Fv-Based Dual-Affinity Re-Targeting Protein Leads To Potent Tumor Cytolysis And In Vivo B-Cell Depletion," J. Molec. Biol. 399(3):436-449; Veri, M.C. *et al.* (2010) "*Therapeutic Control Of B Cell Activation Via Recruitment Of Fcgamma Receptor IIb (CD32B) Inhibitory Function With A Novel Bispecific Antibody Scaffold*," Arthritis Rheum. 62(7):1933-1943; Moore, P.A. *et al.* (2011) "*Application Of Dual Affinity Retargeting Molecules To Achieve Optimal Redirected T-Cell Killing Of B-Cell Lymphoma*," Blood 117(17):4542-4551; US Patent Publications No. 2012/0294796 and 2013/0149236). Such approaches involve engineering one or more cysteine residues into each of the employed polypeptide species. For example, the addition of a cysteine residue to the C-terminus of such constructs has been shown to allow disulfide bonding between the polypeptide chains, stabilizing the resulting heterodimer without interfering with the binding characteristics of the bivalent molecule.

[0017] Diabodies and other immunoglobulins have been described purporting to have specificity for either or both of gpA33 and CD3 (see, *e.g.*, US Patent Publications No. 2012/0014957; 2012/0034160; 2012/0087858; 2012/0189541; 2012/0195900; 2012/0201746; 2012/0237442; 2012/0263722; 2012/0258108; and 2012/0276608).

[0018] Notwithstanding such success, the production of stable, functional heterodimeric, non-monospecific diabodies can be further improved by the careful consideration and placement of the domains employed in the polypeptide chains. The present invention is thus directed to the provision of specific polypeptides that are particularly designed to form, via covalent bonding, heterodimeric diabodies and heterodimeric Fc diabodies that are capable of simultaneously binding gpA33 and CD3.

Summary ~~of the Invention~~:

[0019] The ~~invention~~disclosure is directed to "gpA33 x CD3 bi-specific monovalent diabodies." In particular embodiments, the diabodies of the present ~~invention~~disclosure further have a domain of an immunoglobulin Fc region (*i.e.*, an "Fc Domain") ("gpA33 x CD3 bi-specific monovalent Fc diabodies") or an Albumin-Binding Domain ("ABD")

("gpA33 x CD3 bi-specific monovalent diabodies with ABD") to extend half-life *in vivo*. The gpA33 x CD3 bi-specific monovalent diabodies of the ~~invention~~disclosure and the gpA33 x CD3 bi-specific monovalent Fc diabodies of the ~~invention~~disclosure comprise two different polypeptide chains that associate with one another in a heterodimeric manner to form one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3. The gpA33 x CD3 bi-specific monovalent diabodies and gpA33 x CD3 bi-specific monovalent Fc diabodies of the ~~invention~~disclosure are thus monovalent in that they are capable of binding to only one copy of an epitope of gpA33 and to only one copy of an epitope of CD3, but bi-specific in that a single diabody is able to bind simultaneously to the epitope of gpA33 and to the epitope of CD3.

[0020] The gpA33 x CD3 bi-specific monovalent diabodies of the ~~invention~~disclosure are composed of two polypeptide chains (a "first" and a "second" polypeptide chain), which are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. The gpA33 x CD3 bi-specific monovalent Fc diabodies of the ~~invention~~disclosure are composed of three polypeptide chains (a "first," "second" and "third" polypeptide chain), wherein the first and second polypeptide chains are covalently bonded to one another and the first and third polypeptide chains are covalently bonded to one another. The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present ~~invention~~disclosure are capable of simultaneous binding to gpA33 and CD3. The ~~invention~~disclosure is directed to such gpA33 x CD3 bi-specific monovalent diabodies and bi-specific monovalent gpA33 x CD3 Fc diabodies, and to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The ~~invention~~disclosure is additionally directed to methods for the use of such diabodies in the treatment of cancer and other diseases and conditions.

[0021] In ~~detail~~one aspect, there is ~~invention provides~~ provided a bi-specific monovalent diabody, wherein the bi-specific monovalent diabody is capable of specific binding to an epitope of gpA33 and to an epitope of CD3, wherein the bi-specific monovalent diabody comprises a first polypeptide chain and a second polypeptide chain, wherein the first and second polypeptide chains are covalently bonded to one another, and wherein:

[0022] The ~~invention~~disclosure additionally concerns the embodiment of the above-described bi-specific monovalent diabody wherein the first polypeptide chain or the second polypeptide chain comprises, an Albumin-Binding Domain (**SEQ ID NO:34**), linked C-terminally to Domain 2 or N-terminally to Domain 1A via a Linker 3 (**SEQ ID NO:32**).

[0023] ~~The invention additionally concerns~~In one aspect, there is provided a bi-specific monovalent Fc diabody, wherein the bi-specific monovalent Fc diabody is capable of specific binding to an epitope of gpA33 and to an epitope of CD3, and possesses an IgG Fc Domain, wherein the bi-specific monovalent Fc diabody comprises a first polypeptide chain, a second polypeptide chain and a third polypeptide chain, wherein the first and second polypeptide chains are covalently bonded to one another and the first and third polypeptide chains are covalently bonded to one another, and wherein:

A. the first polypeptide chain comprises, in the N-terminal to C-terminal direction:

   i. a Domain 1, comprising a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to gpA33 (VL_{gpA33}) (**SEQ ID NO:26**) and a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:25**), wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker (**SEQ ID NO:1**);

   ii. a Domain 2, wherein the Domain 2 is an E-coil Domain (**SEQ ID NO:3**) or a K-coil Domain (**SEQ ID NO:4**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:2**); and

   iii. a Domain 3, comprising a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) (**SEQ ID NO:39**) and a sub-Domain (3B), which comprises a polypeptide portion of an IgG Fc Domain having CH2 and CH3 domains of an IgG immunoglobulin Fc Domain; wherein the Domains 3 and 2 are separated from one another by a spacer peptide (Linker 5) (**SEQ ID NO:33**);

B. the second polypeptide chain comprises, in the N-terminal to C-terminal direction:

i. a Domain 1, comprising a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:5**), and a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to gpA33 (VH_{gpA33}) (**SEQ ID NO:27**); wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker (**SEQ ID NO:1**);

ii. a Domain 2, wherein the Domain 2 is a K-coil Domain (**SEQ ID NO:4**) or an E-coil Domain (**SEQ ID NO:3**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:2**); and wherein the Domain 2 of the first polypeptide chain and the Domain 2 of the second polypeptide chain are not both E-coil Domains or both K-coil Domains; and

C. the third polypeptide chain comprises, in the N-terminal to C-terminal direction, a Domain 3 comprising:

(1) a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) (**SEQ ID NO:39**); and

(2) a sub-Domain (3B), which comprises a polypeptide portion of an IgG Fc Domain having CH2 and CH3 domains of an IgG immunoglobulin Fc Domain;

and wherein:

(a) the polypeptide portions of the IgG Fc domains of the first and third polypeptide chain form the IgG Fc Domain;

(b) the VL Domain of the first polypeptide chain and the VH Domain of the second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of CD3; and

(c) the VH Domain of the first polypeptide chain and the VL Domain of the second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of gpA33.

[0024] ~~The invention additionally concerns~~In one aspect, there is provided a bi-specific monovalent Fc diabody, wherein the bi-specific monovalent Fc diabody is capable of specific binding to an epitope of gpA33 and to an epitope of CD3, and possesses an IgG Fc Domain, wherein the bi-specific monovalent Fc diabody comprises a first polypeptide chain, a

wherein the Domain 2 of the first polypeptide chain and the Domain 2 of the second polypeptide chain are not both E-coil Domains or both K-coil Domains; and

C. the third polypeptide chain comprises, in the N-terminal to C-terminal direction, a Domain 3 comprising:

(1) a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) (**SEQ ID NO:39**); and

(2) a sub-Domain (3B), which comprises a polypeptide portion of an IgG Fc Domain having CH2 and CH3 domains of an IgG immunoglobulin Fc Domain;

and wherein:

(a) the polypeptide portions of the IgG Fc domains of the first and third polypeptide chain form the IgG Fc Domain;

(b) the VL Domain of the first polypeptide chain and the VH Domain of the second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of CD3; and

(c) the VH Domain of the first polypeptide chain and the VL Domain of the second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of gpA33.

[0025] The ~~invention~~disclosure further concerns the embodiments of any of the above-described bi-specific monovalent Fc diabodies wherein the sub-Domain (3B) of the first polypeptide chain comprises a sequence different from that of the sub-Domain (3B) of the third polypeptide chain.

[0026] The ~~invention~~disclosure further concerns the embodiments of such above-described bi-specific monovalent Fc diabodies wherein the sub-Domain (3B) of the first polypeptide chain has the amino acid sequence of **SEQ ID NO:40**, and the sub-Domain (3B) of the third polypeptide chain has the amino acid sequence of **SEQ ID NO:41**.

[0027] The ~~invention~~disclosure further concerns the embodiments of such above-described bi-specific monovalent Fc diabodies wherein the sub-Domain (3B) of the first

polypeptide chain has the amino acid sequence of **SEQ ID NO:41**, and the sub-Domain (3B) of the third polypeptide chain has the amino acid sequence of **SEQ ID NO:40**.

[0028] The ~~invention~~disclosure further concerns the embodiments of such above-described bi-specific monovalent Fc diabodies wherein the Domain 3 of the first polypeptide chain and/or the Domain 3 of the third polypeptide chain comprises a variant CH2-CH3 sequence that exhibits altered binding to an Fcγ receptor.

[0029] The ~~invention~~disclosure further concerns the embodiments of any of the above-described bi-specific monovalent diabodies or of any of the above-described bi-specific monovalent Fc diabodies, wherein the Domain 2 of the first polypeptide chain comprises an E-coil (**SEQ ID NO:3**), and the Domain 2 of the second polypeptide chain comprises a K-coil (**SEQ ID NO:4**).

[0030] The ~~invention~~disclosure further concerns the embodiments of any of the above-described bi-specific monovalent diabodies or of any of the above-described bi-specific monovalent Fc diabodies, wherein the Domain 2 of the first polypeptide chain comprises a K-coil (**SEQ ID NO:4**), and the Domain 2 of the second polypeptide chain comprises an E-coil (**SEQ ID NO:3**).

[0031] ~~The invention further concerns~~In one aspect, there is provided a bi-specific monovalent diabody, wherein the bi-specific monovalent diabody is capable of specific binding to an epitope of CD3 and to an epitope of gpA33, wherein the bi-specific monovalent diabody comprises:

(1) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:28**, and a second polypeptide chain having the amino acid sequence of **SEQ ID NO:30**; or

(2) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:35**, and a second polypeptide chain having the amino acid sequence of **SEQ ID NO:30**;

wherein the first and the second polypeptide chains are covalently bonded to one another by a disulfide bond.

[0032] ~~The invention further concerns~~In another aspect, there is provided a bi-specific monovalent Fc diabody, wherein the bi-specific monovalent Fc diabody is capable of specific binding to an epitope of CD3 and to an epitope of gpA33, and possesses an IgG Fc Domain, wherein the bi-specific monovalent Fc diabody comprises:

(1) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:42**, a second polypeptide chain having the amino acid sequence of **SEQ ID NO:44**, and a third polypeptide chain having the amino acid sequence of **SEQ ID NO:46**; or

(2) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:48**, a second polypeptide chain having the amino acid sequence of **SEQ ID NO:28**, and a third polypeptide chain having the amino acid sequence of **SEQ ID NO:46**;

wherein the first and the second polypeptide chains are covalently bonded to one another by a first disulfide bond and the first and third polypeptide chains are covalently bonded to one another by a second disulfide bond.

[0033] ~~The invention further concerns~~In one aspect, there is provided a pharmaceutical composition comprising any of the above-described bi-specific monovalent diabodies or any of the above-described bi-specific monovalent Fc diabodies; and a physiologically acceptable carrier.

[033a] In one aspect, there is provided use of the above-described pharmaceutical composition in the manufacture of a medicament for treatment of a cancer characterized by the expression of gpA33.

[0034] The ~~invention~~disclosure further concerns the use of the above-described pharmaceutical composition in the treatment of a cancer characterized by the expression of gpA33, and especially such use wherein the cancer is colorectal cancer, colon cancer, gastric cancer or pancreatic cancer.

[0035] The ~~invention~~disclosure further concerns a cell that expresses a polypeptide chain of any of the above-described bi-specific monovalent diabodies or any of the above-described bi-specific monovalent Fc diabodies, as well as a polynucleotide that encodes such expressed polypeptide.

[0035a] In one aspect, there is provided a cell that expresses a polypeptide chain of any of the above-described bi-specific monovalent diabodies or a polypeptide chain of any of the above-described bi-specific monovalent Fc diabodies.

[0035b] In one aspect, there is provided a polynucleotide that encodes the above-described polypeptide.

[0036] ~~The invention further concerns~~In one aspect, there is provided a cell that expresses an antibody or a polypeptide portion or fragment thereof, wherein the antibody binds to gpA33, and wherein the antibody or polypeptide portion or fragment thereof comprises:

CD3 and to an epitope of gpA33, wherein said bi-specific monovalent diabody comprises:

(1) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:28**, and a second polypeptide chain having the amino acid sequence of **SEQ ID NO:30**; or

(2) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:35**, and a second polypeptide chain having the amino acid sequence of **SEQ ID NO:30**;

wherein said first and said second polypeptide chains are covalently bonded to one another by a disulfide bond.

Claim 12. A bi-specific monovalent Fc diabody, wherein said bi-specific monovalent Fc diabody is capable of specific binding to an epitope of CD3 and to an epitope of gpA33, and possesses an IgG Fc Domain, wherein said bi-specific monovalent Fc diabody comprises:

(1) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:42**, a second polypeptide chain having the amino acid sequence of **SEQ ID NO:44**, and a third polypeptide chain having the amino acid sequence of **SEQ ID NO:46**; or

(2) a first polypeptide chain having the amino acid sequence of **SEQ ID NO:48**, a second polypeptide chain having the amino acid sequence of **SEQ ID NO:28**, and a third polypeptide chain having the amino acid sequence of **SEQ ID NO:46**;

wherein said first and said second polypeptide chains are covalently bonded to one another by a first disulfide bond and said first and third polypeptide chains are covalently bonded to one another by a second disulfide bond.

Claim 13. A pharmaceutical composition comprising the bi-specific monovalent diabody of any of claims 1-2 or 9-~~12~~11 or the bi-specific monovalent Fc diabody of any of claims 3-10 or 12; and a physiologically acceptable carrier.

Claim 14. Use of the pharmaceutical composition of claim 13 in the manufacture of a medicament for treatment of a cancer characterized by the expression of gpA33.

Claim 15. The use of claim 14, wherein said cancer is colorectal cancer, colon cancer, gastric cancer or pancreatic cancer.

Claim 16. A cell that expresses a polypeptide chain of any of the bi-specific monovalent diabodies of claims 1-2 or 9-~~12~~ 11 or a polypeptide chain of any of the bi-specific monovalent Fc diabodies of claims 3-10 or 12.

Claim 17. A polynucleotide that encodes the polypeptide of claim 16.

Claim 18. A cell that expresses an antibody or a polypeptide portion or fragment thereof, wherein the antibody binds to gpA33, and wherein the antibody or polypeptide portion or fragment thereof comprises:

(1) CDR1 (**SEQ ID NO:14**), CDR2 (**SEQ ID NO:15**) and CDR3 (**SEQ ID NO:16**) of a light chain of an anti-human gpA33 antibody;

(2) CDR1 (**SEQ ID NO:18**), CDR2 (**SEQ ID NO:19**) and CDR3 (**SEQ ID NO:20**) of a heavy chain of an anti-human gpA33 antibody; or

(3) both (1) and (2).