Title of the Invention:

Bi-Specific Monovalent Diabodies That Are Capable Of Binding to gpA33 And CD3, And Uses Thereof

Cross-Reference to Related Applications:

[0001] This Application claims priority to United States Patent Applications No. 61/869,528 (filed on August 23, 2013; pending) and 61/907,691 (filed on November 22, 2013; pending), and to European Patent Application No. 13198859 (filed on December 20, 2013), each of which applications is herein incorporated by reference in its entirety.

Reference to Sequence Listing:

[0002] This application includes one or more Sequence Listings pursuant to 37 C.F.R. 1.821 *et seq.*, which are disclosed in both paper and computer-readable media, and which paper and computer-readable disclosures are herein incorporated by reference in their entirety.

Background of the Invention:

Field of the Invention:

[0003] The present invention is directed to bi-specific monovalent diabodies that comprise two polypeptide chains and which possess one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3 (*i.e.*, a "gpA33 x CD3 bi-specific monovalent diabody"). The present invention also is directed to bi-specific monovalent diabodies that comprise an immunoglobulin Fc Domain ("bi-specific monovalent Fc diabodies") and are composed of three polypeptide chains and which possess one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3 (i.e., a "gpA33 x CD3 bi-specific monovalent Fc diabody"). The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present invention are capable of simultaneous binding to gpA33 and CD3. The invention is directed to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The

invention is additionally directed to methods for the use of such diabodies in the treatment of cancer and other diseases and conditions.

Description of Related Art:

I. gpA33

[0004] Colorectal cancer is among the most common malignancies of the Western world and is a leading cause of cancer deaths (Silverberg, E. *et al.* (1989) "*Cancer Statistics, 1989*," CA Cancer J Clin. 39(1):3-20). One potentially useful target for colon cancer is the 43kD transmembrane glycoprotein A33 (gpA33) ((Heath, J.K. *et al.* (1997) "*The Human A33 Antigen Is A Transmembrane Glycoprotein And A Novel Member Of The Immunoglobulin Superfamily*," Proc. Natl. Acad. Sci. (U.S.A.) 94(2):469-474; Ritter, G. *et al.* (1997) "*Characterization Of Posttranslational Modifications Of Human A33 Antigen, A Novel Palmitoylated Surface Glycoprotein Of Human Gastrointestinal* Epithelium," Biochem. Biophys. Res. Commun. 236(3):682-686). gpA33 was first discovered through raising monoclonal murine antibodies against the human pancreatic carcinoma derived cell line ASPC1. One antibody (MAb A33) was found to react with a surface cell protein of 43 kDa, which was therefore designated "gpA33" (Wong, N.A. *et al.* (2006) "*EpCAM and gpA33 Are Markers Of Barrett's Metaplasia*," J. Clin. Pathol. 59(3):260-263).

[0005] gpA33 is a transmembrane protein of the junctional adhesion molecule family; Abud, H.E. et al. (2000) "*The Murine A33 Antigen Is Expressed At Two Distinct Sites During Development, The ICM Of The Blastocyst And The Intestinal Epithelium*," Mech. Dev. 98(1-2):111-114; Barendswaard, E.C. et al. (1998) "*Rapid And Specific Targeting Of Monoclonal Antibody A33 To A Colon Cancer Xenograft In Nude Mice*," Int. J. Oncol. 12(1):45-53; Panjideh, H. et al. (2008) "*Biodistribution And Efficacy Of [131I]A33scFv::CDy, A Recombinant Antibody-Enzyme Protein For Colon Cancer*," Int. J. Oncol. 32(4):925-930). Although the functional significance of the A33 antigen is not yet understood, it has been shown to mediate colonic mucosal repair in an animal model of colitis and is homogeneously expressed in >95% of all colorectal carcinomas. A33 expression is uniform across both disease stage and degree of histological differentiation, and the antigen is not detectably secreted or

shed into the blood stream (Infante, J.R. et al. (2013) "*Safety, Pharmacokinetics And Pharmacodynamics Of The Anti-A33 Fully-Human Monoclonal Antibody, KRN330, In Patients With Advanced Colorectal Cancer*," Eur. J. Cancer. 49(6):1169-1175; Panjideh, H. et al. (2008) "*Biodistribution And Efficacy Of [131I]A33scFv::CDy, A Recombinant Antibody-Enzyme Protein For Colon Cancer*,"Int. J. Oncol. 32(4):925-930). Conversely, only a few instances of non-gastrointestinal A33 antigen expression have been identified (Johnstone, C.N. et al. (2000) "*Characterization Of Mouse A33 Antigen, A Definitive Marker For Basolateral Surfaces Of Intestinal Epithelial Cells*," Am. J. Physiol. Gastrointest. Liver Physiol. 279(3):G500-G510).

[0006] In light of the highly restricted expression of the A33 antigen, researchers have explored the possibility of treating A33-associated cancers with antibodies (Infante, J.R. *et al.* (2013) "*Safety, Pharmacokinetics And Pharmacodynamics Of The Anti-A33 Fully-Human Monoclonal Antibody, KRN330, In Patients With Advanced Colorectal Cancer*," Eur. J. Cancer. 49(6):1169-1175; Ackerman, M.E. *et al.* (2008) "*A33 Antigen Displays Persistent Surface Expression*," Cancer Immunol. Immunother. 57(7):1017-1027;Barendswaard, E.C. *et al.* (2001) "*Relative Therapeutic Efficacy Of (125)I- And (131)I-Labeled Monoclonal Antibody A33 In A Human Colon Cancer Xenograft*," J. Nucl. Med. 42(8):1251-1256; Carrasquillo, J.A. *et al.* (2011) "*(124)I-huA33 Antibody PET Of Colorectal Cancer*," J. Nucl. Med. 52(8):1173-1180; Chong, G. *et al.* (2005) "*Phase I Trial Of 131I-HuA33 In Patients With Advanced Colorectal Carcinoma*," Clin. Cancer Res. 11(13):4818-4826; Deckert, P.M. *et al.* (2000) "*Pharmacokinetics And Microdistribution Of Polyethylene Glycol-Modified Humanized A33 Antibody Targeting Colon Cancer Xenografts*," Int. J. Cancer. 87(3):382-390; Johnston, A.P. *et al.* (2012) "*Targeting Cancer Cells: Controlling The Binding And Internalization Of Antibody-Functionalized Capsules*" ACS Nano. 6(8):6667-6674; Koppe, M.J. *et al.* (2005) "*Radioimmunotherapy And Colorectal Cancer*," Br. J. Surg. Mar;92(3):264-276; Sakamoto, J. *et al.* (2006) "*A Phase I Radioimmunolocalization Trial Of Humanized Monoclonal Antibody HuA33 In Patients With Gastric Carcinoma*," Cancer Sci. 97(11):1248-1254; Scott, A.M. *et al.* (2005) "*A Phase I Trial Of Humanized Monoclonal Antibody A33 In Patients With Colorectal Carcinoma: Biodistribution, Pharmacokinetics, And Quantitative Tumor Uptake*," Clin. Cancer Res. 11(13):4810-4817; Tschmelitsch, J. *et al.* (1997)

"*Enhanced Antitumor Activity Of Combination Radioimmunotherapy (^{131}I-Labeled Monoclonal Antibody A33) With Chemotherapy (Fluorouracil),*"Cancer Res. 57(11):2181-2186). Likewise fragments of such antibodies have also been evaluated for their potential therapeutic role (Coelho, V. *et al.* (2007) "*Design, Construction, And In Vitro Analysis Of A33scFv::CDy, A Recombinant Fusion Protein For Antibody-Directed Enzyme Prodrug Therapy In Colon Cancer,*" Int. J. Oncol. 31(4):951-957).

II. CD3

[0007] CD3 is a T cell co-receptor composed of four distinct chains (Wucherpfennig, K.W. et al. (2010) "*Structural Biology Of The T-Cell Receptor: Insights Into Receptor Assembly, Ligand Recognition, And Initiation Of Signaling,*" Cold Spring Harb. Perspect. Biol. 2(4):a005140; pages 1-14; Chetty, R. *et al.* (1994) "*CD3: Structure, Function And The Role Of Immunostaining In Clinical Practice,*" J. Pathol. 173:303-307).

[0008] In mammals, the CD3 complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with a molecule known as the T cell receptor (TCR) in order to generate an activation signal in T lymphocytes. In the absence of CD3, TCRs do not assemble properly and are degraded (Thomas, S. et al. (2010) "*Molecular Immunology Lessons From Therapeutic T-Cell Receptor Gene Transfer,*" Immunology 129(2):170–177). CD3 is found bound to the membranes of all mature T cells, and in virtually no other cell type (see, Janeway, C.A. et al. (2005) In: IMMUNOBIOLOGY: THE IMMUNE SYSTEM IN HEALTH AND DISEASE," 6th ed. Garland Science Publishing, NY, pp. 214- 216; Sun, Z. J. et al. (2001) "*Mechanisms Contributing To T Cell Receptor Signaling And Assembly Revealed By The Solution Structure Of An Ectodomain Fragment Of The CD3ε:γ Heterodimer,*" Cell 105(7):913-923; Kuhns, M.S. et al. (2006) "*Deconstructing The Form And Function Of The TCR/CD3 Complex,*" Immunity. 2006 Feb;24(2):133-139).

III. Bi-Specific Diabodies

[0009] The ability of an intact, unmodified antibody (*e.g.*, an IgG) to bind an epitope of an antigen depends upon the presence of variable domains on the immunoglobulin

light and heavy chains (*i.e.*, the VL and VH domains, respectively). The design of a diabody is based on the single chain Fv construct (scFv) (see, *e.g.*, Holliger *et al.* (1993) "*'Diabodies': Small Bivalent And Bispecific Antibody Fragments,*" Proc. Natl. Acad. Sci. (U.S.A.) 90:6444-6448; US Patent Publication No. 2004/0058400 (Hollinger *et al.*); US 2004/0220388 (Mertens *et al.*); Alt *et al.* (1999) FEBS Lett. 454(1-2):90-94; Lu, D. *et al.* (2005) "*A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor Activity,*" J. Biol. Chem. 280(20):19665-19672; WO 02/02781 (Mertens *et al.*); Olafsen, T. *et al.* (2004) "*Covalent Disulfide-Linked Anti-CEA Diabody Allows Site-Specific Conjugation And Radiolabeling For Tumor Targeting Applications,*" Protein Eng. Des Sel. 17(1):21-27; Wu, A. *et al.* (2001) "*Multimerization Of A Chimeric Anti-CD20 Single Chain Fv-Fv Fusion Protein Is Mediated Through Variable Domain Exchange,*" Protein Engineering 14(2):1025-1033; Asano *et al.* (2004) "*A Diabody For Cancer Immunotherapy And Its Functional Enhancement By Fusion Of Human Fc Region,*" Abstract 3P-683, J. Biochem. 76(8):992; Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System,*" Protein Eng. 13(8):583-588; Baeuerle, P.A. *et al.* (2009) "*Bispecific T-Cell Engaging Antibodies For Cancer Therapy,*" Cancer Res. 69(12):4941-4944).

[0010] Interaction of an antibody light chain and an antibody heavy chain and, in particular, interaction of its VL and VH domains forms one of the epitope binding sites of the antibody. In contrast, the scFv construct comprises a VL and VH Domain of an antibody contained in a single polypeptide chain wherein the domains are separated by a flexible linker of sufficient length to allow self-assembly of the two domains into a functional epitope binding site. Where self-assembly of the VL and VH domains is rendered impossible due to a linker of insufficient length (less than about 12 amino acid residues), two of the scFv constructs interact with one another other to form a bivalent molecule in which the VL of one chain associates with the VH of the other (reviewed in Marvin *et al.* (2005) *"Recombinant Approaches To IgG-Like Bispecific Antibodies,"* Acta Pharmacol. Sin. 26:649-658).

[0011] Natural antibodies are capable of binding to only one epitope species (*i.e.*, mono-specific), although they can bind multiple copies of that species (*i.e.*, exhibiting bi-valency or multi-valency). The art has noted the capability to produce diabodies that differ from such natural antibodies in being capable of binding two or more different epitope species (*i.e.*, exhibiting bi-specificity or multispecificity in addition to bi-valency or multi-valency) (see, *e.g.*, Holliger *et al.* (1993) "*'Diabodies': Small Bivalent And Bispecific Antibody Fragments,*" Proc. Natl. Acad. Sci. (U.S.A.) 90:6444-6448; US 2004/0058400 (Hollinger *et al.*); US 2004/0220388 (Mertens *et al.*); Alt *et al.* (1999) FEBS Lett. 454(1-2):90-94; Lu, D. *et al.* (2005) "*A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor Activity,*" J. Biol. Chem. 280(20):19665-19672; WO 02/02781 (Mertens *et al.*); Mertens, N. *et al.*, "*New Recombinant Bi- and Trispecific Antibody Derivatives,*" In: NOVEL FRONTIERS IN THE PRODUCTION OF COMPOUNDS FOR BIOMEDICAL USE, A. VanBroekhoven *et al.* (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands (2001), pages 195-208; Wu, A. *et al.* (2001) "*Multimerization Of A Chimeric Anti-CD20 Single Chain Fv-Fv Fusion Protein Is Mediated Through Variable Domain Exchange,*" Protein Engineering 14(2):1025-1033; Asano *et al.* (2004) "*A Diabody For Cancer Immunotherapy And Its Functional Enhancement By Fusion Of Human Fc Region,*" Abstract 3P-683, J. Biochem. 76(8):992; Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System,*" Protein Eng. 13(8):583-588; Baeuerle, P.A. *et al.* (2009) "*Bispecific T-Cell Engaging Antibodies For Cancer Therapy,*" Cancer Res. 69(12):4941-4944).

[0012] The provision of non-monospecific diabodies provides a significant advantage: the capacity to co-ligate and co-localize cells that express different epitopes. Bivalent diabodies thus have wide-ranging applications including therapy and immunodiagnosis. Bi-valency allows for great flexibility in the design and engineering of the diabody in various applications, providing enhanced avidity to multimeric antigens, the cross-linking of differing antigens, and directed targeting to specific cell types relying on the presence of both target antigens. Due to their increased valency, low dissociation rates and rapid clearance from the circulation (for

diabodies of small size, at or below ~50 kDa), diabody molecules known in the art have also shown particular use in the field of tumor imaging (Fitzgerald *et al.* (1997) *"Improved Tumour Targeting By Disulphide Stabilized Diabodies Expressed In Pichia pastoris,"* Protein Eng. 10:1221). Of particular importance is the co-ligating of differing cells, for example, the cross-linking of cytotoxic T cells to tumor cells (Staerz *et al.* (1985) *"Hybrid Antibodies Can Target Sites For Attack By T Cells,"* Nature 314:628-631, and Holliger *et al.* (1996) *"Specific Killing Of Lymphoma Cells By Cytotoxic T-Cells Mediated By A Bispecific Diabody,"* Protein Eng. 9:299-305).

[0013] Diabody epitope binding domains may also be directed to a surface determinant of any immune effector cell such as CD3, CD16, CD32, or CD64, which are expressed on T lymphocytes, natural killer (NK) cells or other mononuclear cells. In many studies, diabody binding to effector cell determinants, *e.g.*, Fcγ receptors (FcγR), was also found to activate the effector cell (Holliger *et al.* (1996) *"Specific Killing Of Lymphoma Cells By Cytotoxic T-Cells Mediated By A Bispecific Diabody,"* Protein Eng. 9:299-305; Holliger *et al.* (1999) *"Carcinoembryonic Antigen (CEA)-Specific T-cell Activation In Colon Carcinoma Induced By Anti-CD3 x Anti-CEA Bispecific Diabodies And B7 x Anti-CEA Bispecific Fusion Proteins,"* Cancer Res. 59:2909-2916; WO 2006/113665; WO 2008/157379; WO 2010/080538; WO 2012/018687; WO 2012/162068). Normally, effector cell activation is triggered by the binding of an antigen bound antibody to an effector cell via Fc-FcγR interaction; thus, in this regard, diabody molecules of the invention may exhibit Ig-like functionality independent of whether they comprise an Fc Domain (*e.g.*, as assayed in any effector function assay known in the art or exemplified herein (*e.g.*, ADCC assay)). By cross-linking tumor and effector cells, the diabody not only brings the effector cell within the proximity of the tumor cells but leads to effective tumor killing (see *e.g.*, Cao *et al.* (2003) *"Bispecific Antibody Conjugates In Therapeutics,"* Adv. Drug. Deliv. Rev. 55:171-197).

[0014] However, the above advantages come at salient cost. The formation of such non-monospecific diabodies requires the successful assembly of two or more distinct and different polypeptides (*i.e.*, such formation requires that the diabodies be formed through the heterodimerization of different polypeptide chain species). This fact is in

contrast to mono-specific diabodies, which are formed through the homodimerization of identical polypeptide chains. Because at least two dissimilar polypeptides (*i.e.*, two polypeptide species) must be provided in order to form a non-monospecific diabody, and because homodimerization of such polypeptides leads to inactive molecules (Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System,*" Protein Eng. 13(8):583-588), the production of such polypeptides must be accomplished in such a way as to prevent covalent bonding between polypeptides of the same species (Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System,*" Protein Eng. 13(8):583-588). The art has therefore taught the non-covalent association of such polypeptides (see, *e.g.*, Olafsen *et al.* (2004) *"Covalent Disulfide-Linked Anti-CEA Diabody Allows Site-Specific Conjugation And Radiolabeling For Tumor Targeting Applications,"* Prot. Engr. Des. Sel. 17:21-27; Asano *et al.* (2004) "*A Diabody For Cancer Immunotherapy And Its Functional Enhancement By Fusion Of Human Fc Region,*" Abstract 3P-683, J. Biochem. 76(8):992; Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System,*" Protein Eng. 13(8):583-588; Lu, D. *et al.* (2005) "*A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor Activity,*" J. Biol. Chem. 280(20):19665-19672).

[0015] However, the art has recognized that bi-specific monovalent diabodies composed of non-covalently-associated polypeptides are unstable and readily dissociate into non-functional monomers (see, *e.g.*, Lu, D. *et al.* (2005) "*A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor Activity,*" J. Biol. Chem. 280(20):19665-19672).

[0016] In the face of this challenge, the art has succeeded in developing stable, covalently bonded heterodimeric non-monospecific diabodies (see, *e.g.*, WO 2006/113665; WO/2008/157379; WO 2010/080538; WO 2012/018687; WO/2012/162068; Johnson, S. *et al.* (2010) "*Effector Cell Recruitment With Novel*

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:

[0019] The disclosure is directed to "gpA33 x CD3 bi-specific monovalent diabodies." In particular embodiments, the diabodies of the present 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 disclosure and the gpA33 x CD3 bi-specific monovalent Fc diabodies of the 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 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 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 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 disclosure are capable of simultaneous binding to gpA33 and CD3. The 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 disclosure is additionally directed to methods for the use of such diabodies in the treatment of cancer and other diseases and conditions.

[0021] 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 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:

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 CD3 (VL_{CD3}) having the amino acid sequence of 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}) having the amino acid sequence of SEQ ID NO:27; wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;

ii. a Domain 2, wherein the Domain 2 is a K-coil Domain having the amino acid sequence of SEQ ID NO:4 or an E-coil Domain having the amino acid sequence of SEQ ID NO:3, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of SEQ ID NO:2;

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 gpA33 (VL_{gpA33}) having the amino acid sequence of 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}) having the amino acid sequence of SEQ ID NO:25, wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;

ii. a Domain 2, wherein the Domain 2 is an E-coil Domain having the amino acid sequence of SEQ ID NO:3 or a K-coil Domain having the amino acid sequence of SEQ ID NO:4, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of 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 wherein:

(a) 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

(b) 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.

[0022] The 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] 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}) and having the amino acid sequence of 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}) and having the amino acid sequence of SEQ ID NO:25, wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;

   ii. a Domain 2, wherein the Domain 2 is an E-coil Domain having the amino acid sequence of SEQ ID NO:3 or a K-coil Domain having the amino acid sequence of SEQ ID NO:4, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of SEQ ID NO:2; and

   iii. a Domain 3, comprising a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) having the amino acid sequence of 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 having sequence GGG (Linker 5);

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}) and having the amino acid sequence of 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}) and having the amino acid sequence of SEQ ID NO:27; wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;

ii. a Domain 2, wherein the Domain 2 is a K-coil Domain having the amino acid sequence of SEQ ID NO:4 or an E-coil Domain having the amino acid sequence of SEQ ID NO:3, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of 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) having the amino acid sequence of 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 gpA33; 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 CD3.

[0024] 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 3, comprising a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) having the amino acid sequence of 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;
   ii. a Domain 1, comprising a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to gpA33 (VL_{gpA33}) and having the amino acid sequence of 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}) and having the amino acid sequence of SEQ ID NO:25, wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;
   wherein the Domains 1 and 3 are separated from one another by a spacer peptide (Linker 4) having the amino acid sequence of SEQ ID NO:38;
   iii. a Domain 2, wherein the Domain 2 is an E-coil Domain having the amino acid sequence of SEQ ID NO:3 or a K-coil Domain having the amino acid sequence of SEQ ID NO:4, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of SEQ ID NO:2; and

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}) and having the amino acid sequence of 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}) and having the amino acid sequence of SEQ ID NO:27; wherein the sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of SEQ ID NO:1;
   ii. a Domain 2, wherein the Domain 2 is a K-coil Domain having the amino acid sequence of SEQ ID NO:4 or an E-coil Domain having the amino acid sequence of SEQ ID NO:3, wherein the Domain 2 is separated from the Domain 1 by a peptide linker having the amino acid sequence of 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) having the amino acid sequence of 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 gpA33; 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 CD3.

[0025] The 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 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 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 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 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 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] 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] 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] In one aspect, there is provided a pharmaceutical composition comprising any one of the above-described bi-specific monovalent diabodies or any one 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, wherein said cancer is colorectal cancer, colon cancer, gastric cancer or pancreatic cancer.

[0034] The 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 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 said first polypeptide chain and/or said second polypeptide chain of any of the above-described bi-specific monovalent diabodies or 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] Disclosed is 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).

Brief Description of the Drawings:

[0037] **Figure 1** illustrates the structures of the first and second polypeptide chains of a two chain gpA33 x CD3 bi-specific monovalent diabody of the present invention.

[0038] **Figures 2A and 2B** illustrate the structures of two versions of the first, second and third polypeptide chains of a three chain gpA33 x CD3 bi-specific monovalent Fc diabody of the present invention (Version 1, **Figure 2A**; Version 2, **Figure 2B**).

[0039] **Figure 3** demonstrates that the diabodies of the present invention are capable of simultaneously binding to CD3 and to gpA33.

[0040] **Figure 4** illustrates the ability of the diabodies of the present invention to treat cancer. Colorectal or pancreatic cancer cells were incubated in the presence of the gpA33 x CD3 bi-specific monovalent diabody ("DART-1) and either human PBMC (E:T = 25:1) or activated human T cells (E:T = 10:1), and cytotoxicity was measured (**Figure 4A** (Colon CSCL colorectal cells), **Figure 4B** (Colo205 colorectal cells), and **Figure 4C** (ASPC-1 pancreatic cancer cells).

[0041] **Figures 5A-5F** show that activation of CD8 T cells occurred in the presence of the CD3 bi-specific monovalent diabody ("DART-1) only in the presence of cancer cells (**Figures 5A-5C**: CD8 T cells + colo205 cells (**Figure 5A**), CD8 T cells + ASPC-1 cells (**Figure 5B**), CD8 T cells alone (**Figure 5C**); **Figures 5D-5F**: CD4 T cells + colo205 cells (**Figure 5D**), CD4 T cells + ASPC-1 cells (**Figure 5E**), CD8 T cells alone (**Figure 5F**).

[0042] **Figures 6A-6D** demonstrate that gpA33 x CD3 bi-specific monovalent diabodies (DART-1 and DART-2) mediated equivalent cytotoxicity for SW948

colorectal adenocarcinoma cells (**Figure 6A**) and colo205 cells (**Figure 6B**) and Colo205-Luc cells (**Figure 6C**), and that neither diabody mediated cytotoxicity of the gpA33-negative cancer cell line, HCT116 (**Figure 6D**).

[0043] **Figures 7A-7D** demonstrate the ability of the gpA33 x CD3 bi-specific monovalent diabody (DART-2), the gpA33 x CD3 bi-specific monovalent diabody having an Albumin-Binding Domain (DART-2 with ABD "w/ABD") and the gpA33 x CD3 bi-specific monovalent diabody having an immunoglobulin IgG Fc Domain (DART-2 with Fc "w/Fc") to promote the cytotoxicity of cancer cells in the presence of human or cynomolgus monkey PBMCs.

[0044] **Figure 8** demonstrates the *in vivo* ability of the gpA33 x CD3 bi-specific monovalent diabody (DART-1) to decrease tumor volume in a murine Colo205 colon cancer model.

[0045] **Figures 9A-9D** shows tumor imaging data of NOD scid gamma (NSG) mice implanted with Colo205 cells two days after receiving Vehicle (**Figure 9A**) or the gpA33 x CD3 bi-specific monovalent diabody (DART-1) (**Figure 9B**), and 12 days after receiving Vehicle (**Figure 9C**) or the DART-1 (**Figure 9D**).

[0046] **Figure 10** demonstrates the *in vivo* ability of the gpA33 x CD3 bi-specific monovalent diabody (DART-1) to decrease tumor volume in a murine ASPC-1 pancreatic cancer model.

[0047] **Figure 11** shows the ability of the gpA33 x CD3 bi-specific monovalent diabody having an immunoglobulin IgG Fc Domain (DART-2 w/Fc Version 1) to mediate a dramatic reduction in tumor volume in an *in vivo* colon cancer model.

[0048] **Figure 12** shows the ability of the gpA33 x CD3 bi-specific monovalent diabody having an immunoglobulin IgG Fc Domain (DART-2 w/Fc Version 1) to mediate a reduction in tumor volume in an *in vivo* colon cancer model even at extremely low doses.

[0049] **Figure 13** shows the pharmacokinetics of the gpA33 x CD3 bi-specific monovalent diabody (DART-2), and gpA33 x CD3 bi-specific monovalent diabody

having an immunoglobulin IgG Fc Domain (DART-2 w/Fc Version 1) diabodies in cynomolgus monkeys.

[0050] **Figures 14A-14B** show SPR analysis of the binding of DART-2 w/Fc Version 1 to immobilized human and cynomolgus monkey CD3. The black dashed lines represent the global fit to a 1:1 Langmuir model of binding curves obtained at DART-2 w/Fc concentrations of 0, 6.25, 12.5, 25, 50 or 100 nM. The data are representative of three independent experiments.

[0051] **Figures 15A-15B** show SPR analysis of the binding of DART-2 w/Fc Version 1 to captured human and cynomolgus monkey gpA33. The black dashed lines represent the global fit to a 1:1 Langmuir model of binding curves obtained at DART-2 w/Fc Version 1 concentration of 0, 6.25, 12.5, 25, 50 or 100 nM. The data are representative of three independent experiments.

Detailed Description of the Invention:

[0052] The present invention is directed to bi-specific monovalent diabodies that comprise two polypeptide chains and which possess one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3 (*i.e.*, a "gpA33 x CD3 bi-specific monovalent diabody"). The present invention also is directed to bi-specific monovalent diabodies that comprise an immunoglobulin Fc Domain ("bi-specific monovalent Fc diabodies") and are composed of three polypeptide chains and which possess one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3 (*i.e.*, a "gpA33 x CD3 bi-specific monovalent Fc diabody"). The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies the present invention are capable of simultaneous binding to gpA33 and CD3. The invention is directed to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The invention is additionally directed to methods for the use of such diabodies in the treatment of cancer and other diseases and conditions.

[0053] The gpA33 x CD3 bi-specific monovalent diabodies of the present invention are composed of two polypeptide chains that associate with one another to form one binding site specific for an epitope of gpA33 and one binding site specific for an

epitope of CD3. The individual polypeptide chains of the diabody are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. Each polypeptide chain contains an Antigen Binding Domain of a Light Chain Variable Domain, an Antigen Binding Domain of a Heavy Chain Variable Domain and a heterodimerization Domain. An intervening linker peptide (Linker 1) separates the Antigen Binding Domain of the Light Chain Variable Domain from the Antigen Binding Domain of the Heavy Chain Variable Domain. The Antigen Binding Domain of the Light Chain Variable Domain of the first polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the second polypeptide chain in order to form a first functional antigen binding site that is specific for the first antigen (*i.e.*, either gpA33 or CD3). Likewise, the Antigen Binding Domain of the Light Chain Variable Domain of the second polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the first polypeptide chain in order to form a second functional antigen binding site that is specific for the second antigen (*i.e.*, either gpA33 or CD3, depending upon the identity of the first antigen). Thus, the selection of the Antigen Binding Domain of the Light Chain Variable Domain and the Antigen Binding Domain of the Heavy Chain Variable Domain of the first and second polypeptide chains are coordinated, such that the two polypeptide chains collectively comprise Antigen Binding Domains of Light and Heavy Chain Variable Domains capable of binding to gpA33 and CD3.

[0054] The gpA33 x CD3 bi-specific monovalent Fc diabodies of the present invention are composed of a first polypeptide chain, a second polypeptide chain and a third polypeptide chain. The first and second polypeptide chains associate with one another to form one binding site specific for an epitope of gpA33 and one binding site specific for an epitope of CD3. The first polypeptide chain and the third polypeptide chain associate with one another to form an immunoglobulin Fc Domain. The first and second polypeptide chains of the bi-specific monovalent Fc diabody are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. The first and third polypeptide chains are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. The first and second polypeptide

chains each contain an Antigen Binding Domain of a Light Chain Variable Domain, an Antigen Binding Domain of a Heavy Chain Variable Domain and a heterodimerization Domain. An intervening linker peptide (Linker 1) separates the Antigen Binding Domain of the Light Chain Variable Domain from the Antigen Binding Domain of the Heavy Chain Variable Domain. The Antigen Binding Domain of the Light Chain Variable Domain of the first polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the second polypeptide chain in order to form a first functional antigen binding site that is specific for the first antigen (*i.e.*, either gpA33 or CD3). Likewise, the Antigen Binding Domain of the Light Chain Variable Domain of the second polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the first polypeptide chain in order to form a second functional antigen binding site that is specific for the second antigen (*i.e.*, either gpA33 or CD3, depending upon the identity of the first antigen). Thus, the selection of the Antigen Binding Domain of the Light Chain Variable Domain and the Antigen Binding Domain of the Heavy Chain Variable Domain of the first and second polypeptide chains are coordinated, such that the two polypeptide chains collectively comprise Antigen Binding Domains of light and Heavy Chain Variable Domains capable of binding to gpA33 and CD3. The first and third polypeptide chains each contain a cysteine-containing peptide (Peptide 1) **SEQ ID NO:39**: and some or all of the CH2 Domain and/or some or all of the CH3 Domain of a complete immunoglobulin Fc Domain and a cysteine-containing peptide. The some or all of the CH2 Domain and/or the some or all of the CH3 Domain associate to form the immunoglobulin Fc Domain of the bi-specific monovalent Fc diabodies of the present invention. The first and third polypeptide chains of the bi-specific monovalent Fc diabodies of the present invention are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within the cysteine-containing peptide of the polypeptide chains.

[0055] The formation of heterodimers of the first and second polypeptide chains of the bi-specific monovalent diabody or bi-specific monovalent Fc diabody can be driven by the heterodimerization domains. Such domains include GVEPKSC (**SEQ ID NO:54**) (or VEPKSC; **SEQ ID NO:55**) on one polypeptide chain and GFNRGEC

(**SEQ ID NO:56**) (or FNRGEC; **SEQ ID NO:57**) on the other polypeptide chain (US2007/0004909). Alternatively, such domains can be engineered to contain coils of opposing charges. The heterodimerization Domain of one of the polypeptide chains comprises a sequence of at least six, at least seven or at least eight positively charged amino acids, and the heterodimerization Domain of the other polypeptide chain comprises a sequence of at least six, at least seven or at least eight negatively charged amino acids. For example, the first or the second heterodimerization Domain may comprise a sequence comprising eight positively charged amino acids and the other of the heterodimerization domains may comprise a sequence comprising eight negatively charged amino acids. The positively charged amino acid may be lysine, arginine, histidine, etc. and/or the negatively charged amino acid may be glutamic acid, aspartic acid, etc. The positively charged amino acid is preferably lysine and/or the negatively charged amino acid is preferably glutamic acid.

[0056] The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present invention are engineered so that such first and second polypeptide chains covalently bond to one another via cysteine residues along their length. Such cysteine residues may be introduced into the intervening linker that separates the VL and VH domains of the polypeptides. Alternatively, and more preferably, a second peptide (Linker 2) is introduced into each polypeptide chain, for example, at the amino-terminus of the polypeptide chains or at a position that places Linker 2 between the heterodimerization Domain and the Antigen Binding Domain of the Light Chain Variable Domain or Heavy Chain Variable Domain.

[0057] As indicated above, gpA33 is expressed by colorectal cells. Antibodies capable of immunospecifically binding to gpA33 are capable of binding to such cells. CD3 is expressed on T cells. Thus, antibodies capable of immunospecifically binding to both gpA33 and CD3 are capable of targeting T cells to colorectal and other cancer cells that express gpA33 (*e.g.*, colon carcinoma cells, pancreatic cancer cells, *etc.*) and of thus providing an improved therapy for such cancers.

I. Preferred gpA33 x CD3 Bi-Specific Monovalent Diabodies of the Present Invention

A. gpA33 x CD3 Bi-Specific Monovalent Diabodies

[0058] One embodiment of the present invention relates to gpA33 x CD3 bi-specific monovalent diabodies that are composed of a first polypeptide chain and a second polypeptide chain, whose sequences permit the polypeptide chains to covalently bind to each other to form a covalently-associated complex that is capable of simultaneously binding to both gpA33 and CD3.

[0059] The first polypeptide chain of preferred gpA33 x CD3 bi-specific monovalent diabodies comprise, in the N-terminal to C-terminal direction, an N-terminus, the VL Domain of a monoclonal antibody capable of binding to either CD3 or gpA33 (*i.e.*, either VL_{CD3} or VL_{gpA33}), a first intervening spacer peptide (Linker 1), a VH Domain of a monoclonal antibody capable of binding to either gpA33 (if such first polypeptide chain contains VL_{CD3}) or CD3 (if such first polypeptide chain contains VL_{gpA33}), a cysteine-containing second intervening spacer peptide (Linker 2), a heterodimer-promoting Domain and a C-terminus (**Figure 1**).

[0060] The second polypeptide chain of preferred gpA33 x CD3 bi-specific monovalent diabodies comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL Domain of a monoclonal antibody capable of binding to either gpA33 or CD3 (*i.e.*, either VL_{gpA33} or VL_{CD3}, depending upon the VL Domain selected for the first polypeptide chain of the diabody), an intervening linker peptide (Linker 1), a VH Domain of a monoclonal antibody capable of binding to either CD3 (if such second polypeptide chain contains VL_{gpA33}) or CD3 (if such second polypeptide chain contains VL_{CD3}), a cysteine-containing spacer peptide (Linker 2), a heterodimer-promoting Domain, and a C-terminus (**Figure 1**).

[0061] The VL Domain of the first polypeptide chain of preferred gpA33 x CD3 bi-specific monovalent diabodies interacts with the VH Domain of the second polypeptide chain of preferred gpA33 x CD3 bi-specific monovalent diabodies in order to form a first functional antigen binding site that is specific for a first antigen (*i.e.*, either CD3 or gpA33). Likewise, the VL Domain of the second polypeptide

chain interacts with the VH Domain of the first polypeptide chain in order to form a second functional antigen binding site that is specific for a second antigen (*i.e.*, either gpA33 or CD3, depending upon the identity of the first antigen). Thus, the selection of the VL and VH domains of the first and second polypeptide chains are coordinated, such that the two polypeptide chains of preferred gpA33 x CD3 bi-specific monovalent diabodies collectively comprise VL and VH domains capable of binding to gpA33 and CD3 (*i.e.*, they comprise VL_{CD3}/VH_{CD3} and VL_{gpA33}/VH_{gpA33}).

[0062] Most preferably, the length of the intervening linker peptide (Linker 1, which separates such VL and VH domains) is selected to substantially or completely prevent the VL and VH domains of the polypeptide chain from binding to one another. Thus the VL and VH domains of the first polypeptide chain are substantially or completely incapable of binding to one another. Likewise, the VL and VH domains of the second polypeptide chain are substantially or completely incapable of binding to one another. A preferred intervening spacer peptide (Linker 1) has the sequence (**SEQ ID NO:1**): GGGSGGGG.

[0063] The cysteine-containing second intervening spacer peptide (Linker 2) will contain 1, 2, 3 or more cysteines. A preferred cysteine-containing spacer peptide (Linker 2) has the sequence is **SEQ ID NO:2**: GGCGGG.

[0064] The heterodimer-promoting domains of the first and second polypeptides differ from one another and are designed to associate with one another so as to promote association of the first and second polypeptide chains. Thus, in a preferred embodiment, one of these polypeptide chains will be engineered to contain a heterodimer-promoting "E-coil" Domain (**SEQ ID NO:3**):

EVAALEKEVAALEKEVAALEKEVAALEK

whose residues will form a negative charge at pH 7, while the other of the two polypeptide chains will be engineered to contain a heterodimer-promoting "K-coil" Domain (**SEQ ID NO:4**):

KVAALKEKVAALKEKVAALKEKVAALKE

whose residues will form a positive charge at pH 7. The presence of such charged domains promotes association between the first and second polypeptides, and thus fosters heterodimerization. It is immaterial which coil is provided to which chain, as long as the coils employed on the first and second polypeptide chains differ so as to foster heterodimerization between such chains.

1. The gpA33 x CD3 Bi-Specific Monovalent Diabody, "DART-1"

[0065] The first and second polypeptide chains of a preferred gpA33 x CD3 bi-specific monovalent diabody, designated herein as "DART-1" comprise polypeptide domains having the following sequences:

[0066] The VL Domain of an antibody that binds CD3 (VL_{CD3}) **(SEQ ID NO:5)**:

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG

[0067] The Antigen Binding Domain of VL_{CD3} comprises CDR1 having the sequence: **(SEQ ID NO:6)** RSSTGAVTTSNYAN; CDR2 having the sequence **(SEQ ID NO:7)**: GTNKRAP; and CDR3 having the sequence **(SEQ ID NO:8)**: ALWYSNLWV.

[0068] The VH Domain of an antibody that binds CD3 (VH_{CD3}) **(SEQ ID NO:9)**:

EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKY
NNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVS
WFAYWGQGTLVTVSS

[0069] The Antigen Binding Domain of VH_{CD3} comprises: CDR1 having the sequence **(SEQ ID NO:10)**: TYAMN; CDR2 having the sequence **(SEQ ID NO:11)** RIRSKYNNYATYYADSVKD; and CDR3 having the sequence **(SEQ ID NO:12)**: HGNFGNSYVSWFAY.

[0070] The VL Domain of a murine antibody that binds gpA33 (VL_{gpA33}) **(SEQ ID NO:13)**:

QIVLTQSPAIMSASPGERVTMTCSARSSISFMYWYQQKPGSSPRLLIYDTSNLAS
GVPVRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSSYPLTFGSGTKLELK

[0071] The Antigen Binding Domain of VL_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:14**): SARSSISFMY; CDR2 having the sequence (**SEQ ID NO:15**): DTSNLAS; and CDR3 having the sequence (**SEQ ID NO:16**): QQWSSYPLT.

[0072] The VH Domain of a murine antibody that binds gpA33 (VH_{gpA33}) (**SEQ ID NO:17**):

QVQLQQSGPELVKPGASVKISCKASGYTFSGSWMNWVKQRPGQGLEWIGRIYPGD
GETNYNGKFKDKATLTADKSSTTAYMELSSLTSVDSAVYFCARIYGNNVYFDVWG
AGTTVTVSS

[0073] The Antigen Binding Domain of VH_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:18**): GSWMN; CDR2 having the sequence (**SEQ ID NO:19**): RIYPGDGETNYNGKFKD; and CDR3 having the sequence (**SEQ ID NO:20**): IYGNNVYFDV.

[0074] The first intervening spacer peptide (Linker 1) has the sequence (**SEQ ID NO:1**): GGGSGGGG. The cysteine-containing spacer peptide (Linker 2) has the sequence is **SEQ ID NO:2**: GGCGGG.

[0075] The heterodimer-promoting Domain of the first polypeptide chain is the "E-coil" Domain (**SEQ ID NO:3**). The heterodimer-promoting Domain of the second polypeptide chain is the "K-coil" Domain (**SEQ ID NO:4**).

[0076] Thus, the first polypeptide chain of DART-1 has the sequence (**SEQ ID NO:21**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG
GGGSGGGGQVQLQQSGPELVKPGASVKISCKASGYTFSGSWMNWVKQRPGQGLEW
IGRIYPGDGETNYNGKFKDKATLTADKSSTTAYMELSSLTSVDSAVYFCARIYGN
NVYFDVWGAGTTVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEK

[0077] As will be appreciated, residues 1-110 of **SEQ ID NO:21** are the VL Domain of an antibody that binds CD3 (VL_{CD3}) (**SEQ ID NO:5**); residues 111-118 of **SEQ ID NO:21** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 119-237 of **SEQ ID NO:21** are the VH Domain of a murine antibody that binds gpA33 (VH_{gpA33}) (**SEQ ID NO:17**), residues 238-243 of **SEQ ID NO:21** are

the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**) and residues 244-271 of **SEQ ID NO:21** are the heterodimer-promoting "E-coil" Domain (**SEQ ID NO:3**).

[0078] A preferred polynucleotide that encodes the first polypeptide chain of DART-1 has the sequence (**SEQ ID NO:22**):

caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactgtga
ccctgacatgcagatccagcacaggcgcagtgaccacatctaactacgccaattg
ggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggtacaaacaaa
agggctccctggacccctgcacggttttctggaagtctgctgggcggaaaggccg
ctctgactattaccggggcacaggccgaggacgaagccgattactattgtgctct
gtggtatagcaatctgtgggtgttcgggggtggcacaaaactgactgtgctggga
ggtggtggatccggcggaggtggacaggtccagctgcagcagtctggacctgagc
tggtgaagcctggggcctcagtgaagatttcctgcaaagcttcaggctacacatt
cagtggctcttggatgaactgggtgaagcagaggcctggacagggtcttgagtgg
attggacggatctaccctggagatggagaaactaactacaatgggaagtttaagg
acaaggccacactgactgcagacaaatcatccaccacagcctacatggagctcag
cagcctgacctctgtggactctgcggtctatttctgtgcaagaatctatggtaat
aacgtttacttcgatgtctggggcgcagggaccacggtcaccgtgtcttccggag
gatgtggcggtggagaagtggccgcactggagaaagaggttgctgctttggagaa
ggaggtcgctgcacttgaaaaggaggtcgcagccctggagaaa

[0079] The second polypeptide chain of DART-1 has the sequence (**SEQ ID NO:23**):

QIVLTQSPAIMSASPGERVTMTCSARSSISFMYWYQQKPGSSPRLLIYDTSNLAS
GVPVRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSSYPLTFGSGTKLELKRGGG
SGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVAR
IRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFG
NSYVSWFAYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKE

[0080] As will be appreciated, residues 1-107 of **SEQ ID NO:23** are the VL Domain of a murine antibody that binds gpA33 ($\mathbf{VL_{gpA33}}$) (**SEQ ID NO:13**); residues 108-115 of **SEQ ID NO:23** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 116-240 of **SEQ ID NO:23** are the VH Domain of an antibody that binds CD3 ($\mathbf{VH_{CD3}}$) (**SEQ ID NO:9**), residues 241-246 of **SEQ ID NO:23** are the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**) and residues 247-274 of **SEQ ID NO:23** are the heterodimer-promoting "K-coil" Domain (**SEQ ID NO:4**).

[0081] A preferred polynucleotide that encodes the second polypeptide chain of DART-1 has the sequence **(SEQ ID NO:24)**:

caaattgttctcacccagtctccagcaatcatgtctgcatctccaggggagaggg
tcaccatgacctgcagtgccaggtcaagtataagtttcatgtactggtaccagca
gaagccaggatcctcccccagactcctgatttatgacacatccaacctggcttct
ggagtccctgttcgcttcagtggcagtgggtctgggacctcttattctctcacaa
tcagccgaatggaggctgaagatgctgccacttattactgccagcagtggagtag
ttacccactcacgttcggttctgggaccaagctggagctgaaacggggtggagga
tccggcggaggcggagaggtgcagctggtggagtctgggggaggcttggtccagc
ctggagggtccctgagactctcctgtgcagcctctggattcaccttcaacacata
cgctatgaattgggtccgccaggctccagggaaggggctggagtgggttgcaagg
atcaggtccaagtacaacaattatgcaacctactatgccgactctgtgaaggata
gattcaccatctcaagagatgattcaaagaactcactgtatctgcaaatgaacag
cctgaaaaccgaggacacggccgtgtattactgtgtgagacacggtaacttcggc
aattcttacgtgtcttggtttgcttattggggacaggggacactggtgactgtgt
cttccggaggatgtggcggtggaaaagtggccgcactgaaggagaaagttgctgc
tttgaaagagaaggtcgccgcacttaaggaaaaggtcgcagccctgaaagag

2. The gpA33 x CD3 Bi-Specific Monovalent Diabody, "DART-2"

[0082] The first and second polypeptide chains of a second preferred gpA33 x CD3 bi-specific monovalent diabody, designated herein as "DART-2," comprise polypeptide domains having the following sequences:

[0083] The VL Domain of an antibody that binds CD3 (VL_{CD3}) **(SEQ ID NO:5)**:

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG

[0084] The Antigen Binding Domain of VL_{CD3} comprises CDR1 having the sequence: **(SEQ ID NO:6)** RSSTGAVTTSNYAN; CDR2 having the sequence **(SEQ ID NO:7)**: GTNKRAP; and CDR3 having the sequence **(SEQ ID NO:8)**: ALWYSNLWV

[0085] The VH Domain of an antibody that binds CD3 (VH_{CD3}) **(SEQ ID NO:25)**:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKY
NNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVS
WFAYWGQGTLVTVSS

[0086] The Antigen Binding Domain of VH_{CD3} comprises CDR1 having the sequence (**SEQ ID NO:10**): TYAMN; CDR2 having the sequence (**SEQ ID NO:11**): RIRSKYNNYATYYADSVKD; and CDR3 having the sequence:(**SEQ ID NO:12**) HGNFGNSYVSWFAY.

[0087] The above-discussed murine antibody that binds to human gpA33 was humanized to provide the VL and VH domains of preferred diabody DART-2. These humanized domains are as follows:

[0088] The VL Domain of a humanized antibody that binds gpA33 (VL_{gpA33}) (**SEQ ID NO:26**):

DIQLTQSPSFLSASVGDRVTITCSARSSISFMYWYQQKPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTEFTLTISSLEAEDAATYYCQQWSSYPLTFGQGTKLEIK

[0089] The Antigen Binding Domain of VL_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:14**): SARSSISFMY; CDR2 having the sequence (**SEQ ID NO:15**): DTSNLAS; and CDR3 having the sequence (**SEQ ID NO:16**): QQWSSYPLT.

[0090] The VH Domain of a humanized antibody that binds gpA33 (VH_{gpA33}) (**SEQ ID NO:27**):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGSWMNWVRQAPGQGLEWIGRIYPGD
GETNYNGKFKDRVTITADKSTSTAYMELSSLRSEDTAVYYCARIYGNNVYFDVWG
QGTTVTVSS

[0091] The Antigen Binding Domain of VH_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:18**): GSWMN; CDR2 having the sequence (**SEQ ID NO:19**): RIYPGDGETNYNGKFKD; and CDR3 having the sequence (**SEQ ID NO:20**): IYGNNVYFDV.

[0092] The first intervening spacer peptide (Linker 1) has the sequence (**SEQ ID NO:1**): GGGSGGGG. The cysteine-containing spacer peptide (Linker 2) has the sequence is **SEQ ID NO:2**: GGCGGG.

[0093] The heterodimer-promoting Domain of the first polypeptide chain is the "E-coil" Domain (**SEQ ID NO:3**). The heterodimer-promoting Domain of the second polypeptide chain is the "K-coil" Domain (**SEQ ID NO:4**).

[0094] Thus, the first polypeptide chain of DART-2 has the sequence (**SEQ ID NO:28**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG
GGGSGGGGQVQLVQSGAEVKKPGASVKVSCKASGYTFTGSWMNWVRQAPGQGLEW
IGRIYPGDGETNYNGKFKDRVTITADKSTSTAYMELSSLRSEDTAVYYCARIYGN
NVYFDVWGQGTTVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEK

[0095] As will be appreciated, residues 1-110 of **SEQ ID NO:28** are the VL Domain of an antibody that binds CD3 (**VL_{CD3}**) (**SEQ ID NO:5**); residues 111-118 of **SEQ ID NO:28** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 119-237 of **SEQ ID NO:28** are the VH Domain of an antibody that binds gpA33 (**VH_{gpA33}**) (**SEQ ID NO:27**), residues 238-243 of **SEQ ID NO:28** are the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**) and residues 244-271 of **SEQ ID NO:28** are the heterodimer-promoting "E-coil" Domain (**SEQ ID NO:3**).

[0096] A preferred polynucleotide that encodes the first polypeptide chain of DART-2 has the sequence (**SEQ ID NO:29**):

caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactgtga
ccctgacatgcagatccagcacaggcgcagtgaccacatctaactacgccaattg
ggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggtacaaacaaa
agggctccctggacccctgcacggttttctggaagtctgctgggcggaaaggccg
ctctgactattaccggggcacaggccgaggacgaagccgattactattgtgctct
gtggtatagcaatctgtgggtgttcgggggtggcacaaaactgactgtgctggga
ggtggtggatccggcggaggtggacaggtccagctggtccagagcggggccgaag
tcaaaaaacccggagcaagcgtgaaggtctcctgcaaagcatcaggctatacatt
tacaggcagctggatgaactgggtgaggcaggctccaggacagggactggagtgg
atcgggcgcatctaccctggagacggcgaaactaactataatggaaagttcaaag
accgagtgaccatcacagccgataagtctactagtaccgcctacatggagctgag
ctccctgcggtctgaagataccgccgtctactattgcgctagaatttacggaaac
aatgtctattttgacgtgtgggggcagggaacaactgtgactgtctcctccggag
gatgtggcggtggagaagtggccgcactggagaaagaggttgctgctttggagaa
ggaggtcgctgcacttgaaaaggaggtcgcagccctggagaaa

[0097] The second polypeptide chain of DART-2 has the sequence **(SEQ ID NO:30)**:

DIQLTQSPSFLSASVGDRVTITCSARSSISFMYWYQQKPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTEFTLTISSLEAEDAATYYCQQWSSYPLTFGQGTKLEIKGGGS
GGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRI
RSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGN
SYVSWFAYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKE

[0098] As will be appreciated, residues 1-106 of **SEQ ID NO:30** are the VL Domain of an antibody that binds gpA33 (VL_{gpA33}) **(SEQ ID NO:26)**; residues 107-114 of **SEQ ID NO:30** are the first intervening spacer peptide (Linker 1) **(SEQ ID NO:1)**; residues 115-239 of **SEQ ID NO:30** are the VH Domain of an antibody that binds CD3 (VH_{CD3}) **(SEQ ID NO:25)**, residues 240-245 of **SEQ ID NO:30** are the cysteine-containing spacer peptide (Linker 2) **(SEQ ID NO:2)** and residues 246-273 of **SEQ ID NO:30** are the heterodimer-promoting "K-coil" Domain **(SEQ ID NO:4)**.

[0099] A preferred polynucleotide that encodes the second polypeptide chain of DART-2 has the sequence **(SEQ ID NO:31)**:

gacattcagctgactcagtccccctcttttctgtccgcatccgtcggagatcgag
tgactattacttgctctgctaggtcctcaatcagcttcatgtactggtatcagca
gaagcccggcaaagcacctaagctgctgatctacgacacaagcaacctggcctcc
ggggtgccatctcggttctctggcagtgggtcaggaactgagtttaccctgacaa
ttagctccctggaggctgaagatgccgctacctactattgccagcagtggagcag
ctatcctctgaccttcggacaggggactaaactggaaatcaagggtggaggatcc
ggcggcggaggcgaggtgcagctggtggagtctgggggaggcttggtccagcctg
gagggtccctgagactctcctgtgcagcctctggattcaccttcagcacatacgc
tatgaattgggtccgccaggctccagggaaggggctggagtgggttggaaggatc
aggtccaagtacaacaattatgcaacctactatgccgactctgtgaaggatagat
tcaccatctcaagagatgattcaaagaactcactgtatctgcaaatgaacagcct
gaaaaccgaggacacggccgtgtattactgtgtgagacacggtaacttcggcaat
tcttacgtgtcttggtttgcttattggggacaggggacactggtgactgtgtctt
ccggaggatgtggcggtggaaaagtggccgcactgaaggagaaagttgctgcttt
gaaagagaaggtcgccgcacttaaggaaaaggtcgcagccctgaaagag

3. The gpA33 x CD3 Bi-Specific Monovalent Diabody Having An Albumin-Binding Domain (ABD) ("DART-2 w/ABD")

[00100] In another embodiment of the invention, the gpA33 x CD3 bi-specific monovalent diabody will comprise an Albumin-Binding Domain ("ABD") (gpA33 x CD3 bi-specific monovalent diabody with ABD").

[00101] As disclosed in WO 2012/018687, in order to improve the *in vivo* pharmacokinetic properties of diabody molecules, the molecules may be modified to contain a polypeptide portion of a serum-binding protein at one or more of the termini of the diabody molecule. Most preferably, such polypeptide portion of a serum-binding protein will be installed at the C-terminus of the diabody molecule. A particularly preferred polypeptide portion of a serum-binding protein for this purpose is the albumin binding domain (ABD) from streptococcal protein G. The albumin binding domain 3 (ABD3) of protein G of Streptococcus strain G148 is particularly preferred.

[00102] The albumin binding domain 3 (ABD3) of protein G of Streptococcus strain G148 consists of 46 amino acid residues forming a stable three-helix bundle and has broad albumin binding specificity (Johansson, M.U. *et al.* (2002) "*Structure, Specificity, And Mode Of Interaction For Bacterial Albumin-Binding Modules*," J. Biol. Chem. 277(10):8114-8120). Albumin is the most abundant protein in plasma and has a half-life of 19 days in humans. Albumin possesses several small molecule binding sites that permit it to non-covalently bind to other proteins and thereby extend their serum half-lives.

[00103] Thus, the first polypeptide chain or second polypeptide chain of a gpA33 x CD3 bi-specific monovalent diabody having an Albumin-Binding Domain contains a third linker (Linker 3), which separates the E-coil (or K-coil) of such polypeptide chain from the Albumin-Binding Domain. A preferred sequence for such Linker 3 is GGGS (**SEQ ID NO:32**) or GGGNS (**SEQ ID NO:33**). A preferred Albumin-Binding Domain (**ABD**) has the amino acid sequence (**SEQ ID NO:34**):

LAQAKEAAIRELDKYGVSDYYKNLIDNAKSAEGVKALIDEILAALP

[00104] In order to illustrate this aspect of the invention, the first polypeptide chain of the above-described DART-2 was modified to contain an Albumin-Binding Domain, resulting in a gpA33 x CD3 bi-specific monovalent diabody having an ABD, designated herein as "DART-2 w/ABD."

[00105] The first polypeptide chain of such DART-2 w/ABD has the amino acid sequence (**SEQ ID NO:35**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG
GGGSGGGGQVQLVQSGAEVKKPGASVKVSCKASGYTFTGSWMNWVRQAPGQGLEW
IGRIYPGDGETNYNGKFKDRVTITADKSTSTAYMELSSLRSEDTAVYYCARIYGN
NVYFDVWGQGTTVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKGGGS
LAQAKEAAIRELDKYGVSDYYKNLIDNAKSAEGVKALIDEILAALP

[00106] As will be recognized, residues 1-271 of **SEQ ID NO:35** are identical to residues 1-271 of DART-2, and thus provide, in the N-terminal to C-terminal direction, the VL Domain of an antibody that binds CD3 (VL_{CD3}) (**SEQ ID NO:5**); the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); the VH Domain of an antibody that binds gpA33 (VH_{gpA33}) (**SEQ ID NO:27**), the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**), the heterodimer-promoting "E-coil" Domain (**SEQ ID NO:3**) and a C-terminus. Residues 272-275 are Linker 3 (**SEQ ID NO:32**), and residues 276-321 are an Albumin-Binding Domain (**SEQ ID NO:34**).

[00107] A preferred polynucleotide that encodes the first polypeptide chain of DART-2 w/ABD has the sequence (**SEQ ID NO:36**):

caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactgtga
ccctgacatgcagatccagcacaggcgcagtgaccacatctaactacgccaattg
ggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggtacaaacaaa
agggctccctggacccctgcacggttttctggaagtctgctgggcggaaaggccg
ctctgactattaccggggcacaggccgaggacgaagccgattactattgtgctct
gtggtatagcaatctgtgggtgttcgggggtggcacaaaactgactgtgctggga
gggggtggatccggcggaggtggacaggtccagctggtccagagcggggccgaag
tcaaaaaacccggagcaagcgtgaaggtctcctgcaaagcatcaggctatacatt
tacaggcagctggatgaactgggtgaggcaggctccaggacagggactggagtgg
atcgggcgcatctaccctggagacggcgaaactaactataatggaaagttcaaag
accgagtgaccatcacagccgataagtctactagtaccgcctacatggagctgag
ctccctgcggtctgaagataccgccgtctactattgcgctagaatttacggaaac
aatgtctattttgacgtgtgggggcagggaacaactgtgactgtctcctccggag
gatgtggcggtggagaagtggccgcactggagaaagaggttgctgctttggagaa
ggaggtcgctgcacttgaaaaggaggtcgcagccctggagaaaggcggcgggtct

```
ctggcccaggcaaaagaggcagccatccgcgaactggataaatatggcgtgagcg
attattataagaacctgattgacaacgcaaaatccgcggaaggcgtgaaagcact
gattgatgaaattctggccgccctgcct
```

[00108] The second polypeptide chain of DART-2 w/ABD is the same as the above-discussed second polypeptide chain of DART-2 (**SEQ ID NO:30**).

B. The gpA33 x CD3 Bi-Specific Monovalent Diabodies Having An IgG Fc Domain ("DART-2 w/Fc")

[00109] In a further embodiment, the invention provides gpA33 x CD3 bi-specific monovalent diabodies having an IgG Fc Domain. Such diabodies are accordingly referred to herein as "gpA33 x CD3 bi-specific monovalent Fc diabodies." The Fc Domain of the Fc diabodies of the present invention may be either a complete Fc region (*e.g.*, a complete IgG Fc region) or only a fragment of a complete Fc region. Although the Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may possess the ability to bind to one or more Fc receptors (*e.g.*, FcγR(s)), more preferably such Fc Domain will cause reduced binding to FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by a wild-type Fc region) or will substantially eliminate the ability of such Fc Domain to bind to such receptor(s). The Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may include some or all of the CH2 Domain and/or some or all of the CH3 Domain of a complete Fc region, or may comprise a variant CH2 and/or a variant CH3 sequence (that may include, for example, one or more insertions and/or one or more deletions with respect to the CH2 or CH3 domains of a complete Fc region). The Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may comprise non-Fc polypeptide portions, or may comprise portions of non-naturally complete Fc regions, or may comprise non-naturally occurring orientations of CH2 and/or CH3 domains (such as, for example, two CH2 domains or two CH3 domains, or in the N-terminal to C-terminal direction, a CH3 Domain linked to a CH2 Domain, etc.).

[00110] In a first embodiment, denoted as "Version 1" and shown in **Figure 2A**, the first polypeptide chain of an exemplary gpA33 x CD3 bi-specific monovalent Fc diabody will comprise, in the N-terminal to C-terminal direction, an N-terminus, the VL Domain of a monoclonal antibody capable of binding to either gpA33 or CD3

(*i.e.*, either VL_{gpA33} or VL_{CD3}), an intervening spacer peptide (Linker 1), a VH Domain of a monoclonal antibody capable of binding to either gpA33 (if such first polypeptide chain contains VL_{CD3}) or CD3 (if such first polypeptide chain contains VL_{gpA33}), a cysteine-containing second intervening spacer peptide (Linker 2), a heterodimer-promoting Domain, a spacer peptide (Linker 5), a cysteine-containing peptide (Peptide 1), an IgG Fc Domain (preferably, all or a portion of the CH2 and CH3 domains of an antibody Fc region), and a C-terminus.

[00111] In a second embodiment, denoted as "Version 2" and shown in **Figure 2B**, the first polypeptide chain of an exemplary gpA33 x CD3 bi-specific monovalent Fc diabody will comprise, in the N-terminal to C-terminal direction, an N-terminus, a cysteine-containing peptide (Peptide 1), an IgG Fc Domain (preferably, all or a portion of the CH2 and CH3 domains of an antibody Fc region), an intervening spacer peptide (Linker 4); the VL Domain of a monoclonal antibody capable of binding to either gpA33 or CD3 (*i.e.*, either VL_{gpA33} or VL_{CD3}), an intervening spacer peptide (Linker 1), a VH Domain of a monoclonal antibody capable of binding to either gpA33 (if such first polypeptide chain contains VL_{CD3}) or CD3 (if such first polypeptide chain contains VL_{gpA33}), a cysteine-containing second intervening spacer peptide (Linker 2), a heterodimer-promoting Domain, and a C-terminus.

[00112] Preferably, in either embodiment, the Fc Domain of the first polypeptide chain will cause reduced binding to FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by a wild-type Fc region) or will substantially eliminate the ability of such Fc Domain to bind to such receptor(s). Fc variants and mutant forms capable of mediating such altered binding are well known in the art and include amino acid substitutions at positions 234 and 235, a substitution at position 265 or a substitution at position 297 (see, for example, US Patent No. 5,624,821, herein incorporated by reference). In a preferred embodiment the CH2 and CH3 Domain includes a substitution at position 234 with alanine and 235 with alanine.

[00113] The second polypeptide chain of such exemplary gpA33 x CD3 bi-specific monovalent Fc diabodies (Version 1 and Version 2) will comprise, in the N-terminal

to C-terminal direction, an N-terminus, a VL Domain of a monoclonal antibody capable of binding to either gpA33 or CD3 (*i.e.*, either VL_{gpA33} or VL_{CD3}, depending upon the VL Domain selected for the first polypeptide chain of the diabody), an intervening linker peptide (Linker 1), a VH Domain of a monoclonal antibody capable of binding to either CD3 (if such second polypeptide chain contains VL_{gpA33}) or CD3 (if such second polypeptide chain contains VL_{CD3}), a cysteine-containing spacer peptide (Linker 2), a heterodimer-promoting Domain (preferably a K-coil Domain), and a C-terminus.

[00114] The exemplary gpA33 x CD3 bi-specific monovalent Fc diabodies (Version 1 and Version 2) will additionally comprise a third polypeptide chain that will comprise, in the N-terminal to C-terminal direction, an N-terminus, a cysteine-containing peptide (Peptide 1), an IgG Fc Domain (preferably, all or a portion of the CH2 and CH3 domains of an antibody Fc region) having the same isotype as that of the Fc Domain of the first polypeptide chain and a C-terminus. Preferably, the Fc Domain of the third polypeptide chain will cause reduced binding to FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by a wild-type Fc region) or will substantially eliminate the ability of such Fc Domain to bind to such receptor(s), as discussed above, with respect to the first polypeptide chain of the exemplary gpA33 x CD3 bi-specific monovalent Fc diabodies.

[00115] The optionally present intervening spacer peptide (Linker 4) will preferably comprise the amino acid sequence (**SEQ ID NO:37**): APSSS, and more preferably have the amino acid sequence (**SEQ ID NO:38**): APSSSPME.

[00116] The cysteine-containing peptide (Peptide 1) of the first and third polypeptide chains may be comprised of the same amino acid sequence or of different amino acid sequences, and will contain 1, 2, 3 or more cysteine residues. A particularly preferred Peptide 1 has the amino acid sequence (**SEQ ID NO:39**): DKTHTCPPCP.

[00117] The intervening spacer peptide (Linker 1) preferably has the sequence of **SEQ ID NO:1**, described above. The cysteine-containing second intervening spacer peptide (Linker 2) preferably has the sequence of **SEQ ID NO:2**, described above.

[00118] The heterodimer-promoting Domain of the first and second polypeptide chains of the gpA33 x CD3 bi-specific monovalent Fc diabodies will preferably by the above-described E-coil Domain (**SEQ ID NO:3**) and K-coil Domain (**SEQ ID NO:4**), and will be selected so that one of such polypeptide chains possesses an E-coil Domain, whereas the other possesses a K-coil Domain, as discussed above.

[00119] A preferred spacer peptide (Linker 5) has the sequence GGG.

[00120] The CH2 and/or CH3 domains of the first and third polypeptides need not be identical, and advantageously are modified to foster complexing between the two polypeptides. For example, an amino acid substitution (preferably a substitution with an amino acid comprising a bulky side group forming a 'knob', *e.g.*, tryptophan) can be introduced into the CH2 or CH3 Domain such that steric interference will prevent interaction with a similarly mutated Domain and will obligate the mutated Domain to pair with a Domain into which a complementary, or accommodating mutation has been engineered, *i.e.*, 'the hole' (*e.g.*, a substitution with glycine). Such sets of mutations can be engineered into any pair of polypeptides comprising the bi-specific monovalent Fc diabody molecule, and further, engineered into any portion of the polypeptides chains of said pair. Methods of protein engineering to favor heterodimerization over homodimerization are well known in the art, in particular with respect to the engineering of immunoglobulin-like molecules, and are encompassed herein (see *e.g.*, Ridgway *et al.* (1996) *"'Knobs-Into-Holes' Engineering Of Antibody CH3 Domains For Heavy Chain Heterodimerization,"* Protein Engr. 9:617-621, Atwell *et al.* (1997) *"Stable Heterodimers From Remodeling The Domain Interface Of A Homodimer Using A Phage Display Library,"* J. Mol. Biol. 270: 26-35, and Xie *et al.* (2005) *"A New Format Of Bispecific Antibody: Highly Efficient Heterodimerization, Expression And Tumor Cell Lysis,"* J. Immunol. Methods 296:95-101; each of which is hereby incorporated herein by reference in its entirety). Preferably the 'knob' is engineered into the CH2-CH3 domains of the first polypeptide chain and the 'hole' is engineered into the CH2-CH3 domains of the third polypeptide chain. Thus, the 'knob' will help in preventing the first polypeptide chain from homodimerizing via its CH2 and/or CH3 domains. As

the third polypeptide chain preferably contains the 'hole' substitution it will heterodimerize with the first polypeptide chain as well as homodimerize with itself. A preferred knob is created by modifying an Fc Domain of a native IgG Fc region to contain the modification T366W. A preferred hole is created by modifying an Fc Domain of a native IgG Fc region to contain the modification T366S, L368A and Y407V. To aid in purifying the third polypeptide chain homodimer from the final bi-specific monovalent Fc diabody comprising the first, second and third polypeptide chains, the protein A binding site of the CH2 and CH3 domains of the third polypeptide chain is preferably mutated by amino acid substitution at position 435 (H435R). To aid in purifying the third polypeptide chain homodimer from the final bi-specific monovalent Fc diabody comprising the first, second and third polypeptide chains, the protein A binding site of the CH2 and CH3 domains of the third polypeptide chain is preferably mutated by amino acid substitution. Thus the third polypeptide chain homodimer will not bind to protein A, whereas the bi-specific monovalent Fc diabody will retain its ability to bind protein A via the protein A binding site on the first polypeptide chain.

[00121] A preferred sequence for the CH2 and CH3 domains of an antibody Fc Domain present in the first polypeptide chain is **(SEQ ID NO:40)**:

APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[00122] A preferred sequence for the CH2 and CH3 domains of an antibody Fc Domain present in the third polypeptide chain is **(SEQ ID NO:41)**:

APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRYTQKSLSLSPGK

1. DART-2 w/Fc Version 1

[00123] The first, second and third polypeptide chains of a preferred gpA33 x CD3 bi-specific monovalent Fc diabody, designated herein as "DART-2 w/Fc Version 1," comprise polypeptide domains having the following sequences:

[00124] The first polypeptide chain of such DART-2 w/Fc Version 1 has the amino acid sequence (**SEQ ID NO:42**):

DIQLTQSPSFLSASVGDRVTITCSARSSISFMYWYQQKPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTEFTLTISSLEAEDAATYYCQQWSSYPLTFGQGTKLEIKGGGS
GGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRI
RSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGN
SYVSWFAYWGQGTLVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKGG
GDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGK

[00125] As will be appreciated, residues 1-106 of **SEQ ID NO:42** are the VL Domain of an antibody that binds gpA33 ($\mathbf{VL_{gpA33}}$) (**SEQ ID NO:26**); residues 107-114 of **SEQ ID NO:42** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 115-239 of **SEQ ID NO:42** are the VH Domain of an antibody that binds CD3 ($\mathbf{VH_{CD3}}$) (**SEQ ID NO:25**); residues 240-245 of **SEQ ID NO:42** are the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**); residues 246-273 of **SEQ ID NO:42** are the heterodimer-promoting "E-coil" Domain (**SEQ ID NO:3**); residues 274-276 are the spacer peptide (Linker 5) (GGG); residues 277-286 are Peptide 1 (**SEQ ID NO:39**), residues 277-503 are the sequence for the CH2 and CH3 domains of an antibody Fc Domain (**SEQ ID NO:40**).

[00126] A preferred polynucleotide that encodes the first polypeptide chain of DART-2 w/Fc Version 1 has the sequence (**SEQ ID NO:43**):

gacattcagctgactcagtccccctcttttctgtccgcatccgtcggagatcgag
tgactattacttgctctgctaggtcctcaatcagcttcatgtactggtatcagca
gaagcccggcaaagcacctaagctgctgatctacgacacaagcaacctggcctcc
ggggtgccatctcggttctctggcagtgggtcaggaactgagtttaccctgacaa
ttagctccctggaggctgaagatgccgctacctactattgccagcagtggagcag
ctatcctctgaccttcggacaggggactaaactggaaatcaagggtggaggatcc
ggcggcggaggcgaggtgcagctggtggagtctgggggaggcttggtccagcctg
gagggtccctgagactctcctgtgcagcctctggattcaccttcagcacatacgc
tatgaattgggtccgccaggctccagggaaggggctggagtgggttggaaggatc
aggtccaagtacaacaattatgcaacctactatgccgactctgtgaaggatagat
tcaccatctcaagagatgattcaaagaactcactgtatctgcaaatgaacagcct
gaaaaccgaggacacggccgtgtattactgtgtgagacacggtaacttcggcaat
tcttacgtgtcttggtttgcttattggggacaggggacactggtgactgtgtctt
ccggaggatgtggcggtggagaagtggccgcactggagaaagaggttgctgcttt
ggagaaggaggtcgctgcacttgaaaaggaggtcgcagccctggagaaaggcggc

ggggacaaaactcacacatgcccaccgtgcccagcacctgaagccgcgggggac
cgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccggac
ccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaag
ttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcggg
aggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcacca
ggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctccca
gcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacagg
tgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgtg
gtgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaat
gggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggct
ccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaa
cgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaag
agcctctccctgtctccgggtaaa

[00127] The second polypeptide chain of such DART-2 w/Fc Version 1 has the amino acid sequence (**SEQ ID NO:44**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNK
RAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG
GGGSGGGGQVQLVQSGAEVKKPGASVKVSCKASGYTFTGSWMNWVRQAPGQGLEW
IGRIYPGDGETNYNGKFKDRVTITADKSTSTAYMELSSLRSEDTAVYYCARIYGN
NVYFDVWGQGTTVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKE

[00128] As will be appreciated, residues 1-110 of **SEQ ID NO:44** are the VL Domain of an antibody that binds CD3 ($\mathbf{VL_{CD3}}$) (**SEQ ID NO:5**); residues 111-118 of **SEQ ID NO:44** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 119-237 of **SEQ ID NO:44** are the VH Domain of an antibody that binds gpA33 ($\mathbf{VH_{gpA33}}$) (**SEQ ID NO:27**), residues 238-243 of **SEQ ID NO:44** are the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**) and residues 244-271 of **SEQ ID NO:44** are the heterodimer-promoting "K-coil" Domain (**SEQ ID NO:4**).

[00129] A preferred polynucleotide that encodes the second polypeptide chain of DART-2 w/Fc Version 1 has the sequence (**SEQ ID NO:45**):

caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactgtga
ccctgacatgcagatccagcacaggcgcagtgaccacatctaactacgccaattg
ggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggtacaaacaaa
agggctccctggacccctgcacggttttctggaagtctgctgggcggaaaggccg
ctctgactattaccggggcacaggccgaggacgaagccgattactattgtgctct
gtggtatagcaatctgtgggtgttcgggggtggcacaaaactgactgtgctggga
gggggtggatccggcggaggtggacaggtccagctggtccagagcggggccgaag
tcaaaaaacccggagcaagcgtgaaggtctcctgcaaagcatcaggctatacatt
tacaggcagctggatgaactgggtgaggcaggctccaggacagggactggagtgg
atcgggcgcatctaccctggagacggcgaaactaactataatggaaagttcaaag

accgagtgaccatcacagccgataagtctactagtaccgcctacatggagctgag
ctccctgcggtctgaagataccgccgtctactattgcgctagaatttacggaaac
aatgtctattttgacgtgtgggggcagggaacaactgtgactgtctcctccggag
gatgtggcggtggaaaagtggccgcactgaaggagaaagttgctgctttgaaaga
gaaggtcgccgcacttaaggaaaaggtcgcagccctgaaagag

[00130] The third polypeptide chain of such DART-2 w/Fc Version 1 has the amino acid sequence (**SEQ ID NO:46**):

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRYTQKS
LSLSPGK

[00131] As will be appreciated, residues 1-10 of **SEQ ID NO:46** are Peptide 1 (**SEQ ID NO:39**) and residues 11-227 are the CH2 and CH3 domains of an antibody Fc Domain (**SEQ ID NO:41**).

[00132] A preferred polynucleotide that encodes the third polypeptide chain of DART-2 w/Fc Version 1 has the sequence (**SEQ ID NO:47**):

gacaaaactcacacatgcccaccgtgcccagcacctgaagccgcgggggaccgt
cagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccggacccc
tgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttc
aactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggagg
agcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccagga
ctggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcc
cccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgt
acaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgagttg
cgcagtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatggg
cagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctcct
tcttcctcgtcagcaagctcaccgtggacaagagcaggtggcagcaggggaacgt
cttctcatgctccgtgatgcatgaggctctgcacaaccgctacacgcagaagagc
ctctccctgtctccgggtaaa

2. DART-2 w/Fc Version 2

[00133] The first, second and third polypeptide chains of a second preferred gpA33 x CD3 bi-specific monovalent Fc diabody, designated herein as "DART-2 w/Fc Version 2," comprise polypeptide domains having the following sequences. Among other differences, DART-2 w/Fc Version 1 differs from DART-2 w/Fc Version 22 in the positioning of the CH2 and CH3 sequences of the first polypeptide chain; these sequences are positioned C-terminal to the VL and VH sequences of DART-2 w/Fc

Version 1, whereas they are positioned N-terminal to the VL and VH sequences of DART-2 w/Fc Version 2.

[00134] The first polypeptide chain of such DART-2 w/Fc Version 2 has the amino acid sequence (**SEQ ID NO:48**):

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
LSLSPGKAPSSSPMEDIQLTQSPSFLSASVGDRVTITCSARSSISFMYWYQQKPG
KAPKLLIYDTSNLASGVPSRFSGSGSGTEFTLTISSLEAEDAATYYCQQWSSYPL
TFGQGTKLEIKGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNW
VRQAPGKGLEWVGRIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTE
DTAVYYCVRHGNFGNSYVSWFAYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEK
VAALKEKVAALKE

[00135] As will be appreciated, residues 1-10 of **SEQ ID NO:48** are Peptide 1 (**SEQ ID NO:39**); residues 11-227 of **SEQ ID NO:48** are the sequence for the CH2 and CH3 domains of an antibody Fc Domain (**SEQ ID NO:40**); residues 228-235 of **SEQ ID NO:48** are intervening spacer peptide (Linker 4) (**SEQ ID NO:38**); residues 236-341 of **SEQ ID NO:48** are the VL Domain of an antibody that binds gpA33 ($\mathbf{VL_{gpA33}}$) (**SEQ ID NO:26**); residues 342-349 of **SEQ ID NO:48** are the first intervening spacer peptide (Linker 1) (**SEQ ID NO:1**); residues 350-474 of **SEQ ID NO:48** are the VH Domain of an antibody that binds CD3 ($\mathbf{VH_{CD3}}$) (**SEQ ID NO:25**); residues 475-480 of **SEQ ID NO:48** are the cysteine-containing spacer peptide (Linker 2) (**SEQ ID NO:2**); and residues 481-508 of **SEQ ID NO:48** are the heterodimer-promoting "K-coil" Domain (**SEQ ID NO:4**).

[00136] The second polypeptide chain of such DART-2 w/Fc Version 2 has the amino acid sequence of the first polypeptide chain of DART-2 (*i.e.*, **SEQ ID NO:28**) (described above).

[00137] The third polypeptide chain of such DART-2 w/Fc Version 2 has the amino acid sequence of **SEQ ID NO:46** (described above).

Pharmaceutical Compositions

[00138] The compositions of the invention include bulk drug compositions useful in the manufacture of pharmaceutical compositions (*e.g.*, impure or non-sterile compositions) and pharmaceutical compositions (*i.e.*, compositions that are suitable for administration to a subject or patient) which can be used in the preparation of unit dosage forms. Such compositions comprise a prophylactically or therapeutically effective amount of the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies disclosed herein and an additional therapeutic agent) and a pharmaceutically acceptable carrier. Preferably, compositions of the invention comprise a prophylactically or therapeutically effective amount of one or more molecules of the invention and a pharmaceutically acceptable carrier.

[00139] The invention also encompasses pharmaceutical compositions comprising such gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies and a second therapeutic antibody (*e.g.*, a cancer-antigen specific monoclonal antibody) that is specific for a particular antigen associated with a cancer, and a pharmaceutically acceptable carrier.

[00140] In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant (*e.g.*, Freund's adjuvant (complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if

desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained release formulations and the like.

[00141] Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[00142] The compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include, but are not limited to those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, *etc.*, and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

[00143] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with such disclosed gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies (alone or with additional therapeutic agent(s)) and such pharmaceutically acceptable carrier. Additionally, one or more other prophylactic or therapeutic agents useful for the treatment of a disease can also be included in the pharmaceutical pack or kit. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00144] The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises one or more molecules of the invention. In another embodiment, a kit further comprises one or more other prophylactic or therapeutic agents useful for the treatment of a cancer, in one or more containers. In another embodiment, a kit further comprises one or more antibodies that bind one or more antigens associated with a cancer. In certain embodiments, the other prophylactic or therapeutic agent is a chemotherapeutic. In other embodiments, the prophylactic or therapeutic agent is a biological or hormonal therapeutic.

Uses of the Compositions of the Invention

[00145] The gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the present invention have the ability to treat any disease or condition associated with or characterized by the expression of gpA33. Thus, without limitation, pharmaceutical compositions comprising such molecules may be employed in the diagnosis or treatment of colon cancers, colorectal cancers, and pancreatic cancers.

Methods of Administration

[00146] The compositions of the present invention may be provided for the treatment, prophylaxis, and amelioration of one or more symptoms associated with a disease, disorder or infection by administering to a subject an effective amount of a pharmaceutical composition of the invention. In a preferred aspect, such compositions are substantially purified (*i.e.*, substantially free from substances that limit its effect or produce undesired side-effects). In a specific embodiment, the subject is an animal, preferably a mammal such as non-primate (*e.g.*, bovine, equine, feline, canine, rodent, *etc.*) or a primate (*e.g.*, monkey such as, a cynomolgus monkey, human, *etc.*). In a preferred embodiment, the subject is a human.

[00147] Various delivery systems are known and can be used to administer the compositions of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the antibody or fusion protein, receptor-mediated endocytosis (See, *e.g.*, Wu *et al.* (1987) *"Receptor-Mediated In Vitro Gene Transformation By A Soluble DNA Carrier System,"* J. Biol. Chem.

262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, *etc.*

[00148] Methods of administering the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the present invention include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral routes). In a specific embodiment, the molecules of the invention are administered intramuscularly, intravenously, or subcutaneously. The compositions may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. See, *e.g.*, U.S. Patent Nos. 6,019,968; 5,985, 320; 5,985,309; 5,934,272; 5,874,064; 5,855,913; 5,290,540; and 4,880,078; and PCT Publication Nos. WO 92/19244; WO 97/32572; WO 97/44013; WO 98/31346; and WO 99/66903, each of which is incorporated herein by reference in its entirety.

[00149] The invention also provides that the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention are packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of such molecules. In one embodiment, the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention are supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, the gpA33 x CD3 diabodies or gpA33 x CD3 Fc diabodies of the invention are supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of at least 5 μg, more preferably at least 10 μg, at least 15 μg, at least 25 μg, at least 50 μg, at least 100 μg, or at least 200 μg.

[00150] The lyophilized gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention should be stored at between 2 and 8°C in their original container and the molecules should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention are supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the molecule, fusion protein, or conjugated molecule. Preferably, the liquid form of such bi-specific monovalent diabodies or bi-specific monovalent Fc diabodies is supplied in a hermetically sealed container in which the molecules are present at a concentration of least 1 μg/ml, more preferably at least 2.5 μg/ml, at least 5 μg/ml, at least 10 μg/ml, at least 50 μg/ml, or at least 100 μg/ml.

[00151] The amount of gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention which will be effective in the treatment, prevention or amelioration of one or more symptoms associated with a disorder can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[00152] For gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies encompassed by the invention, the dosage administered to a patient is typically at least about 0.01 μg/kg, at least about 0.05 μg/kg, at least about 0.1 μg/kg, at least about 0.2 μg/kg, at least about 0.5 μg/kg, at least about 1 μg/kg, at least about 2 μg/kg, at least about 3 μg/kg, at least about 5 μg/kg, at least about 10 μg/kg, at least about 20 μg/kg, at least about 30 μg/kg, at least about 50 μg/kg, at least about 0.1 mg/kg, at least about 0.15 mg/kg, or more of the subject's body weight.

[00153] The dosage and frequency of administration of the bi-specific monovalent diabodies or bi-specific monovalent Fc diabodies of the invention may be reduced or

altered by enhancing uptake and tissue penetration of the bi-specific monovalent Fc diabodies by modifications such as, for example, lipidation.

[00154] In one embodiment, the dosage of the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 Fc bi-specific monovalent diabodies of the invention administered to a patient may be calculated for use as a single agent therapy. In another embodiment the bi-specific monovalent diabodies or bi-specific monovalent Fc diabodies of the invention are used in combination with other therapeutic compositions and the dosage administered to a patient are lower than when such bi-specific monovalent diabodies or bi-specific monovalent Fc diabodies are used as a single agent therapy.

[00155] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion, by injection, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a molecule of the invention, care must be taken to use materials to which the molecule does not absorb.

[00156] In another embodiment, the compositions can be delivered in a vesicle, in particular a liposome (*See* Langer (1990) *"New Methods Of Drug Delivery,"* Science 249:1527-1533); Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, ibid., pp. 3 17-327; see generally ibid.).

[00157] In yet another embodiment, the compositions can be delivered in a controlled release or sustained release system. Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more molecules of the invention. *See, e.g.*, U.S. Patent No. 4,526,938; PCT publication WO 91/05548; PCT publication WO 96/20698; Ning *et al.* (1996) *"Intratumoral Radioimmunotheraphy Of A Human Colon Cancer Xenograft Using A Sustained-Release Gel,"* Radiotherapy & Oncology 39:179-189, Song *et al.* (1995) *"Antibody Mediated Lung Targeting Of Long-Circulating Emulsions,"* PDA Journal

of Pharmaceutical Science & Technology 50:372-397; Cleek *et al.* (1997) *"Biodegradable Polymeric Carriers For A bFGF Antibody For Cardiovascular Application,"* Pro. Int'l. Symp. Control. Rel. Bioact. Mater. 24:853-854; and Lam *et al.* (1997) *"Microencapsulation Of Recombinant Humanized Monoclonal Antibody For Local Delivery,"* Proc. Int'l. Symp. Control Rel. Bioact. Mater. 24:759-760, each of which is incorporated herein by reference in its entirety. In one embodiment, a pump may be used in a controlled release system (*See* Langer, *supra*; Sefton, (1987) *"Implantable Pumps,"* CRC Crit. Rev. Biomed. Eng. 14:201-240; Buchwald *et al.* (1980) *"Long-Term, Continuous Intravenous Heparin Administration By An Implantable Infusion Pump In Ambulatory Patients With Recurrent Venous Thrombosis,"* Surgery 88:507-516; and Saudek *et al.* (1989) *"A Preliminary Trial Of The Programmable Implantable Medication System For Insulin Delivery,"* N. Engl. J. Med. 321:574-579). In another embodiment, polymeric materials can be used to achieve controlled release of antibodies (*see e.g.*, MEDICAL APPLICATIONS OF CONTROLLED RELEASE, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); CONTROLLED DRUG BIOAVAILABILITY, DRUG PRODUCT DESIGN AND PERFORMANCE, Smolen and Ball (eds.), Wiley, New York (1984); Levy *et al.* (1985) *"Inhibition Of Calcification Of Bioprosthetic Heart Valves By Local Controlled-Release Diphosphonate,"* Science 228:190-192; During *et al.* (1989) *"Controlled Release Of Dopamine From A Polymeric Brain Implant: In Vivo Characterization,"* Ann. Neurol. 25:351-356; Howard *et al.* (1989) *"Intracerebral Drug Delivery In Rats With Lesion-Induced Memory Deficits,"* J. Neurosurg. 7(1):105-112); U.S. Patent No. 5,679,377; U.S. Patent No. 5,916,597; U.S. Patent No. 5,912,015; U.S. Patent No. 5,989,463; U.S. Patent No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target (*e.g.*, the lungs), thus requiring only a fraction of the systemic dose (see, *e.g.*,

Goodson, in MEDICAL APPLICATIONS OF CONTROLLED RELEASE, *supra*, vol. 2, pp. 115-138 (1984)). In another embodiment, polymeric compositions useful as controlled release implants are used according to Dunn *et al.* (See U.S. 5,945,155). This particular method is based upon the therapeutic effect of the in situ controlled release of the bioactive material from the polymer system. The implantation can generally occur anywhere within the body of the patient in need of therapeutic treatment. In another embodiment, a non-polymeric sustained delivery system is used, whereby a non-polymeric implant in the body of the subject is used as a drug delivery system. Upon implantation in the body, the organic solvent of the implant will dissipate, disperse, or leach from the composition into surrounding tissue fluid, and the non-polymeric material will gradually coagulate or precipitate to form a solid, microporous matrix (See U.S. 5,888,533).

[00158] Controlled release systems are discussed in the review by Langer (1990, *"New Methods Of Drug Delivery,"* Science 249:1527-1533). Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more therapeutic agents of the invention. See, *e.g.*, U.S. Patent No. 4,526,938; International Publication Nos. WO 91/05548 and WO 96/20698; Ning *et al.* (1996) *"Intratumoral Radioimmunotheraphy Of A Human Colon Cancer Xenograft Using A Sustained-Release Gel,"* Radiotherapy & Oncology 39:179-189, Song *et al.* (1995) *"Antibody Mediated Lung Targeting Of Long-Circulating Emulsions,"* PDA Journal of Pharmaceutical Science & Technology 50:372-397; Cleek *et al.* (1997) *"Biodegradable Polymeric Carriers For A bFGF Antibody For Cardiovascular Application,"* Pro. Int'l. Symp. Control. Rel. Bioact. Mater. 24:853-854; and Lam *et al.* (1997) *"Microencapsulation Of Recombinant Humanized Monoclonal Antibody For Local Delivery,"* Proc. Int'l. Symp. Control Rel. Bioact. Mater. 24:759-760, each of which is incorporated herein by reference in its entirety.

[00159] In a specific embodiment where the composition of the invention is a nucleic acid encoding a bi-specific monovalent diabody or bi-specific monovalent Fc diabody of the invention, the nucleic acid can be administered *in vivo* to promote expression of its encoded bi-specific monovalent diabody or bi-specific monovalent Fc diabody, by constructing it as part of an appropriate nucleic acid expression vector and

administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (*See* U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (See *e.g.*, Joliot *et al.* (1991) *"Antennapedia Homeobox Peptide Regulates Neural Morphogenesis,"* Proc. Natl. Acad. Sci. (U.S.A.) 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination.

[00160] Treatment of a subject with a therapeutically or prophylactically effective amount of the gpA33 x CD3 bi-specific monovalent diabodies or gpA33 x CD3 bi-specific monovalent Fc diabodies of the invention can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with molecules of the invention one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. In other embodiments, the pharmaceutical compositions of the invention are administered once a day, twice a day, or three times a day. In other embodiments, the pharmaceutical compositions are administered once a week, twice a week, once every two weeks, once a month, once every six weeks, once every two months, twice a year or once per year. It will also be appreciated that the effective dosage of the molecules used for treatment may increase or decrease over the course of a particular treatment.

[00161] Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

Example 1
Characteristics of Anti-Human gpA33 Monoclonal Antibody

[00162] A murine monoclonal antibody capable of specific binding to human gpA33 was chimericized and humanized. The VL and VH chains of the original murine antibody have the sequences of **SEQ ID NOs:13** and **17**, respectively. The VL and

VH chains of the humanized antibody have the sequences of **SEQ ID NOs:26 and 27**, respectively.

[00163] The Antigen Binding Domain of VL_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:14**): SARSSISFMY; CDR2 having the sequence (**SEQ ID NO:15**): DTSNLAS; and CDR3 having the sequence (**SEQ ID NO:16**): QQWSSYPLT.

[00164] The Antigen Binding Domain of VH_{gpA33} comprises CDR1 having the sequence (**SEQ ID NO:18**): GSWMN; CDR2 having the sequence (**SEQ ID NO:19**): RIYPGDGETNYNGKFKD; and CDR3 having the sequence (**SEQ ID NO:20**): IYGNNVYFDV.

[00165] **Table 1** shows the effect of such alterations on the kinetics of binding.

Table 1

Antibody	KD	ka	kd
Murine mAb 1	2.3 nM	3.3×10^{5}	7.5×10^{-4}
Chimeric mAb 1	2.4 nM	5.8×10^{5}	1.4×10^{-3}
Humanized mAb 1	3.4 nM	5.6×10^{5}	1.9×10^{-3}

[00166] The data indicates that the modifications resulting in the humanization of the antibody VL and VH domains did not substantially affect gpA33 binding kinetics.

Example 2
Construction Of gpA33 x CD3 Bi-specific Monovalent Diabodies and Fc Diabodies And Control Diabodies

[00167] **Table 2** contains a list of sequences of the polypeptide chains of the preferred gpA33 x CD3 diabodies and gpA33 x CD3 Fc diabodies that were expressed and purified. The diabodies were found to be capable of simultaneously binding to gpA33 and CD3, as judged by the detection of such simultaneous binding by the exemplary gpA33 x CD3 bi-specific monovalent diabodies, DART-1 and DART-2, and by the exemplary gpA33 x CD3 bi-specific monovalent Fc diabody (DART-2 w/Fc). Additionally, a control bi-specific monovalent diabody ("Control DART") was produced that was bi-specific monovalent for CD3 and FITC, and was found to be capable of simultaneously binding to CD3 and FITC.

Table 2	
Diabody	**Substituent Polypeptides (in the N-Terminal to C-Terminal Direction)**
gpA33 x CD3 bi-specific monovalent diabody (DART-1)	**SEQ ID NO:21** **SEQ ID NO:23**
gpA33 x CD3 bi-specific monovalent diabody (DART-2)	**SEQ ID NO:28** **SEQ ID NO:30**
gpA33 x CD3 bi-specific monovalent diabody having an Albumin-Binding Domain (DART-2 w/ABD) Comprises an Albumin-Binding Domain (ABD) for extension of half-life in vivo	**SEQ ID NO:35** **SEQ ID NO:30**
gpA33 x CD3 bi-specific monovalent diabody having an IgG Fc Domain version 1 (DART-2 w/Fc Version 1) Comprises an Fc Domain for extension of half-life *in vivo*	**SEQ ID NO:42** **SEQ ID NO:44** **SEQ ID NO:46**
gpA33 x CD3 bi-specific monovalent diabody having an IgG Fc Domain version 2 (DART-2 w/Fc Version 2) Comprises an Fc Domain for extension of half-life *in vivo*	**SEQ ID NO:48** **SEQ ID NO:28** **SEQ ID NO:46**

[00168] The gpA33 x CD3 bi-specific monovalent diabodies are heterodimers composed of two polypeptide chains (one chain of each recited sequence) and the gpA33 x CD3 bi-specific monovalent Fc diabodies are heterotrimers composed of three polypeptide chains (one chain of each recited amino acid sequence). Methods for forming bi-specific monovalent diabodies are provided in WO 2006/113665, WO 2008/157379, WO 2010/080538, WO 2012/018687, WO 2012/162068 and WO 2012/162067.

[00169] The control CD3 x FITC bi-specific monovalent diabody was found to be capable of simultaneously binding to CD3 and to FITC. The above-described gpA33 x CD3 bi-specific monovalent diabodies and gpA33 x CD3 bi-specific monovalent Fc diabodies were found to be capable of simultaneously binding to gpA33 and to CD3. In order to demonstrate such simultaneous binding, the gpA33 x CD3 bi-specific monovalent diabody DART-1 was incubated in the presence of a soluble CD3

fragment that had been immobilized to a solid support. The detection of binding was assessed by the capacity of immobilized antibodies to additionally bind gpA33. The results confirm the capacity of the above-described gpA33 x CD3 bi-specific monovalent diabodies and gpA33 x CD3 bi-specific monovalent Fc diabodies to mediate simultaneous binding to gpA33 and CD3 (**Figure 3**).

Example 3
gpA33 x CD3 Bi-Specific Monovalent Diabodies Are Cytotoxic to Cancer Cells

[00170] The ability of the gpA33 x CD3 bi-specific monovalent diabodies of the present invention to treat cancer was illustrated by incubating colorectal or pancreatic cancer cells in the presence of the gpA33 x CD3 bi-specific monovalent DART-1 and either human PBMC (E:T = 25:1) or activated human T cells (E:T = 10:1). gpA33 x CD3 bi-specific monovalent diabody DART-1 exhibited potent redirected killing ability with concentrations required to achieve 50% maximal activity (EC50) in the sub-ng/mL to around 1 ng/mL range. In contrast, cytotoxicity was not observed when gpA33-negative cancer cell lines (*e.g.*, HCT116) were employed. The results of the investigation are shown in **Figure 4A** (colorectal cancer stem-like cells (Colon CSCL cells), **Figure 4B** (Colo205 colorectal cells), and **Figure 4C** (ASPC-1 pancreatic cancer cells). Results are summarized in **Table 3**.

Table 3

Target Cell Line	EC50 of gpA33 x CD3 Bi-Specific Monovalent Diabody (ng/mL)	Effector:Target (E:T)	Max % Killing Observed
Colon CSLC	0.9015	25:1	38
Colo205	0.5853	10:1	35
ASPC-1	1.142	10:1	25

Example 4
T cell Activation in the Presence of gpA33 x CD3 Bi-Specific Monovalent Diabodies

[00171] In order to further demonstrate the ability of the diabodies of the present invention to treat cancer, resting human T cells were incubated with the gpA33 x CD3 bi-specific monovalent DART-1 in the presence or absence of cancer cells (colo205

or ASPC-1). To characterize T cell activation during gpA33 x CD3 bi-specific monovalent diabody (DART-1)-mediated redirected killing process, T cells from redirected killing assays were stained for the T cell activation marker CD25 and analyzed by FACS. CD25 was upregulated in CD8 (**Figures 5A-5B**) and CD4 (**Figures 5D-5E**) T cells in a dose-dependent manner indicating that the gpA33 x CD3 bi-specific monovalent diabodies induced T cell activation in the process of redirected killing. Conversely, in the absence of target cells there was no activation of CD8 (**Figure 5C**) or CD4 (**Figure 5F**) T cells indicating the gp-A33 x CD3 diabodies do not activate T cells in the absence of target cells. Likewise, CD8 or CD4 T cells were not activated when incubated with target cells and a control bi-specific monovalent diabody (Control DART) (**Figures 5A-5B**, and **Figures 5D-5F**, respectively) indicating the requirement of cross-linking the T cell and target cell with the gpA33 x CD3 bi-specific monovalent diabodies.

Example 5
Equivalency of gpA33 x CD3 Bi-Specific Monovalent Diabody (DART-1) Having Murine Anti-Human gpA33 Variable Domain Sequences and gpA33 x CD3 Bi-Specific Monovalent Diabody (DART-2) Having Humanized Anti-Human gpA33 Variable Domain Sequences

[00172] As discussed above, the gpA33 x CD3 bi-specific monovalent diabody DART-1 contains VL_{gpA33} and VH_{gpA33} domains of a murine monoclonal antibody, whereas the gpA33 x CD3 bi-specific monovalent diabody DART-2 contains humanized VL_{gpA33} and humanized VH_{gpA33} domains of the same murine antibody. In order to demonstrate the ability of the humanized VL_{gpA33} and VH_{gpA33} domains to promote T cell targeting to gpA33-expressing cancer cells, cancer cells that express gpA33 were incubated in the presence of resting T cells (LDH assay; E:T = 10:1) in the presence of either DART-1, DART-1 or a control bi-specific monovalent diabody (Control DART). The results of this analysis (shown in **Figures 6A-6D**) demonstrate that DART-1 and DART-2 mediated equivalent cytotoxicity for SW948 colorectal adenocarcinoma cells (**Figure 6A**) and colo205 cells (**Figure 6B**). DART-1 and DART-2 both mediated cytotoxicity of a luciferase expressing Colo205 cell line which was stably transfected with firefly luciferase gene (luc2) (Colo205-Luc), as measured by decreased luminescence (**Figure 6C**). Neither DART-1 nor DART-2 mediated cytotoxicity of the gpA33-negative cancer cell line, HCT116 (**Figure 6D**).

As shown in **Table 4**, DART-1 and DART-2 exhibited similar equivalent bioactivity against multiple tumor cell lines.

Table 4

Effector/Target		LDH Assay		Luciferase Assay	
Donor T Cell	**Tumor Cell Line**	**gpA33xCD3 DART-2**	**gpA33xCD3 DART 1**	**gpA33xCD3 DART-2**	**gpA33xCD3 DART 1**
D54677	SW948	0.79	1.34		
D54677	Colo205	1.17	2.52		
D51031	Colo205-Luc	2.29	3.53	2.53	4.55
D41440	Colo205	2.29	3.37		
D41440	Colo205-Luc	2.80	4.26	2.57	3.26

Example 6
Cross-Reactivity of gpA33 x CD3 Bi-Specific Monovalent Diabodies, gpA33 x CD3 Bi-Specific Monovalent Diabodies Having an Albumin-Binding Domain and gpA33 x CD3 Bi-Specific Monovalent Diabodies Having an IgG Fc Domain with PBMCs of Cynomolgus Monkey

[00173] As shown above, the humanized VL_{gpA33} and humanized VH_{gpA33} domains of the gpA33 x CD3 bi-specific monovalent diabody DART-2 mediate the cytotoxicity of gpA33-expressing cancer cells in the presence of human T cells. The VL_{CD3} and VH_{CD3} domains of the gpA33 x CD3 bi-specific monovalent diabodies of the present invention were unexpectedly found to also be capable of binding to the CD3 of cynomolgus monkey T cells and redirect those cells to kill gpA33-expressing cells.

[00174] As shown in **Figures 7A-7D**, the gpA33 x CD3 bi-specific monovalent DART-2 diabody, the gpA33 x CD3 bi-specific monovalent diabody having an Albumin-Binding Domain (DART-2 w/ABD) and the gpA33 x CD3 bi-specific monovalent DART-2 diabody having an IgG Fc Domain (DART-2 w/Fc) were all found to be capable of promoting the cytotoxicity of cancer cells in the presence of human or cynomolgus monkey PBMCs. **Figures 7A-7B** show the ability of the three diabodies to mediate cytotoxicity of Colo205-Luc cells that were incubated with

human PBMC, as measured by LDH assay (**Figure 7A**) or luciferase (**Figure 7B**). **Figures 7C-7D** show the corresponding ability of the three diabodies to mediate cytotoxicity of Colo205-Luc cells that were incubated with cynomolgus monkey PBMC, as measured by LDH assay (**Figure 7A**) or luciferase (**Figure 7B**).

[00175] As shown in **Table 5**, the gpA33 x CD3 bi-specific monovalent diabody DART-2 and the gpA33 x CD3 bi-specific monovalent diabody having an Albumin-Binding Domain (DART-2 w/ABD) displayed comparable CTL activity. The bi-specific monovalent diabodies exhibited consistent activity with both human and cynomolgus monkey (cyno) PBMC effector cells.

Table 5

	EC50 – CTL Activity (ng/mL) Colo205 Target Cells			
	LDH Assay		**Luciferase Assay**	
DART	**Human PBMC**	**Cyno PBMC**	**Human PBMC**	**Cyno PBMC**
gpA33 x CD3 bi-specific monovalent diabody (DART-2)	4.09	3.81	2.73	1.55
gpA33 x CD3 i-specific diabody having an Albumin-Binding Domain (DART-2 w/ABD)	5.52	4.63	3.07	1.63

Example 7
in vivo Reactivity of gpA33 x CD3 diabody in Murine Colon Tumor Model

[00176] In order to demonstrate the *in vivo* ability of the gpA33 x CD3 diabodies of the present invention to provide a treatment for cancer, colo205 cells were co-implanted with activated T cells in immunodeficient NSG (NOD scid gamma) mice (Agliano, A. *et al.* (2008) "*Human Acute Leukemia Cells Injected In NOD/Ltsz-Scid/IL-2Rgamma Null Mice Generate A Faster And More Efficient Disease Compared To Other NOD/Scid-Related Strains*," Int. J. Cancer 123(9):2222-2227; Sanchez, P.V. *et al.* (2009) "*A Robust Xenotransplantation* Model *For Acute Myeloid Leukemia*," Leukemia 23(11):2109-2117; Racki, W.J. *et al.* (2010) "*NOD-Scid IL2rgamma(Null) Mouse Model Of Human Skin Transplantation And Allograft*

Rejection," Transplantation 89(5):527-536; Choi, B. *et al.* (2011) "*Human B Cell Development And Antibody Production In Humanized NOD/SCID/IL-2Rγ(Null) (NSG) Mice Conditioned By Busulfan*," J. Clin. Immunol. 31(2):253-264; Sartelet, H. *et al.* (2012) "*Description Of A New Xenograft Model Of Metastatic Neuroblastoma Using NOD/SCID/Il2rg Null (NSG) Mice*," In Vivo 26(1):19-29; Spranger, S. *et al.* (2012) "*NOD/scid IL-2Rg(null) Mice: A Preclinical Model System To Evaluate Human Dendritic Cell-Based Vaccine Strategies in vivo*," J. Transl. Med. 10:30; von Bonin, M. *et al.* (2013) "*in vivo Expansion Of Co-Transplanted T Cells Impacts On Tumor Re-Initiating Activity Of Human Acute Myeloid Leukemia In NSG Mice*," PLoS One. 8(4):e60680).

[00177] The gpA33 x CD3 bi-specific monovalent diabody DART-1 was administered IV to the mice for once daily for 4 days (QDx4) starting at implantation. Colo205 tumor volume was found to increase in mice receiving the Vehicle control (**Figure 8**). However, animals receiving DART-1 were found to exhibit lower or no Colo205 tumor volume (**Figure 8**).

[00178] Imaging of NSG mice implanted with Colo205 cells showed that at day 2 of treatment mice receiving Vehicle (**Figure 9A**) or the gpA33 x CD3 bi-specific monovalent diabody DART-1 (**Figure 9B**) had significant tumors. However, at day 12 of treatment mice receiving the gpA33 x CD3 bi-specific monovalent diabody DART-1 had dramatically lower tumor volumes (**Figure 9D**). At day 12 of treatment, mice receiving Vehicle showed increased tumor volume (**Figure 9C**).

[00179] As further evidence of the *in vivo* ability of the gpA33 x CD3 diabodies of the present invention to provide a treatment for cancer, the above-described tumor model was conducted using ASPC-1 pancreatic tumor cells and activated human T cells (E:T = 1:1). The gpA33 x CD3 bi-specific monovalent diabody DART-1, a control bi-specific monovalent diabody (Control DART), or Vehicle were administered IV for once daily for 9 days (QDx9) starting at implantation. ASPC-1 tumor volume was found to increase in mice receiving the Vehicle control (**Figure 10**). However, animals receiving DART-1 were found to exhibit lower tumor volume, in a dose-dependent manner (**Figure 10**).

Example 8
Efficacy Determination of gpA33 x CD3 Bi-Specific Monovalent Diabody Having An IgG Fc Domain Version 1 (DART-2 w/Fc Version 1)

[00180] In order to determine the efficacy of the gpA33 x CD3 bi-specific monovalent diabody having an IgG Fc Domain version 1 (DART-2 w/Fc Version 1), mice were infused (using osmotic pumps) for 7 days with the above-described DART-2 w/Fc Version 1 at various dosage levels. 48 h after pump implantation (*i.e.*, in the presence of a steady-state circulating level of DART-2 w/Fc Version 1), a mixture of Colo205 tumor cells and T cells were implanted subcutaneously into the mice, and the extent of tumor growth was monitored. **Table 6** summarizes the design of the study; each group contained 8 female mice.

Table 6

Group	Treatment	Dose (mg/kg)	Route / Schedule	Cell Implant(s)
1	Vehicle	0	IV/QDx5	COLO205 (5E6)
2	gpA33xCD3 bi-specific monovalent diabody having an IgG Fc Domain (DART-2 w/Fc Version 1)	3.1	IP/CIF	COLO205 (5E6) hT-cells (5E6)
3	DART-2 w/Fc Version 1	1.5	IP/CIF	COLO205 (5E6) hT-cells (5E6)
4	DART-2 w/Fc Version 1	0.75	IP/CIF	COLO205 (5E6) hT-cells (5E6)
5	DART-2 w/Fc Version 1	0.375	IP/CIF	COLO205 (5E6) hT-cells (5E6)
6	DART-2 w/Fc Version 1	0.5	IV/QDx5	COLO205 (5E6) hT-cells (5E6)

[00181] The results of this study are shown in **Figure 11**, and indicate that the administration of the above-described gpA33 x CD3 bi-specific monovalent diabodies having an IgG Fc Domain (DART-2 w/Fc Version 1) mediated a dramatic reduction in tumor volume at all tested dosages.

[00182] In light of the dramatic reduction in tumor volume obtained in the above study, a further study was conducted to assess efficacy at much lower doses. **Table 7** summarizes the design of this further study; each group contained 8 female mice.

Table 7

Group	Treatment	Dose (mg/kg)	Route / Schedule	Cell Implant(s)
1	Vehicle	0	IV/QDx5	COLO205 (5E6)
2	gpA33xCD3 bi-specific monovalent diabody having an IgG Fc Domain (DART-2 w/Fc Version 1)	0.2	IP/CIF	COLO205 (5E6) hT-cells (5E6)
3	DART-2 w/Fc Version 1	0.04	IP/CIF	COLO205 (5E6) hT-cells (5E6)
4	DART-2 w/Fc Version 1	0.008	IP/CIF	COLO205 (5E6) hT-cells (5E6)
5	DART-2 w/Fc Version 1	0.0016	IP/CIF	COLO205 (5E6) hT-cells (5E6)
6	DART-2 w/Fc Version 1	0.5	IV/QDx5	COLO205 (5E6) hT-cells (5E6)

[00183] The results of this further study are shown in **Figure 12**. In **Figure 12**, each symbol denotes an animal that received the indicated dosage of the above-described gpA33 x CD3 bi-specific monovalent diabody having an IgG Fc Domain (DART-2 w/Fc Version 1) or Vehicle. The data show efficacy at all tested dosages.

Example 9
Pharmacokinetic Profile of gpA33 x CD3 Bi-Specific Monovalent Diabody (DART-2) and gpA33 x CD3 Bi-Specific Monovalent Diabody Having an IgG Fc Domain (DART-2 w/Fc) in Cynomolgus Monkey

[00184] The ability of the VL_{CD3} and VH_{CD3} domains of the diabodies of the present invention to bind to the CD3 of cynomolgus monkey permits the use of such animals to measure the *in vivo* pharmacokinetics of the diabodies of the present invention.

[00185] To measure such pharmacokinetics, the above-described gpA33 x CD3 bi-specific monovalent diabody (DART-2) or gpA33 x CD3 bi-specific monovalent diabody having an IgG Fc Domain (DART-2 w/Fc Version 1) were injected into cynomolgus monkeys (10 μg/kg/day) and the concentration of such molecules remaining in the circulation was monitored. **Figure 13** shows the result of this study, and indicates that DART-2 and DART-2 w/Fc Version 1 exhibit first-order elimination kinetics.

Example 10
SPR Analysis of gpA33 x CD3 Bi-Specific Monovalent Fc Diabody (DART-1 w/Fc Version 1) Binding to Human and Cynomolgus Monkey CD3 and gpA33

[00186] gpA33 x CD3 bi-specific Fc diabody (DART-2 w/Fc Version 1) binding to soluble versions of human and cynomolgus monkey CD3 receptor was analyzed by SPR on a BIAcore 3000 biosensor (GE, Healthcare). Receptors were immobilized on the CM5 sensor chip according to the procedure recommended by the manufacturer. Briefly, the carboxyl groups on the sensor chip surface were activated with an injection of a solution containing 0.2M N-ethyl-N-(3dietylamino-propyl) carbodiimide and 0.05M N-hydroxy-succinimide. Soluble CD3 receptor (1μg/ml) was then injected over the activated CM5 surface in 10mM sodium-acetate, pH 5.0, at flow rate 5 μL/min, followed by 1 M ethanolamine for deactivation.

[00187] The soluble versions of cynomolgus and human CD3 employed in such analysis were expressed in mammalian cells as a CD3ε / CD3δ heterodimer, stabilized by oppositely charged heterodimer-promoting E-coil and K-coil sequences at their C-termini. The soluble cynomolgus CD3ε contained the first 118 amino acid residues of cynomolgus monkey CD3ε, with the V35 allele (FN18+) followed by the above-described E-coil Domain (**SEQ ID NO:3**) at the carboxy terminus. The amino acid sequence of the V35 allele (FN18+) cynomolgus CD3ε is (**SEQ ID NO:49**):

MQSGTRWRVL GLCLLSIGVW GQDGNEEMGS ITQTPYQVSI SGTTVILTCS
QHLGSEAQWQ HNGKNKEDSG DRLFLPEFSE MEQSGYYVCY PRGSNPEDAS
HHLYLKARVC ENCMEMDVMA VATIVIVDIC ITLGLLLLVY YWSKNRKAKA
KPVTRGAGAG GRQRGQNKER PPPVPNPDYE PIRKGQQDLY SGLNQRRI

[00188] The soluble cynomolgus CD3δ contained the first 101 amino acid residues of cynomolgus monkey CD3δ followed by the above-described K-coil Domain (**SEQ ID NO:4**) at the carboxy terminus. The amino acid sequence of the cynomolgus CD3δ is (**SEQ ID NO:50**):

MEHSTFLSGL VLATLLSQVS PFKIPVEELE DRVFVKCNTS VTWVEGTVGT
LLTNNTRLDL GKRILDPRGI YRCNGTDIYK DKESAVQVHY RMCQNCVELD
PATLAGIIVT DVIATLLLAL GVFCFAGHET GRLSGAADTQ ALLRNDQVYQ
PLRDRDDAQY SRLGGNWARN K

[00189] The two proteins were co-expressed in mammalian CHO-S cells and purified using an anti-E/K-coil mAb coupled to SEPHAROSE®.

[00190] The soluble human CD3ε contained residues 1-127 of human CD3ε with C119S and C122S, followed by the above-described E-coil Domain (**SEQ ID NO:3**) at the carboxy terminus. The amino acid sequence of human CD3ε is (**SEQ ID NO:51**):

MQSGTHWRVL GLCLLSVGVW GQDGNEEMGG ITQTPYKVSI SGTTVILTCP
QYPGSEILWQ HNDKNIGGDE DDKNIGSDED HLSLKEFSEL EQSGYYVCYP
RGSKPEDANF YLYLRARVCE NCMEMDVMSV ATIVIVDICI TGGLLLLVYY
WSKNRKAKAK PVTRGAGAGG RQRGQNKERP PPVPNPDYEP IRKGQRDLYS
GLNQRRI

[00191] The soluble human CD3δ contained residues 1-101 of human CD3δ followed by the above-described K-coil Domain (**SEQ ID NO:4**) at the carboxy terminus. The two proteins were co-expressed in mammalian CHO-S cells and purified using an anti-E/K-coil affinity column. The amino acid sequence of human CD3δ is (**SEQ ID NO:52**):

FKIPIEELE DRVFVNCNTS ITWVEGTVGT LLSDITRLDL GKRILDPRGI
YRCNGTDIYK DKESTVQVHY RMCQSCVELD PATVAGIIVT DVIATLLLAL
GVFCFAGHET GRLSGAADTQ ALLRNDQVYQ PLRDRDDAQY SHLGGNWARN
K

[00192] The soluble human gpA33 contained residues 1-235 of human gpA33 with (**SEQ ID NO:53**) HHHHHH ("6His") repeats at the carboxy terminal end. The soluble cynomolgus gpA33 contained residues 1-314 of cynomolgus monkey gpA33 Met 1 to Gln 314 with 6 His repeats at the carboxy terminal end. The proteins were expressed in mammalian CHO-S cells and purified using Ni SEPHAROSE®.

[00193] Binding experiments were performed in HBS-EP buffer, which contains 10mM HEPES, pH 7.4, 150mM NaCl, 3mM EDTA and 0.005% P20 surfactant. Binding of DART-2 w/Fc Version 1 was analyzed (in duplicate) at concentrations of 0, 6.25, 12.5, 25, 50 and 100 nM, injected for 120 sec at a flow rate of 30 μL/min.

[00194] Regeneration of the immobilized receptor surfaces was performed by pulse injection of 10mM glycine, pH 1.5. Reference curves were obtained by injection of each dilution of DART-2 w/Fc over the treated surface with no immobilized protein. Binding curves at zero concentration were subtracted as a blank. KD values were determined by a global fit of binding curves to the Langmuir 1:1 binding model (BIAevaluation™ software v4.1).

[00195] The SPR analysis of gpA33 x CD3 bi-specific Fc diabody (DART-2 w/Fc Version 1) binding to human and cynomolgus monkey CD3 and gpA33 demonstrated a substantial similarity for the molecules from the two different species (**Figures 14A-14B**; **Figures 15A-15B**). **Table 8** provides the equilibrium dissociation constants (KDs) calculated by global fit to a 1:1 Langmuir model affinity and kinetic constants for DART-2 w/Fc interactions. The KD values of DART-2 w/Fc Version 1 for human and cynomolgus monkey CD3 are nearly identical at 23 and 26 nM, respectively, despite some difference in the maximal binding responses between the two antigens. Random orientation of antigens with different amino acid sequences directly immobilized on the surface can result in different densities of available binding sites on the surface. The KD values for the interaction of DART-2 w/Fc Version 1 with human and monkey gpA33 are 2.2nM and 12nM, respectively (**Table 8**). The difference in affinity is the result of a relatively small decrease in association rate constant and increase in dissociation rate constant for the interaction of DART-2 w/Fc Version 1 with cynomolgus monkey gpA33 (**Table 8**). The data are averages of three independent experiments in duplicates (SD = standard deviation; h, human; cyno, cynomolgus monkey).

Table 8
Equilibrium Dissociation Constants (KD) For The Binding Of DART-2 W/Fc Version 1 To Antigens From Different Species

Antigens	k_a (±SD) ($M^{-1}s^{-1}$)	k_d (±SD) (s^{-1})	K_D (±SD) (nM)
hCD3ε/δ	$1.5(\pm0.1) \times 10^{5}$	$3.5(\pm0.06)\times10^{-3}$	23 ±2.0
cynoCD3ε/δ	$1.3(\pm0.02)\times10^{5}$	$3.4(\pm0.02) \times10^{-3}$	26 ±0.6
hgpA33-His	$4.2(\pm0.3) \times10^{5}$	$9.0(\pm0.5)\times10^{-4}$	2.2±0.2
cynogpA33-His	$2.3(\pm0.2)\times10^{5}$	$2.8(\pm0.1) \times10^{-3}$	12±1.0

[00196] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in its entirety. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

What is claimed is:

Claim 1. A bi-specific monovalent diabody, wherein said 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 said first and second 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 CD3 (VL_{CD3}) having the amino acid sequence of **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}) having the amino acid sequence of **SEQ ID NO:27**; wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**;

ii. a Domain 2, wherein said Domain 2 is a K-coil Domain having the amino acid sequence of **SEQ ID NO:4** or an E-coil Domain having the amino acid sequence of **SEQ ID NO:3**, wherein said Domain 2 is separated from said Domain 1 by a peptide linker having the amino acid sequence of **SEQ ID NO:2**;

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 gpA33 (VL_{gpA33}) having the amino acid sequence of **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}) having the amino acid sequence of **SEQ ID NO:25**, wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**;

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

and wherein:

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

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

Claim 2. The bi-specific monovalent diabody of claim 1, wherein said first polypeptide chain comprises, an Albumin-Binding Domain having the amino acid sequence of **SEQ ID NO:34**, said Albumin-Binding Domain being positioned C-terminally to said Domain 2, and separated from said Domain 2 by a Linker 3 having the amino acid sequence of **SEQ ID NO:32**.

Claim 3. A bi-specific monovalent Fc diabody, wherein said 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 said first and second polypeptide chains are covalently bonded to one another and said 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}) and having the amino acid sequence of **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}) and having the amino acid sequence of **SEQ ID NO:25**, wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**;

ii. a Domain 2, wherein said Domain 2 is an E-coil Domain having the amino acid sequence of **SEQ ID NO:3** or a K-coil Domain having the amino acid sequence of **SEQ ID NO:4**, wherein said Domain 2 is separated from said Domain 1 by a peptide linker having the amino acid sequence of **SEQ ID NO:2**; and

iii. a Domain 3, comprising a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) having the amino acid sequence of **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 said Domains 3 and 2 are separated from one another by a spacer peptide having sequence GGG (Linker 5);

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}) and having the amino acid sequence of **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}) and having the amino acid sequence of **SEQ ID NO:27**; wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**;

ii. a Domain 2, wherein said Domain 2 is a K-coil Domain having the amino acid sequence of **SEQ ID NO:4** or an E-coil Domain having the amino acid sequence of **SEQ ID NO:3**, wherein said Domain 2 is separated from said Domain 1 by a peptide linker having the amino acid sequence of **SEQ ID NO:2**; and wherein said Domain 2 of said first polypeptide chain and said Domain 2 of said 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) having the amino acid sequence of **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) said polypeptide portions of the IgG Fc domains of said first and third polypeptide chain form said IgG Fc Domain;

(b) said VL Domain of said first polypeptide chain and said VH Domain of said second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of gpA33; and

(c) said VH Domain of said first polypeptide chain and said VL Domain of said second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of CD3.

Claim 4. A bi-specific monovalent Fc diabody, wherein said 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 said first and second polypeptide chains are covalently bonded to one another and said 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 3, comprising a sub-Domain (3A), which comprises a cysteine-containing peptide (Peptide 1) having the amino acid sequence of **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;

ii. a Domain 1, comprising a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to gpA33 (VL_{gpA33}) and having the amino acid sequence of **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}) and having the amino acid sequence of **SEQ ID NO:25**, wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**; wherein said Domains 1 and 3 are separated from one another by a spacer peptide (Linker 4) having the amino acid sequence of **SEQ ID NO:38**;

iii. a Domain 2, wherein said Domain 2 is an E-coil Domain having the amino acid sequence of **SEQ ID NO:3** or a K-coil Domain having the amino acid sequence of **SEQ ID NO:4**, wherein said Domain 2 is separated from said Domain 1 by a peptide linker having the amino acid sequence of **SEQ ID NO:2**; and

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}) and having the amino acid sequence of **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}) and having the amino acid sequence of **SEQ ID NO:27**; wherein said sub-Domains (1A) and (1B) are separated from one another by a peptide linker having the amino acid sequence of **SEQ ID NO:1**;

ii. a Domain 2, wherein said Domain 2 is a K-coil Domain having the amino acid sequence of **SEQ ID NO:4** or an E-coil Domain having the amino acid sequence of **SEQ ID NO:3**, wherein said Domain 2 is separated from said Domain 1 by a peptide linker having the amino acid sequence of **SEQ ID NO:2**; and wherein said Domain 2 of said first polypeptide chain and said Domain 2 of said 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) having the amino acid sequence of **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) said polypeptide portions of the IgG Fc domains of said first and third polypeptide chain form said IgG Fc Domain;

(b) said VL Domain of said first polypeptide chain and said VH Domain of said second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of gpA33; and

(c) said VH Domain of said first polypeptide chain and said VL Domain of said second polypeptide chain form an Antigen Binding Domain capable of specific binding to an epitope of CD3.

Claim 5. The bi-specific monovalent Fc diabody of any one of claims 3-4, wherein said sub-Domain (3B) of said first polypeptide chain comprises a sequence different from that of said sub-Domain (3B) of said third polypeptide chain.

Claim 6. The bi-specific monovalent Fc diabody of claim 5, wherein said sub-Domain (3B) of said first polypeptide chain has the amino acid sequence of **SEQ ID NO:40**, and said sub-Domain (3B) of said third polypeptide chain has the amino acid sequence of **SEQ ID NO:41**.

Claim 7. The bi-specific monovalent Fc diabody of claim 5, wherein said sub-Domain (3B) of said first polypeptide chain has the amino acid sequence of **SEQ ID NO:41**, and said sub-Domain (3B) of said third polypeptide chain has the amino acid sequence of **SEQ ID NO:40**.

Claim 8. The bi-specific monovalent Fc diabody of any one of claims 3-4, wherein said Domain 3 of said first polypeptide chain and/or said Domain 3 of said third polypeptide chain comprises a variant CH2-CH3 sequence that exhibits altered binding to an Fcγ receptor.

Claim 9. The bi-specific monovalent diabody of any one of claims 1-2 or the bi-specific monovalent Fc diabody of any of claims 3-8, wherein said Domain 2 of said first polypeptide chain comprises an E-coil having the amino acid sequence of **SEQ ID NO:3**, and said Domain 2 of said second polypeptide chain comprises a K-coil having the amino acid sequence of **SEQ ID NO:4**.

Claim 10. The bi-specific monovalent diabody of any one of claims 1-2 or the bi-specific monovalent Fc diabody of any of claims 3-8, wherein said Domain 2 of said first polypeptide chain comprises a K-coil having the amino acid sequence of **SEQ ID NO:4**, and said Domain 2 of said second polypeptide chain comprises an E-coil having the amino acid sequence of **SEQ ID NO:3**.

Claim 11. A bi-specific monovalent diabody, wherein said bi-specific monovalent diabody is capable of specific binding to an epitope of

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 one of claims 1-2 or 9-11 or the bi-specific monovalent Fc diabody of any one 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, wherein said cancer is colorectal cancer, colon cancer, gastric cancer or pancreatic cancer.

Claim 15. A cell that expresses said first polypeptide chain and/or said second polypeptide chain of any of the bi-specific monovalent diabodies of any one of claims 1-2 or 9-11 or of any of the bi-specific monovalent Fc diabodies of any one of claims 3-10 or 12.

Claim 16. A polynucleotide that encodes the polypeptide of claim 15.