrace™ Calcein Red-Orange, AM) labeled CD20 positive B-cell cell line (Daudi)

Multi-specific binding and multi-specific functional analysis can be performed in a similar manner using techniques known in the art, such as those described above.

Claims:

1. A binding molecule having a penta- or hexameric ring structure comprising five or six bispecific binding units, wherein each of said bispecific binding units has the same two binding specificities and comprises a first chain comprising at least a Cμ4 domain of an IgM heavy chain constant region conjugated to a first binding region to a first binding target, and a second chain comprising at least a Cμ4 domain of an IgM heavy chain constant region and a second binding region to a second binding target, wherein said first and second binding targets are different, and wherein said first and second chains are assembled to create a bispecific binding unit as a result of an asymmetric interface created between their respective IgM heavy chain constant regions.

2. The binding molecule of claim 1, wherein said bispecific binding units are identical.

3. The binding molecule of claim 2, which further comprises an IgM J chain.

4. The binding molecule of claim 3, which has a pentameric ring structure.

5. The binding molecule of claim 2, which has a hexameric structure.

6. The binding molecule of claim 2, wherein said first and second chains further comprise a Cμ3 domain of an IgM heavy chain constant region.

7. The binding molecule of claim 2 or claim 6, wherein said first and second chains further comprise a Cμ2 domain of an IgM heavy chain constant region.

8. The binding molecule of claim 2, wherein said first and second binding targets are selected from peptides, polypeptides, glycoproteins, nucleic acid molecules, and organic and non-organic small molecules.

9. The binding molecule of claim 2, wherein said first and second binding targets are selected from soluble polypeptides, cell surface receptors, ligands, molecular transporters, enzymes and substrates of enzymes.

10. The binding molecule of claim 2, wherein said first and second binding targets bind to two sites on the same soluble target, two sites on the same cell surface receptor target, two different soluble targets, two cell surface receptor targets, one soluble target and one cell surface receptor target, one soluble or cell surface receptor target and one long residence time target, one soluble target and one matrix protein or substrate target, one soluble or receptor target and one molecular transporter target, or two different cell types.

11. The binding molecule of any one of claims 1 to 10, wherein conjugation is by fusion.

12. The binding molecule of claim 11, wherein said first and second binding regions are fused to the N-termini of said first and said second IgM heavy chain constant region, respectively.

13. The binding molecule of claim 12, wherein said first and second binding regions are variable regions of an antibody.

14. The binding molecule of claim 13, wherein said first and second binding targets are two different antigens.

15. The binding molecule of claim 13, wherein said first and second binding targets are different epitopes on the same antigen.

16. The binding molecule of claim 13, wherein said first and second binding regions are two different antibody heavy chain variable regions, binding to said first and said second binding target, respectively.

17. The binding molecule of claim 13, wherein said first and second binding regions are two different antibody heavy chain variable regions, each binding to a different epitope on the same binding target.

18. The binding molecule of claim 16 or claim 17, wherein said antibody heavy chain variable regions are from an IgG, IgA, IgE, or IgM antibody.

19. The binding molecule of claim 18, wherein said antibody heavy chain variable regions are from an IgM antibody.

20. The binding molecule of claim 16 or claim 17, which is a bispecific IgM molecule.

21. The binding molecule of claim 20, further comprising at least one IgM light chain variable region sequence associated with at least one of said two different IgM heavy chain variable regions.

22. The binding molecule of claim 20, further comprising an IgM light chain variable region sequence associated with each of said two different IgM heavy chain variable regions.

23. The binding molecule of any one of claims 1 to 22, wherein at least some of the asymmetric interfaces between the IgM heavy chain constant regions of the two chains of a binding unit are created by a salt bridge formed by pair-wise switches between oppositely charged amino acid residues in at least one of the Cμ2, Cμ3 and/or Cμ4 domains of the two chains of said binding unit.

24. The binding molecule of claim 23, wherein a salt bridge is formed between at least one of the Cμ2-Cμ2, Cμ4-Cμ4, and Cμ2-Cμ3-Cμ4 domains of the two chains of said binding unit.

25. The binding molecule of claim 23, wherein the pair-wise switches are selected from the group consisting of E→K, K→E; R→E, E→R; D→K, K→D; and R→D, D→R.

26. The binding molecule of claim 24, comprising at least one pair-wise charged amino acid residue switch in the Cμ4-Cμ4 domains.

27. The binding molecule of claim 26, wherein said switch is selected from the group consisting of R328E,D↔E339R,K; R344E,D↔S330R,K; K376E,D↔E385R,K; R427E,D↔E339R,K; and T354E,D↔I397R,K.

28. The binding molecule of claim 24, comprising at least one pair-wise charged amino acid switch between the Cμ2-Cμ2 domains.

29. The binding molecule of claim 28, wherein said switch is selected from the group consisting of E167R,K↔K177E,D and K169E,D↔E170R,K.

30. The binding molecule of claim 24, comprising at least one pair-wise charged amino acid residue switch in the Cμ2-Cμ3-Cμ4 domains.

31. The binding molecule of claim 29, wherein said switch is selected from the group consisting of D121K,R↔K315D,E; K150E,D↔E385K,R; and K185D,E↔D360K,R.

32. The binding molecule of any one of claims 1 to 22, wherein at least some of the asymmetric interfaces between the IgM heavy chain constant regions of the two chains of a binding unit are created through knobs-into-holes connections.

33. The binding molecule of claim 32, wherein at least one knobs-into-hole connection is created by mutations selected from the group consisting of knobs: T350→Y,F,W; and H395→Y,F; and holes: L352→ G,A,V,I,M,S,T; H395→A,V,I,L,M,F,Y; F393→W,Y; I397→ A,V,S,T; T350→S,A,V; and T348→S.

34. The binding molecule of any one of claims 21 to 33, wherein said light chain variable region sequences are coupled to their matching heavy chain variable region by creating an asymmetric interface between the light and heavy chains.

35. The binding molecule of claim 34, wherein the asymmetric interface is created by CrossMab technique, knobs-into-holes coupling and/or salt bridges coupling.

36. The binding molecule of any one of claims 21 to 35, comprising a common light chain.

37. The binding molecule of any one of claims 1 to 36, conjugated to a toxin.

38. The binding molecule of any one of claims 1 to 36, conjugated to a chemotherapeutic agent.

39. The binding molecule of claim 37 or claim 38, wherein conjugation is by fusion.

40. The binding molecule of claim 37 or claim 38, wherein conjugation is by a chemical linker.

41. The binding molecule of any one of claims 20 to 40, which is chimeric or humanized.

42. A composition comprising at least about 70% of the binding molecule according to any one of 1 to 41.

43. A composition comprising at least about 80% of the binding molecule according to any one of 1 to 41.

44. A composition comprising at least about 95% of the binding molecule according to any one of claims 1 to 41.

45. A composition comprising at least about 98% of a binding molecule according to any one of claims 1 to 41.

46. A composition comprising at least about 99% of a binding molecule according to any one of claims 1 to 42.

47. The composition according to any one of claims 42 to 46, which is a pharmaceutical composition.

48. A multi-specific binding molecule having a penta- or hexameric ring structure comprising five or six monospecific binding units, wherein (i) each of said monospecific

binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of said monospecific binding units bind to different binding targets, and (iii) an external asymmetric interface is created between the heavy chain constant regions of the neighboring monospecific binding units that bind to different binding targets.

49. The multi-specific binding molecule of claim 48, wherein at least three of said monospecific binding units bind to different binding targets.

50. The multi-specific binding molecule of claim 48, wherein at least four of said monospecific binding units bind to different binding targets.

51. The multi-specific binding molecule of claim 48, wherein the binding molecule has a pentameric ring structure and all five monospecific binding units bind to different targets.

52. The multi-specific binding molecule of claim 48, wherein the binding molecule has a hexameric ring structure and at least five of said monospecific binding units bind to different targets.

53. The multi-specific binding molecule of claim 52, wherein all six monospecific binding units bind to different targets.

54. A multi-specific binding molecule having a penta- or hexameric ring structure comprising five or six bispecific binding units, wherein (i) each of said bispecific binding units comprises two IgM heavy chain constant regions each comprising at least a Cμ3 and Cμ4 domain conjugated to a binding region to a binding target, (ii) at least two of said bispecific binding units bind to different binding targets, (iii) an internal asymmetric interface is created between two IgM heavy chain constant regions of each bispecific binding unit, and (iv) an external asymmetric interface is created between the heavy chain constant regions of the neighboring bispecific binding units binding to different targets.

55. The multi-specific binding molecule of claim 54, wherein at least three of said bispecific binding units bind to different binding targets.

56. The multi-specific binding molecule of claim 54, wherein at least four of said bispecific binding units bind to different binding targets.

57. The multi-specific binding molecule of claim 54, wherein the binding molecule has a pentameric ring structure and all five bispecific binding units bind to different targets.

58. The multi-specific binding molecule of claim 54, wherein the binding molecule has a hexameric ring structure and at least five of said bispecific binding units bind to different targets.

59. The multi-specific binding molecule of claim 52, wherein all six bispecific binding units bind to different targets.

60. The multi-specific binding molecule of claim 48 or claim 54, which further comprises an IgM J chain.

61. The multi-specific binding molecule of claim 48 or claim 54, which has a pentameric ring structure.

62. The multi-specific binding molecule of claim 48 or claim 54, which has a hexameric structure.

63. The multi-specific binding molecule of claim 48 or claim 54, wherein in at least one of said binding units the IgM heavy chain constant regions further comprise a Cμ2 domain.

64. The multi-specific binding molecule of claim 63, wherein in all of said binding units the IgM heavy chain constant regions further comprise a Cμ2 domain.

65. The multi-specific binding molecule of claim 48 or claim 54, wherein in all of said binding units the heavy chain constant regions are identical.

66. The multi-specific binding molecule of claim 48 or claim 54, wherein the binding targets are selected from peptides, polypeptides, glycoproteins, nucleic acid molecules, and organic and non-organic small molecules.

67. The multi-specific binding molecule of claim 48 or claim 54, wherein the binding targets are selected from soluble polypeptides, cell surface receptors, ligands, molecular transporters, enzymes and substrates of enzymes.

68. The multi-specific binding molecule of claim 48 or claim 54, wherein the binding units binding to different targets are selected from the group consisting of binding units binding to sites on the same soluble target; sites on the same cell surface receptor target; different soluble targets; different cell surface receptor targets; soluble and cell surface receptor targets; soluble or cell surface receptor and long residence time targets; soluble and matrix protein or substrate targets; soluble or receptor and molecular transporter targets, and different cell types.

69. The multi-specific binding molecule of any one of claims 48 to 68, wherein conjugation is by fusion.

70. The multi-specific binding molecule of claim 67, wherein said binding regions are fused to the N-termini of the IgM heavy chain constant regions.

71. The multi-specific binding molecule of claim 70, wherein at least one of said binding regions is a variable region of an antibody.

72. The multi-specific binding molecule of claim 71, wherein all of said binding regions are antibody heavy chain variable regions.

73. The multi-specific binding molecule of claim 72, wherein at least two binding targets are different antigens.

74. The multi-specific binding molecule of claim 72, wherein at least two binding targets are different epitopes on the same antigen.

75. The multi-specific binding molecule of claim 71, wherein the antibody heavy chain variable regions are from an IgG, IgA, IgE, or IgM antibody.

76. The multi-specific binding molecule of claim 75, wherein the antibody heavy chain variable regions are from an IgM antibody.

77. The multi-specific binding molecule of claim 76, which is a multi-specific IgM molecule.

78. The multi-specific binding molecule of claim 77, further comprising at least one IgM light chain variable region sequence associated with an IgM heavy chain variable region in at least one of the binding units.

79. The multi-specific binding molecule of claim 78, further comprising an IgM light chain variable region sequence associated with each of the IgM heavy chain variable regions.

80. The multi-specific binding molecule of any one of claims 48 to 79, wherein said internal asymmetric interface is created by a salt bridge formed by pair-wise switches between oppositely charged amino acid residues in at least one of the Cμ2, Cμ3 and/or Cμ4 domains.

81. The multi-specific binding molecule of claim 80, wherein a salt bridge is formed between at least one of the Cμ2-Cμ2, Cμ4-Cμ4, and Cμ2-Cμ3-Cμ4 domains of the two chains of said binding unit.

82. The multi-specific binding molecule of claim 81, wherein the pair-wise switches are selected from the group consisting of E→K, K→E; R→E, E→R; D→K, K→D; and R→D, D→R.

83. The multi-specific binding molecule of claim 80, comprising at least one pair-wise charged amino acid residue switch in the Cμ4-Cμ4 domains.

84. The multi-specific binding molecule of claim 83, wherein said switch is selected from the group consisting of R328E,D↔E339R,K; R344E,D↔S330R,K; K376E,D↔E385R,K; R427E,D↔E339R,K; and T354E,D↔I397R,K.

85. The multi-specific binding molecule of claim 80, comprising at least one pair-wise charged amino acid switch between the Cμ2-Cμ2 domains.

86. The multi-specific binding molecule of claim 85, wherein said switch is selected from the group consisting of E167R,K↔K177E,D and K169E,D↔E170R,K.

87. The multi-specific binding molecule of claim 80, comprising at least one pair-wise charged amino acid residue switch in the Cμ2-Cμ3-Cμ4 domains.

88. The multi-specific binding molecule of claim 87, wherein said switch is selected from the group consisting of D121K,R↔K315D,E; K150E,D↔E385K,R; and K185D,E↔D360K,R.

89. The multi-specific binding molecule of any one of claims 48 to 88, wherein the external asymmetric interface is created by at least one pair-wise charged amino acid residue switch in the Cμ3-Cμ3 domains.

90. The multi-specific binding molecule of claim 89, wherein the pair-wise changed amino acid switch is K238 ↔ D293 or K268 ↔ D294.

91. The multi-specific binding molecule of any one of claims 48 to 79, wherein at least some of the external and/or internal asymmetric interfaces between the IgM heavy chain constant regions are created through knobs-into-holes connections.

92. The multi-specific binding molecule of claim 91, wherein at least one knobs-into-hole connection is created by mutations selected from the group consisting of knobs:

T350→Y,F,W; and H395→Y,F; and holes: L352→ G,A,V,I,M,S,T; H395→A,V,I,L,M,F,Y; F393→W,Y; I397→ A,V,S,T; T350→S,A,V; and T348→S.

93. The multi-specific binding molecule of any one of claims 79 to 92, wherein said light chain variable region sequences are coupled to their matching heavy chain variable region by creating an asymmetric interface between the light and heavy chains.

94. The multi-specific binding molecule of claim 93, wherein the asymmetric interface is created by CrossMab technique, knobs-into-holes coupling and/or salt bridges coupling.

95. The multi-specific binding molecule of any one of claims 79 to 94, comprising a common light chain.

96. The multi-specific binding molecule of any one of claims 48 to 95, conjugated to a toxin.

97. The multi-specific binding molecule of any one of claims 48 to 95, conjugated to a chemotherapeutic agent.

98. The multi-specific binding molecule of claim 96 or claim 97, wherein conjugation is by fusion.

99. The multi-specific binding molecule of claim 96 or claim 97, wherein conjugation is by a chemical linker.

100. The multi-specific binding molecule of any one of claims 79 to 99, which is chimeric or humanized.

101. A composition comprising at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 98%, or at least about 99% of the multi-specific binding molecule according to any one of 48 to 100.

102. The composition of claims 101, which is a pharmaceutical composition.

FIG. 1

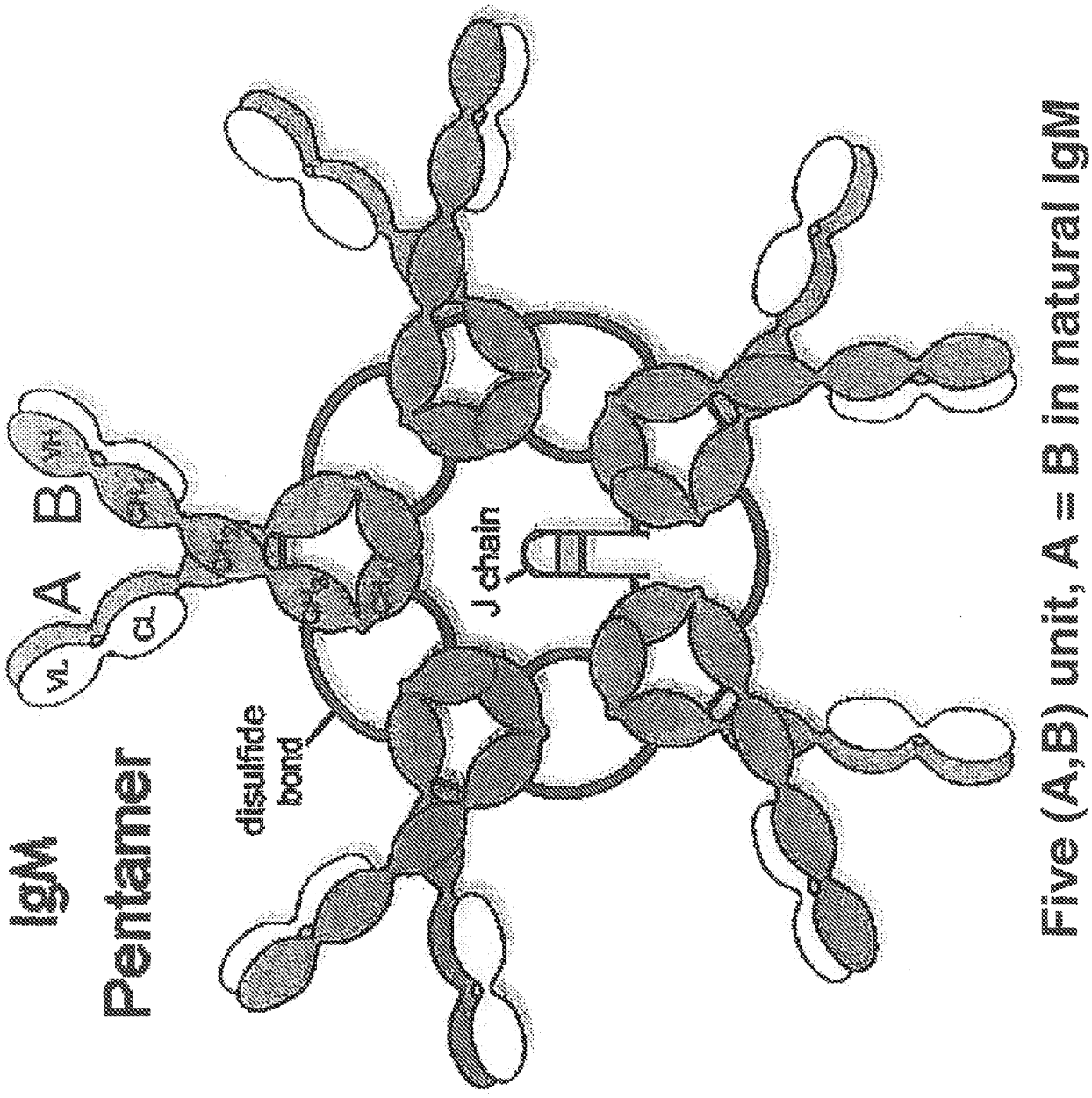
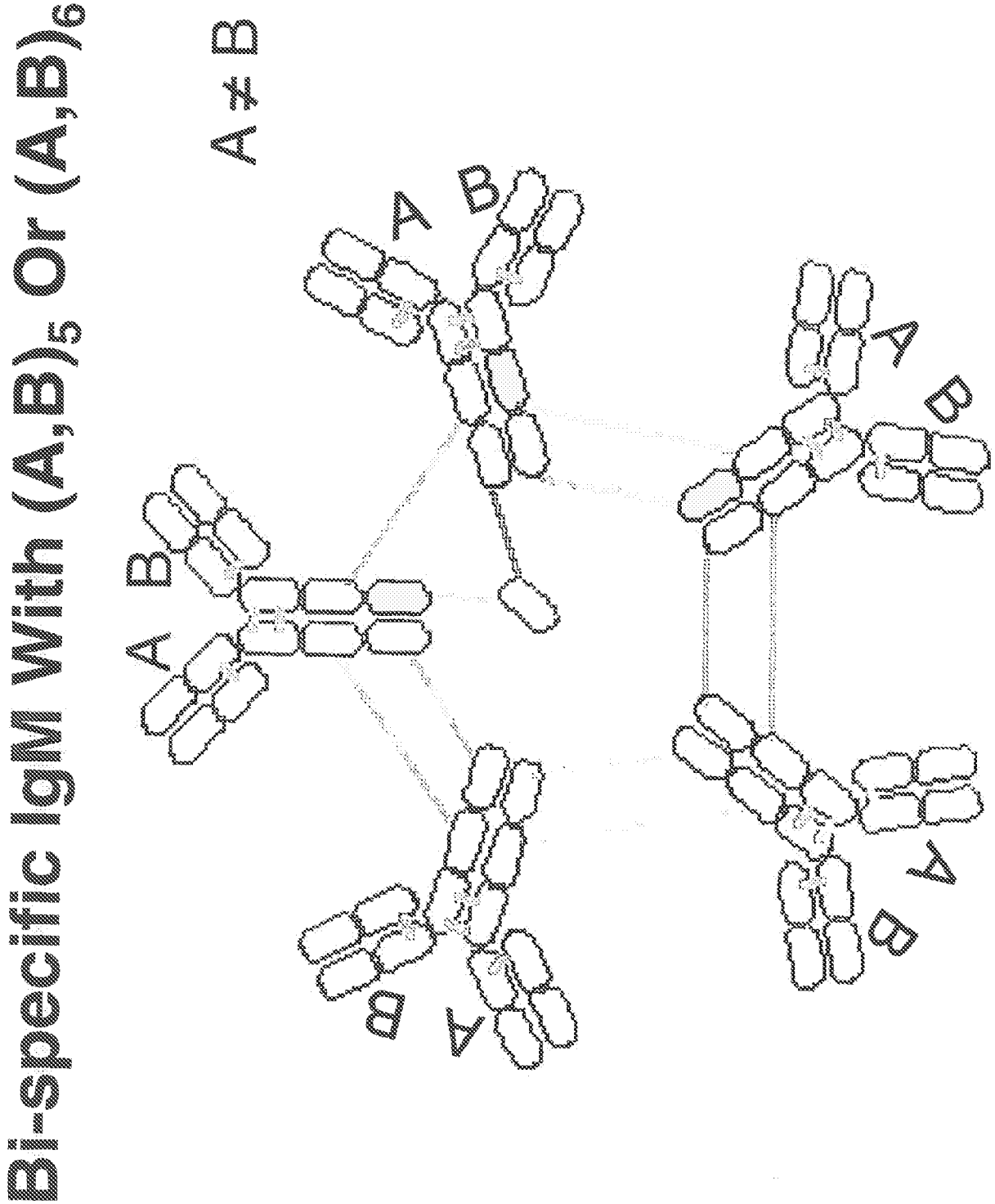

IgM
Pentamer
A
B
μ
α
disulfide bond
J chain
Five (A,B) unit, A = B in natural IgM

FIG. 2A



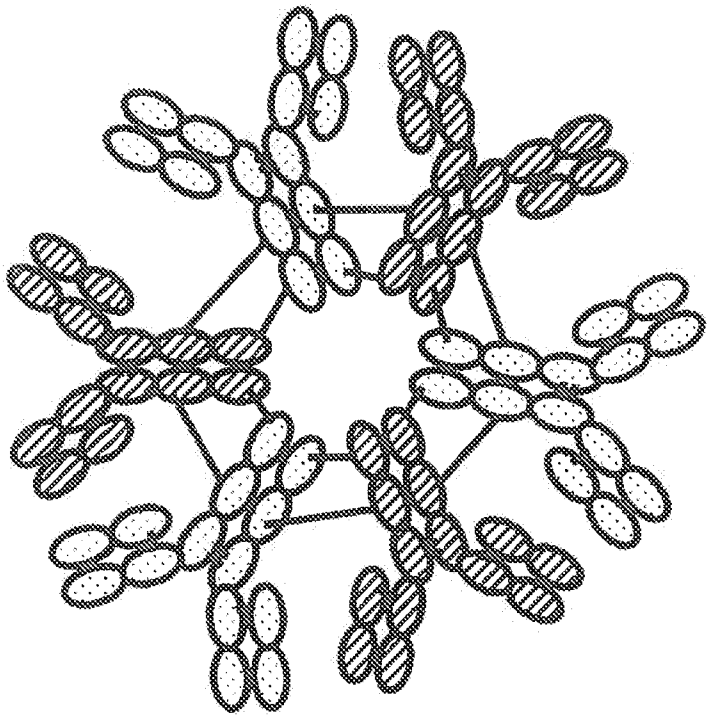


FIG. 2B

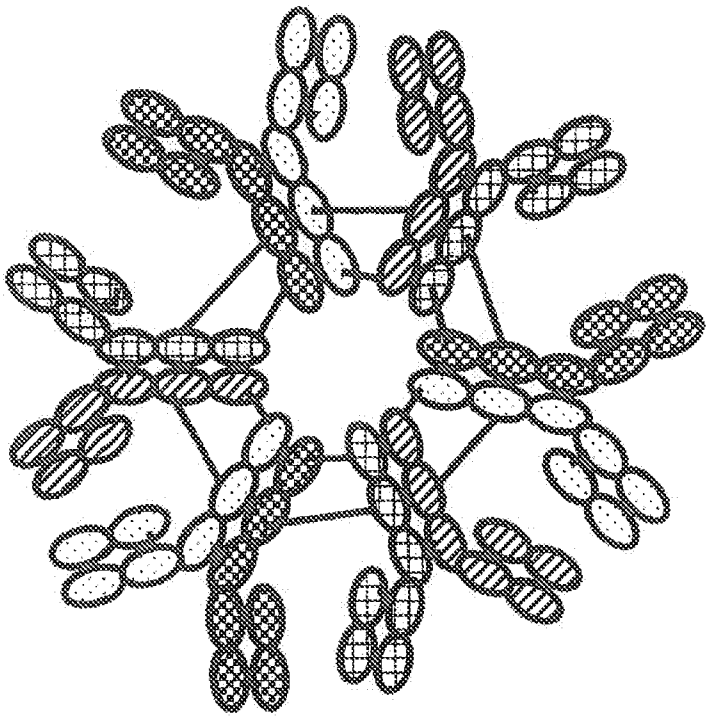


FIG. 2C

FIG. 3

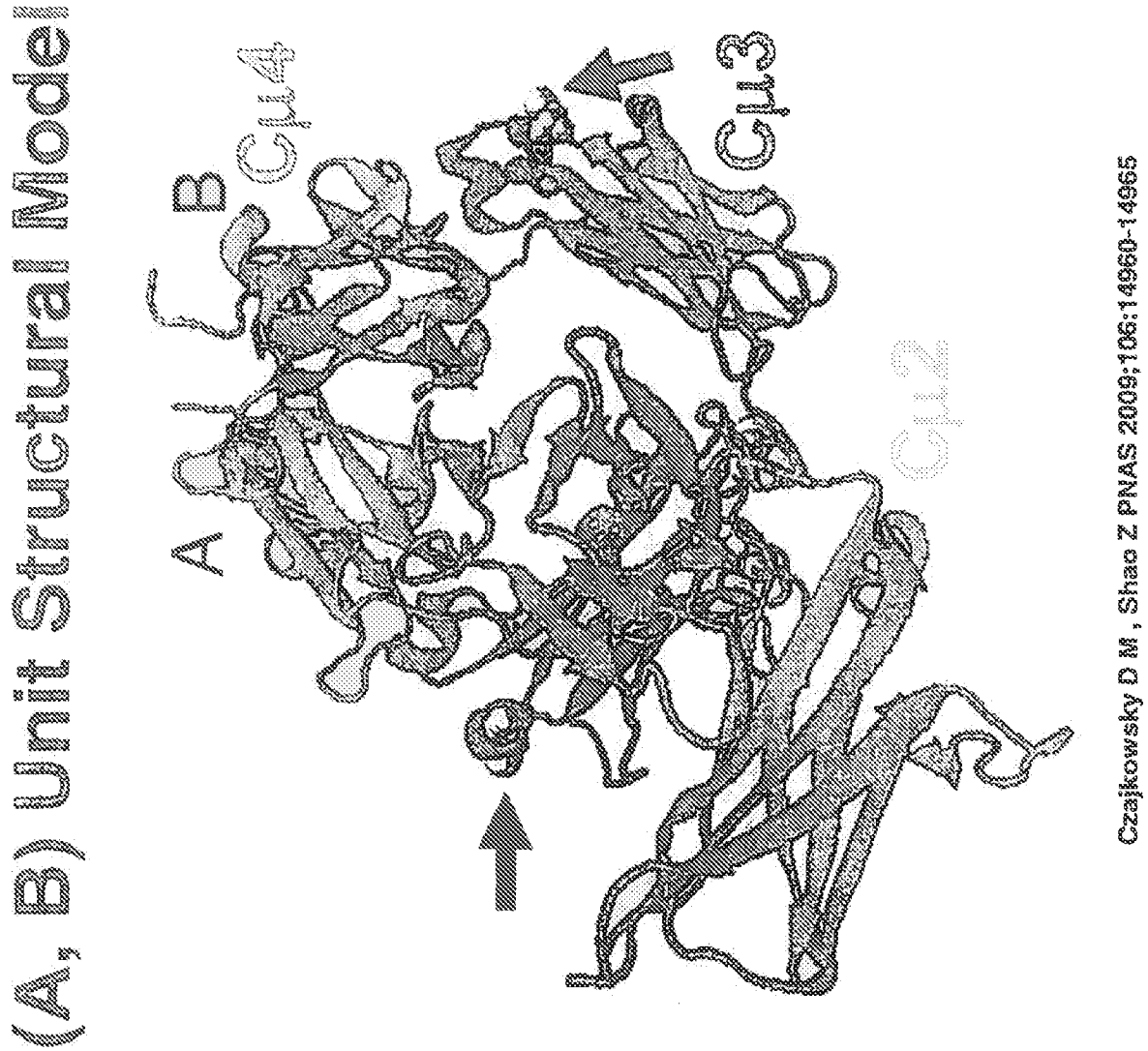

(A, B) Unit Structural Model
A
B
Cu4
Cu3
Cu2
Czajkowsky D M , Shao Z PNAS 2009;106:14960-14965

FIG. 4A

```
Constant domains  CG1 /CE1/CM1

CE1   ASTQSPSVFPLTRCCKNIPSNATSVTLGCLATGYFPSPVMVTYCDTGSLNGTT  --MTLPATTLTL
CG1   ASTKGPSVFPLAPSSKSTS --GGTAALGCLVKDYFPEPVTVSWNSGALTSGV  --HTFPAVLQSS
          1      10        20        30        40        50        60
          *      *         *         *         *         *         *
CM1   GSASAPTLFPLVSCENSPSD -TSSVAVGCLAQDFLPDSITLSWKYKNNSDISSTRGFPSVLRGGKY
                                                                    H
                                                                    L

CE1   SGHYATISLLTVSGAWAKQMFTCRVAHTPSSTDWVDNKTFS
CG1   GLYSLSSVVTVPSS SLGTQTYICNVNHKPSNTKVDKKV
          70        80        90        100
          *         *         *         *
CM1   AATSQVLLFSKDVMQGTDEHVVCKVQHPNGNKEKNVPLP
```

FIG.4B

Constant domains CE2/CM2

```
             230       240       250  b    260       270
              *         *         *         *         *
                 ssssss          ssssssss      sssssss     hhh
CE2    VCSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVD   -----L
              110       120       130       140       150       160
               *         *         *         *         *         *
CM2    --VIAELPPKVSVFVPPRDGFFGNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQV

        280       290       300       310       320
         *         *         *         *         *
        sssssss    sssssssssshhhhhh  sssssss   ssssss
CE2    STASTTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKC
           170       180       190       200       210
            *         *         *         *         *
CM2    QAEAKESGPTTYKVTSTLTIKESDWLGQSMFTCRVDHRGLTFQQNA   SSMC
               T                 S
```

IgG1 hinge

CG-H EPKSCDKTHTCPPCP

FIG. 4C

Constant domains CG2/CE3/CM3

```
                330       340       350       360       370       380
                 *         *         *         *         *         *
                ssss    hhhhhhh    ssssssssss      ssssss
CE3  -ADSNPRGVSAYLSRPSPFDLFIRKSPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTR
                240       250       260       270       280       290
                 *         *         *         *         *         *
                sssss   hhhhhhh     ssssss          ssssss   sss   sss
CG2  APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
                220       230       240       250       260       270
                 *         *         *         *         *         *
CM3  -VPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYD  -SVTISWTRQNGEAVKTHTN
                 G    I    S
                390       400       410       420       430
                 *         *         *         *         *
                ssss     ssss ssss   hhhhhh    ssssss     sssss
CE3  KEEKQRNGTLTVTSTLPVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTS
                          300       310       320       330       340
                           *         *         *         *         *
                          ssssssss hhhhhhh    ssssss          sssss
CG2  PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
                          280       290       300       310       320
                           *         *         *         *         *
CM3  ISESHPNATFSAVGEASICEDDWNSGERFTCTVTHTDLPSPLKQTISRPK
```

FIG. 4D

Constant domains CG3/ CE4/CM4

```
         440       450        460       470       480       490
          *         *          *         *         *         *
              ssssss          sssssssss       ssssss     hhh sssss
CE4      -GPRAAPEVYAFATPEWPGSR -DKRTLACLIQN FMPEDISVQWLHNEVQLPDARHSTTQPRKTK
                 350        360        370       380       390       400
                  *          *          *         *         *         *
              sssss  hhhhh    ssssssssss     ssssss  sss          ss
CG3      -GQPREPQVYTLPPSRDELTK -NQVSLTCLVKGFYPSDIAVEWESNGQPENN  --YKTTPPVLDS
            330        340        350       360       370       380
             *          *          *         *         *         *
CM4      GVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSPEK   YVTSAPMPEP

         500       510       520       530       540
          *         *         *         *         *
            sssssssssshhhhhhh  ssssss          ssssss
CE4      --GSGFFVFSRLEVTRAEWEQKDEFICRAVHEAASPSQTVQRAVSVNPGK
                 410       420       430       440
                  *         *         *         *
            sssssssssshhhhhh   ssssss   hhhhhsssss
CG3      --DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
         390       400       410       420       430       440       450
          *         *         *         *         *         *         *
CM4      QAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRVTERTVDKSTGKPTLYNVSLVMSDTAGTCY
```

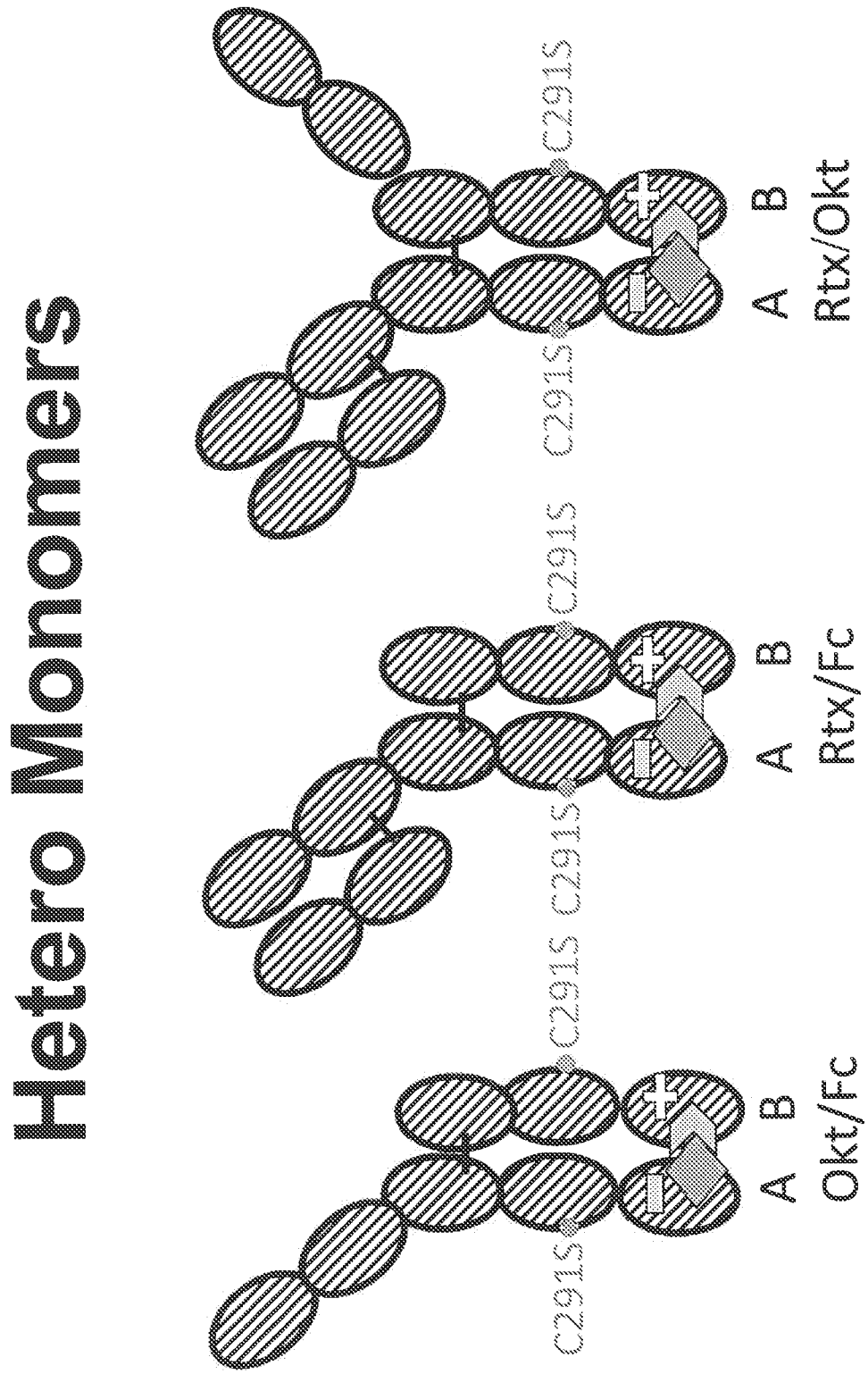

Hetero Monomers
C2915
C2915
C2915
C2915
C2915
C2915
A B
Rtx/Okt
A B
Rtx/Fc
A B
Okt/Fc

FIG. 5

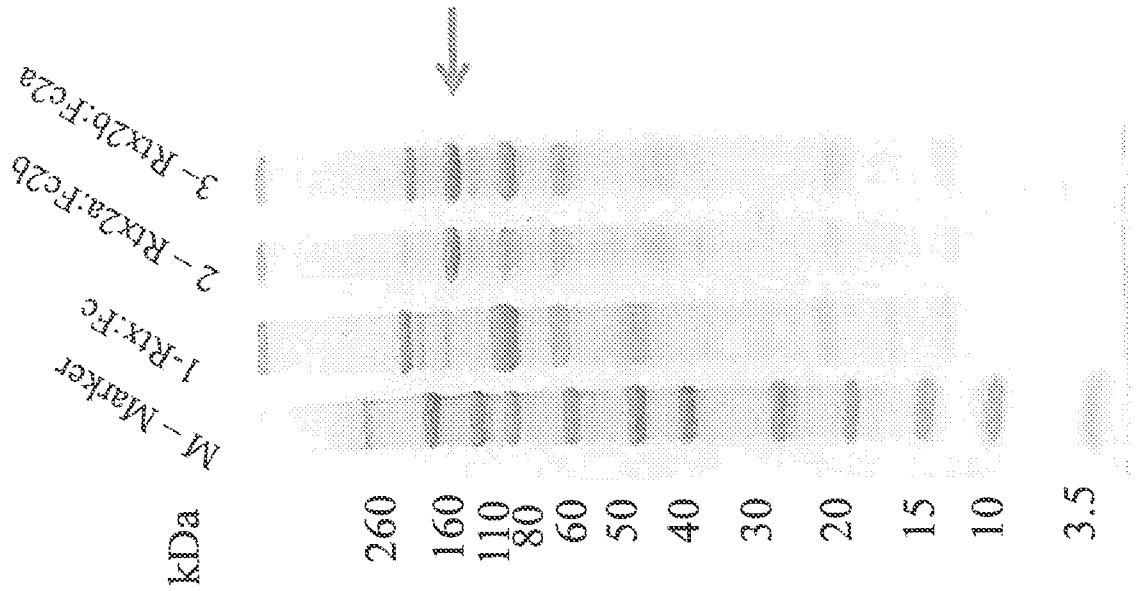

kDa
260
160
110
80
60
50
40
30
20
15
10
3.5
M – Marker
1-Rtx:Fc
2 – Rtx2a:Fc2b
3 – Rtx2b:Fc2a

FIG. 6

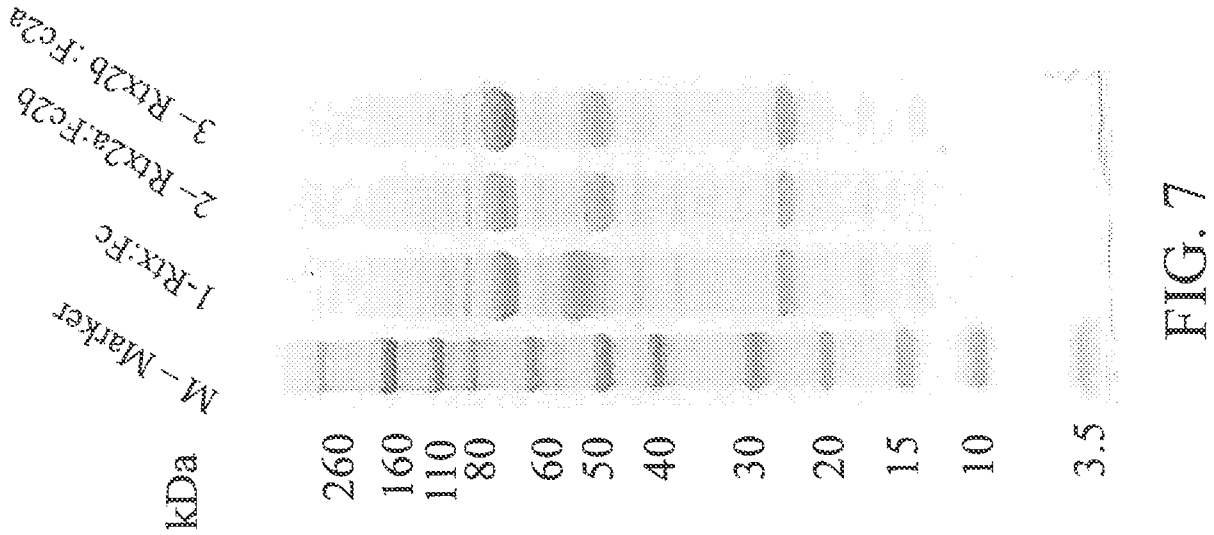

kDa
260
160
110
80
60
50
40
30
20
15
10
3.5
M – Marker
1-Rtx:Fc
2- Rtx2a:Fc2b
3- Rtx2b :Fc2a

FIG. 7

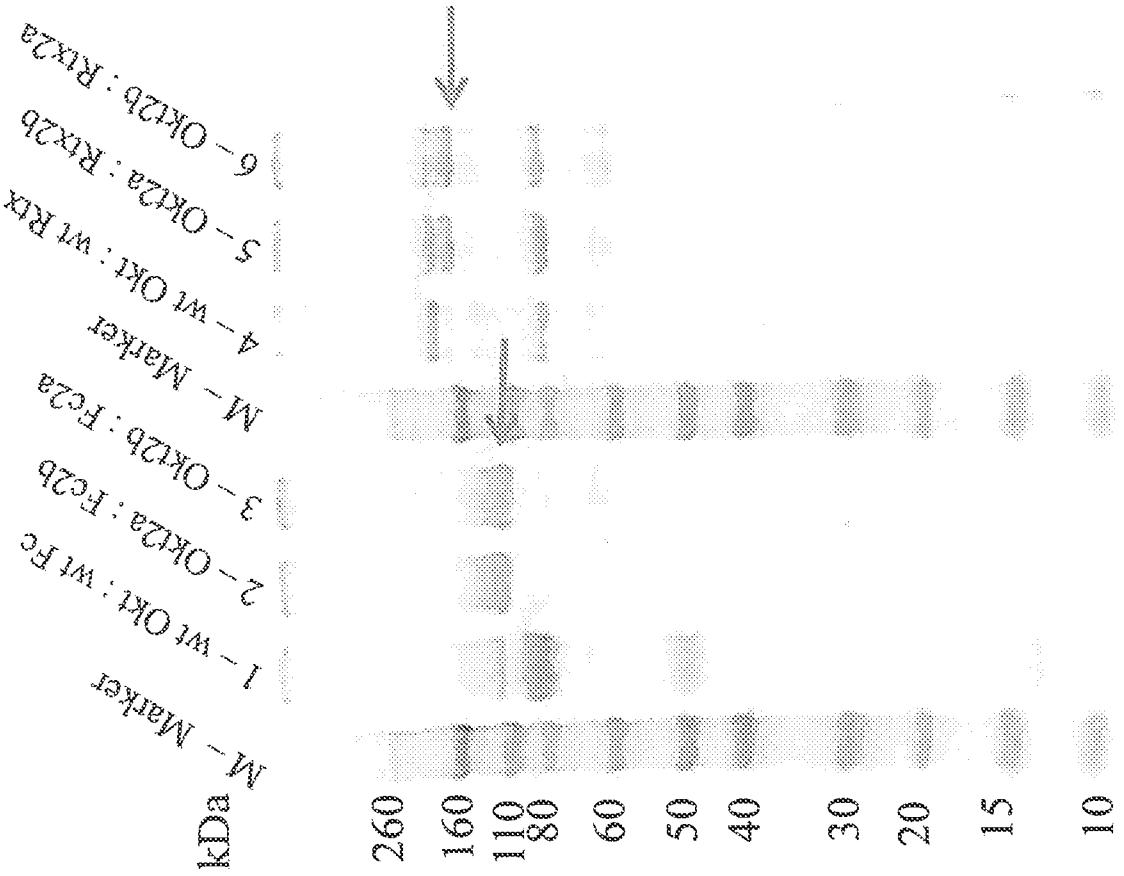

kDa
260
160
110
80
60
50
40
30
20
15
10
M – Marker
1 – wt Okt : wt Fc
2 – Okt2a : Fc2b
3 – Okt2b : Fc2a
M – Marker
4 – wt Okt : wt Rtx
5 – Okt2a : Rtx2b
6 – Okt2b : Rtx2a

FIG. 8

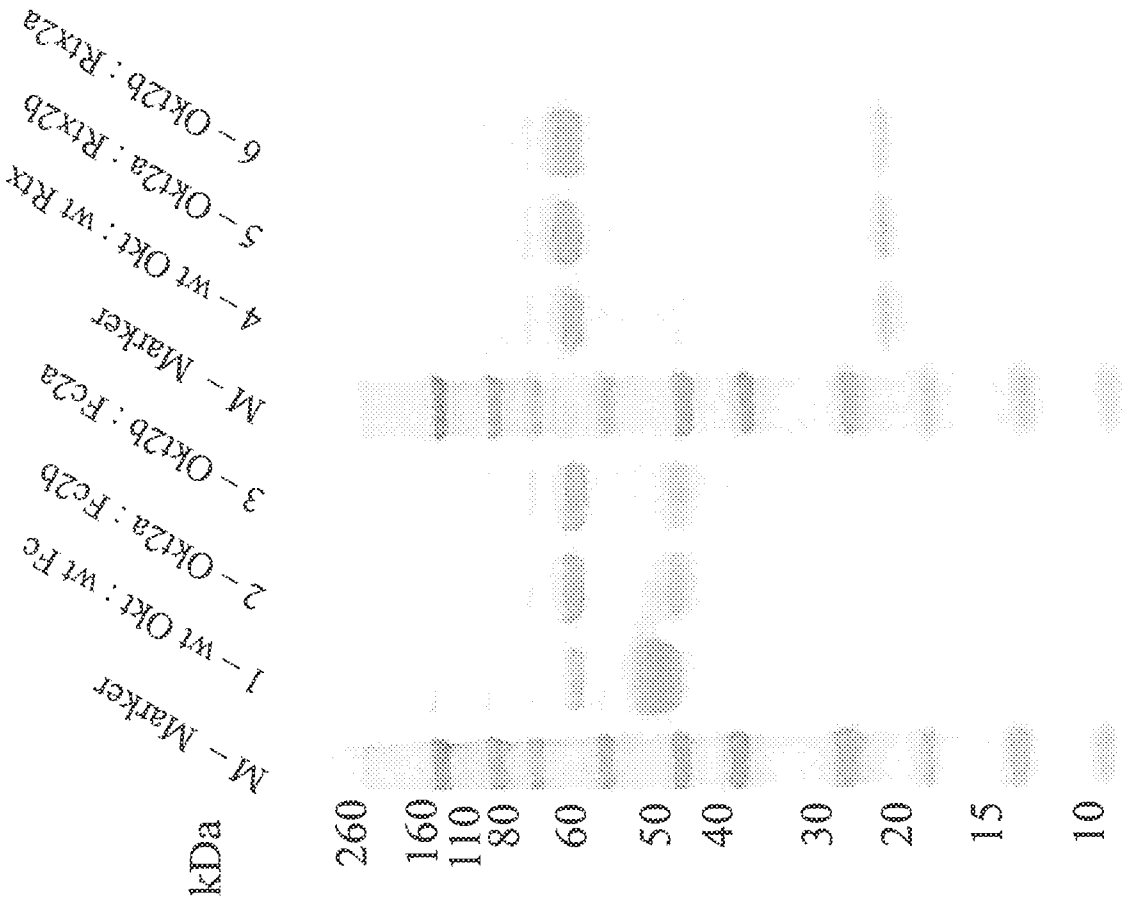

kDa
260
160
110
80
60
50
40
30
20
15
10
M – Marker
1 – wt Okt : wt Fc
2 – Okt2a : Fc2b
3 – Okt2b : Fc2a
M – Marker
4 – wt Okt : wt Rtx
5 – Okt2a : Rtx2b
6 – Okt2b : Rtx2a

FIG. 9

FIG. 10

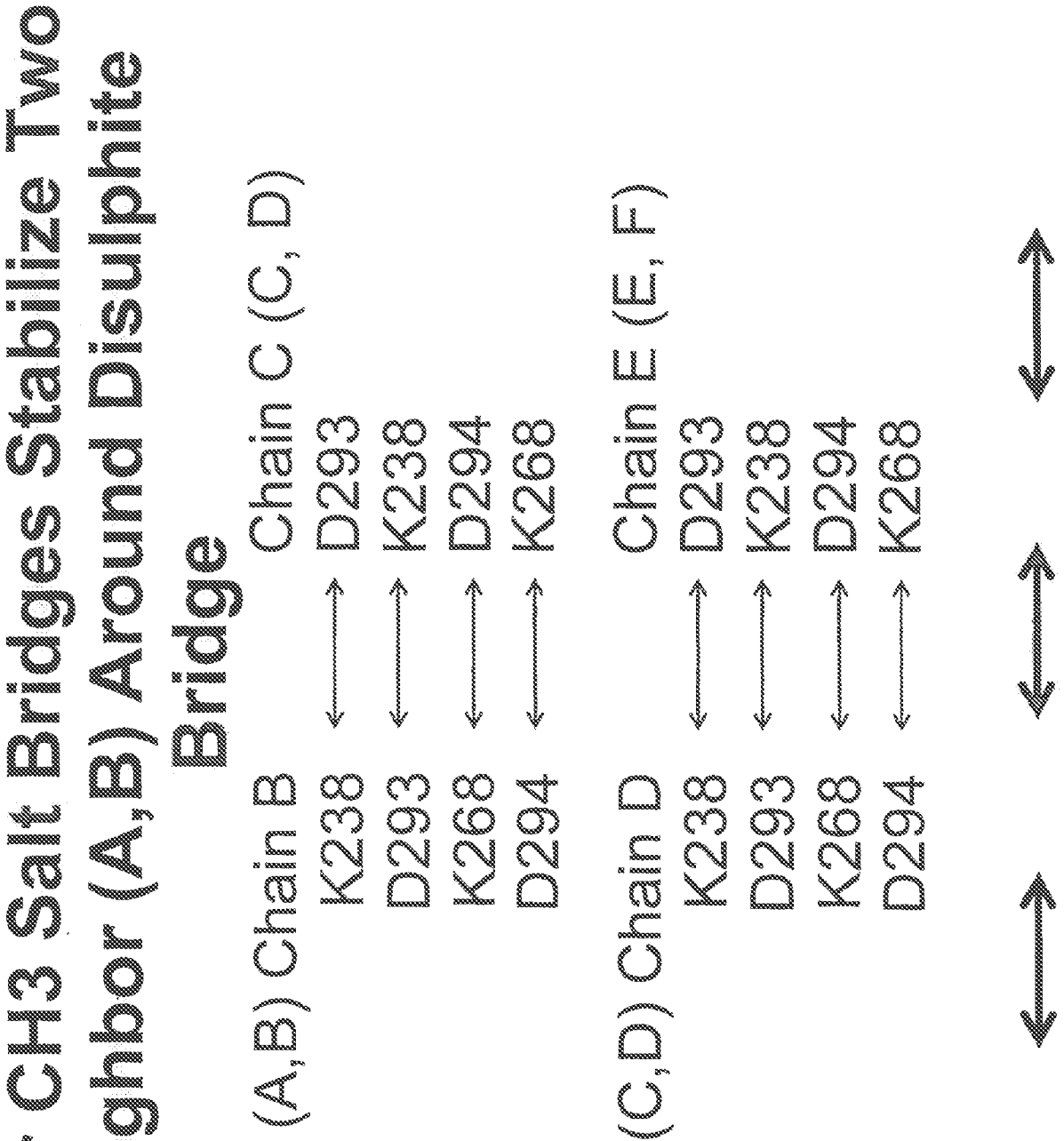

Four CH3 Salt Bridges Stabilize Two Neighbor (A,B) Around Disulphite Bridge
(A,B) Chain B
K238
D293
K268
D294
Chain C (C, D)
D293
K238
D294
K268
(C,D) Chain D
K238
D293
K268
D294
Chain E (E, F)
D293
K238
D294
K268

Table A

Human IgM CM4 domain interface residues
Knobs-Holes and charge change positions

Set #1		
Knob (CM4 #1)		
Residue #	Native Seq	Potential Mutations
350	T	Y
Hole (CM4 #2): any combination		
Residue #	Native Seq	Potential Mutations
352	L	S
393	F	F (W)
395	H	A (V,I,L,S,T)

Set #2 (additions to set #1)		
Knob (CM4 #1)		
Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E
Hole (CM4 #2)		
Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Set #3 (additions to set #1)		
Knob (CM4 #1)		
Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R
Hole (CM4 #2)		
Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Set #4		
Knob (CM4 #1)		
Residue #	Native Seq	Potential Mutations
350	T	Y
395	H	Y
Hole (CM4 #2): any combination		
Residue #	Native Seq	Potential Mutations
352	L	S

393	P	F (W)
395	H	A (V,I,L)
397	I	T (S)

Set #5 (additions to set #4)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Set #6 (additions to set #4)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Set #7

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	Y
395	H	F

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
350	T	V (A,I,L)
352	L	S
393	P	F (W)
395	H	A (V,I,L)

Set #8 (additions to set #7)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations

354	T	K,R
397	I	K,R

Set #9 (additions to set #7)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Set #10

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	F

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
352	L	A
393	F	F (W)
395	M	A (V,I,L,S,T)

Set #11 (additions to set #10)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Set #12 (additions to set #10)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Set #13
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	F
395	H	Y

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
352	L	A
393	F	F (W)
395	H	A (V,I,L)
397	I	T (S)

==========

Set #14 (additions to set #13)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========

Set #15 (additions to set #13)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========

Set #16
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	F
395	H	F

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
350	T	V (A,I,L)
352	L	A
393	F	F (W)
395	H	A (V,I,L)

==========
Set #17 (additions to set #16)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========
Set #18 (additions to set #16)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========
Set #19
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	W

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
352	L	G (A)
393	F	F (W)
395	H	A (V,S,T)

==========
Set #20 (additions to set #19)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========
Set #21 (additions to set #19)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========

Set #22

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
350	T	W
395	H	Y

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
352	L	G (A)
393	F	F (W)
395	H	A (V,I,L)
397	I	T (S)

==========

Set #23 (additions to set #22)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========

Set #24 (additions to set #22)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========

Set #25

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations

350	T	W
395	H	F

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
350	T	V (A,I,L)
352	L	G (A)
393	F	F (W)
395	H	A (V,I,L)

==========

Set #26 (additions to set #25)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========

Set #27 (additions to set #25)

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========

Set #28

Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
334	L	C
350	T	W (F)
395	H	F

Hole (CM4 #2): any combination

Residue #	Native Seq	Potential Mutations
334	L	C
350	T	M
352	L	A (V)
393	F	F (W)
395	H	M (I)

==========

Set #29 (additions to set #28)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

==========

Set #30 (additions to set #28)
Knob (CM4 #1)

Residue #	Native Seq	Potential Mutations
354	T	K,R
397	I	K,R

Hole (CM4 #2)

Residue #	Native Seq	Potential Mutations
354	T	D,E
397	I	D,E

==========

Table 8

Human IgM CM4 domain interface residues
Potential Charge Swaps in Cm4 domain

Set #1		
CM4 Chain #1		
Residue #	Native Seq	Potential Mutations
328	R	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
339	E	R (K)
Set #2		
CM4 Chain #1		
Residue #	Native Seq	Potential Mutations
344	R	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
330	D	R (K)
Set #3		
CM4 Chain #1		
Residue #	Native Seq	Potential Mutations
376	K	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
385	E	R (K)
Set #4		
CM4 Chain #1		
Residue #	Native Seq	Potential Mutations
427	R	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
339	E	R (K)

Table C

Human IgM CM2 domain interface residues
Charge Introductions

Set #1		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
116	F	E (D)
133	I	E (D)
CM2 Chain #2		
Residue #	Native Seq	Potential Mutations
116	F	R (K)
133	I	R (K)

Table D

Human IgM CM2 domain interface residues
Knobs-Holes

Set #1		
Knob (CM2 #1)		
Residue #	Native Seq	Potential Mutations
119	P	W
Hole (CM2 #2): any combination		
Residue #	Native Seq	Potential Mutations
115	V	V (I)
117	V	G (A,S)
132	L	M (A)
189	W	V (I,L,M,F)
210	A	G
Set #2		
Knob (CM2 #1)		
Residue #	Native Seq	Potential Mutations
116	F	E (D)
119	P	W
133	I	E (D)
Hole (CM2 #2): any combination		
Residue #	Native Seq	Potential Mutations
115	V	V (I)
116	F	R (K)
117	V	G (A,S)
132	L	M (A)
133	I	R (K)
189	W	V (I,L,M,F)
210	A	G
Set #3		
Knob (CM2 #1)		
Residue #	Native Seq	Potential Mutations
116	F	R (K)
119	P	W
133	I	R (K)
Hole (CM2 #2): any combination		
Residue #	Native Seq	Potential Mutations
115	V	V (I)
116	F	E (D)
117	V	G (A,S)

132	L	M (A)
133	I	E (D)
189	W	V (I,L,M,P)
210	A	G

Table E

Human IgM CM2 domain interface residues
Charge Exchanges

Set #1		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
167	E	K (R)
CM2 Chain #2		
Residue #	Native Seq	Potential Mutations
177	K	E (D)
Set #2		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
169	K	E (D)
CM2 Chain #2		
Residue #	Native Seq	Potential Mutations
170	E	K (R)
Set #3		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
170	E	K (R)
CM2 Chain #2		
Residue #	Native Seq	Potential Mutations
177	K	E (D)
Set #4		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
167	E	K (R)
169	K	E (D)
CM2 Chain #2		
Residue #	Native Seq	Potential Mutations
170	E	K (R)
177	K	E (D)

Table F

Human IgM CM2, CM3 and CM4 domain interface residues
Charge Exchanges

Set #1		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
121	D	K (R)
CM3 Chain #2		
Residue #	Native Seq	Potential Mutations
315	K	D (E)
Set #2		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
150	K	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
385	E	K (R)
Set #3		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
185	K	E (D)
CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
360	D	K (R)
Set #4		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
121	D	K (R)
150	K	E (D)
CM3/CM4 Chain #2		
Residue #	Native Seq	Potential Mutations
315	K	D (E)
385	E	K (R)
Set #5		
CM2 Chain #1		
Residue #	Native Seq	Potential Mutations
121	D	K (R)

185	K	E (D)

CM3/CM4 Chain #2

Residue #	Native Seq	Potential Mutations
315	K	D (E)
360	D	K (R)

****

Set #6

CM2 Chain #1

Residue #	Native Seq	Potential Mutations
150	K	E (D)
185	K	E (D)

CM4 Chain #2

Residue #	Native Seq	Potential Mutations
360	D	K (R)
385	E	K (R)

****

Set #7

CM2 Chain #1

Residue #	Native Seq	Potential Mutations
121	D	K (R)
150	K	E (D)
185	K	E (D)

CM3/CM4 Chain #2

Residue #	Native Seq	Potential Mutations
315	K	D (E)
360	D	K (R)
385	E	K (R)

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/054079

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00 C07K16/28
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/129723 A1 (BLANKENSHIP JOHN W [US] ET AL) 23 May 2013 (2013-05-23) paragraphs [0008], [0044], [0199]; claims 1, 2	1-26,28, 30,32, 34-83, 85,87, 89,91, 93-102

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

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 January 2015	02/02/2015

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International application No
PCT/US2014/054079

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