**Title:** TRI-SPECIFIC BINDING MOLECULES THAT SPECIFICALLY BIND TO MULTIPLE CANCER ANTIGENS AND METHODS OF USE THEREOF

**Abstract:** The present invention relates to Tri-Specific Binding Molecules, which are multi-chain polypeptide molecules that possess three Binding Domains and are thus capable of mediating coordinated binding to three epitopes. The Tri-Specific Binding Molecule is preferably characterized in possessing binding domains that permit it to immunospecifically bind to: (1) an epitope of a first Cancer Antigen, (2) an epitope of a second Cancer Antigen, and (3) an epitope of a molecule that is expressed on the surface of an immune system effector cell, and are thus capable of localizing an immune system effector cell to a cell that expresses a Cancer Antigen, so as to thereby facilitate the killing of such cancer cell.

![Figure 10C](image-url)

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Title of the Invention:

Tri-Specific Binding Molecules That Specifically Bind to Multiple Cancer Antigens and Methods of Use Thereof

Cross-Reference to Related Applications

[0001] This application claims priority to United States Patent Applications No. 62/107,824 (filed January 26, 2015), 62/008,229 (filed June 5, 2014; pending), and 62/004,571 (filed May 29, 2014; pending), 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 computer-readable media (file name: 1301_01_19PCT_Sequence_Listing_ST25.txt, created on 18 May 2015, and having a size of 416,408 bytes), which file is herein incorporated by reference in its entirety.

Background of the Invention:

Field of the Invention:

[0003] The present invention relates to Tri-Specific Binding Molecules, which are multi-chain polypeptide molecules that possess three Binding Domains and are thus capable of mediating coordinated binding to three epitopes. The Tri-Specific Binding Molecule is preferably characterized in possessing binding domains that permit it to immunospecifically bind to: (1) an epitope of a first Cancer Antigen, (2) an epitope of a second Cancer Antigen, and (3) an epitope of a molecule that is expressed on the surface of an immune system effector cell, and are thus capable of localizing an immune system effector cell to a cell that expresses a Cancer Antigen, so as to thereby facilitate the killing of such cancer cell.
Description of Related Art:

I. The Mammalian Immune System


[0005] The mammalian immune system is mediated by two separate but interrelated systems: the cellular and humoral immune systems. Generally speaking, the humoral system is mediated by soluble products (antibodies or immunoglobulins) that have the ability to combine with and neutralize products recognized by the system as being foreign to the body. In contrast, the cellular immune system involves the mobilization of certain cells, termed "T cells," that serve a variety of therapeutic roles. T cells are lymphocytes that are derived from the thymus and circulate between the tissues, lymphatic system and the circulatory system. In response to the presence and recognition of foreign structures (antigens), T cells become "activated" to initiate an immune response. In many instances these foreign antigens are expressed on host cells as a result of neoplasia or infection. Although T cells do not themselves secrete antibodies, they are usually required for antibody secretion by the second class of lymphocytes, B cells (which derive from bone marrow). Critically, T cells exhibit extraordinary immunological specificity so as to be capable of discerning one antigen from another. Two types of T cells, "T helper cells" and "cytotoxic T cells," are of particular relevance.

[0006] T helper cells are characterized by their expression of the glycoprotein, CD4 (i.e., they are "CD4+"). CD4+ T cells are the essential organizers of most mammalian immune and autoimmune responses (Dong, C. et al. (2003) "Immune
Regulation by Novel Costimulatory Molecules," Immunolog. Res. 28(1):39-48). The activation of CD4+ T cells has been found to be mediated through co-stimulatory interactions between an antigen:major histocompability class II (MHC II) molecule complex that is arrayed on the surface of an Antigen Presenting Cell (such as a B cell, a macrophage or a dendritic cell) and a complex of two molecules, the T Cell Receptor ("TCR") and a CD3 cell surface receptor ligand, that are arrayed on surface of a naive CD4+ T cell. Activated T helper cells are capable of proliferating into Th1 cells that are capable of mediating an inflammatory response to the target cell.

[0007] Cytotoxic T cells are characterized by their expression of CD8 (i.e., they are "CD8+" as well as CD3+). The activation of CD8+ T cells has been found to be mediated through co-stimulatory interactions between an antigen:major histocompability class I (MHC I) molecule complex that is arrayed on the surface of a target cell and a complex of CD8 and the T Cell Receptor, that are arrayed on surface of the CD8+ T cell. Unlike MHC II molecules, which are expressed by only certain immune system cells, MHC I molecules are very widely expressed. Thus, cytotoxic T cells are capable of binding to a wide variety of cell types. Activated cytotoxic T cells mediate cell killing through their release of the cytotoxins perforin, granzymes, and granulysin. Through the action of perforin, granzymes enter the cytoplasm of the target cell and their serine protease function triggers the caspase cascade, which is a series of cysteine proteases that eventually lead to apoptosis (programmed cell death) of targeted cells.

[0008] The T cell receptor ("TCR") is a covalently linked heterodimer of a and β chains ("TCRaβ"). These chains are class I membrane polypeptides of 259 (α) and 296 (β) amino acids in length. The CD3 molecule is a T cell co-receptor composed of five distinct polypeptide chains (a CD3 γ chain, a CD3 δ chain, two CD3 ε chains and two zeta chains). The individual polypeptide chains associate to form a complex of three dimers (εγ, εδ, γζ) (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; Guy, C.S. et al. (2009) "Organization


[0009] The TCR and CD3 complex, along with the CD3 ζ chain zeta chain (also known as T cell receptor T3 zeta chain or CD247) comprise the TCR complex (van der Merwe, P.A. etc. (epub Dec. 3, 2010) "Mechanisms For T Cell Receptor Triggering," Nat. Rev. Immunol. 11:47-55; 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:a005140). The complex is particularly significant since it contains a large number (ten) of immunoreceptor tyrosine-based activation motifs (ITAMs).


In pathologic states, T cells are the key players of various organ-specific autoimmune diseases, such as type I diabetes, rheumatoid arthritis, and multiple sclerosis (Dong, C. et al. (2003) "Immune Regulation by Novel Costimulatory Molecules," Immunolog. Res. 28(l):39-48).

[0011] The need for two signals to activate T cells such that they achieve an adaptive immune response is believed to provide a mechanism for avoiding responses to self-antigens that may be present on an Antigen Presenting Cell at locations in the system where it can be recognized by a T cell. Where contact of a T cell with a Cell results in the generation of only one of two required signals, the T cell does not become activated and an adaptive immune response does not occur.

II. Antibodies and Other Epitope-Binding Molecules

A. Antibodies

[0012] "Antibodies" are immunoglobulin molecules capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the Variable Domain of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, camelized antibodies, single-chain antibodies, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id and anti-anti-Id antibodies to antibodies of the invention), but also mutants thereof, naturally occurring variants, fusion proteins comprising an antibody portion with an antigen recognition site of the required specificity, humanized antibodies, and chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity. Throughout this
application, the numbering of amino acid residues of the light and heavy chains of
antibodies is according to the EU index as in Kabat et al. (1992) SEQUENCES OF
PROTEINS OF IMMUNOLOGICAL INTEREST, National Institutes of Health Publication
No. 91-3242. As used herein, an "antigen-binding fragment of an antibody" is a
portion of an antibody that possesses an at least one antigen recognition site. As used
herein, the term encompasses fragments (e.g., Fab, Fab', F(ab')_2 Fv), disulfide-linked
bispecific Fvs (sdFv), intrabodies, and single-chain molecules (e.g., scFv). In
particular, antibodies include immunoglobulin molecules and immunologically active
fragments of immunoglobulin molecules, i.e., molecules that contain an antigen-
binding site. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD,
IgA and IgY), class (e.g., IgGl, IgG2, IgG3, IgG4, IgAl and IgA2) or subclass.

[0013] Natural antibodies (such as IgG antibodies) are composed of two Light
Chains complexed with two Heavy Chains. Each Light Chain contains a Variable
Domain (VL) and a Constant Domain (CL). Each heavy chain contains a Variable
Domain (VH), three Constant Domains (CHI, CH2 and CH3), and a Hinge Domain
located between the CHI and CH2 Domains. The basic structural unit of naturally
occurring immunoglobulins (e.g., IgG) is thus a tetramer having two light chains and
two heavy chains, usually expressed as a glycoprotein of about 150,000 Da. The
amino-terminal ("N") portion of each chain includes a variable region of about 100 to
110 or more amino acids primarily responsible for antigen recognition. The carboxy-
terminal ("C") portion of each chain defines a constant region, with light chains
having a single Constant Domain and heavy chains usually having three Constant
Domains and a hinge region. Thus, the structure of the light chains of an IgG
molecule is n-VL-CL-c and the structure of the IgG heavy chains is
n-VH-CHI-H-CH2-CH3-c (where H is the hinge region, and n and c represent,
respectively, the N-terminus and the C-terminus of the polypeptide).

[0014] The ability of an intact, unmodified antibody (e.g., an IgG antibody) 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 Domain and VH Domain,
respectively). 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. The variable regions of an IgG molecule consist of the complementarity determining regions (CDR), which contain the residues in contact with epitope, and non-CDR segments, referred to as framework segments (FR), which in general maintain the structure and determine the positioning of the CDR loops so as to permit such contacting (although certain framework residues may also contact antigen). Thus, the VL and VH Domains have the structure n-FRI-CDR1-FR2-CDR2-FR3-CDR3-FR4-c. Polypeptides that are (or may serve as) the first, second and third CDR of an antibody Light Chain are herein respectively designated CDR L1 Domain, CDR L2 Domain, and CDR L3 Domain. Similarly, polypeptides that are (or may serve as) the first, second and third CDR of an antibody heavy chain are herein respectively designated CDR H1 Domain, CDR H2 Domain, and CDR H3 Domain. Thus, the terms CDR L1 Domain, CDR L2 Domain, CDR L3 Domain, CDR H1 Domain, CDR H2 Domain, and CDR H3 Domain are directed to polypeptides that when incorporated into a protein cause that protein to be able to bind to an specific epitope regardless of whether such protein is an antibody having light and heavy chains or a diabody or a single-chain binding molecule (e.g., an scFv, a BiTe, etc.), or is another type of protein. In contrast to such antibodies, 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 may 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).

[0015] In addition to their known uses in diagnostics, antibodies have been shown to be useful as therapeutic agents. The last few decades have seen a revival of interest in the therapeutic potential of antibodies, and antibodies have become one of the leading classes of biotechnology-derived drugs (Chan, C.E. et al. (2009) "Z34e Use Of Antibodies In The Treatment Of Infectious Diseases," Singapore Med. J. 50(7):663-666). Nearly 200 antibody-based drugs have been approved for use or are under development.
The term "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an antigen. Monoclonal antibodies are highly specific, being directed against a single epitope (or antigenic site). The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')_2 Fv), single-chain (scFv), mutants thereof, fusion proteins comprising an antibody portion, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity and the ability to bind to an antigen. It is not intended to be limited as regards to the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody." Methods of making monoclonal antibodies are known in the art. One method which may be employed is the method of Kohler, G. et al. (1975) "Continuous Cultures Of Fused Cells Secreting Antibody Of Predefined Specificity," Nature 256:495-497 or a modification thereof. Typically, monoclonal antibodies are developed in mice, rats or rabbits. The antibodies are produced by immunizing an animal with an immunogenic amount of cells, cell extracts, or protein preparations that contain the desired epitope. The immunogen can be, but is not limited to, primary cells, cultured cell lines, cancerous cells, proteins, peptides, nucleic acids, or tissue. Cells used for immunization may be cultured for a period of time (e.g., at least 24 hours) prior to their use as an immunogen. Cells may be used as immunogens by themselves or in combination with a non-denaturing adjuvant, such as Ribi (see, e.g., Jennings, V.M. (1995) "Review of Selected Adjuvants Used in Antibody Production," ILAR J. 37(3): 119-125).

In general, cells should be kept intact and preferably viable when used as immunogens. Intact cells may allow antigens to be better detected than ruptured cells by the immunized animal. Use of denaturing or harsh adjuvants, e.g., Freud's adjuvant, may rupture cells and therefore is discouraged. The immunogen may be administered multiple times at periodic intervals such as, bi weekly, or weekly, or
may be administered in such a way as to maintain viability in the animal (e.g., in a tissue recombinant). Alternatively, existing monoclonal antibodies and any other equivalent antibodies that are immunospecific for a desired pathogenic epitope can be sequenced and produced recombinantly by any means known in the art. In one embodiment, such an antibody is sequenced and the polynucleotide sequence is then cloned into a vector for expression or propagation. The sequence encoding the antibody of interest may be maintained in a vector in a host cell and the host cell can then be expanded and frozen for future use. The polynucleotide sequence of such antibodies may be used for genetic manipulation to generate a chimeric antibody, a humanized antibody, or a caninized antibody, or to improve the affinity, or other characteristics of the antibody. The term "humanized" antibody refer to a chimeric molecule, generally prepared using recombinant techniques, having an antigen-binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule based upon the structure and/or sequence of a human immunoglobulin. The polynucleotide sequence of the variable domains of such antibodies may be used for genetic manipulation to generate such derivatives and to improve the affinity, or other characteristics of such antibodies. The general principle in humanizing an antibody involves retaining the basic sequence of the antigen-binding portion of the antibody, while swapping the non-human remainder of the antibody with human antibody sequences. There are four general steps to humanize a monoclonal antibody. These are: (1) determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy variable Domains (2) designing the humanized antibody or caninized antibody, i.e., deciding which antibody framework region to use during the humanizing or canonizing process (3) the actual humanizing or caninizing methodologies/techniques and (4) the transfection and expression of the humanized antibody. See, for example, U.S. Patents Nos. 4,816,567; 5,807,715; 5,866,692; and 6,331,415.

[0018] The epitope-binding domain of such antibodies may comprise either complete Variable Domains fused onto Constant Domains or only the complementarity determining regions (CDRs) grafted onto appropriate framework regions in the Variable Domains. Antigen-binding sites may be wild-type or modified by one or more amino acid substitutions. This eliminates the constant region as an
In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, or six) which differ in sequence relative to the original antibody.

B. Bi-Specific Antibodies, Multi-Specific Diabodies and DART™ Diabodies

Natural antibodies are capable of binding to only one epitope species (i.e., they are "mono-specific"), although they may be able to bind multiple copies of that species (i.e., they may exhibit bi-valency or multi-valency). A wide variety of recombinant bi-specific antibody formats have been developed (see, e.g., PCT Publications Nos. WO 2008/003116, WO 2009/132876, WO 2008/003103, WO 2007/146968, WO 2007/146968, WO 2009/018386, WO 2012/009544, WO 2013/070565), most of which use linker peptides either to fuse the antibody core (IgA, IgD, IgE, IgG or IgM) to a further binding protein (e.g., scFv, VL VH, etc.) to, or within, the antibody core, or to fuse multiple antibody portions or to fuse (e.g. two Fab fragments or scFv) to a Heterodimerization-Promoting Domain such as the CH2-CH3 Domain or alternative polypeptides (WO 2005/070966, WO 2006/107786A WO 2006/107617A, WO 2007/046893). Typically, such approaches involve compromises and trade-offs. For example, PCT Publications Nos. WO 2013/174873, WO 2011/133886 and WO 2010/136172 disclose that the use of linkers may cause problems in therapeutic settings, and teaches a tri-specific antibody in which the CL and CHI Domains are switched from their respective natural positions and the VL and VH Domains have been diversified (WO 2008/027236; WO 2010/108127) to allow them to bind to more than one antigen. Thus, the molecules disclosed in these documents trade binding specificity for the ability to bind additional antigen species. PCT Publications Nos. WO 2013/163427 and WO 2013/119003 disclose modifying the CH2 Domain to contain a fusion protein adduct comprising a binding domain. The document notes that the CH2 Domain likely plays only a minimal role in mediating effector function. PCT Publications Nos. WO 2010/028797, WO2010028796 and WO 2010/028795 disclose recombinant antibodies whose Fc Domains have been replaced with additional VL and VH Domains, so as to form trivalent binding molecules. PCT Publications Nos. WO 2003/025018 and WO2003012069 disclose recombinant diabodies whose individual chains contain scFv domains. PCT Publications No. WO 2013/006544 discloses multi-valent Fab molecules that are synthesized as a single polypeptide chain and then subjected to proteolysis to yield heterodimeric structures. Thus, the molecules disclosed in these
documents trade all or some of the capability of mediating effector function for the ability to bind additional antigen species. PCT Publications Nos. WO 2014/022540, WO 2013/003652, WO 2012/162583, WO 2012/156430, WO 2011/086091, WO 2007/075270, WO 1998/002463, WO 1992/022583 and WO 1991/003493 disclose adding additional Binding Domains or functional groups to an antibody or an antibody portion (e.g., adding a diabody to the antibody's Light Chain, or adding additional VL and VH Domains to the antibody's light and heavy chains, or adding a heterologous fusion protein or chaining multiple Fab Domains to one another). Thus, the molecules disclosed in these documents trade native antibody structure for the ability to bind additional antigen species.

The design of a diabody is based on the structure of single-chain Variable Domain fragments (scFv). Such molecules are made by linking light and/or Heavy Chain Variable Domains to one another via a short linking peptide. Bird et al. (1988) ("Single-Chain Antigen-Binding Proteins," Science 242:423-426) describes an example of linking peptides which bridge approximately 3.5 nm between the carboxy terminus of one Variable Domain and the amino terminus of the other Variable Domain. Linkers of other sequences have been designed and used (Bird et al. (1988) "Single-Chain Antigen-Binding Proteins," Science 242:423-426). Linkers can in turn be modified for additional functions, such as attachment of drugs or attachment to solid supports. The single-chain variants can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as E. coli. Polynucleotides encoding the scFv of interest can be made by routine manipulations such as ligation of polynucleotides. The resultant scFv can be isolated using standard protein purification techniques known in the art.


The provision of non-mono-specific 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).

[0025] Diabody epitope-binding domains may be directed to a surface determinant of any immune effector cell such as CD3, CD16, CD32, CD64, etc., 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., Fey 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 2006/1 3665; 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 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).
For example, United States Patent No. 6,171,586, concerns the production of bi-specific antibodies by proteolytically cleaving two antibodies to obtain their F(\text{ab}')_2 fragments, reducing such fragments under conditions for preventing intermolecular disulfide bond formation, and then mixing the fragments to generate the bi-specific antibody). United States Patents No. 6,551,592; 6,994,853 and 8,277,806 and PCT Publications Nos. WO 2012/156430, WO 2002/020039, WO 2000/018806 and WO 1998/003670 concern the production of tri-specific antibodies capable of simultaneously binding to T cells and other antigens on a tumor cell, and, via the Fc portion of the bi-specific antibody, to the Fc receptor of cells possessing such a receptor. PCT Publications Nos. WO 2000/018806, WO 1998/003670 and WO 2006/072152 concern the production of tri-specific antibodies capable of simultaneously binding to T cells and other antigens. United States Patent Publication No. 2008-0057054 discloses bi-specific conjugates specific for a binding element against amyloid beta oligomers and a binding element against transmembrane protein telencephalin. United States Patent Publication No. 2010-0291112 concerns bi-specific and tri-specific single-chain Fv molecules that specifically bind to a one (or two) tumor antigen(s) and an effector cell antigen (such as CD3, CD16 CD32, CD64, etc.).

PCT Publication Nos. WO 1999/042597 and WO 1998/006749 disclose antibody derivatives that comprise human Major Histocompatibility Complex binding domains, with or without bound MHC binding peptides. PCT Publication No. WO 02/072141 concerns multi-specific binding molecules whose on-rates (rates at which they bind to target molecules) and off-rates (rates at which they release target molecules) differ so as to preferentially bind to one target compared to their binding to the other such target molecule. Tri-specific molecules, for example molecules having a monovalent first portion which is an Anti-CD3 or anti-CD28 antibody, and a second portion comprising a divalent immune function exerting moiety which immunospecifically binds to one or more target ligands on a target diseased cell or immune cell.

United States Patent No. 7,695,936 and Patent Publication 2007/0196363 concern bi-specific antibodies that are formed from the heavy chains of two
antibodies, one of which possess a protuberance engineered into its heavy chain and the second of which possess a complementary cavity engineered into its heavy chain. The presence of such complementary "knobs" and "holes" is taught to preferentially form bi-specific hetero-antibodies (having one heavy chain of each such antibody) relative to mono-specific homo-antibodies that contain two heavy chains of the same antibody. Various bi-specific hetero-antibodies are proposed, including those that are immunospecific for CD3 and a tumor cell antigen. Various tri-specific hetero-antibodies are also proposed, including some that are immunospecific for CD3, CD8 and CD37 (a transmembrane protein expressed predominantly on B cells that is involved the regulation of T cell proliferation (Robak, T. et al. (2014) "Anti-CD37 Antibodies For Chronic Lymphocytic Leukemia," Expert Opin. Biol. Ther. 14(5):651-661), however, no mechanism for their production and no disclosure of their structure is provided.

PCT Publication WO2012-162561 concerns bi-specific, tetravalent binding molecules that comprise two polypeptides, each of which comprises two diabody structures, separated by an intervening CH2-CH3 Domain. The document also concerns tetravalent binding molecules composed of four polypeptide chains in which two of the polypeptide chains contain variable light and variable heavy Domains for two antigens, and in which the other two polypeptide chains contain the complementary variable heavy and variable light Domains for the antigens and a terminal CH2-CH3 Domain. The bi-specific, tetravalent binding molecules form through the association of their respective CH2-CH3 Domains. In the four polypeptide chain construct, the "light" chains are not covalently bound to the heavy chains, thus leading to instability (see, 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). The document discloses a third construct in which the chains are altered to provide such covalent bonding, but at the cost of eliminating their bi-specificity (i.e., the molecules are mono-specific). Molecules having specificity for CD2, CD3, CD4, CD8, CD161, a chemokine receptor, CD95, CCR5, etc. are disclosed. A bi-specific molecule capable of binding to both CD3 and CD8 is not disclosed.
However, the above advantages come at salient cost. The formation of such non-mono-specific 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-mono-specific 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 Domain," 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).

However, the art has recognized that bi-specific 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).
In the face of this challenge, the art has succeeded in developing stable, covalently bonded heterodimeric non-mono-specific diabodies, termed DARTs™ (see, e.g., United States Patent Publications No. 2013-0295121; 2010-0174053 and 2009-0060910; European Patent Publication No. EP 2714079; EP 2601216; EP 2376109; EP 2158221 and PCT Publications No. WO 2012/162068; WO 2012/018687; WO 2010/080538; and 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; Veri, M.C. et al. (2010) "Therapeutic Control Of B Cell Activation Via Recruitment Of Fcgamma Receptor lib (CD32B) Inhibitory Function With A Novel Bispecific Antibody Scaffold," Arthritis Rheum. 62(7): 1933-1943; 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. Mol. Biol. 399(3):436-449). Such diabodies comprise two or more covalently complexed polypeptides and involve engineering one or more cysteine residues into each of the employed polypeptide species that permit disulfide bonds to form and thereby covalently bond two polypeptide chains. 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.

There are many DART™ embodiments. Each of the two polypeptides of the simplest DART™ embodiment comprises three Domains (Figure 1). The first polypeptide comprises: (i) a first domain that comprises a binding region of a Light Chain Variable Domain of the a first immunoglobulin (VL1), (ii) a second domain that comprises a binding region of a Heavy Chain Variable Domain of a second immunoglobulin (VH2), and (iii) a third domain that contains a cysteine residue (or a Cysteine-Containing Domain) and a Heterodimerization-Promoting Domain that serves to promote heterodimerization with the second polypeptide chain. The cysteine residue (or a Cysteine-Containing Domain) of the third domain serves to promote the covalent bonding of the first polypeptide chain to the second polypeptide chain of the diabody. The second polypeptide contains: (i) a complementary first domain (a VL2-containing Domain), (ii) a complementary second domain (a VH1-
containing Domain) and (iii) a third domain that contains a cysteine residue (or a Cysteine-Containing Domain) and, optionally, a complementary Heterodimerization-Promoting Domain that complexes with the Heterodimerization-Promoting Domain of the first polypeptide chain in order to promote heterodimerization with the first polypeptide chain. The cysteine residue (or a Cysteine-Containing Domain) of the third domain of the second polypeptide chain serves to promote the covalent bonding of the second polypeptide chain to the first polypeptide chain of the diabody. Such molecules are stable, potent and have the ability to simultaneously bind two or more antigens. They are able to promote re-directed T cell mediated killing of cells expressing target antigens.

[0034] In one embodiment, the third domains of the first and second polypeptides each contain a cysteine residue, which serves to bind the polypeptides together via a disulfide bond. The third domain of one or both of the polypeptides may additionally possess the sequence of a CH2-CH3 Domain, such that complexing of the diabody polypeptides forms an Fc Domain that is capable of binding to the Fc receptor of cells (such as B lymphocytes, dendritic cells, Natural Killer cells, macrophages, neutrophils, eosinophils, basophils and mast cells) (Figures 2A-2B).

[0035] Many variations of such molecules have been described (see, e.g., United States Patent Publications No. 2013-0295121; 2010-0174053 and 2009-0060910; European Patent Publication No. EP 2714079; EP 2601216; EP 2376109; EP 2158221 and PCT Publications No. WO 2012/162068; WO 2012/018687; WO 2010/080538). These Fc-bearing DARTs may comprise three polypeptide chains (e.g., Figure 2B). The first polypeptide chain of such a diabody contains three domains: (i) a VL1-containing Domain, (ii) a VH2-containing Domain and (iii) a domain containing a cysteine residue (or a Cysteine-Containing Domain) and a Heterodimerization-Promoting Domain, and (iv) a cysteine residue (or a Cysteine-Containing Domain and a CH2-CH3 Domain. The second polypeptide chain of such DART™ contains: (i) a VL2-containing Domain, (ii) a VH1-containing Domain and (iii) a Domain that contains a cysteine residue (or a Cysteine-Containing Domain) and a Heterodimerization-Promoting Domain that promotes heterodimerization with the first polypeptide chain. The cysteine residue (or a Cysteine-Containing Domain) of
the third domain of the second polypeptide chain serves to promote the covalent bonding of the second polypeptide chain to the first polypeptide chain of the diabody. The third polypeptide of such DART™ comprises a cysteine residue (or a Cysteine-Containing Domain) and a CH2-CH3 Domain. Thus, the first and second polypeptide chains of such DART™ associate together to form a VL1/VH1 binding site that is capable of binding to the epitope, as well as a VL2/VH2 binding site that is capable of binding to the second epitope. The first and second polypeptides are bonded to one another through a disulfide bond involving cysteine residues in their respective third domains. Notably, the first and third polypeptide chains complex with one another to form an Fc Domain that is stabilized via a disulfide bond. Such diabodies have enhanced potency. Such Fc-bearing DARTs™ may have either of two orientations (Table 1):

| Table 1 |
|-----------------|-----------------|
| **First Orientation** | **Second Orientation** |
| 3rd Chain | NH2-CH2-CH3-COOH |
| 1st Chain | NH2-VL1-VH2-Cys-Heterodimer-Promoting Domain-CH2-CH3-COOH |
| 2nd Chain | NH2-VL2-VH1-Cys-Heterodimer-Promoting Domain-COOH |
| 3rd Chain | NH2-CH2-CH3-COOH |
| 1st Chain | NH2-CH2-CH3-VL1-VH2-Cys-Heterodimer-Promoting Domain-COOH |
| 2nd Chain | NH2-VL2-VH1-Cys-Heterodimer-Promoting Domain-COOH |

[0036] Even more complex DART™ diabodies, termed Ig-DART™ (Figures 3A-3B) and Fc-DART™ diabodies (Figure 3C) have been described (WO 2012/018687). Fc-DARTs™ have four polypeptide chains. The first and third polypeptide chains of such a diabody contain three Domains: (i) a VL1-containing Domain, (ii) a VH2-containing Domain and (iii) a Domain containing a CH2-CH3 sequence. The second and fourth polypeptide of the Fc-DART™ contain: (i) a VL2-containing Domain, (ii) a VH1-containing Domain and (iii) a Domain that promotes heterodimerization and covalent bonding with the Fc-DART’s™ first polypeptide chain. The third and fourth, and the first and second polypeptide chains may be the same or different so as to permit tetravalent binding that is either mono-specific, bispecific or terra-specific. Such more complex DART™ molecules also possess Cysteine-Containing Domains which function to form a covalently bonded complex. Fc-DART™ diabodies contain CHI and CL Domains.
Alternative constructs are known in the art for applications where a tetravalent molecule is desirable but an Fc is not required including, but not limited to, tetravalent tandem antibodies, also referred to as "TandAbs" (see, e.g. United States Patent Publications Nos. 2005-0079170, 2007-0031436, 2010-0099853, 2011-020667 2013-0189263; European Patent Publication Nos. EP 1078004, EP 2371866, EP 2361936 and EP 1293514; PCT Publications Nos.WO 1999/057150, WO 2003/025018, and WO 2013/013700) which are formed by the homo-dimerization of two identical chains each possessing a VH1, VL2, VH2, and VL2 Domain.

However, despite all prior advances, a need remains for compositions that could provide improved therapeutic value to patients suffering from cancer or other diseases and conditions. The present invention is directed to this and other goals.

**Summary of the Invention:**

The present invention relates to Tri-Specific Binding Molecules, which are multi-chain polypeptide molecules that possess three Binding Domains and are thus capable of mediating coordinated binding to three epitopes. The Tri-Specific Binding Molecule is preferably characterized in possessing binding domains that permit it to immunospecifically bind to: (1) an epitope of a first Cancer Antigen, (2) an epitope of a second Cancer Antigen, and (3) an epitope of a molecule that is expressed on the surface of an immune system effector cell, and are thus capable of localizing an immune system effector cell to a cell that expresses a Cancer Antigen, so as to thereby facilitate the killing of such cancer cell.

In detail, the invention provides a Tri-Specific Binding Molecule capable of immunospecifically binding to three different epitopes, said Epitopes being Epitope I, Epitope II, and Epitope III, wherein two of three epitopes are epitopes of Cancer Antigen(s), and the third of said epitopes is an epitope of an Effector Cell Antigen.

The invention particularly concerns the embodiment of such Tri-Specific Binding Molecule wherein the molecule comprises four different polypeptide chains covalently complexed together and comprises:

(I) an Antigen-Binding Domain I that is capable of immunospecifically binding to an Epitope I present on a first antigen, and an Antigen-Binding Domain II that
is capable of immunospecifically binding to an Epitope II present on a second antigen, wherein the Antigen-Binding Domain I and the Antigen-Binding Domain II are both Diabody-Type Binding Domains;

(II) an Antigen-Binding Domain III that is capable of immunospecifically binding to an Epitope III present on a third antigen; and

(III) an Fc Domain that is formed by the complexing of two CH2-CH3 Domains to one another;

wherein one of Epitope I, Epitope II or Epitope III is an epitope of an Effector Cell Antigen, a second of Epitope I, Epitope II or Epitope III is an epitope of a first Cancer Antigen, and the third of Epitope I, Epitope II or Epitope III is an epitope of a second Cancer Antigen, and wherein the Antigen-Binding Domains I, II and III of the Binding Molecules mediate coordinated binding of an immune system effector cell expressing the Effector Cell Antigen and a cancer cell expressing the first and second Cancer Antigens.

[0042] The invention particularly concerns the embodiment of such Tri-Specific Binding Molecules wherein the Fc Domain is capable of binding to an Fc Receptor arrayed on the surface of a cell.

[0043] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the Effector Cell Antigen is arrayed on the surface of an effector cell and wherein the Cancer Antigens are arrayed on the surface of a cancer cell, and wherein the immunospecific binding is sufficient to co-localize the Effector Cell Antigen, and the Cancer Antigens, thereby facilitating the activation of the effector cell against the cancer cell.

[0044] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the Effector Cell Antigen is selected from the group consisting of: CD2, CD3, CD16, CD19, CD20, CD22, CD32B, CD64, the B cell Receptor (BCR), the T cell Receptor (TCR), and the NKG2D Receptor.

[0045] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the first and second Cancer Antigens are independently selected from the group consisting of: colon cancer antigen 19.9; a gastric cancer
mucin; antigen 4.2; glycoprotein A33 (gpA33); ADAM-9; gastric cancer antigen AH6; ALCAM; malignant human lymphocyte antigen APO-1; cancer antigen Bl; B7-H3; beta-catenin; blood group ALLa/Leb; Burkitt’s lymphoma antigen-38.13, colonic adenocarcinoma antigen C14; ovarian carcinoma antigen CA125; Carboxypeptidase M; CD5; CD19; CD20; CD22; CD23; CD25; CD27; CD30 ; CD33; CD36; CD45; CD46; CD52; CD79a/CD79b; CD103; CD317; CDK4; carcinoembryonic antigen (CEA); CEACAM5; CEACAM6; C017-1A; CO-514 (blood group Le\(^b\)); CO-43 (blood group Le\(^a\)); CTA-1; CTLA4; Cytokeratin 8; antigen Dl.l; antigen Di56-22; DR5; Ei series (blood group B); EGFR (Epidermal Growth Factor Receptor); Ephrin receptor A2 (EphA2); ErbBl; ErbB3; ErbB4; GAGE-1; GAGE-2; GD2/GD3/GM2; lung adenocarcinoma antigen F3; antigen FC10.2; G49, ganglioside GD2; ganglioside GD3; ganglioside GM2; ganglioside GM3; G\(_{D2}\); G\(_{D3}\); GICA 19-9; G\(_{M2}\); gp100; human leukemia T cell antigen Gp37; melanoma antigen gp75; gpA33; HER2 antigen (pI85 \(^{HFr2}\)); human milk fat globule antigen (HMFG); human papillomavirus-E6/human papillomavirus-E7; high molecular weight melanoma antigen (HMW-MAA); I antigen (differentiation antigen) I(Ma); Integrin Alpha-V-Beta-6 IntegrinP6 (ITGB6); Interleukin-13; Receptor a2 (IL13Ra2); JAM-3; KID3; KID31; KS 1/4 pancreaticoma antigen; human lung carcinoma antigens L6 and L20; LEA; LUCA-2; Ml:22:25:8; M18; M39; MAGE-1; MAGE-3; MART; MUC-1; MUM-1; Myl; N-acetylglucosaminyltransferase; neoglycoprotein; NS-10; OFA-1; OFA-2; Oncostatin M; p15; melanoma-associated antigen p97; polymorphic epithelial mucin (PEM); polymorphic epithelial mucin antigen (PEMA); PIPA; prostate-specific antigen (PSA); prostate-specific membrane antigen (PSMA); prostatic acid phosphate; R\(_{24}\); ROR1; sphingolipids; SSEA-1; SSEA-3; SSEA-4; sTn; T cell receptor derived peptide; T\(_5\)A5; TAG-72; TL5 (blood group A); TNF-a receptor; TNF-\(\beta\) receptor; TNF-\(\gamma\) receptor; TRA-1-85 (blood group H); Transferrin Receptor; tumor-specific transplantation antigen (TSTA), oncofetal antigen-alpha-fetoprotein (AFP); VEGF; VEGFR, VEP8; VEP9; VIM-D5; and Y hapten, Le\(^y\).

[0046] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the first and second Cancer Antigens are selected from the group consisting of: CD2, CD317, CEACAM5, CEACAM6, DR5, EphA2, gpA33, Her2, B7-H3; EGF, EGFR, VEGF and VEGFR.
The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the Non-Diabody-Type Binding Domain III comprises the Fab-Type Binding Domain (VL\textsubscript{III}/VH\textsubscript{III}) that is capable of immunospecifically binding to an Epitope III, wherein the molecule comprises:

(A) a first polypeptide chain that comprises in the N-terminus to C-terminus direction:
   (1) a light chain variable Domain of an immunoglobulin capable of binding to a first of the three epitopes (VL\textsubscript{i});
   (2) a heavy chain variable Domain of an immunoglobulin capable of binding to a second of the three epitopes (VH\textsubscript{n});
   (3) a Heterodimer-Promoting Domain; and
   (4) CH\textsubscript{2} and CH\textsubscript{3} Domains of an IgG;

(B) a second polypeptide chain that comprises, in the N-terminus to C-terminus direction:
   (1) a light chain variable Domain of an immunoglobulin capable of binding to the second of the three epitopes (VL\textsubscript{n});
   (2) a heavy chain variable Domain of an immunoglobulin capable of binding to the first of the three epitopes (VH\textsuperscript{a}); and
   (3) a complementary Heterodimer-Promoting Domain;

(C) a third polypeptide chain that comprises, in the N-terminus to C-terminus direction:
   (1) a heavy chain variable Domain of an immunoglobulin capable of binding to a third of the three epitopes (VH\textsubscript{in}); and
   (2) a CH\textsubscript{I} Domain, a Hinge Domain, and a CH\textsubscript{2}-CH\textsubscript{3} Domain of an IgG;

and

(D) a fourth polypeptide chain that comprises, in the N-terminus to C-terminus direction:
   (1) a light chain variable Domain of an immunoglobulin capable of binding to the third of the three epitopes (VL\textsubscript{a}); and
   (2) a light chain constant Domain (CL);

wherein:
(i) the VLi and $V^{3/4}$ Domains associate to form a Domain capable of binding the first epitope;
(ii) the $V_{L_{II}}$ and $VH_n$ Domains associate to form a Domain capable of binding the second epitope;
(iii) the $V_{L_{III}}$ and $VH_m$ Domains associate to form a Domain capable of binding the third epitope;
(iv) the CH2-CH3 Domain of the first polypeptide chain and the CH2-CH3 Domain of the third polypeptide chain associate to form an Fc Domain;
(v) the first and second polypeptide chains are covalently bonded to one another;
(vi) the first and third polypeptide chains are covalently bonded to one another; and
(vii) the third and fourth polypeptide chains are covalently bonded to one another.

[0048] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein:

(A) the Heterodimer-Promoting Domain is an E-coil and the complementary Heterodimer-Promoting Domain is a K-coil; or
(B) the Heterodimer-Promoting Domain is a K-coil and the complementary Heterodimer-Promoting Domain is an E-coil.

[0049] The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein:

(A) the CH2-CH3 Domains of the first and third polypeptide chains each have the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits normal FcyR-mediated effector function; or
(B) the CH2-CH3 Domain of the first and third polypeptide chains comprise at least one amino acid substitution, relative to the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits altered FcyR-mediated effector function.
The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the at least one amino acid substitution comprises at least one amino acid substitution selected from the group consisting of: L235V, F243L, R292P, Y300L, V305I, and P396L, wherein the numbering is that of the EU index as in Kabat.

The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the at least one amino acid substitution comprises:

(A) at least one substitution selected from the group consisting of F243L, R292P, Y300L, V305I, and P396L;

(B) at least two substitutions selected from the group consisting of:
   (1) F243L and P396L;
   (2) F243L and R292P; and
   (3) R292P and V305I;

(C) at least three substitutions selected from the group consisting of:
   (1) F243L, R292P and Y300L;
   (2) F243L, R292P and V305I;
   (3) F243L, R292P and P396L; and
   (4) R292P, V305I and P396L;

(D) at least four substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L and P396L; and
   (2) F243L, R292P, V305I and P396L;

or

(E) at least the five substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L, V305I and P396L; and
   (2) L235V, F243L, R292P, Y300L and P396L.

The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein the CH2-CH3 Domain of the first and third polypeptide chains differ from one another and have an amino acid sequence selected from the group consisting of SEQ ID NO:52 and SEQ ID NO:53.
The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein:

(A) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of the first Cancer Antigen, an epitope of the second Cancer Antigen and an epitope of the Effector Cell Antigen;

(B) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of the first Cancer Antigen, an epitope of the Effector Cell Antigen and an epitope of the second Cancer Antigen;

(C) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of second Cancer Antigen, an epitope of the first Cancer Antigen, and an epitope of the Effector Cell Antigen;

(D) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of the second Cancer Antigen, an epitope of the Effector Cell Antigen and an epitope of the first Cancer Antigen;

(E) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of the Effector Cell Antigen, an epitope of the first Cancer Antigen, and an epitope of the second Cancer Antigen;

and

(F) the Epitope I, Epitope II and Epitope III are, respectively, an epitope of the Effector Cell Antigen, an epitope of second Cancer Antigen, and an epitope of the first Cancer Antigen.

The invention additionally concerns the embodiment of such Tri-Specific Binding Molecules wherein:

(A) the epitope of an Effector Cell Antigen is a CD2 epitope recognized by antibody Lo-CD2a;

(B) the epitope of an Effector Cell Antigen is a CD3 epitope recognized by antibody OKT3, M291, YTH12.5, Anti-CD3 mAb 1 or Anti-CD3 mAb 2;

(C) the epitope of an Effector Cell Antigen is a CD16 epitope recognized by antibody 3G8 or A9;

(D) the epitope of an Effector Cell Antigen is a CD19 epitope recognized by antibody MD1342, MEDI-551, blinatumomab or HD37;
(E) the epitope of an Effector Cell Antigen is a CD20 epitope recognized by antibody rituximab, ibritumomab, ofatumumab, and tositumomab;

(F) the epitope of an Effector Cell Antigen is a CD22 epitope recognized by antibody epratuzumab;

(G) the epitope of an Effector Cell Antigen is a CD32B epitope recognized by antibody CD32B mAb 1;

(H) the epitope of an Effector Cell Antigen is a CD64 epitope recognized by antibody CD64 mAb 1;

(I) the epitope of an Effector Cell Antigen is a BCR/CD79 epitope recognized by antibody CD79 mAb 1;

(J) the epitope of an Effector Cell Antigen is a TCR epitope recognized by antibody BMA 031;

or

(K) the epitope of an Effector Cell Antigen is a NKG2D Receptor epitope recognized by antibody KYK-2.0.

[0055] The invention additionally concerns a pharmaceutical composition that comprises any of the above-described Tri-Specific Binding Molecules, and a pharmaceutically acceptable carrier, excipient or diluent.

[0056] The invention additionally concerns the embodiment of such pharmaceutical composition or of any such Tri-Specific Binding Molecules wherein the Tri-Specific Binding Molecule is used in the treatment of cancer.

[0057] The invention additionally concerns the embodiment of such pharmaceutical compositions or such Tri-Specific Binding Molecules wherein the cancer is characterized by the presence of a cancer cell selected from the group consisting of a cell of: an adrenal gland tumor, an AIDS-associated cancer, an alveolar soft part sarcoma, an astrocytic tumor, bladder cancer, bone cancer, a brain and spinal cord cancer, a metastatic brain tumor, a breast cancer, a carotid body tumors, a cervical cancer, a chondrosarcoma, a chordoma, a chromophobe renal cell carcinoma, a clear cell carcinoma, a colon cancer, a colorectal cancer, a cutaneous benign fibrous histiocytoma, a desmoplastic small round cell tumor, an ependymoma,
a Ewing's tumor, an extraskeletal myxoid chondrosarcoma, a fibrogenesis imperfecta ossium, a fibrous dysplasia of the bone, a gallbladder or bile duct cancer, gastric cancer, a gestational trophoblastic disease, a germ cell tumor, a head and neck cancer, hepatocellular carcinoma, an islet cell tumor, a Kaposi's Sarcoma, a kidney cancer, a leukemia, a lipoma/benign lipomatous tumor, a liposarcoma/malignant lipomatous tumor, a liver cancer, a lymphoma, a lung cancer, a medulloblastoma, a melanoma, a meningioma, a multiple endocrine neoplasia, a multiple myeloma, a myelodysplasia syndrome, a neuroblastoma, a neuroendocrine tumors, an ovarian cancer, a pancreatic cancer, a papillary thyroid carcinoma, a parathyroid tumor, a pediatric cancer, a peripheral nerve sheath tumor, a phaeochromocytoma, a pituitary tumor, a prostate cancer, a posterior uveal melanoma, a rare hematologic disorder, a renal metastatic cancer, a rhabdoid tumor, a rhabdomysarcoma, a sarcoma, a skin cancer, a soft-tissue sarcoma, a squamous cell cancer, a stomach cancer, a synovial sarcoma, a testicular cancer, a thymic carcinoma, a thymoma, a thyroid metastatic cancer, and a uterine cancer.

[0058] The invention additionally concerns the embodiment of such pharmaceutical compositions or such Tri-Specific Binding Molecules wherein the cancer is acolorectal cancer, hepatocellular carcinoma, glioma, kidney cancer, breast cancer, multiple myeloma, bladder cancer, neuroblastoma; sarcoma, non-Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer, pancreatic cancer or a rectal cancer.

[0059] The invention additionally concerns the embodiment of such pharmaceutical compositions or such Tri-Specific Binding Molecules the cancer is acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), acute B lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin's lymphomas (NHL), including mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, or Burkitt's lymphoma.
Brief Description of the Drawings:

[0060] Figures 1A-1B show diagrammatic representation of the Domains of DART™ diabodies. Figure 1A shows a diagrammatic representation of the Domains of a basic DART™ diabody. Figure 1B provides a schematic of a covalently bonded diabody composed of two polypeptide chains, each having a Heterodimer-Promoting Domain VL and VH domains that recognize the same epitope are shown using the same shading.

[0061] Figures 2A-2B provide a schematic of covalently bonded diabodies composed of two polypeptide chains, each having a CH2 and CH3 Domain (Figure 2A) or in which only one has a CH2 and CH3 Domain (Figure 2B), such that the associated chains form an Fc Domain that comprises all or part of a naturally occurring Fc Domain. VL and VH domains that recognize the same epitope are shown using the same shading.

[0062] Figures 3A-3C provide schematics showing tetravalent diabodies composed of two pairs of polypeptide chains. The pairs are different, thus resulting in a bi-specific molecule that is bivalent with respect to each of two epitopes, in which one is an epitope of DR5 and the other is an epitope of a molecule present on the surface of an effector cell. One polypeptide of each pair possesses a CH2 and CH3 Domain, such that the associated chains form an Fc Domain that comprises all or part of a naturally occurring Fc Domain. VL and VH domains that recognize the same epitope are shown using the same shading. Only one pair of epitopes (shown with the same shading) is capable of binding to DR5. Figure 3A shows an Ig diabody. Figure 3B shows an Ig diabody, which contains E-coil and K-coil heterodimer-promoting domains. Figure 3C, shows an Fc-DART™ diabody that contains antibody CHI and CL domains. The notation "VL1" and "VH1" denote respectively, the Variable Light Chain Domain and Variable Heavy Chain Domain that bind the "first" epitope. Similarly, the notation "VL2" and "VH2" denote respectively, the Variable Light Chain Domain and Variable Heavy Chain Domain that bind the "second" epitope.
[0063] Figures 4A-4G provide a diagrammatic representation of the Domains of preferred Tri-Specific Binding Molecules of the present invention. The Figures illustrate schematically the order and orientation of the Domains of embodiments of the preferred Tri-Specific Binding Molecules of the present invention. Figures 4A, 4B and 4G illustrate embodiments in which the Tri-Specific Binding Molecule is composed of four polypeptide chains. Figures 4C, 4D, 4E and 4F illustrate embodiments in which the binding molecule is composed of three polypeptide chains. The molecule may possess Hinge and/or CL domains (Figures 4A, 4B, 4C, 4E) or may contain an alternative linker peptide (Figure 4D, 4F, 4G).

[0064] Figures 5A-5E provide a diagrammatic representation of the Domains of an alternative embodiment of the Tri-Specific Binding Molecules of the present invention, in which the Effector Cell-Binding Domain is composed of an Effector Cell Receptor-Type Binding Domain rather than a Diabody-Type Binding Domain or a Fab-Type Binding Domain. Figures 5A and 5B illustrate embodiments in which the Tri-Specific Binding Molecule is composed of four polypeptide chains. Figure 5C and Figure 5E illustrate an embodiment in which the binding molecule is composed of three polypeptide chains. Figure 5D illustrates an embodiment in which the binding molecule is composed of five polypeptide chains. The molecule may possess Hinge and/or CL domains or may contain alternative linker peptides.

[0065] Figure 6 shows the ability of anti-human DR5 monoclonal antibodies DR5 mAb 1 and DR5 mAb 2 to bind to human DR5 and to the DR5 of cynomolgus monkey.

[0066] Figure 7, Panels A-H, show the kinetics of binding of DR5 mAb 1 (Panels A and E), DR5 mAb 2 (Panels B and F), DR5 mAb 3 (Panels C and G) and DR5 mAb 4 (Panels D and H) for human DR 5 (Panels A-D) and for cynomolgus monkey DR5 (Panels E-H).

[0067] Figure 8 shows the unexpected superiority of DR5 mAb 1 and DR5 mAb 2. Superiority was assessed by comparing the ability of DR5 x CD3 diabodies having the VL and VH Domains of DR5 mAb 1, DR5 mAb 2, DR5 mAb 3, or DR5 mAb 4,
to mediate the cytotoxicity of A549 adenocarcinomic human alveolar basal epithelial tumor cells.

[0068] Figures 9A-9C demonstrate the synergistic enhancement in target cell binding that is attained when both of the two Cancer Antigen-Binding Domains of a Tri-Specific Binding Molecule of the present invention are able to bind to a target cell. Figure 9A shows the binding obtained when trispecific molecules: EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1; EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1; and gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 are incubated in the presence of EphA2-expressing CHO cells. Figure 9B shows the binding obtained when such trispecific molecules are incubated in the presence of DR5-expressing CHO cells. Figure 9C shows the binding obtained when such trispecific molecules are incubated in the presence of DU145 human prostate cells that express EphA2 and DR5, but not gpA33.

[0069] Figures 10A-IOC demonstrate the synergistic enhancement in target cell cytotoxicity that is attained when both of the two Cancer Antigen-Binding Domains of a Tri-Specific Binding Molecule of the present invention are able to bind to a target cell. Figure 10A shows the percent cytotoxicity obtained by incubating trispecific molecules: EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1; EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1; and gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 are incubated in the presence of EphA2-expressing CHO cells and cytotoxic lymphocytes. Figure 10B shows the percent cytotoxicity obtained when such trispecific molecules are incubated in the presence of DR5-expressing CHO cells and cytotoxic lymphocytes. Figure 10C shows the cytotoxicity obtained when such trispecific molecules are incubated in the presence of DU145 human prostate cells and cytotoxic lymphocytes. DU145 cells express EphA2 and DR5, but not gpA33. Cytotoxicity was measured by the increase in luminescence caused by the release of luciferase upon cell lysis.

Detailed Description of the Invention:

[0070] The present invention relates to Tri-Specific Binding Molecules, which are multi-chain polypeptide molecules that possess three Binding Domains and are thus capable of mediating coordinated binding to three epitopes. The Tri-Specific Binding
Molecule is preferably characterized in possessing binding domains that permit it to immunospecifically bind to: (1) an epitope of a first Cancer Antigen, (2) an epitope of a second Cancer Antigen, and (3) an epitope of a molecule that is expressed on the surface of an immune system effector cell, and are thus capable of localizing an immune system effector cell to a cell that expresses a Cancer Antigen, so as to thereby facilitate the killing of such cancer cell.

[0071] The Tri-Specific Binding Molecules of the present invention may include Epitope-Binding Domains of humanized, chimeric or caninized derivatives of the above-discussed antibodies, for example, DR5 mAb 1 or DR5 mAb 2.

I. General Techniques and General Definitions

II. Preferred Tri-Specific Binding Molecules of the Present Invention

A. Binding Capabilities

The preferred Tri-Specific Binding Molecules of the present invention are able to coordinately and simultaneously bind to three different epitopes. The preferred Tri-Specific Binding Molecules of the present invention comprise:

(I) a "Binding Domain I" that is capable of immunospecifically binding to an "Epitope I" present on a first antigen, and a "Binding Domain II" that is capable of immunospecifically binding to an "Epitope II" present on a second antigen, wherein said Binding Domain I and said Binding Domain II are both "Diabody-Type Binding Domains;"

(II) a "Binding Domain III" that is capable of immunospecifically binding to an "Epitope III" present on a third antigen; and

(III) an Fc Domain that is formed by the complexing of two CH2-CH3 Domains to one another;

wherein:

(A) one of Epitope I, Epitope II or Epitope III is an epitope of a first "Cancer Antigen" Cancer Antigen;

(B) a second of Epitope I, Epitope II or Epitope III is an epitope of a second Cancer Antigen; and
(C) the third of Epitope I, Epitope II or Epitope III is an epitope of a molecule expressed on the surface of an immune system effector cell ("Effector Cell Antigen"); and wherein the Binding Domains I, II and III of the binding molecules mediate coordinated binding of the immune system effector cell and a cell expressing both the first and second Cancer Antigens to thereby co-localize such cells.

[0074] Diabody Epitope-Binding Domains may also be directed to a surface determinant of a B cell, such as CD19, CD20, CD22, CD30, CD37, CD40, and CD74 (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; Cheson, B.D. et al. (2008) "Monoclonal Antibody Therapy For B-Cell Non-Hodgkin's Lymphoma," N. Engl. J. Med. 359(6):613-626; Castillo, J. et al. (2008) "Newer monoclonal antibodies for hematological malignancies," Exp. Hematol. 36(7):755-768. In many studies, diabody binding to effector cell determinants, e.g., Fey receptors (FcyR), was also found to activate the effector cell (Holliger et al. 1996) "Specific Killing Of Lymphoma Cells By Cytotoxic T-Cells Mediated By A Bi-specific Diabody," Protein Eng. 9:299-305; Holliger et al. (1999) "Carcinoembryonic Antigen (CEA)-Specific T-Cell Activation In Colon Carcinoma Induced By Anti-CDS x Anti-CEA Bi-specific Diabodies And B7 x Anti-CEA Bi-specific Fusion Proteins," Cancer Res. 59:2909-2916; WO 2006/13665; 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-FcyR interaction; thus, in this regard, diabody molecules 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) "Bi-specific Antibody Conjugates In Therapeutics," Adv. Drug. Deliv. Rev. 55:171-197).

[0075] Although such Tri-Specific Binding Molecules are particularly preferred, the invention additionally specifically contemplates Tri-Specific Binding Molecules
that comprise any combination of Binding Domains sufficient to produce a molecule having three binding specificities, of which two are binding specificities directed against Cancer Antigens, and one is a binding specificity directed against an Effector Cell Antigen. Thus, for example, the invention contemplates: a Tri-Specific Binding Molecule that comprises three Fab-Type Binding Domains, a Tri-Specific Binding Molecule that comprises one bivalent, bi-specific antibody domain (formed for example, by complexing two different light chains and two different heavy chains) and one Fab-Type Binding Domain, a Tri-Specific Binding Molecule that comprises two bivalent, bi-specific antibody domains (formed for example, by complexing four different light chains and two different heavy chains), but in which one of antibody domains has been rendered inactive, etc.

[0076] The terms "polypeptide," "polypeptide chain," and "peptide" are used interchangeably herein to refer to polymers of amino acids of any length, but especially lengths greater than 3, 5, 10, 15, 20 or 25 amino acid residues, in which two, and more preferably all, amino acid residues are joined via an amide (peptide) bond (-NH-C(O)-). The polymer may however be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. The polypeptides of this invention can occur as single-chains or as complexed chains.

[0077] A "Diabody-Type Binding Domain" is the Epitope-Binding Domain of a diabody, and especially, a DART® diabody. The terms "diabody" and "DART® diabody" has been discussed above, and refers to a molecule that comprises at least two polypeptide chains that preferably complex with one another through a covalent interaction to form at least two epitope binding sites, which may recognize the same or different epitopes. Two of the polypeptide chains of a diabody or DART® diabody
each comprise immunoglobulin Light Chain Variable Region and an immunoglobulin Heavy Chain Variable Region, but these regions do not interact to form an epitope binding site (*i.e.*, they are not mutually *"complementary"*). Rather, the immunoglobulin Heavy Chain Variable Region of one (*e.g.*, the first) of the diabody, or DART® diabody, chains interacts with the immunoglobulin Light Chain Variable Region of a different (*e.g.*, the second) diabody or, DART® diabody, polypeptide chain to form an epitope binding site. Similarly, the immunoglobulin Light Chain Variable Region of one (*e.g.*, the first) of the diabody, or DART® diabody, polypeptide chains interacts with the immunoglobulin Heavy Chain Variable Region of a different (*e.g.*, the second) diabody, or DART® diabody, polypeptide chain to form an epitope binding site. DART® diabody molecules are disclosed in United States Patent Publications No. 2013-0295121; 2010-0174053 and 2009-0060910; European Patent Publication No. EP 2714079; EP 2601216; EP 2376109; EP 2158221 and PCT Publications No.WO 2012/162068; WO 2012/018687; WO 2010/080538; WO 2006/13665, WO 2008/157379 and 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; Veri, M.C. *et al.* (2010) *"Therapeutic Control Of B Cell Activation Via Recruitment Of Fcgamma Receptor lib (CD32B) Inhibitory Function With A Novel Bi-specific Antibody Scaffold,"* Arthritis Rheum. 62(7): 1933-1943; and 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. Mol. Biol. 399(3):436-449.

[0078] Binding Domain III is preferably a *"Non-Diabody-Type"* Binding Domain, which is intended to denote that Binding Domain III does not have the structure of a Diabody-Type Binding Domain. Preferably, Binding Domain III is a Non-Diabody-Type Binding Domain that is a Fab-Type Binding Domain or an Effector Cell Receptor-Type Binding Domain. Thus, in one embodiment, exemplified in Figures 4A-4G, the Binding Domain III is a Fab-Type Binding Domain. Figures 5A-5E exemplify the embodiment in which Binding Domain III is an Effector Cell Receptor-Type Binding Domain. As used herein, the term an *"Fab-Type Binding Domain"* refers to an epitope Binding Domain that is formed by the interaction of the VL Domain of an immunoglobulin light chain and a complementing
VH Domain of an immunoglobulin heavy chain. Fab-Type Binding Domains differ from Diabody-Type Binding Domain in that the two polypeptide chains that form a Fab-Type Binding Domain comprise only a single epitope Binding Domain, whereas the two polypeptide chains that form a Diabody-Type Binding Domain comprise at least two epitope Binding Domains. Thus, as used herein Fab-Type Binding Domains are distinct from Diabody-Type Binding Domain. When a binding domain is a Fab-Type Binding Domain or a Diabody-Type Binding Domain, it will be composed of a VL Domain and a VH Domain, which may be located on the same or on different polypeptide chains. The selection of such VL and VH domains is coordinated, such that the domains form an epitope binding domain. As used herein, the term "Effector Cell Receptor-Type Binding Domain" refers to an epitope binding domain that is formed by the interaction of a variable domain of a T Cell Receptor alpha chain and a variable domain of a T Cell Receptor beta chain. Such receptors recognize peptides displayed in the context of MHC and are thus capable of recognizing intracellular epitopes.

[0079] The Tri-Specific Binding Molecules of the present invention are thus distinguished from tetravalent binding molecules, such as those produced from the dimerization of a bivalent antibody, and preferably possess three and not four Binding Domains. As discussed below, the trispecific molecules of the present invention may possess additional binding domains (such as an Albumin-Binding Domain, an FcyR-Binding Domain, etc.). Such additional Binding Domains are not intended to be considered or counted as being one of the three Binding Domains of the Tri-Specific Binding Molecules of the present invention.

[0080] As used herein, the terms "association" or "associating," with regard to polypeptides (e.g., one diabody polypeptide to another, an immunoglobulin light chain to an immunoglobulin heavy chain, one CH2-CH3 Domain to another CH2-CH3 Domain, etc.) is intended to denote a non-covalent combining of the polypeptides. The terms "complexes" or "complexing" are intended to denote a covalent combining of the polypeptides.
As used herein, Binding Domains of a Binding Molecule of the invention is said to mediate "coordinated binding" if at least two of its Binding Domains and preferably all of its Binding Domains, are capable of concurrently being bound to their respective recognized epitopes or binding ligand. Such binding may be simultaneous. However, one aspect of the present invention relates to modifying the "on" and/or "off" rates with which such Binding Domains bind to their recognized epitopes. As used here, the "on rate" of binding is a measure of the affinity with which such Binding Domains recognize and initiate binding to their recognized epitopes. In contrast, the "off rate" of binding is a measure of the degree of stability of the Binding Domain:epitope complex. The "on" and/or "off" rates of binding can be modified by altering the amino acid sequence of the CDRs of a Binding Domain.

As discussed below, independent of any CDR modifications, the extent of coordinated binding of the molecules of the present invention may be modulated by changing the configuration of the their Binding Domains so that a particular Binding Domain (i.e., a VLx/VHx Domain) is present as Binding Domain III or as an internal or external Diabody-Type Binding Domain relative to Binding Domain III (discussed in detail below).


[0083] The Binding Domains of the the Tri-Specific Binding Molecules of the present invention bind to epitopes in an "immunospecific" manner. As used herein, an antibody, diabody or other epitope binding molecule is said to "immunospecifically" bind a region of another molecule (i.e., an epitope) if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with that epitope relative to alternative epitopes. For example, an antibody that immunospecifically binds to a viral epitope is an antibody that binds this viral epitope with greater affinity, avidity, more readily, and/or with greater duration than it immunospecifically binds to other viral epitopes or non-viral epitopes. It is also understood by reading this definition that, for example, an antibody (or moiety or epitope) that immunospecifically binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means "specific" binding. Two molecules are said to be capable of binding to one another in a "physiospecific" manner, if such binding exhibits the specificity with which receptors bind to their respective ligands.

[0084] The functionality of antibodies can be enhanced by generating multispecific antibody-based molecules that can simultaneously bind two separate and distinct antigens (or different epitopes of the same antigen) and/or by generating antibody-based molecule having higher valency (i.e., more than two binding sites) for the same epitope and/or antigen.

[0085] Thus, in their simplest embodiment, the preferred binding molecules of the present invention are at least trispecific. Significantly, such molecules have at least
three Sites that are capable of binding antigen: an "external" Diabody-Type Binding Domain that is located furthest from Binding Domain III, an "internal" Diabody-Type Binding Domain that is located nearest to Binding Domain III, and Binding Domain III itself. The positions of such Domains are respectively designated as "Site A," Site B" and "Site C" (Figures 4A-4G; Figures 5A-5E).

[0086] The Tri-Specific Binding Molecules of the present invention are able to coordinately bind to three different epitopes by comprising three binding domains. Two of the binding domains of such molecules are capable of binding to epitopes of "Cancer Antigens," such that the molecule is capable of binding to two different Cancer Antigens. The third binding domain of such molecules is capable of binding to an epitope of a molecule expressed on the surface of an immune system effector cell (i.e., an "Effector Cell Antigen"). Thus, the Tri-Specific Binding Molecules of the present invention are able to mediate coordinated and simultaneous binding to a cancer cell expressing two Cancer Antigens and to an immune system effector cell expressing the Effector Cell Antigen. The epitopes recognized by the Tri-Specific Binding Molecules of the present invention may be continuous or discontinuous (e.g., conformational).

[0087] The first and second Cancer Antigens that are bound by the Cancer Antigen-Binding Domains of the trispecific binding molecules of the present invention may be selected from any molecule that is characteristically present on the surface of a cancer cell. One aspect of the present invention relates to the ability to target "Low Expression Cancer Antigens" (i.e., a Cancer Antigen that may be expressed on a cancer cell at a level too low to permit a monospecific binding molecule to provide an effective cancer therapy). In contrast to such monospecific binding molecules, the Tri-Specific Binding Molecules of the present invention, by targeting two Cancer Antigens instead of one, exhibit synergistic and cooperative enhanced binding avidity that may compensate for low affinity of binding and thus may be advantageously used to target cancers characterized even by a Low Expression Cancer Antigen. A second aspect of the present invention relates to the ability to target "Low Specificity Cancer Antigens" (i.e., a Cancer Antigen that may be expressed on a normal cell in addition to being expressed on a cancer cell). The Tri-Specific Binding Molecules of the
present invention, by providing synergistic and cooperative enhanced binding avidity to two Cancer Antigens, exhibits higher avidity of binding even for Low Specificity Cancer Antigens and thus provides a means for treating cancers that are characterized by such Cancer Antigens. Thus, the Tri-Specific Binding Molecules of the present invention may be used to impart an anti-cancer therapy even in circumstances where one or both of the target Cancer Antigens is ineffective on its own to provide such therapy.

[0088] For example, CD32B (the FcγRIIB receptor) is widely expressed on hematopoietic cells, including monocytes, macrophages, B cells, NK cells, neutrophils, mast cells, and platelets. Upon binding to IgG Fc Domain, CD32B inhibits the host immune system to thereby depress an ongoing immune response. Although such inhibition is desirable in helping the host recover from inflammatory reactions, it serves to exacerbate the immune deficiencies of subjects suffering from cancer or infectious disease. Antibodies that bind to CD32B, so as to block the binding of IgG Fc molecules, serve to prevent such inhibition and thus have utility as adjunct molecules in the treatment of cancer and infectious disease (Veri, M.C. et al. (2007) "Monoclonal Antibodies Capable Of Discriminating The Human Inhibitory Fcgamma-Receptor IIB (CD32B) From The Activating Fcgamma-Receptor IIA (CD32A): Biochemical, Biological And Functional Characterization," Immunology 121(3):392-404). Unfortunately, CD32B is also expressed on liver sinusoidal endothelial cells ("LSE cells") (Shahani, T. et al. (2014) "Human Liver Sinusoidal Endothelial Cells But Not Hepatocytes Contain Factor VIII," J. Thromb. Haemost. 12(1):36-42; Geraud, C. et al. (2013) "Endothelial Transdifferentiation In Hepatocellular Carcinoma: Loss Of Stabilin-2 Expression In Peri-Tumourous Liver Correlates With Increased Survival," Liver Int. 33(9): 1428-1440; Takabe, Y. et al. (2012) "Immunomagnetic Exclusion Of E-Cadherin-Positive Hepatoblasts In Fetal Mouse Liver Cell Cultures Impairs Morphogenesis And Gene Expression Of Sinusoidal Endothelial Cells," J. Anat. 221(3):229-239). Thus, antibodies that bind CD32B attack LSE cells. However, by forming a Tri-Specific Binding Molecule of the present invention that binds to CD32B and to antigens (i.e., the first and second Cancer Antigens) that are not expressed on LSE cells, or are expressed at low levels by such cells (i.e., Low Expression Cancer Antigen(s)), or are expressed with low...
specificity on cancer cells and such LSE cells (i.e., Low Specificity Cancer Antigen(s)), the present invention provides compositions and methods that would be used to depress CD32B-mediated immune system inhibition.

B. Exemplary Cancer Antigen-Binding Domains

Human Anti-Idiotypic Antibody That Mimics The GD2 Antigen, " J. Immunol., 151, 3390-3398); ganglioside GD3 (Shitara et al. (1993) "A Mouse/Human Chimeric Anti-
(Ganglioside GD3) Antibody With Enhanced Antitumor Activities, " Cancer Immunol. Immunother. 36:373-380); ganglioside GM2 (Livingston et al. (1994) "Improved
Survival In Stage III Melanoma Patients With GM2 Antibodies: A Randomized Trial Of Adjuvant Vaccination With GM2 Ganglioside, " J. Clin. Oncol. 12:1036-1044);
53:5244-5250); G\textsubscript{D\textsubscript{2}}; G\textsubscript{D\textsubscript{3}}; GICA 19-9 (Herlyn et al. (1982) "Monoclonal Antibody Detection Of A Circulating Tumor-Associated Antigen. I. Presence Of Antigen In Sera Of Patients With Colorectal, Gastric, And Pancreatic Carcinoma, " J. Clin. Immunol.
2:135-140); G\textsubscript{M\textsubscript{2}}; gp100 (Lotem, M. et al. 2006 J Immunother. 29(6):616-27); Gp37 (human leukemia T cell antigen) (Bhattacharya-Chatterjee et al. (1988) "Idiotype Vaccines Against Human T Cell Leukemia. II. Generation And Characterization Of A Monoclonal Idiotype Cascade (Ab1, Ab2, and Ab3), " J. Immunol. 141:1398-1403);
gp75 (melanoma antigen) (Vijayasardahl et al. (1990) "The Melanoma Antigen Gp75 Is The Human Homologue Of The Mouse B (Brown) Locus Gene Product, " J.
(Ragupathi, G. 2005 Cancer Treat Res. 123:157-180); prostatic acid phosphate
(Tailor et al. (1990) "Nucleotide Sequence Of Human Prostatic Acid Phosphatase
Determined From A Full-Length cDNA Clone," Nucl. Acids Res. 18(16):4928; R24
as found in melanoma; ROR1 (United States Patent No. 5,843,749); sphingolipids;
SSEA-1; SSEA-3; SSEA-4; sTn (Holmberg, L.A. 2001 Expert Opin Biol Ther.
1(5):881-91); T cell receptor derived peptide from a cutaneous T cell lymphoma
(see Edelson (1998) "Cutaneous T-Cell Lymphoma: A Model For Selective
Immunotherapy," Cancer J Sci Am. 4:62-71); T2Aγ found in myeloid cells; TAG-72
(Yokota et al. (1992) "Rapid Tumor Penetration Of A Single-Chain Fv And
Comparison With Other Immunoglobulin Forms," Cancer Res. 52:3402-3408); TL5
(blood group A); TNF-receptor (TNF-a receptor, TNF-β receptor; TNF-γ receptor
CurrDrug Targets. l(4):327-64); TRA-1-85 (blood group H); Transferrin Receptor
(United States Patent No. 7,572,895; PCT Publication No. WO 05/121 179); TSTA
(tumor-specific transplantation antigen) such as virally-induced tumor antigens
including T-antigen DNA tumor viruses and envelope antigens of RNA tumor viruses,
oncofetal antigen-alpha-fetoprotein such as CEA of colon, bladder tumor oncofetal
antigen (Hellstrom et al. (1985) "Monoclonal Antibodies To Cell Surface Antigens
Shared By Chemically Induced Mouse Bladder Carcinomas," Cancer. Res. 45:2210-
2188); VEGF receptor (O’Dwyer. P.J. 2006 Oncologist. 11(9):992-998); VEP8;
VEP9; VIM-D5; and Y hapten, Le3 as found in embryonal carcinoma cells.

1. Campath-1 (CD52) Binding Domain (Alemtuzumab)

The amino acid sequence of the VL Domain of the humanized anti-CD52
antibody "Alemtuzumab" (SEQ ID NO:205) is shown below (CDR residues are
shown underlined):

DIQMTQSPSS LSASVGDRVT ITCKASQND KYLNYWQKP GKAQKLLIYN
TNNLQTGVPF RFSGSGSGTD PTFTISSLQP EDIATYYCLQ HISPRRTFGQ
GTKVEIKR

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The amino acid sequence of the VH Domain of the humanized anti-CD52 antibody "Alemtuzumab" (SEQ ID NO:206) is shown below (CDR residues are shown underlined):

\[
\text{QVQLQESGPGLVRPSQTLSTCTVGFTFTDFYMNWVRQPGRGLEWIGFIRDKAKGYTT_EYNPSVKGRVTMLVDTSKNQFSLRLSSVTAADTAIVYCAR} \]

\[
\text{EGHTAAPFDYWGGSLVTVS} \]

2. CD317 (BMST2)-Binding Domains


\[
\text{QVQLQESGPGLVRPSQTLSTCTVGFTFTDFYMNWVRQPGRGLEWIGFIRDKAKGYTT_EYNPSVKGRVTMLVDTSKNQFSLRLSSVTAADTAIVYCAR} \]

\[
\text{EGHTAAPFDYWGGSLVTVS} \]
The amino acid sequence of the VH Domain of the anti-CD317 antibody "HM1.24" (SEQ ID NO:303) is shown below (CDR residues are shown underlined):

QVQLQQSGAE LARPGASVKL SCKASGYTFT PYWMQWVKQR PGQGLEWIGS

IFPGDGDTRY SQKFKGKATL TADKSSSTAY MQSILAFED SAVYYCARGL

RRGYYFDPYW GQGTTLTVSS

3. CEACAM5- and CEACAM6-Binding Domains

Carcinoembryonic Antigen-Related Cell Adhesion Molecules 5 (CEACAM5) and 6 (CEACAM6) have been found to be associated with various types of cancers including medullary thyroid cancer, colorectal cancer, pancreatic cancer, hepatocellular carcinoma, gastric cancer, lung cancer, head and neck cancers, urinary bladder cancer, prostate cancer, uterine cancer, endometrial cancer, breast cancer, hematopoietic cancer, leukemia and ovarian cancer (PCT Publication No. WO 2011/034660), and particularly colorectal, gastrointestinal, pancreatic, non-small cell lung cancer (NSCL), breast, thyroid, stomach, ovarian and uterine carcinomas (Zheng, C. et al. (2011) "A Novel Anti-CEACAM5 Monoclonal Antibody, CC4, Suppresses Colorectal Tumor Growth and Enhances NK Cells-Mediated Tumor Immunity," PLoS One 6(6):e21146, pp. 1-11).

CEACAM5 has been found to be overexpressed in 90% of gastrointestinal, colorectal and pancreatic cancers, 70% of non-small cell lung cancer cells and 50% of breast cancers (Thompson, J.A. et al. (1991) "Carcinoembryonic Antigen Gene Family: Molecular Biology And Clinical Perspectives," J. Clin. Lab. Anal. 5:344-366).

Overexpressed carcinoembryonic antigen-related cellular adhesion molecule 6 (CEACAM6) plays important roles in the invasion and metastasis of a variety of human cancers, including medullary thyroid cancer, colorectal cancer, pancreatic cancer, hepatocellular carcinoma, gastric cancer, lung cancer, head and neck cancers, urinary bladder cancer, prostate cancer, uterine cancer, endometrial cancer, breast cancer, hematopoietic cancer, leukemia and ovarian cancer (PCT Publication No. WO 2011/034660; Deng, X. et al. (2014) "Expression Profiling Q

```
DI QMTQS P S S L SASVGDRVT ITCGASENY GALN WYQRKP GKS PKLL IWG ASNLADGMP S RFSGSGSGGRQ YTLT I S SLQP EDVATYY CNQ VLSSPYTFGG GTKLE 1 K
```

[0097] The amino acid sequence of the VH Domain of the humanized anti-CEACAM5 / ANTI-CEACAM6 antibody 16C3 (EP 2585476) (SEQ ID NO:305) is shown below (CDR residues are shown underlined):

```
QVQLQQS GPE VVRPGVS VKI SCKGSYFTFT DYAMHHVKQ S HAKS LEWI GL ISTYSGDTKY NQNFKGKATM TVDKS TAY MEL S SLRSE D TAVYYCARGD YSGSRYWFAY WGGGTLVTS S
```

[0098] The amino acid sequence of the VL Domain of the humanized anti-CEACAM5 / CEACAM6 antibody hMN15 (WO 2011/034660) (SEQ ID NO:306) is shown below (CDR residues are shown underlined):

```
DI QLTQS P S S L SASVGDRVT MTCSASSRVS VIH WYQQKPG KAPKRWI YGT STLASGVPAR F S GSGSGTDF TFTI S SLQPE DIATYYC QOW SYNPPTFQGG TKVE I KR
```
The amino acid sequence of the VH Domain of the humanized anti-
CEACAM5 / CEACAM6 antibody hMN15 (WO 201/034660) (SEQ ID NO:307) is shown below (CDR residues are shown underlined):

QVQLVESGGG VVQPGRSLRL SCSSSGALT DYYMSWVRQA PGKGLEWLGF IANKANGHTT DYPSVKGRF TISRDNSKNT LFLQMDSLRP EDTGVYFCAR DMGRIRWNFDV WGGGTPVTS

4. DR5-Binding Domains

DR5 is a preferred Cancer Antigen of the present invention. The preferred anti-human DR5-binding molecules of the present invention possess the VL and/or VH Domains of murine anti-human DR5 monoclonal antibodies "DR5 mAb 1" and/or "DR5 mAb 2," and more preferably possess 1, 2 or all 3 of the CDRs of the VL Domain and/or 1, 2 or all 3 of the CDRs of the VH Domain of such anti-human DR5 monoclonal antibodies. Alternatively, any anti-human DR5 monoclonal antibody may be employed, particularly: drozitumab (designated herein as "DR5 mAb 3"), conatumumab (designated herein as "DR5 mAb 4"), tigatumumab (designated herein as "DR5 mAb 5"), LBY135-1 (designated herein as "DR5 mAb 6"), LBY135-2 (designated herein as "DR5 mAb 7") and KMTR2 (designated herein as "DR5 mAb 8").

a. The Anti-Human DR5 Antibody DR5 mAb 1

DR5 has potential utility in the treatment of a wide range of cancers (e.g., colorectal cancer, hepatocellular carcinoma, glioma, kidney cancer, breast cancer, multiple myeloma, bladder cancer, neuroblastoma; sarcoma, non-Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer, pancreatic cancer and rectal cancer. The amino acid sequence of human DR5 precursor (NCBI Sequence NP_003833.4) (SEQ ID NO:2) is:

MEQRGQNAPA ASGARKRHGP GPREARGARP GLRVPKTLVL VVAAVLLLVS AESALITQOD LAPQQRVAPQ QKRSSPSEGL CPPGHISIED GRDCI SCKYG QDYSTHWNDL LFCLRCTRCD SGEVELSPCT TTRNTVCQCE EGTFFREEDSP EMCRKCRTGC PRGVMKVGD CTPWDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS LSIIGGVTVT AVAVLVIAVF VCKSSLWKKV LPYLKIGCSG GGGDPERVDR SSQRPAGEDN VLNIIEVSILQ PTQVIPGEQME VQEPAEPTGV NMLSPGESEH LLEFAPAAERS QRRRLLVPAN EGDPTETLRQ CFDDFADLVP FDSWEPILMRK LGMLDNEIKV AKAEEAGHRD TLYTMLIKWV NKTGRDASVH TLLDALETLG ERLAKQKIED HLLSSGKFMY LEGNADSAMS
The amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3) is shown below (CDR residues are shown underlined):

\[
\text{DIVLTSQPAS LAVSLQRAT ISCRASKVS SSGYSYMHWY QQKPGQP}}\] 
\[
\text{LIFLLSNLDS GVPARFSGSG SGTDFTLNIH PVEDGDAATY YCOHSRDLP}}\] 
\[
\text{TFGGGKTLEI K}
\]

CDR\textsubscript{L} 1 of DR5 mAb 1 (SEQ ID NO:4): RASKSVSSSGYSYMHWY

CDR\textsubscript{L} 2 of DR5 mAb 1 (SEQ ID NO:5): LSSNLDs

CDR\textsubscript{L} 3 of DR5 mAb 1 (SEQ ID NO:6): QHSRDLPPT

The VL Domain of DR5 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO:7) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

\[
g\text{acattgtgc} \text{tgacacagtc} \text{tcctgcttcc} \text{ttagctgtat} \text{ctctcgggca} \\
g\text{agggccacc} \text{atctcatgca} \underline{g\text{gcccagca}} \underline{a\text{aggtgcagt}} \text{tctctctggct} \\
\text{taatgtatat} \text{gcac tggagc} \text{caacagaaac} \text{cagcacgcc acccaagtc} \\
\text{ctcatccttc} \underline{tttcatccaa} \underline{cctagattct} \\
\text{tgccagttgg} \text{tctgggacag} \text{acttcaccc} \text{caacatccat} \text{ctctgagg} \\
\text{atggggatgtg} \text{gacg} \text{ttcggtg} \text{gaggcacca} \text{gctggaaatc} \text{aaa}
\]

The VH Domain of DR5 mAb 1 (SEQ ID NO:8) is shown below (CDR residues are shown underlined). The C-terminal amino acid may be substituted with alanine to facilitate subcloning of this VH Domain.

\[
\text{EVKFLESGGG LVQPGGSLKL SCVASFDFS RYWMSWVRQA PGKGLEWIGE} \\
\underline{INPDSNTIN} \underline{TPSLKD}I \underline{SRDNAKNTLY LQM}TKVRSED \underline{TALLYCTRRA} \\
\underline{YYGNP}AWFAY \underline{WGQ}GLTVTS S
\]

CDR\textsubscript{H} 1 of DR5 mAb 1 (SEQ ID NO:9): GFDFSRYWMS

CDR\textsubscript{H} 2 of DR5 mAb 1 (SEQ ID NO:10): EINPDSNTINYTPSLKD

CDR\textsubscript{H} 3 of DR5 mAb 1 (SEQ ID NO:11): RAYYGNPFAWFAY

The VH Domain of DR5 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO:12) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

\[
g\text{aggtgaagt} \text{ttctcgagtc} \text{tgaggtggc} \text{ctgtggcagc} \text{ctggaggatc} \\
g\text{cttgaaactc} \text{ttctctgtag} \underline{ctcgagatt} \underline{cgatttttagt} \underline{agatactgga} \\
\text{tgaggttgg} \text{cggcaggtc} \text{caggagaaag} \text{ggctgaatag} \text{gatt} \text{gaa} \\
\underline{attaatccag} \underline{atagcaat} \underline{ac} \underline{gataaaatc} \underline{agc} \underline{cat} \underline{ctc} \underline{a} \underline{aaagataa}
\]
The Anti-Human DR5 Antibody DR5 mAb 2

(1) Murine Anti-Human Antibody DR5 mAb 2

[00106] The amino acid sequence of the VL Domain of DR5 mAb 2 (SEQ ID NO: 13) is shown below (CDR residues are shown underlined):

DIVMTQSHKF MSTSVGDRVS **ITCKASQDVN** TAVA**YQQKP** GQSPKLLIYW ASTRHTGVPD RFTGSGSTGD YTLTIKSVQA EDLTLYYCQQ HYITPWTGG GTKLEIK

**CDR_L**1 of DR5 mAb 2 (SEQ ID NO: 14): **KASQDVNTAVA**

**CDR_L**2 of DR5 mAb 2 (SEQ ID NO: 15): **WASTRHT**

**CDR_L**3 of DR5 mAb 2 (SEQ ID NO: 16): **QQHYITPWT**

[00107] The VL Domain of DR5 mAb 2 is preferably encoded by a polynucleotide (SEQ ID NO: 17) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

gacattgtga tgacccagtc tcacaaatcc atgtccactt cagtaggaga cagggtcagc atcacctgca aggexcagtea ggtgtggaat actgtgtag cctgttatca acaaaaaacct gggcaatctct ctaaactact gatttactg gcatccccacc gcacactgg agttccctgtat cgcttccagag gcagtggtgc tgggacagat tatacactca ccatcaaaag tgtgcagcttg gaagatctg gacatccatca ctgtcagcaa cactatatca ctccgggtgac gtcttggtgga ggcacaagct ggaatcaaa

[00108] The amino acid sequence of the VH Domain of DR5 mAb 2 (SEQ ID NO: 18) is shown below (CDR residues are shown underlined):

KVQLQSGSAE LVKFGASVKL **SCKASYFTFT EYILHWVKQK** SGQGLEWIGW **FYPPGNNIKY** NEKFDKATL TADKSSTVY MELSRTLSED SAVYFCAHRE **QGPGYFDYG** QGTTTLVSS

**CDR_H**1 of DR5 mAb 2 (SEQ ID NO: 19): **GYTFTEYILH**

**CDR_H**2 of DR5 mAb 2 (SEQ ID NO: 20): **WFYPGNNIKYNEKFKD**

**CDR_H**3 of DR5 mAb 2 (SEQ ID NO: 21): **HEQGPGYFDY**
The VH Domain of DR5 mAb 2 is preferably encoded by a polynucleotide (SEQ ID NO: 22) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

aaggtccagc tgcacgcttc tggagctgaa ctgggagaac ccggggcatc
agtgaagctg tcctgcaagct gggctgatat ccggtgaggt gattggggtgg
ctctcctcctctctcctct ctaataaata aatgaggttag cagagtctat
caagggacag gttacttggta ctactgggcc caaggccac ctcctacag tctcctcc

(2) Humanized DR5 mAb 2 ("hDR5 mAb 2")

The above-described murine anti-human DR5 antibody DR5 mAb 2 was humanized in order to demonstrate the capability of humanizing an anti-human DR5 antibody so as to decrease its antigenicity upon administration to a human recipient. The humanization yielded four humanized VL Domains designated herein as "hDR5 mAb 2 VL-2," "hDR5 mAb 2 VL-3," "hDR5 mAb 2 VL-4," and "hDR5 mAb 2 VL-5," and one humanized VH Domain, designated herein as "hDR5 mAb 2 VH-2." Any of the humanized VL Domains may be paired with the humanized VH Domain. Accordingly, any antibody comprising one of the humanized VL Domains paired with the humanized VH Domain is referred to generically as "hDR5 mAb 2," and particular combinations of humanized VL/VH Domains are referred to by reference to the VL domain.

The amino acid sequence of the VL Domain of hDR5 mAb 2 VL-2 (SEQ ID NO: 23) is shown below (CDR residues are shown underlined):

DIQMTQSPSF LSASVGRDVT ITCKASQDVN TAVAHYQQYP GKAPKLHLYW ASTRHIGVPS RFSGSGSGTD FTLTISLQPP EDVATYYCQQ HYIPWTFGG GTKLEIK

hDR5 mAb 2 VL-2 is preferably encoded by a polynucleotide (SEQ ID NO: 24) having the sequence shown below:

gatattcagt gttacccagag tccctctatgg ctggctgcctt cccgtcggtga
ccgctgctgt attacttggta aagctttccac ggagctcaac accggcctgg
cttggctacca gcacagcacc cggtaagcag ctaaagctctt gatctacttg
gccagcacttc ggccacccag cgcctcctctg aggtttctctg gcagttggatc
agggacagac ttaccctctga caatgtgcct ctcgacccag gaggtgtgg
The amino acid sequence of the VL Domain of hDR5 mAb 2 VL-3 (SEQ ID NO: 25) is shown below (CDR residues are shown underlined):

```
DIQMTQSPSF LSASVGDRVT ITCRASQDVN TAVA WYQQKP GKAPKLLIYW ASTRHTGVDP RFSGSGSGTD FTTLISSLQP EDVATYYCQQ HYITPWTFGG GTKLEIK
```

hDR5 mAb 2 VL-3 is preferably encoded by a polynucleotide (SEQ ID NO: 26) having the sequence shown below:

```
gatattcaga tgCCCCcagag tccctcattt ctgtccgctt ccgctcggtga cccgctgact attacctgtc ggctctctca ggatgtcaac accggcgtgg ctttgtagca gcagaagcgc ggtaagcagc ctaagctgtc gatctaatgg gcacagactc gcacacccgg agttccagat aggtctcttg gcagtgtagc agggacacac tttagcttc gaattaagcgtc cttgacagcgc gaggatgtgg ctaacctaca ttgtcagcag cactacatca ctccttggac cttcgccggg ggcacaaaaa cggaaatccaa
```

The amino acid sequence of the VL Domain of hDR5 mAb 2 VL-4 (SEQ ID NO: 27) is shown below (CDR residues are shown underlined):

```
DIQMTQSPSF LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYW ASTRHTGVPS RFSGSGSGTD FTTLISSLQP EDIATYYCQQ HYITPWTFGG GTKLEIK
```

hDR5 mAb 2 VL-4 is preferably encoded by a polynucleotide (SEQ ID NO: 28) having the sequence shown below:

```
gatattcaga tgCCCCcagag tccctcattt ctgtccgctt ccgctcggtga cccgctgact attacctgtc ggctctctca ggatgtcaac accggcgtgg ctttgtagca gcagaagcgc ggtaagcagc ctaagctgtc gatctaatgg gcacagactc gcacacccgg agttccagat aggtctcttg gcagtgtagc agggacacac tttagcttc gaattaagcgtc cttgacagcgc gaggatgtgg ctaacctaca ttgtcagcag cactacatca ctccttggac cttcgccggg ggcacaaaaa cggaaatccaa
```

The amino acid sequence of the VL Domain of hDR5 mAb 2 VL-5 (SEQ ID NO: 29) is shown below (CDR residues are shown underlined):

```
DIQMTQSPSF LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYW ASTRHTGVPD RFSGSGSGTD FTTLISSLQP EDIATYYCQQ HYITPWTFGG GTKLEIK
```

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**hDR5 mAb 2 VL-5** is preferably encoded by a polynucleotide (SEQ ID NO:30) having the sequence shown below:

```
gatattcaga tgacccagag tccctcattt ctgtccgacct cctgctggta
cgcggctgact attacttgtc gggcttctca gatggtcaac accgccgtag
ttggtacca tttacccctg cttaagtgct ctttgctgtt gcaggtgctg
gccagcact gcggacccgg agtcccgagat aggtctcttg gcagtggtgct
gagggcagac tttaccctga caattagctc cctgcagccc gaggatatcg
gaagtttcccag cggacacgcc aggttctctg gcagttggact
ggcacaaaac tggaaatcag a
```

**hDR5 mAb 2 VH-2** is preferably encoded by a polynucleotide (SEQ ID NO:32) having the sequence shown below:

```
caggtccagc tggtgcagag tggggcagag gttgaaaaagc caggggcatc
agtgaaagtg tcttgtaaag catcaggtta ttcatttact gagtacatcc
tgcactgggt gcgacaggca ccaggacagg gactggaatg gatggggtgg
ttctaccctg gcaacaacaa cattaagttc aacgagaagtt ttaaagaccg
ggtgaccatc acacgaggata agtctacgag tagtctatag atggagctga
gctcccttgag aagcagcagc accgccgctct acttatgcgc tccccacagaa
caggtcagcag ttatcttggg cagggagactc tggctacagt cagctcc
```

The CDR1 of the VL Domain of **hDR5 mAb 2 VL-3**, **hDR5 mAb 2 VL-4** and **hDR5 mAb VL-5** has the amino acid sequence: **RASQDVNTAVA** (SEQ ID NO:196).

**Drozitumab ("DR5 mAb 3")**

**hDR5 mAb 2 VL-2** is preferably encoded by a polynucleotide (SEQ ID NO:31) having the sequence shown below:

```
QVQLVQSGAE VKKPGASVKV SCKASGYTFT_EYILHVRQA PGQGLEWMGW FY PonN M I K NEF K D RVT T AD KSTSTVY MELSSLRSED TAVYYCARHE
```

The amino acid sequence of the VL Domain of **drozitumab ("DR5 mAb 3")** (SEQ ID NO:54) is shown below (CDR residues are shown underlined):

```
SELTQDPAVS VALGQTVRIT CSGDSLRSYY ASWYQQKPG QAPVVLVIYGA
NNRPSGI PDR FSQSSSNGIA SLTITGAQAE DEADYCYNSA DSSGNHWFG
GGTKLTVLG
```

CDR₁₁ of DR5 mAb 3 (SEQ ID NO:55): **SGDSLRSYYAS**

CDR₁₂ of DR5 mAb 3 (SEQ ID NO:56): **GANNRPS**

CDR₁₃ of DR5 mAb 3 (SEQ ID NO:57): **NSADSSGNHW**
The amino acid sequence of the VH Domain of **drozitumab** ("DR5 mAb 3") (SEQ ID NO:58) is shown below (CDR residues are shown underlined):

```
EVQLVQSGG GVERPGGSRL SCAASGFTFD DYAMS WVRQA P GKGLEWVSG
IN WQG GGSTGY ADSVK GRTV I SRDNAKNSL YLQMNSLRAED TAVYYCAKIL
GAGRGWYFDY W GKGTTTVS S
```

- CDR<sub>H</sub>1 of DR5 mAb 3 (SEQ ID NO:59): GFTFDDYAMS
- CDR<sub>H</sub>2 of DR5 mAb 3 (SEQ ID NO:60): INWQGGSTGYADSVKG
- CDR<sub>H</sub>3 of DR5 mAb 3 (SEQ ID NO:61): ILGAGRGWYFDY

**d. Conatumumab** ("DR5 mAb 4")

The amino acid sequence of the VL Domain of **conatumumab** ("DR5 mAb 4") (SEQ ID NO:62) is shown below (CDR residues are shown underlined):

```
EIVLTQSPGT LS LSLPGERAT LS C RASQGISRSYLA RGS QAPSSLLYG 
GASSRATGIP DRS FGSGSGT DFTLTISRLE PEDFAVVYYCQ QFGSSPWTFG
QGTKVEIK
```

- CDR<sub>L</sub>1 of DR5 mAb 4 (SEQ ID NO:63): RASQGISRSYLA
- CDR<sub>L</sub>2 of DR5 mAb 4 (SEQ ID NO:64): GASSRAT
- CDR<sub>L</sub>3 of DR5 mAb 4 (SEQ ID NO:65): QFGSSPWTFG

**e. Tigatumumab** ("DR5 mAb 5")

The amino acid sequence of the VH Domain of **tigatumumab** ("DR5 mAb 5") (SEQ ID NO:70) is shown below (CDR residues are shown underlined):

```
QVQIQGSGPG LVKPSQTLSSL TCTVSGGSIS SGDYW SFRQIR QPGK GLEWIS
GHIHSMDYYV YNPLSKRS VRTI ISVDTSKKQF SLRLSSVTAA DTAVYYCARD
RGG DYYYGMD V W GG TTV SS
```

- CDR<sub>H</sub>1 of DR5 mAb 4 (SEQ ID NO:67): GSISSGDYFWS
- CDR<sub>H</sub>2 of DR5 mAb 4 (SEQ ID NO:68): HIRNSGTYYNPSLK S
- CDR<sub>H</sub>3 of DR5 mAb 4 (SEQ ID NO:69): DRGG DYYYGMDV

**f. Tigatumumab** ("DR5 mAb 5")
CDR L 1 of DR5 mAb 5 (SEQ ID NO:71): KASQDVGTAVA
CDR L 2 of DR5 mAb 5 (SEQ ID NO:72): WASTRHT
CDR L 3 of DR5 mAb 5 (SEQ ID NO:73): QQYSSYRT

[00127] The amino acid sequence of the VH Domain of tigatumumab ("DR5 mAb5") (SEQ ID NO:74) is shown below (CDR residues are shown underlined):

EVQLVESGGG LVQPGGSLRL SCAAASGFTFS SYVMSWVRQA PGKGLEWVAT ISGGGSYTYY PDSVKGRTFTI SRDNAKNTLY LQMNSLRAED TAVYYCARRG DSMITTDYG QGTLTVSS

CDR H 1 of DR5 mAb 5 (SEQ ID NO:75): GFTSSYVMS
CDR H 2 of DR5 mAb 5 (SEQ ID NO:76): TISSGGSYTTYYPDSVKG
CDR H 3 of DR5 mAb 5 (SEQ ID NO:77): RGDSMITTDY

f. LBY135-1 ("DR5 mAb 6")

[00128] The amino acid sequence of the VL Domain of LBY135-1 ("DR5 mAb 6") (SEQ ID NO:78) is shown below (CDR residues are shown underlined):

DIAMTQSHKF MSTLVGDRTIS TICASQDVNTAIA YQQKPGQSPKLLIYWL FYPGGGYIKY NEKFDRATL
ASTRHTGVPD RFYGSGSGTD YTLTISMEAYEDAAYTYCQQ WSSNPLTFGATKLEKLRA

CDR L 1 of DR5 mAb 6 (SEQ ID NO:79): QDVNTAIA
CDR L 2 of DR5 mAb 6 (SEQ ID NO:80): WASTRHT
CDR L 3 of DR5 mAb 6 (SEQ ID NO:81): QQWSSNPLT

[00129] The amino acid sequence of the VH Domain of LBY135-1 ("DR5 mAb 6") (SEQ ID NO:82) is shown below (CDR residues are shown underlined):

KVQLQQSGAE LVKPGASVKL SCKASGTYFT DTIHWVKQR SGQGLEWGW FYPGGGYIKY NEKFDRATL TADKSSNTVY MELSRTLSEGSAVyyFCARHE EGTYFDYWGQ GTTLTVSS

CDR H 1 of DR5 mAb 6 (SEQ ID NO:83): GYTFDYTEIH
CDR H 2 of DR5 mAb 6 (SEQ ID NO:84): WFYPGGGYIKYNEKFKD
CDR H 3 of DR5 mAb 6 (SEQ ID NO:85): HEEGIYFDY
g. LBY135-2 ("DR5 mAb 7")

The amino acid sequence of the VL Domain of LBY135-2 ("DR5 mAb 7") (SEQ ID NO:86) is shown below (CDR residues are shown underlined):

```
DIVMTQS HKF MSTSVDGRVS ITCKASQDVY TAIA NYYQQKP GQS PKLL IYW ASTRHTGYPD RTFGS GSGTD YTLT I S SVQA EDLALYYC QQ_HYITPFTFGS GTKL
```

CDR L1 of DR5 mAb 7 (SEQ ID NO:87): KASQDVNTAIA
CDR L2 of DR5 mAb 7 (SEQ ID NO:88): WASRHT
CDR L3 of DR5 mAb 7 (SEQ ID NO:89): QQHYTTPFT

h. KMTR2 ("DR5 mAb 8")

The amino acid sequence of the VH Domain of KMTR2 ("DR5 mAb 8") (SEQ ID NO:90) is shown below (CDR residues are shown underlined):

```
KVQLQQS GAE LVKPGAVKL SCKASGYTFT DYTIIHWVQKR SGQGLEGW FTLP FLYGK YN HKF KD TADKS SNTVY MELSRLL SED SAVYFCARHE EGIGYDFYWGQ GTKLVKIR
```

CDR H1 of DR5 mAb 7 (SEQ ID NO:91): GYTFIDYTH
CDR H2 of DR5 mAb 7 (SEQ ID NO:92): WFPYG GGY IKYNEKF KD
CDR H3 of DR5 mAb 7 (SEQ ID NO:93): HEEGIYFDY

The amino acid sequence of the VH Domain of KMTR2 ("DR5 mAb 8") (SEQ ID NO:90) is shown below (CDR residues are shown underlined):

```
QVQLQQS GAE MKKPGAVKL SCKASGYTFT_NYKINWVRQA PGQGLEGW MNPDTSSTGY PKFKFOGRVTL TRNT STAY MELSSLRS E D TAVYFCARSY GSGSYYRQYY YGMDVWGQGT GTKLVKLR
```

The amino acid sequence of the VH Domain of KMTR2 ("DR5 mAb 8") (SEQ ID NO:90) is shown below (CDR residues are shown underlined):

```
QVQLQQS GAE MKKPGAVKL SCKASGYTFT_NYKINWVRQA PGQGLEGW MNPDTSSTGY PKFKFOGRVTL TRNT STAY MELSSLRS E D TAVYFCARSY GSGSYYRQYY YGMDVWGQGT GTKLVKLR
```
CDRH\textsubscript{1} of DR5 mAb 8 (SEQ ID NO:99): GYTFTNYKIN
CDRH\textsubscript{2} of DR5 mAb 8 (SEQ ID NO: 100): WMNPDTDSTGYPQKFQG
CDRH\textsubscript{3} of DR5 mAb 8 (SEQ ID NO: 101): SYGSGSYYRDYYYGMDV

5. EphA2-Binding Domains

[00134] The receptor tyrosine kinase, ephrin type-A receptor 2 (EphA2) is a preferred cancer antigen of the present invention. EphA2 is normally expressed at sites of cell-to-cell contact in adult epithelial tissues, however, recent studies have shown that it is also overexpressed in various types of epithelial carcinomas, with the greatest level of EphA2 expression observed in metastatic lesions. High expression levels of EphA2 have been found in a wide range of cancers and in numerous tumor cell lines, including prostate cancer, breast cancer, non-small cell lung cancer and melanoma (Xu, J. et al. (2014) “High Epha2 Protein Expression In Renal Cell Carcinoma Is Associated With A Poor Disease Outcome,” Oncol. Lett. Aug 2014; 8(2): 687-692; Miao, B. et al. (2014) "EphA2 is a Mediator of Vemurafenib Resistance and a Novel Therapeutic Target in Melanoma," Cancer Discov. pii: CD-14-0295. EphA2 does not appear to be merely a marker for cancer, but rather appears to be persistently overexpressed and functionally changed in numerous human cancers (Chen, P. et al. (2014) "Epha2 Enhances The Proliferation And Invasion Ability Of Lncap Prostate Cancer Cells," Oncol. Lett. 8(1):41-46).

[00135] The invention particularly contemplates the selection of EphA2 as a Cancer Antigen, and the use of anti-EphA2 antibodies to provide the Cancer Antigen-Binding Domain of the Tri-Specific Binding Molecules of the present invention. Exemplary anti-EphA2 antibodies include "EphA2 mAb 1," "EphA2 mAb 2" and "EphA2 mAb 3."
a. EphA2 mAb 1

[00136] The amino acid sequence of the VL Domain of a preferred anti-human EphA2 antibody ("EphA2 mAb 1") (SEQ ID NO:153) is shown below (CDR residues are shown underlined):

```
DIQMTQTSS LSASLGDRIT ISCRASQDIS NYLNWYQQKP DGTKLILYY
TSRLHSGVPS RFSGSGSGID YSLTISNLEQ EDIATYFCQO GYLTLYTFGGG
TKLEIK
```

CDR$_L^1$ of EphA2 mAb 1 (SEQ ID NO:154): **RASQDISNYLN**

CDR$_L^2$ of EphA2 mAb 1 (SEQ ID NO:155): **YTSRLHS**

CDR$_L^3$ of EphA2 mAb 1 (SEQ ID NO:156): **QQGYTLYT**

[00137] The VL Domain of EphA2 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO:157) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
gatatccaga tgacacagac tacatctccc ctgctcgctt ctctgggaga
cagaatcacc atcagttgc a gggcaagtca ggaattatttaa
actggtatac gcagaaacca gatggaactg ttaaacctct gatctac tac
acataaagat tacactcagg agtcccatca aggttcagtg gcagttcggc
tggaacagat tattctctca ccattagcaa cctggagcaa gaagatattg
caccaagctgg aaataaaa
```

[00138] The amino acid sequence of the VH Domain of EphA2 mAb 1 (SEQ ID NO:158) is shown below (CDR residues are shown underlined):

```
QVQLKESGPGL VAPSQSLST TCTVSGFSLSL RYSVHWWQRQP PKGKLEWLG
IWGGGSTDYN SALKRSRLSI KDNSKSQVFL KMNSLQDTDT AMYYCARKHG
NYTMDYWGQ GTSVTVSS
```

CDR$_H^1$ of EphA2 mAb 1 (SEQ ID NO:159): **GFSLSRYSVH**

CDR$_H^2$ of EphA2 mAb 1 (SEQ ID NO:160): **MIWGGGSTDYNSAIKS**

CDR$_H^3$ of EphA2 mAb 1 (SEQ ID NO:161): **KHGNYYTMDY**

[00139] The VH Domain of EphA2 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO:162) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
caggtgccagc tgagaggtgc aggacctggc ctgcttgccac cctcagacag
ccttgccagc acatgcacgt tcctcaggtt ctcattaccc agatataagc
tacactgagtt tgcagctgct cccagaaagg gtaataagtt gcctggagta agtagagag
```

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The amino acid sequence of the VL Domain of a second preferred anti-human EphA2 antibody ("EphA2 mAb 2") (SEQ ID NO: 163) is shown below (CDR residues are shown underlined):

```
DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSSGNTYLHW YLQKPGQSPK
LII YKVSNRFS GGVPRFSGSI GSGTDFTLKI SVREAEDLGV YFCSQSTHVP
TFGSGKLEI K
```

CDR\(_L\)1 of EphA2 mAb 2 (SEQ ID NO: 164): RSSQSLVHSSGNTYLH

CDR\(_L\)2 of EphA2 mAb 2 (SEQ ID NO: 165): KVSNRFS

CDR\(_L\)3 of EphA2 mAb 2 (SEQ ID NO: 166): SQSTHVPT

The VL Domain of EphA2 mAb 2 is preferably encoded by a polynucleotide (SEQ ID NO:318) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
gatgttgtga tgacccaaac tccactctcc ctcgctgtca gtctttggaga
tcaagccctcc atctctgcca gatctagctca gaqccgtaat cacagtagtg
gaaacaccta tttacattgg tacgctgaag agccagggca gtcctcagaag
tctctgagct caacagtttt caacccgattt tctggtggtcc cagacaggtt
cagttgcaag ggtcatggga cagatcattc actcaagatc agcagagtg
gaggctgaga tctgggagtt tattttctgt ctcagagctt acatggattt
acgggctgct cgggggaaat gttggaataa
```

The amino acid sequence of the VH Domain of EphA2 mAb 2 (SEQ ID NO: 167) is shown below (CDR residues are shown underlined):

```
QIQLVQSGPE LKKPGETVKSI SCKASGFTFT NYGMNWVKQA PGKGLKWMGW
INTYIGEPTY ADDFGKRFVF SLETSASTAY LGIINLNKED MATYFCAREL
GPYYFDYWQG GTTLTVSS
```

CDR\(_H\)1 of EphA2 mAb 2 (SEQ ID NO: 168): GFTFTNYGMN

CDR\(_H\)2 of EphA2 mAb 2 (SEQ ID NO: 169): WINTYIGEPTYADDFKG

CDR\(_H\)3 of EphA2 mAb 2 (SEQ ID NO: 170): ELGPYYFDY
The VH Domain of EphA2 mAb 2 is preferably encoded by a polynucleotide (SEQ ID NO: 171) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

cagatccagt tggctccagtc tggaccttgag ctgaagaagc ctggagagac
agtcagatcc tcctgcaagg cttctgggtt taccttcaca aactatggaa
tggaactggtt gaagcagcagct ccagggaaag gttaaagtgt gatgggcttg
atataacacct atatatgagaa ccgcaacat atcgagtagct tcaagggacg
gtcttgccttc tctttggaaa cctctgccag cactgcctat ttgcagatca
acaacatcaa aaatggagac atggccacat atttctgtgc aagagaactg
ggaccatact acctttgac ta cttgggcctaa gccaccacac tcaagttgctc
cctcc

EphA2 mAb 3

The amino acid sequence of the VL Domain of a further preferred anti-human EphA2 antibody ("EphA2 mAb 3") (SEQ ID NO: 172) is shown below (CDR residues are shown underlined):

DIVLTQSHRS MSTDVGDRVN ITKASQDVVT TAVAWYYQXP GQSPKLIFW
ASTRHAGVPD RFTGSGSTGD FTLTISSVQA GDLALYYCQG HYSTPYTFGG
GTKLEIK

CDR1 of EphA2 mAb 3 (SEQ ID NO: 173): KASQDVTTAVA
CDR2 of EphA2 mAb 3 (SEQ ID NO: 174): WASTRHA
CDR3 of EphA2 mAb 3 (SEQ ID NO: 175): QQHSTPYT

The VL Domain of EphA2 mAb 3 is preferably encoded by a polynucleotide (SEQ ID NO: 176) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

gacattgtgc tgaccatgtc tcacagatcc atgtccacat cagtaggaga
cagggtcaac atccacctgca aggctccagtc gatgtcagct actgtcgttag
cctgtcatca acaaaaaacc gggcaatctc taaattact gattttctgg
gcatccaccc ggcacgctgg agttccctgtc gcttccacag gcagtgggtc
tgggacagat tttactctca ccacagcag tggccagagct ggaagcttgg
cacttatta cgtgtcaacaa cattatagc a caccgtacac atctggaggag
gggaccaagc tggaaaaaa a

c.

The amino acid sequence of the VH Domain of EphA2 mAb 3 (SEQ ID NO: 177) is shown below (CDR residues are shown underlined):

EVQLVESGGG SVKPGSSLKL SCAASGFTFT DHMYWVRQT PEKRLEWVAT
ISDGGPSFTYS PDSVKGRTFT SRDIAPNNLY LQMSLKSED TAMYCTRDE
SDRPFPYWGQ GTLVTVSS

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CDR<sub>H</sub>1 of EphA2 mAb 3 (SEQ ID NO: 178): GFTFTDHYMY
CDR<sub>H</sub>2 of EphA2 mAb 3 (SEQ ID NO:179): TISDGGSFTSPDSVKG
CDR<sub>H</sub>3 of EphA2 mAb 3 (SEQ ID NO: 180): DESDRPFPY

[00147] The VH Domain of EphA2 mAb 3 is preferably encoded by a polynucleotide (SEQ ID NO:319) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggaggggtc
ccctgaaactc ttctgtgcaag cctctgagatt cactttcact gaccattaca
tgtattggtt tgcctgcagct cgggaagaaga ggctggagtg ggtcgcaacc
attagtgatgg gcggtagttt cacctcctat cccagacagtg tgaaggggcgtga
gacttgatgg gcggtagttt cacctcctat cccagacagtg tgaaggggcgtga
```

6. gpA33-Binding Domains


[00149] The invention particularly contemplates the selection of gpA33 as a Cancer Antigen, and the use of anti-gpA33 antibodies to provide the Cancer Antigen-Binding Domain of the Tri-Specific Binding Molecules of the present invention. An exemplary anti-gpA33 antibody is "gpA33 mAb 1."
The amino acid sequence of the VL Domain of a preferred anti-human gpA33 antibody ("gpA33 mAb 1") (SEQ ID NO: 181) is shown below (CDR residues are shown underlined):

**CDR L1** of gpA33 mAb 1 (SEQ ID NO: 182): SARSSISFMY

**CDR L2** of gpA33 mAb 1 (SEQ ID NO: 183): DTSNLAS

**CDR L3** of gpA33 mAb 1 (SEQ ID NO: 184): QQWSSYPLT

The VL Domain of gpA33 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO: 185) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
gacattcagc tgactcagtc cccctctttt ctgtccgcat ccgtcggaga
tcgagtgact attacttgct ctgctaggtc cctcaatcagc ttcatgtact
ggtatcagca gaagccggcg aaagacacct aagctgctgca gttgggtcaggg
tcctactattg ccagcagtgg agcagctatc ctctgacctt cggacagggg
```

The amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO: 186) is shown below (CDR residues are shown underlined):

**CDR H1** of gpA33 mAb 1 (SEQ ID NO: 187): GYTFTGSWMN

**CDR H2** of gpA33 mAb 1 (SEQ ID NO: 188): RIYPGDGETNYNGKFKD

**CDR H3** of gpA33 mAb 1 (SEQ ID NO: 189): IYGNVYFDV

The VH Domain of gpA33 mAb 1 is preferably encoded by a polynucleotide (SEQ ID NO: 190) having the sequence shown below (polynucleotides encoding the CDRs are shown in underline):

```
caggtccagc tggtccagag cggggccgaa gtcaaagacc cccgagcag
```

"gpA33 mAb 1" refers to the anti-human gpA33 antibody. CDRs (Complementarity-Determining Regions) are critical parts of the antibody that determine its specificity. The polynucleotides encode the amino acid sequences shown above. CDR residues are highlighted in bold and underlined.
7. Her2-Binding Domains

[00154] The invention also particularly contemplates the selection of Her2 as a Cancer Antigen, and the use of anti-Her2 antibodies to provide the Cancer Antigen-Binding Domain of the Tri-Specific Binding Molecules of the present invention. Exemplary anti-Her2 antibodies include "Her2 mAb 1" and Trastuzumab.

a. Her2 mAb 1

[00155] The amino acid sequence of the VL Domain of anti-Her2 antibody "Her2 mAb 1" (SEQ ID NO: 191) is shown below (CDR residues are shown underlined):

DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVA WQKYQ KAPKLLIYS ASFLESGVPS RFSGRSQGDFTLTISSLQPEFATYYCQQ HYTPPTFGQ GTKVEIKRT

[00156] The amino acid sequence of the VH Domain of anti-Her2 antibody "Her2 mAb 1" (SEQ ID NO: 192) is shown below (CDR residues are shown underlined):

QVQLQSGPE LVKPGASLKL SCTASGFIK DTYIHWQKR PEQGLEWIGR IYPTNGYTRY DPKFQDKAT I TADTSSNTAY LQSRLTSED TAVYCSRWG GDGFYAMDYW GQGTVTSS

b. Trastuzumab

[00157] The amino acid sequence of the VL Domain of the humanized anti-Her2 antibody "Trastuzumab" (SEQ ID NO: 193) is shown below (CDR residues are shown underlined):

DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVA WQKYQ KAPKLLIYS ASFLYSGVPS RFSGRSQGDFTLTISSLQPEFATYYCQQ HYTPPTFGQ GTKVEIKRT

[00158] The amino acid sequence of the VH Domain of the humanized anti-Her2 antibody "Trastuzumab" (SEQ ID NO: 194) is shown below (CDR residues are shown underlined):

EVQLVESGGG LVQPGGSLRL SCAASGFIK DTYIHWQRA PKGKLEWVAR IYPTNGYTRY ADSVKGRFT I SADTSKNTAY LQMNLRATED TAVYCSRWG GDGFYAMDYW QGTLTVTSS
8. **B7-H3-Binding Domains**


[00160] The invention also particularly contemplates the selection of B7-H3 as a Cancer Antigen, and the use of anti-B7-H3 antibodies to provide the Cancer Antigen-Binding Domain of the Tri-Specific Binding Molecules of the present invention. Exemplary anti-B7-H3 antibodies include "B7-H3 mAb 1," "B7-H3 mAb 2," and "B7-H3 mAb 3."

a. **B7-H3 mAb 1**

[00161] The amino acid sequence of the VL Domain of anti-B7-H3 antibody "B7-H3 mAb 1" (SEQ ID NO: 195) is shown below (CDR residues are shown underlined):
The amino acid sequence of the VH Domain of anti-B7-H3 antibody "B7-H3 mAb 1" (SEQ ID NO: 196) is shown below (CDR residues are shown underlined):

```
DVQLVESGGG LVQPGGSRKLS CAASGFTFS SFGMHWWVRQA PEKGLEWAVY
SFDSSAIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCGGR
ENIYYSRLD YWGQGTTLTV SS
```

b. B7-H3 mAb 2

The amino acid sequence of the VL Domain of anti-B7-H3 antibody "B7-H3 mAb 2" (SEQ ID NO: 197) is shown below (CDR residues are shown underlined):

```
DIQMTQITSS LSASLGDRVTS CRASQDIS NYLNWYQQKP DGTVKLLIYY
TSRLHSQVPS RFS GSGSCTD YSLTI DNLEQ EDIATYFC QQ GNLPPFTGG
GTKLEIK
```

The amino acid sequence of the VH Domain of anti-B7-H3 antibody "B7-H3 mAb 2" (SEQ ID NO: 198) is shown below (CDR residues are shown underlined):

```
QVQLQQSGAE LARPASVKL SCKASGYTFT SYWMQWVKQR PQQGLEWI GT
YPGDGDTRY TQKFKGKATL TADKS S STAY MQLS SLASE D SAVYCCARRG
IPRLWYFDVW GAGTTVTVS S
```

c. B7-H3 mAb 3

The amino acid sequence of the VL Domain of anti-B7-H3 antibody "B7-H3 mAb 3" (SEQ ID NO: 199) is shown below (CDR residues are shown underlined):

```
DIQMTQS PAS LSVSVGAVTVT ITCRASESIV SYLAWYQQKQ GKS PQLLVYN
TKILPEGVPS RFS GSGSCTQ FSLKINS LQP EDFGRRYCQH HYGTPPWTFG
GGTNLEIK
```

The amino acid sequence of the VH Domain of anti-B7-H3 antibody "B7-H3 mAb 3" (SEQ ID NO: 200) is shown below (CDR residues are shown underlined):

```
EVQQVE SGDD LVKPGGSLKL SCAASGFTFS SYGMSWVRQ TDKRLEWVAT
INSQGSSNTTY PDSLKGRFTI SRDNAKNTLY LQMRLKSE D TAMYYCARRH
GGAMDWGQG TSVTVS S
```

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9. EGF Receptor-Binding Domains (Cetuximab)

[00167] The amino acid sequence of the VL Domain of the chimeric anti-EGFR antibody "Cetuximab" (SEQ ID NO:201) is shown below (CDR residues are shown underlined):

```
DILLTQSPVI LSVSPGERVS FSCRASQSIG TNIH WYQQRT NGSPRLLIKYGASESIGIPS RFSGSGSGTD FTLSINSVES EDIADYYC QQ NNNWPTTFGA GTKLELR
```

[00168] The amino acid sequence of the VH Domain of the chimeric anti-EGFR antibody "Cetuximab" (SEQ ID NO:202) is shown below (CDR residues are shown underlined):

```
QVQLKQSGPG LVQPSQSLSI TCTVSGFSLT NYGVHWVRQS PGKGLEWLGVTWGGNTDYN TPFTSRLSI N KDNSKSQVFF KMNSLQSNDAIYYCARALTYYDYEFAWYGQGTLVTVSA
```

[00169] Panitumumab (e.g., Vectibix®, Amgen) is an alternative EGF receptor-binding antibody that may be used in accordance with the present invention.

10. VEGF-Binding Domains (Bevacizumab)

[00170] The amino acid sequence of the VL Domain of the humanized anti-VEGF antibody "Bevacizumab" (SEQ ID NO:203) is shown below (CDR residues are shown underlined):

```
DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLN WYQQKP GKAPKVLIELG TSSLHSGVPS RFSGSGSGTD FTLTISLQP EDFATYYC QQ YSTVPWTFGQ GTKVEIKR
```

[00171] The amino acid sequence of the VH Domain of the humanized anti-VEGF antibody "Bevacizumab" (SEQ ID NO:204) is shown below (CDR residues are shown underlined):

```
EVQLVESGGG LVQPGGSLRL SCAASYTFT NYGMN WVRQA PGKGLEWVGWTINTYTGEPTY AADFKRRFTF SLDTSKSTAY LQMNSLRAED TAVYYCAKYP HYYGSSSHWF DVWGQGTLVTVSS
```

11. 5T4-Binding Domains

[00172] The oncofetal protein, 5T4, is a tumor-associated protein displayed on the cell membrane of many carcinomas, including kidney, colon, prostate, lung, carcinoma and in acute lymphoblastic leukemia (see, Boghaert, E.R. et al. (2008) “The Oncofetal Protein, 5T4, Is A Suitable Target For Antibody-Guided Anti-Cancer...

```
DIQMTQSPSS  LSASVGVDRVT  ITCRASQGIS  NYLAWFQQKPGAPKSLIYR
ANRLQSGVPS  RFSGSGSGTD  FTLTISSLQP  EDVATYYCLQ  YDDFPWTFGQ
GTKLEIK
```

[00173] The amino acid sequence of the Heavy Chain Variable Domain of such exemplary anti-5T4 antibody is shown below (CDR residues are shown underlined): (SEQ ID NO:309):

```
QVQLVQSGAE  VVKPGASVKV  SCKASGYTFT  SFWMHWVRQA  PGQGLEWMGR
IDPNRGGTEY  NEKAKSRTVM  TADKSTSTAY  MELSSLRSED  TAVYYCAGGN
PYYPMDYWGQ  GTTTVSS
```

[00174] The amino acid sequence of the Light Chain Variable Domain of a second exemplary anti-5T4 antibody ("5T4 mAb 2") is shown below (CDR residues are shown underlined): (SEQ ID NO:310):

```
DVLMQTQTPLS  LPVSLGDQAS  ISCRRSSQIV  YSNNGNTYLEW  YLQKPGQSPK
LL1  YKVSNRF  SGPDRFSGS  GSGTDFTLKI  SRVEAEDLGV  YYCFQGSHVP
FTFGSGTKL1  IK
```

[00175] The amino acid sequence of the Heavy Chain Variable Domain of such second exemplary anti-5T4 antibody is shown below (CDR residues are shown underlined) (SEQ ID NO:311):

```
QVQLQQPGAE  LVKPGASVKM  SCKASGYTFT  SYWITWVKQR  PGQGLEWIGD
IYPGSSGRANY  NEKFKSKATL  TVDTDSSSTAY  MQLSSLTSED  SAVYNCARYG
PLFTTWDPN  SYAMDYWGQG  TSIVTVSS
```

12. IL13Ra2-Binding Domains

[00176] Interleukin-13 Receptor α2 (IL13Ra2) is overexpressed in a variety of cancers, including glioblastoma, colorectal cancer, cervical cancer, pancreatic cancer, multiple melanoma, osteosarcoma, leukemia, lymphoma, prostate cancer and lung cancer (PCT Pubmication No. WO 2008/146911:


DIQMTQSPSS LSASVGVDRVT ITCKASQDVG TAVAHYQQKP GKAPKLLIYS ASYRSTGVPS RFSGSGSGTD FTLTISLQP EDFATYYCQGH HYSAPWTFGG GTKVEIK

[00178] The amino acid sequence of the Heavy Chain Variable Domain of such exemplary anti-IL13Ra2 antibody ("hu08," PCT Publication No. WO 2014/072888) is shown below (CDR residues are shown underlined): (SEQ ID NO:309):

EVQLVESGGG LVQPGGLSLRL SCAASGFTFS RNGMSWVRQA PGKGLEWVT ASVSGSYIYY ADSDKVGRFT I SDRNAKNSLY LQMNLAED TAVYYCARQG TTALATRFDD VWGGTLVLIV SS

13. Integrin Beta6-Binding Domains

[00179] IntegrinP6 (ITGB6) is a subtype of integrin that is expressed exclusively on the surfaces of epithelial cells and is a receptor for extracellular matrix (ECM) proteins. ITGB6 expression is specifically expressed in tumor tissues (such as those of colon, prostate, kidney cancer), but is generally undetectable in healthy epithelial tissue (Liang, B. et al. (2014) "Integriν6-targeted Immunoliposomes Mediate Tumor Specific Drug Delivery and Enhance Therapeutic Efficacy in Colon Carcinoma," Clin. Cancer Res. Dec 30. pii: clincanres.1194.2014). Monoclonal antibodies that immunospecifically bind to ITGB6 are available commercially (e.g., MAB2075Z clone R6G9,EMD Millipore; see also, Weinacker, A. et al. (1994) ‘Role Of The
Integrin Alpha V Beta 6 In Cell Attachment To Fibronectin. Heterologous Expression Of Intact And Secreted Forms Of The Receptor;’ J. Biol. Chem. 269:6940-6948).

Anti-ITGB6 monoclonal antibodies 3G9 and 8G6, and variants thereof are disclosed in PCT Publication Nos. WO 03/100033 and WO 2007/008712.

[00180] The amino acid sequence of the Light Chain Variable Domain of an exemplary humanized anti-ITGB6 antibody (derived from antibody 3G9, PCT Publication No. WO 2007/008712) is shown below (CDR residues are shown underlined): (SEQ ID NO:312):

EIVLTQS PAT LSLSPGERAT **LSCSASSSVS** SSYLYWYQQK PGQAPRL I Y
STSNLASGIP ARRS GSGS GT GFTLT ISSLE PEDFAVYYC H **QWSTYPPTFG**
GGTKVE I K

[00181] The amino acid sequence of the Heavy Chain Variable Domain of such exemplary humanized anti-ITGB6 antibody (derived from antibody 3G9, PCT Publication No. WO 2007/008712) is shown below (CDR residues are shown underlined): (SEQ ID NO:313):

EVQLVE SGGG LVQPGGS LRL SCAASGFTFS **RYWMSWVRQA** PGKGLEWVAS
ISSGRMNYYP **P**TVKGRFTIS RDNAKNS LYL QMNSLRAE DT AVYYCARGSI
YDGYYVFYWP GQGTLVTVS S

[00182] The amino acid sequence of the Light Chain Variable Domain of an exemplary anti-ITGB6 antibody (derived from antibody 8G6, PCT Publication No. WO 2007/008712) is shown below (CDR residues are shown underlined): (SEQ ID NO:314):

EIVLTQS PAT LSLSPGERAT **LSCRASQSVS TSSYMYW** QKPQGQAPRL
LYYASNLGS GI PARFS GSG **SGTDFTLT I S** SLEPEDFAVY **YCQHNWEIPF**
TFGGGTKVE I K

[00183] The amino acid sequence of the Heavy Chain Variable Domain of such exemplary anti-ITGB6 antibody (derived from antibody 8G6, PCT Publication No. WO 2007/008712) is shown below (CDR residues are shown underlined): (SEQ ID NO:315):

QVQLVQS GAEVKPGASVKS Ckas GlytFdyahm WVRQAPGQGLEWMGVISTYY
GNTNYQFKGRVTMTRDT S SI STAYMELSRLRSDDTAVYYCARGGLRRGDRPSLO
YAMDYWGQGTLVTVS S
14. Additional Anti-Cancer Antigen-Binding Domains

[00184] Additional anti-cancer antigen antibodies that may be used in accordance with the present invention include the following commercially available antibodies: Brentuximab (e.g., Adcetris®), which binds to CD30; Gemtuzumab (e.g., Mylotarg®, Wyeth), which binds to CD33; and Ipilimumab (e.g., Yervoy®), which binds to CTLA-4.

C. Exemplary Effector Cell-Binding Domains

[00185] Antibodies that are capable of binding to immune system effector cells may be used to provide the Effector Cell-Binding Domains of the Tri-Specific Binding Molecules of the present invention. Particularly suitable are antibodies that bind to CD2, CD3, CD16, CD19, CD20, CD22, CD32B, CD64, the B cell Receptor (BCR), the T cell Receptor (TCR), and the NKG2D Receptor.

1. CD2-Binding Domains


```
DVVL TQT PPT LLAT I GQS VS [TSRCSSQSL L HSSGNTYLNW LLQRTGQS PQ LPIYLVSKL] SGVPNRF S GS GSGTDFLKI SGEAE DLGV YVCQMTFTYP YTFGAGTKLE LK
```

[00187] The amino acid sequence of the VH Domain of anti-CD2 antibody (Lo-CD2a; ATCC Accession No: 11423) is (SEQ ID NO: 103) (CDR residues are shown underlined):

```
EVQLQQS GPE LQRPGAVK L SCKAS GY I FT [EYMYVWKQR PKQGLELVGR IDPEDGSIDY VEKFKKKATL] TADT S SNTAY MQLS S LT SE D TATYFCARGK FNYRFAYWGQ GTLVTVS S
```
2. CD3-Binding Domains


As discussed below, in order to illustrate the present invention, bi-specific anti-human CD3 x anti-human DR5-binding molecules were produced. An anti-human CD3 antibody used for such constructs is designated herein as "CD3 mAb 2." The amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO: 104) is shown below (CDR residues are shown underlined):

QAVVTVQPSL TVSPGGTVPD TCRSTGAVT TSNAYNQVQ KPGQAPRGLI
GGTNKRAPTW PARFSGSLLG GAALITITGA QAEDADYYC ALWYNLWVF

CDR_L1 of CD3 mAb 2 (SEQ ID NO: 105): RSSTGAVTTSNYAN
CDR_L2 of CD3 mAb 2 (SEQ ID NO: 106): GTNKRAP
CDR_L3 of CD3 mAb 2 (SEQ ID NO: 107): ALWYNLWVF
The amino acid sequence of the VH Domain of CD3 mAb 2 (SEQ ID NO: 108) is shown below (CDR residues are shown underlined):

```
EVQLVE SGGG LVQPGGS LRL SCAAS GFT F S  **TYAMN**VRQA PGKGLEWVGR_
IRSKYNNYAT YYADSVKDRF T1 SRDDSKNS LYLQMN LKT EDTAVYYCVR
HGNFGNSYVS WFA**YWGQGTL** VTVS S
```

CDRH1 of CD3 mAb 2 (SEQ ID NO:109):  **TYAMN**

CDRH2 of CD3 mAb 2 (SEQ ID NO:110):  **RIRSKYNNYATYYADSVKG**

CDRH3 of CD3 mAb 2 (SEQ ID NO:111):  **HGNFGNSYVSWFAY**

In some of the CD3 constructs, a variant VH Domain was employed for CD3 mAb 2. The variant VH domain possesses a D65G substitution, thus having the amino acid sequence shown below (SEQ ID NO:112) (CDR residues are shown underlined):

```
EVQLVE SGGG LVQPGGS LRL SCAAS GFT F S  **TYAMNWVRQ**A PGKGLEWVGR_
IRSKYNNYAT YYADSVK**G**RF T1 SRDDSKNS LYLQMN LKT EDTAVYYCVR
HGNFGNSYVS WFA**YWGQGTL** VTVS S
```

The substitution causes the CDRH2 to have the amino acid sequence (SEQ ID NO:113) **RIRSKYNNYATYYADSVKG**_. The substituted position (D65G) is shown in double underline.


```
QIVLTQ**S** PAI MSAS PGK**E**KVT MTCSASS**SV**S YMN**W**YQQKS G TSPKRWI YDT
S**K**LASGV**PA**H FRGS GGT**S**TY SLTI SGMEAE DAA**T**YCYQQW S**S**NPFTFGSG
TKLE INR
```

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The amino acid sequence of the VH Domain of OKT3 (SEQ ID NO: 115) is shown below (CDR residues are shown underlined):

```
QVQLQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLUDYGQ QGTTLVSS
```

3. CD16-Binding Domains


The amino acid sequence of a Variable Light Chain Domain of anti-CD16 antibody 3G8 is (SEQ ID NO: 116) (CDR residues are shown underlined):

```
DTVLTQSPAS LAVSLGQRAT ISCKASQSVD FDGDSFMNWY QQKPGQPFPKLI YYTTSNLES GIPARFASG SGTDTLNIH PVEEEDTATY YCQQSNEDPY TFGGGTKEIK
```

The amino acid sequence of the Variable Heavy Chain Domain of anti-CD16 antibody 3G8 is (SEQ ID NO:117) (CDR residues are shown underlined):

```
QVTLKESGPFG ILQPSQTILSL TCSFSFGFSLR TSGMGVGWLR QPSKGLEWL AHITWDDDKKR YNPALKSRTL ISKDTSNQVF LKIASVDTADTATYYCAQI NPAWFAYWGQ GTLVTVSA
```

The amino acid sequence of a Variable Light Chain Domain of anti-CD16 antibody A9 is (SEQ ID NO: 118) (CDR residues are shown underlined):

```
DIQAVVTQES ALTTPSGETV TLTCRSNTGT VTTNSYANWV QEKPDHLFTG LIGHTNNRAPP GVPARFSGSL IGDKAALTIT GAQTEDEAIY FICALWYNNHW VFGGGTKTLTVL
```
The amino acid sequence of the Variable Heavy Chain Domain of anti-CD16 antibody A9 is (SEQ ID NO: 119) (CDR residues are shown underlined):

```
QVQLQGS GAE IYPGGGYTNY SWYFDVWGAR
LVPRGT SVKI NAKKFKGATV LVRPGT
SCKAS GYTF TADTD SSTAY
NYWLGVVKQR VQVRS LTSED
PGHLEWI GD SAVYFCARSA
GAE IYPGGGYTNY SWYFDVWGAR
```

4. CD19-Binding Domains

CD19 antigen is a type I transmembrane glycoprotein belonging to the immunoglobulin Ig superfamily. CD19 is expressed on follicular dendritic cells and B cells. It is considered a pan B cell marker expressed throughout B cell development but with threefold higher expression in mature cells as compared to immature B cells (Raufi A. et al. (2013) “Targeting CD19 In B-Cell Lymphoma: Emerging Role Of SAR3419,” Cancer Manag. Res. 5:225-233). Many CD19 antibodies have been described (e.g., MD1342, MEDI-551, etc.) (Mei, H.E. et al. (2012) "Rationale Of Anti-CD19 Immunotherapy: An Option To Target Autoreactive Plasma Cells In Autoimmunity,” Arthritis Res. Ther. 14(Suppl 5):S1:1-16). The anti-CD19 binding molecule "blinatumomab” is disclosed in EP 2186527.

The amino acid sequence of the VL Domain of a preferred anti-CD19 antibody (HD37) is (SEQ ID NO: 120) (CDR residues are shown underlined):

```
DI L1 TQS PKS ASNRYTGVPD
MSMSVGERTV RFTGS GSAT D
LTKASENW TTVS DTVQK
GAE IYPGGGYTNY SWYFDVWGAR
```

5. CD20-Binding Domains

of a chimeric anti-CD20 antibody (rituximab) is (SEQ ID NO: 122) (CDR residues are shown underlined):

QIVL S Q S PAI LSAS PGEKVT **MTCRASSVS** YIH WFQKPG **SSKPWIYAT**
**SNLASGPVPVR** FS GSGS GTS Y SLT1 SRVEAE **DAATYYCQOW** TSNPPTFGGG
TKLE I KR

[00204] The amino acid sequence of the VH Domain of anti-CD20 antibody (rituximab) is (SEQ ID NO: 123) (CDR residues are shown underlined):

QVQLQQPGAE LVKPGASVKM **SCKASGYTFT** S YN**M**H W**V**KQT PGRGLEWI GA
**TYPNGDTSY** NQKFGKATL TADKS S STAY MQ SLSLTSED SAV**YYC**ARST
**YYGGDWYFNV** WAGTTTVS A

[00205] Alternative anti-CD20 antibodies that may be used in accordance with the present invention include the following commercially available antibodies:

Ibritumomab (e.g., Zevalin®, Spectrum Pharmaceuticals, Inc.), Ofatumumab (e.g., Arzerra®, SmithKlineGlaxo) and Tositumomab (e.g., Bexxar®, GlaxoSmithKline).

6. CD22-Binding Domains


[00207] The amino acid sequence of the VL Domain of anti-CD22 antibody (epratuzumab) is (SEQ ID NO: 124) (CDR residues are shown underlined):

DI QLTQS P S S LSAS VGDRVT **MSCKSSQSVL** YSANHKNYLA WYQKPGKAP
**KLL I YWASTR** ES GGP GSR S G SGS GTDFFT T S LQPE DI A **TYYCHQYLSS**
WTFGGGTKVQ **IKR**

[00208] The amino acid sequence of the VH Domain of anti-CD22 antibody (epratuzumab) is (SEQ ID NO: 125) (CDR residues are shown underlined):

QVQLVQPGAE VKKPGSSVKV **SCKASYTF** SYWL HWRQQA PGGLEWIGY
**INPRNDYTE Y NOFKD KAT I** T A D E S T N T A Y M E L S S L R S E D T A F Y F C A R R D
**ITTFYWQGQT** TVTVS S
7. CD32B-Binding Domains

[00209] A preferred sequence for the VL domain of an antibody that binds to human CD32B is CD32B mAb 1 (SEQ ID NO: 126) (CDR residues are shown underlined):

DIQMTQSPSS LLAALGERVS LTCRASQEIS GYLSWLQQXP DGTIKRLIYA
ASTLDGVPK RFSGSESGSD YSLTISSLES EDFADYYCLQ YFSYPLTFGA
GTKLELK

[00210] A preferred sequence for the VH domain of the CD32B mAb 1 antibody that binds to human CD32B is (SEQ ID NO: 127) (CDR residues are shown underlined):

EVKLEESGGG LVQPGGSMKL SCEASGFTFS DAMDWVRQS PEKGLEWVAE
IRNKRKHAT YYAESVIRGF TISRDDSKSS VYLQMNLSRA EDTGI YYCGA
LGLDYGQQTTLTVSS

8. CD64-Binding Domains

[00211] CD64 is the FcγRI receptor and is expressed on monocytes/macrophages, dendritic cells, and activated granulocytes. The expression can be upregulated by IFN-γ stimulation. CD64 binds IgG immune complex. CD64 plays a role in antigen capture, phagocytosis of IgG/antigen complexes, and antibody-dependent cellular cytotoxicity (WO 2006/002438).

[00212] A preferred sequence for the VL domain of an antibody that binds to human CD64 is CD64 mAb 1 (SEQ ID NO: 128) (CDR residues are shown underlined):

EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAYQQKP GQAPRLLIYD
ASSRATGI PA RFGGSGGSGT DFTLTISSLE PEDFAVYYCQLRSNWPPYTF
GQGTLKLEIK

[00213] A preferred sequence for the VH domain of an antibody that binds to human CD64 is (SEQ ID NO: 129) (CDR residues are shown underlined):

QVQLVESGGG VVQPGRSLRL SCAASGFIFS GYGMHWVRQA PGKLEWVTV
IWYGDSNKKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDET
GDRFFDYWGQ GTLVTVSS

9. BCR/CD79-Binding Domains

[00214] The BCR is composed of a membrane immunoglobulin which, together with non-covalently associated α and β subunits of CD79 ("CD79a" and "CD79b,"

[00215] A preferred sequence for the VL domain of an antibody that binds to the human B Cell Receptor (CD79) is CD79 mAb 1 (SEQ ID NO:130) (CDR residues are shown underlined):

\[
\begin{aligned}
DVVMQTQLT & I\underline{S}VN\ I\ GQPAS \\
RL\ I\ YLVSKLD & S\ \underline{GVPDRTGS} \\
\underline{LTFGAGTKLE} & L\ K
\end{aligned}
\]

[00216] A preferred sequence for the VH domain of the CD79 mAb 1 antibody that binds to the human B Cell Receptor (CD79) is (SEQ ID NO:131) (CDR residues are shown underlined):

\[
\begin{aligned}
QVQLQ\underline{QPGAE} & LVRPGASVKL \underline{SCK\underline{AGY\underline{T}}FT SY\underline{W}MN\underline{WVQK\underline{R}}} \ PGQGLEW1 \ GM \\
\underline{VPDSDESETHY NQMF\underline{DKATL}} & TVDKS S\ STAY MQLS SLTSED SAVYYCARAM \\
GYWGQGT SVT & V S S
\end{aligned}
\]

10. T Cell Receptor-Binding Domains

[00217] In an alternate embodiment, the second epitope that is bound by the Tri-Specific Binding Molecules of the present invention will be an epitope of the T cell

[00219] The amino acid sequence of the VL Domain of anti-TCR antibody BMA 031 is (SEQ ID NO:132) (CDR residues are shown underlined):

\[
\begin{aligned}
\text{EILVTQS } & \text{PAT } \text{LSLSGERAT } \underline{\text{LSCSATSSVS YMHYQQKPG}} \text{ KAPKRWI YDT} \\
\text{SKLASGVP} & \text{SR } \text{FSGSFIGTE } \text{F} \text{TLT ISSLQPE } \underline{\text{DFATYYCQW SSNPLTFGQQ}} \\
\text{TKE} & \text{I} \text{K}
\end{aligned}
\]

[00220] The amino acid sequence of a VH Domain of anti-TCR antibody BMA 031 is (SEQ ID NO:133) (CDR residues are shown underlined):

\[
\begin{aligned}
\text{QVQLVQS GAE } & \text{VKKPGASVKV } \text{SCKAS GYKFT} \underline{\text{SVMHVRQA}} \text{ PGQGLEW1 GY} \\
\text{INPYNDVTKY NEKFGRVT } & \text{TADKS TSTAY} \text{ LQMNS LRSE D} \text{TAVHYCARGS} \\
\text{YYDYDFVXW } & \text{GQGTLVTVS} \text{ S}
\end{aligned}
\]

11. NKG2D Receptor-Binding Domains

The amino acid sequence of the VL Domain of anti-NKG2D antibody KYK-1.0 is (SEQ ID NO: 134) (CDR residues are shown underlined):

QPVLTQP SSVV SVAPGE TARID PCGGDDIETK SVHWWYQQKPG QAPVLVI YDDDDRPSG IPER FFGSNSSNTA TL S1 SRVEAG DEADYYVQW DDNNDEWVF
GQTQLTLV

The amino acid sequence of the VH Domain of anti-NKG2D antibody KYK-1.0 is (SEQ ID NO: 135) (CDR residues are shown underlined):

EVQLVE SGGG VVQPGGS LRLG SCAAS GFT FS SYGMHWVRQA PGKGLEWVAF
IRYGDSNKKY ADSVKGRFTI SRDNSKNTKY LQMNS LRAE D TAVYYCAKDR
FGYYLWDYGQ GTLVTVS S

The amino acid sequence of a VL Domain of anti-NKG2D antibody KYK-2.0 is (SEQ ID NO: 136) (CDR residues are shown underlined):

QSALTQPASV SGPSQS 1 TI SCSGSSSNIG NNANWVWYQQL PGKAPKL I Y
YDDLPSGVS GDSK GTS RDFSRSAFLAI SGLQ SEDEADYYCA AWDDSLNGPV
FGGGTTLTVL

The amino acid sequence of a VH Domain of anti-NKG2D antibody KYK-2.0 is (SEQ ID NO: 137) (CDR residues are shown underlined):

QVQLVE SGGG LVKPGGS LRLG SCAAS GFT FS SYGMHWVRQA PGKGLEWVAF
IRYGDSNKKY ADSVKGRFTI SRDNSKNTLY LQMNS LRAE D TAVYYCAKDR
GLGDDGTYFDY WQGGTTVTVS S

D. Preferred Trispecific Binding Molecules of the Present Invention

1. Preferred Fc Domains

The CH2 and CH3 Domains of the two heavy chains interact to form the Fc Domain, which is a domain that is recognized by cellular Fc Receptors (FcRs). As used herein, the term "Fc Domain" is used to define a C-terminal region of an IgG heavy chain. The amino acid sequence of the CH2-CH3 domain of an exemplary human IgG1 is (SEQ ID NO:1):
Throughout the present specification, the numbering of the residues in an IgG heavy chain is that of the EU index as in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, NH1, MD (1991), expressly incorporated herein by references. The "EU index as in Kabat" refers to the numbering of the human IgG1 EU antibody. Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated by the position of an amino acid in the chain. Kabat described numerous amino acid sequences for antibodies, identified an amino acid consensus sequence for each subgroup, and assigned a residue number to each amino acid. Rabat's numbering scheme is extendible to antibodies not included in his compendium by aligning the antibody in question with one of the consensus sequences in Kabat by reference to conserved amino acids. This method for assigning residue numbers has become standard in the field and readily identifies amino acids at equivalent positions in different antibodies, including chimeric or humanized variants. For example, an amino acid at position 50 of a human antibody light chain occupies the equivalent position to an amino acid at position 50 of a mouse antibody light chain.

Although boundaries may vary slightly, the CH2 domain of a human IgG Fc Domain usually extends from amino acids 231 to amino acid 341 of a human IgG according to the numbering system of Kabat. The CH3 domain of a human IgG usually extends from amino acids 342 to 447 according to the numbering system of Kabat. The "hinge region" or "hinge domain" is generally defined as stretching from Glu216 to Pro230 of human IgG1.
Polymorphisms have been observed at a number of different positions within antibody constant regions (e.g., Fc positions, including but not limited to positions 270, 272, 312, 315, 356, and 358 as numbered by the EU index as set forth in Kabat), and thus slight differences between the presented sequence and sequences in the prior art can exist. Polymorphic forms of human immunoglobulins have been well-characterized. At present, 18 Gm allotypes are known: Glm (1, 2, 3, 17) or Glm (a, x, f, z), G2m (23) or G2m (n), G3m (5, 6, 10, 11, 13, 14, 15, 16, 21, 24, 26, 27, 28) or G3m (bl, c3, b3, bO, b3, b4, s, t, gl, c5, u, v, g5) (Lefranc, et al., The human IgG subclasses: molecular analysis of structure, function and regulation. Pergamon, Oxford, pp. 43-78 (1990); Lefranc, G. et al., 1979, Hum. Genet.: 50, 199-211). It is specifically contemplated that the antibodies of the present invention may be incorporate any allotype, isoallotype, or haplotype of any immunoglobulin gene, and are not limited to the allotype, isoallotype or haplotype of the sequences provided herein.

Activating and inhibitory signals are transduced through the Fc Receptors (FcγRs) following their ligation to an Fc Domain. These diametrically opposing functions result from structural differences among the different receptor isoforms. Two distinct domains within the cytoplasmic signaling domains of the receptor called immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) account for the different responses. The recruitment of different cytoplasmic enzymes to these structures dictates the outcome of the FcγR-mediated cellular responses. ITAM-containing FcγR complexes include FcγRI, FcγRIIA, FcγRIIB, whereas ITIM-containing complexes only include FcγRIIB. Human neutrophils express the FcγRIIA gene. FcγRIIA clustering via immune complexes or specific antibody cross-linking serves to aggregate ITAMs along with receptor-associated kinases which facilitate ITAM phosphorylation. ITAM phosphorylation serves as a docking site for Syk kinase, activation of which results in activation of downstream substrates (e.g., PI3K). Cellular activation leads to release of proinflammatory mediators. The FcγRIIB gene is expressed on B lymphocytes; its extracellular domain is 96% identical to FcγRIIA and binds IgG complexes in an indistinguishable manner. The presence of an ITIM in the cytoplasmic domain of FcγRIIB defines this inhibitory subclass of FcγR. Recently
the molecular basis of this inhibition was established. When co-ligated along with an
activating FcyR, the ITIM in FcyRIIB becomes phosphorylated and attracts the SH2
domain of the inositol polyphosphate 5'-phosphatase (SHIP), which hydrolyzes
phosphoinositol messengers released as a consequence of ITAM-containing FcyR-
mediated tyrosine kinase activation, consequently preventing the influx of
intracellular Ca++. Thus cross-linking of FcyRIIB dampens the activating response to
FcyR ligation and inhibits cellular responsiveness. B cell activation, B cell
proliferation and antibody secretion is thus aborted.

[00231] The Fc Domain of the binding molecules of the present invention may be
either a complete Fc Domain (e.g., a complete IgG Fc Domain) or only a fragment of
a complete Fc Domain. 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., FcyR(s)), more preferably such Fc Domain will cause altered binding
to FcyRIA (CD64), FcyRIIA (CD32A), FcyRIIB (CD32B), FcyRIIIA (CD16a) or
FcyRIIIB (CD16b) (relative to the binding exhibited by a wild-type Fc Domain) or
will substantially eliminate the ability of such Fc Domain to bind to inhibitory
receptor(s). Thus, the Fc Domain of the Fc Domain-containing 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 Domain, 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
Domain). Such Fc Domains may comprise non-Fc polypeptide portions, or may
comprise portions of non-naturally complete Fc Domains, 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.).

[00232] Fc Domain modifications identified as altering effector function are known
in the art, including modifications that increase binding to activating receptors (e.g.,
FcyRIIA (CD16A) and reduce binding to inhibitory receptors (e.g., FcyRIIB (CD32B)
(see, e.g., Stavenhagen, J.B. et al. (2007) "Fc Optimization Of Therapeutic Antibodies
Enhances Their Ability To Kill Tumor Cells In Vitro And Controls Tumor Expansion

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In Vivo Via Low-Affinity Activating Fcgamma Receptors," Cancer Res. 57(18):8882-8890).

[00233] In particular, it is preferred for the CH2-CH3 domains of the polypeptide chains of the Fc Domain-containing diabodies of the present invention to exhibit decreased (or substantially no) binding to FcyRIIA (CD64), FcyRIIA (CD32A), FcyRIIB (CD32B), FcyRIIIA (CD16a) or FcyRIIIB (CD16b) (relative to the binding exhibited by the wild-type Fc Domain (SEQ ID NO:1).)Fc variants and mutant forms capable of mediating such altered binding are described above. In a preferred embodiment the CH2-CH3 Domain of the first and/or third polypeptide chains of such diabodies include any 1, 2, 3, 4, 5, 6, or 7 of the substitutions: L234A, L235A, F243L, R292P, Y300L, V305I and P396L. Exemplary variants of human IgGl Fc Domains with reduced binding to CD32B and/or increased binding to CD16A contain F243L, R292P, Y300L, V305I or P296L substitutions. These amino acid substitutions may be present in a human IgGl Fc Domain in any combination. In one embodiment, the human IgGl Fc Domain variant contains a F243L, R292P and Y300L substitution. In another embodiment, the human IgGl Fc Domain variant contains a F243L, R292P, Y300L, V305I and P296L substitution. In one embodiment the CH2-CH3 Domain of the first and/or third polypeptide chains of such diabodies include any 1, 2, 3, or 3 of the substitutions: L234A, L235A, N297G, N297Q. In another embodiment, the human IgGl Fc Domain variant contains an N297Q substitution, L234A and L235A substitutions or a D265A substitution, as these mutations abolish FcR binding. Alternatively, a CH2-CH3 domain which inherently exhibits decreased (or substantially no) binding to FcyRIIIA (CD16a) and/or reduced effector function (relative to the binding exhibited by the wild-type IgGl Fc Domain (SEQ ID NO:1)) is utilized. In a specific embodiment, the Fc Domain-containing diabodies of the present invention comprise an IgG2 Fc Domain or an IgG4 Fc Domain. Where an IgG4 Fc Domain in utilized the instant invention also encompasses the introduction of a stabilizing mutation such as S228P, as numbered by the EU index as set forth in Kabat (Lu et al. (2008) "7¾e Effect Of A Point Mutation On The Stability Of IgG As Monitored By Analytical Ultracentrifugation," J Pharmaceutical Sciences 97:960-969) to reduce the incidence of strand exchange. Other stabilizing mutations known in the art may be introduced into an IgG4 Fc Domain (Peters, P et al., (2012)

[00234] The CH2 and/or CH3 Domains of such polypeptide chains need not be identical in sequence, and advantageously are modified to foster complexing between the two polypeptide chains. 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 two of the polypeptides of the Tri-Specific Binding Molecule. 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 Bi-specific 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 other CH2-CH3-containing polypeptide chain. Thus, the "knob" will help in preventing the first polypeptide chain from homodimerizing via its CH2 and/or CH3 Domains. The CH2-CH3 "hole-bearing" polypeptide chain will heterodimerize with the CH2-CH3 "knob-bearing" polypeptide chain, and will also homodimerize with itself. A preferred knob is created by modifying a native IgG Fc
Domain to contain the modification T366W. A preferred hole is created by modifying a native IgG Fc Domain to contain the modification T366S, L368A and Y407V. To aid in purifying the "hole-bearing" polypeptide chain homodimer from the final Tri-Specific Binding Molecule, the protein A binding site of the CH2 and CH3 Domains of the "hole-bearing" Fc Domain is preferably mutated by amino acid substitution at position 435 (H435R). Thus, the "hole-bearing" Fc Domain homodimer will not bind to protein A, whereas the desired Tri-Specific Binding Molecule will retain its ability to bind protein A via the protein A binding site on the first polypeptide chain.

[00235] A preferred sequence for the CH2 and CH3 Domains of the first polypeptide chain of an Fc Domain-containing diabody of the present invention will have the "knob-bearing" sequence (SEQ ID NO:52):

APEAAAGGP SV FLFP PKPKDT LMI SRT PEVT CVVDVS HE D PEVKFNWYVD
GVEVHNAKT G PREEQNS TY RVVS VLT V LH QDW LNGKE Y CKVSNKAL PA
PLI EKT I SKAK GQP PRE PQVYT L P P SREEMTK NQVS LW CLV K G FY P S D I A V E
WE SNGQPENN YKT T P PVLDS DGS FFL Y SKL T VDKSRWQQG NVFS C SVMHE
ALHNHYTQKS L S L S PGK

[00236] A preferred sequence for the CH2 and CH3 Domains of the second polypeptide chain of an Fc Domain-containing diabody of the present invention having two polypeptide chains (or the third polypeptide chain of an Fc Domain-containing diabody having three polypeptide chains) will have the "hole-bearing" sequence (SEQ ID NO:53):

APEAAAGGP SV FLFP PKPKDT LMI SRT PEVT CVVDVS HE D PEVKFNWYVD
GVEVHNAKT G PREEQNS TY RVVS VLT V LH QDW LNGKE Y CKVSNKAL PA
PLI EKT I SKAK GQP PRE PQVYT L P P SREEMTK NQVS LW CLV K G FY P S D I A V E
WE SNGQPENN YKT T P PVLDS DGS FFL Y SKL T VDKSRWQQG NVFS C SVMHE
ALHNRYTQKS L S L S PGK

[00237] As will be noted, the CH2-CH3 Domains of SEQ ID NO:52 and SEQ ID NO:53 include a substitution at position 234 with alanine and 235 with alanine, and thus form an Fc Domain exhibit decreased (or substantially no) binding to FcyRIA (CD64), FcyRIIA (CD32A), FcyRIIB (CD32B), FcyRIIIB (CD16a) or FcyRIIIB (CD16b) (relative to the binding exhibited by the wild-type Fc Domain (SEQ ID NO:1).
It is preferred that the first polypeptide chain will have a "knob-bearing" CH2-CH3 sequence, such as that of SEQ ID NO: 52. However, as will be recognized, a "hole-bearing" CH2-CH3 Domain (e.g., SEQ ID NO: 53) could be employed in the first polypeptide chain, in which case, a "knob-bearing" CH2-CH3 Domain (e.g., SEQ ID NO: 52) would be employed in the second polypeptide chain of an Fc Domain-containing diabody of the present invention having two polypeptide chains (or the third polypeptide chain of an Fc Domain-containing diabody having three polypeptide chains).

2. Preferred First Polypeptide Chain

A first polypeptide chain of a preferred binding molecule of the present invention will comprise a Variable Light Chain Domain capable of binding to Epitope I (VLi), a Variable Heavy Chain Domain capable of binding to Epitope II (VHi), a Heterodimer-Promoting Domain and a CH2-CH3 Domain.

Since the Variable Light Chain and Variable Heavy Chain Domains of the first polypeptide are directed toward different epitopes, they cannot associate together to form a Binding Domain that is able to bind either Epitope I or Epitope II. The Variable Light Chain and Variable Heavy Chain Domains of the first polypeptide are spaced apart from one another by an intervening linker peptide that is sufficiently short as to substantially prevent the association of these Domains. An exemplary linker, termed "Linker 1," has the sequence (SEQ ID NO: 33): GGGSGGGG.

The Variable Heavy Chain Domain of the first polypeptide and the Heterodimer-Promoting Domain of that polypeptide are preferably spaced apart from one another by an intervening linker peptide that contains 1, 2, 3 or more cysteine residues. A preferred cysteine-containing spacer peptide ("Linker 2") has the sequence is SEQ ID NO: 34: GGCYGG.

Linkers that may be employed to link a CH2-CH3 Domain to a polypeptide chain of the molecules of the present invention include: AS\(\ddot{T}\)K (SEQ ID NO: 47), DKTHTCPPCP (SEQ ID NO: 48), LEPKSS (SEQ ID NO: 49), and APSSSPME (SEQ ID NO: 50), APSSS (SEQ ID NO: 152) and GGG or GCG. SEQ
ID NO:49 may be used in lieu of GGG or GCG for ease of cloning. Additionally, SEQ ID NO:49 may be immediately followed by SEQ ID NO:47 to form an alternate linker (LE PKS SDKTHC PPCP: SEQ ID NO:51).

[00243] The Heterodimer-Promoting Domain of the first polypeptide and the Heterodimer-Promoting Domain of the second polypeptide are coordinately selected. The Domains differ from one another and are designed to associate with one another so as to promote the association of the first and second polypeptide chains. For example, one of the Heterodimer-Promoting Domains will be engineered to have a negative charge at pH 7, while the other of the two polypeptide chains will be engineered to have 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 Heterodimer-Promoting Domains is provided to which chain, as long as the Domains employed on the first and second polypeptide chains differ so as to foster heterodimerization between such chains.

[00244] The Heterodimer-Promoting Domains may be the IgG CL and CHI domains or may be a peptide having the amino acid sequence GVEPKSC (SEQ ID NO:35) or VEPKSC (SEQ ID NO:36), derived from the hinge domain of a human IgG, and in lieu of the CL domain, one may employ the C-terminal 6 amino acids of the human kappa light chain, GFNRGEC (SEQ ID NO:37) or FNRGEC (SEQ ID NO:38).


[00246] Such repeated coil domains may be exact repeats or may have substitutions. For example, the Heterodimer-Promoting Domain of the first polypeptide chain may comprise a sequence of eight negatively charged amino acid residues and the Heterodimer-Promoting Domain of the second polypeptide chain may comprise a sequence of eight negatively charged amino acid residues. It is
immaterial which coil is provided to the first or second polypeptide chains, provided that a coil of opposite charge is used for the other polypeptide chain. 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. It is possible for only a single Heterodimer-Promoting Domain to be employed (since such domain will inhibit homodimerization and thereby promote heterodimerization), however, it is preferred for both the first and second polypeptide chains of the diabodies of the present invention to contain Heterodimer-Promoting Domains.

[00247] In a preferred embodiment, one of the Heterodimer-Promoting Domains will comprise four tandem "E-coil" helical domains (SEQ ID NO:39: _EVAALEK_ _EVAALEK_ _EVAALEK_ _EVAALEK_), whose glutamate residues will form a negative charge at pH 7, while the other of the Heterodimer-Promoting Domains will comprise four tandem "K-coil" domains (SEQ ID NO:40: _KVAALKE_ _KVAALKE_ _KVAALKE_ _KVAALKE_), whose lysine 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. Especially preferred is a Heterodimer-Promoting Domain in which one of the four tandem "E-coil" helical domains of SEQ ID NO:39 has been modified to contain a cysteine residue: _EVAACEK_ _EVAALEK_ _EVAALEK_ _EVAALEK_ (SEQ ID NO:41). Likewise, especially preferred is a Heterodimer-Promoting Domain in which one of the four tandem "K-coil" helical domains of SEQ ID NO:40 has been modified to contain a cysteine residue: _KVAALKE_ _KVAALKE_ _KVAALKE_ _KVAALKE_ (SEQ ID NO:42).

[00248] As disclosed in WO 2012/018687, in order to improve the in vivo pharmacokinetic properties of diabodies, a diabody may be modified to contain a polypeptide portion of a serum-binding protein at one or more of the termini of the diabody. Most preferably, such polypeptide portion of a serum-binding protein will be installed at the C-terminus of the diabody. 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. 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. Thus, a particularly preferred polypeptide portion of a serum-binding protein for improving the in vivo pharmacokinetic properties of a diabody is the Albumin-Binding Domain (ABD) from streptococcal protein G, and more preferably, the Albumin-Binding Domain 3 (ABD3) of protein G of Streptococcus strain G148 (SEQ ID NO:43): LAEAKVLANR ELDKYGVSDY YKNLI DNAKS AEGVKAL IDE I LAAL P.

[00249] As disclosed in WO 2012/162068 (herein incorporated by reference), "deimmunized" variants of SEQ ID NO:43 have the ability to attenuate or eliminate MHC class II binding. Based on combinational mutation results, the following combinations of substitutions are considered to be preferred substitutions for forming such a deimmunized albumin-binding domain: 66S/70S +71A; 66S/70S +79A; 64A/65A/71A+66S; 64A/65A/71A+66D; 64A/65A/71A+66E; 64A/65A/79A+66S; 64A/65A/79A+66D; 64A/65A/79A+66E. Variant ABDs having the modifications L64A, I65A and D79A or the modifications N66S, T70S and D79A. Variant deimmunized ABD having the amino acid sequence:

L AEAKVLANR ELDKYGVS DY YKNL I DNAK S AEGVKAL IDE I LAAL P (SEQ ID NO: 44),

or the amino acid sequence:

L AEAKVLANR ELDKYGVS DY YKNLI SNAKS VEGVKAL I A79E I LAAL P (SEQ ID NO: 45),

are particularly preferred as such deimmunized Albumin-Binding Domains exhibit substantially wild-type binding while providing attenuated MHC class II binding. Thus, the first polypeptide chain of such a diabody having an Albumin-Binding Domain contains a third linker (Linker 3) preferably positioned C-terminally to the E-coil (or K-coil) Domain of such polypeptide chain so as to intervene between the E-coil (or K-coil) Domain and the Albumin-Binding Domain (which is preferably a
deimmunized Albumin-Binding Domain). A preferred sequence for such Linker 3 is
SEQ ID NO:46: GGGS.

[00250] Thus, in sum, a preferred first polypeptide chain of a preferred Tri-Specific
Binding Molecule of the present invention will comprise the Domains and linkers:
(VLi Domain)—(Linker 1)—(VHn Domain)—(Linker 2)—(E-coil Heterodimer-
Promoting Domain)—(Linker 3)—(Knob-Bearing CH2-CH3 Domain).

3. Alternative First Polypeptide Chain

[00251] In one embodiment, the orientations of the above-described Domains will
be in the N-terminal to C-terminal direction. The present invention, however, also
contemplates a variation thereof, wherein the orientations of the Domains of the first
polypeptide chain are: NH2-(Knob-Bearing CH3-CH2 Domain)-(VLi
Domain)—(Linker 1)—(VHn Domain)—(Linker 2)—(E-coil Heterodimer-
Promoting Domain). Preferably, a cysteine-containing peptide is present, N-terminal
to such CH2-CH3 Domain. The sequence of an exemplary peptide is sequence (SEQ
ID NO:48): DKTHTCPPCP. Preferably in this embodiment, the CH3 Domain is
spaced apart from the VLi Domain by an intervening peptide linker (Linker 4), such
as one having the amino acid sequence of (SEQ ID NO:152): APSSS, and more
preferably, the amino acid sequence (SEQ ID NO:50): APSSSPME.

4. Preferred Second Polypeptide Chain

[00252] A second polypeptide chain of such preferred Tri-Specific Binding
Molecules will comprise, in the N-terminal to C-terminal direction, a Variable Light
Chain Domain capable of binding to Epitope II (VLn), a Variable Heavy Chain
Domain capable of binding to Epitope I (VHi), and a Heterodimer-Promoting
Domain.

[00253] Since the Variable Light Chain and Variable Heavy Chain Domains of the
second polypeptide are directed toward different epitopes, they cannot associate
together to form a Binding Domain that is able to bind either Epitope I or Epitope II.
The Variable Light Chain and Variable Heavy Chain Domains of the second
polypeptide are spaced apart from one another by an intervening linker peptide that is
sufficiently short as to substantially prevent the association of these Domains. "Linker 1," having the sequence (SEQ ID NO:33): GGGSGGGG is an exemplary linker for this purpose.

[00254] As in the case of the first polypeptide chain, the Variable Heavy Chain Domain of the second polypeptide and the Heterodimer-Promoting Domain of that polypeptide are preferably spaced apart from one another by an intervening linker peptide that contains 1, 2, 3 or more cysteine residues. "Linker 2," having the sequence (SEQ ID NO:34) GCGGGG is an exemplary linker for this purpose. Such cysteine residues can form disulfide bonds with cysteine residues in the cysteine-containing spacer peptide that separates the Variable Heavy Chain Domain of the first polypeptide and the Heterodimer-Promoting Domain of that polypeptide. Thus, the first and second polypeptides of the Binding Molecules of the present invention are covalently bonded to one another.

[00255] As discussed above, the Heterodimer-Promoting Domain of the second polypeptide chain is selected so as coordinate with the Heterodimer-Promoting Domain of the first polypeptide chain. Thus, in a preferred embodiment, the Heterodimer-Promoting Domain of the first polypeptide chain is either a "K-coil" Domain (SEQ ID NO:40) or an "E-coil" Domain (SEQ ID NO:39). If the cysteine-containing E-coil (SEQ ID NO:41) is employed in the first polypeptide chain, then the cysteine-containing K-coil (SEQ ID NO:42) is preferably employed in the second polypeptide chain. Conversely, if the cysteine-containing K-coil (SEQ ID NO:42) is employed in the first polypeptide chain, then the cysteine-containing E-coil (SEQ ID NO:41) is preferably employed in the second polypeptide chain. Since the first polypeptide chain will preferably possess an "E-coil" Domain, the second polypeptide chain will preferably contain a "K-coil" Domain.

[00256] As the first and second polypeptide chains are polypeptide chains of a diabody, they are able to associate together to form a Domain I Binding Domain (VLA/VHA) that recognizes and immunospecifically binds to Epitope I, and a Domain II Binding Domain (VL B/VH B) that recognizes and immunospecifically binds to Epitope II.
Thus, in sum, a preferred second polypeptide chain of a preferred Binding Molecule of the present invention will comprise the Domains and linkers: (VLII Domain)—(Linker 1)—(VHi Domain)—(Linker 2)—(K-coil Heterodimer-Promoting Domain).

5. Preferred Third Polypeptide Chain

A third polypeptide chain of a preferred Binding Molecule of the present invention is a polypeptide that comprises, in the N-terminal to C-terminal direction, a Binding Domain, an optional CHI-Hinge Domain, and a CH2-CH3 Domain. The Binding Domain of the third polypeptide chain of a preferred Binding Molecule of the present invention may be a Variable Heavy Chain Domain capable of binding to Epitope III (VHm), in which case, the fourth polypeptide chain of the preferred Binding Molecules of the present invention (discussed below) is a polypeptide that comprises a Variable Light Chain Domain capable of binding to Epitope III (VLm), such that the Binding Domain is capable of immunospecific binding to an antigen possessing Epitope III. Alternatively, the Binding Domain of the third polypeptide chain of the preferred Binding Molecules of the present invention may comprise an Effector Cell Receptor-Type Binding Domain, in which case, the fourth polypeptide chain of the preferred Binding Molecules of the present invention (discussed below) is a polypeptide that comprises a complementary Effector Cell Receptor-Type Binding Domain, such that the interaction of two polypeptide chains forms a Binding Domain that is capable of physiospecific binding to molecule present on the surface of the effector cell. The third polypeptide chain may be isolated from naturally occurring antibodies. Alternatively, it may be constructed recombinantly. An exemplary CHI Domain is a human IgGl CHI Domain having the amino acid sequence (SEQ ID NO:207):

ASTKGPSVFPLPSSKSTSGAALGCLVQDYFPEPVTVSNNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSQTLQKLVATISADSKVQPRELSEDCRQSNPPNGSFYGSQYQVNGQ

A variant of the human IgGl CHI Domain of SEQ ID NO:207 is (SEQ ID NO:208):

ASTKGPSVFPLPSSKSTSGAALGCLVQDYFPEPVTVSNNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSQTLQKLVATISADSKVQPRELSEDCRQSNPPNGSFYGSQYQVNGQ
[00260] An exemplary Hinge Domain is a human IgG1 Hinge Domain having the amino acid sequence (SEQ ID NO:209): EPKS CDKTHTC PPCP. As will be recognized, the exemplary Hinge Domain comprises multiple cysteine residues (Elkabetz et al. (2005) "Cysteines In CHI Underlie Retention Of Unassembled Ig Heavy Chains," J. Biol. Chem. 280:14402-14412) that may participate in interchain covalent bonding.

[00261] Although a wild-type CH2-CH3 Domain may be employed, it is preferred, as described above, to employ a modified CH2-CH3 Domain that promotes heterodimerization with the CH2-CH3 Domain of the first polypeptide chain.

[00262] Preferably, therefore the CH2-CH3 Domain of the third polypeptide chain will be a "hole-bearing" CH2-CH3 Domain whose amino acid sequence is complementary to the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52) employed in the first polypeptide. As discussed above, the "hole-bearing" CH2-CH3 domain preferably should comprise a substitution at position 435 (H435R) to remove the Protein A binding site. An exemplary "hole-bearing" CH2-CH3 Domain with the H435R substitution for the third polypeptide is SEQ ID NO:53.

[00263] As will be recognized, a "knob-bearing" CH2-CH3 Domain (e.g., SEQ ID NO:52) could be employed in the third polypeptide chain, in which case, a "hole-bearing" CH2-CH3 Domain (e.g., SEQ ID NO:53) would be employed in the first polypeptide chain.

[00264] In the embodiment in which the third (and fourth) polypeptide chains of the preferred Tri-Specific Binding Molecules of the present invention each comprise a polypeptide chain of an Effector Cell Receptor-Type Binding Domain, methods for producing such Effector Cell Receptor-Type Binding Domains are well-known (e.g., US2012/0294874A1).

[00265] Thus, in sum, a third polypeptide chain of the preferred Binding Molecules of the present invention will comprise the Domains and linkers: (VHm Domain)—(Optional CHI Domain)—(Optional Hinge Domain)—("Hole-Bearing" CH2-CH3 Domain), or (T Cell Receptor-Type Binding Domain; first or
second polypeptide thereof)—(Optional CHI Domain)—(Optional Hinge Domain)—("Hole-Bearing" CH2-CH3 Domain).

6. Preferred Fourth Polypeptide Chain

[00266] A fourth polypeptide chain of the preferred Tri-Specific Binding Molecules of the present invention is either a polypeptide of an Effector Cell Receptor-Type Binding Domain (wherein the third and fourth polypeptides form a ligand for a receptor found on the surface of an effector cell, or more preferably, a light chain of the above-indicated antibody that immunospecifically binds to Epitope III or which are complementary to the binding domain of the third polypeptide chain.

[00267] Thus, wherein the third and fourth polypeptides form a Fab-Type Binding Domain such fourth polypeptide chain comprises, in the N-terminal to C-terminal direction, a Variable Light Chain Domain capable of binding to Epitope III (VLm), and a Domain for promoting covalent bonding to the third polypeptide chain or a Binding Domain and such Domain for promoting covalent bonding to the third polypeptide chain. Such Domain may be a CL Domain, or a cysteine-containing portion thereof, such as (SEQ ID NO:38) FNRGEC or a linker such as Linker 2 (having the sequence (SEQ ID NO:34) GGCYGGY. An exemplary a cysteine-containing peptide that forms disulfide bonds with such Linker 2 comprises the amino acid sequence VEPKS C (SEQ ID NO:36) or a Hinge Domain.

[00268] The fourth polypeptide chain may be isolated from naturally occurring antibodies. Alternatively, it may be constructed recombinantly. A preferred CL Domain is a human IgGl CL Kappa Domain having the amino acid sequence (SEQ ID NO:210):

RTVAAP SVF I F P P S DEQLKS GTASV V C L N NF Y PRE AK VQ WKVDNALQS G
NS Q E SVTE QD SKDS T Y S L S S TLTL SK AD Y E KHKVY A C EV T HQ GL S S PV TK
S FNRGEC

[00269] Alternatively, an exemplary CL Domain is a human IgGl CL Lambda2 Domain having the amino acid sequence (SEQ ID NO:211):

QPKAAP SVTL F P P S SEELQA NK AT L V CL I S D F Y PGA VT VA γ K A D S S PV KA
G V E T T P SK QS NN K YA AS SY L S L T PE QW K S H R SY S C Q V T H E G S T V E K TV AP
TEC S

- 100 -
[00270] As will be noticed, the CL Domain, or other Cysteine-Containing Domain, of the fourth polypeptide chain comprises cysteine residues. Such cysteine residues are able to covalently bond to cysteine residues of the CHI Domain of the third polypeptide chain to thereby covalently complex the third and fourth polypeptide chains of the binding molecules of the present invention to one another. Thus the third and fourth polypeptide chains are covalently bonded to one another.

[00271] Additionally, cysteine residues of the CH2-CH3 Domain of the first polypeptide chain can form disulfide bonds with cysteine residues of the CH2-CH3 Domain of the third polypeptide chain. Thus the first and third polypeptide chains are covalently bonded to one another.

E. Variant Fc Domains

[00272] In traditional immune function, the interaction of antibody-antigen complexes with cells of the immune system results in a wide array of responses, ranging from effector functions such as antibody-dependent cytotoxicity, mast cell degranulation, and phagocytosis to immunomodulatory signals such as regulating lymphocyte proliferation and antibody secretion. All of these interactions are initiated through the binding of the Fc Domain of antibodies or immune complexes to specialized cell surface receptors on hematopoietic cells. The diversity of cellular responses triggered by antibodies and immune complexes results from the structural heterogeneity of the three Fc receptors: FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD16). FcyRI (CD64), FcyRIIA (CD32A) and FcyRIII (CD16) are activating (i.e., immune system enhancing) receptors; FcyRIIB (CD32B) is an inhibiting (i.e., immune system dampening) receptor. The amino acid sequence of an exemplary IgG1 Fc Domain (SEQ ID NO:1) is presented above.

[00273] Modification of the Fc Domain normally leads to an altered phenotype, for example altered serum half-life, altered stability, altered susceptibility to cellular enzymes or altered effector function. It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. Reduction or elimination of effector function is desirable in certain cases, for example in the case of antibodies whose
mechanism of action involves blocking or antagonism, but not killing of the cells bearing a target antigen. Increased effector function is generally desirable when directed to undesirable cells, such as tumor and foreign cells, where the FcγRs are expressed at low levels, for example, tumor-specific B cells with low levels of FcγRIIB (e.g., non-Hodgkins lymphoma, CLL, and Burkitt's lymphoma). In said embodiments, molecules of the invention with conferred or altered effector function activity are useful for the treatment and/or prevention of a disease, disorder or infection where an enhanced efficacy of effector function activity is desired.

[00274] In certain embodiments, the Tri-Specific Binding Molecules of the present invention comprise an Fc Domain that possesses one or more modifications (e.g., substitutions, deletions, or insertions) to the sequence of amino acids of a wild-type Fc Domain (SEQ ID NO:1), which reduce the affinity and avidity of the Fc Domain and, thus, the molecule of the invention, for one or more FcγR receptors. In other embodiments, the molecules of the invention comprise an Fc Domain that possesses one or more modifications to the amino acids of the wild-type Fc Domain, which increase the affinity and avidity of the Fc Domain and, thus, the molecule of the invention, for one or more FcγR receptors. In other embodiments, the molecules comprise a variant Fc Domain wherein said variant confers or mediates increased ADCC activity and/or an increased binding to FcγRIIA, relative to a molecule comprising no Fc Domain or comprising a wild-type Fc Domain. In alternate embodiments, the molecules comprise a variant Fc Domain wherein said variant confers or mediates decreased ADCC activity (or other effector function) and/or an increased binding to FcγRIIB, relative to a molecule comprising no Fc Domain or comprising a wild-type Fc Domain. In some embodiments, the invention encompasses Tri-Specific Binding Molecules comprising a variant Fc Domain, which variant Fc Domain does not show a detectable binding to any FcγR, relative to a comparable molecule comprising the wild-type Fc Domain. In other embodiments, the invention encompasses Tri-Specific Binding Molecules comprising a variant Fc Domain, which variant Fc Domain only binds a single FcγR, preferably one of FcγRIIA, FcγRIIB, or FcγRIIIA. Any such increased affinity and/or avidity is preferably assessed by measuring in vitro the extent of detectable binding to the FcγR or FcγR-related activity in cells that express low levels of the FcγR when binding.
activity of the parent molecule (without the modified Fc Domain) cannot be detected in the cells, or in cells which express non-Fcy receptor target antigens at a density of 30,000 to 20,000 molecules/cell, at a density of 20,000 to 10,000 molecules/cell, at a density of 10,000 to 5,000 molecules/cell, at a density of 5,000 to 1,000 molecules/cell, at a density of 1,000 to 200 molecules/cell or at a density of 200 molecules/cell or less (but at least 10, 50, 100 or 150 molecules/cell).

[00275] The Tri-Specific Binding Molecules of the present invention may comprise altered affinities for an activating and/or inhibitory Fey receptor. In one embodiment, the Tri-Specific Binding Molecule comprises a variant Fc Domain that has increased affinity for FcyRIIB and decreased affinity for FcyRIIIA and/or FcyRIIA, relative to a comparable molecule with a wild-type Fc Domain. In another embodiment, the Tri-Specific Binding Molecule of the present invention comprise a variant Fc Domain, which has decreased affinity for FcyRIIB and increased affinity for FcyRIIIA and/or FcyRIIA, relative to a comparable molecule with a wild-type Fc Domain. In yet another embodiment, the Tri-Specific Binding Molecules of the present invention comprise a variant Fc Domain that has decreased affinity for FcyRIIB and decreased affinity for FcyRIIIA and/or FcyRIIA, relative to a comparable molecule with a wild-type Fc Domain. In still another embodiment, the Tri-Specific Binding Molecules of the present invention comprise a variant Fc Domain, which has unchanged affinity for FcyRIIB and decreased (or increased) affinity for FcyRIIIA and/or FcyRIIA, relative to a comparable molecule with a wild-type Fc Domain.

[00276] In certain embodiments, the Tri-Specific Binding Molecules of the present invention comprise a variant Fc Domain having an altered affinity for FcyRIIIA and/or FcyRIIA such that the immunoglobulin has an enhanced effector function, e.g., antibody-dependent cell-mediated cytotoxicity. Non-limiting examples of effector cell functions include antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent phagocytosis, phagocytosis, opsonization, opsonophagocytosis, cell binding, rosetting, Clq binding, and complement dependent cell-mediated cytotoxicity.
In a preferred embodiment, the alteration in affinity or effector function is
at least 2-fold, preferably at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold,
at least 8-fold, at least 9-fold, at least 10-fold, at least 50-fold, or at least 100-fold,
relative to a comparable molecule comprising a wild-type Fc Domain. In other
embodiments of the invention, the variant Fc Domain immunospecifically binds one
or more FcRs with at least 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%,
100%, 125%, 150%, 175%, 200%, 225%, or 250% greater affinity relative to a
molecule comprising a wild-type Fc Domain. Such measurements can be in vivo or
in vitro assays, and in a preferred embodiment are in vitro assays such as ELISA or
surface plasmon resonance assays.

In different embodiments, the Tri-Specific Binding Molecules of the
present invention comprise a variant Fc Domain wherein said variant agonizes at least
one activity of an FcyR receptor, or antagonizes at least one activity of an FcyR
receptor. In a preferred embodiment, the molecules comprise a variant that
agonizes one or more activities of FcyRIIB, for example, B cell receptor-mediated
signaling, activation of B cells, B cell proliferation, antibody production, intracellular
calcium influx of B cells, cell cycle progression, FcyRIIB-mediated inhibition of
FcRs signaling, phosphorylation of FcyRIIB, SHIP recruitment, SHIP
phosphorylation and association with She, or activity of one or more downstream
molecules (e.g., MAP kinase, INK, p38, or Akt) in the FcyRIIB signal transduction
pathway. In another embodiment, the Tri-Specific Binding Molecules of the present
invention comprise a variant that agonizes one or more activities of FcsRI, for
example, mast cell activation, calcium mobilization, degranulation, cytokine
production, or serotonin release.

In certain embodiments, the molecules comprise an Fc Domain comprising
regions from two or more IgG isotypes (e.g., IgGl, IgG2, IgG3 and IgG4). The
various IgG isotypes exhibit differing physical and functional properties including
serum half-life, complement fixation, FcyR binding affinities and effector function
activities (e.g., ADCC, CDC, etc.) due to differences in the amino acid sequences of
their hinge and/or Fc Domains, for example as described in Flesch and Neppert
25131; Chappel et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88:9036-9040; or Bruggemann et al. (1987) J. Exp. Med 166:1351-1361. This type of variant Fc Domain may be used alone, or in combination with an amino acid modification, to affect Fc-mediated effector function and/or binding activity. In combination, the amino acid modification and IgG hinge/Fc Domain may display similar functionality (e.g., increased affinity for FcγRIIA) and may act additively or, more preferably, synergistically to modify the effector functionality in the molecule of the invention, relative to a molecule of the invention comprising a wild-type Fc Domain. In other embodiments, the amino acid modification and IgG Fc Domain may display opposite functionality (e.g., increased and decreased affinity for FcγRIIA, respectively) and may act to selectively temper or reduce a specific functionality in the molecule of the invention, relative to a molecule of the invention not comprising an Fc Domain or comprising a wild-type Fc Domain of the same isotype.

[00280] In a preferred specific embodiment, the Tri-Specific Binding Molecules of the present invention comprise a variant Fc Domain, wherein said variant Fc Domain comprises at least one amino acid modification relative to a wild-type Fc Domain, such that said molecule has an altered affinity for an FcR, provided that said variant Fc Domain does not have a substitution at positions that make a direct contact with FcγR based on crystallographic and structural analysis of Fc-FcR interactions such as those disclosed by Sondermann et al. (2000) Nature 406:267-73. Examples of positions within the Fc Domain that make a direct contact with FcγR are amino acid residues 234-239 (hinge region), amino acid residues 265-269 (B/C loop), amino acid residues 297-299 (C/E loop), and amino acid residues 327-332 (F/G loop). In some embodiments, the molecules of the invention comprise variant Fc Domains comprise modification of at least one residue that does not make a direct contact with an FcγR based on structural and crystallographic analysis, e.g., is not within the Fc-FcγR binding site.

[00281] Variant Fc Domains are well-known in the art, and any known Fc variant may be used in the present invention to confer or modify the effector function exhibited by a molecule of the invention comprising an Fc Domain (or portion thereof) as functionally assayed, e.g., in an NK dependent or macrophage dependent
assay. For example, Fc Domain variants identified as altering effector function are disclosed in the Antibody Engineering Technology Art, and any suitable variant disclosed therein may be used in the present molecules.

[00282] In certain embodiments, the Tri-Specific Binding Molecules of the present invention comprise a variant Fc Domain, having one or more amino acid modifications in one or more sites, which modification(s) alter (relative to a wild-type Fc Domain) the Ratio of Affinities of the variant Fc Domain to an activating FcyR (such as FcyRIIA or FcyRIIIA) relative to an inhibiting FcyR (such as FcyRIIB):

\[
\text{Ratio of Affinities} = \frac{\text{Wild-Type to Variant Change in Affinity to } \gamma R_{\text{Activating}}}{\text{Wild-Type to Variant Change in Affinity to } \gamma R_{\text{Inhibiting}}}
\]

[00283] Particularly preferred are Tri-Specific Binding Molecules of the present invention that possess a variant Fc Domain (relative to the wild-type Fc Domain) in which the Fc variant has a Ratio of Affinities greater than 1. Such molecules have particular use in providing a therapeutic or prophylactic treatment of a disease, disorder, or infection, or the amelioration of a symptom thereof, where an enhanced efficacy of effector cell function (e.g., ADCC) mediated by FcyR is desired, e.g., cancer or infectious disease. In contrast, an Fc variant having a Ratio of Affinities less than 1 mediates decreased efficacy of effector cell function. Table 1 lists exemplary single, double, triple, quadruple and quintuple mutations by whether their Ratio of Affinities is greater than or less than 1.

<table>
<thead>
<tr>
<th>Exemplary Single and Multiple Mutations Listed by Ratio of Affinities</th>
<th>Single</th>
<th>Double</th>
<th>Triple</th>
<th>Quadruple</th>
<th>Quintuple</th>
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<tr>
<td>Ratio of Affinities &gt; 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F243L</td>
<td>F243L &amp;</td>
<td>F243L,</td>
<td>L234F,</td>
<td>L235V,</td>
<td>L235V,</td>
</tr>
<tr>
<td>D270E R292G</td>
<td>R292P</td>
<td>P247L &amp;</td>
<td>F243L,</td>
<td>F243L,</td>
<td>F243L,</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Y300L</td>
<td>Y300L</td>
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Table 1
Exemplary Single and Multiple Mutations Listed by Ratio of Affinities

<table>
<thead>
<tr>
<th>Single</th>
<th>Double</th>
<th>Triple</th>
<th>Quadruple</th>
<th>Quintuple</th>
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<tr>
<td>R292P &amp; V305I</td>
<td>P247L, D270E &amp; N421K</td>
<td>F243L, D270E, G316D &amp; R416G</td>
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<td></td>
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<tr>
<td>R292P &amp; P396L</td>
<td>R255L, D270E &amp; P396L</td>
<td>F243L, D270E, K392T &amp; P396L</td>
<td></td>
<td></td>
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<tr>
<td>Y300L &amp; P396L</td>
<td>D270E, G316D &amp; R416G</td>
<td>F243L, D270E, P396L &amp; Q419H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P396L &amp; Q419H</td>
<td>D270E, K392T &amp; P396L</td>
<td>F243L, R292P, V300L &amp; P396L</td>
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<td></td>
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<tr>
<td>R292P, Y300L &amp; P396L</td>
<td>D270E, P396L &amp; Q419H</td>
<td>F243L, R292P, V305I &amp; P396L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V284M, R292L &amp; K370N</td>
<td>F243L, D270E, Y300L &amp; N421K</td>
<td>P247L, D270E, R292G &amp; P396L</td>
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<td></td>
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<tr>
<td></td>
<td>D270E, G316D, P396L &amp; R416G</td>
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Ratio of Affinities < 1

<table>
<thead>
<tr>
<th>Y300L</th>
<th>F243L &amp; P396L</th>
<th>F243L, R292P &amp; V305I</th>
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<tr>
<td>P396L</td>
<td>P247L &amp; N421K</td>
<td>F243L, R292P &amp; V305I</td>
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<td>R255L &amp; P396L</td>
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<tr>
<td></td>
<td>R292P &amp; V305I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K392T &amp; P396L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P396L &amp; Q419H</td>
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</table>

[00284] In a specific embodiment, in variant Fc Domains, any amino acid modifications (e.g., substitutions) at any of positions 235, 240, 241, 243, 244, 247, 262, 263, 269, 298, 328, or 330 and preferably one or more of the following residues: A240, 1240, L241, L243, H244, N298, 1328 or V330. In a different specific embodiment, in variant Fc Domains, any amino acid modifications (e.g., substitutions) at any of positions 268, 269, 270, 272, 276, 278, 283, 285, 286, 289, 292, 293, 301, 303, 305, 307, 309, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376,
416, 419, 430, 434, 435, 437, 438 or 439 and preferably one or more of the following residues: H280, Q280, Y280, G290, S290, T290, Y290, N294, K295, P296, D298, N298, P298, V298, 1300 or L300.

[00285] In a preferred embodiment, in variant Fc Domains that bind an FcyR with an altered affinity, any amino acid modifications (e.g., substitutions) at any of positions 255, 256, 258, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 300, 301, 303, 305, 307, 309, 312, 320, 322, 326, 329, 330, 332, 331, 333, 334, 335, 337, 338, 339, 340, 359, 360, 373, 376, 416, 419, 430, 434, 435, 437, 438 or 439. Preferably, the variant Fc Domain has any of the following residues: A256, N268, Q272, D286, Q286, S286, A290, S290, A298, M301, A312, E320, M320, Q320, R320, E322, A326, D326, E326, N326, S326, K330, T339, A333, A334, E334, H334, L334, M334, Q334, V334, K335, Q335, A359, A360 or A430.

[00286] In a different embodiment, in variant Fc Domains that bind an FcyR (via its Fc Domain) with a reduced affinity, any amino acid modifications (e.g., substitutions) at any of positions 252, 254, 265, 268, 269, 270, 278, 289, 292, 293, 294, 295, 296, 299, 300, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439.

[00287] In a different embodiment, in variant Fc Domains that bind an FcyR (via its Fc Domain) with an enhanced affinity, any amino acid modifications (e.g., substitutions) at any of positions 280, 283, 285, 286, 290, 294, 295, 298, 300, 301, 305, 307, 309, 312, 315, 331, 333, 334, 337, 340, 360, 378, 398 or 430. In a different embodiment, in variant Fc Domains that binds FcyRIIA with an enhanced affinity, any of the following residues: A255, A256, A258, A267, A268, N268, A272, Q272, A276, A280, A283, A285, A286, D286, Q286, S286, A290, S290, M301, E320, M320, Q320, R320, E322, A326, D326, E326, S326, K330, A331, Q335, A337 or A430.

[00288] Preferred variants include one or more modifications at any of positions: 228, 230, 231, 232, 233, 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264,
Particularly preferred variants include one or more modifications selected from groups A-AI:

<table>
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<td>B</td>
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<td>C</td>
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<td>E</td>
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<td>G</td>
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Still more particularly preferred variants include one or more modifications selected from Groups 1-105:

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</table>
In one embodiment, a multivalent DR5 binding molecule of the invention will comprise a variant Fc Domain having at least one modification in the Fc Domain. In certain embodiments, the variant Fc Domain comprises at least one substitution selected from the group consisting of L235V, F243L, R292P, Y300L, V305I, and P396L, wherein said numbering is that of the EU index as in Kabat.

In a specific embodiment, the variant Fc Domain comprises:

(A) at least one substitution selected from the group consisting of F243L, R292P, Y300L, V305I, and P396L;
(B) at least two substitutions selected from the group consisting of:
   (1) F243L and P396L;
   (2) F243L and R292P; and
   (3) R292P and V305I;
(C) at least three substitutions selected from the group consisting of:
   (1) F243L, R292P and Y300L;
   (2) F243L, R292P and V305I;
   (3) F243L, R292P and P396L; and
   (4) R292P, V305I and P396L;
(D) at least four substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L and P396L; and
   (2) F243L, R292P, V305I and P396L; or
(E) at least the five substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L, V305I and P396L; and
   (2) L235V, F243L, R292P, Y300L and P396L.
In another specific embodiment, the variant Fc Domain comprises substitutions of:

(A) F243L, R292P, and Y300L;

(B) L235V, F243L, R292P, Y300L, and P396L; or

(C) F243L, R292P, Y300L, V305I, and P396L.


[00295] In some embodiments, the molecules of the invention further comprise one or more glycosylation sites, so that one or more carbohydrate moieties are covalently attached to the molecule. Preferably, the molecules of the invention with one or more glycosylation sites and/or one or more modifications in the Fc Domain confer or have an enhanced antibody-mediated effector function, e.g., enhanced ADCC activity, compared to a parent antibody. In some embodiments, the invention further comprises molecules comprising one or more modifications of amino acids that are directly or indirectly known to interact with a carbohydrate moiety of the antibody, including but not limited to amino acids at positions 241, 243, 244, 245, 245, 249, 256, 258, 260, 262, 264, 265, 296, 299, and 301. Amino acids that directly or indirectly interact with a carbohydrate moiety of an antibody are known in the art, see, e.g., Jefferis et al, 1995 Immunology Letters, 44: 111-7, which is incorporated herein by reference in its entirety.

[00296] In another embodiment, the invention encompasses molecules that have been modified by introducing one or more glycosylation sites into one or more sites of the molecules, preferably without altering the functionality of the molecules, e.g., binding activity to target antigen or FcyR. Glycosylation sites may be introduced into the variable and/or constant region of the molecules of the invention. As used herein, "glycosylation sites" include any specific amino acid sequence in an antibody to which an oligosaccharide (i.e., carbohydrates containing two or more simple sugars linked together) will specifically and covalently attach. Oligosaccharide side chains
are typically linked to the backbone of an antibody via either N- or O-linkages. N-linked glycosylation refers to the attachment of an oligosaccharide moiety to the side chain of an asparagine residue. O-linked glycosylation refers to the attachment of an oligosaccharide moiety to a hydroxyamino acid, e.g., serine, threonine. The molecules of the invention may comprise one or more glycosylation sites, including N-linked and O-linked glycosylation sites. Any glycosylation site for N-linked or O-linked glycosylation known in the art may be used in accordance with the instant invention. An exemplary N-linked glycosylation site that is useful in accordance with the methods of the present invention is the amino acid sequence: Asn-X-Thr/Ser, wherein X may be any amino acid and Thr/Ser indicates a threonine or a serine. Such a site or sites may be introduced into a molecule of the invention using methods well-known in the art to which this invention pertains (see for example, IN VITRO MUTAGENESIS, RECOMBINANT DNA: A SHORT COURSE, J. D. Watson, et al. W.H. Freeman and Company, New York, 1983, chapter 8, pp. 106-116, which is incorporated herein by reference in its entirety. An exemplary method for introducing a glycosylation site into a molecule of the invention may comprise: modifying or mutating an amino acid sequence of the molecule so that the desired Asn-X-Thr/Ser sequence is obtained.

[00297] In some embodiments, the invention encompasses methods of modifying the carbohydrate content of a molecule of the invention by adding or deleting a glycosylation site. Methods for modifying the carbohydrate content of antibodies (and molecules comprising antibody domains) are well-known in the art and encompassed within the invention, see, e.g., U.S. Patent No. 6,218,149; EP 0 359 096 Bl; U.S. Publication No. US 2002/0028486; WO 03/035835; U.S. Publication No. 2003/01 15614; U.S. Patent No. 6,218,149; U.S. Patent No. 6,472,511; all of which are incorporated herein by reference in their entirety. In other embodiments, the invention encompasses methods of modifying the carbohydrate content of a molecule of the invention by deleting one or more endogenous carbohydrate moieties of the molecule. In a specific embodiment, the invention encompasses shifting the glycosylation site of the Fc Domain of an antibody, by modifying positions adjacent to 297. In a specific embodiment, the invention encompasses modifying position 296 so that position 296 and not position 297 is glycosylated.
Effector function can also be modified by techniques such as by introducing one or more cysteine residues into the Fc Domain, thereby allowing interchain disulfide bond formation in this region to occur, resulting in the generation of a homodimERIC antibody that may have improved internalization capability and/or increased complement-mediated cell killing and ADCC (Caron, P.C. et al. (1992) "Engineered Humanized Dimeric Forms Of IgG Are More Effective Antibodies," J. Exp. Med. 176:1 191-1 195; Shopes, B. (1992) "A Genetically Engineered Human IgG Mutant With Enhanced Cytolytic Activity," J. Immunol. 148(9):2918-2922. Homodimeric antibodies with enhanced antitumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff, E.A. et al. (1993) "Monoclonal Antibody Homodimers: Enhanced Antitumor Activity In Nude Mice," Cancer Research 53:2560-2565. Alternatively, an antibody can be engineered which has dual Fc Domains and may thereby have enhanced complement lysis and ADCC capabilities (Stevenson, G.T. et al. (1989) "A Chimeric Antibody With Dual Fc Domains (bisFabFc) Prepared By Manipulations At The IgG Hinge," Anti-Cancer Drug Design 3:219-230).

III. Exemplary Trispecific Binding Molecules

F. gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1

[00299] An exemplary Tri-Specific Binding Molecule composed of four polypeptide chains was constructed. The Tri-Specific Binding Molecule comprises the VL and VH domains of gpA33 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of DR5 mAb 1, and was accordingly designated "gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:212):

```
D1 QLTQS P S F LSASVGDRVT I TC SARS S I S FMYWYQQKPG KAPKLL I YDT
SNLAS GVP SR FSGSGSGTEF TLT I S S LEAE DAATYYCQQW S SYPLT FGQQ
TKLE I KGGGS GGGLVQPGG SGGLVQPGG SLRL SCAAS G FT F S T Y AMN W
VRQAPKGLE \[ VGR I RS KYN NYATYYADSV KGRF T I SRDD SKNS LYLQM N
SLKTE DTA VY YCVR HGNFGN S YV S W F AY W G QGTLVTVS SG GCGGGEVAAL
EKEVAALEKE VALEKEVFAA LEKGGGDKTH T C P P C A P EA AGGP SVFLEP
PKPKDTLM S R T PE VTCV VV DVS HE D PE VK FNWYVDGEV HNA TK KPREE
QYNS TYR VVS VLTV LHQD WL NGKEYKCKVS NKAL PAP I E K T1 SKAKQQP R
EPQV YTL P P S REEMTKNQVS LWCLVKGFY P S DIAVE WE SN GQPENNYK T
```
[00300] In SEQ ID NO:212, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GGC GG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00301] A polynucleotide that encodes SEQ ID NO:212 is SEQ ID NO:213:

gacctcagc tcgacctcagc ccctctcttccc ctgttccgcat ccgttggaga
tcgagtgcact attaactcgtg ctgctaggtcc ctcatacatgc ttcatgtgct

gtattacagc gcagccgctcg aagccgccttg ctacgctgcctg ctcgcccctag
agcaactgg ccctcgggggt gccatctcgg ttcctctgca gtgggtcaggg
acagtacctgta aatacataggg ttggagatcgg gccgggagag gcggggagag
 tcctctgctgc agggtttggtgc caccacactcg ctgggcggag gcggttgtgg
tcctctgtgc agccctctgga ttcacctctca gcacatatgc tataatgagc
gtccggcagg ctcaggggaa ggggtgggag tgggttgaga gatcaggtgc
caagggcgtg ccgtcgcttg ccgtcagctg ctgctggaggg gctgggctgg
 gatggttgga tcgctggaggg ctggggcgggc ggtgggctgg gcggggcagg
gggctggaggg ggtgggggag gcggggcgggc ggtgggctgg gctgggctgg
ggtaatgagc gcggggagag gcggggcgggc ggtgggctgg gctgggctgg
ggtcggcagcc ctggaagagac gcgggagag gcggggcgggc ggtgggctgg
ggtaatgagc gcggggagag gcggggcgggc ggtgggctgg gctgggctgg
gtccggcagg ctcaggggaa ggggtgggag tgggttgaga gatcaggtgc
caagggcgtg ccgtcgcttg ccgtcagctg ctgctggaggg gctgggctgg
ggtaatgagc gcggggagag gcggggcgggc ggtgggctgg gctgggctgg
gtccggcagg ctcaggggaa ggggtgggag tgggttgaga gatcaggtgc
caagggcgtg ccgtcgcttg ccgtcagctg ctgctggaggg gctgggctgg
ggtaatgagc gcggggagag gcggggcgggc ggtgggctgg gctgggctgg
ggtaatgagc gcggggagag gcggggcgggc ggtgggctgg gctgggctgg
The amino acid sequence of the second polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:214):

QAVVTQEPSL TVSPGGTVTL TCRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPPT PARFSGSLLG GKAALTITGA QAEDADYYAC ALYYSNLWVF
GGGKLTVLGG GGGSGGGQQVQLVQGSKAEK KPGASVKKVSC KASYFTTGS
WMNVMRQAAPQ QGLEGWIGRY PGQGETNYNG KFKDRTVITA DKSTSTAYME
LSSLRSEDATA VYYCARIYGN NVYFVWGQGGG TTVTSSGGGC GGGKVAALKE
KVAALKEKVAALK

In SEQ ID NO:214, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO: 104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO: 33), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 238-243 correspond to the linker GCCGGG (SEQ ID NO:34), and residues 244-271 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:214 is (SEQ ID NO:215):

caggctgttggtgcctcagga gcttcctactg accgttgtcc cgaggccggaac
tgtgaccccttg ccacacagag cgcactcactg accgtgtcag ccacacacag
ggggtacaa ctatgtccgtg ccacacacag cgcactcactg accgtgtcag
tctgctggcc ggaaggccg ctctgcagctg tttctggaag
tctgctgggcc ggaaggccg ctctgcagctg tttctggaag
tggtggtgcca aaaaactgac tgtctgggga ggggttggtg ccaggccgaggg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg
tggaagtggtc gaggcctgtgg gaggcctgtgg gaggcctgtgg

The amino acid sequence of the third polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:216):

EVVFLESGGG LVQPFGSLLK SCVASGFDFS RYWMSWVQA PGKLEGWIGE
INPDSNTINY TPSLKDKFI1 SRDNAKNTLY LQMTKVRSED TALYYCTRR
YYGNPAWFAY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV
KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
TYICNVNHKP SNTVDKRVE PKSCDKHTC PPCPAPEAAG GFSVFLFFPK
PKD1TLMI SRT PEVTCCVVVD SHEDPEVKFN WYDVGEVHNN AKTPKREEQY
NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQREP
QVYTLPPSRE EMTKQNQVLS CAVKGFYPSD IAWEWESNGQ PENNYKTTYP
VLDSDGSFL VSKLTVDSLKR WQQGNVFSCS VMHEALHNRY TQKSLSLSPG
K

[00306] In SEQ ID NO:216, amino acid residues 1-121 correspond to the amino acid sequence of the VH Domain of DR5 mAb 1 (SEQ ID NO:8), residues 122-219 correspond to a modified CHI Domain (SEQ ID NO:208), residues 220-234 correspond to a linker (SEQ ID NO:209), and residues 235-451 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

[00307] A polynucleotide that encodes SEQ ID NO:216 is (SEQ ID NO:217):

gaggtgaaagt ttctcagagtct tggaaggtgagc ctgggtcagct ctggaggatc
cctgaaactc tcctgtgtag cctcaggatt cgattttagt agatacttgg
ctgagttggt tcggcaggtct ccaagggaag ggctagatgt gattgggaga
attaatccag atagcaatac gataaactat acggcatctc taaaggataa
attcatcatc tccagagaca acgccaaaaa taccgtgtat tgcbaaatga
cacaagtagg atctgagggac acagccctttt attattgtac aagaagggcc
tactatggta accgggccctgt gttgtacctc tggggcaag agactctgggt
caccgtccttc tccgctctca caaagggccc atcggtctgc cccctggcac
cctcctccaa gagacacctct ggggcaagag cggcctgggt ctgctgtgac
aagcactacct ccgccagaaac ggtgacggttc tctggaactc cagggccctt
gaccgacggc gtcgcacacct tccgggtctg ccaggactctc tcagagcact
actccctcag cagcgtggttg accgtgcccc taccgagttc gggacccag
acccatatct gcacggctgaa tccagccagtc agcacaccca agggtggaca
agagcttgag ccaaatcctt tgacaaaaac tcaacagtcg ccaccggctcc
caccactctg accggccggag ggaccgctag tctctctctt cccccccaaa
cccaagaccg ccctctagct ctccggctgc ccaggaggtc tctgcatctg
gtagagagtg tggcagcagag accctgaggt caagttcaac tggtaagttgg
accgcggtaa gttgcatag taacagcagcc agggccggcgg gaggcaagtac
accacgcaat cagcgcgctgc gccgctggcag acgccctggc ccaccggctcc
gctagatgcc aaggaactac agttcaagtc tccacagcgg ccctcctcgag
ccccctcaga cagaccccaac tccctgctgtcc aaggccggcc cgagacccca
ccatcgata cacccctcatg cccgtctggc atctgaggtt ggtggcagcag
accttgaagagt cagcggcttg ccagccctgg ccacccctgg ccaccggctcc
cgccagtta cagcggcttc ccacagcggt cggcctggg ccaccggctcc
caggttgtac cccgctgctcc atccggggag gagatggacca agaaccaggt
cacgctgact tcggcagttc taacgcattct cccacgcccc ccacggccag
gattgggagag caatggcagcc cccgagacca cttcaagaggg cccgcaaccc
caggtgtctg ccctgctgctc atccggggag gagatggacca agaaccaggt
cagctgact gcagcgcgctct cttctgctct gccgctggg ccaccggctcc
cagcgcgaag gtggcgatggt gggctgctgc cttctgctct gccgctggg ccaccggctcc

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The amino acid sequence of the fourth polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:218):

DIVLTQSPAS LAVSLGQRAT ISCRASKVS SSGSYMHWW YQKPGQPDKV
LIFLSINLDS GVPARFSGSG STGDFTLNIH PVEDGDAATY YCQHSDRLPP
TFGGGTKLEI KRTVAAPSVF IFPFSDEQLK SGTASVCLL NNFPYREAV
QWKVVDNALQS GNSQESVTEQ DSKDSTYSL SLTLSKADY EKHKVYACEV
THQGLSSPVT KSFRNREGC

In SEQ ID NO:218, amino acid residues 1-111 correspond to the amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3), and residues 112-218 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes SEQ ID NO:218 is (SEQ ID NO:219):

gaccttggtgc tgacacagtc tcctgcttcc ttagctgtat ctctcgggca
gagggccacc atctcatgcg gggccagcaa aagtgtcagt tcctctggct
atatgttagc gcacacctat tactgtcagc acagtgagaa tcttcctggc
agtggagatgc tgcaacctat tactgtcagc acagtgagaa tcttcctggc
cctctgttggt ggcctctgtg tggctgctg aataacttct atcccagaga
cagtggaagg tggataacgc cctccaatcg ggtaactccc aggagagtgt
cacagagcag gacagcaagg acagcaccta cagcctcagc acagccttcg
cgctgagcaag cgagactac gagaacaaca aagtctcagc cgctgagcac
acctgactgg gcctgagtc gcctcagcag aagagcttca acaggggaga
gtgyG.

gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 2

A second exemplary Tri-Specific Binding Molecule composed of four polypeptide chains was constructed. The Tri-Specific Binding Molecule comprises the VL and VH domains of gpA33 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of DR5 mAb 2, and was accordingly designated "gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 2." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:220):

DIQLTQSPSF LSASVGVDRVT ITCSARSSIS FMYWYQQKPG KAPKLLI YDT
SNLASGVPSP FSGGSGSTEF TLTISSLAEE DAATYYCQWW SSYPLTFGQG
TKLEIKGGS GGGGEVQLVE SGGGLVQPPG SLRLSCAASG TFTPSTYAMNW
VRQAPGKgle WVGRIRSKYN NYATTYADSV KGRFTISRDD SNKSLYLMN
SLKTEDTAVY YCVRHGNFNG SYV5W FYW GQTLTVVSSG GGCGGEVAAAL
EKEVALEEKE VAALEKEVAA LEKGGGDKTH TCPCPAPEA AGGPSVFLFP
PKPKDTLMIS RTPEVTCVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKLAPAPIEK TISKAKQOPR EPOVYTLPPS REEMTNQVVS LWCLVKGYFP SDIAVEWEN SGPENNYKTT PPVLDSDGSF FLYSKLTVDK SRWQQQNVFS CSVMHEALHN HYTQKSLSSL

[00312] In SEQ ID NO:220, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GGCDDD linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00313] A polynucleotide that encodes SEQ ID NO:220 is (SEQ ID NO:221):

gacattcagc tgactcagtc cccctctttt ctgatcagtc ccgtcggaga
tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggc

ggtacctgca gaagcgggag ccggcagttct cctagtctgga cggacaggg

tagcacagtgt cctccgaggt ggcctcggga tcgcttcggc ctccttgga

ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga

tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggg
tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggg
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga

tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggg
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga

tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggg
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga

tcgagtgact attacttgct ctgctaggtc ctcacctgcga ccgacaggg
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
ggcgtcggta cccctctttt ctgatcagtc ccgtcggaga
The amino acid sequence of the second polypeptide chain of gpA33 mAb is (SEQ ID NO:222):

QAVVTQEPSL TVSPGGTVTL TCRSSTGAVT TS NYANW VQQ KPGQAPRGLI
GGGTKLTVLG GGGSGGGGQV QLVQSGAEVK KPGASVKVSC KASGYTFTGS
WMNWVRQAPG QGLEWIGRY PGDGETYNG KFKDRVTIT A DKSTSTAYME
LSSLRSEDTA VYYCARI YGN NVYFDVWQG TTTVTSSGGC GGGKVAALKE
KVAALKEKVA ALKEKVAALK E

In SEQ ID NO:222, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 238-243 correspond to the linker GGC GGG (SEQ ID NO:34), and residues 244-271 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:222 is (SEQ ID NO:223):

caggctgttg tgactcagga gccttcactg accgtgtccc caggccggaac
tgtgaccctg acatcgacag ccacagcag ccacagtgac accatcact
acgcacattt ggtgcagcag aagccaggac aggcccatag gggccctgac
ccgggtacaa cacaagaaggg tccctggaac cctgcaagct tttctggaag
tctgccttggc gggaagggcg ctctgtgact atggacattc ggtagggttc
ggggtgtga caaaaacctac tgtgcttgga ggggctgtgat cccggcgag
ccgcatctac cttggaagac gcagaaacta ctaataatgt aagttcagag
acccggtgac ctcctgcctg ctaaatcatt ctagacggcg ctaggctgg
cttacaggct ggtgaccgcc gagcagtcag ccaatagccg cccggcgtgag
ctgagctccc tggggcgcc ggggagtgct cttgatcatt gccgagcttg
cttgggctcc cttgcagagcc ggtgagctgg ctgggggctgc atcagttcag
ccttgacggtg ggtgagctgg gttgagctgg ctgctgagtt ccggtggtgt
caatctggtc ggtgagctgg cctgagctgg atggagctgg ctgattgcga
tggagctgg gcggcgcggt ggtgggtggt gggcggtggcc cgcggcggag
tggctgctgct tggggcgcc gcggcgcggt ggtgggtggt gggcggtggcc
tggctgctgct tggggcgcc gcggcgcggt ggtgggtggt gggcggtggcc
tggctgctgct tggggcgcc gcggcgcggt ggtgggtggt gggcggtggcc
tggctgctgct tggggcgcc gcggcgcggt ggtgggtggt gggcggtggcc
tggctgctgct tggggcgcc gcggcgcggt ggtgggtggt gggcggtggcc

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The amino acid sequence of the third polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 2 is (SEQ ID NO:224):

```
KVQLQQSGAE LVKPGASVKL SCKASQYPTF EYILHWVKQK SGQGLEMWG
FYQGNNKKY NEKFKDKatL TADKSSSTVY MELSRLTSED SAVYFCARHE
QGPFYDFWGQ STKGPSVFPL APSSKSTSSG TAAALCGLVKD
YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLLSVDTV TSSSLGTQTY
ICVNYNKHPSN TKVDKRVFEPK SCDRTHTCPF CEPAPAAAGGP SVFLFPFPKPK
DVTMI SRTPE VTCVVDVSH EDPEVFKFNWY VDGVEVNHAK TKPREEQYNS
TYRVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIKTISK AKGQPREOPV
YTLPPREEM TKNQVSLSCA VKGFPYDDIA VEWEENQOPE NNYKTTPVPL
DSDGSFLVLS KLTVDKSRWQ QGNVFSCSVM HEALHNRYTN KSLSSLPGK
```

In SEQ ID NO:224, amino acid residues 1-119 correspond to the amino acid sequence of the VH Domain of DR5 mAb 2 (SEQ ID NO:18), residues 120-217 correspond to a modified CHI Domain (SEQ ID NO:208), residues 218-232 correspond to a linker (SEQ ID NO:209), and residues 233-449 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

A polynucleotide that encodes SEQ ID NO:224 is (SEQ ID NO:225):

```
aaggtccagtc tgccagactg tcggagctgaa ctggtgtaac ccggggcac tcatgatgtcc tctctgggtg cagatatatttt
tacactgggt aacgcagaaag tctggacaggt catttggagcg ctggtgatgc cgggtgggtgc cgtctagcgac
ttttatcttg gaatataataa tataaagtac aatggaatatt ccaagcgac ggtgatgtgc cggctgagttc
gaagcagtcg ttctcgagcg ctgctggacag cctctgcccg cggggttcag ccagttttgag cattttggagc
tactctccgg ccaccagcgg ccaccagcgg ccaccccgcc ccaggccagc ccagtttctt gttgttgctg cggcctgagc
tccctccg ccaccagcgg gcacccctgg cgcctttccg cgcctggac cgcctggagcc cggggttcag cctctcgagc
tccctccgg ccaccagcgg ccaccagcgg ccaccccgcc ccaggccagc ccagtttctt gttgttgctg cggcctgagc
tcagtttgag cgtgatgtcc tctctgggtg cagatatatttt
tacactgggt aacgcagaaag tctggacaggt catttggagcg ctggtgatgc cgggtgggtgc cgtctagcgac
ttttatcttg gaatataataa tataaagtac aatggaatatt ccaagcgac ggtgatgtgc cggctgagttc
gaagcagtcg ttctcgagcg ctgctggacag cctctgcccg cggggttcag ccagttttgag cattttggagc
tactctccgg ccaccagcgg ccaccagcgg ccaccccgcc ccaggccagc ccagtttctt gttgttgctg cggcctgagc
tccctccgg ccaccagcgg ccaccagcgg ccaccccgcc ccaggccagc ccagtttctt gttgttgctg cggcctgagc
tcagtttgag cgtgatgtcc tctctgggtg cagatatatttt
tacactgggt aacgcagaaag tctggacaggt catttggagcg ctggtgatgc cgggtgggtgc cgtctagcgac
ttttatcttg gaatataataa tataaagtac aatggaatatt ccaagcgac ggtgatgtgc cggctgagttc
gaagcagtcg ttctcgagcg ctgctggacag cctctgcccg cggggttcag ccagttttgag cattttggagc
```
The amino acid sequence of the fourth polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 2 is (SEQ ID NO:226):

```
DIVMTQSHKF MSTSVMGRDS VITCKASQDVNL TAVAWYQQKPGQ SQPKLILY
ASTRHTGVPD RFTGSSSGTDL YTLTVSKVQA EDELTLYYCQQHY ITPTWFTGGG
GTKLEIKRTTV AAPSVFIFPP SDEQLKSGTAA SVCCLNNFY PREAKYQWKV
DNALQSSNSQ ESVTEQDSDKL STYSLSTSLTL SKADYEKHK VYACEVTHQG
LSSPVTKSFN RGECC
```

In SEQ ID NO:226, amino acid residues 1-107 correspond to the amino acid sequence of the VL Domain of DR5 mAb 2 (SEQ ID NO:13), and residues 108-214 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes SEQ ID NO:226 is (SEQ ID NO:227):

```
gacattgtga tgacccagtc tcacaaattc atgtccactt cagtaggaga
cagggtcagc atcacctgca aggccagtca ggatgtgaat actgctgtag
cctggtatca acaaaaacca gggcaatctc cttaaactact gatttactgg
gcatccaccc ggcacactgg agtccctgat cgcttcacag gcagtggatc
tgggacagat tatacactca ccatcaaaag tgtgcaggct gaagacctga
cactttatta ctgtcagcaa cactatatca ctccgtggac gttcggtgga
ggaccaagcc tggaaatcag acgtacggtc tgccagcacat ccgctctcat
cctccggcca tctgtagagc agttgaatct tggacttcaag ctcttggtgt
```

H. EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1

A further exemplary Tri-Specific Binding Molecule composed of four polypeptide chains was constructed. The Tri-Specific Binding Molecule comprises the VL and VH domains of EphA2 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of DR5 mAb 1, and was accordingly designated "EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:228):

```
DIQMTQTSS LSASLGDRIT ISCRASQDIS NYLNWYQQKP DGTKTLIYY
TSRLHSGVPS RFSGSGSTGT YSLITSNLQEDIATYFCQQGY TLIYTFGGG
TKLEIKGGGS GGGGEVQLVLE SGGGLVQPGG SRLSCLAASG FTFSYAMNW
VRQAPKGKLE WVGIRSKYN NYATYYADSV KGRFTISRD DKSLSLQMN
SLKTEDTAVY YCVRHGNFGN SYVSWFAYWG QGTLTVSSG GCGGGEVAAL
```
[00324] In SEQ ID NO:228, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 1 (SEQ ID NO:153), residues 107-114 correspond to the intervening spacer peptide GGGGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GGGGG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00325] A polynucleotide that encodes SEQ ID NO:228 is (SEQ ID NO:229):

gatatccaga tgacacagac tacatcttcc ctgtctgctt ctctgggaga
cagaatcacc atcagtgtca gggcaagtca gacacattgc aattatattg
cactgttatt gccagaaaa gatggaactg ttaaacctct gcattacactc
cattcactcc gacgccatc agtctccatc gcgtactcgc gagatctgctg
tggtgactgt ctcattcggga gggtgctgagtg ggtggtggag ggtggtggag
cagtacaca cattatgcaa ccctactatc gcacggtcag gcacggtcag
taatatgtat ttaaatctgc gcgaggtgca gcgtgtggac ggtatatattg
ttcgctgctg cggcgggaga ggggtgctgag tgggtggtga gcggatgtgc
caatgactaag ataatgctta cctactatgc gcacggtcag gcacggtcag
tccatctct ccgacgtgat tcaaaaagtg actagttatc gcagagttga
tcgactgaa acacgtgatc gtttattttg gcagagttga gcagagttga
ttcgacgact cggatgcaggt ggtgtgagtc gatggtggag gggcggcctg
gagaaagag gattattatt tgggaaggtggtc gattattatt tgggaaggtggtc
gttttttttt ggtttttttt aacatggagt aacatggagt aacatggagt
cgagaggtga cagcctctct ggtggtggtg ggtggtggtg ggtggtggtg

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The amino acid sequence of the second polypeptide chain of EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:230):

QAVVTQEPSL TVSPGTVIL TCRSSTGAVT TSNYANWQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALTITGA QAEDEADYYC ALWYSNLWVF
GGTKLTVLGG GGGSGGGGQV QLKEBGPGLV APSQSLSTIC TVSGFSLRYP
SVHWVRQPPG KLEWLGMNM GGGSTDYNSA LKLSRLSISKD NSKSQVFLKM
NSLQDTDDTM YYCARHKHNY YTMDYWGQGT SVTVSSGGCG GGKVVALKEK
VAALKEKVAALKEK

In SEQ ID NO:230, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 1 (SEQ ID NO:158), residues 237-242 correspond to the linker GGGCGGG (SEQ ID NO:34), and residues 243-270 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:230 is (SEQ ID NO:231):

caggctgtgg tgacctcagga gcctctcactg accgttgtccc caggccgaac
ttgtaccctg catactgcagat ccagcagagac cgcagtggacc accatctaact
acgcacatgg ggactcagnagc aagccagagac aggccaccaag gggctgtgatc
ggggtgctaca accaaggtgc ccctctggtggt ctctctctctg cctctggtgatg
cttcgctgggc ggaaagggcc cttcggctgg ctctctctctg cctctcggctgg
acgacaagcag ttcagactggt gctctctcagt aggaggtgatc tcctcctgctg
cagacctcgtc acctcagactg accctggtggt gctctctgctg cctctcggctgg
tagttactctc atctctcagc gctctctcagt cctctcggctgg cctctcggctgg
aatagtaggtg ggtctctgcta gcacacagca cctctcggctgg cctctcggctgg
actactactct cctcctcggc cctcctcggc cctcctcggc cctcctcggc
tgcacctgag ctctctcggc cctcctcggc cctcctcggc cctcctcggc
agtttactctc atctctcagc cctcctcggc cctcctcggc cctcctcggc
The amino acid sequence of the third polypeptide chain of EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:232):

EVKFLESGGG LVQPGGSLKL SCVAGFDFFS RYWMSWRVRQA PGKGLEWIGE

[00330] In SEQ ID NO:232, amino acid residues 1-121 correspond to the amino
acid sequence of the VH Domain of DR5 mAb 1 (SEQ ID NO:8), residues 122-219
 correspond to a modified CH1 Domain (SEQ ID NO:208), residues 220-234
 correspond to a linker (SEQ ID NO:209), and residues 235-451 correspond to the
 "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

A polynucleotide that encodes SEQ ID NO:232 is (SEQ ID NO:233):

gaggtgaagt ttctcgagttc ttgaggtgcc ccgtgcagtc ccggaggatc
cctgaacctc tccctgttagc cctcaggatt cgatttttagt agatactgga
tgatggtggt ccgccagctc ccagggagag ggtcagatag gattggygaa
attaatccag atacgaacta gataacttac accccacttc taaaggtaa
atttcatcatt cttcagagaca acgcacaaaa taagctgtat ctgcacattg
ccaaagttgag atctgaggac acagccccct attattgtac aagaagggcc
tactatgctg acgccggcttg gttgctttac tggggccaag gaggctgtgt
cactgctttt cccgcctcatc ccagggagag atcgccagtct cccctggcac
ccctctccaa gaccacccct gggcgccacag cggccctggg ctgcctggtc
gaagtactct cttccagaaac ccgtgcagtt gcgtgagcgtg cgtggaactc
gaccagcgcc gtcgacaccct tccggcctgt ctcagccttc ctggacactt
actcctctag accgcggattg acctggtctct cccgacgttt ggccacccag
acctatctct gcaacttgga tccacagcgg agaacaaccc aagctgagcc
agagttgag cccaaatctt tgtcaataac ttgacaagcc tcacacgattc ccaccggtcc
ccagcacttg acccgccgggg ggacggtctag ctctttcttt ctcctacaac
ccaaagttgag atctgaggac acagccccct attattgtac aagaagggcc
tactatgctg acgccggcttg gttgctttac tggggccaag gaggctgtgt
cactgctttt cccgcctcatc ccagggagag atcgccagtct cccctggcac
ccctctccaa gaccacccct gggcgccacag cggccctggg ctgcctggtc
gaagtactct cttccagaaac ccgtgcagtt gcgtgagcgtg cgtggaactc
gaccagcgcc gtttcgagtg gaccgccagc gccgtcttgct gccgaaaaa
tccctcatac cttccaagcgc cttggccgct catgctgtggt
gtgggagcttg accgccgaag acccttaggt cagattcaac tgtgctggtg
cagcgcgtgga gttgcttaaat gcaccagcgg aagcggccgg gaagctgagc
daagcgcatgt accgtgctgt caccgtctctt accgctcagc accaggactg
agctatgagc aagagatcgc agtcgaagtt ccataccaaac gcccctccag
cccccatacgac gaaaaaccttc tcacagcgcgcc aagggaggcc cggacaacca
caggtgatac ccctcgcccc atccgggagc gacagccaca gcagaacaccg
cgcgcctggt tcacagcttca caccgagc tggacacttcc cccgctcgga
- 126 -
The amino acid sequence of the fourth polypeptide chain of EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:234):

DIVLTLQSPAS LASVLGQHRT ISCRASKSVS SGYSYMHKY QQKPGQPPKV
LIFLSNNLDS GPAPRFSGSG SGTFTLNIIH PVEDDAATY YQC Habitat LPP
TFGGGTVKLEI KRTVAAPSVGFPFSEDQLK SGTVAVGCLL NNFYPREKAV
QWVKVDNLQSGNSQESVTEQ DSKDSTYSLSTLTLTSKAY EKHKVYACEV
THQGLSSPVTRKSFNRECGE

The amino acid residues 1-11 of (SEQ ID NO:234) correspond to the amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3), and residues 112-218 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes (SEQ ID NO:234) is (SEQ ID NO:235):

gacattgtgc tgacacagtgc ttctgctttcc ttagctgtat tctcctgggca
gagggccacc atctcatgca gggccagc aaagtgtcagtg tctcttggtct
atagttatat gactggtagtca cacacagacac cagagacacccc acccaaaatc
ttctaggttcttct caacagc aagagcttca cagcctcagc atcccagaga
gagaaacaca ctgcgaagtc tctggactgtgctg gctggaaatc gcagttgaaa
gctggactgctgc ttagctggtaa tctccttttc tctcctgggtat cttcctcgtc
gcctctctgtctct atcccacatcc accacagatct cctggtgtttat ggctgaaatc
gggaagctg atagttatat gactggtagtca cacacagacac cagagacacccc acccaaaatc
ttctaggttcttct caacagc aagagcttca cagcctcagc atcccagaga
gagaaacaca ctgcgaagtc tctggactgtgctg gctggaaatc gcagttgaaa
gctggactgctgc ttagctggtaa tctccttttc tctcctgggtat cttcctcgtc
gcctctctgtctct atcccacatcc accacagatct cctggtgtttat ggctgaaatc
gggaagctg atagttatat gactggtagtca cacacagacac cagagacacccc acccaaaatc
ttctaggttcttct caacagc aagagcttca cagcctcagc atcccagaga
gagaaacaca ctgcgaagtc tctggactgtgctg gctggaaatc gcagttgaaa
gctggactgctgc ttagctggtaa tctccttttc tctcctgggtat cttcctcgtc
gcctctctgtctct atcccacatcc accacagatct cctggtgtttat ggctgaaatc
gggaagctg atagttatat gactggtagtca cacacagacac cagagacacccc acccaaaatc
ttctaggttcttct caacagc aagagcttca cagcctcagc atcccagaga
gagaaacaca ctgcgaagtc tctggactgtgctg gctggaaatc gcagttgaaa
gctggactgctgc ttagctggtaa tctccttttc tctcctgggtat cttcctcgtc
gcctctctgtctct atcccacatcc accacagatct cctggtgtttat ggctgaaatc
gggaagctg atagttatat gactggtagtca cacacagacac cagagacacccc acccaaaatc

I. EphA2 mAb 2 x CD3 mAb 2 x DR5 mAb 1

A further exemplary Tri-Specific Binding Molecule comprised of four polypeptide chains was constructed. The Tri-Specific Binding Molecule comprises the VL and VH domains of EphA2 mAb 2, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of DR5 mAb 1, and was accordingly designated "EphA2 mAb 2 x CD3 mAb 2 x DR5 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:236):

DVMTQTPLS LPVSLGDQAS ICSRSSQSLV HSSGNTYLMH YLQKPGQSPK
LLYKVSNSRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHVP
TFGSGTLKLEI KGGGSGGGGE VQLVESGGGL VQPGGSLRSL CAASGFTFST
[00336] In SEQ ID NO:236, amino acid residues 1-11 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 2 (SEQ ID NO:163), residues 112-119 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 120-244 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 245-250 correspond to the GGCGGG linker (SEQ ID NO:34), residues 251-278 correspond to an E-coil Domain (SEQ ID NO:39), residues 279-281 are the linker GGG, residues 282-291 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 292-508 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00337] A polynucleotide that encodes SEQ ID NO:236 is (SEQ ID NO:237):

gatgttgtgta tgacccaaac tccactctcc ctgcctgtca

tcaagcctcc atctcttgca tgacccaaac tccactctcc
gaaacaccta ttacattgg tccactctcc ctgcctgtca
tctctgatct acaaagtttc caaccgattt tctggggtcc
cagtggcagt ggatcaggga cagatttcac actcaagatc
ttacgtgagga ttggaggtca aggtcagact ctggtcttgg
tgtgagacac ggtaacttcg gcaattctta cgtgtcttgg
tgcacttgaa aaggaggtcg cagccctgga gaaaggcggc

tatctgcaaa tgaacagcct gaaaaccgag gacaggccgg

tgtaacacg gttaacttcc ctaaatttta etttgcctatt
gggacagg gacactggtc actgtgttct ctggtcttgg

gaatgccccg ccctggtgtc tggagttgcc ggtgctttgg
gcctgaggtc acatgtggtc cctttagctt atctctcgtg
tacgctatga attgggtcc gccgctcca ggaagggttt
	ggaaggagtc tgtgagacac ggatactttca cgggtgcttt
tcccctgaga cagggggtcg ccctggttca gcaagccccg

tgctcttgct caccgtcctg caccaggact ggctgaatgg

gagctcaccat gatttcccag gttgcaatgg tattttgcctt
gggacaggg gacactggtc ccagctgactt ccccctcmaa

tgctctctct tccccctaa accaagagc acctctcatct ctcccttggc

tcaatggctg aacatgggtt aagtgggttc agggaggttcc
tgatctgccgt aagctgctag gactgtggtc aagctgctag

gattctgctg caccgtcctg caccaggact ggctgaatgg
gagctcaccat gatttcccag gttgcaatgg tattttgcctt
gggacaggg gacactggtc ccagctgactt ccccctcmaa

tgctctctct tccccctaa accaagagc acctctcatct ctcccttggc

tcaatggctg aacatgggtt aagtgggttc agggaggttcc
tgatctgccgt aagctgctag gactgtggtc aagctgctag

gattctgctg caccgtcctg caccaggact ggctgaatgg
gagctcaccat gatttcccag gttgcaatgg tattttgcctt
gggacaggg gacactggtc ccagctgactt ccccctcmaa

tgctctctct tccccctaa accaagagc acctctcatct ctcccttggc

tcaatggctg aacatgggtt aagtgggttc agggaggttcc
tgatctgccgt aagctgctag gactgtggtc aagctgctag

gattctgctg caccgtcctg caccaggact ggctgaatgg
tctccaacaa agccctccca gcccccatcg agaaaaccat ctccaaagcc
aaagggcagc cccgagaacc acaggtgtac accctgcccc ... aggatgtggc ggtggaaaag tggccgcact gaaggagaaa
gttgctgctt tgaaagagaa ggtcgccgca cttaaggaaa aggtcgcagc
cctgaaagag

[00338] The amino acid sequence of the second polypeptide chain of EphA2 mAb 2 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:238):
QAVVTQEPSL TVSPGVTVL TCRSTGAVT TSNYANWQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALTITGA QAEDADYYC ALWYSNLWVF
GGGKTVLVG GGGGSGGGQI QLVQSGPELK KPGETVKISC KASGFTFTNY
GMNWKQAPG KGLKMWGWIN TYIGEPTYAD DFKGRFVFL ETASASTAYLQ
INNLKEDMA TYFCARELGP YYYDYGQQT TLTVSSGCGG GGTKVAILKE
VAALKEKVAA LKEKVAALKE

[00339] In SEQ ID NO:238, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 2 (SEQ ID NO:167), residues 237-242 correspond to the linker GGCAGG (SEQ ID NO:34), and residues 243-270 are a K-coil Domain (SEQ ID NO:40).

[00340] A polynucleotide that encodes SEQ ID NO:238 is (SEQ ID NO:239):
caggctgcttg tgaccccgga gccctctactg acctgtctcc caggccggaac
ttgaccccgat acatgcaacat ccacgcagcg cgcgttgacc acatctaaact
acggccaaatg ggctgcagcag aagccaggac aggcaccaag gggcctgtac
GGggttcaca acaaaacgtg ctccgtaaggct ttcgcttggaag
ttgctgctggc ggaaagcgcc tctctgactat taccgggga caggccgagg
acggccagca ttactatgtt gctctgtggt tatacagact atagcttggtgc
acacggctta cagaaaatcgct atggtcttgga ggggttggtg ccggcggaggg
tggacagatgc agttgttggtc tgtctgggac tcagtcctag aagccttgag
acagacgctag atatctctcg aagcctcttg gtgtcttccttc cacaatact
ggagatgacgtt ggtgagacga ggctccagga aagggctttaa aaggtgtggg
cagctgataac acatataattt gagaagccgac atatgctgtg gacttcaggg
acagcgctttt ctccttcttg gaaactctcg ccacagctag ctatgctccg
atcaacaacc tcacaaggata ggcactaggcc acatatcttc gcgttccagga
actggtgacc aactacatttt ggtctggccc ccaagggacc aactctcagac
tgttggctgtt tgggagagaa gggcgagagcg ct.taaggaga aaggtgacgc
ctcggagag
The amino acid sequence of the third polypeptide chain of EphA2 mAb 2 x CD3 mAb 2 x DR5 mAb 1 is (SEQ_ID: NO:240):

EVKFLSEGGE LVQPGGLLKL SCVAVGFDFS RYWMSWVRQA PGKGLEWIGE
INPDSTNTY TPSSLKDFKI SRDNANAKNTLY LQMVKVSRD TALXYCTRRRA
YYGNNPAFWAY WGGGTLTVTS SASTKGPSVF PLAPSSKSTS GTGAALGCVLV
KDFYFPEPVTV SWNSGALTSG VHFTFPALVQS SGLYSSLSSV TVPSSSLTGG
TYICNKNHPK SNKVDKVRVE PKSCKDTHTC PPCPAPEAAG GSPVFLFPFPK
PKDTLMI SRT PEVDPEVFKN WYVGDVEVHN AKTKPRPQKY
NSTYRVSVL TVLHHDWNLG KEYKCKVSNK ALPAPIEKTI SKAKQPPREP
QVYTLPPSRE EMTKNVQVLS CAVKGFGYPSD IAVEVSSGTPV PENNYTTTP
VLDSDGSFL VSKLTVDKSR WQOQGNSFSVM VMHEALHNY TQKSLSLSPG K

In SEQ ID NO:240, amino acid residues 1-121 correspond to the amino acid sequence of the VH Domain of DR5 mAb 1 (SEQ ID NO:8), residues 122-219 correspond to a modified CHI Domain (SEQ ID NO:208), residues 220-234 correspond to a linker (SEQ ID NO:209), and residues 235-451 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

A polynucleotide that encodes SEQ ID NO:240 is (SEQ ID NO:241):

gaggtgaagt ttctcgagtc tgaggtgtgc ctggctgagc ctggaggatc
cctgaaactc ttctctttagct cctcagatgtg ctgattttgtg agatactgga
tgagttggtt cggcgaggtt ctgaagggaga ggtctagatg gatttgagaa
attatatcctt atagcaaatat gataaactat acggtcatct taaaggtata
tattcatcatt ctcagagaga acgcctaaaa tacgtctgat ctgcataagta
ccaaaagttg gttctagggactc acagcccctt attattgtac aaggagggcc
tactatgta accggcctgt gtttgccctat tgggccaagag gactctggt
tatttggcctt ccgtccccat ctggagggcac accgtctgcc taaggtctgc
acatttact gctctggtttt tctggccttgt gtcggtcttact cttcatgcct
acacccctcag accgctgctgct cagctgccttg cgccctgctgctgctgctg
caccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
caccctagct ccctctggcctg ccctctggcctg ccctctggcctg ccctctggcctg
gctctggtgt cgaacgctgctgctgctgctg ccggccgaggg ggcgctgcctct
cagctgctgctgctgctgctg ccggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cagctgctgctgctgctgctg ccggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg
cagccacccct cggccgaggg ggcgctgcctct cttctccctt cccccccaaa
cccaagatag cccctcgagc ctggctgagc ctggctgagc gcctggtgtgt
gtagtctgtag gacccgaggg cccctctctg ccgctgtgctgctgctgctg

- 130 -
The amino acid sequence of the fourth polypeptide chain of EphA2 mAb 2 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:242):

```
DIVLTQSPAS LAVSLGQRAT ISCRASKSVS SSGYSYMHWY QQKPGQPPKV
LIFLSSNLD5 GVPARFGSGS SGDFTLNIH PVEDGDAATY YCQHSRLDPP
TFGGGTKLEI KRTVAAPSVF IFPPSDEQLK SGTASVVCCL NNFYPREAKV
QWKVDNALQS GNSQESVTEQ DSKDSTYSLG EKHKVYACEV
THQGLSSPVT KSFNRGEC
```

In SEQ ID NO:242, amino acid residues 1-11 correspond to the amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3), and residues 112-218 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes SEQ ID NO:242 is (SEQ ID NO:243):

```
gacattgtgc tgacacagtc tctctgcttcc ttagctgtat ctctcgggca
gagggccacc atctcatgca ggccagcaa aagtgtcagt tcctctggct
atagttatat gcactggtac caacagaaac caggacagcc acccaaaatc
tctcatctttctttccttcaacctgcttctctggct tctctgcttcc
tgacattgtgc tcctctggct ttagctgtat ctctcgggca
```

J. EphA2 mAb 3 x CD3 mAb 2 x DR5 mAb 1

A further exemplary Tri-Specific Binding Molecule composed of four polypeptide chains was constructed. The Tri-Specific Binding Molecule comprises the VL and VH domains of EphA2 mAb 3, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of DR5 mAb 1, and was accordingly designated "EphA2 mAb 3 x CD3 mAb 2 x DR5 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:244):

```
DIVLTQSHRS MSTSVDRIVN ITCKASQDVT TAVAWYQKRP GQSPKLLIFW
ASTRHAGVPD RFTGSSGGTD FTLTSSVQA GDLALYYCQQ HYSTPYTFGG
GTKLEIKGGG SGGGGEVQLV ESGGGLVQPQ GSLRSLCAAS GTFSTYAMN
```
In SEQ ID NO:244, amino acid residues 1-107 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 3 (SEQ ID NO:172), residues 108-115 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 116-240 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 241-246 correspond to the GGGCGG linker (SEQ ID NO:34), residues 247-274 correspond to an E-coil Domain (SEQ ID NO:39), residues 275-277 are the linker GGG, residues 278-287 are the linker DKTHTCPCPCP (SEQ ID NO:48), and residues 288-504 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

A polynucleotide that encodes SEQ ID NO:244 is (SEQ ID NO:245):

gacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg

gcatccaccc gcagcagctgg aggccagtca ggatgtgact actgctgtag
tgggacagat tttaacttctca ccacacccag ttggacaggg gggacacagt
cacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg

gcatccaccc gcagcagctgg aggccagtca ggatgtgact actgctgtag
tgggacagat tttaacttctca ccacacccag ttggacaggg gggacacagt
cacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg

gcatccaccc gcagcagctgg aggccagtca ggatgtgact actgctgtag
tgggacagat tttaacttctca ccacacccag ttggacaggg gggacacagt
cacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg

gcatccaccc gcagcagctgg aggccagtca ggatgtgact actgctgtag
tgggacagat tttaacttctca ccacacccag ttggacaggg gggacacagt
cacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg

gcatccaccc gcagcagctgg aggccagtca ggatgtgact actgctgtag
tgggacagat tttaacttctca ccacacccag ttggacaggg gggacacagt
cacattgtgc tgaccacagtcc tcaacagatcc atgtccacat cagtagggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggtctgcaag atctttctgg
The amino acid sequence of the second polypeptide chain of EphA2 mAb 3 x CD3 Ab 2 x DR5 mAb 1 is (SEQ ID NO:246):

```
QAVVTQEPSL TVSPGGTVTL TCRSSTGAVT TSNYANWQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALTITGA QAEDEADYYC ALWYSNLWVF
GGGKLTVLG GGGSGGGGGEV QLVESGGGSV KPGGLKLSC AASGFTFDDH
YMYWVRQTPE KRLEWVATIS DGSFTSYPD SVKGRFTISR DIAKNNLYLQ
MSSLKEDETA MYCTRDESD RPFPYWQGT LVTVSSGGCG GKKVAALKE
VAALKEKVAA LKEKVAALKE
```

In SEQ ID NO:246, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 3 (SEQ ID NO:177), residues 237-242 correspond to the linker GCCGGG (SEQ ID NO:34), and residues 243-270 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:246 is (SEQ ID NO:247):

```
caggctgttg tgacctcagga gctctcaactg accgtgctcc caggccggaac
tgtgaccctg acatgcagat ccagcacagg cgcagtgacc acatctaact
acgccaatttg ggtgcagcag aagccaggac aggcaccaag gggctgtacg
gggggtacaa caaaaaaggg cttccctgacc cctgcagctt tttctggaag
tctgctgagg ccagcggcag ctctgactat taccggggac caggccgagg
acggaagccttg taactattgt gctctgttgt atagcaacctt tgtggctgttc
gggggtgacc ctaaactgac tgtgtctgga ggggttgctt cccggcgagg
 tgtgagagtct aacattctgt tggcgaggct ggctctgagg cagtcccttg
ctctctctcttg gagcctcctgt gatttacatt ctctgagcct
```

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The amino acid sequence of the third polypeptide chain of EphA2 mAb 3 x CD3 mAb 2 x DR5 mAb 1 is (SEQ_ID NO:248):

EVKFLESGGG LVQPFGSLLKL SCVASFDFPS RWMSWVRQA PGKGLEWIGE
INPDSNTINY TPSSLKDFKI SRDNAKNTLY LQMTKVRSED TALLYCTRRA
YYGNPAWFAV WQGTQLTTVS SASTKGPSVF PLAPSSKSTS GTTAALGCLV
KDYFPEPVTIV SWNSGALTSG VHTPFAPLQS SLGYLSLSSV TVPSSSLGTQ
TYICNVTNKP SNTKVDRKVE PKSCKDTHTC PPCPAPEAAG GPSVFLFPFPK
PKDITLMIRT PEVSHCVDV VSHEDPEVKFN WYVGDVEVHNN AKTPPREEQY
NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKQPREP
QVYTLPSRE EMTKQVSLV CAVKGFPAPD IAWEWESNNT PENYKTTMP
VLDSDGSSFIL VSKLTVDRSR WQQGQFVSCS VMHEALHNRQ TQKSLSLSPG
K

In SEQ_ID NO:248, amino acid residues 1-121 correspond to the amino acid sequence of the VH Domain of DR5 mAb 1 (SEQ_ID NO:8), residues 122-219 correspond to a modified CHI Domain (SEQ_ID NO:208), residues 220-234 correspond to a linker (SEQ_ID NO:209), and residues 235-451 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ_ID NO:53).

A polynucleotide that encodes SEQ_ID NO:248 is (SEQ_ID NO:249):

gaggtgaagt ttctcagagt tggagttggc ctggtcgacg ctggagggatc
cctgaaaactc ttctgtgtag ccctcaggatt cgattttagt agatactgga
tgagttgggt ccggcacagct tggaggtggc ctggtgcagc ctggaggatc
cctgaaactc tcctgtgtag ccctcaggatt cgattttagt agatactgga
tgagttgggt ccggcacagct tggaggtggc ctggtgcagc ctggaggatc
The amino acid sequence of the fourth polypeptide chain of EphA2 mAb 3 x CD3 mAb 2 x DR5 mAb 1 is (SEQ ID NO:250):

```
DIVLTQSPAS LAVSLGQRAT ISCRASKSVS SSGYSYMHWY QQKPGGPPKV
LIFLSSNLDS GPVRASQGSR SGDTFTLNHI PVEDGDAATY YQCHSRLDPP
TFGGGTKLEI KRVAAPSVC IFPPSDEQLK SGTASVVCLL NNFYPREAKV
QWKVDNALQS GNSQESVTEQ DSKDSTYSLS STTLKSADYE EHKKVYACEV
THQGLSSPVT KSFNRGEC
```

In SEQ ID NO:250, amino acid residues 1-11 correspond to the amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3), and residues 112-218 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes SEQ ID NO:250 is (SEQ ID NO:251):

```
gacattgtgc tgacacagtc tcctgcttcc ttagctgtat ctctcgggca
gaggccccca atctcatgca ggccagcaca aagtgctcgt tctcttggct
atagttatat gcactgggtac caacagaaca caggagaccc accaaaagtcc
tctatcttttc ttctacccaa cctagatctt ggggtccttg ccaggtttagc
tggcagtggg tctggtgacc acctccacct caacatccat cttggtagg
atgggtgtgc tcgacagctg ctctgcttcc tcctgcttcc ttagctgtat ctctcgggca
acacagcacc acgcagcact acgcagaaga gcctctccct ctctccgggt
aggctctgca caaccgctac acgcagaaga gcctctccct ctctccgggt
3.3.3.
```

Although the exemplary Tri-Specific Binding Molecules described above comprise three Light Chain (VL) CDRs and three Heavy Chain (VH) CDRs for each binding domain, it will be recognized that the invention also includes Tri-Specific Binding Molecules that possess:

1. at least one of the CDRs of the VL Domain of any such binding domain;
2. at least two of the CDRs of the VL Domain of any such binding domain;
3. the three CDRs of the VL Domain of any such binding domain;
(4) at least one of the CDRs of the VH Domain of any such binding domain;
(5) at least two of the CDRs of the VH Domain of any such binding domain;
(6) the three CDRs of the VH Domain of any such binding domain;
(7) at least one of the CDRs of the VL Domain of any such binding domain and at least one of the CDRs of the VH Domain of that binding domain;
(8) at least two of the CDRs of the VL Domain of any such binding domain and at least two of the CDRs of the VH Domain of that binding domain;
(9) the three CDRs of the VL Domain of any such binding domain and the three CDRs of the VH Domain of that binding domain;
(10) the VL Domain of any such binding domain;
(11) the VH Domain of any such binding domain; or
(12) the VL and VH Domains of any such binding domain.

K. gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 1

[00360] A Tri-Specific Binding Molecule composed of four polypeptide chains was constructed that comprises the VL and VH domains of gpA33 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of EphA2 mAb 1. The Tri-Specific Binding Molecule was accordingly designated "gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:252):

```
DIQLTQSPSF LSASVGDRVT ITCSARSSIS FMWYQQKPG KAPKLLYD
SNLASEGVR SRPSGSSGTEF TLTISSLEAE DAATYVCQGQ SSYPLTFCGQ
TKDLEIKGGS GGGVQVLVLE SGGVLQPGG SLRSLCAASG FTFSYAMNW
VRQAPGKLE KVGRIRSKVN NYATYADSV KGRFTISRDD SKNSLYLQMN
SLKTEDTAVY YCVRGHNFGN SYVSLFYAFFG QGLTVTVSSG GCCGGEVAAL
EKEVAALEKE VAALEKEVAA LEKGGGDKTH TCPCPAPEA AGGPSVFLFP
PKPKDLMIS RTPEVTCCVV DVSHEDPEVK FNWYVDGVEV HNAKTPREE
QYNSTYRVS VLTVLHVIDWL NGKEYCKKVS NKAIPAPIEK TISAKGQPR
EPQYITLPPS REEMTNQVS LWCLVKGFP SDIAVEWESN QGPPENNYKT
PPVLDSGDGF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSL
PGK
```
In SEQ ID NO:252, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GGCAGG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

A polynucleotide that encodes SEQ ID NO:252 is (SEQ ID NO:253):

```
  gacattcagc  tgactcagtc  cccctctttt  ctgtccgcat  ccgtcggaga
tcgagtgact  attacttgct  ctgctaggtc  ctcaatcagc  ttcatgtact
gtagtagttt  atctctgcag  gcacatccgg  gcgttcgagg  tcgagtgact
tccctgtgc  agcctctgga  tctctgactt  gggtctggag  aactgagttt
gtccgccaag  ctccagggaa  ggggtcggag  tggggcgcgc  gctggtggag
gagaagagg  tgccttcgttt  ggaagaggag  tgctgcagcc  ggtgcagcc
tgcggacagc  ctggagaaag  gcgccgaggg  caaaaactcc  ggtgcgcagc
tggcgccagc  acctgaagcc  ccgggggacc  ccgtcagtctt  cctcctcccc
tgagagttgg  gcaagcaggg  cggccgtgtat  tctgtgtgca  ggttcgccag
cagtacaaac  gcacaggggc  ccgggtaaa  gagaggtggtc  ggtgactgt
```

[00362]
The amino acid sequence of the second polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 1 is (SEQ ID NO:254):

```
QAVVTQEPESL TVSPGGTTVL TCRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTKLTVLGC GGGSSGGGQV QLVQSGAEVK KGAVSKVSC KASGYFTGGS
WMNWVRGAG QGLEYWGRYI PGDGETNYNG KFKDRTVTITA DKSTSTAYME
LSSLRESDTA VYVCARIYGN NVYFDVWGQG TTNTTVSSGCG GGGKAALKE
KVAALKVAAKLK E
```

In SEQ ID NO:254, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSSGGG (Linker 1) (SEQ ID NO:33), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 238-243 correspond to the linker GGGG (SEQ ID NO:34), and residues 244-271 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:254 is (SEQ ID NO:255):

```
caggctgtgg tgactcagga gccttcactg accgtgtccc caggcggaac
ttgaccctg acatgcagat ccagcacagg cgcagtgacc acatctaact
acgccaattg ggtcgacagc aagccagggc cgcagtgacc acatctaact
ggggtacaa acaaaagggc tccctggacc cctgcacggt tttctggaag
tctgctgggc ggaaaggcc ctctgactat taccggggca caggccgagg
acgaagccga ttactattgt gctctgtggt atagcaatct gtgggtgttc
ggggtggca caaaaactgac tgtgctggga gggggtggat ccggcggagg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
caagcgtgaa ggtctcctgc aaagcatcag gctatacatt tacagcgcg
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
ccgcatctac cctggagccg gggggtggca caggccgagg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
tggatgaact gggtgaggca ggctccagga cagggactgg agtgatcgg
tggacaggtc cagctggtcc agagcggggc cgaagtcaaa aaacccggag
```

The amino acid sequence of the third polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 1 is (SEQ ID NO:256):

```
QVQLKE58GGP LVAPQ58LSL TCT58GSF58LS RYS58VHWVRQP PGKGEWLG58M
IW58G58STDYN SALKR58LSIS KD58NS58SQVFL KM58NSLQTD58DT AM58Y58CARKH58G
NY58Y58M58DYWGG TG58S58TVWSSAS TK58G58PSF58P58L58A58 S58K58ST58S58GT AAL58G58LCLVKD58Y
FPE58PVTV58W58N SG58ALT58SV58HT FP58AV58L58Q58SS58GL Y58S58LS5858S58V5858T58P Y58S58LS5858L58G58T58Q58YI
```

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In SEQ ID NO:256, amino acid residues 1-118 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 1 (SEQ ID NO: 158), residues 119-216 correspond to a modified CH1 Domain (SEQ ID NO:208), residues 217-231 correspond to a linker (SEQ ID NO:209), and residues 232-448 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

A polynucleotide that encodes SEQ ID NO:256 is (SEQ ID NO:257):

caggtgcagc tgaaggagtc aggacctggc ctgggtggac cctcacagag cctgtccatc acatgcactg tctctgggtt ctcattatcc agatatagtg tacactgggt tcgccagcct ccaggaaagg gtctggagtg gctgggaatg atatggggtg gtggaagcac agactataat tcagctctca aatccagact gagtatcagc aaggacaact ccaagagcct aaatccgatc agatatagtg tacactgggt tcgccagcct ccaggaaagg gtctggagtg gctgggaatg atatggggtg gtggaagcac agactataat tcagctctca aatccagact gagtatcagc aaggacaact ccaagagcct aaatccgatc agatatagtg tacactgggt tcgccagcct ccaggaaagg gtctggagtg gctgggaatg atatggggtg gtggaagcac agactataat tcagctctca aatccagact gagtatcagc aaggacaact ccaagagcct aaatccgatc

The amino acid sequence of the fourth polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 1 is (SEQ ID NO:258):

DIQMTQITSS LSALSGDRIT ISCRASQDIS NYLNYWQQKP DTGVKLLIYY
TSRLHSVGPS RFSGSSSGTD YSLTISNLEQ EDIATYFCQQ GYTLYTFGGG
TKLEIKRTVA APSVFIFPPSS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD
In SEQ ID NO:258, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 1 (SEQ ID NO: 153), and residues 107-213 correspond to the CL Kappa Domain (SEQ ID NO:210).

A polynucleotide that encodes SEQ ID NO:258 is (SEQ ID NO:259):

gatatccaga tgacacagac tacatctccc ctgtctgcct ctctgggaga
cagaatcacc atcagttgca gggcaagtca ggacattagc aattatttaa
actggtatca gcagaaacca gatggaactg ttaaactcct gatctactac
accagctgga aatcattggac ctgctggctc ggctcctgcc gctctgggaga
ttgatccagag tcgagttcagtg caaagggaga gcacctggagc aattatttaa
ccatctctca ccattttctg tatcctctct ctcttcctgc cgcctctgga
cgccctctcc atctcgacat ttatcacatg ctctctcctc ctcctgatcg
caagacccca aacctagcgc tctctgatct gcctctg cccctctctg
cctcctgcag gccgcagact gcctgctgta cagctctctc ctcctgatcg
actagctggga cagctctcct ctagctctct ctctctcctc ctcctgatcg
agctcggcagg ctctcagag ccctctcctc ctcctgatcg

A Tri-Specific Binding Molecule composed of four polypeptide chains was constructed that comprises the VL and VH domains of gpA33 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of EphA2 mAb 2. The Tri-Specific Binding Molecule was accordingly designated "gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 2." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:260):

DIQLTQSPSF LSASVGVPRV ITCSARSSIS FMWYQQKPG KAPKLLIYDT
SNLASGVPFS FGSGGSGTEF TLTISSLEAE DAAAYCQQW SSYPLTFQGG
TLKEIKGGGS GGGGIVQLVE SGGVLQPGG SLRLSAAGS FTFSYAMNW
VRQAPGKGLE WVGIRSKYN NYAYYNADSV KGRFTISRDD SNKSLYLMN
SLKTEDTAVY YCVRHRGFGN SYVSWFAYWG QGLTVTSSG GCCGGEVGAAL
EKEVALEKE VAALEKEVAA LEKGGGDKTH TCPCAPAPEA AGGPVSFLFP
PKPKDILIMS RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTPREE
QYNSTYRVS VLTLYHQDVL NGKEYKCKVS NKLAPPAEKP TSKAKQGPR
EPQVYTLPPS REEMTKQVQ WWLVLKGFYP SDIAVEWESN GQPENNYKTT
PPVLDSDGSF FLYSKLTVDK SRWQQGVNSF CSVMHEALHN HYTQKSLSLSPGK
In SEQ ID NO:260, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GCCGGG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKHTTCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

A polynucleotide that encodes SEQ ID NO:260 is (SEQ ID NO:261):

gacattcagc tgactccagtgc cccctctttt tctgccgcat cggctggaga
tcaggtgact atattaattgct ctgctaggtgc tccaatcagc ttcattgtact
ggatcagcga aagacacgac caagcctctct ctgcagtctt tctgcgctt
gagcaacttg cctccgggttg gccatctcggt tttcttggca gtgggtcagg
aacgtagtttacctgacaa ttacctccttt gcaggtgctag tactgtctgg
ccctactattt ccagctagtg agcagctatc ctctgacctt cggacagggg
actaaacttg aaatcaaggg tggaggtact ggcgcggcag gcaggtgctg
agtcttgagg tctgctggag tctgggttgc gctctctgtgc tggtgtcgg
ctcctctgtgc acgtctcggta ttccacctct gccctcaagt ttacctgttg
gtctggccagt ctccacggaa ggggtctggag tggggtggaag gcatcagttc
gaagaaaggg ttcggctggtt ggagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt
tcttcccgg agaaccgtag tctctcggag gctcgtcgcct caggtgcttt
catggctgggt gtcgtctgtt gctctcggag gactaagagcc gcaggtgctg
cgcttactac acgtctcggac gtcgtgcaag gctggggtggt gtcggcagc
agtaactcag gcacagcttt tctctgcagc cccttgagga gtcggtctc
ccctcttcgc ctgctcaactt acgtctcggag gactaagagc gcacagcttt
gagagagag ggcggctgct ttgggttgtt
agttcgagcc cttttgtaggtt gcaagacagc acgagttgtt gcggtggttt

cggggtaaaa
The amino acid sequence of the second polypeptide chain of gpA33 mAb:

1 x CD3 mAb 2 x EphA2 mAb 2 is (SEQ ID NO:262):

```
QAVVTQEPLSL TVSPGGTVTL TCRSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALITITGA QAEEDADYYC ALWYSNLWVF
GGGGTTLVGLGGGSGGGGQVQLVQGSAEVKKPGASKVSKCASKYFTGGS
WMMWNVQRAPG QGLEGWIRY PGDGETNYNG KFKDRVTITADKSTAYME
LSSLSREDTA VYQGARIYGN NVYFDVWGQGTTTVSSSGGC GGGKVAALK
KVAALKEKVA ALKEKVAALK E
```

In SEQ ID NO:262, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 238-243 correspond to the linker GGCGGG (SEQ ID NO:34), and residues 244-271 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:262 is (SEQ ID NO:263):

```
caggctgtgg tgaactcagga gccttcacctg acctgtcctcc caggccggaac
ttgacacctg acatgcaagat ccaacggagacc gcagctggagccc ttcactaatctt
gggggtgcga caaactctgctctgcatcatttgggacttgg
tttgttgccc cagggctgggt cgggggccgt gaggctggacionales
taccctagttc tttctgttgt gggcctgactg gggctgttttcttgagtttgagttgga
tttgttgccc tttctgttgtg gggcctgactg gggctgttttcttgagtttgagttgga
```

The amino acid sequence of the third polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 2 is (SEQ ID NO:264):

```
QIQLVQSGPE LKKGPETVKI SCKASGFTFT NYGMNWVKA PGKGLKWMG
INTYIGEPTY ADFDKGRFVF SLETSASTAY LQINNLKNEH MATYFCAREL
GPYYFDYWGG GTTLTVSSAS HKPSVSFPLA PSSKSTSGGT AALGCLVKDY
FPEPVTWWSN SGALTSGVHT FPAVLQSSGL YLSLSSVTVP SSSLGTQTYI
CVNHNKPSNT KVDKRVPEKS CDKTHCPCP PAPEAAGGPS VFLFPPPKPD
```
[00379] In SEQ ID NO:264, amino acid residues 1-118 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 2 (SEQ ID NO:167), residues 119-216 correspond to a modified CHI Domain (SEQ ID NO:208), residues 217-231 correspond to a linker (SEQ ID NO:209), and residues 232-448 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

[00380] A polynucleotide that encodes SEQ ID NO:264 is (SEQ ID NO:265):
cagatccagt tggtgcagtc tgagacctgag ctgaagaagc ctggagagac
agtcaagatc ttctgcaagg ttctctgagt ttacttcaca aacctagggaa
tgaactggtt gaagcagccg ccagaaagag gttaaaaagt gatgggctgg
ataaacacct atattggaga gccgacatgt gtgtgagact tcagaggagc
gttgttgcttc cttttggaaa cctttgccag cactgcctat ttgcaagatca
acaaacctaa aatgagggac atggccacat atttcctgtgc aaggaggactg
 ggaccatact acctcggact tttgcccaga gacacgacttc tcacagcttc
cctgcctctcc ccaagggccc cactcggcttc cccctggtca cccccctcctca
gcaagctggt gacccgtgcc tgggccctga cctgcgctttg ctggaagactc
tgcaacgta atccacaccc cagcaacccc aaggtggaca agagagttga
gcacaatttt tgtgacaaaa ctcaacacat cccacggtgc ccaccacgctg
aagccgcgggg gggacgctca gctcttcctct tccacaacaa cccacagcag
acccctatga ttcctccgag cctctgaggct acatgcgtgg ttgtggagct
 garccagcag gaacctgagg tcaatgctca ttggtacgtg gacgcgctgg
aggtgcataa gcggccccaa gaccctgagg tcaacgccct gacggtggga
taccgctggt tcacggctct ccacgctcgt cccacgactt gctgtaagtg
 caagagcgtac aagttgacag ccctctccccc ccctcagcctt ccccccctccag
agaaaaactt ttcacaagcc aaaggccagc ccggagacc accagttgtac
 acctgcctcc ttcctgggaa cggagacggt aagaaacgcg tccacgcctg
ttggagagtc aaggtgcttc atccacagca cttcggcttg gatggagagga
gcaatgaggca ggcaggcagc aactacaaga ccacgcctcc ccggtgtggc
 tccagccggt cttctctcct cctgagctca cttcggcttg acaagacgcg
gtgggcagtc gggagcgctt cctcactgtg cttgactgat gggagctccc
gcaacgtaga cccagcagag cctcccctca gcccccaggt ccccccaggg
[00381] The amino acid sequence of the fourth polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 2 is (SEQ ID NO:266):
DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSSGNYLHW YLQKPGQSPK
LLIYKVSNSRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHPV
TFGSGTKLEI KRTVAAPSVF IFPPSDEQLK GTASVVCPLL NNFYPREAKV
[00382] In SEQ ID NO:266, amino acid residues 1-111 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 1 (SEQ ID NO:163), and residues 112-218 correspond to the CL Kappa Domain (SEQ ID NO:210).

[00383] A polynucleotide that encodes SEQ ID NO:266 is (SEQ ID NO:267):

```
gatgttgtga tgacccaaac tccactctcc ctgcctgtca gtcttggaga
tcaagcctcc atctcttgca gatctagtca gagccttgta cacagttagtg
gaaacaccta ttcatatggg tacctgcaga agccaggcca gtctccaaag
tctctgtgct cagagtagtg gaaacaccta ttttacattgg tacctgcaga
agccaggcca gtctccaaag cttcctgatct acaaagtttc caaccgattt
tctggggtcc cagacaggtt cagtggcagt ggaatcaggga cagatttcac
actcaagatc agcagagtgg aggctgagga tctgggagtt tatttctgct
tcaaagtttc acgttcggct cggggacaaa gttggaaata aaacgtacgg tggctgcacc
atcggtcttc atcttcccgc catctgatga gcagttgaaa tctggaactg
cctctgttgt gtgcctgctg aataacttct atcccagaga ggccaaagta
cagtggaagg tggataacgc cctccaatcg ggtaactccc aggagagtgt
cacagagcag gacagcaagg acacgaccta cagccctcgc acgcctcctga
cagctgacaa agcagactac gagaacaca aagtctacgc ctgcgaagtct
accatcagg gcctgagctc gccgctcaca aagagcttca acaggggaga
gtgt
```

[00384] A Tri-Specific Binding Molecule composed of four polypeptide chains was constructed that comprises the VL and VH domains of gpA33 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of EphA2 mAb 3. The Tri-Specific Binding Molecule was accordingly designated "gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 3." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:268):

```
DIQLTQSPSF LSASVGDRVT ITCSARSSIS FMYWYQQKPG KAPKLLIYDT
SNLASGVPSR FSGSSGTEF TLTISSLEAE DAATYYQCOQ SYSLFAFGQG
TKLEIKGGGS GGGEVQLVTE SGGLVQPQG SLRLSCAASG TFTPSTMANY
VRQAPGKGLK WVGIRSKYK NYATYYADSV KGRFTISRDD SKNSLYLQMN
SLKTEDTAVY YCVRHGFNQ SYSVSFAYYW QGTLYTVSSG GCGGGEVAAL
EKEVAALEKE VAIAEKEVAA LEKGGGDKTH TCPCPAPEA AGGPSVFLFP
PKPDTLMIS RTPEVTCCVV DVSHDPEVK FNWYVDGVEV HNATKPREE
QYNSTYRVS VLTVLHQDWM NGKEYKCKVS NAKLPAPIEK TISKAKGQPR
EPQYTLPPS REEMTKNQVS LWCLVKGYPF SDIAVEWESN GQPENNYKTT
PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSMHEALHN HYTQKSLSLSPGK
```
[00385] In SEQ ID NO:268, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GCCGGG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00386] A polynucleotide that encodes SEQ ID NO:268 is (SEQ ID NO:269):

gacattcagc tgacttcagtc cccctctttt ctgctcggcat cccgtcggaga
tcggacttgtc attactttgc tctgcttggca ttggtctcagagctggtcgtc

ggccggcggg ccagctcgcga aagagctgct gccagctggtc gtcgcttcctt
tcgggtgaga atctcgatga tgttgtggtgcc gctgtggtgtt ccgggggtgt
tctcttctgg ctcggctcgg gctctggctgg gccaagctgc cggcgggtgt
ctggtgctgc ggtgtggctgg ggtgttcgtgt gcgtgtggtgt ggtgtggtgg
tgccgggcat cgggtggtgg gccgggtggt gcgtggtgggt ggtggtgggt
tccggggtggt cgggtggtgg gccgtggtgg gcgtggtgggt gcgtggtgggt
tccggggtggt cgggtggtgg gccgtggtgg gcgtggtgggt gcgtggtgggt
tccggggtggt cgggtggtgg gccgtggtgg gcgtggtgggt gcgtggtgggt
tccggggtggt cgggtggtgg gccgtggtgg gcgtggtgggt gcgtggtgggt
The amino acid sequence of the second polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 3 is (SEQ ID NO:270):

```
QAVVTQEPSSL TVSPGTVTL TCRRSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALITTGA QAEDDEDDYYC ALWYSNLWVF
GGGKTLTVLG GGGSGGGQV QLVQSGAEVK KPASVYKSC KASGYTFGLS
WMNVVRQAPG QGLEWIGRY PGDGETNYNG KFKDRVTITA DKSTSTAYME
LSSLRESDTA VYYCARIYGN NVYFDRVQGG TTVTSSGGC GGGKVAALK
KVAALKEKVA ALKEKVAALK E
```

In SEQ ID NO:270, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO: 104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 238-243 correspond to the linker GCCG (SEQ ID NO:34), and residues 244-271 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:270 is (SEQ ID NO:271):

```
caggctgtgg tgactcagga gccttcactg accgtgtccc caggcggaac
ttgaccctg acatgcagat ccagcacagg cgcagtgacc acatctaact
acgccaattg gcctggacc accgtgtccc ggggtacaa acaaaagggc
tgcacggttc cgcaggggca caggccgagg acgaagccga
tttgacgtgtg gcctttgaaaga ggtcgccgc cacttaagg aaaaggtcgc
tgtgctggga ggggtggat ccggcggagg tggacaggtc cagctggtcc
```

The amino acid sequence of the third polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 3 is (SEQ ID NO:272):

```
EVQLVESGGG SVKPGGSGLL SCAASGFTFT DHMYMYWVRQT PEKRLEWVAT
ISDGGSFHTS PDVSKGRFTI SRDIAKNNLY LQMSSLKSED TAMYCTRDE
SDRPFPYWGG GTLVTVSSAS TGKPSVFPPLA PSSKSTSGGT AAAGLCLVKYD
FEPFVTVWGN SGALTSGVHT FPAVLQSSGL YLSLSVVTVP SSSLGTQTYI
CNVNHKPSNT KVDKRVPEKS CDKTHCPCP PAPEAAGGPS VFLFPPKPD
```
TLMI SRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST
YRVVSTTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY
TLPPSREEMT KNQVSLSCAV KGFYPSDIAV EWESENGQPEN NYKTPFPVLQ
SDGSFLLVSK LTVDKSRWQQ GNVFSCSVMH EALHNRYTQK SLSLSPGK

[00391] In SEQ ID NO:272, amino acid residues 1-118 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 3 (SEQ ID NO: 177), residues 119-216 correspond to a modified CHI Domain (SEQ ID NO:208), residues 217-231 correspond to a linker (SEQ ID NO:209), and residues 232-448 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

[00392] A polynucleotide that encodes SEQ ID NO:272 is (SEQ ID NO:273):

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

gaagtgcagc tggtggagtc tgggggaggc tcagtgaagc ctggagggtc
cctgaaactc tcctgtgcag cctctggatt cactttcact gaccattaca
ttcacctac ttcagagagc ttgcaacaga aagatgatgagttt ccagacagtg
tgcagctgagc gttactgtac gctgggaccg ggtcgcaacc

[00393] The amino acid sequence of the fourth polypeptide chain of gpA33 mAb 1 x CD3 mAb 2 x EphA2 mAb 3 is (SEQ ID NO:274):

DIVLVTQSHRS MSTSVDGRVN ITCKASQDVT TAVAWYQQKP GQSPKLLIFW
ASTRHAGVPD RFTGGSSGTD FTLITISSVQA GDLALYCCQ HYSTPYTFGG
GTKLEIKRTIV AAPSVFIFPP SDEQLKSGTA SVVCLFNFFY PREAKVQNKV

- 147 -
DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

[00394] In SEQ ID NO:274, amino acid residues 1-107 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 3 (SEQ ID NO: 172), and residues 108-214 correspond to the CL Kappa Domain (SEQ ID NO:210).

[00395] A polynucleotide that encodes SEQ ID NO:274 is (SEQ ID NO:275):

gacattgtgc tgacccagtgc tcacagatcc atgtccacat cagtaggaga
cagggtcaac atcacctgca aggccagtca ggatgtgact actgctgtag
cctggtatca acaaaaacca gggcaatctc ctaaattact gattttctgg
gcatccaccc ggcacgctgg agtccctgat cgcttcacag gcagtggatc
tgggacagat tttactctca ccatcagcag tgtgcaggct ggagacctgg
cactttatta ctgctcaaaa cattatagca caccgtacac attcggaggg
gggaccaagc tggaaataaa acgtacggtg gctgcaccat cggtcttcat
cctcccgcca tctgatgagc agttgaaatc tggaactgcc tctgttgtgt
gcctgctgaa taacttctat cccagagagg ccaaagtaca gtggaaggtg
gataacgcct tccaatcggg taactcccag gagagtgtca cagagcagga
cagcaaggac agaccttaca gcctcagcag tgtgcaggct ggagacctgg
gctctctcctc taactttcat cccagagagg ccaaagtaca gtggaaggtg
gggaccaagc tggaaataaa acgtacggtg gctgcaccat cggtcttcat
cctcccgcca tctgatgagc agttgaaatc tggaactgcc tctgttgtgt
gcctgctgaa taacttctat cccagagagg ccaaagtaca gtggaaggtg
gataacgcct tccaatcggg taactcccag gagagtgtca cagagcagga
cagcaaggac agaccttaca gcctcagcag tgtgcaggct ggagacctgg
gctctctcctc taactttcat cccagagagg ccaaagtaca gtggaaggtg
gggaccaagc tggaaataaa acgtacggtg gctgcaccat cggtcttcat
cctcccgcca tctgatgagc agttgaaatc tggaactgcc tctgttgtgt
gcctgctgaa taacttctat cccagagagg ccaaagtaca gtggaaggtg
gataacgcct tccaatcggg taactcccag gagagtgtca cagagcagga
cagcaaggac agaccttaca gcctcagcag tgtgcaggct ggagacctgg
gctctctcctc taactttcat cccagagagg ccaaagtaca gtggaaggtg
gggaccaagc tggaaataaa acgtacggtg gctgcaccat cggtcttcat
cctcccgcca tctgatgagc agttgaaatc tggaactgcc tctgttgtgt
gcctgctgaa taacttctat cccagagagg ccaaagtaca gtggaaggtg
gataacgcct tccaatcggg taactcccag gagagtgtca cagagcagga
cagcaaggac agaccttaca gcctcagcag tgtgcaggct ggagacctgg
gctctctcctc taactttcat cccagagagg ccaaagtaca gtggaaggtg
gggaccaagc tggaaataaa acgtacggtg gctgcaccat cggtcttcat
cctcccgcca tctgatgagc agttgaaatc tggaactgcc tctgttgtgt
gcctgctgaa taacttctat cccagagagg ccaaagtaca gtggaaggtg

N. EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1

[00396] An alternative EphA2 / CD3 / gpA33 Tri-Specific Binding Molecule was constructed. The molecule was composed of four polypeptide chains and comprises the VL and VH domains of EphA2 mAb 1, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of gpA33 mAb 1. The molecule was designated "EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:276):

DIQMTQMTSS LSALGLDRIT ISCRASQDIS NYLNWYQQKP DGTVKILLYY
TSRLHSGVPS RFSGSGSMTD YSLTISNLNEQ EDIATYFCQQ GYTLYTFGGG
TKLEIKGGS GGGEVQVLVE SGGGLVQPGG SLRLSCAASG FTFSFTAMNW
VRQAPGKGLE WVGRIRSKYN NYATYYADSV KGRFTISRDD SKNSLYLQMN
SLKTEDTAVY YCVRHNFGN SYVSFWAYWG QGTLVTVSSG GCGGGEVAAL
EKEVAALEKE VALEKEVAEE LEKGGGDKTH TCPCPAPEA AGGPSVFLFP
PKPKDILIMIS RTPEVTICVVV DVSHEDPEVK FWYWDVGEV HNATKFPREE
QYNSTYVRVS VTLTVHQDVL NGKEYCKKS VNKALPAPIEK TISKAKGQPR
EPQVYTLPPS REEMTKNQVS LWCLVKGFPY SDIAVEWESN GQPENNYKTT
PPVLDSDGKF FLYSKLTVDK SRWQQNVF V CSVMHEALHN HYTEQKSLSLSPGK
In SEQ ID NO:276, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 1 (SEQ ID NO:153), residues 107-114 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-245 correspond to the GCCGGG linker (SEQ ID NO:34), residues 246-273 correspond to an E-coil Domain (SEQ ID NO:39), residues 274-276 are the linker GGG, residues 277-286 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 287-503 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

A polynucleotide that encodes SEQ ID NO:276 is (SEQ ID NO:277):

```plaintext
gatatccaga tgacacagac tacatcctcc ctgtctgctt ctctgggaga
cagaatcacc atcagttgca gggcaagtcg ggcaggtgac gcagtggtgc
gactgatcga ttgatggttc gttcctgctg ttctgggagc
ttgacaggat tcgcaaggtc cggcctgctg cggcgtgtag
tcgtggtgat ggcggtcttc gctggtttaa
tggtattata ggcggtcttc cggcctgctg
tcggctgca cggcggcggc
tctgtgaatt gctctgattct
tgactgctct ccgcgctgct
tgctggactg ggcggtcttc gctggtttaa
tcgtggtgat ggcggtcttc
tggtattata ggcggtcttc
tcggctgca cggcggcggc
```

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The amino acid sequence of the second polypeptide chain of EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:278):

QAVVTQEPSSL TVSPGGTVTL TCRSTSGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRPAWT PARFSGSLLG GKAALTITGA QAEEDADYYC ALWYSNLWVF
GGGTLKTVLG GGGSGGGGQV QLKEBSPGLV APSQSLSTTC TVSGFSLSRY
SVHWVRQPPG KGLEWLGMWV GGSGTDYNSA LKSRLSISKD NSKSVFLLKM
NSLQTDDTAM YTCARKHGNY YTMDYWGGQT SVTVSUGGCC GGGVALKKE
VAALKEKVA LKEKVAALKE

In SEQ ID NO:278, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 1 (SEQ ID NO:158), residues 237-242 correspond to the linker GGCGGG (SEQ ID NO:34), and residues 243-270 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:278 is (SEQ ID NO:279):

caggctgtgg tgactcagga gccttcactg accgtgtccc caggcggaac
tgggaccctg acatgcagat ccagcacagg cgcagtgacc acatctaact
acgccaagtgc caaaggggcc tccctggacc cctgctcgg tctgctgggc
ggggtacaa acaaaagggc tccctggacc cctgctcgg tctgctgggc
tctgctgggc gggaaggccc tggcctggtg caggccgagg acgaagccga
taatatagtatg ggtggtggaa gcacagacta taattcagct cttaaatcca
gactgatagat cagcagagac aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca
ggggtacaa acaatcagat ccacagacag aatcactaagaa gcaagtttt cttaaaaatg
ccacagacta taattcagct cttaaatcca

The amino acid sequence of the third polypeptide chain of EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:280):

QVQLVQSGAE VKPQGASVK NEW SCKASGYTFT GSWMNWVRQA PQQGLEWIGR
IYPGDGETNY NGKFKDRVII TADKSTSTAY MELOSLRSED TAVYYCARIY
GNVYFDWV QGTTVTVSSA STKGPSVFPL APSSKSTSSG TAAALGCLVKD
YFPFEPVTIS NWSGALTSGVH TFPQVLQQSS LYSLSVVTVT PSSSLGTQTY
ICNVNHKPSN TKVDKRVEPK SCDKTHTCPP CPAEEAAAGP SVFLFPPKP
[00403] In SEQ ID NO:280, amino acid residues 1-119 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 120-217 correspond to a modified CHI Domain (SEQ ID NO:208), residues 218-232 correspond to a linker (SEQ ID NO:209), and residues 233-449 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

[00404] A polynucleotide that encodes SEQ ID NO:280 is (SEQ ID NO:281):

caggtccagc tggtccagag cggggccgaa gtcaaaaaac ccggagcaag
cgtgaaggtc tcctgcaaag catcaggcta tacatttaca ggcagctgga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
ccgaagacg cccttggtggg cagcggcgcct tgggtgctgt gctcaagagc
tacttccccc aaccggtgac ggtgtcgtgg aactcaggcg ccctgaccag
tcgccagct cccttggtgg gacccctcgg tgggctgcct ggtcaaggac
tctctgggggc acagcggccc tgggctgcct ggtcaaggac
tctccgccg ttccttcgcg cttcccagca gcttgggcac ccagacctac
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcaggaagct cattggctcag gttgtggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
tctcctccgg cccaccaagg gcccatcggt cttccccctg gcacccctct
cgaaccctca tgatctcccg gaccttcgag gtcacatgct gtttggtggga
tgtgaccatc acagccgata agtctactag taccgcctac atggagctga
tgaactgggt gaggcaggct ccaggacagg gactggagtg gatcgggcgc
[00406] In SEQ ID NO:282, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), and residues 107-213 correspond to the CL Kappa Domain (SEQ ID NO:210).

[00407] A polynucleotide that encodes SEQ ID NO:282 is (SEQ ID NO:283):

gaacattcagc tgactcagtc cccctctttt ctgtccgcat ccgtcggaga
tcgagtgact attacttgct ctgctaggtc ctcaatcagc ttcatgtact
aggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaactctgg aaatcaacgc tccctgacac cctccggggt gccatctcgg
ttgctgaataa ctgctaggtc ctcaatcagc ttcatgtact

O. EphA2 mAb 2 x CD3 mAb 2 x gpA33 mAb 1

[00408] A second alternative EphA2 / CD3 / gpA33 Tri-Specific Binding Molecule was constructed. The molecule was composed of four polypeptide chains and comprises the VL and VH domains of EphA2 mAb 2, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of gpA33 mAb 1. The molecule was designated "EphA2 mAb 2 x CD3 mAb 2 x gpA33 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:284):

DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSSGNYLHW YLQKPGQSPK
LLI YKVSNRF SGVDFRSFGS GSGTDFTLKI SRVEAEHDFV YFCSQSTHV
TFGSSTKLEI KGKGGSGSGGE VQLVESGGGL VPQGSRLRS CAASGFTFST
YAMNWRVQAP KGKLEGWVRI RSKNYNAYAT YADSVKGRFT ISRDDSKNSL
YLQMNSLKTE DTAVYYCVRH GNFGNSYVSW FAYWQGTLV TVSSGCGCGG
EVAALEKEVA ALEKEVAALG KEVALEKGK GDRKTHCPPC PAPEAAGGPS
VFLFPPKPKD TLMRTSRPVE TVVVDVSHE DPEVFKFNYV DGVEVNAKT
KPREQYNST YRVVSVTLVL HQDWNKVEY KCKVSNKALP APIEKTIKSA
KGQPREPQVY TLPSREEMT KNQVSLWCLV KGFPDPIDAV EWSNQQDEN
NYKTPPVVLG SDGSFFLYSK LTDKSRWQQ GNVFSCSVMH EALHNHYTQK
SLSLSRGK
[00409] In SEQ ID NO:284, amino acid residues 1-11 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 2 (SEQ ID NO:163), residues 112-119 correspond to the intervening spacer peptide GGGS (Linker 1) (SEQ ID NO:33), residues 120-244 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 245-250 correspond to the GGCGGG linker (SEQ ID NO:34), residues 251-278 correspond to an E-coil Domain (SEQ ID NO:39), residues 279-281 are the linker GGG, residues 282-291 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 292-508 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

[00410] A polynucleotide that encodes SEQ ID NO:284 is (SEQ ID NO:285):

gatgttgtgta tgacccaaac tccacttctct ctgctctgtca gtcttggaga
tcaagcctcc atctctttcc gattctgtca gacagtctga cagcactggg
gaaacaccta ttttaacttt gcattgatca cagcagctgg cagacagggg
tgacctttctt gatctgttgta cagtcctcttc cagactttctc ctgctcttct
gaggctactgt gcttttttctt caggtacagc aatcactgtctc acctgtctct
agctactgtga cagctctctct cagcactttct caggttgtgtg gatgttgaca
gagctttgaaa tctctctggg taaa
The amino acid sequence of the second polypeptide chain of EphA2 mAb
2 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO: 286):

QAVVTQEPSSL TVSPGGTVTL TCRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALTITGA QAEDADYYC ALWYSNLWVF
GGGKTLTVLG GGS5GGGGQI QLVQSGPELK KPGETVKSIC KAGSTFTTNY
GMNWVQKAPG KGLKQMGW IN TYIGEPTYAD DFKGRFVFSL ETSASTAYLQ
INNLKNEDMA TYFCARELGP YYFDYWGQGT TLTVSSGGCG GKKVAALKEK
VAALKEKVAA LKEKVAALKE

In SEQ ID NO: 286, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO: 104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO: 33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 2 (SEQ ID NO: 167), residues 237-242 correspond to the linker GGC6GG (SEQ ID NO: 34), and residues 243-270 are a K-coil Domain (SEQ ID NO: 40).

A polynucleotide that encodes SEQ ID NO: 286 is (SEQ ID NO: 287):
caggctgtgg tgacactcagga gccttccttg accgctgtccc cagccggaaac
ttgaccctgg acatgcagat ccagcagcag ccacgtagc acatctaatc
acgccaattgg ggtgcacagc aagccaggac aggcaccaag gggcctgatc
ggggtctcag taacaagctgcc tccctgagcc cctgcagcggct tttctggaag
tctggcgcttg gggaaggccg ggcctgacat taccggggc cagccggaaac
tggacagtgg acatatggtgc cattctgggc atagcatact ctgggtgtggc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc
gggggggtca gcaaaactgc gttcgtgtgg atagcaatct atgggtgtgctc

definition of the third polypeptide chain of EphA2 mAb 2
x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO: 288):
QVQLVQSGAE VVKPGASVTV SCKASGTYFT GSWMVNVRQA PGQGLEWIGR
IYPGDGETNY NGKFDRVTI TADKSTSTAY MELSSLRSED TAVYYYCARIY
GNVYFDWVG QTGTQTVSSA STKGPSVPFL APSSKSTSGG TAALGCVLKD
YFPEPVTWS NSGALTSGVH TFPAVLQSSG LYSLLSSVT TPSSSLGTQTY
ICNVNHKPSN TKVDRKEFK SCDKTHTCPP CPAPEAAGGP SVFLFPFPFPK

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In SEQ ID NO:288, amino acid residues 1-119 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 120-217 correspond to a modified CHI Domain (SEQ ID NO:208), residues 218-232 correspond to a linker (SEQ ID NO:209), and residues 233-449 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

A polynucleotide that encodes SEQ ID NO:288 is (SEQ ID NO:289):

caggtccagc tggtccagag ccggggccgaa gtcaaataac ccggagacaag
cgtaagctgc tcctgcaaaag catcaggcagc tacattaacc gcgcaggtga
tgaaactgacct ggccagcgtg ccaggacagc gactggatgc gatcgagggcgc
actcatctcg gacagccgag aacactaat attaaggact tcaagagcagc
tagcctgccg tccaccaaggg gcccatcgg tccctccctg gcaccctccct
ccaagacgag ctcctgagag aacagggcctg tgggtgctcg ccagacacagc
taccctccgc aacccggtgag ggtgtgctgg agtctagcag cccttgacagc
cggcgctgca acctccctcg ctgctctaca gctctcagga ctctactccc
tcaagccgct ggtgacgctg ccctcagacg tgggtgctcg ccagacacagc
atctgcaagc tgaatcacaag gccacagcagacaagttcg acaagaaggt

tgacacccaa tctctgcaac aaacctcaac atgccacacc tggccacacc
cacaagggcg ctcgactcttc tctctcccttc aaaaaccaag
gacacccctca tgaattcccc gcacccctcg gtcaactgcg tgggtgtggga
cgtaagccac ggaagccctg agtcaacttg ctacgtctac gttgagggcgcc
tggaggctga taatgccaga acaagccgcg cggagaggag cttacaacgtcg
gtacctgctg tggctgcagc cctccagcgc ctgacacagcg actgtgtggaa
tgacacaagc cagaagcctc tctccagccaa aacaagccct ccagccgccca
tcgagaaacac catctcccaaa gcaccaaggg acggcgcgag acacaggtg

tacacccctcg cccacccctg ggagagagct tcaagagcctc aggccgggca
gagttgctgc tctctccag gcacatcgcg gttgagtttgag

caggtgagcag caggggacag tctctctcag tctgctgctg tggagctgta

tgacacaagc caggggacag tctctctcag tctgctgctg tggagctgta

diamino acid sequence of the fourth polypeptide chain of EphA2 mAb 2 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:290):

DIQLTQSPSFLASAVGDRVTTITCSARSSISMHWYQQKPGKAPKLLIYDT
SNLASSVPSRFSGSGSGTSEFTLTISSLEAEAAATYYQQWSSYPLLTFQGG
TKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQQKWD

- 155 -
NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFN R GEC

[00418] In SEQ ID NO:290, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO: 181), and residues 107-213 correspond to the CL Kappa Domain (SEQ ID NO:210).

[00419] A polynucleotide that encodes SEQ ID NO:290 is (SEQ ID NO:291):

gacattcagc tgactcagtc cccccttttt ctgtccgcat ccgtcggaga
tcgagtgtct attaacctgt ctgcttggttc ctctcggctt

ggtatcagca gaagcccggc aagcaccta agctgctgat
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

tccttgtaata cttctatccc agagagcacc aagtacagcgg
tacaatactgg aatctcaagcg tacgtggttg ccacatgctg
tcagctaggtc ctcaatcagct tttcatgtacct
ggtatcagca gaagcccggc aaagcacctta gcctggccat

P. EphA2 mAb 3 x CD3 mAb 2 x gpA33 mAb 1

[00420] A third alternative EphA2 / CD3 / gpA33 Tri-Specific Binding Molecule was constructed. The molecule was composed of four polypeptide chains and comprises the VL and VH domains of EphA2 mAb 3, the VL and VH domains of antibody CD3 mAb 2 and the VL and VH domains of gpA33 mAb 1. The molecule was designated "EphA2 mAb 3 x CD3 mAb 2 x gpA33 mAb 1." The amino acid sequence of the first polypeptide chain of this Tri-Specific Binding Molecule is (SEQ ID NO:292):

DIVLTSQRSHS MSTSVGDRVN ITCKASQDTVT TAVAWYQQKP QGSPKLLIFW
ASTRHAGVPCP RFTGSGSGTD FTLSISSVQA SDLALYYCQQ HYSTPYTFGG
GTKLEIKGGS GGSSGEVQLV ESGGLVQPG GLSRPLSCAS GFTFSTYAMN
WVRQAPGKSQL EWVGRIRSKY NNYATYYADVS VAKRFTRISDS DSKNSLYLMQ
NSLKTEDAV YYCRHGNF GSYVSWFAYW GQGTLVTSSS GCGGGEVAA
LEKEVALEK EVALEEKEVA ALEKGGGDYT HCPCCPAPE AAGPSVLFL
PPKPKSDLILM SRTPETVPCTVY VDTSREDPEV KFNWYVMDGE VNHAHTKPRE
EQYNSTYRVV SVLTVLHQDW LNQEKYKCKV SNKKLAPAPIE KTISKAKQGP
REPQYTLLEP SREEMTKNOHD SLWCLLVKGFLY PSDIAVEWES NGSPENYKST
TPPVLDSDGSS FFLYSLKTVD KSRWQQGNVF SCFSVMHEALH NHYTQKSLSL
SPGK
In SEQ ID NO:292, amino acid residues 1-107 correspond to the amino acid sequence of the VL Domain of EphA2 mAb 3 (SEQ ID NO:172), residues 108-115 correspond to the intervening spacer peptide GGGSGGG (Linker 1) (SEQ ID NO:33), residues 116-240 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 241-246 correspond to the GCCGGG linker (SEQ ID NO:34), residues 247-274 correspond to an E-coil Domain (SEQ ID NO:39), residues 275-277 are the linker GGG, residues 278-287 are the linker DKTHTCPPCP (SEQ ID NO:48), and residues 288-504 are the "knob-bearing" CH2-CH3 Domain (SEQ ID NO:52).

A polynucleotide that encodes SEQ ID NO:292 is (SEQ ID NO:293):

gacattgtgc tgacccagtc tcacagatcc atgtccacat cagtaggaga
cagggtcaca atcactgctga aggccaaggga tggagcaggg
cctgtgatca aaaaatctca gggcgagctg tggagcgcgag
gcatccaccc ggccacgctgg gcttcagcag ggtcgagcag
tgggctgcac caggtcaggc gggcgagctg tggagcgcgag
gtgctactca ccatcagcag tgtgcagctg ggtcgagcag
tgggacagaac ggtgtctgctc ttttgagaag gaggtcgtgc

ggaggtgca gcctggagag aagccggcgg ggacaaact cacaatcgc
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cccccaccaac ccagacgac cactcatgatc cccgggagac ctggagcagc
atcgctgggtg gtggagcctg ggcacagaga cctgttggct aagtccatct

gctgcaagag cggggcttttgtgaggg gaggttcgctc ttttgagaag
ggaggtgca gcctggagag aagccggcgg ggacaaact cacaatcgc
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
cacctgccca gcggcggcgg cggcggcgg cggcggcgg
The amino acid sequence of the second polypeptide chain of EphA2 mAb 3 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:294):

QAVVTQEPSSL TVSPGGT VTL TCRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALITGA QAEDADYYC ALWYSNLWVF
GGGTTLVLG GGSSGGGGEV QLVESGGGSV KPGGLKLSC AASGFTFTDH
YMYWVRQTEPE KRLEWVRTAT DGGSFTSYPD SVKGRFTISR DIAKNNLYQ
MSSLKEDTA MYCYCTREDSE RFPFYWGQGT LVTVSSGCGG GKKVAALKE
VAALKKEKVA LKEKVAALKE

In SEQ ID NO:294, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 3 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGS (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the amino acid sequence of the VH Domain of EphA2 mAb 3 (SEQ ID NO:177), residues 237-242 correspond to the linker GGCG (SEQ ID NO:34), and residues 243-270 are a K-coil Domain (SEQ ID NO:40).

A polynucleotide that encodes SEQ ID NO:294 is (SEQ ID NO:295):

caggctgttg gtagctcagga gcccttcactg accgtgtccc caggccggaac
ttgacccctg acatgcagat ccaagccagcc cgcagtcgcc acatatcaact
acgccaatttg ggtgcacagc aagccaggac aggccaccaag gggcctgtatc
gggggttaca acaaaactgc tccttgagcc cctgcacggt tttctggaag
tctgctttgg gcaagcggcg ctctgactat cccctggacc tggccgttgc
acgaagccga ttactattgt gctcttgtgg atagcaatct gtgggtgttc
ggggttgcac caaaaactgc tgggtctcag taccggggca cagccgtagc
tggagaatgt cagcctgttg agtctggggt aggctcctgtg aagctctgag
agtctggggt tagctcctga cagacccagcc caggtgcttgct ggtgggtgct
aaccattagt gatggcgcgta gtttacacct catatccagac atgtgaagg
agccagtcct catcctcagg cagacctgca tagatctggc tggccttcag
atgagactgt ctaaagcttg ggcagcgcag agtgggtgct gattctgctg
ctggtcagct cagatgtctg gttgagaaag tggccggcctg aagccctgag
agtctgccagt cagctgccag tttactgggg ccaagggact tggctcactg
tcccttcggc aggatgttgc ggtggaagaa tgtgacccccag aagccctgag
ctggtcagct cagctgccag tttactgggg ccaagggact tggctcactg

The amino acid sequence of the third polypeptide chain of EphA2 mAb 3 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:296):

QVQLVQSGAE VKPQGSSKS KYFASGGTYF GGWSMNWVRQA PGQGLEWIGR
IYPGDGETNY NGKFKDRTVI TADKSTSTAY MELSSLRSED TAVYCYCRIY
GNNYFDVWG QTTYVTVSSA STKGPSVFPPL APEGSKTSQG TAAAGGLVJKD
YFPEPVTVSV NGSALTSVH TFPYAILQSSG LYSLLSVTVT PSSSLGTQTY
ICNVNHKPSN TKVDRKEFCK SCDKHTTCPP CPAPAEAGGP SVFLFPKPK

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[00427] In SEQ ID NO:296, amino acid residues 1-119 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (SEQ ID NO:186), residues 120-217 correspond to a modified CHI Domain (SEQ ID NO:208), residues 218-232 correspond to a linker (SEQ ID NO:209), and residues 233-449 correspond to the "hole-bearing" CH2-CH3 Domain (SEQ ID NO:53).

[00428] A polynucleotide that encodes SEQ ID NO:296 is (SEQ ID NO:297):

caggtccagc tggctccagag cggggccgaa gtcaaaaaac ccggagcaag
cgtgacacgc tcctgcgaag catcaggctg tataatttta gccagcaggg
tgaactctggt gaggcagct tcagagcagg gactgaggtg gatcgagggcgc
actacctctg gacagcggga aacaactaat aatggaagat tcgaagaccgc
agtcacaccac tctatggggc caacaggcccc ttgtgtctgt actgtaagcag
gctcctcgcc ggcacccagg gcctcctgct gttctccctgc gcacccctgct
tcagcagcct ggtgtcctcgt gcttcccaag gcctgcctcg ccaacccagg
tactttccgg acctccagct gattctccgg gcctctctgt ccctgctcag
ccgccgcgac atctctctcg tctcttctctgc ccaacccagg ccctgcctcg
tgacagcagc tcctcctcg gttcctccag gcctctctct gcctgcctcg
gagcctctgc tctctcctgc ccaacccagg ccctgcctcg ccaacccagg
tcgagctcag cagctcctcg tgtctcctcg tggctttcctgc ccaacccagg
tacccctcag tcccttttgc cttgcctgcag ccaacccagg ccctgcctcg
tagcctcctcg gctgtcttctg ccaacccagg ccctgcctcg ccaacccagg
tggccagcag tcctcctcct ggtgtcttgga cggtcctcctgc tggctttcctgc
tcgagctcag cagctcctcg gctgtcttctg ccaacccagg ccctgcctcg
tgacagcagc tcctcctcct ggtgtcttgga cggtcctcctgc tggctttcctgc

dcagcgtgttg ggtgtcttgga ggtgtcttgga cggtcctcctgc tggctttcctgc

dcagcgtgttg ggtgtcttgga ggtgtcttgga cggtcctcctgc tggctttcctgc

dcagcgtgttg ggtgtcttgga ggtgtcttgga cggtcctcctgc tggctttcctgc

[00429] The amino acid sequence of the fourth polypeptide chain of EphA2 mAb 3 x CD3 mAb 2 x gpA33 mAb 1 is (SEQ ID NO:298):

DIQLTQPSPSL LASAVGDRTV ITCSARSSIS FMYWYQQKPG KAPKLLIYDT
SNLASGVPSRS FSGSGSSTEF TLTISSELAEE DAATYYCQQW SSYPLTFQGQG
TKLEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYF REALKVQWKVD
NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL
SSPVTKSFNR GEC

[00430] In SEQ ID NO:298, amino acid residues 1-106 correspond to the amino acid sequence of the VL Domain of gpA33 mAb 1 (SEQ ID NO: 181), and residues 107-213 correspond to the CL Kappa Domain (SEQ ID NO:210).

[00431] A polynucleotide that encodes SEQ ID NO:298 is (SEQ ID NO:299):

```
gacattcagc tgactcagtc cccctctttt ctgtccgcat ccgtcggaga
tcgagtgact attacttgct ctgctaggtc ctcaatcagc ttcatgtact
ggtatcagca gaagcccggc aaagcaccta agctgctgat ctacgacaca
actaaacttg aaatcagcc tggctgcgtt aacgtgcttt gaaagccagc
```

IV. Reference Antibodies and Diabodies

[00432] In order to assist in the characterization of the Tri-Specific Binding Molecules of the present invention, the following reference diabodies were constructed.

Q. DR5 mAb 1 x CD3 mAb 2 Diabody

[00433] An exemplary bi-specific diabody composed of two polypeptide chains was constructed having the VL and VH domains of anti-human DR5 antibody DR5 mAb 1 and the VL and VH domains of CD3 mAb 2. The diabody was designated "DR5 mAb 1 x CD3 mAb 2 diabody." The amino acid sequence of the first polypeptide chain of this diabody is (SEQ ID NO: 140):

```
DIVLTQSPAS LAVSLGQRAT ISCRASKVS SSGYSYMHWY QQKPGQPPKV
LIFLSSNLDS GVPARFSGG SGTDFTLINH PVEDDAATY YCQHSRDLPF
TFGGGTKLEI KGGSGGSGGE VQLVESGGGL VQPGGSLRLS CAASTFST
YAMNWVRQAP GKGLEWVGRI RSKYNNATY YADSVKGRFT ISRDDSKNSL
YLQMNLSKTE DTAVYVCRHH GNFGNSYVSW FAYWGQGTLV TVSSASTKGE
VAACEKEVAA LEKEVAALEK EVAALEK
```
In SEQ ID NO:140, amino acid residues 1-11 correspond to the amino acid sequence of the VL Domain of DR5 mAb 1 (SEQ ID NO:3), residues 112-119 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 120-244 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 245-249 correspond to the ASTKG linker (SEQ ID NO:47) and residues 250-277 correspond to a cysteine-containing E-coil Domain (SEQ ID NO:41). A polynucleotide that encodes SEQ ID NO:140 is SEQ ID NO:141:

```
gacattgtgc tgacacagtG tcctgctttcc ttagctgtatat ctctcgggca
aggggcaccc atctctagcga gggccagcag aagtgaagtctctcgtgtgtc
atgtttatat gcactgtgtac caacagaaca cacagacagc acccaagtct
ctcatactttct aaaaaaaaagg ggggtctcttg cccagtttccag
aggggttcccact aagcttcattc tcttcacccct ctctcgagag
agtcatctga attggttctcc ccaagttcaca gggaagaggg gcgttaggtgtg
```

The amino acid sequence of the second polypeptide chain of the DR5 mAb 1 x CD3 mAb 2 diabody is (SEQ ID NO:142):

```
QAVVTQEPSL TVSPGGTVTL ICRSSTGAVT TSNYANWQQQ KPGQAPRGLI
GGTNKRAPWT PAFRSGLLGG GKAALTITGA QAEDEDYDF ALWYSNLWVF
GGGTCLTVLG GGGSGGGGGEV KFLESGLGGL VQPGSGLLSC VASGFDFSRY
\WMSWVRQAPG KGLEWIEGNE PDSNTINYTP SLKDKFI ISR DANKNTLYLQ
MTKVRSEDTAA LYYCTRRAAY GNPAPFAYWG QGTVLTVSAAL STKGVAACK
EKVAALKEKV AALKEVAA AL
```

In SEQ ID NO:142, amino acid residues 1-110 correspond to the amino acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 119-239 correspond to the amino acid sequence of the VH Domain of DR5 mAb 1 (SEQ ID NO:8), except that the C-terminal serine residue of SEQ ID NO:8 has been replaced with an alanine residue), residues 240-244 correspond to an
ASTKG linker (SEQ ID NO:47), and residues 245-272 correspond to a cysteine-containing K-coil Domain (SEQ ID NO:42). A polynucleotide that encodes SEQ ID NO:142 is SEQ ID NO:143:

caggtcgtgga tgacccagga gccttcactg accgtgtcct cagggcggaac
ttgaccccttg acatgcagat ccagccaggg cgaggtgcacc acatctact
cgacccatttg ggtgcagcag aacgcaagca gcgggacgag cggctgtatc
ggggttgca aaaaactgac tgtgtgagga gttgtgagat ccggcgccgg
aggcagagtg aagtttcctcg aaggctgagg tggcctgttg cagcctgag
agtctctctgaa atctctcctgt ttagccccag gattgcatat tagtagacat
tggatgagtt ggctccagca ggctccaggg aagggtctag aatggttagg
aaagaatatt catctcctaga gacaacgcct ccaaatagctgt atatctgcna
atgaccaagaa gtagacccag ggacacacga ctttttagtatg atacaagaga
ggctctctatt ggttaacccgg cctgtcttttc ttatctgagagr cagggacatc
tgtctctctat tcgcttcagc tccacacagc gcaggctgag cgcagacatc
gagagatttc tgtcttctgaa agagacagtc gcccacactta aggaagaggt
cgcagcccctg aaagag

R. DR5 mAb 2 x CD3 mAb 2 Diabody

[00437] An exemplary bi-specific diabody composed of two polypeptide chains was constructed having the VL and VH domains of anti-human DR5 antibody DR5 mAb 2 and the VL and VH domains of CD3 mAb 2. The diabody was designated "DR5 mAb 2 x CD3 mAb 2 diabody." The amino acid sequence of the first polypeptide chain of this diabody is (SEQ ID NO:144):

DIVMTQSHKF MSTSVGDRVS ITCKASQDVN TAVAAYQQKP GQSPKLIYYP
ASTRHTGVPD RFTGSSGSTD YTLITKSVQA EDLTLYCCQ HYITPHVGGY
GTKLEIKGGG SGGGGEVQLV ESSGGGLVQPG GSLRLSCAAS GFTFSTYAMNN
WVRQAPKGKL EWVGRIRSKY NNYATYYADS VKGRTFISRD DSKNSLYQMM
NSLKTEDTAV YYCVRHGNFG NSYVSFWFAYW GQGTLVTVSS ASTKGEVAAC
EKEVALEKE VAALEKEVAA LEK

[00438] In SEQ ID NO:144, amino acid residues 1-107 correspond to the amino acid sequence of the VL Domain of DR5 mAb 2 (SEQ ID NO:13), residues 108-115 correspond to intervening spacer peptide (Linker 1) (SEQ ID NO:33), residues 116-240 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 241-245 correspond to an ASTKG linker (SEQ ID NO:47) and residues 246-273 correspond to a cysteine-containing E-
coil Domain (SEQ ID NO:41). A polynucleotide that encodes SEQ ID NO:144 is
SEQ ID NO:145:

gacattgtga tgacccagtc ... ggaaaggccg ctctgactat taccggggca caggccgagg
acgaagccga ttactattgt gctctgtggt atagcaatct gtgggtgttc

[00439] The amino acid sequence of the second polypeptide chain of the DR5 mAb
2 x CD3 mAb 2 diabody is (SEQ ID NO:146):

QAVVTQEPSL TVSPGGTVTIL TCRSSTGAVT TSNYANWVQQ KPGQAPRLGI
GGTNKRAPWT PARFSGSLLLG GKAALTITGA QAEDDEAYYC ALWYSNLWVIF
GGGTKTIVLGG GGGSGGGGKV QLQQSGAELV KPGASVKLSC KSGQFTFEY
ILHWVQKWSG QGLEWIGWIFG PGNNINKYNE KFKDKATLTA DKSSSTVYMEE
LSRLTSEDSAA VYFCARHEQQ PGYFDYWQGQTTLTVSSAST KGKVAACKEK
VAALKEKVAALKE

[00440] In SEQ ID NO:146, amino acid residues 1-110 correspond to the amino
acid sequence of the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-
118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID
NO:33), residues 119-237 correspond to the amino acid sequence of the VH Domain
of DR5 mAb 2 (SEQ ID NO:18), residues 238-242 correspond to an ASTKG linker
(SEQ ID NO:47), and residues 243-270 correspond to a cysteine-containing K-coil
Domain (SEQ ID NO:42). A polynucleotide that encodes SEQ ID NO:146 is SEQ
ID NO:147:

caggtctgtgg tgactcagga gccttcactg accgtgtccc caggccggaac
tgtgacccctg atcagcagat ccagcacagc ccagctgacc acatcctaact
acgccaattct ggtgcagcag cagagccgta cagacagcat ccagcacagc
ggggtcaca acaaagggcg tccctggacc ccctgccagtc tgtgtggagt
ctgctgtggc ggtgcagcag cagagccgta cagacagcat ccagcacagc
**S. DR5 mAb 3 x CD3 mAb 2 Diabody**

[00441] An exemplary bi-specific diabody composed of two polypeptide chains was constructed having the VL and VH domains of anti-human DR5 antibody DR5 mAb 3 and the VL and VH domains of CD3 mAb 2. The amino acid sequence of the first polypeptide chain of the diabody had the sequence (SEQ ID NO:148) (CDR residues are shown underlined):

```
SLETPQDPAVS VALGQTVRIT CSGDSLRSYY ASWYQQKPGQ APVLVIYGAN
NRPSGI PDRF SGSSSGNTAS LTITGAQAED EADYCNASD SSGNHWFGG
GTKLVTGGG GSGGGGEVQL VESGGGLVQP GSSLRSCA SGFTFSYAM
NWVRQAPGK KLEWVRIRSK YNNVATYYAD SVKGRFTISR DDSKNSLYLQ
MNSLKTEDTA VYYCVRHGNF GNSVSWVFAY WGGQTLVTVS SASTKEVAA
CEKEVAALLEK EVAALKEVA ALEK
```

[00442] In SEQ ID NO:148, amino acid residues 1-108 correspond to the VL Domain of DR5 mAb 3 (SEQ ID NO:54), residues 109-116 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO:33), residues 117-241 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 242-246 correspond to an ASTKG linker (SEQ ID NO:47), and residues 247-275 correspond to a cysteine-containing K-coil Domain (SEQ ID NO:42).

[00443] The amino acid sequence of the second polypeptide chain of the diabody had the sequence (SEQ ID NO:149) (CDR residues are shown underlined):

```
QAVVTQEPSL TVSPGGTVIL TCRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSSLG GKAALTITGA QAEDADYYC ALWYSNLWVF
GGGTKLTVLG GGSGGGGGGEV QLVQSGGGVE RPPGGSLRSLC AASGFTFDDY
AMSWFQAPG KLEGWVSG IN WQGGSSTGYAD SVKGRFTISR DNAKNSLYLQ
```
In SEQ ID NO: 149, amino acid residues 1-110 correspond to the VL Domain of CD3 mAb 2 (SEQ ID NO: 104), residues 111-118 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO: 33), residues 119-239 correspond to the amino acid sequence of the VH Domain of DR5 mAb 3 (SEQ ID NO: 58), residues 240-244 correspond to an ASTKG linker (SEQ ID NO: 47), and residues 245-272 correspond to a cysteine-containing K-coil Domain (SEQ ID NO: 42).

T. DR5 mAb 4 x CD3 mAb 2 Diabody

An exemplary bi-specific diabody composed of two polypeptide chains was constructed having the VL and VH domains of anti-human DR5 antibody DR5 mAb 4 and the VL and VH domains of CD3 mAb 2. The amino acid sequence of the first polypeptide chain of the diabody had the sequence (SEQ ID NO: 150) (CDR residues are shown underlined):

EIVLTQSPGT LSLSPGERAT LS.RCASQGIS RSYLAWYQQK PFGQAPSLLY
GASSRATG 1P DRFSGSQGSGT DFTLTISRLE PEDFAVYYEQ QFGSSPWTFG
QGTRKVEIKG GSGGGEQVL VESGGGLVQP GGSRLSCAA SGFTFSTYAM
NWVRQAPGKG LEWVGRIRSK VNNVATYYAD SVKGRFT ISR DDDKNLSYLQ
MNSLKTEDTA VYCYVRHGF GNSY VSWFAY WQQGTLYTVS SASTKGEVAA
CEKEVAALEK EVAALKEKVA ALKEK

In SEQ ID NO: 150, amino acid residues 1-108 correspond to the VL Domain of DR5 mAb 4 (SEQ ID NO: 62), residues 109-116 correspond to the intervening spacer peptide GGGSGGGG (Linker 1) (SEQ ID NO: 33), residues 117-241 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO: 112), residues 242-246 correspond to an ASTKG linker (SEQ ID NO: 47), and residues 247-275 correspond to a cysteine-containing E-coil Domain (SEQ ID NO: 41).

The amino acid sequence of the second polypeptide chain of the diabody had the sequence (SEQ ID NO: 151) (CDR residues are shown underlined):

QAVVTQEPSL TVSPGGTVTL TCRSTGAVT TSNYANWYQQ KPGQAPRGLI
GGTNKRAPW7T PARFSQGLLG GKAALTITGA QAEDADYYC ALWYSNLWVF
GGTKLTVLGG GGGGGGGQV QLQESGPGLV KPSQTLSLTC TVSGGISISSG
In SEQ ID NO:151, amino acid residues 1-110 correspond to the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGS GGGS (Linker 1) (SEQ ID NO:33), residues 119-240 correspond to the amino acid sequence of the VH Domain of DR5 mAb 4 (SEQ ID NO:66), residues 241-245 correspond to an ASTKG linker (SEQ ID NO:47), and residues 246-273 correspond to a cysteine-containing K-coil Domain (SEQ ID NO:42).

Reference gpA33 x CD3 mAb 2 Dibody

To further exemplify the bi-specific Tri-Specific Binding Molecules of the present invention, a diabody composed of two polypeptide chains was constructed using the VL and VH domains of gpA33 mAb 1 and CD3 mAb 2. The amino acid sequence of the first polypeptide chain of the diabody had the sequence (SEQ ID NO:316) (CDR residues are shown underlined):

```
DIQLTQSPSFLASVGDRVTVTCSARSSISFMYWYQQKPGKAPKLLIYDT
SNLASGVPSTRFSGSGSTLEFDTLTISSLLEEDAATYQCQWSYYPLTFQGG
TKLEIKGGGSGGGGEVQLVESGGLVQPGGSLRSLCAASGFTFSTYAMN
VRQAPGKGLEWVGRIRSKNYATYYADSVKGRFTISRDDSNSLYLQMN
SLKTEDTAYVYCVRHGNGFNYSYVSFWFAYWGQTLVTVSSASTKGEVAACE
KEVAALEKEVAAALEKEVALEEK
```

In SEQ ID NO:316, amino acid residues 1-106 correspond to the VL Domain of gpA33 mAb 1 (SEQ ID NO:181), residues 107-114 correspond to the intervening spacer peptide GGGS GGGS (Linker 1) (SEQ ID NO:33), residues 115-239 correspond to the amino acid sequence of the VH Domain of CD3 mAb 2 having the D65G substitution (SEQ ID NO:112), residues 240-244 correspond to an ASTKG linker (SEQ ID NO:47), and residues 245-272 correspond to a cysteine-containing E-coil Domain (SEQ ID NO:41).

The amino acid sequence of the second polypeptide chain of the diabody had the sequence (SEQ ID NO:317) (CDR residues are shown underlined):

```
QAVVTVQEPSTVSPGGTVTTLTCTRSSGAVTTSSNYANLQQ
GQTNKRAPWTPARFSGSLLGKGAALTIQGAADEADYYCERALWYSMLNWVF
```
In **SEQ ID NO:317**, amino acid residues 1-110 correspond to the VL Domain of CD3 mAb 2 (**SEQ ID NO:104**), residues 111-118 correspond to the intervening spacer peptide GGGS GGGS (Linker 1) (**SEQ ID NO:33**), residues 119-237 correspond to the amino acid sequence of the VH Domain of gpA33 mAb 1 (**SEQ ID NO:186**), residues 238-242 correspond to an ASTKG linker (**SEQ ID NO:47**), and residues 243-270 correspond to a cysteine-containing K-coil Domain (**SEQ ID NO:42**).

**V. Reference Anti-Fluorescein Antibody**

The anti-fluorescein antibody 4-4-20 (Gruber, M. et al. (1994) "Efficient Tumor Cell Lysis Mediated By A Bi-specific Single Chain Antibody Expressed In Escherichia coli," J. Immunol. 152(1):5368-5374; Bedzyk, W.D. et al. (1989) "Comparison Of Variable Region Primary Structures Within An Anti-Fluorescein Idiotype Family," J. Biol. Chem. 264(3): 1565-1569) was used in control diabodies. The amino acid sequences of the variable light and variable heavy Domains of anti-fluorescein antibody 4-4-20 are as follows:

**V. Methods of Production**

The Tri-Specific Binding Molecules of the present invention can be created from the polynucleotides and/or sequences of antibodies that are
immunospecific for DR5, a desired Cancer Antigen, and a desired Effector Cell by methods known in the art, for example, synthetically or recombinantly. One method of producing such peptide agonists, antagonists and modulators involves chemical synthesis of the polypeptide, followed by treatment under oxidizing conditions appropriate to obtain the native conformation, that is, the correct disulfide bond linkages. This can be accomplished using methodologies well-known to those skilled in the art (see, e.g., Kelley, R. F. et al. (1990) In: GENETIC ENGINEERING PRINCIPLES AND METHODS, Setlow, J.K. Ed., Plenum Press, N.Y., vol. 12, pp 1-19; Stewart, J.M et al. (1984) SOLID PHASE PEPTIDE SYNTHESIS, Pierce Chemical Co., Rockford, IL; see also United States Patents Nos. 4,105,603; 3,972,859; 3,842,067; and 3,862,925).


[00458] In yet another alternative, suitable antibodies having one or more of the CDRs of a desired anti-DR5 antibody, anti-Cancer Antigen antibody or anti-Effector Cell antibody may be obtained through the use of commercially available mice that have been engineered to express specific human immunoglobulin proteins. Transgenic animals that are designed to produce a more desirable (e.g., fully human antibodies) or more robust immune response may also be used for generation of humanized or human antibodies. Examples of such technology are XENOMOUSE™ (Abgenix, Inc., Fremont, CA) and HUMAB-MOUSE ® and TC MOUSE™ (both from Medarex, Inc., Princeton, NJ).

[00459] In an alternative, antibodies may be made recombinantly and expressed using any method known in the art. Antibodies may be made recombinantly by first isolating the antibodies made from host animals, obtaining the gene sequence, and using the gene sequence to express the antibody recombinantly in host cells (e.g.,
CHO cells). Another method that may be employed is to express the antibody sequence in plants (e.g., tobacco) or transgenic milk. Suitable methods for expressing antibodies recombinantly in plants or milk have been disclosed (see, for example, Peeters et al. (2001) "Production Of Antibodies And Antibody Fragments In Plants," Vaccine 19:2756; Lonberg, N. et al. (1995) "Human Antibodies From Transgenic Mice," Int. Rev. Immunol 13:65-93; and Pollock et al. (1999) "Transgenic Milk As A Method For The Production Of Recombinant Antibodies," J. Immunol Methods 231:147-157). Suitable methods for making derivatives of antibodies, e.g., humanized, single-chain, etc. are known in the art. In another alternative, antibodies may be made recombinantly by phage display technology (see, for example, U.S. Patent Nos. 5,565,332; 5,580,717; 5,733,743; 6,265,150; and Winter, G. et al. (1994) "Making Antibodies By Phage Display Technology," Annu. Rev. Immunol. 12:433-455).

[00460] The antibodies or protein of interest may be subjected to sequencing by Edman degradation, which is well-known to those of skill in the art. The peptide information generated from mass spectrometry or Edman degradation can be used to design probes or primers that are used to clone the protein of interest.

[00461] An alternative method of cloning the protein of interest is by "panning" using purified DR5, and/or a desired Cancer Antigen, and/or a molecule expressed on the surface of a desired Effector Cell (or portions of any such molecules), for cells expressing an antibody or protein of interest that possesses one or more CDRs so as to be capable of binding to DR5, or such desired Cancer Antigen or Effector Cell molecule. The "panning" procedure may be conducted by obtaining a cDNA library from tissues or cells that express DR5, overexpressing the cDNAs in a second cell type, and screening the transfected cells of the second cell type for a specific binding to DR5 in the presence or absence of a known antibody that is capable of binding to such molecule (e.g., DR5 mAb 1 or DR5 mAb 2 in the case of panning for new anti-DR5 antibodies, etc.). Detailed descriptions of the methods used in cloning mammalian genes coding for cell surface proteins by "panning" can be found in the art (see, for example, Aruffo, A. et al. (1987) "Molecular Cloning Of A CD28 cDNA By A High-Efficiency COS Cell Expression System," Proc. Natl. Acad. Sci. (U.S.A.)

[00462] Vectors containing polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (e.g., where the vector is an infectious agent such as vaccinia virus). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

[00463] Any host cells capable of overexpressing heterologous DNAs can be used for the purpose of isolating the genes encoding the antibody, polypeptide or protein of interest. Non-limiting examples of suitable mammalian host cells include but are not limited to COS, HeLa, and CHO cells. Preferably, the host cells express the cDNAs at a level of about 5-fold higher, more preferably 10-fold higher, even more preferably 20-fold higher than that of the corresponding endogenous antibody or protein of interest, if present, in the host cells. Screening the host cells for a specific binding to DR5 is effected by an immunoassay or FACS. A cell overexpressing the antibody or protein of interest can be identified.

[00464] The invention includes polypeptides comprising an amino acid sequence of the antibodies of this invention. The polypeptides of this invention can be made by procedures known in the art. The polypeptides can be produced by proteolytic or other degradation of the antibodies, by recombinant methods (i.e., single or fusion polypeptides) as described above or by chemical synthesis. Polypeptides of the antibodies, especially shorter polypeptides up to about 50 amino acids, are conveniently made by chemical synthesis. Methods of chemical synthesis are known in the art and are commercially available. For example, an anti-DR5 polypeptide could be produced by an automated polypeptide synthesizer employing the solid phase method.
The invention includes modifications to any such antibodies (or to any of their polypeptide fragments that bind to DR5, the Cancer Antigen or the effector cell, as the case may be) and the agonists, antagonists, and modulators of such molecules, including functionally equivalent antibodies and fusion polypeptides that do not significantly affect the properties of such molecules as well as variants that have enhanced or decreased activity. Modification of polypeptides is routine practice in the art and need not be described in detail herein. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity, or use of chemical analogs. Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; serine/threonine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; lysine/arginine; and phenylalanine/tyrosine. These polypeptides also include glycosylated and non-glycosylated polypeptides, as well as polypeptides with other post-translational modifications, such as, for example, glycosylation with different sugars, acetylation, and phosphorylation. Preferably, the amino acid substitutions would be conservative, i.e., the substituted amino acid would possess similar chemical properties as that of the original amino acid. Such conservative substitutions are known in the art, and examples have been provided above. Amino acid modifications can range from changing or modifying one or more amino acids to complete redesign of a region, such as the variable region. Changes in the variable region can alter binding affinity and/or specificity. Other methods of modification include using coupling techniques known in the art, including, but not limited to, enzymatic means, oxidative substitution and chelation. Modifications can be used, for example, for attachment of labels for immunoassay, such as the attachment of radioactive moieties for radioimmunoassay. Modified polypeptides are made using established procedures in the art and can be screened using standard assays known in the art.

The invention encompasses fusion proteins comprising one or more of the polypeptides of this invention. In one embodiment, a fusion polypeptide is provided that comprises a light chain, a heavy chain or both a light and heavy chain. In another embodiment, the fusion polypeptide contains a heterologous immunoglobulin
constant region. In another embodiment, the fusion polypeptide contains a light chain variable region and a heavy chain variable region of an antibody produced from a publicly-deposited hybridoma. For purposes of this invention, an antibody fusion protein contains one or more polypeptide domains that specifically bind to DR5, a Cancer Antigen, or an effector cell (as the case may be) and another amino acid sequence to which it is not attached in the native molecule, for example, a heterologous sequence or a homologous sequence from another region.

VI. Uses of the Tri-Specific Binding Molecules of the Present Invention

[00467] The Tri-Specific Binding Molecules of the present invention provide a general therapy for cancer. The cancers that may be treated by such molecules include cancers characterized by the presence of a cancer cell selected from the group consisting of a cell of: an adrenal gland tumor, an AIDS-associated cancer, an alveolar soft part sarcoma, an astrocytic tumor, bladder cancer, bone cancer, a brain and spinal cord cancer, a metastatic brain tumor, a breast cancer, a carotid body tumors, a cervical cancer, a chondrosarcoma, a chordoma, a chromophobe renal cell carcinoma, a clear cell carcinoma, a colon cancer, a colorectal cancer, a cutaneous benign fibrous histiocytoma, a desmoplastic small round cell tumor, an ependymoma, a Ewing's tumor, an extraskeletal myxoid chondrosarcoma, a fibrogenesis imperfecta ossium, a fibrous dysplasia of the bone, a gallbladder or bile duct cancer, gastric cancer, a gestational trophoblastic disease, a germ cell tumor, a head and neck cancer, hepatocellular carcinoma, an islet cell tumor, a Kaposi’s Sarcoma, a kidney cancer, a leukemia, a lipoma/benign lipomatous tumor, a liposarcoma/malignant lipomatous tumor, a liver cancer, a lymphoma, a lung cancer, a medulloblastoma, a melanoma, a menigioma, a multiple endocrine neoplasia, a multiple myeloma, a myelodysplasia syndrome, a neuroblastoma, a neuroendocrine tumors, an ovarian cancer, a pancreatic cancer, a papillary thyroid carcinoma, a parathyroid tumor, a pediatric cancer, a peripheral nerve sheath tumor, a phaeochromocytoma, a pituitary tumor, a prostate cancer, a posterior uveal melanoma, a rare hematologic disorder, a renal metastatic cancer, a rhabdoid tumor, a rhabdomyosarcoma, a sarcoma, a skin cancer, a soft-tissue sarcoma, a squamous cell cancer, a stomach cancer, a synovial sarcoma, a testicular cancer, a thymic carcinoma, a thymoma, a thyroid metastatic cancer, and a uterine
cancer. The Tri-Specific Binding Molecules of the present invention may be used in the treatment of colorectal cancer, hepatocellular carcinoma, glioma, kidney cancer, breast cancer, multiple myeloma, bladder cancer, neuroblastoma; sarcoma, non-Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer, pancreatic cancer and rectal cancer.

[00468] The Tri-Specific Binding Molecules of the present invention augment the cancer therapy provided by an antibody directed to a Cancer Antigen that is characteristic of cells of a target tumor by being additionally able to bind to DR5 molecules arrayed on the surface of such tumor cells. The utility of the invention is particularly seen in circumstances in which the density of the Cancer Antigen is low, or when the binding kinetics of the anti-Cancer Antigen antibody is suboptimal (or insufficient) to promote a clinically sufficient therapeutic response. In such cases, the ability of the molecules of the present invention to bind both the Cancer Antigen and DR5 of the tumor cells provides enhanced binding (via avidity) that is sufficient to promote a clinically sufficient therapeutic response. Additionally, by also possessing a Binding Domain capable of binding to a molecule on the surface of an immune system effector cell, the Tri-Specific Binding Molecules of the present invention permit the co-localization of such immune system cells to the tumor cells, thereby promoting a cytotoxic response against the tumor cells via redirected killing.

[00469] As shown in Table 2, Tri-Specific Binding Molecules of the present invention that possess particular combinations of Cancer Antigen-Binding Domains have preferred utility in the treatment of specific cancers.

<table>
<thead>
<tr>
<th>Cancer Antigen-Binding Domains</th>
<th>Preferred Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>gpA33</td>
<td>DR5</td>
</tr>
<tr>
<td>gpA33</td>
<td>EphA2</td>
</tr>
<tr>
<td>gpA33</td>
<td>B7-H3</td>
</tr>
<tr>
<td>gpA33</td>
<td>BST2</td>
</tr>
<tr>
<td>5T4</td>
<td>EphA2</td>
</tr>
<tr>
<td>5T4</td>
<td>CEACAM5</td>
</tr>
<tr>
<td>5T4</td>
<td>B7-H3</td>
</tr>
<tr>
<td>5T4</td>
<td>DR5</td>
</tr>
<tr>
<td>B7-H3</td>
<td>CEACAM5</td>
</tr>
<tr>
<td>B7-H3</td>
<td>CEACAM6</td>
</tr>
</tbody>
</table>
In addition to their utility in therapy, the Tri-Specific Binding Molecules of the present invention may be detectably labeled and used in the diagnosis of cancer or in the imaging of tumors and tumor cells.

VII. Pharmaceutical Compositions

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 Tri-Specific Binding Molecules of the present invention, or a combination of such agents and a pharmaceutically acceptable carrier. Preferably, compositions of the invention comprise a prophylactically or therapeutically effective amount of the Tri-Specific Binding Molecules of the present invention and a pharmaceutically acceptable carrier. The invention particularly encompasses such pharmaceutical compositions in which the Tri-Specific Binding Molecule has a DR5-Binding Domain of:

1. a DR5 mAb 1 antibody;
2. a DR5 mAb 2 antibody;
3. a DR5 mAb 3 antibody;
4. a DR5 mAb 4 antibody;
5. a DR5 mAb 5 antibody;
6. a DR5 mAb 6 antibody;
7. a DR5 mAb 7 antibody; or

<table>
<thead>
<tr>
<th>Cancer Antigen-Binding Domains</th>
<th>Preferred Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-H3</td>
<td>IL1Ra2</td>
</tr>
<tr>
<td>EphA2</td>
<td>IL1Ra2</td>
</tr>
<tr>
<td>EphA2</td>
<td>DR5</td>
</tr>
<tr>
<td>EphA2</td>
<td>CEACAM5</td>
</tr>
<tr>
<td>EphA2</td>
<td>CEACAM6</td>
</tr>
<tr>
<td>ITGβ6</td>
<td>B7-H3</td>
</tr>
<tr>
<td>ITGβ6</td>
<td>DR5</td>
</tr>
<tr>
<td>ITGβ6</td>
<td>BST2</td>
</tr>
<tr>
<td>BST2</td>
<td>CEACAM5</td>
</tr>
<tr>
<td>BST2</td>
<td>EGFR</td>
</tr>
</tbody>
</table>

Broadly Applicable To Treatment Of Many Types Of Cancers
(8) a DR5 mAb 8 antibody (or a humanized derivative of any such antibodies).

[00472] The invention further particularly encompasses such pharmaceutical compositions in which the Tri-Specific Binding Molecule has a Cancer Antigen-Binding Domain that:

(A) binds to an epitope of EphA2, especially wherein the Tri-Specific Binding Molecule has a Cancer Antigen-Binding Domain of EphA2 mAb 1, EphA2 mAb 2 or EphA2 mAb 3, or a humanized or chimeric variant thereof; or

(B) binds to an epitope of gpA33, especially wherein the Tri-Specific Binding Molecule has a Cancer Antigen-Binding Domain of gpA33 mAb 1, or a humanized or chimeric variant thereof; or

(C) binds to an epitope of Her2, especially wherein the Tri-Specific Binding Molecule has a Cancer Antigen-Binding Domain of Her2 mAb 1 or trastuzumab, or a humanized or chimeric variant thereof; or

(D) binds to an epitope of B7-H3, especially wherein the Tri-Specific Binding Molecule has a Cancer Antigen-Binding Domain of B7-H3 mAb 1, B7-H3 mAb 2, or B7-H3 mAb 3, or a humanized or chimeric variant thereof.

[00473] The invention further particularly encompasses such pharmaceutical compositions in which the Tri-Specific Binding Molecule has an Effector Cell-Binding Domain that binds to CD2, CD3, CD17, CD20, CD22, CD32B, CD64, BCR/CD79, the T cell Receptor or the NKG2D Receptor. The invention further particularly encompasses such pharmaceutical compositions in which the Tri-Specific Binding Molecule has an Effector Cell-Binding Domain of antibody: Lo-CD2a, CD3 mAb 2, OKT3, 3G8, A9, HD37, rituximab, epratuzumab, CD32B mAb 1, CD64 mAb 1, CD79 mAb 1, BMA 031, KYK-1.0, or KYK-2.0.

[00474] The invention specifically contemplates Tri-Specific Binding Molecules, pharmaceutical compositions that comprise such binding molecule and uses of such Tri-Specific Binding Molecules, in which:
[00475] The invention further specifically contemplates Tri-Specific Binding Molecules, pharmaceutical compositions that comprise such binding molecule and uses of such Tri-Specific Binding Molecules, in which:

(1) the DR5 Binding Domain is a DR5 binding domain of any anti-DR5 antibody;
(2) the Cancer Binding Domain is any of: EphA1, gpA33, Her2, or B7-H3;
and
(3) the Effector Cell-Binding Domains binds to any of CD2, CD3, CD17, CD20, CD22, CD32B, CD64, BCR/CD79, the T cell Receptor or the NKG2D Receptor.

[00476] The invention particularly contemplates each of the Tri-Specific Binding Molecules, as well as pharmaceutical compositions that comprise such binding molecule and uses of such Tri-Specific Binding Molecules, in which:

(1) the DR5 Binding Domain is a DR5 binding domain of any of: a DR5 mAb 1 antibody, a DR5 mAb 2 antibody, a DR5 mAb 3 antibody, a DR5 mAb 4 antibody, a DR5 mAb 5 antibody, a DR5 mAb 6 antibody, a DR5 mAb 7 antibody, or a DR5 mAb 8 antibody;
(2) the Cancer Antigen-Binding Domain is a binding domain of any of: EphA2 mAb 1, EphA2 mAb 2, EphA2 mAb 3, gpA33 mAb 1, Her2 mAb 1, trastuzumab, B7-H3 mAb 1, B7-H3 mAb 2, or B7-H3 mAb 3;
and
(3) the Effector Cell-Binding Domain is a binding domain of any of: Lo-CD2a, CD3 mAb 2, OKT3, 3G8, A9, HD37, rituximab, epratuzumab,
CD32B mAb 1, CD64 mAb 1, CD79 mAb 1, BMA 031, KYK-1.0, or KYK-2.0.

As 8 anti-DR5 Binding Domain antibodies, 9 anti-Cancer Antigen-Binding Domain antibodies and 14 anti-Effector Cell-Binding Domain antibodies are listed, such specific contemplation encompasses all (8 x 9 x 14 =) 1,008 combinations of such binding domains.

[00477] The invention also encompasses such pharmaceutical compositions that additionally include a second therapeutic antibody (e.g., tumor-specific monoclonal antibody) that is specific for a particular cancer antigen, and a pharmaceutically acceptable carrier.

[00478] 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.

[00479] 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.

[00480] 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.

[00481] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with a Tri-Specific Binding Molecule of the present invention (and more preferably, any of the specific binding molecules discussed or exemplified above). 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.

[00482] The present invention provides kits that can be used in the above methods. A kit can comprise any of the Tri-Specific Binding Molecules of the present invention. The kit can further comprise one or more other prophylactic and/or therapeutic agents useful for the treatment of cancer, in one or more containers; and/or the kit can further comprise one or more cytotoxic antibodies that bind one or more cancer antigens associated with 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.

VIII. Methods of Administration

[00483] 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 fusion protein or a conjugated molecule of the invention, or a pharmaceutical composition comprising a fusion protein or a conjugated molecule 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.

[00484] 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.

[00485] Methods of administering a molecule of the 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 Tri-Specific Binding Molecules of the present 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.

[00486] The invention also provides that the Tri-Specific Binding Molecules of the present invention are packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of the molecule. In one embodiment, such molecules 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 Tri-Specific Binding Molecules of the present 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.

[00487] The lyophilized Tri-Specific Binding Molecules of the present 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, such molecules are supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the molecule, fusion protein, or conjugated molecule. Preferably, such Tri-Specific Binding Molecules when provided in liquid form are 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.

[00488] The amount of the composition 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.

[00489] For the Tri-Specific Binding Molecules monovalent diabodies encompassed by the invention, the dosage administered to a patient is preferably determined based upon the body weight (kg) of the recipient subject. The dosage administered is typically from at least about 0.3 ng/kg per day to about 0.9 ng/kg per day, from at least about 1 ng/kg per day to about 3 ng/kg per day, from at least about 3 ng/kg per day to about 9 ng/kg per day, from at least about 10 ng/kg per day to about 30 ng/kg per day, from at least about 30 ng/kg per day to about 90 ng/kg per day, from at least about 100 ng/kg per day to about 300 ng/kg per day, from at least about 200 ng/kg per day to about 600 ng/kg per day, from at least about 300 ng/kg per day to about 900 ng/kg per day, from at least about 400 ng/kg per day to about 800 ng/kg per day, from at least about 500 ng/kg per day to about 1000 ng/kg per day, from at least about 600 ng/kg per day to about 1000 ng/kg per day, from at least about 700 ng/kg per day to about 1000 ng/kg per day, from at least about 800 ng/kg per day to about 1000 ng/kg per day, from at least about 900 ng/kg per day to about 1000 ng/kg per day, or at least about 1,000 ng/kg per day.

[00490] In another embodiment, the patient is administered a treatment regimen comprising one or more doses of such prophylactically or therapeutically effective amount of a Tri-Specific Binding Molecule of the present invention, wherein the treatment regimen is administered over 2 days, 3 days, 4 days, 5 days, 6 days or 7 days. In certain embodiments, the treatment regimen comprises intermittently administering doses of the prophylactically or therapeutically effective amount of the Tri-Specific Binding Molecules of the present invention (for example, administering a dose on day 1, day 2, day 3 and day 4 of a given week and not administering doses of the prophylactically or therapeutically effective amount of the Tri-Specific Binding Molecule

[00491] Especially encompassed is the administration of such Tri-Specific Binding Molecules that comprise any of the specific combinations of DR5 Binding Domains,
Cancer Antigen-Binding Domains and Effector Cell-Binding Domains discussed above, on day 5, day 6 and day 7 of the same week). Typically, there are 1, 2, 3, 4, 5 or more courses of treatment. Each course may be the same regimen or a different regimen.

[00492] In another embodiment, the administered dose escalates over the first quarter, first half or first two-thirds or three-quarters of the regimen(s) (e.g. over the first, second, or third regimens of a 4 course treatment) until the daily prophylactically or therapeutically effective amount of the Tri-Specific Binding Molecule is achieved. Table 3 provides 5 examples of different dosing regimens described above for a typical course of treatment.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Day</th>
<th>Diabody Dosage (ng diabody per kg subject weight per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 2, 3, 4</td>
<td>100 100 100 100</td>
</tr>
<tr>
<td></td>
<td>5, 6, 7</td>
<td>none none none none</td>
</tr>
<tr>
<td>2</td>
<td>1, 2, 3, 4</td>
<td>300 500 700 900 1,000</td>
</tr>
<tr>
<td></td>
<td>5, 6, 7</td>
<td>none none none none</td>
</tr>
<tr>
<td>3</td>
<td>1, 2, 3, 4</td>
<td>300 500 700 900 1,000</td>
</tr>
<tr>
<td></td>
<td>5, 6, 7</td>
<td>none none none none</td>
</tr>
<tr>
<td>4</td>
<td>1, 2, 3, 4</td>
<td>300 500 700 900 1,000</td>
</tr>
<tr>
<td></td>
<td>5, 6, 7</td>
<td>none none none none</td>
</tr>
</tbody>
</table>

[00493] The dosage and frequency of administration of a Tri-Specific Binding Molecule of the present invention may be reduced or altered by enhancing uptake and tissue penetration of the molecule by modifications such as, for example, lipidation.

[00494] The dosage of a Tri-Specific Binding Molecule of the invention administered to a patient may be calculated for use as a single agent therapy. Alternatively, the molecule may be used in combination with other therapeutic compositions and the dosage administered to a patient are lower than when said molecules are used as a single agent therapy.

[00495] The pharmaceutical compositions of the invention may be administered 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.


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): 105-1 12; 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. 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)).

Polymeric compositions useful as controlled-release implants can be 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. A non-polymeric sustained delivery system can be 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).

[00498] 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

[00499] Where the composition of the invention is a nucleic acid encoding a Tri-Specific Binding Molecule of the present invention, the nucleic acid can be administered in vivo to promote expression of its encoded Tri-Specific Binding Molecule 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.

[00500] Treatment of a subject with a therapeutically or prophylactically effective amount of a Tri-Specific Binding Molecule of the present invention can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with such a diabody 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. The pharmaceutical compositions of the invention can be administered once a day, twice a day, or three times a day. Alternatively, the pharmaceutical compositions can be 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.

Examples:

[00501] 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. It will be apparent to those skilled in the art that many modifications, both to materials and methods, can be practiced without departing from the scope of the present disclosure.

W. Example 1: Characterization of Anti-Human DR5 Monoclonal Antibodies DR5 mAb 1 and DR5 mAb 2

[00502] Two monoclonal antibodies were isolated as being capable of immunospecifically binding to human DR5, and accorded the designations "DR5 mAb 1" and "DR5 mAb 2". As discussed above, the CDRs of these antibodies were found to differ. In order to determine whether the antibodies bound to different DR5 epitopes, a human DR5-Fc fusion protein was prepared and was coated to an immobilized surface. DR5 mAb 1 (1 µg/mL) was biotinylated and incubated with either a control IgG or with DR5 mAb 2 (10 µg/mL), and the ability of the IgG or DR5 mAb 2 antibody to compete for binding (to human DR5-Fc fusion protein) with DR5 mAb 1 was assessed by measuring the amount of immobilized biotinylated antibody. Additionally, the ability of the IgG or DR5 mAb 1 antibody to compete for binding with biotinylated DR5 mAb 2 was assessed. The results of this experiment are shown in Table 4.

<table>
<thead>
<tr>
<th>1 µg/mL DR5-Fc Fusion coat</th>
<th>10 µg/mL Competitor mAb</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mIgG</td>
<td>DR5 mAb 1</td>
</tr>
<tr>
<td>1 µg/mL biotinylated DR5 mAb</td>
<td>DR5 mAb 1</td>
<td>2.162</td>
</tr>
<tr>
<td></td>
<td>DR5 mAb 2</td>
<td>2.102</td>
</tr>
</tbody>
</table>
The results of this experiment indicate that the biotinylated antibody was capable of binding to the DR5 protein even in the presence of excess amounts of the non-biotinylated antibody. Thus, the results show that DR5 mAb 1 and DR5 mAb 2 bind to different epitopes of DR5.

In order to further characterize the DR5 mAb 1 and DR5 mAb 2 antibodies, their ability to block binding between DR5 and the TRAIL ligand as assessed. Thus, biotinylated DR5 mAb 1, biotinylated DR5 mAb 2 or biotinylated DR5-Fc fusion (each at 2 µg/mL) were separately incubated with immobilized DR5-Fc fusion (1 µg/mL) in the presence of either buffer or histidine tagged TRAIL (20 µg/mL). The amount of immobilized biotinylated antibody was assessed. The results of this experiment are shown in Table 5.

<table>
<thead>
<tr>
<th>2 µg/mL Biotinylated DR5 mAb</th>
<th>1 µg/mL DR5-Fc fusion coat</th>
<th>1 µg/mL TRAIL-His coat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 µg/mL TRAIL-His</td>
<td>Buffer</td>
</tr>
<tr>
<td>DR5 mAb 1</td>
<td>1.939</td>
<td>2.118</td>
</tr>
<tr>
<td>DR5 mAb 2</td>
<td>2.052</td>
<td>2.052</td>
</tr>
<tr>
<td>DR5-Fc fusion</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

The results show that the amount of DR5 mAb 1 or DR5 mAb 2 bound to the immobilized DR5-Fc was not affected by the presence of the histidine tagged TRAIL, thus indicating that neither DR5 mAb 1 nor DR5 mAb 2 block the TRAIL ligand binding site of DR5. Additionally, neither antibody was capable of binding to the histidine tagged TRAIL ligand.

In order to assess the species specificity of anti-human DR5 monoclonal antibodies DR5 mAb 1 and DR5 mAb 2, the ability of the antibodies to bind to human DR5 was compared with their ability to bind cynomolgus monkey (Macaca fascicularis) DR5. The results of this experiment are shown in Figure 6. The results show that both antibodies are capable of binding to cynomolgus monkey DR5, but that they each exhibit higher binding affinity for human DR5.
The kinetics of binding was investigated using Biacore Analysis, as shown in Figure 7. Bi-specific DR5 x CD3 diabodies were incubated with His-tagged DR5 and the kinetics of binding was determined via Biacore analysis. The diabodies employed were DR5 mAb 1 x CD3 mAb 2 (Figure 7, Panels A and E), DR5 mAb 2 x CD3 mAb 2 (Figure 7, Panels B and F), DR5 mAb 3 x CD3 mAb 2 (Figure 7, Panels C and G), and DR5 mAb 4 x CD3 mAb 2 (Figure 7, Panels D and H). Figure 7, Panels A-D show the results for human DR5. Figure 7, Panels E-H show the results for cynomolgus monkey DR5. The calculated ka, kd and KD are presented in Table 6.

<table>
<thead>
<tr>
<th>Anti-DR Antibody</th>
<th>Human</th>
<th>Cynomolgus Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ka</td>
<td>kd</td>
</tr>
<tr>
<td>DR mAb 1</td>
<td>8.5 x 10^6</td>
<td>1.2 x 10^{-3}</td>
</tr>
<tr>
<td>DR mAb 2</td>
<td>3.4 x 10^5</td>
<td>2.1 x 10^{-4}</td>
</tr>
<tr>
<td>DR mAb 3</td>
<td>4.2 x 10^6</td>
<td>3.7 x 10^{-2}</td>
</tr>
<tr>
<td>DR mAb 4</td>
<td>5.4 x 10^6</td>
<td>1.7 x 10^{-2}</td>
</tr>
</tbody>
</table>

The results demonstrate that DR5 mAb 1 and DR5 mAb 2 exhibit altered kinetics of binding relative to reference antibodies DR5 mAb 3 and DR5 mAb 4.

Y. Example 3: Unexpected Superiority of DR5 mAb 1 and DR5 mAb 2

The ability of DR5-binding molecules DR5 mAb 1 and DR5 mAb 2 of the present invention to mediate cytotoxicity was compared with that of the reference anti-DR5 antibodies: DR5 mAb 3 and DR5 mAb 4. In order to make such a comparison, a bi-specific DR5 x CD3 diabody containing the VL and VH Domains of these antibodies and the VL and VH Domains of CD3 mAb 2 were prepared. The prepared diabodies were: DR5 mAb 1 x CD3 mAb 2; DR5 mAb 2 x CD3 mAb 2; DR5 mAb 3 x CD3 mAb 2; and DR5 mAb 4 x CD3 mAb 2.

The employed control diabody contained the VL and VH domains of anti-fluorescein antibody 4-4-20 (respectively, SEQ ID Nos:138 and 139) and the VL and VH domains of CD3 mAb 2 (respectively, SEQ ID Nos:102 and 108), and was designated as the anti-fluorescein x anti-CD3 control diabody "4-4-20 x CD3 mAb 2." The diabody was composed of two polypeptide chains. The first polypeptide
chain of the diabody had the amino acid sequence (SEQ ID NO:300) (CDRs are shown in underline):

```
DVVMTQT PFS  L PVS LGDQAS  I SCRSSQLV  HSNGNTYLRW  YLQKPGQS  PK
VL I YKVSNRFT  S GVPDRFS GS GSGTDFTLKI  SRVEAE DLGV  VFCSQSTHVP
WT FGQGTLKB  I KGGG GGGG  EVQVLVE S GGG  LVQPQGS  LRL  SCAAS GFT FN
TYANNVRQOA  PGKGLEWVAP  I RS KYNNYAT YYA DSVKDF  T I SRDDSNS
LYLQMNS LKT  E DTA VYYCVR  HGNF GNSYS  WFA YWGQGTL  VT VS S GGC GG
GEVAALEKEV  AALEKEVAAL  EKEVAALEK
```

[00511] In SEQ ID NO:300, amino acid residues 1-112 correspond to the VL Domain of anti-fluorescein antibody 4-4-20 (SEQ ID NO:138), residues 113-120 correspond to the intervening spacer peptide GGGS GGGG (Linker 1) (SEQ ID NO:33), residues 121-245 correspond to the VH Domain of CD3 mAb 2 (SEQ ID NO:108), residues 246-251 are a cysteine-containing spacer peptide (GGCGGG) (SEQ ID NO:34), and residues 252-280 correspond to an E-coil Domain (SEQ ID NO:39).

[00512] The second polypeptide chain of the diabody had the amino acid sequence (SEQ ID NO:301) (CDRs are shown in underline):

```
QAVV TQE PSL  TVS PGGTVT L  TCR S TGA V T  TS NYANWVQQ  KPGQ APRGL I
GGTNKRAP W T  PARF S GLLG  GKA ALTI TG A  QAE DEADYYC  ALW YSNLWV F
GGGTKLTVL G  GGGG GGGGE V  KLDE TGGL LV  QPRPMKL SC  VAS GFT FSD Y
WMN WVR Q ES P E  KGLEWA QIR  N KP Y NYETYY  SDS VKGRFT I  SR DDSK S SV Y
LQMNNLRVE D  M GI Y YC T G S Y  YG MDYW GQGT  SVTV S S GG CG  G GKV AAL KEK
VAALKEKVA A  LKE KVA AL KE
```

[00513] In SEQ ID NO:301, amino acid residues 1-110 correspond to the VL Domain of CD3 mAb 2 (SEQ ID NO:104), residues 111-118 correspond to the intervening spacer peptide GGGS GGGG (Linker 1) (SEQ ID NO:33), residues 119-236 correspond to the VH Domain of anti-fluorescein antibody 4-4-20 (SEQ ID NO:139), residues 237-242 are a cysteine-containing spacer peptide (GGCGGG) (SEQ ID NO:34), and residues 243-270 correspond to a K-coil Domain (SEQ ID NO:40).

[00514] Target tumor cells were incubated with one of these diabodies or with the control diabody (4-4-20 x CD3 mAb 2) in the presence of peripheral blood mononuclear cells (PBMC) and A549 adenocarcinomic human alveolar basal epithelial cells for 24 hours at an effector to target cell ratio of 20:1. The percentage cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) into the media by damaged cells.
The results of this investigation are shown in Figure 8. Similar results were obtained using SKMES human lung cancer cells, DU145 human prostate cancer cells, A375 human malignant melanoma cells, SKBR3 human HER2-overexpressing breast carcinoma cells, and JIMT human breast carcinoma cells. The results indicate that the VL and VH domains of DR5 mAb 1 and DR5 mAb 2 are significantly and unexpectedly more potent in inducing cytotoxicity than those of the reference DR5 mAbs.

Z. Example 4: The Trispecific Binding Molecules Mediate Coordinated and Simultaneous Binding to Target Cells

The ability of Tri-Specific Binding Molecules of the present invention to bind to target cells was investigated. The employed trispecific molecules were: EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1; EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1; and gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1. As shown in Figure 9A, those Tri-Specific Binding Molecules that comprise an EphA2 Cancer Antigen-Binding Domain were found to be capable of binding to EphA2-expressing CHO target cells. As shown in Figure 9B, those Tri-Specific Binding Molecules that comprise a DR5 Cancer Antigen-Binding Domain were found to be capable of binding to DR5-expressing CHO target cells. As shown in Figure 9C, those Tri-Specific Binding Molecules that comprise an EphA2 Cancer Antigen-Binding or a DR5 Cancer Binding Domain were found to be capable of binding to DU145 cells. DU145 cells are a human prostate cell line that express both EphA2 and DR5, but not gpA33. The above-described reference gpA33 mAb 1 x CD3 mAb 2 diabody was used as a control.

Significantly, the data show that when both of the two Cancer Antigen-Binding Domains of a Tri-Specific Binding Molecule of the present invention are able to bind to a target cell, such dual binding is associated with a synergistic (e.g., a 5-25 fold) enhancement in target binding.
AA. Example 5: The Trispecific Binding Molecules Mediate Cytotoxicity of Bound Target Cells

[00518] The ability of Tri-Specific Binding Molecules of the present invention to mediate the cytotoxicity of bound target cells in the presence of cytotoxic lymphocytes was investigated. The employed trispecific molecules were: EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1; EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1; and gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1. The above-described reference gpA33 mAb 1 x CD3 mAb 2 diabody and the 4-4-20 x CD3 mAb 2 diabody were used as controls.

[00519] As shown in Figure 10A, those Tri-Specific Binding Molecules that comprise an EphA2 Cancer Antigen-Binding Domain, and thus were able to bind to bind to EphA2-expressing CHO cells, were able to mediate the cytotoxicity of such cells in the presence of the cytotoxic lymphocytes. As shown in Figure 10B, those Tri-Specific Binding Molecules that comprise a DR5 Cancer Antigen-Binding Domain, and thus were able to bind to bind to DR5-expressing CHO cells, were found to be capable of mediating cytotoxicity of DR5-expressing CHO target cells in the presence of the cytotoxic lymphocytes.

[00520] As shown in Figure 10C, those Tri-Specific Binding Molecules that comprise an EphA2 Cancer Antigen-Binding or a DR5 Cancer Binding Domain, and thus are capable of binding to DU145 cells, were able to mediate the cytotoxicity of such cells in the presence of the cytotoxic lymphocytes. Significantly, the data show that when both of the two Cancer Antigen-Binding Domains of a Tri-Specific Binding Molecule of the present invention are able to bind to a target cell, such dual binding is associated with a synergistic enhancement in target binding. Thus, EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1, which is capable of binding to both EphA2 and DR5, mediated substantially greater cytotoxicity than EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1 or gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1, which were capable of binding to only EphA2 or DR5 molecules of the DU146 cells (since such cells lack gpA33).
In this regard, at approximately the EC50 of EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1, no cytotoxic lymphocyte response is seen for either EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1 or gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1. At approximately the EC90 of EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1, EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1 shows only an EC15, and gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1 shows no cytotoxic lymphocyte response at all.

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 Tri-Specific Binding Molecule capable of immunospecifically binding to three different epitopes, said Epitopes being Epitope I, Epitope II, and Epitope III, wherein two of said three epitopes are epitopes of Cancer Antigen(s), and the third of said three epitopes is an epitope of an Effector Cell Antigen.

Claim 2. The Tri-Specific Binding Molecule of claim 1, capable of immunospecifically binding to three different epitopes, wherein said binding molecule comprises four different polypeptide chains covalently complexed together and comprises:

(I) an Antigen-Binding Domain I that is capable of immunospecifically binding to an Epitope I present on a first antigen, and an Antigen-Binding Domain II that is capable of immunospecifically binding to an Epitope II present on a second antigen, wherein said Antigen-Binding Domain I and said Antigen-Binding Domain II are both Diabody-Type Binding Domains;

(II) an Antigen-Binding Domain III that is capable of immunospecifically binding to an Epitope III present on a third antigen; and

(III) an Fc Domain that is formed by the complexing of two CH2-CH3 Domains to one another;

wherein one of Epitope I, Epitope II or Epitope III is an epitope of an Effector Cell Antigen, a second of Epitope I, Epitope II or Epitope III is an epitope of a first Cancer Antigen, and the third of Epitope I, Epitope II or Epitope III is an epitope of a second Cancer Antigen, and wherein the Antigen-Binding Domains I, II and III of the Binding Molecules mediate coordinated binding of an immune system effector cell expressing said Effector Cell Antigen and a cancer cell expressing said first and second Cancer Antigens.
Claim 3. The Tri-Specific Binding Molecule of claim 2, wherein said Fc Domain is capable of binding to an Fc Receptor arrayed on the surface of a cell.

Claim 4. The Tri-Specific Binding Molecule of any one of claims 1-3, wherein said Effector Cell Antigen is arrayed on the surface of an effector cell and wherein said Cancer Antigens are arrayed on the surface of a cancer cell, and wherein said immunospecific binding is sufficient to co-localize said Effector Cell Antigen, and said Cancer Antigens, thereby facilitating the activation of said effector cell against said cancer cell.

Claim 5. The Tri-Specific Binding Molecule of any one of claims 1-4, wherein said Effector Cell Antigen is selected from the group consisting of: CD2, CD3, CD16, CD19, CD20, CD22, CD32B, CD64, the B cell Receptor (BCR), the T cell Receptor (TCR), and the NKG2D Receptor.

Claim 6. The Tri-Specific Binding Molecule of any one of claims 1-5, wherein said first and second Cancer Antigens are independently selected from the group consisting of: colon cancer antigen 19.9; a gastric cancer mucin; antigen 4.2; glycoprotein A33 (gpA33); ADAM-9; gastric cancer antigen AH6; ALCAM; malignant human lymphocyte antigen APO-1; cancer antigen Bl; B7-H3; beta-catenin; blood group ALe\(^{b}\)/Le\(^{y}\); Burkitt’s lymphoma antigen-38.13, colonic adenocarcinoma antigen C14; ovarian carcinoma antigen CA125; Carboxypeptidase M; CD5; CD19; CD20; CD22; CD23; CD25; CD27; CD30; CD33; CD36; CD45; CD46; CD52; CD79a/CD79b; CD103; CD317; CDK4; carcinoembryonic antigen (CEA); CEACAM5; CEACAM6; CO17-1A; CO-43 (blood group Le\(^{b}\)); CO-514 (blood group Le\(^{a}\)); CTA-1; CTLA4; Cytokeratin 8; antigen D1.l; antigen Di56-22; DR5; Ei series (blood group B); EGFR (Epidermal Growth Factor Receptor); Ephrin receptor A2 (EphA2); ErbBl; ErbB3; ErbB4; GAGE-1; GAGE-2; GD2/GD3/GM2; lung adenocarcinoma antigen F3; antigen FC10.2;
G49, ganglioside GD2; ganglioside GD3; ganglioside GM2; ganglioside GM3; G_{d2}; G_{d3}; GICA 19-9; G_{M2}; gp100; human leukemia T cell antigen Gp37; melanoma antigen gp75; gpA33; HER2 antigen (pl85_{HER2}); human milk fat globule antigen (HMFG); human papillomavirus-E6/human papillomavirus-E7; high molecular weight melanoma antigen (HMW-MAA); I antigen (differentiation antigen) I(Ma); Integrin Alpha-V-Beta-6 Integrin6 (ITGB6); Interleukin-13; Receptor a2 (IL13Ra2); JAM-3; KID3; KID31; KS 1/4 pan-carcinoma antigen; human lung carcinoma antigens L6 and L20; LEA; LUCA-2; Ml:22:25:8; M18; M39; MAGE-1; MAGE-3; MART; MUC-1; MUM-1; Myl; N-acetylgalactosaminytransferase; neoglycoprotein; NS-10; OFA-1; OFA-2; Oncostatin M; p15; melanoma-associated antigen p97; polymorphic epithelial mucin (PEM); polymorphic epithelial mucin antigen (PEMA); PIPA; prostate-specific antigen (PSA); prostate-specific membrane antigen (PSMA); prostatic acid phosphate; R_{24}; ROR1; sphingolipids; SSEA-1; SSEA-3; SSEA-4; sTn; T cell receptor derived peptide; T_{A7}; TAG-72; TL5 (blood group A); TNF-a receptor; TNF-β receptor; TNF-γ receptor; TRA-1-85 (blood group H); Transferrin Receptor; tumor-specific transplantation antigen (TSTA), oncofetal antigen-alpha-fetoprotein (AFP); VEGF, VEGFR; VEP8; VEP9; VIM-D5; and Y hapten, Le^{y}.

Claim 7. The Tri-Specific Binding Molecule of claim 6, wherein said first and second Cancer Antigens are selected from the group consisting of: CD2, CD317, CEACAM5, CEACAM6, DR5, EphA2, gpA33, Her2, B7-H3; EGF, EGFR, VEGF and VEGFR.

Claim 8. The Tri-Specific Binding Molecule of any one of claims 1-7, wherein said Non-Diabody-Type Binding Domain III comprises said Fab-Type Binding Domain (VLin/VHin) that is capable of immunospecifically binding to an Epitope III, wherein said molecule comprises:

(A) a first polypeptide chain that comprises in the N-terminus to C-terminus direction:
a light chain variable Domain of an immunoglobulin capable of binding to a first of said three epitopes (VI^);

(2) a heavy chain variable Domain of an immunoglobulin capable of binding to a second of said three epitopes (VH_n);

(3) a Heterodimer-Promoting Domain; and

(4) CH2 and CH3 Domains of an IgG;

(B) a second polypeptide chain that comprises, in the N-terminus to C-terminus direction:

(1) a light chain variable Domain of an immunoglobulin capable of binding to said second of said three epitopes (VL_n);

(2) a heavy chain variable Domain of an immunoglobulin capable of binding to said first of said three epitopes (VH_I);

(3) a complementary Heterodimer-Promoting Domain;

(C) a third polypeptide chain that comprises, in the N-terminus to C-terminus direction:

(1) a heavy chain variable Domain of an immunoglobulin capable of binding to a third of said three epitopes (VH_II); and

(2) a CHI Domain, a Hinge Domain, and a CH2-CH3 Domain of an IgG;

and

(D) a fourth polypeptide chain that comprises, in the N-terminus to C-terminus direction:

(1) a light chain variable Domain of an immunoglobulin capable of binding to said third of said three epitopes (VL_III); and

(2) a light chain constant Domain (CL);

wherein:
(i) said VLi and V⅓ Domains associate to form a Domain capable of binding said first epitope;
(ii) said VLn and VHn Domains associate to form a Domain capable of binding said second epitope;
(iii) said VL⅓ and VHm Domains associate to form a Domain capable of binding said third epitope;
(iv) said CH2-CH3 Domain of said first polypeptide chain and said CH2-CH3 Domain of said third polypeptide chain associate to form an Fc Domain;
(v) said first and second polypeptide chains are covalently bonded to one another;
(vi) said first and third polypeptide chains are covalently bonded to one another; and
(vii) said third and fourth polypeptide chains are covalently bonded to one another.

Claim 9. The Tri-Specific Binding Molecule of claim 8, wherein:
(A) said Heterodimer-Promoting Domain is an E-coil and said complementary Heterodimer-Promoting Domain is a K-coil; or
(B) said Heterodimer-Promoting Domain is a K-coil and said complementary Heterodimer-Promoting Domain is an E-coil.

Claim 10. The Tri-Specific Binding Molecule of any one of claims 7-9, wherein:
(A) said CH2-CH3 Domains of said first and third polypeptide chains each have the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits normal FcyR-mediated effector function; or
(B) said CH2-CH3 Domain of said first and third polypeptide chains comprise at least one amino acid substitution, relative to the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits altered FcyR-mediated effector function.

(ii) said VLn and VHn Domains associate to form a Domain capable of binding said second epitope;
(iii) said VL⅓ and VHm Domains associate to form a Domain capable of binding said third epitope;
(iv) said CH2-CH3 Domain of said first polypeptide chain and said CH2-CH3 Domain of said third polypeptide chain associate to form an Fc Domain;
(v) said first and second polypeptide chains are covalently bonded to one another;
(vi) said first and third polypeptide chains are covalently bonded to one another; and
(vii) said third and fourth polypeptide chains are covalently bonded to one another.

Claim 9. The Tri-Specific Binding Molecule of claim 8, wherein:
(A) said Heterodimer-Promoting Domain is an E-coil and said complementary Heterodimer-Promoting Domain is a K-coil; or
(B) said Heterodimer-Promoting Domain is a K-coil and said complementary Heterodimer-Promoting Domain is an E-coil.

Claim 10. The Tri-Specific Binding Molecule of any one of claims 7-9, wherein:
(A) said CH2-CH3 Domains of said first and third polypeptide chains each have the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits normal FcyR-mediated effector function; or
(B) said CH2-CH3 Domain of said first and third polypeptide chains comprise at least one amino acid substitution, relative to the sequence of SEQ ID NO:1, such that the Fc Domain formed from their association exhibits altered FcyR-mediated effector function.
Claim 11. The Tri-Specific Binding Molecule of claim 10, wherein said at least one amino acid substitution comprises at least one amino acid substitution selected from the group consisting of: L235V, F243L, R292P, Y300L, V305I, and P396L, wherein said numbering is that of the EU index as in Kabat.

Claim 12. The Tri-Specific Binding Molecule of claim 10 or 11, wherein said at least one amino acid substitution comprises:
(A) at least one substitution selected from the group consisting of F243L, R292P, Y300L, V305I, and P396L;
(B) at least two substitutions selected from the group consisting of:
   (1) F243L and P396L;
   (2) F243L and R292P; and
   (3) R292P and V305I;
(C) at least three substitutions selected from the group consisting of:
   (1) F243L, R292P and Y300L;
   (2) F243L, R292P and V305I;
   (3) F243L, R292P and P396L; and
   (4) R292P, V305I and P396L;
(D) at least four substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L and P396L; and
   (2) F243L, R292P, V305I and P396L;
or
(E) at least the five substitutions selected from the group consisting of:
   (1) F243L, R292P, Y300L, V305I and P396L; and
   (2) L235V, F243L, R292P, Y300L and P396L.

Claim 13. The Tri-Specific Binding Molecule of any one of claims 8-9, wherein said CH2-CH3 Domain of said first and third polypeptide chains differ from one another and have an amino acid sequence selected from the group consisting of SEQ ID NO:52 and SEQ ID NO:53.
Claim 14. The Tri-Specific Binding Molecule of any one of claims 7-13, wherein:

(A) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of said first Cancer Antigen, an epitope of said second Cancer Antigen and an epitope of said Effector Cell Antigen;

(B) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of said first Cancer Antigen, an epitope of said Effector Cell Antigen and an epitope of said second Cancer Antigen;

(C) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of second Cancer Antigen, an epitope of said first Cancer Antigen, and an epitope of said Effector Cell Antigen;

(D) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of said second Cancer Antigen, an epitope of said Effector Cell Antigen and an epitope of said first Cancer Antigen;

(E) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of said Effector Cell Antigen, an epitope of said first Cancer Antigen, and an epitope of said second Cancer Antigen;

and

(F) said Epitope I, Epitope II and Epitope III are, respectively, an epitope of said Effector Cell Antigen, an epitope of second Cancer Antigen, and an epitope of said first Cancer Antigen.

Claim 15. The Tri-Specific Binding Molecule of any of claims 1-14, wherein:

(A) said epitope of an Effector Cell Antigen is a CD2 epitope recognized by antibody Lo-CD2a;

(B) said epitope of an Effector Cell Antigen is a CD3 epitope recognized by antibody OKT3, M291, YTH12.5, Anti-CD3 mAb 1 or Anti-CD3 mAb 2;

(C) said epitope of an Effector Cell Antigen is a CD16 epitope recognized by antibody 3G8 or A9;
(D) said epitope of an Effector Cell Antigen is a CD19 epitope recognized by antibody MD1342, MEDI-551, blinatumomab or HD37;

(E) said epitope of an Effector Cell Antigen is a CD20 epitope recognized by antibody rituximab, ibritumomab, ofatumumab, and tositumomab;

(F) said epitope of an Effector Cell Antigen is a CD22 epitope recognized by antibody epratuzumab;

(G) said epitope of an Effector Cell Antigen is a CD32B epitope recognized by antibody CD32B mAb 1;

(H) said epitope of an Effector Cell Antigen is a CD64 epitope recognized by antibody CD64 mAb 1;

(I) said epitope of an Effector Cell Antigen is a BCR/CD79 epitope recognized by antibody CD79 mAb 1;

(J) said epitope of an Effector Cell Antigen is a TCR epitope recognized by antibody BMA 031;

or

(K) said epitope of an Effector Cell Antigen is a NKG2D Receptor epitope recognized by antibody KYK-2.0.

Claim 16. A pharmaceutical composition that comprises the Tri-Specific Binding Molecule of any of claims 1-15, and a pharmaceutically acceptable carrier, excipient or diluent.

Claim 17. The Tri-Specific Binding Molecule of any one of claims 1-15, or the pharmaceutical composition of claim 15, wherein said molecule is used in the treatment of cancer.

Claim 18. The Tri-Specific Binding Molecule of any one of claims 1-15, or the pharmaceutical composition of claim 15, wherein said cancer is characterized by the presence of a cancer cell selected from the group consisting of a cell of: an adrenal gland tumor, an AIDS-associated cancer, an alveolar soft part sarcoma, an astrocytic tumor, bladder...
cancer, bone cancer, a brain and spinal cord cancer, a metastatic brain
tumor, a breast cancer, a carotid body tumors, a cervical cancer, a
chondrosarcoma, a chordoma, a chromophobe renal cell carcinoma, a
clear cell carcinoma, a colon cancer, a colorectal cancer, a cutaneous
benign fibrous histiocytoma, a desmoplastic small round cell tumor, an
ependymoma, a Ewing's tumor, an extraskeletal myxoid chondrosarcoma,
a fibrogenesis imperfecta ossium, a fibrous dysplasia of the bone, a
gallbladder or bile duct cancer, gastric cancer, a
gestational trophoblastic disease, a germ cell tumor, a head and neck
cancer, hepatocellular carcinoma, an islet cell tumor, a Kaposi's Sarcoma,
a kidney cancer, a leukemia, a lipoma/benign lipomatous
tumor, a liposarcoma/malignant lipomatous tumor, a liver cancer, a
lymphoma, a lung cancer, a medulloblastoma, a melanoma, a
meningioma, a multiple endocrine neoplasia, a multiple myeloma, a
myelodysplasia syndrome, a neuroblastoma, a neuroendocrine tumors,
an ovarian cancer, a pancreatic cancer, a papillary thyroid carcinoma, a
parathyroid tumor, a pediatric cancer, a peripheral nerve sheath tumor,
a phaeochromocytoma, a pituitary tumor, a prostate cancer, a
posterior uveal melanoma, a rare hematologic disorder, a renal
metastatic cancer, a rhabdoid tumor, a rhabdomyosarcoma, a sarcoma, a
skin cancer, a soft-tissue sarcoma, a squamous cell cancer, a stomach
cancer, a synovial sarcoma, a testicular cancer, a thymic carcinoma, a
thymoma, a thyroid metastatic cancer, and a uterine cancer.

Claim 19. The Tri-Specific Binding Molecule or pharmaceutical composition of
any one of claims 17-18, wherein said cancer is colorectal cancer,
hepatocellular carcinoma, glioma, kidney cancer, breast cancer,
multiple myeloma, bladder cancer, neuroblastoma; sarcoma, non-
Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer,
pancreatic cancer or a rectal cancer.
Claim 20. The Tri-Specific Binding Molecule or pharmaceutical composition of any one of claims 17-18, wherein said cancer is acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), acute B lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin's lymphomas (NHL), including mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, or Burkitt's lymphoma.
Figure 1A

Figure 1B
Figure 3B
First Polypeptide Chain

Second Polypeptide Chain

Third Polypeptide Chain

Fourth Polypeptide Chain

Two Diabody-Type Binding Domains

Non Diabody-Type Binding Domain

Figure 4A
Figure 4B
Figure 5A
Figure 5B
Figure 5C

Figure 5D
Figure 5E
Figure 6

- mIgG
- DR5 mAb 1 (Human)
- DR5 mAb 1 (Cyno)
- DR5 mAb 2 (Human)
- DR5 mAb 2 (Cyno)
**Figure 7**

### Human DR5/His

- **A**: DR5 mAb 1 x CD3 mAb 2
  - $K_D = 0.14\,\text{nM}$
  - $k_a = 8.5 \times 10^6$, $k_d = 1.2 \times 10^{-3}$

- **B**: DR5 mAb 2 x CD3 mAb 2
  - $K_D = 0.62\,\text{nM}$
  - $k_a = 3.4 \times 10^5$, $k_d = 2.1 \times 10^{-4}$

- **C**: DR5 mAb 3 x CD3 mAb 2
  - $K_D = 8.8\,\text{nM}$
  - $k_a = 4.2 \times 10^6$, $k_d = 3.7 \times 10^{-2}$

- **D**: DR5 mAb 4 x CD3 mAb 2
  - $K_D = 3.2\,\text{nM}$
  - $k_a = 5.4 \times 10^6$, $k_d = 1.7 \times 10^{-2}$

---

### Cyno DR5/His

- **E**: DR5 mAb 1 x CD3 mAb 2
  - $K_D = 32.5\,\text{nM}$
  - $k_a = 4.0 \times 10^6$, $k_d = 1.3 \times 10^{-1}$

- **F**: DR5 mAb 2 x CD3 mAb 2
  - $K_D = 0.42\,\text{nM}$
  - $k_a = 2.4 \times 10^5$, $k_d = 1.0 \times 10^{-4}$

- **G**: DR5 mAb 3 x CD3 mAb 2
  - $K_D = 13.3\,\text{nM}$
  - $k_a = 3.3 \times 10^6$, $k_d = 4.4 \times 10^{-2}$

- **H**: DR5 mAb 4 x CD3 mAb 2
  - $K_D = 16.4\,\text{nM}$
  - $k_a = 2.5 \times 10^6$, $k_d = 4.1 \times 10^{-2}$
A549 + PBMC (LDH) 24h  
E:T=20:1

% Cytotoxicity

Concentration (nM)

- Control Diabody (4-4-20 x CD3 mAb 2)
- DR5 mAb 1 x CD3 mAb 2 Diabody
- DR5 mAb 2 x CD3 mAb 2 Diabody
- DR5 mAb 3 x CD3 mAb 2 Diabody
- DR5 mAb 4 x CD3 mAb 2 Diabody

Figure 8
Figure 9A
Figure 9B
Du145 (gpA33⁻ EphA2⁺ DR5⁺) FACS

DU145 (Log MFI)

Concentration (nM)

- EphA2 mAb 1 x CD3 mAb 2 x DR5 mAb 1
- EphA2 mAb 1 x CD3 mAb 2 x gpA33 mAb 1
- gpA33 mAb 1 x CD3 mAb 2 x DR5 mAb 1
- gpA33 mAb 1 x CD3 mAb 2

Figure 9C
Figure 10A
Figure 10B
Figure 10C
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395, C07K 16/00, C12P 21/08 (2015.01)
CPC - A61K 39/39558, C07K 2317/626, 2317/31

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 39/395, 39/00, 39/38, 2039/505, 39/39; C07K16/00, C12P 21/08 (2015.01)
CPC: A61K 38/00, 39/39558; C07K 16/18, 2317/626, 2317/31; USPC: 530/388.2, 388.1, 387.1, 386, 380, 388.3, 350; 424/184.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; PubMed; Dialog ProQuest; trispecific, 'Binding molecule,' antibody, 'cancer antigen,' 'tumor antigen,' 'effector cell antigen,' 'B cell antigen,' 'T cell antigen,' 'Fc domain'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2010/02911 112 A1 (KELLNER, C et al.) November 18, 2010; abstract; figures 1, 3A, 5; paragraphs [0030], [0037], [0038], [0039], [0041], [0042], [0057], [0107], [0120], [0150]</td>
<td>1, 4/1</td>
</tr>
<tr>
<td>Y</td>
<td>WO 2012/018687 A1 (MACROGENICS, INC.) February 09, 2012; abstract; paragraphs [0099], [00125]; figure 32</td>
<td>2, 3, 4/2, 4/3</td>
</tr>
<tr>
<td>A</td>
<td>US 6994853 B1 (LINDHOFER, H et al.) February 07, 2006; abstract</td>
<td>1-4</td>
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* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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  "P" document published prior to the international filing date but later than the priority date claimed
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  "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "X" document member of the same patent family

Date of the actual completion of the international search
17 August 2015 (17.08.2015)

Date of mailing of the international search report
02 SEP 2015

Name and mailing address of the ISA:
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Authorized officer Shane Thomas
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PCT OSP: 571-272-7774

Form PCT/ISA A/2 (second sheet) (January 2015)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. forming part of the international application as filed:
      □ in the form of an Annex C/ST.25 text file.
      □ on paper or in the form of an image file.
   b. furnished together with the international application under PCT Rule 13(1)(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. furnished subsequent to the international filing date for the purposes of international search only:
      □ in the form of an Annex C/ST.25 text file (Rule 13(1)(a)).
      □ on paper or in the form of an image file (Rule 13(1)(b) and Administrative Instructions, Section 713).

2. □ In addition, in the case that more than one version or copy of a sequence listing has, been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
# INTERNATIONAL SEARCH REPORT

**Observations where certain claims were found unsearchable (Continuation of item 2 of First sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:
   - because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.:
   - because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. 12SJ Claims Nos. 5-20
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- □ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)