| | | | |
|---|---|---|---|
| **FcγRIII (CD16)** | IgG1 $5 \times 10^5$ $M^{-1}$ | NK cells<br>Eosinophil<br>Macrophages<br>Neutrophils<br>Mast Cells | Induction of Killing |
| **FcεRI** | IgE $10^{10}$ $M^{-1}$ | Mast cells<br>Eosinophil<br>Basophils | Secretion of granules |
| **FcαRI (CD89)** | IgA1, IgA2 $10^7$ $M^{-1}$ | Macrophages<br>Neutrophils<br>Eosinophils | Uptake Induction of Killing |

## 3. SUMMARY ~~OF THE INVENTION~~

**[0016]** The present disclosure~~invention~~ particularly relates to covalent diabodies and/or covalent diabody molecules and to their use in the treatment of a variety of diseases and disorders including cancer, autoimmune disorders, allergy disorders and infectious diseases caused by bacteria, fungi or viruses. Preferably, the diabody of the present disclosure~~invention~~ can bind to two different epitopes on two different cells wherein the first epitope is expressed on a different cell type than the second epitope, such that the diabody can bring the two cells together.

**[0017]** In detail, the disclosure~~invention~~ provides a polypeptide (and especially an antigen-binding molecule) comprising a polypeptide chain that comprises a portion of a deimmunized serum-binding protein capable of binding to said serum protein; wherein said serum-binding protein portion extends the serum half-life of said polypeptide, relative to the serum half-life of said polypeptide if lacking said portion of said deimmunized serum-binding protein.

**[0018]** The disclosure~~invention~~ also concerns the uses of (or methods involving the use of) such polypeptide portion of a deimmunized serum-binding protein to extend the serum half-life of a polypeptide covalently linked thereto, the extension of serum half-life being relative to the serum half-life of the antigen-binding molecule if lacking the albumin binding protein.

**[0019]** The disclosure~~invention~~ additionally concerns the embodiment of the above-described polypeptide or uses, wherein the polypeptide chain comprises an additional portion of a deimmunized serum-binding protein, wherein the portions of the deimmunized serum-binding protein are both capable of binding to the serum protein.

**[0020]** The disclosure~~invention~~ particularly concerns the embodiment of the above-described polypeptides or uses wherein the polypeptide portion of the deimmunized serum-binding protein is an albumin-binding domain (ABD) of a Streptococcal Protein G, or albumin-binding variant thereof, and especially wherein the albumin-binding domain (ABD) of the Streptococcal Protein G is an albumin-binding domain 3 (ABD3) of protein G of Streptococcus strain G148.

**[0020a]** In one aspect, there is provided a polypeptide that comprises a portion of a deimmunized serum-binding protein capable of binding to said serum protein; wherein said deimmunized serum-binding protein portion is a variant of a wild-type albumin-binding domain (ABD) of a Streptococcal Protein G, said wild-type ABD having the amino acid sequence of SEQ ID NO:304; wherein said variant ABD has an amino acid sequence that differs from that of SEQ ID NO:304 in comprising: (A) a valine to alanine variation at ABD position 71, wherein said variant ABD position is position 31 of SEQ ID NO:304; (B) a leucine to alanine variation at ABD position 64, an isoleucine to alanine variation at ABD position 65, and a valine to alanine variation at ABD position 71; wherein said variant ABD positions are respectively positions 24, 25, and 31 of SEQ ID NO:304; or (C) an asparagine to aspartic acid variation at ABD position 66, a threonine to serine substitution at ABD position 70, and a valine to alanine variation at ABD position 71; wherein said variant ABD positions are respectively positions 26, 30, and 31 of SEQ ID NO:304; wherein said deimmunized serum-binding protein portion extends the serum half-life of said polypeptide, relative to the serum half-life of said polypeptide if lacking said portion of said deimmunized serum-binding protein.

**[0021]** The disclosure~~invention~~ particularly concerns the embodiment of the above-described polypeptides or uses wherein the albumin binding domain comprises:

(A) a valine to alanine variation at position 71, a valine to alanine variation at position 74, or an aspartate to alanine variation at position 79;

(B) a leucine to alanine variation at position 64, isolucine to alanine variation at position 65, and valine to alanine variation at position 71; or

(C) an asparagine to aspartic acid variation at position 66, a threonine to serine substitution at position 70, and a valine to alanine variation at position 71.

**[0022]** The disclosure~~invention~~ further concerns the embodiment of the above-described polypeptides or uses wherein the albumin-binding domain has the sequence of SEQ ID NO: 304, SEQ ID NO: 323, SEQ ID NO: 324, SEQ ID NO: 325, SEQ ID NO: 326, SEQ ID NO: 327, SEQ ID NO: 328, or SEQ ID NO: 329.

**[0022a]** In another aspect, there is provided the use of a portion of a deimmunized serum-binding protein capable of binding to said serum protein to extend the serum half-life of a covalently linked polypeptide, wherein said deimmunized serum-binding protein portion is a variant of a wild-type albumin-binding domain (ABD) of a Streptococcal Protein G, said wild-type ABD having the amino acid sequence of SEQ ID NO:304, and wherein said variant ABD has an amino acid sequence that differs from that of SEQ ID NO:304 in comprising: (A) a valine to alanine variation at ABD position 71, wherein said variant ABD position is position 31 of SEQ ID NO:304; (B) a leucine to alanine variation at ABD position 64, an isoleucine to alanine variation at ABD position 65, and a valine to alanine variation at ABD position 71; wherein said variant ABD positions are respectively positions 24, 25, and 31 of SEQ ID NO:304; or (C) an asparagine to aspartic acid variation at ABD position 66, a threonine to serine substitution at ABD position 70, and a valine to alanine variation at ABD position 71; wherein said variant ABD positions are respectively positions 26, 30, and 31 of SEQ ID NO:304; wherein said extension of serum half-life is relative to the serum half-life of said antigen-binding molecule if lacking said deimmunized serum-binding protein portion.

**[0023]** The disclosure~~invention~~ further concerns the embodiment of the above-described antigen-binding molecules or uses wherein the antigen-binding molecule is an antigen-binding molecule. The disclosure~~invention~~ further concerns the embodiment in which the antigen-binding molecule is a diabody composed of at least a first and a second polypeptide chain which interact with one another to form two antigen-binding sites, wherein at least one of the polypeptide chains comprises the second polypeptide portion that comprises the portion of the deimmunized serum-binding protein that is capable of binding to the serum protein. The disclosure~~invention~~ further concerns the embodiments of such diabodies wherein both the first and the second polypeptide chains comprise second polypeptide portions that comprise the portion of the deimmunized serum-binding

protein capable of binding to the serum protein and/or wherein the first and the second polypeptide chains are covalently linked to one another.

**[0024]** The disclosure~~invention~~ particularly concerns the embodiment of the above-described diabodies or uses, wherein the diabody binds to:

(A) the Natural Killer Group 2D (NKG2D) receptor or the T-cell receptor (TCR); and

(B) a tumor-associated antigen

[0024a] In another aspect, there is provided the use of the diabody as defined herein in the manufacture of a medicament for the treatment of a cancer expressing a tumor-associated antigen, wherein said diabody binds to: (A) the Natural Killer Group 2D (NKG2D) receptor or the T-cell receptor (TCR); and (B) said tumor-associated antigen.

**[0025]** The disclosure~~invention~~ particularly concerns the embodiment of all of the above-described diabodies or uses, wherein the antigen is a breast cancer antigen, an ovarian cancer antigen, a prostate cancer antigen, a cervical cancer antigen, a pancreatic carcinoma antigen, a lung cancer antigen, a bladder cancer antigen, a colon cancer antigen, a testicular cancer antigen, a glioblastoma cancer antigen, an antigen associated with a B cell malignancy, an antigen associated with multiple myeloma, an antigen associated with non-Hodgkins lymphoma, or an antigen associated with chronic lymphocytic leukemia.

**[0026]** In one embodiment, the present disclosure~~invention~~ is directed to a covalent bispecific diabody, which diabody comprises a first and a second polypeptide chain, which first polypeptide chain comprises (i) a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, (ii) a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope, and, optionally, (iii) a third domain comprising at least one cysteine residue, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; which second polypeptide chain comprises (i) a fourth domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), (ii) a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and, optionally, (iii) a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL2)(VH2) that binds the second epitope.

**[0027]** In another embodiment, the present disclosure~~invention~~ is directed to a covalent bispecific diabody, which diabody comprises a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain

variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; which second polypeptide chain comprises (i) a fourth domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), which fourth and fifth domains are covalently linked such that the third and fourth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL2)(VH2) that binds the second epitope.

**[0028]** In certain embodiment~~aspects~~, the present disclosure~~invention~~ is directed to diabody molecule, which molecule comprises a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; which second polypeptide chain comprises **(i)** a fourth domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain

and the fourth domain associate to form a second binding site (VL2)(VH2) that binds the second epitope.

**[0029]** In certain embodiments, the present disclosure~~invention~~ is directed to a covalent bispecific diabody, which diabody is a dimer of diabody molecules, each diabody molecule comprising a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; and which second polypeptide chain comprises **(i)** a fourth domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain of each diabody molecule are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain of each diabody molecule associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain of each diabody molecule associate to form a second binding site (VL2)(VH2) that binds the second epitope.

**[0030]** In yet other embodiments, the present disclosure~~invention~~ is directed to a covalent tetrapecific diabody, which diabody is a dimer of diabody molecules, the first diabody molecule comprising a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; and which second polypeptide chain comprises **(i)** a fourth domain comprising a binding

region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL2)(VH2) that binds the second epitope; and the second diabody molecule comprising a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a third immunoglobulin (VL3) specific for a third epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a fourth immunoglobulin (VH4) specific for a fourth epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site; and which second polypeptide chain comprises **(i)** a fourth domain comprising a binding region of a light chain variable domain of the fourth immunoglobulin (VL4), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the third immunoglobulin (VH3), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL3)(VH3) that binds the third epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL4)(VH4) that binds the fourth epitope.

**[0031]** In certain embodiment~~aspect~~s of the disclosure~~,invention~~ the first epitope, second epitope, and where applicable, third epitope and fourth epitope can be the same. In other embodiment~~aspect~~s, the first epitope, second epitope, and where applicable, third epitope and fourth epitope can each different from the other. In certain embodiment~~aspect~~s of the disclosure~~invention~~ comprising a third epitope binding domain, the first epitope and third epitope can be the same. In certain embodiment~~aspect~~s of

the disclosureinvention comprising a fourth epitope binding domain, the first epitope and fourth epitope can be the same. In certain embodimentaspects of the disclosureinvention comprising a third epitope binding domain, the second epitope and third epitope can be the same. In certain embodimentaspects of the disclosureinvention comprising a fourth epitope binding domain, the second epitope and fourth epitope can be the same. In preferred embodimentaspects of the disclosureinvention, the first eptitope and second epitope are different. In yet other embodimentaspects of the disclosureinvention comprising a third epitope binding domain and a fourth epitope binding domain, the third epitope and fourth epitope can be different. It is to be understood that any combination of the foregoing is encompassed in the present disclosureinvention.

**[0032]** In particular embodimentaspects of the disclosureinvention, the first domain and the fifth domain of the diabody or diabody molecule can be derived from the same immunoglobulin. In another embodimentaspect, the second domain and the fourth domain of the diabody or diabody molecule can be derived from the same immunoglobulin. In yet another embodimentaspect, the first domain and the fifth domain of the diabody or diabody molecule can be derived from a different immunoglobulin. In yet another embodimentaspect, the second domain and the fourth domain of the diabody or diabody molecule can be derived from a different immunoglobulin. It is to be understood that any combination of the foregoing is encompassed in the present disclosureinvention.

**[0033]** In certain embodimentaspects of the disclosureinvention, the covalent linkage between the first polypeptide chain and second polypeptide chain of the diabody or diabody molecule can be via a disulfide bond between at least one cysteine residue on the first polypeptide chain and at least one cysteine residue on the second polypeptide chain. The cysteine residues on the first or second polypeptide chains that are responsible for disulfide bonding can be found anywhere on the polypeptide chain including within the first, second, third, fourth, fifth and sixth domains. In a specific embodiment the cysteine residue on the first polypeptide chain is found in the first domain and the cysteine residue on the second polypeptide chain is found in the fifth domain. The first, second, fourth and fifth domains correspond to the variable regions responsible for binding. In preferred embodiments, the cysteine residues responsible for the disulfide bonding between the first and second polypeptide chains are located within the third and sixth domains, respectively. In a particular aspect of this embodiment, the third domain of the first polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**, which can be encoded by the amino acid sequence **(SEQ ID NO: 17)**. In

another ~~aspect of this~~ embodiment, the sixth domain of the second polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**, which can be encoded by the amino acid sequence **(SEQ ID NO: 17)**. In still another ~~aspect of this~~ embodiment, the third domain of the first polypeptide chain comprises the amino acid sequence VEPKSC **(SEQ ID NO: 77)**, derived from the hinge domain of a human IgG, and which can be encoded by the nucleotide sequence **(SEQ ID NO: 78)**. In another ~~aspect of this~~ embodiment, the sixth domain of the second polypeptide chain comprises the amino acid sequence VEPKSC **(SEQ ID NO: 77)**, derived from the hinge domain of a human IgG, and which can be encoded by the nucleotide sequence **(SEQ ID NO: 78)**. In certain ~~aspects of this~~ embodiments, the third domain of the first polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the sixth domain of the second polypeptide chain comprises the amino acid sequence VEPKSC **(SEQ ID NO: 77)**. In other ~~aspects of this~~ embodiments, the sixth domain of the second polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the third domain of the first polypeptide chain comprises the amino acid sequence VEPKSC **(SEQ ID NO: 77)**. In yet other ~~aspects of this~~ embodiments, the third domain of the first polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the sixth domain of the second polypeptide chain comprises a hinge domain. In other ~~aspects of this~~ embodiments, the sixth domain of the second polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the third domain of the first polypeptide chain comprises the hinge domain. In yet other ~~aspects of this~~ embodiments, the third domain of the first polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the sixth domain of the first polypeptide chain comprises an Fc domain, or portion thereof. In still other ~~aspects of this~~ embodiments, the sixth domain of the second polypeptide chain comprises the C-terminal 6 amino acids of the human kappa light chain, FNRGEC **(SEQ ID NO: 23)**; and the third domain of the first polypeptide chain comprises an Fc domain, or portion thereof.

**[0034]** In other embodiments, the cysteine residues on the first or second polypeptide that are responsible for the disulfide bonding can be located outside of the first, second or

third domains on the first polypeptide chain and outside of the fourth, fifth and sixth domain on the second polypeptide chain. In particular, the cysteine residue on the first polypeptide chain can be N-terminal to the first domain or can be C-terminal to the first domain. The cysteine residue on the first polypeptide chain can be N-terminal to the second domain or can be C-terminal to the second domain. The cysteine residue on the first polypeptide chain can be N-terminal to the third domain or can be C-terminal to the third domain. Further, the cysteine residue on the second polypeptide chain can be N-terminal to the fourth domain or can be C-terminal to the fourth domain. The cysteine residue on the second polypeptide chain can be N-terminal to the fifth domain or can be C-terminal to the fifth domain. Accordingly, the cysteine residue on the second polypeptide chain can be C-terminal to the sixth domain or can be N-terminal to the sixth domain. In a particular embodiment~~aspect~~, disulfide bond can between at least two cysteine residues on the first polypeptide chain and at least two cysteine residues on the second polypeptide chain. In a particular embodiment~~aspect~~, wherein the third domain and sixth domain do not comprise an Fc domain, or portion thereof, the cysteine residue can be at the C-terminus of the first polypeptide chain and at the C-terminus of the second polypeptide chain. It is to be understood that any combination of the foregoing is encompassed in the present disclosure~~invention~~.

**[0035]** In specific embodiments of the disclosure~~invention~~ described *supra*, the covalent diabody of the disclosure~~invention~~ encompasses dimers of diabody molecules, wherein each diabody molecule comprises a first and second polypeptide chain. In certain ~~aspects of this~~ embodiments, the diabody molecules can be covalently linked to form the dimer, with the proviso that the covalent linkage is not a peptide bond. In preferred ~~aspects of this~~ embodiments, the covalent linkage is a disulfide bond between at least one cysteine residue on the first polypeptide chain of each of the diabody molecules of the dimer. In yet more preferred embodiment~~aspects~~ of this disclosure~~invention~~, the covalent linkage is a disulfide bond between at least one cysteine residue on the first polypeptide chain of each of the diabody molecules forming the dimer, wherein said at least one cysteine residue is located in the third domain of each first polypeptide chain.

**[0036]** In certain embodiment~~aspects~~ of the disclosure~~invention~~, the first domain on the first polypeptide chain can be N-terminal to the second domain or can be C-terminal to the second domain. The first domain on the first polypeptide chain can be N-terminal to the third domain or can be C-terminal to the third domain. The second domain on the first polypeptide chain can be

N-terminal to the first domain or can be C-terminal to the first domain. Further, the second domain on the first polypeptide chain can be N-terminal to the third domain or can be C-terminal to the third domain. Accordingly, the third domain on the first polypeptide chain can be N-terminal to the first domain or can be C-terminal to the first domain. The third domain on the first polypeptide chain can be N-terminal to the second domain or can be C-terminal to the second domain. With respect to the second polypeptide chain, the fourth domain can be N-terminal to the fifth domain or can be C-terminal to the fifth domain. The fourth domain can be N-terminal to the sixth domain or can be C-terminal to the sixth domain. The fifth domain on the second polypeptide chain can be N-terminal to the fourth domain or can be C-terminal to the fourth domain. The fifth domain on the second polypeptide chain can be N-terminal to the sixth domain or can be C-terminal to the sixth domain. Accordingly the sixth domain on the second polypeptide chain can be N-terminal to the fourth domain or can be C-terminal to the fourth domain. The sixth domain on the second polypeptide chain can be N-terminal to the fifth domain or can be C-terminal to the fifth domain. It is to be understood that any combination of the foregoing is encompassed in the present disclosure~~invention~~.

**[0037]** In certain embodiments, first domain and second domain can be located C-terminal to the third domain on the first polypeptide chain; or the first domain and second domain can be located N-terminal to the third domain on the first polypeptide chain. With respect to the second polypeptide chain, the fourth domain and fifth domain can be located C-terminal to the sixth domain, or the fourth domain and fifth domain can be located N-terminal to the sixth domain. In certain ~~aspects of this~~ embodiments, the present disclosure~~invention~~ is directed to a covalent bispecific diabody, which diabody is a dimer of diabody molecules, each diabody molecule comprising a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site and wherein the third domain is located N-terminal to both the first domain and second domain; and which second polypeptide chain comprises **(i)** a fourth

domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain of each diabody molecule are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain of each diabody molecule associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain of each diabody molecule associate to form a second binding site (VL2)(VH2) that binds the second epitope.

**[0038]** In yet another embodiment, the present disclosure~~invention~~ is directed to a covalent tetrapecific diabody, which diabody is a dimer of diabody molecules, the first diabody molecule comprising a first and a second polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site and wherein the third domain is located N-terminal to both the first domain and second domain; and which second polypeptide chain comprises **(i)** a fourth domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL1)(VH1) that binds the first epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL2)(VH2) that binds the second epitope; and the second diabody molecule comprises a first and a second

polypeptide chain, which first polypeptide chain comprises **(i)** a first domain comprising a binding region of a light chain variable domain of a third immunoglobulin (VL3) specific for a third epitope, **(ii)** a second domain comprising a binding region of a heavy chain variable domain of a fourth immunoglobulin (VH4) specific for a fourth epitope and **(iii)** a third domain comprising an Fc domain or portion thereof, which first and second domains are covalently linked such that the first and second domains do not associate to form an epitope binding site and wherein the third domain is located N-terminal to both the first domain and second domain; and which second polypeptide chain comprises **(i)** a fourth domain comprising a binding region of a light chain variable domain of the fourth immunoglobulin (VL4), **(ii)** a fifth domain comprising a binding region of a heavy chain variable domain of the third immunoglobulin (VH3), and **(iii)** a sixth domain comprising at least one cysteine residue, which fourth and fifth domains are covalently linked such that the fourth and fifth domains do not associate to form an epitope binding site; and wherein the first polypeptide chain and the second polypeptide chain are covalently linked, with the proviso that the covalent link is not a peptide bond; wherein the first domain and the fifth domain associate to form a first binding site (VL3)(VH3) that binds the third epitope; wherein the second domain and the fourth domain associate to form a second binding site (VL4)(VH4) that binds the fourth epitope.

**[0039]** As discussed above, the domains on the individual polypeptide chains are covalently linked. In specific embodiment~~aspects~~, the covalent link between the first and second domain, first and third domain, second and third domain, fourth and fifth domain, fourth and sixth domain, and/or fifth and sixth domain can be a peptide bond. In particular, the first and second domains, and the fourth and fifth domains can be separated by the third domain and sixth domain, respectively, or by additional amino acid residues, so long as the first and second, and fourth and fifth domains do not associate to form a binding site. The number of amino acid residues can be 0, 1, 2, 3, 4, 5, 6, 7, 8 or 9 amino acid residues. In one preferred embodiment~~aspect~~, the number of amino acid residues between the domains is 8.

**[0040]** In certain embodiments of the disclosure~~aspects of the invention~~, the domains of the first and second polypeptid chain comprising an Fc domain, *i.e.*, optionally, the third and sixth domains, respectively, can further comprise a hinge domain such that the domain comprises a hinge-Fc region. In alternative embodiments, the first polypeptide chain or the second polypeptide chain can comprise a hinge domain without also comprising an Fc domain. The heavy chains,

light chains, hinge regions, Fc domains, and/or hinge-Fc domains for use in the invention can be derived from any immunoglobulin type including IgA, IgD, IgE, IgG or IgM. In a preferred embodimentaspect, the immunoglobulin type is IgG, or any subtype thereof, *i.e.*, $IgG_1$, $IgG_2$, $IgG_3$ or $IgG_4$. In other embodimentaspects, the immunoglobulin from which the light and heavy chains are derived is humanized or chimerized.

**[0041]** Further, the first epitope and second epitopes, and, where applicable, third epitope and fourth epitope, to which the diabody or diabody molecule binds can be different epitopes from the same antigen or can be different epitopes from different antigens. The antigens can be any molecule to which an antibody can be generated. For example, proteins, nucleic acids, bacterial toxins, cell surface markers, autoimmune markers, viral proteins, drugs, etc. In particular embodimentaspects, at least one epitope binding site of the diabody is specific for an antigen on a particular cell, such as a B-cell, a T-cell, a phagocytic cell, a natural killer (NK) cell or a dendritic cell.

**[0042]** In certain aspects of the present embodiments, at least one epitope binding site of the diabody or diabody molecule is specific for a Fc receptor, which Fc receptor can be an activating Fc receptor or an inhibitory Fc receptor. In particular embodimentaspects, the Fc receptor is a Fcγ receptor, and the Fcγ receptor is a FcγRI, FcγRII or FcγRIII receptor. In more preferred embodimentaspects, the FcγRIII receptor is the FcγRIIIA (CD16A) receptor or the FcγRIIIB (CD16B) receptor, and, more preferably, the FcγRIII receptor is the FcγRIIIA (CD16A) receptor. In another preferred embodimentaspect, the FcγRII receptor is the FcγRIIA (CD32A) receptor or the FcγRIIB (CD32B) receptor, and more preferably the FcγRIIB (CD32B) receptor. In a particularly preferred embodimentaspect, one binding site of the diabody is specific for CD32B and the other binding site is specific for CD16A. In a specific embodiment of the disclosureinvention, at least one epitope binding site of the diabody or diabody molecule is specific for an activating Fc receptor and at least one other site is specific for an inhibitory Fc receptor. In certain aspects of this embodiments, the activating Fc receptor is CD32A and the inhibitory Fc receptor is CD32B. In other aspects of this embodiments, the activating Fc receptor is BCR and the inhibitory Fc receptor is CD32B. In still other aspects of this embodiments, the activating Fc receptor is IgERI and the inhibitory Fc receptor is CD32B.

**[0043]** In cases where one epitope binding site is specific for CD16A, the VL and VH domains can be the same as or similar to the VL and VH domains of the mouse antibody

3G8, the sequence of which has been cloned and is set forth herein. In other cases where one epitope binding site is specific for CD32A, the VL and VH domains can be the same as or similar to the VL and VH domains of the mouse antibody IV.3. In yet other cases where one epitope binding site is specific for CD32B, the VL and VH domains can be the same as or similar to the VL and VH domains of the mouse antibody 2B6, the sequence of which has been cloned and is set forth herein. It is to be understood that any of the VL or VH domains of the 3G8, 2B6 and IV.3 antibodies can be used in any combination. The present disclosure~~invention~~ is also directed to a bispecific diabody or diabody molecule wherein the first epitope is specific for CD32B, and the second epitope is specific for CD16A.

**[0044]** In other embodiment~~aspects~~, an epitope binding site can be specific for a pathogenic antigen. As used herein, a pathogenic antigen is an antigen involved in a specific pathogenic disease, including cancer, infection and autoimmune disease. Thus, the pathogenic antigen can be a tumor antigen, a bacterial antigen, a viral antigen, or an autoimmune antigen. Exemplary pathogenic antigens include, but are not limited to lipopolysaccharide, viral antigens selected from the group consisting of viral antigens from human immunodeficiency virus, Adenovirus, Respiratory Syncitial Virus, West Nile Virus (e.g., E16 and/or E53 antigens) and hepatitis virus, nucleic acids (DNA and RNA) and collagen. Preferably, the pathogenic antigen is a neutralizing antigen. In a preferred embodiment~~aspect~~, where one epitope binding site is specific for CD16A or CD32A, the other epitope binding site is specific for a pathogenic antigen excluding autoimmune antigens. In yet another preferred embodiment~~aspect~~, where one epitope binding site is specific for CD32B, the other epitope binding site is specific for any pathogenic antigen. In specific embodiments, the diabody molecule of the disclosure~~invention~~ binds two different antigens on the same cell, for example, one antigen binding site is specific for an activating Fc receptor while the other is specific for an inhibitory Fc receptor. In other embodiments, the diabody molecule binds two distinct viral neutralizing epitopes, for example, but not limited to, E16 and E53 of West Nile Virus.

**[0045]** In yet another embodiment of the present disclosure~~invention~~, the diabodies of the disclosure~~invention~~ can be used to treat a variety of diseases and disorders. Accordingly, the present disclosure~~invention~~ is directed to a method for treating a disease or disorder comprising administering to a patient in need thereof an effective amount of a covalent diabody or diabody molecule of the disclosure~~invention~~ in which at least one binding site is specific for a

pathogenic antigen, such as an antigen expressed on the surface of a cancer cell or on the surface of a bacterium or virion and at least one other binding site is specific for a Fc receptor, *e.g.*, CD16A.

**[0046]** In yet another embodiment, the ~~invention~~disclosure is directed to a method for treating a disease or disorder comprising administering to a patient in need thereof an effective amount of a diabody or diabody molecule of the ~~invention~~disclosure, in which at least one binding site is specific for CD32B and at least one other binding site is specific for CD16A.

**[0047]** In yet another embodiment, the ~~invention~~disclosure is directed to a method for inducing immune tolerance to a pathogenic antigen comprising administering to a patient in need there an effective amount of a covalent diabody or dovalent diabody molecule of the ~~invention~~disclosure, in which at least one binding site is specific for CD32B and at least one other binding site is specific for said pathogenic antigen. In these~~aspects of this~~ embodiments, the pathogenic antigen can be an allergen or another molecule to which immune tolerance is desired, such as a protein expressed on transplanted tissue.

**[0048]** In yet another embodiment, the present ~~invention~~disclosure is directed to a method for detoxification comprising administering to a patient in need thereof an effective amount of a covalent diabody or diabody molecule of the ~~invention~~disclosure, in which at least one binding site is specific for a cell surface marker and at least one other binding site is specific for a toxin. In particular embodiment~~aspect~~s, the diabody of the ~~invention~~disclosure administered is one where one binding site is specific for a cell surface marker such as an Fc and the other binding site is specific for a bacterial toxin or for a drug. In one embodiment~~aspect~~, the cell surface marker is not found on red blood cells.

**[0049]** The present ~~invention~~disclosure additionally provies a method for increasing the half life of a molecule that comprises an antigen-binding domain of an antibody, wherein the method comprises linking (*e.g.*, directly or indirectly via conjugation, or by bonding, *etc.*), two or more albumin binding domains to the molecule. The ~~invention~~disclosure particularly concerns the embodiment of such method wherein the linking of two or more albumin binding domains comprises linking 2, 3, 4, 5, 6, 7, 8, 9, 10 or more albumin binding domains to the molecule, and more particularly concerns the embodiment of such method wherein the linking of two or more albumin binding domains comprises linking 2 albumin binding domains to the molecule. Such two or more albumin binding domains may be the same or

different and are preferably independently selected from the group consisting of the sequence of SEQ ID NO: 304, SEQ ID NO: 323, SEQ ID NO: 324, SEQ ID NO: 325, SEQ ID NO: 326, SEQ ID NO: 327, SEQ ID NO: 328, or SEQ ID NO: 329. The disclosure~~invention~~ particularly concerns the embodiment of such methods wherein the two or more albumin binding domains are the same or wherein 2 of the albumin binding domains are the same. The disclosure~~invention~~ particularly concerns the embodiment of such method wherein the molecule that comprises an antigen-binding domain of an antibody is a diabody.

**[0049a]** In another aspect, there is provided a composition comprising the polypeptide as defined herein and a pharmaceutically acceptable carrier.

### 3.1 DEFINITIONS

**[0050]** Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Current Protocols in Immunology (J. E. Coligan et al., eds., 1999, including supplements through 2001); Current Protocols in Molecular Biology (F. M. Ausubel et al., eds., 1987, including supplements through 2001); Molecular Cloning: A Laboratory Manual, third edition (Sambrook and Russel, 2001); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); The Immunoassay Handbook (D. Wild, ed., Stockton Press NY, 1994); Bioconjugate Techniques (Greg T. Hermanson, ed., Academic Press, 1996); Methods of Immunological Analysis (R. Masseyeff, W. H. Albert, and N. A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993), Harlow and Lane Using Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999; and Beaucage et al. eds., Current Protocols in Nucleic Acid Chemistry John Wiley & Sons, Inc., New York, 2000).

**[0051]** As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, synthetic antibodies, chimeric antibodies, polyclonal antibodies, camelized antibodies, single-chain