

Title of the Invention:**Bi-Specific Monovalent Diabodies That Are Capable of Binding CD19 and CD3, and Uses Thereof****Cross-Reference To Related Applications**

[0001] This Application claims priority to U.S. Provisional Patent Appln. Serial No. 62/055,695 (filed on September 26, 2014; pending), which application is hereby incorporated by reference herein 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_0116PCT_Sequence_Listing_ST25.txt, created on September 18, 2015, and having a size of 47,329 bytes), which file is herein incorporated by reference in its entirety.

Field of the Invention:

[0003] The present invention is directed to bi-specific monovalent diabodies that comprise two polypeptide chains and which possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 (*i.e.*, a “CD19 x CD3 bi-specific monovalent diabody”). Most preferably, such CD19 x CD3 bi-specific monovalent diabodies are composed of three polypeptide chains and possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 and additionally comprise an immunoglobulin Fc Domain (*i.e.*, a “CD19 x CD3 bi-specific monovalent Fc diabody”). The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present invention are capable of simultaneous binding to CD19 and CD3. The invention is directed to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The invention is additionally directed to methods for the use of such diabodies in the treatment of disease, in particular hematologic malignancies.

Background of the Invention:

A. CD19

[0004] CD19 (B lymphocyte surface antigen B4, Genbank accession number M28170) is a 95 kDa type I transmembrane glycoprotein of the immunoglobulin superfamily (Stamenkovic, I. *et al.* (1988) “*CD19, The Earliest Differentiation Antigen Of The B Cell Lineage, Bears Three Extracellular Immunoglobulin-Like Domains And An Epstein-Barr Virus-Related Cytoplasmic Tail,*” J. Exper. Med. 168(3):1205-1210; Tedder, T.F. *et al.* (1989) “*Isolation Of cDNAs Encoding The CD19 Antigen Of Human And Mouse B Lymphocytes. A New Member Of The Immunoglobulin Superfamily,*” J. Immunol. 143(2):712-717; Zhou, L.J. *et al.* (1991) “*Structure And Domain Organization Of The CD19 Antigen Of Human, Mouse, And Guinea Pig B Lymphocytes. Conservation Of The Extensive Cytoplasmic Domain,*” J. Immunol. 147(4):1424-1432). CD19 is expressed on follicular dendritic cells and on all B cells from early pre-B cells at the time of heavy chain rearrangement up to the plasma cell stage, when CD19 expression is down regulated. CD19 is not expressed on hematopoietic stem cells or on B cells before the pro-B cell stage (Sato *et al.* (1995) “*The CD19 Signal Transduction Molecule Is A Response Regulator Of B-Lymphocyte Differentiation,*” Proc. Natl. Acad. Sci. (U.S.A.) 92:11558-62; Loken *et al.* (1987) “*Flow Cytometric Analysis of Human Bone Marrow. II. Normal B Lymphocyte Development,*” Blood, 70:1316- 1324; Wang *et al.* (2012) “*CD19: A Biomarker For B Cell Development, Lymphoma Diagnosis And Therapy,*” Exp. Hematol. and Oncol. 1:36).

[0005] CD19 is a component of the B cell-receptor (BCR) complex, and is a positive regulator of B cell signaling that modulates the threshold for B cell activation and humoral immunity. CD19 interacts with CD21 (CR2, C3d fragment receptor) and CD81 through two extracellular C2-type Ig-like domains, forming together with CD225 the BCR complex. The intracellular domain of CD19 is involved in intracellular signaling cascades, primarily but not exclusively regulating signals downstream of the BCR and CD22 (Mei *et al.* (2012) “*Rationale of Anti-CD19 Immunotherapy: An Option To Target Autoreactive Plasma Cells In Autoimmunity,*” Arthritis Res and Ther. 14(Suppl. 5):S1; Wang *et al.* (2012) “*CD19: A Biomarker For*

B Cell Development, Lymphoma Diagnosis And Therapy,” Exp. Hematol. and Oncol. 1:36); Del Nagro et al. (2005) “CD19 Function in Central and Peripheral B-Cell Development,” Immunologic Res. 31:119-131).

[0006] Several properties of CD19 suggest its possible potential as a target for immunotherapy. CD19 is one of the most ubiquitously expressed antigens in the B cell lineage and is expressed on >95% of B cell malignancies, including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and non-Hodgkin’s Lymphoma (NHL) (Wilson, K. et al. (2010) “Flow Minimal Residual Disease Monitoring Of Candidate Leukemic Stem Cells Defined By The Immunophenotype, CD34+CD38lowCD19+ In B-Lineage Childhood Acute Lymphoblastic Leukemia,” Haematologica 95(4):679-683; Maloney, D.G. et al. (1997) “IDEC-C2B8 (Rituximab) Anti-CD20 Monoclonal Antibody Therapy In Patients With Relapsed Low-Grade Non-Hodgkin’s Lymphoma,” Blood 90(6):2188-2195; Vose, J.M. (1998) “Current Approaches To The Management Of Non-Hodgkin’s Lymphoma,” Semin. Oncol. 25(4):483-491; Nagorsen, D. et al. (2012) “Blinatumomab: A Historical Perspective,” Pharmacol. Ther. 136(3):334-342; Topp, M.S. et al. (2011) “Targeted Therapy With The T-Cell-Engaging Antibody Blinatumomab Of Chemotherapy-Refractory Minimal Residual Disease In B-Lineage Acute Lymphoblastic Leukemia Patients Results In High Response Rate And Prolonged Leukemia-Free Survival,” J. Clin Oncol. 2011 Jun 20;29(18):2493-2498; Nadler, et al. (1983) “B4, A Human B Lymphocyte-Associated Antigen Expressed On Normal, Mitogen-Activated, And Malignant B Lymphocytes.” J. Immunol.;131:244-250; Ginaldi et al. (1998) “Levels Of Expression Of CD19 And CD20 In Chronic B Cell Leukaemias,” J. Clin. Pathol. 51:364-369; Anderson et al. (1984) “Expression of Human B Cell-Associated Antigens on Leukemias and Lymphomas: A Model of Human B Cell Differentiation,” Blood 63:1424-1433). CD19 is expressed on few if any other cell types and is also not expressed on terminally differentiated plasma cells, which thus may be spared by CD19-directed therapies. CD19 is not shed into the circulation, and can rapidly internalize (Ma et al. (2002) “Radioimmunotherapy For Model B Cell Malignancies Using 90Y-Labeled Anti-CD19 And Anti-CD20 Monoclonal Antibodies,” Leukemia 16:60-66; Raufi et al. (2013) “Targeting CD19 in B-cell lymphoma: emerging role of SAR3419,” Cancer Management Res 3:225-233).

Notably, CD19 expression is maintained on B cell lymphomas that become resistant to anti-CD20 therapy (Davis *et al.* (1999) “*Therapy of B-cell Lymphoma With Anti-CD20 Antibodies Can Result In The Loss Of CD20 Antigen Expression.*” Clin Cancer Res, 5:611-615, 1999). CD19 has also been suggested as a target to treat autoimmune diseases (Tedder (2009) “*CD19: A Promising B Cell Target For Rheumatoid Arthritis,*” Nat. Rev. Rheumatol. 5:572-577).

[0007] B cell malignancies represent a heterogeneous group of disorders with widely varying characteristics and clinical behavior. Historically, most patients with symptomatic disease received a combination of non-cross-reactive genotoxic agents with the intent of achieving a durable remission and, in some cases, a cure. Although effective, many traditional regimens are also associated with considerable acute and long-term toxicities (see, e.g., Stock, W. *et al.* (2013) “*Dose Intensification Of Daunorubicin And Cytarabine During Treatment Of Adult Acute Lymphoblastic Leukemia: Results Of Cancer And Leukemia Group B Study 19802,*” Cancer. 2013 Jan 1;119(1):90-98). The anti-CD20 monoclonal antibody **rituximab** is used for the treatment of many B cell disorders, where it may be employed in combination with “standard of care” agents or employed as a single agent (Fowler *et al.* (2013) “*Developing Novel Strategies to Target B-Cell Malignancies,*” Targeted Pathways, B-Cell Lymphoma in ASCO Educational Book, pp. 366-372; Bargou, R. *et al.* (2008) “*Tumor Regression In Cancer Patients By Very Low Doses Of A T Cell-Engaging Antibody,*” Science 321(5891):974-977; Thomas, D.A. *et al.* (2010) “*Chemoimmunotherapy With A Modified Hyper-CVAD And Rituximab Regimen Improves Outcome In De Novo Philadelphia Chromosome-Negative Precursor B-Lineage Acute Lymphoblastic Leukemia,*” J. Clin. Oncol. 28(24):3880-3889). Despite encouraging clinical results, retreatment of patients with indolent lymphomas with single agent rituximab has been associated with a response rate of only 40%, suggesting that resistance may occur in malignant B cells as a response to prolonged exposure to rituximab (Smith (2003) “*Rituximab (Monoclonal Anti-CD20 Antibody): Mechanisms Of Action And Resistance.*” Oncogene. 22:7359-68; Davis *et al.* (1999) “*Therapy of B-cell Lymphoma With Anti-CD20 Antibodies Can Result In The Loss Of CD20 Antigen Expression,*” Clin Cancer Res, 5:611-615, 1999; Gabrilovich, D. *et al.* (2003) “*Tumor Escape From Immune Response: Mechanisms And Targets Of*

Activity,” Curr. Drug Targets 4(7):525-536). Rituximab-resistant cell lines generated in vitro have been reported to display cross-resistance against multiple chemotherapeutic agents (Czuczman et al. (2008) “Acquirement Of Rituximab Resistance In Lymphoma Cell Lines Is Associated With Both Global CD20 Gene And Protein Down-Regulation Regulated At The Pretranscriptional And Post-transcriptional Levels,” Clin Cancer Res. 14:1561-1570; Olejniczak et al. (2008) “Acquired Resistance To Rituximab Is Associated With Chemotherapy Resistance Resulting From Decreased Bax And Bak Expression,” Clin Cancer Res. 14:1550-1560).

[0008] Additional B cell lymphoma surface antigens targeted by therapeutic antibodies include CD19 (Hoelzer (2013) “*Targeted Therapy With Monoclonal Antibodies In Acute Lymphoblastic Leukemia*, Curr. Opin. Oncol. 25:701-706; Hammer (2012) “*CD19 As An Attractive Target For Antibody-Based Therapy*,” mAbs 4:571-577). However, despite the potent anti-lymphoma activities associated with complex internalization and intracellular release of free drug were reported for various anti-CD19 antibodies bi-specific antibodies having an anti-CD19 binding portion or antibody drug conjugates (ADCs) the anti-tumor effects of these anti-CD19 antibodies or ADCs were variable, suggesting that antibody specific properties, such as epitope binding, the ability to induce CD19 oligomerization or differences in intracellular signaling affect their potencies (Du et al. (2008) “*Differential Cellular Internalization Of Anti-CD19 And -CD22 Immunotoxins Results In Different Cytotoxic Activity*,” Cancer Res. 68:6300-6305; Press et al.. (1994) “*Retention Of B-Cell-Specific Monoclonal Antibodies By Human Lymphoma Cells. Blood*,” 83:1390-1397; Szatrowski et al. (2003) “*Lineage Specific Treatment Of Adult Patients With Acute Lymphoblastic Leukemia In First Remission With Anti-B4-Blocked Ricin Or High-Dose Cytarabine: Cancer And Leukemia Group B Study 9311*,” Cancer 97:1471-1480; Frankel et al. (2013) “*Targeting T Cells To Tumor Cells Using Bispecific Antibodies*,” Curr. Opin. Chem. Biol. 17:385-392).

[0009] Thus, despite improvements in survival for many, the majority of patients suffering from B cell malignancies continue to relapse following standard chemo-immunotherapy and more than 15,000 patients still die annually in the United States

from B cell cancers. Accordingly, treatments with new non-cross-resistant compounds are needed to further improve patient survival.

B. CD3

[0010] CD3 is a T cell co-receptor composed of four distinct chains (Wucherpfennig, K.W. et al. (2010) “*Structural Biology Of The T-Cell Receptor: Insights Into Receptor Assembly, Ligand Recognition, And Initiation Of Signaling*,” Cold Spring Harb. Perspect. Biol. 2(4):a005140; pages 1-14; Chetty, R. et al. (1994) “*CD3: Structure, Function, And Role Of Immunostaining In Clinical Practice*,” J. Pathol. 173(4):303-307; Guy, C.S. et al. (2009) “*Organization Of Proximal Signal Initiation At The TCR:CD3 Complex*,” Immunol. Rev. 232(1):7-21).

[0011] In mammals, the complex contains a CD3 γ chain, a CD3 δ chain, and two CD3 ϵ chains. These chains associate with a molecule known as the T cell receptor (TCR) in order to generate an activation signal in T lymphocytes (Smith-Garvin, J.E. et al. (2009) “*T Cell Activation*,” Annu. Rev. Immunol. 27:591-619). In the absence of CD3, TCRs do not assemble properly and are degraded (Thomas, S. et al. (2010) “*Molecular Immunology Lessons From Therapeutic T-Cell Receptor Gene Transfer*,” Immunology 129(2):170–177). CD3 is found bound to the membranes of all mature T cells, and in virtually no other cell type (see, Janeway, C.A. et al. (2005) In: IMMUNOBIOLOGY: THE IMMUNE SYSTEM IN HEALTH AND DISEASE,” 6th ed. Garland Science Publishing, NY, pp. 214- 216; Sun, Z. J. et al. (2001) “*Mechanisms Contributing To T Cell Receptor Signaling And Assembly Revealed By The Solution Structure Of An Ectodomain Fragment Of The CD3 ϵ : γ Heterodimer*,” Cell 105(7):913-923; Kuhns, M.S. et al. (2006) “*Deconstructing The Form And Function Of The TCR/CD3 Complex*,” Immunity. 2006 Feb;24(2):133-139).

[0012] The invariant CD3 ϵ signaling component of the T cell receptor (TCR) complex on T cells, has been used as a target to force the formation of an immunological synapse between T cells and tumor cells. Co-engagement of CD3 and the tumor antigen activates the T cells, triggering lysis of tumor cells expressing the tumor antigen (Baeuerle et al. (2011) “*Bispecific T Cell Engager For Cancer Therapy*,” In: BISPECIFIC ANTIBODIES, Kontermann, R.E. (Ed.) Springer-Verlag;

2011:273-287). This approach allows bi-specific antibodies to interact globally with the T cell compartment with high specificity for tumor cells and is widely applicable to a broad array of cell-surface tumor antigens.

C. Antibodies and Other Binding Molecules

[0013] **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 region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, 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.

[0014] The ability of an intact, unmodified antibody (e.g., an IgG) to bind an epitope of an antigen depends upon the presence of Variable Domains on the immunoglobulin light and heavy chains (i.e., the VL and VH Domains, respectively). Interaction of an antibody light chain and an antibody heavy chain and, in particular, interaction of its VL and VH Domains forms one of the epitope binding sites of the antibody. In contrast, the scFv construct comprises a VL and VH Domain of an antibody contained in a single polypeptide chain wherein the Domains are separated by a flexible linker of sufficient length to allow self-assembly of the two Domains into a functional epitope binding site. Where self-assembly of the VL and VH Domains is rendered impossible due to a linker of insufficient length (less than about 12 amino acid residues), two of the scFv constructs interact with one another 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) “*The 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.

[0016] Natural antibodies are capable of binding to only one epitope species (*i.e.*, mono-specific), although they can bind multiple copies of that species (*i.e.*, exhibiting bi-valency or multi-valency). A wide variety of recombinant bi-specific antibody formats have been developed (see, *e.g.*, PCT Publication Nos. WO 2008/003116, WO 2009/132876, WO 2008/003103, WO 2007/146968), 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) or to fuse *e.g.* two Fab fragments or scFv. 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 CH1 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 2013/119903 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 Regions have been replaced with additional VL and VH Domains, so as to form tri-valent 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.

[0017] The art has additionally noted the capability to produce **diabodies** that differ from such natural antibodies in being capable of binding two or more different epitope species (i.e., exhibiting bi-specificity or multispecificity in addition to bi-valency or multi-valency) (see, e.g., Holliger *et al.* (1993) “*Diabodies’: Small Bivalent And Bispecific Antibody Fragments,*” Proc. Natl. Acad. Sci. (U.S.A.) 90:6444-6448; US 2004/0058400 (Hollinger *et al.*); US 2004/0220388 (Mertens *et al.*); Alt *et al.* (1999) FEBS Lett. 454(1-2):90-94; Lu, D. *et al.* (2005) “*A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor Activity,*” J. Biol. Chem. 280(20):19665-19672; WO 02/02781 (Mertens *et al.*); Olafsen, T. *et al.* (2004) “*Covalent Disulfide-Linked Anti-CEA Diabody Allows Site-Specific Conjugation And Radiolabeling For Tumor Targeting Applications,*” Protein Eng Des Sel. 17(1):21-27; Wu, A. *et al.* (2001) “*Multimerization Of A Chimeric Anti-CD20 Single Chain Fv-Fv Fusion Protein Is Mediated Through Variable Domain Exchange,*” Protein Engineering 14(2):1025-1033; Asano *et al.* (2004) “*A Diabody For Cancer Immunotherapy And Its Functional Enhancement By Fusion Of Human Fc 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; Baeuerle, P.A. *et al.* (2009) “*Bispecific T-Cell Engaging Antibodies For Cancer Therapy,*” Cancer Res. 69(12):4941-4944).

[0018] The design of a diabody is based on the single chain variable region fragments (**scFv**). Such molecules are made by linking light and/ or heavy chain variable regions by using a short linking peptide. Bird *et al.* (1988) (“*Single-Chain*

Antigen-Binding Proteins,” Science 242:423-426) describes example of linking peptides which bridge approximately 3.5 nm between the carboxy terminus of one variable region and the amino terminus of the other variable region. 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.

[0019] United States Patent No. 7,585,952 and United States Patent Publication No. 2010-0173978 concern scFv molecules that are immunospecific for ErbB2. Bi-specific T cell engagers (“**BiTEs**”), a type of scFv molecule has been described (WO 05/061547; Baeuerle, P et al. (2008) “*BiTE: A New Class Of Antibodies That Recruit T Cells*,” Drugs of the Future 33: 137-147; Bargou, et al. 2008) “*Tumor Regression in Cancer Patients by Very Low Doses of a T Cell-Engaging Antibody*,” Science 321: 974-977). Such molecules are composed of a single polypeptide chain molecule having two antigen-binding domains, one of which immunospecifically binds to a CD3 epitope and the second of which immunospecifically binds to an antigen present on the surface of a target cell.

[0020] Bi-specific molecules that target both CD19 and CD3 have been described. Blinatumomab, a bi-specific scFv-CD19 x CD3 BiTE is in clinical trials and is reported to have an affinity for CD3 of ~100 nM, and an affinity for CD19 of ~1 nM. Blinatumomab exhibits an EC₅₀ for target cell lysis *in vitro* of ~10-100 pg/ml (Frankel et al. (2013) “*Targeting T Cells To Tumor Cells Using Bispecific Antibodies*,” Curr. Opin. Chem. Biol. 17:385-392). A bi-specific CD19 x CD3 dual affinity retargeting (DART) has also been developed (Moore 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). Compared with the bi-specific scFv-CD19 x CD3 BiTE, this molecule exhibited similar binding affinities but had a lower EC₅₀ for target cell lysis *in vitro* of ~0.5 to 5 pg/ml and showed consistently higher levels of maximum lysis. AFM11, a CD19 x CD3 a bi-specific Tandab has also been described. AFM11 is reported to have an EC₅₀ for target cell lysis *in vitro* of ~0.5 to 5 pg/ml (Zhukovsky *et al.* (2013) “A CD19/CD3 Bispecific Tandab, AFM11, Recruits T Cells To Potently and Safely Kill CD19+ Tumor Cells,” J Clin Oncol 31, 2013 (suppl; abstr 3068)). None of these constructs include an Fc domain.

[0021] Notwithstanding such success, the production of stable, functional heterodimeric, non-mono-specific diabodies can be further optimized by the careful consideration and placement of cysteine residues in one or more of the employed polypeptide chains. Such optimized diabodies can be produced in higher yield and with greater activity than non-optimized diabodies. The present invention is thus directed to the problem of providing polypeptides that are particularly designed and optimized to form heterodimeric diabodies. The invention solves this problem through the provision of exemplary, optimized CD19 x CD3 diabodies.

Summary:

[0022] The present disclosure is directed to bi-specific monovalent diabodies that comprise two polypeptide chains and which possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 (*i.e.*, a “CD19 x CD3 bi-specific monovalent diabody”). Most preferably, such CD19 x CD3 bi-specific monovalent diabodies are composed of three polypeptide chains and possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 and additionally comprise an immunoglobulin Fc Domain (*i.e.*, a “CD19 x CD3 bi-specific monovalent Fc diabody”). The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present disclosure are capable of simultaneous binding to CD19 and CD3. The disclosure is directed to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The disclosure is additionally directed to

methods for the use of such diabodies in the treatment of disease, in particular hematologic malignancies.

[0023] The CD19 x CD3 bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the disclosure thus comprise at least two different polypeptide chains. The polypeptide chains of such diabodies associate with one another in a heterodimeric manner to form one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3. A CD19 x CD3 bi-specific monovalent diabody or bi-specific monovalent Fc diabody of the present disclosure is thus monovalent in that it is capable of binding to only one copy of an epitope of CD19 and to only one copy of an epitope of CD3, but bi-specific in that a single diabody is able to bind simultaneously to the epitope of CD19 and to the epitope of CD3. Each of the polypeptide chains of the diabodies is covalently bonded to another polypeptide chain of the diabody, for example by disulfide bonding of cysteine residues located within such polypeptide chains, so as to form a covalently bonded complex. In particular embodiments, the diabodies of the present disclosure further have an immunoglobulin Fc Domain and/or an Albumin-Binding Domain to extend half-life in vivo.

[0024] In one aspect, there is provided a CD19 x CD3 bi-specific Fc diabody capable of specific binding to CD19 and to CD3, wherein the diabody comprises a first, a second and a third polypeptide chain, wherein the polypeptide chains form a covalently bonded complex, and wherein:

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

A. a Domain IA, comprising

- (1) a sub-Domain (IA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and
- (2) a sub-Domain (IA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});

wherein the sub-Domains IA1 and IA2 are separated from one another by a polypeptide linker, and are either

- (a) VL_{CD19} and VH_{CD3}; or

- (b) VL_{CD3} and VH_{CD19} ;
 - B. a Domain IB, comprising a charged Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**, wherein the Domain IB is separated from the Domain IA by a polypeptide linker;
 - C. a Domain IC, comprising a CH2-CH3 Domain of an antibody; and
 - II. the second polypeptide chain comprises, in the N-terminal to C-terminal direction:
 - A. a Domain IIA, comprising
 - (1) a sub-Domain (IIA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and
 - (2) a sub-Domain (IIA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});
 wherein the sub-Domains IIA1 and IIA2 are separated from one another by a polypeptide linker, and are:
 - (a) VL_{CD19} and VH_{CD3} , if the sub-Domains IA1 and IA2 are VL_{CD3} and VH_{CD19} ; or
 - (b) VL_{CD3} and VH_{CD19} , if the sub-Domains IA1 and IA2 are VL_{CD19} and VH_{CD3} ;
 - B. a Domain IIB, comprising a charged Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**, wherein the Domain IIB is separated from the Domain IIA by a polypeptide linker, and wherein the charged Heterodimer-Promoting Domain of the Domain IB and the charged Heterodimer-Promoting Domain of the Domain IIB have opposite charges; and
 - III. the third polypeptide chain comprises, in the N-terminal to C-terminal direction a Domain IIIC that comprises a CH2-CH3 Domain of an antibody;
- wherein the VL_{CD19} and the VH_{CD19} domains form a monovalent CD19 binding domain, and the VL_{CD3} and VH_{CD3} domains form a monovalent CD3 binding domain; and the CH2-CH3 Domains of the first and third polypeptide chains form an Fc domain capable of binding to an Fc receptor, thereby forming the CD19 x CD3 bi-specific Fc diabody.

[0025] The disclosure additionally provides the embodiment of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabody, wherein:

- (A) the Domains IB and IIB each comprise a cysteine residue that covalently bonds the first polypeptide chain to the second polypeptide chain via a disulfide bond; and
- (B) the Domains IC and IIC each comprise a cysteine residue that covalently bonds the first polypeptide chain to the third polypeptide chain via a disulfide bond.

[0026] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, wherein the VL_{CD19} has the amino acid sequence of **SEQ ID NO:17** and the VH_{CD19} has the amino acid sequence of **SEQ ID NO:21**.

[0027] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, wherein the VL_{CD3} has the amino acid sequence of **SEQ ID NO:25** and the VH_{CD3} has the amino acid sequence of **SEQ ID NO:29**.

[0028] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, wherein the CH2-CH3 Domain of the Domain IC has the amino acid sequence of **SEQ ID NO:15** and the CH2-CH3 Domain of the Domain IIC has the amino acid sequence of **SEQ ID NO:16**.

[0029] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, wherein

- (A) the charged Heterodimer-Promoting Domain of the Domain IB has the amino acid sequence of **SEQ ID NO:10** and the charged Heterodimer-Promoting Domain of the Domain IIB has the amino acid sequence of **SEQ ID NO:11**; or
- (B) the charged Heterodimer-Promoting Domain of the Domain IB has the amino acid sequence of **SEQ ID NO:12** and the charged Heterodimer-Promoting Domain of the Domain IIB has the amino acid sequence of **SEQ ID NO:13**.

[0030] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, wherein:

- (A) the first polypeptide chain has the amino acid sequence of **SEQ ID NO:35**;
- (B) the second polypeptide chain has the amino acid sequence of **SEQ ID NO:37**;
and
- (C) the third polypeptide chain has the amino acid sequence of **SEQ ID NO:39**.

[0031] The disclosure additionally provides the embodiment of any of the above-indicated CD19 x CD3 bi-specific monovalent Fc diabodies, which is capable of cross-reacting with both human and primate CD19 and CD3.

[0032] Disclosed is any of the above-described CD19 x CD3 bi-specific monovalent Fc diabodies for use as a pharmaceutical.

[0033] Disclosed is any of the above-described CD19 x CD3 bi-specific monovalent Fc diabodies for use in the treatment of a disease or condition associated with or characterized by the expression of CD19. Also disclosed is a method of treating a disease or condition characterized by the expression of CD19, particularly wherein the disease or condition associated with or characterized by the expression of CD19 is cancer, and more particularly, wherein the cancer is selected from the group consisting of: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), including blastic crisis of CML and Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter's syndrome or Richter's transformation of CLL, hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), including mantle cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, and Burkitt's lymphoma.

[0034] In one aspect, there is provided a covalently associated polypeptide complex, wherein the polypeptide complex comprises a first polypeptide chain and a second polypeptide chain, wherein:

- (A) the first polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:2** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**; and

(B) the second polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:2** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**;

wherein the Heterodimer-Promoting Domain of the first polypeptide chain and the Heterodimer-Promoting Domain of the second polypeptide chain are covalently bonded to one another via a disulfide bond.

[0034a] In another aspect, there is provided a covalently associated polypeptide complex, wherein said polypeptide complex comprises a first polypeptide chain and a second polypeptide chain, wherein:

(A) said first polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:3** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**; and

(B) said second polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:3** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**;

wherein said Heterodimer-Promoting Domain of said first polypeptide chain and said Heterodimer-Promoting Domain of said second polypeptide chain are covalently bonded to one another via a disulfide bond.

[0035] In another aspect, there is provided a pharmaceutical composition comprising any of the above-described CD19 x CD3 bi-specific Fc diabodies and a physiologically acceptable carrier. In another aspect, there is provided a pharmaceutical composition comprising any one of the above-described covalently associated polypeptide complex and a physiologically acceptable carrier. In another aspect, there is provided use of any of the above-described CD19 x CD3 bi-specific Fc diabody, or the pharmaceutical composition above-described in the manufacture of a medicament for the treatment of a disease or condition associated with or characterized by the expression of CD19, wherein said disease or condition is cancer. The disclosure additionally concerns the use of such a pharmaceutical composition in the treatment of a disease or condition associated with or characterized by the expression of CD19, particularly wherein the disease or condition associated with or characterized by the expression of CD19 is cancer, and more particularly, wherein the cancer is selected from the group consisting of: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), including blastic crisis of CML and Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter's syndrome or Richter's transformation of CLL, hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), including mantle cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, and Burkitt's lymphoma.

[0036] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of exhibiting similar binding affinity to soluble human and cynomolgus monkey CD3 and a 10-fold difference in binding to CD19, as analyzed by surface plasmon resonance (SPR) technology (BIAcore), or as analyzed by flow cytometry using a CD4+ and CD8+ gated T cell population and a CD20+ gated B cell population.

[0037] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of

mediating redirected killing of target tumor cells using human T cells in an assay employing any of 3 target B lymphoma cell lines, Raji/GF (Burkitt's lymphoma), HBL-2 (mantle cell lymphoma), or Jeko-1 (mantle cell lymphoma), and using purified human primary T cells as effector cells. In such an assay, target tumor cell killing is measured using a lactate dehydrogenase (LDH) release assay in which the enzymatic activity of LDH released from cells upon cell death is quantitatively measured, or by a luciferase assay in which luciferase relative light unit (RLU) is the read-out to indicate relative viability of Raji/GF target cells, which have been engineered to express both the green fluorescent protein (GFP) and luciferase reporter genes. The observed EC50 of such redirected killing is about 5 pM or less, about 3 pM or less, about 1 pM or less, about 0.5 pM or less, about 0.3 pM or less, about 0.2 pM or less, about 0.1 pM or less, about 0.05 pM or less, about 0.04 pM or less, about 0.03 pM or less, about 0.02 pM or less or about 0.01 pM or less.

[0038] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of mediating cytotoxicity in human and cynomolgus monkey peripheral blood mononuclear cells (PBMCs) so as to cause a dose-dependent CD20+ B cell depletion in both such cell systems at an Effector cell to T cell ratio of 2:1, 3:1, 4: 1, 5:1, 6:1, 7:1, 8:1 or 9:1. The observed EC50 of human CD20+ B cell depletion is about 7 pM or less, about 5 pM or less, about 3 pM or less, about 1 pM or less, about 0.5 pM or less, about 0.3 pM or less, about 0.2 pM or less, about 0.1 pM or less, about 0.05 pM or less, about 0.04 pM or less, about 0.03 pM or less, about 0.02 pM or less or about 0.01 pM or less. The observed EC50 of cynomolgus CD20+ B cell depletion is about 1000 pM or less, about 900 pM or less, about 800 pM or less, about 500 pM or less, about 300 pM or less, about 100 pM or less, about 50 pM or less, about 20 pM or less, about 10 pM or less, about 5 pM or less, about 2 pM or less, or about 1 pM or less, such that the ratio of autologous human B cell depletion to cynomolgus monkey B cell depletion is about 500:1 or less, about 300:1 or less, about 100:1 or less, about 50:1 or less, about 20:1 or less or about 10:1 or less.

[0039] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of

mediating the release of cytokines (*e.g.*, IFN- γ , TNF- α , IL-2, IL-4, IL-6, and IL-10 and especially IFN- γ and TNF- α) by human and cynomolgus monkey PBMCs. Such release is about 5000 pg/mL or less, about 4000 pg/mL or less, about 3000 pg/mL or less, about 2000 pg/mL or less, about 1000 pg/mL or less, about 500 pg/mL or less, about 2000 pg/mL or less, or about 100 pg/mL or less. The mean EC₅₀ of such cytokine release from human PBMCs is about 100 pM or less, about 90 pM or less, about 80 pM or less, about 50 pM or less, about 30 pM or less, about 20 pM or less, about 10 pM or less, or about 5 pM or less. The mean EC₅₀ of such cytokine release from cynomolgus monkey PBMCs is about 3000 pM or less, about 2000 pM or less, about 1000 pM or less, about 500 pM or less, about 300 pM or less, about 200 pM or less, about 100 pM or less, or about 50 pM or less.

[0040] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of mediating the inhibition of human B cell lymphoma tumor growth in a co-mix xenograft in which such molecules are introduced into NOD/SCID mice along with HBL-2 (a human mantle cell lymphoma) or Raji (Burkitt's lymphoma) tumor cells and activated human T cells at a ratio of 1:5. Preferably, such diabodies are capable of inhibiting tumor growth when provided at a concentration of greater than 100 μ g/ml, at a concentration of about 100 μ g/kg, at a concentration of about 80 μ g/kg, at a concentration of about 50 μ g/kg, at a concentration of about 20 μ g/kg, at a concentration of about 10 μ g/kg, at a concentration of about 5 μ g/kg, at a concentration of about 2 μ g/kg, at a concentration of about 1 μ g/kg, at a concentration of about 0.5 μ g/kg, at a concentration about 0.2 μ g/kg, at a concentration of about 0.1 μ g/kg, at a concentration of about 0.05 μ g/kg, at a concentration of about 0.02 μ g/kg, at a concentration of about 0.01 μ g/kg, or at a concentration of about 0.005 μ g/kg, or at a concentration less than 0.005 μ g/kg.

[0041] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of exhibiting anti-tumor activity in an HBL-2 (human mantle cell lymphoma) xenograft model in female NSG B2m^{-/-} mice, implanted with HBL-2 tumor cells intradermally (ID) on Day 0 followed by intraperitoneal (IP) injection of PBMCs on Day 4 and

administration of diabody on Day 17, so as to cause a decrease in tumor volume of about 10%, about 20%, about 40%, about 60%, about 80%, about 90% or more than 90%.

[0042] The CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent Fc diabodies of the present disclosure are preferably capable of exhibiting prolonged half-lives upon introduction into a recipient human or non-human animal. Such half-lives can be measured by introducing the diabody into a human FcRn transgenic mouse (United States Patents No. 6,992,234 and 7,358,416; Haraya, K. (2014) “*Application Of Human FcRn Transgenic Mice As A Pharmacokinetic Screening Tool Of Monoclonal Antibody*,” *Xenobiotica* 2014 Jul 17:1-8; Proetzel, G. *et al.* (2014) “*Humanized FcRn mouse models for evaluating pharmacokinetics of human IgG antibodies*,” *Methods* 65(1):148-153; Stein, C. *et al.* (2012) “*Clinical Chemistry Of Human FcRn Transgenic Mice*,” *Mamm. Genome* 23(3-4):259-269; Roopenian, D.C. *et al.* (2010) “*Human FcRn Transgenic Mice For Pharmacokinetic Evaluation Of Therapeutic Antibodies*,” *Methods Molec. Biol.* 602:93-104). The measurement is conducted using a B6.Cg-Fcgrt^{tm1Dcr} (CAG-FCGRT)276Dcr/DcrJ mouse (Stock Number 004919), available from the Jackson Laboratories, Bar Harbor, Maine, US.

[0043] For the avoidance of any doubt, the diabodies of the disclosure may exhibit one, two, three, more than three or all of the functional attributes described herein. Thus the diabodies of the disclosure may exhibit any combination of the functional attributes described herein.

[0043a] In another aspect, there is provided a polynucleotide encoding a polypeptide comprising the amino acid sequences of SEQ ID NO: 35, SEQ ID NO: 37, and SEQ ID NO: 39. In another aspect, there is provided an expression vector comprising the above-described polynucleotide.

[0044] The diabodies of the present disclosure may be for use as a pharmaceutical. Preferably, the diabodies are for use in the treatment of a disease or condition associated with or characterized by the expression of CD19. The disclosure also relates to the use of diabodies of the disclosure in the manufacture of a pharmaceutical composition, preferably for the treatment of a disease or condition associated with or characterized by the expression of CD19 as further defined herein.

[0045] The disease or condition associated with or characterized by the expression of CD19 may be a B cell malignancy (Campo, E. *et al.* (2011) “*The 2008 WHO*

Classification Of Lymphoid Neoplasms And Beyond: Evolving Concepts And Practical Applications,” Blood 117(19):5019-5032). For example, the cancer may be selected from the group consisting of: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), including blastic crisis of CML and Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter’s syndrome or Richter’s transformation of CLL, hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), including mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin’s lymphoma, systemic mastocytosis, and Burkitt’s lymphoma.

[0046] The disclosure additionally provides a pharmaceutical composition comprising any of the above-described diabodies and a physiologically acceptable carrier. The disclosure particularly pertains to such a pharmaceutical composition wherein the treated disease or condition is refractory to treatment with rituximab.

[0047] The disclosure additionally provides a use of the above-described pharmaceutical composition in the treatment of a disease or condition associated with or characterized by the expression of CD19.

[0048] The disclosure is particularly directed to the embodiment of such use, wherein the disease or condition associated with or characterized by the expression of CD19 is a B cell malignancy (especially a cancer selected from the group consisting of: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), including blastic crisis of CML and Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter’s syndrome or Richter’s transformation of CLL, hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), including mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin’s lymphoma, systemic mastocytosis, and Burkitt’s lymphoma).

[0049] Terms such as “about” should be taken to mean within 10%, more preferably within 5%, of the specified value, unless the context requires otherwise.

Brief Description of the Drawings:

[0050] **Figure 1** illustrates the structure of a covalently associated bi-specific monovalent diabody composed of two polypeptide chains.

[0051] **Figures 2A and 2B** illustrate the structures of two versions of the first, second and third polypeptide chains of a three chain CD19 x CD3 bi-specific monovalent Fc diabody of the present invention (Version 1, **Figure 2A**; Version 2, **Figure 2B**).

[0052] **Figure 3** shows the simultaneous engagement of both CD19 and CD3 by **DART-A** (●). Neither **Control DART 1** (■) nor **Control DART 2** (▲) were capable of binding to both targets simultaneously.

[0053] **Figures 4A-4B** show the binding of a CD19 x CD3 bi-specific molecule (**DART-A**) to human (**Figure 4A**) and cynomolgus monkey (**Figure 4B**) CD20+ B cells.

[0054] **Figures 5A-5B** show the binding of the CD19 x CD3 bi-specific molecule (**DART-A**) to human (**Figure 5A**) and cynomolgus monkey (**Figure 5B**) CD4+ and CD8+ T cells.

[0055] **Figures 6A-6F** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated redirected killing of target cells relative to that of **Control DART 1**. Dose-response curves of **DART-A**-mediated cytotoxicity using primary human T cells as effector cells for target cell lines: HBL-2 (**Figure 6A**), Raji/GF (green fluorescent) (**Figure 6B**), Jeko-1 (**Figure 6C**), Molm-13 (**Figure 6D**), and Colo205/Luc (**Figure 6F**) with target cell killing measured using the LDH assay. **Figure 6E** shows the killing of Raji/GF cells as determined using a luciferase assay (RLU) to measure relative viable cells. EC50 values for **DART-A** are shown on each graph for 1 representative experiment. **DART-A**: ▲; **Control DART 1**: ▼.

[0056] **Figures 7A-7D** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated redirected killing of target Raji/GF cells at varying E:T cell ratios (10:1 (●), 5:1 (■), and 2.5:1 (▲)). Dose-response curves of **DART-A**-mediated cytotoxicity (percent cytotoxicity) with primary human T cells as effector cells for CD19+ target Raji/GF cells determined after 24 hours incubation (**Figure 7A**) and after 48 hours incubation (**Figure 7B**). Dose-response curves of **DART-A**-mediated cytotoxicity (percent cytotoxicity) with primary human T cells as effector cells for CD19-negative JIMT-1 cells determined after 24 hours incubation (**Figure 7C**) and after 48 hours incubation (**Figure 7D**) were included as controls. Shown is 1 of 3 representative experiments, each of which used T cells from independent donors.

[0057] **Figures 8A-8D** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated redirected killing of target Raji/GF Cells at lower E:T cell ratios of 2.5:1 (●) or 1:1 (■). Dose-response curves of **DART-A**-mediated cytotoxicity (percent cytotoxicity) with primary human T cells as effector cells for CD19+ target Raji/GF cells determined after 72 hours incubation (**Figure 8A**) and after 96 hours incubation (**Figure 8B**). Dose-response curves of CD19 x CD3 bi-specific **DART-A**-mediated cytotoxicity (percent cytotoxicity) with primary human T cells as effector cells for CD19-negative JIMT-1 cells determined after 72 hours incubation (**Figure 8C**) and after 96 hours incubation (**Figure 8D**) were included as controls. Shown is 1 of 3 representative experiments, each of which used T cells from independent donors.

[0058] **Figures 9A-9B** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated autologous B cell depletion in human or cynomolgus monkey PBMCs. Dose-response curves are presented for DART (**DART-A** or **Control DART 1**)-mediated autologous B cell depletion. **Figure 9A**: percent of human CD20+ cells after PBMC treatment with various doses of **DART-A** or **Control DART 1**. **Figure 9B**: percent of cynomolgus monkey CD20+ cells after PBMC treatment with various doses of **DART-A** or **Control DART 1**. EC50 values for **DART-A** are shown on each graph. The percent of CD3+ cells was analyzed in parallel and used as an internal reference control – no changes were observed at any concentration tested. **DART-A**: ▲; **Control DART 1**: ▼.

[0059] **Figure 10** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated redirected killing of target cells with cynomolgus monkey PBMCs at E:T = 30:1. **DART-A: ▲; Control DART 1: ▼**.

[0060] **Figures 11A-11C** show CD19 x CD3 bi-specific molecule (**DART-A**)-mediated cytokine release from human T cells in the presence of target cells. Human T cells were treated with **DART-A** or **Control DART 1** for about 24 hours in the presence of Raji/GF target cells. Culture supernatants were collected and subjected to ELISA-based cytokine measurement. Shown is one representative experiment of 2 using different donors. **Figure 11A: γ -interferon (IFN γ) release; Figure 11B: tumor necrosis factor- α (TNF- α) release; Figure 11C: Interleukin 2 (IL-2) release. DART-A: ▲; Control DART 1: ▼**.

[0061] **Figures 12A-12D** show human T cell activation during redirected cell killing by a CD19 x CD3 bi-specific molecule (**DART-A**) relative to that of **Control DART 1**. Dose-response of **DART-A**-mediated induction of T cell activation markers CD25 (**Figures 12A-12B**) and CD69 (**Figures 12C-12D**) on CD8+ (**Figures 12A and 12C**) and CD4+ (**Figures 12B and 12D**) human T cells using Raji/GF target cells. Shown is one representative experiment of 2, each of which used T cells from different donors. **DART-A: ▲; Control DART 1: ▼**.

[0062] **Figures 13A-13D** show cynomolgus monkey T cell activation during redirected cell killing by a CD19 x CD3 bi-specific molecule (**DART-A**) relative to that of **Control DART 1**. Dose-response of **DART-A**-mediated induction of T cell activation markers CD25 (**Figures 13A and 13B**) and CD69 (**Figures 13C and 13D**) on CD8+ (**Figures 13A and 13C**) and CD4+ (**Figures 13B and 13D**) cynomolgus monkey T cells or PBMCs using Raji/GF target cells. Shown is one representative experiment using T cells. **DART-A: ▲; Control DART 1: ▼**.

[0063] **Figure 14A-14D** show that the CD19 x CD3 bi-specific molecule (**DART-A**) and **Control DART 1** fail to mediate human T cell activation in the presence of CD19-negative JIMT1 cells. Presented is a dose-response of **DART-A**-mediated induction of T cell activation markers CD25 (**Figures 14A and 14B**) and CD69 (**Figures 14C and 14D**) on CD8+ (**Figures 14A and 14C**) and CD4+ (**Figures 14B**

and 14D) from the same donor as in **Figures 12A-12D**. **DART-A**: ▲; **Control DART 1**: ▼.

[0064] **Figures 15A-15B** show the intracellular levels of granzyme B (**Figure 15A**) and perforin (**Figure 15B**) staining in CD4⁺ and CD8⁺ human T cells after incubation with Raji/GF target cells and **DART-A** or **Control DART 1** at varying concentrations for 24 hours at an E:T ratio of 10:1.

[0065] **Figures 16A and 16B** show the proliferation of CFSE-labeled human T cells by FACS analysis after co-culturing with HBL-2 target cells at an E:T ratio of 10:1 in the presence of **DART-A** or **Control DART 1** at 200 ng/mL for 24 hours (**Figure 16A**) or 72 hours (**Figure 16B**). Staining profiles after co-culture with HBL-2 cells in the presence of **DART-A** (black lines) or in the presence of **Control DART 1** (grey fill) are shown.

[0066] **Figure 17** shows inhibition of tumor growth by the CD19 x CD3 bi-specific molecule (**DART-A**) in mice implanted with HBL-2 tumor cells in the presence of activated human T cells. Female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 8/group) were implanted subcutaneously (SC) with HBL-2 tumor cells + activated human T cells on Day 0 followed by treatment with vehicle control, **Control DART 1**, or **DART-A** on Days 0-3 for a total of 4 doses, administered IV. The human T cells and HBL-2 cells were incubated at a ratio of 1:5 (1×10^6 and 5×10^6 cells, respectively). The tumor volume is shown as group mean $\pm$ SEM.

[0067] **Figure 18** shows inhibition of tumor growth by the CD19 x CD3 bi-specific molecule (**DART-A**) in mice implanted with Raji tumor cells in the presence of activated human T cells. Female NOD/SCID mice (n = 8/group) were implanted SC with Raji tumor cells + activated human T cells on Day 0 followed by treatment with vehicle control, **Control DART 1**, or **DART-A** on Days 0-3 for a total of 4 doses administered IV. The human T cells and Raji cells were incubated at a ratio of 1:5 (1×10^6 and 5×10^6 cells, respectively). Tumor volume is shown as group mean $\pm$ SEM.

[0068] **Figure 19A-19B** shows the change in tumor volume over time in mice implanted with HBL-2 tumor cells. **Figure 19A:** Female NSG B2m^{-/-} mice (n = 8 per group) were implanted intradermally (ID) with HBL-2 tumor cells (5×10^6) on Day 0. On Day 4, mice were implanted with PBMCs (5×10^7) intraperitoneally (IP). The mice were then treated intravenously (IV) with vehicle, **Control DART 1**, or the CD19 x CD3 bi-specific **DART-A** as indicated (see Rx arrows). Tumor volume is shown as a group mean $\pm$ SEM. **Figure 19B:** Female NSG B2m^{-/-} mice (n = 8 per group) were implanted intradermally (ID) with HBL-2 tumor cells (5×10^6) on Day 0. On Day 4, mice were implanted with PBMCs (5×10^7) intraperitoneally (IP). The mice were then treated intravenously (IV) with vehicle, **Control DART 1** or the CD19 x CD3 bi-specific **DART-A** as indicated (see Rx arrows). Tumor volume is shown as a group mean $\pm$ SEM.

[0069] **Figures 20A-20B** show circulating B cell levels over time in cynomolgus monkeys following IV administration of up to 4 cycles of weekly escalating doses of the CD19 x CD3 bi-specific **DART-A**. Cynomolgus monkeys in Group 1 (n = 4) were treated with vehicle $\rightarrow$ 0.5 $\rightarrow$ 5 $\rightarrow$ 50 $\rightarrow$ 50 μ g/kg **DART-A** administered IV on Days 1, 8, 15, 22, and 29 (indicated by dotted vertical lines) (**Figure 20A**). Cynomolgus monkeys in Group 2 (n = 4) were treated with vehicle $\rightarrow$ 2 $\rightarrow$ 10 $\rightarrow$ 100 $\rightarrow$ 100 μ g/kg **DART-A** administered IV on Days 1, 8, 15, 22, and 29 (indicated by dotted vertical lines) (**Figure 20B**). Data for each cynomolgus monkey are shown.

[0070] **Figures 21A-21D** shows circulating B cell levels over time in cynomolgus monkeys following IV administration of up to 3 cycles of weekly fixed doses or up to 5 cycles of every 3-day fixed doses of **DART-A**. Cynomolgus monkeys in Groups 3-5 (n = 2 per group) were treated with CD19 x CD3 bi-specific **DART-A**. Group 3 (**Figure 21A**) received 5 ng/kg **DART-A**; Group 4 (**Figure 21B**) received 50 ng/kg **DART-A**; and Group 5 (**Figure 21C**) received 500 ng/kg **DART-A**. **DART-A** was administered IV on Days 1, 8, and 15 (indicated by dotted vertical lines). Cynomolgus monkeys in Group 6 (n = 2) (**Figure 21D**) were treated with 500 ng/kg **DART-A** administered IV on Days 1, 4, 8, 11, and 15 (indicated by dotted vertical lines). Data for each cynomolgus monkey are shown.

[0071] **Figure 22** shows **DART-A** human FcRn transgenic mice pharmacokinetic (PK) analysis. mAb 1, mAb 2 and mAb 3 are irrelevant humanized IgG1 antibodies employed as a control to permit determination of typical antibody half-life in the transgenic animals.

[0072] **Figure 23** shows the survival curves in a disseminated Raji-luc cell leukemia model in human PBMC-reconstituted mice following treatment with 500 µg/kg **DART-A** (■), 500 µg/kg **Control DART 1** (▲) or vehicle control (●).

[0073] **Figure 24** shows the survival curves in a disseminated Raji-luc cell leukemia model in human PBMC-reconstituted mice following treatment with 500 µg/kg (●), 100 µg/kg (■), 20 µg/kg (○), 4 µg/kg (▲), 0.8 µg/kg (▼) or 0.16 µg/kg (□) **DART-A**, 500 mg/kg (Δ) **Control DART 1** or vehicle control (◆).

[0074] **Figure 25** shows the survival curves in a Raji.luc cell lymphoma model in human PBMC-reconstituted mice following treatment with 0.5 mg/kg **DART-A** (▲), 0.1 mg/kg **DART-A** (■) or vehicle control (●).

[0075] **Figure 26** shows the survival curves in a Raji.luc cell lymphoma model in human PBMC-reconstituted mice following treatment with 500 µg/kg (■), 100 µg/kg (▲), 20 µg/kg (▼), 4 µg/kg (◆), 0.8 µg/kg (□) or 0.16 µg/kg (Δ) **DART-A**, 0.1 mg/kg **Control DART 1** (○) or vehicle control (●).

[0076] **Figure 27** shows the serum concentration-time profile of **DART-A** in a cynomolgus monkey dosed with 10 µg/kg **DART-A**.

[0077] **Figure 28** shows the mean ± SEM number of CD45+/CD20+ cells/µL as determined by flow cytometry for a Study Day and Group. The dashed vertical lines on Days 8, 15, 22 and 29 indicate each dose (D) of **DART-A** by 2-hour IV infusion for animals of Groups 2-5; while animals of Group 1 continued to receive vehicle control. The x-axis indicating Study Day is split into 3 segments covering Day -7 to -1, Day 0 to 63 and Day 64-121.

[0078] **Figures 29A-29I** show representative FACS analysis of malignant B cells (CD20+/CD5+) (**Figures 29A-29C**), T cells (CD4+ or CD8+) (**Figures 29D-29E**)

and T cell activation (CD25 in CD4+ (**Figures 29F-29G**) or CD8+ (**Figures 29H-29I**) T cells) in CLL PBMCs (E:T = 1:23) following No treatment (**Figure 29A**), **DART-A** (**Figures 29C, 29E, 29G and 29I**) or **Control DART 1** (**Figures 29B, 29D, 29F and 29H**) treatment for 6 days.

Detailed Description of the Invention:

[0079] The present invention is directed to bi-specific monovalent diabodies that comprise two polypeptide chains and which possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 (*i.e.*, a “CD19 x CD3 bi-specific monovalent diabody”). Most preferably, such CD19 x CD3 bi-specific monovalent diabodies are composed of three polypeptide chains and possess one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 and additionally comprise an immunoglobulin Fc Domain (*i.e.*, a “CD19 x CD3 bi-specific monovalent Fc diabody”). The bi-specific monovalent diabodies and bi-specific monovalent Fc diabodies of the present invention are capable of simultaneous binding to CD19 and CD3. The invention is directed to pharmaceutical compositions that contain such bi-specific monovalent diabodies or such bi-specific monovalent Fc diabodies. The invention is additionally directed to methods for the use of such diabodies in the treatment of disease, in particular hematologic malignancies.

A. Antibodies and Other Binding Molecules

1. Antibodies

[0080] As used herein, the term “**Antibodies**” encompasses not only intact polyclonal or monoclonal antibodies, 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 at least one antigen recognition site. As used herein, the term encompasses fragments (such as Fab, Fab', F(ab')₂ Fv), and single chain (scFv).

[0081] 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 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. 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 the bi-specific molecules of the invention as well as a chimeric antibody, a humanized antibody, or a caninized antibody, to improve the affinity, or other characteristics of the antibody. 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.

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

[0082] The provision of non-mono-specific “**diabodies**” provides a significant advantage over antibodies: 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).

[0083] 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.*, Fcγ receptors (FcγR), was also found to activate the effector cell (Holliger *et al.* (1996) “*Specific Killing Of Lymphoma Cells By Cytotoxic T-Cells Mediated By A Bispecific Diabody*,” Protein Eng. 9:299-305; Holliger *et al.* (1999) “*Carcinoembryonic Antigen (CEA)-Specific T-Cell Activation In Colon Carcinoma Induced By Anti-CD3 x Anti-CEA Bispecific Diabodies And B7 x Anti-CEA Bispecific Fusion Proteins*,” Cancer Res. 59:2909-2916; WO 2006/113665; WO 2008/157379; WO 2010/080538; WO 2012/018687; WO 2012/162068). Normally, effector cell activation is triggered by the binding of an antigen bound antibody to an effector cell via Fc-FcγR interaction; thus, in this regard, diabody molecules 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).

[0084] However, the above advantages come at a 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 (*i.e.*, so as to prevent homodimerization) (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).

[0085] 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).

[0086] 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 Fcγ Receptor IIb (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. 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.

[0087] Each of the two polypeptides of the simplest **DART™** comprises three Domains (**Figure 1**). The first polypeptide comprises: (i) a 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 serves to promote heterodimerization with the second polypeptide and to covalently bond the first polypeptide to the second polypeptide of the diabody. The second polypeptide contains a complementary first Domain (a VL2 Domain), a complementary second Domain (a VH1 Domain) and a third Domain that complexes with the third Domain of the first polypeptide chain in order to promote heterodimerization and covalent bonding with the first polypeptide chain. Such molecules are stable, potent and have the ability to simultaneously bind two or more antigens. They are able to promote redirected T cell mediated killing of cells expressing target antigens.

[0088] 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**). 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. The first polypeptide of such a diabody contains three Domains: (i) a VL1-containing Domain, (ii) a VH2-containing Domain, (iii) a Domain that promotes heterodimerization and covalent bonding with the diabody's first polypeptide chain and (iv) a Domain containing a CH2-CH3 sequence. The second polypeptide of such DARTTM contains: (i) a VL2-containing Domain, (ii) a VH1-containing Domain and (iii) a Domain that promotes heterodimerization and covalent bonding with the diabody's first polypeptide chain. The third polypeptide of such DARTTM comprises a CH2-CH3 sequence. Thus, the first and second polypeptide chains of such DARTTM 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 **DARTsTM** may have either of two orientations (**Table 1**):

Table 1		
First Orientation	3 rd Chain	NH ₂ -CH ₂ -CH ₃ -COOH
	1 st Chain	NH ₂ -VL1-VH2-Heterodimer-Promoting Domain-CH ₂ -CH ₃ -COOH
	2 nd Chain	NH ₂ -VL2-VH1-Heterodimer-Promoting Domain-COOH
Second Orientation	3 rd Chain	NH ₂ -CH ₂ -CH ₃ -COOH
	1 st Chain	NH ₂ -CH ₂ -CH ₃ -VL1-VH2-Heterodimer-Promoting Domain-COOH
	2 nd Chain	NH ₂ -VL2-VH1-Heterodimer-Promoting Domain-COOH

B. Preferred CD19 x CD3 Bi-Specific Monovalent Diabodies Of The Present Invention

[0089] The present invention is particularly directed to CD19 x CD3 bi-specific monovalent diabodies that are capable of simultaneous binding to CD19 and CD3, and to the uses of such molecules in the treatment of hematologic malignancies. Although non-optimized CD19 x CD3 bi-specific monovalent diabodies are fully functional, analogous to the improvements obtained in gene expression through codon optimization (see, e.g., Grosjean, H. *et al.* (1982) “*Preferential Codon Usage In Prokaryotic Genes: The Optimal Codon-Anticodon Interaction Energy And The Selective Codon Usage In Efficiently Expressed Genes*” *Gene* 18(3):199-209), it is possible to further enhance the stability and/or function of CD19 x CD3 bi-specific monovalent diabodies by modifying or refining their sequences.

[0090] The preferred CD19 x CD3 bi-specific monovalent diabodies of the present invention are CD19 x CD3 bi-specific monovalent Fc diabodies that are composed of three polypeptide chains which associate with one another to form one binding site specific for an epitope of CD19 and one binding site specific for an epitope of CD3 (see, **Figure 2A-2B**), so as to be capable of simultaneously binding to CD19 and to CD3. Thus, such diabodies bind to a “first antigen,” which may be either CD3 or CD19, and a “second antigen,” which is CD19 when the first epitope is CD3, and is CD3 when the first epitope is CD19.

[0091] Preferably, as shown in **Figure 2A**, the first of such three polypeptide chains will contain, in the N-terminal to C-terminal direction, an N-terminus, the Antigen-Binding Domain of a Light Chain Variable Domain (VL) of a “first” antigen (either CD3 or CD19), the Antigen-Binding Domain of a Heavy Chain Variable Domain

(**VH**) of a second antigen (CD19, if the first antigen was CD3; CD3, if the first antigen was CD19), a Heterodimer-Promoting Domain, and a C-terminus. An intervening linker peptide (**Linker 1**) separates the Antigen-Binding Domain of the Light Chain Variable Domain from the Antigen-Binding Domain of the Heavy Chain Variable Domain. Preferably, the Antigen-Binding Domain of the Heavy Chain Variable Domain is linked to the Heterodimer-Promoting Domain by an intervening linker peptide (**Linker 2**). In the case of a CD19 x CD3 bi-specific monovalent Fc diabody, the C-terminus of the Heterodimer-Promoting Domain is linked to the CH2-CH3 domains of an Fc region ("Fc Domain") by an intervening linker peptide (**Linker 3**) or by an intervening spacer-linker peptide (**Spacer-Linker 3**). Most preferably, the first of the three polypeptide chains will thus contain, in the N-terminal to C-terminal direction: VL_{First Antigen} – Linker 1 – VH_{Second Antigen} – Linker 2 – Heterodimer-Promoting Domain – Spacer-Linker 3 – Fc Domain.

[0092] Alternatively, as shown in **Figure 2B**, the first of such three polypeptide chains will contain, in the N-terminal to C-terminal direction, an N-terminus, **Linker 3**, the CH2-CH3 domains of an Fc region ("Fc Domain"), an intervening spacer peptide (**Linker 4**), having, for example the amino acid sequence: APSSS (**SEQ ID NO:47**) or the amino acid sequence APSSSPME (**SEQ ID NO:48**), the Antigen-Binding Domain of a Light Chain Variable Domain (**VL**) of the first antigen (either CD3 or CD19), the Antigen-Binding Domain of a Heavy Chain Variable Domain (**VH**) of the second antigen (CD19, if the first antigen was CD3; CD3, if the first antigen was CD19), a Heterodimer-Promoting Domain, and a C-terminus. An intervening linker peptide (**Linker 1**) separates the Antigen-Binding Domain of the Light Chain Variable Domain from the Antigen-Binding Domain of the Heavy Chain Variable Domain. Preferably, the Antigen-Binding Domain of the Heavy Chain Variable Domain is linked to the Heterodimer-Promoting Domain by an intervening linker peptide (**Linker 2**). Most preferably, the first of the three polypeptide chains will thus contain, in the N-terminal to C-terminal direction: **Linker 3** – Fc Domain – **Linker 4** – VL_{First Antigen} – Linker 1 – VH_{Second Antigen} – Linker 2 – Heterodimer-Promoting Domain.

[0093] Preferably, the second of such three polypeptide chains will contain, in the N-terminal to C-terminal direction, an N-terminus, the Antigen-Binding Domain of a Light Chain Variable Domain (**VL**) of the second antigen, the Antigen-Binding Domain of a Heavy Chain Variable Domain (**VH**) of the first antigen, a Heterodimer-Promoting Domain and a C-terminus. An intervening linker peptide (**Linker 1**) separates the Antigen-Binding Domain of the Light Chain Variable Domain from the Antigen-Binding Domain of the Heavy Chain Variable Domain. Preferably, the Antigen-Binding Domain of the Heavy Chain Variable Domain is linked to the Heterodimer-Promoting Domain by an intervening linker peptide (**Linker 2**). Most preferably, the second of the three polypeptide chains will thus contain, in the N-terminal to C-terminal direction: VL_{Second Antigen} – Linker 1 – VH_{First Antigen} – Linker 2 – Heterodimer-Promoting Domain.

[0094] Preferably, the third of such three polypeptide chains will contain the linker peptide (**Linker 3**) and the CH2-CH3 domains of an Fc region (“Fc Domain”).

[0095] The Antigen-Binding Domain of the Light Chain Variable Domain of the first polypeptide chain interacts with the Antigen-Binding Domain of the Heavy Chain Variable Domain of the second polypeptide chain in order to form a functional antigen-binding site that is specific for the first antigen (*i.e.*, either CD19 or CD3). Likewise, the Antigen-Binding Domain of the Light Chain Variable Domain of the second polypeptide chain interacts with the Antigen-Binding Domain of the Heavy Chain Variable Domain of the first polypeptide chain in order to form a second functional antigen-binding site that is specific for the second antigen (*i.e.*, either CD3 or CD19, depending upon the identity of the first antigen). Thus, the selection of the Antigen-Binding Domain of the Light Chain Variable Domain and the Antigen-Binding Domain of the Heavy Chain Variable Domain of the first and second polypeptide chains are coordinated, such that the two polypeptide chains collectively comprise Antigen-Binding Domains of light and Heavy Chain Variable Domains capable of binding to CD19 and CD3.

1. Preferred Linkers

[0001] Most preferably, the length of **Linker 1**, which separates such VL and VH domains of a polypeptide chain is selected to substantially or completely prevent such VL and VH domains from binding to one another. Thus the VL and VH domains of the first polypeptide chain are substantially or completely incapable of binding to one another. Likewise, the VL and VH domains of the second polypeptide chain are substantially or completely incapable of binding to one another. A preferred intervening spacer peptide (**Linker 1**) has the sequence (**SEQ ID NO:1**): GGGSGGGG.

[0096] The purpose of **Linker 2** is to separate the VH Domain of a polypeptide chain from the Heterodimer-Promoting Domain of that polypeptide chain. Any of a variety of linkers can be used for the purpose of **Linker 2**. A preferred sequence for such **Linker 2** has the amino acid sequence: ASTKG (**SEQ ID NO:2**), which is derived from the IgG CH1 domain, or GGCGGG (**SEQ ID NO:3**), which possesses a cysteine residue that may be used to covalently bond the first and second polypeptide chains to one another via a disulfide bond. Since the **Linker 2**, ASTKG (**SEQ ID NO:2**) does not possess such a cysteine, the use of such **Linker 2** is preferably associated with the use of a cysteine-containing Heterodimer-Promoting Domain, such as the E-coil of **SEQ ID NO:12** or the K-coil of **SEQ ID NO:13** (see below).

[0097] The purpose of **Linker 3** is to separate the Heterodimer-Promoting Domain of a polypeptide chain from the Fc Domain of that polypeptide chain. Any of a variety of linkers can be used for the purpose of **Linker 3**. A preferred sequence for such **Linker 3** has the amino acid sequence: DKTHTCPPCP (**SEQ ID NO:4**). A preferred sequence for **Spacer-Linker 3** has the amino acid sequence: GGGDKTHTCPPCP (**SEQ ID NO:5**).

2. Preferred Heterodimer-Promoting Domains

[0098] The formation of heterodimers of the first and second polypeptide chains can be driven by the inclusion of Heterodimer-Promoting Domains. Such domains include GVEPKSC (**SEQ ID NO:6**) or VEPKSC (**SEQ ID NO:7**) on one polypeptide chain and GFNRGEC (**SEQ ID NO:8**) or FNRGEC (**SEQ ID NO:9**) on the other polypeptide chain (US2007/0004909).

[0099] More preferably, however, the Heterodimer-Promoting Domains of the present invention are formed from one, two, three or four tandemly repeated coil domains of opposing charge that comprise a sequence of at least six, at least seven or at least eight charged amino acid residues (Apostolovic, B. *et al.* (2008) “*pH-Sensitivity of the E3/K3 Heterodimeric Coiled Coil*,” *Biomacromolecules* 9:3173–3180; Arndt, K.M. *et al.* (2001) “*Helix-stabilized Fv (hsFv) Antibody Fragments: Substituting the Constant Domains of a Fab Fragment for a Heterodimeric Coiled-coil Domain*,” *J. Molec. Biol.* 312:221-228; Arndt, K.M. *et al.* (2002) “*Comparison of In Vivo Selection and Rational Design of Heterodimeric Coiled Coils*,” *Structure* 10:1235-1248; Boucher, C. *et al.* (2010) “*Protein Detection By Western Blot Via Coiled–Coil Interactions*,” *Analytical Biochemistry* 399:138-140; Cachia, P.J. *et al.* (2004) “*Synthetic Peptide Vaccine Development: Measurement Of Polyclonal Antibody Affinity And Cross-Reactivity Using A New Peptide Capture And Release System For Surface Plasmon Resonance Spectroscopy*,” *J. Mol. Recognit.* 17:540-557; De Crescenzo, G.D. *et al.* (2003) “*Real-Time Monitoring of the Interactions of Two-Stranded de novo Designed Coiled-Coils: Effect of Chain Length on the Kinetic and Thermodynamic Constants of Binding*,” *Biochemistry* 42:1754-1763; Fernandez-Rodriquez, J. *et al.* (2012) “*Induced Heterodimerization And Purification Of Two Target Proteins By A Synthetic Coiled-Coil Tag*,” *Protein Science* 21:511-519; Ghosh, T.S. *et al.* (2009) “*End-To-End And End-To-Middle Interhelical Interactions: New Classes Of Interacting Helix Pairs In Protein Structures*,” *Acta Crystallographica D* 65:1032-1041; Grigoryan, G. *et al.* (2008) “*Structural Specificity In Coiled-Coil Interactions*,” *Curr. Opin. Struc. Biol.* 18:477-483; Litowski, J.R. *et al.* (2002) “*Designing Heterodimeric Two-Stranded α -Helical Coiled-Coils: The Effects Of Hydrophobicity And α -Helical Propensity On Protein Folding, Stability, And Specificity*,” *J. Biol. Chem.* 277:37272-37279; Steinkruger, J.D. *et al.* (2012) “*The d'--d' Vertical Triad is Less Discriminating Than the a'--a'-a' Vertical Triad in the Antiparallel Coiled-coil Dimer Motif*,” *J. Amer. Chem. Soc.* 134(5):2626–2633; Straussman, R. *et al.* (2007) “*Kinking the Coiled Coil – Negatively Charged Residues at the Coiled-coil Interface*,” *J. Molec. Biol.* 366:1232-1242; Tripet, B. *et al.* (2002) “*Kinetic Analysis of the Interactions between Troponin C and the C-terminal Troponin I Regulatory Region and Validation of a New Peptide Delivery/Capture*

System used for Surface Plasmon Resonance,” J. Molec. Biol. 323:345–362; Woolfson, D.N. (2005) “The Design Of Coiled-Coil Structures And Assemblies,” Adv. Prot. Chem. 70:79-112; Zeng, Y. et al. (2008) “A Ligand-Pseudoreceptor System Based On de novo Designed Peptides For The Generation Of Adenoviral Vectors With Altered Tropism,” J. Gene Med. 10:355-367).

[00100] 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 positively charged amino acid residues (or *vice versa*). 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. However, a preferred CD19 x CD3 bi-specific monovalent diabody of the present invention has a first polypeptide chain having a negatively charged coil. 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.

[00101] In a preferred embodiment, one of the Heterodimer-Promoting Domains will comprise four tandem “E-coil” helical domains (**SEQ ID NO:10: EVAALEEK-EVAALEEK-EVAALEEK-EVAALEEK**), 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:11: KVAAALKE-KVAAALKE-KVAAALKE-KVAAALKE**), 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:10** has been modified to contain a cysteine residue: **EVAACEK-EVAALEK-EVAALEK-EVAALEK (**SEQ ID NO:12**). Likewise, especially preferred is a Heterodimer-Promoting Domain in which one of the four tandem “K-coil” helical domains of **SEQ ID NO:11** has been modified to contain a cysteine residue: **KVAACKE-KVAALKE-KVAALKE-KVAALKE (**SEQ ID NO:13**).****

3. Covalent Bonding of the Polypeptide Chains

[00102] The CD19 x CD3 bi-specific monovalent diabodies of the present invention are engineered so that their first and second polypeptide chains covalently bond to one another via one or more cysteine residues positioned along their length. Such cysteine residues may be introduced into the intervening linker that separates the VL and VH domains of the polypeptides. Alternatively, **Linker 2** may contain a cysteine residue. Additionally or alternatively, **Linker 3** may contain a cysteine residue, as in **SEQ ID NO:4** or **SEQ ID NO:5**. Most preferably, one or more coil domains of the Heterodimer-Promoting Domain will be substituted to contain a cysteine residue as in **SEQ ID NO:12** or **SEQ ID NO:13**.

[00103] In particular embodiments, the CD19 x CD3 bi-specific monovalent diabodies of the present invention may further have an Albumin-Binding Domain to extend half-life *in vivo*.

4. Preferred Fc Domains

[00104] The Fc Domain of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention may be either a complete Fc region (*e.g.*, a complete IgG Fc region) or only a fragment of a complete Fc region. Although the Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may possess the ability to bind to one or more Fc receptors (*e.g.*, FcγR(s)), more preferably such Fc Domain will cause reduced binding to FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by a wild-type Fc region) or will substantially eliminate the ability of such Fc Domain to bind to such receptor(s). The Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may include some or all of the CH2 Domain and/or some or all of the CH3 Domain of a complete Fc region, or may comprise a variant

CH2 and/or a variant CH3 sequence (that may include, for example, one or more insertions and/or one or more deletions with respect to the CH2 or CH3 domains of a complete Fc region). The Fc Domain of the bi-specific monovalent Fc diabodies of the present invention may comprise non-Fc polypeptide portions, or may comprise portions of non-naturally complete Fc regions, or may comprise non-naturally occurring orientations of CH2 and/or CH3 domains (such as, for example, two CH2 domains or two CH3 domains, or in the N-terminal to C-terminal direction, a CH3 Domain linked to a CH2 Domain, etc.).

[00105] In a preferred embodiment the first and third polypeptide chains of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention each comprise CH2-CH3 domains that complex together to form an immunoglobulin (IgG) Fc Domain. The amino acid sequence of the CH2-CH3 domain of human IgG1 is (**SEQ ID NO:14**):

```

APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA
PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE
WESNGQPENN YKTTTPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCVMHE
ALHNHYTQKS LSLSPGK

```

Thus the CH2 and/or CH3 Domains of the first and third polypeptide chains may both be composed of **SEQ ID NO:14**, or a variant thereof.

[0002] In particular, it is preferred for the CH2-CH3 domains of the first and third polypeptide chains of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention to exhibit decreased (or substantially no) binding to FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by the wild-type Fc region (**SEQ ID NO:14**)). Fc variants and mutant forms capable of mediating such altered binding are well known in the art and include amino acid substitutions at positions 234 and 235, a substitution at position 265 or a substitution at position 297 (see, for example, US Patent No. 5,624,821, herein incorporated by reference). In a preferred embodiment the CH2-CH3 Domain of the first and/or third polypeptide chains of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention include a substitution at position 234 with alanine and 235 with alanine.

[00106] The CH2 and/or CH3 Domains of the first and third 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 pair of polypeptides comprising the bi-specific monovalent Fc diabody molecule, and further, engineered into any portion of the polypeptides chains of said pair. Methods of protein engineering to favor heterodimerization over homodimerization are well known in the art, in particular with respect to the engineering of immunoglobulin-like molecules, and are encompassed herein (see *e.g.*, Ridgway *et al.* (1996) "'Knobs-Into-Holes' Engineering Of Antibody CH3 Domains For Heavy Chain Heterodimerization," Protein Engr. 9:617-621, Atwell *et al.* (1997) "Stable Heterodimers From Remodeling The Domain Interface Of A Homodimer Using A Phage Display Library," J. Mol. Biol. 270: 26-35, and Xie *et al.* (2005) "A New Format Of Bispecific Antibody: Highly Efficient Heterodimerization, Expression And Tumor Cell Lysis," J. Immunol. Methods 296:95-101; each of which is hereby incorporated herein by reference in its entirety). Preferably the 'knob' is engineered into the CH2-CH3 Domains of the first polypeptide chain and the 'hole' is engineered into the CH2-CH3 Domains of the third polypeptide chain. Thus, the 'knob' will help in preventing the first polypeptide chain from homodimerizing via its CH2 and/or CH3 Domains. As the third polypeptide chain preferably contains the 'hole' substitution it will heterodimerize with the first polypeptide chain as well as homodimerize with itself. A preferred knob is created by modifying 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 third polypeptide chain homodimer from the final bi-specific monovalent Fc diabody comprising the first, second and third polypeptide chains, the protein A binding site of the CH2 and CH3 Domains of the third polypeptide chain is preferably

mutated by amino acid substitution at position 435 (H435R). Thus, the third polypeptide chain homodimer will not bind to protein A, whereas the bi-specific monovalent Fc diabody will retain its ability to bind protein A via the protein A binding site on the first polypeptide chain.

[00107] A preferred sequence for the CH2 and CH3 Domains of the first polypeptide chain of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention will have the “**knob-bearing**” sequence (**SEQ ID NO:15**):

```
APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA
PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLWCLVK GFYPSDIAVE
WESNGQPENN YKTTTPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE
ALHNHYTQKS LSLSPGK
```

[00108] A preferred sequence for the CH2 and CH3 Domains of the third polypeptide chain of the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention will have the “**hole-bearing**” sequence (**SEQ ID NO:16**):

```
APEAAGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA
PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLSCAVK GFYPSDIAVE
WESNGQPENN YKTTTPVLDS DGSFFLVSKL TVDKSRWQQG NVFSCSVMHE
ALHNRYTQKS LSLSPGK
```

[00109] As will be noted, the CH2-CH3 Domains of **SEQ ID NO:15** and **SEQ ID NO:16** 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 FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIIA (CD16a) or FcγRIIIB (CD16b) (relative to the binding exhibited by the wild-type Fc region (**SEQ ID NO:14**)).

[00110] It is preferred that the first polypeptide chain will have a “knob-bearing” CH2-CH3 sequence, such as that of **SEQ ID NO:15**. However, as will be recognized, a “hole-bearing” CH2-CH3 Domain (*e.g.*, **SEQ ID NO:16**) could be employed in the first polypeptide chain, in which case, a “knob-bearing” CH2-CH3 Domain (*e.g.*, **SEQ ID NO:15**) would be employed in the third polypeptide chain.

5. Preferred CD19 Variable Domains

[00111] The Antigen-Binding Domain of the Light Chain Variable Domain of any anti-CD19 antibody may be used in accordance with the present invention. Preferably, however, such VL_{CD19} Domain will have the amino acid sequence (SEQ ID NO:17):

ENVLTQSPAT LSVTPGEKAT ITCRASQSVS YMHWYQQKPG QAPRLLIYDA
SNRASGVPSR FSGSGSGTDH TLTSSLEAE DAATYYCFQG SVYPFTFGQG
 TKLEIK

wherein the underlined sequences are, respectively, CDR1, CDR2, and CDR3, thereof:

VL _{CD19} CDR1:	RASQSVSYM	(SEQ ID NO:18)
VL _{CD19} CDR2:	DASNRAS	(SEQ ID NO:19)
VL _{CD19} CDR3:	FQGSVYPFT	(SEQ ID NO:20)

[00112] Likewise, the Antigen-Binding Domain of the Heavy Chain Variable Domain of any anti-CD19 antibody may be used in accordance with the present invention. Preferably, however, such VH_{CD19} Domain will have the amino acid sequence (SEQ ID NO:21):

QVTLRESGPA LVKPTQTLTL TCTFSGFSL TSGMGVGWIR QPPGKALEWL
AHIWDDDKR YNPALKSRLT ISKDTSKNQV FLTMTNMDPV DTATYYCARM
ELWSYYFDYW GQGTTVTVSS

wherein the underlined sequences are, respectively, CDR1, CDR2, and CDR3, thereof:

VH _{CD19} CDR1:	TSGMGVG	(SEQ ID NO:22)
VH _{CD19} CDR2:	HIWDDDKRYNPALKS	(SEQ ID NO:23)
VH _{CD19} CDR3:	MELWSYYFDY	(SEQ ID NO:24)

6. Preferred CD3 Variable Domains

[00113] The Antigen-Binding Domain of the Light Chain Variable Domain of any anti-CD3 antibody may be used in accordance with the present invention. Preferably, however, such VL_{CD3} Domain will have the amino acid sequence (SEQ ID NO:25):

QAVVTQEPSL TVSPGGTVTL TCRRSSTGAVT TSNYANWVQQ KPGQAPRGLI
GGTNKRAPWT PARFSGSLLG GKAALTITGA QAEDEADYYC ALWYSNLWVF
 GGGTKLTVLG

wherein the underlined sequences are, respectively, CDR1, CDR2, and CDR3, thereof:

VL_{CD3} CDR1: RSSTGAVTTSNYAN (SEQ ID NO:26)
 VL_{CD3} CDR2: GTNKRAP (SEQ ID NO:27)
 VL_{CD3} CDR3: ALWYSNLWV (SEQ ID NO:28)

[00114] Likewise, the Antigen-Binding Domain of the Heavy Chain Variable Domain of any anti-CD3 antibody may be used in accordance with the present invention. Preferably, however, such VH_{CD3} Domain will have the amino acid sequence (SEQ ID NO:29):

EVQLVESGGG LVQPGGSLRL SCAASGFTFS TYAMNWVRQA PGKGLEWVGR
IRSKYNNYAT YYADSVKGRF TISRDDSKNS LYLQMNSLKT EDTAVYYCVR
HGNFGNSYVS WFAYWGQGTLL VTVSS

wherein the underlined sequences are, respectively, CDR1, CDR2, and CDR3, thereof:

VH_{CD3} CDR1: TYAMN (SEQ ID NO:30)
 VH_{CD3} CDR2: RIRSKYNNYATYYADSVKG (SEQ ID NO:31)
 VH_{CD3} CDR3: HGNFGNSYVSWFAY (SEQ ID NO:32)

7. Optional Albumin-Binding Domain

[00115] As disclosed in WO 2012/018687, in order to further improve the *in vivo* pharmacokinetic properties of the CD19 x CD3 bi-specific monovalent diabodies and CD19 x CD3 bi-specific monovalent diabodies Fc diabodies of the present invention, such diabodies 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. A particularly preferred polypeptide portion of a serum-binding protein for this purpose is the Albumin-Binding Domain (ABD) from streptococcal protein G. The Albumin-Binding Domain 3 (ABD3) of protein G of Streptococcus strain G148 is particularly preferred.

[00116] The Albumin-Binding Domain 3 (ABD3) of protein G of Streptococcus strain G148 consists of 46 amino acid residues forming a stable three-helix bundle and has broad albumin-binding specificity (Johansson, M.U. *et al.* (2002) "Structure,

Specificity, And Mode Of Interaction For Bacterial Albumin-Binding Modules,” J. Biol. Chem. 277(10):8114-8120). Albumin is the most abundant protein in plasma and has a half-life of 19 days in humans. Albumin possesses several small molecule binding sites that permit it to non-covalently bind to other proteins and thereby extend their serum half-lives.

[00117] Thus, the second polypeptide chain of such a CD19 x CD3 bi-specific monovalent diabody or bi-specific monovalent Fc diabody having an Albumin-Binding Domain contains a linker (**Linker 4**), which separates the E-coil (or K-coil) of such polypeptide chain from the Albumin-Binding Domain. A preferred sequence for such **Linker 4** is **SEQ ID NO:33**: GGGG. A preferred Albumin-Binding Domain (**ABD**) has the sequence (**SEQ ID NO:34**): LAEAKVLANRELDKYGVSDYYKNLIDNAKSAEGVKALIDEILAALP.

C. Illustrative CD19 x CD3 Bi-Specific Monovalent Fc Diabody, “DART-A”

[00118] The invention provides an illustrative CD19 x CD3 bi-specific monovalent Fc diabody capable of simultaneously and specifically binding to CD19 and to CD3. This illustrative diabody is denoted as “**DART-A**.” The diabodies of the present invention were found to exhibit enhanced functional activity relative to other CD19 x CD3 diabodies.

[00119] As indicated above, the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention comprise three polypeptide chains. The first polypeptide chain of **DART-A** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD19 (VL_{CD19}) (**SEQ ID NO:17**), an intervening linker peptide (**Linker 1**; GGGSGGGG (**SEQ ID NO:1**)), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:29**), an intervening linker peptide (**Linker 2**; ASTKG (**SEQ ID NO:2**)), a Heterodimer-Promoting (E-coil) Domain (EVAACEK-EVAALEK-EVAALEK-EVAALEK (**SEQ ID NO:12**)), an intervening linker peptide (**Spacer-Linker 3**; GGGDKTHTCPPCP (**SEQ ID NO:5**)), a polypeptide that contains the “knob-

bearing” CH2 and CH3 Domains (Fc Domain sequence, SEQ ID NO:15) and a C-terminus.

[00120] Thus, the first polypeptide chain of **DART-A** is composed of: **SEQ ID NO:17 — SEQ ID NO:1 — SEQ ID NO:29 — SEQ ID NO:2 — SEQ ID NO:12 — SEQ ID NO:5 — SEQ ID NO:15**.

[00121] The amino acid sequence of the first polypeptide of **DART-A** is (SEQ ID NO:35):

```
ENVLTQSPATLSVTPGEKATITCRASQSVSYMHYQQKPGQAPRLLIYDASN
RASGVPSRFSGSGSGTDHTLTISLSLEAEDAATYYCFQGSVYPFTFGQGTKLE
IKGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWRQAPG
KGLEWVGRIRSKYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLKTEDTA
VYYCVRHGNFGNSYVSWFAYWGQGLTVTVSSASTKGEVAACEKEVAALEKEV
AALEKEVAALEKGGGDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPE
VTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSREEMTKN
QVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK
```

[00122] A preferred polynucleotide encoding such a polypeptide is (SEQ ID NO:36):

```
gagaatgtgctcacacagtcacctgcaactctgagcgttaactccaggggaga
aggccaccatcacgtgtagagcctcccagagtgtagctacatgcactggta
tcagcagaaacctggacaagctcccaggttgctgatctatgacgcgagcaac
cgggctagtggcgttccatcccgggtttctggctcaggatctggcactgacc
acacctcaccatatccagccttgaagccgaagatgccgcaacctactactg
ctttcaggggagtggtatcccttcacattcggtcaggggtacaaagctggag
attaaggggtggaggatccggcggcggaggcgaggtgcagctgggtggagtctg
ggggaggcttggtccagcctggaggggtccctgagactctcctgtgcagcctc
tggattcaccttcagcacatacgctatgaattgggtccgccaggctccaggg
aaggggctggagtgggttggaggatcaggtccaagtacaacaattatgcaa
cctactatgccgactctgtgaagggttagattcaccatctcaagagatgattc
aaagaactcactgtatctgcaaatgaacagcctgaaaaccgaggacacggcc
gtgtattactgtgtgagacacggtaacttcggcaattcttacgtgtcttggt
ttgcttattggggacaggggacactggtgactgtgtcttccgcctccaccaa
gggcgaagtggccgcatgtgagaaagaggttgctgctttggagaaggaggtc
gctgcacttgaaaaggaggtcgagccctggagaaaggcggcggggacaaaa
ctcacacatgcccaccgtgcccagcacctgaagccgcggggggaccgtcagt
cttcctcttccccccaaaacccaaggacacctcatgatctcccggaccctc
gaggtcacatgcgtggtggtggacgtgagccacgaagacctgaggtcaagt
tcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcg
ggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctg
caccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaag
```

ccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccg
 agaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaac
 caggtcagcctgtggtgcttgggtcaaaggcttctatcccagcgacatcgccg
 tggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcc
 cgtgctggactccgacggctccttcttctctacagcaagctcaccgtggac
 aagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgagg
 ctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaa

[00123] The second polypeptide chain of **DART-A** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:25**), an intervening linker peptide (**Linker 1**; GGGSGGGG (**SEQ ID NO:1**)), a VH domain of a monoclonal antibody capable of binding to CD19 (VH_{CD19}) (**SEQ ID NO:21**), an intervening linker peptide (**Linker 2**; ASTKG (**SEQ ID NO:2**)), a Heterodimer-Promoting (K-coil) Domain (**KVAALKE-KVAALKE-KVAALKE** (**SEQ ID NO:13**)) and a C-terminus.

[00124] Thus, the second polypeptide of **DART-A** is composed of: **SEQ ID NO:25** — **SEQ ID NO:1** — **SEQ ID NO:21** — **SEQ ID NO:2** — **SEQ ID NO:13**.

[00125] The amino acid sequence of the second polypeptide of **DART-A** is (**SEQ ID NO:37**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGG
 TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
 KLTVLGGGSGGGGQVTLRESGPALVKPTQTLTLCTFSGFSLSTSGMGVGV
 IRQPPGKALEWLAHIWDDDKRYNPALKSRLTISKDTSKNQVFLTMTNMMDPV
 DTATYYCARMELWSYYFDYWGGTQTTVTVSSASTKGKVAACKEKVAALKEKVA
 ALKEKVAALKE

[00126] A preferred polynucleotide encoding such a polypeptide has the sequence (**SEQ ID NO:38**):

caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactg
 tgaccctgacatgcagatccagcacaggcgcagtgaccacatctaactacgc
 caattgggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggg
 acaaacaaaagggtccctggacccctgcacggttttctggaagtctgctgg
 gcggaaaggccgctctgactattaccggggcacaggccgaggacgaagccga
 ttactattgtgctctgtggtatagcaatctgtgggtgttcgggggtggcaca
 aaactgactgtgctgggaggggggtggatccggcggagggtggacaggtgacac
 tgagggaatctggtccagctctggtgaaaccactcagacgctcactctcac
 ttgcacctttagtgggttctcactgtccacatctggcatgggagtaggctgg
 attcgacagccacctgggaaagccttggagtggcttgccacatctggtggg
 atgacgacaagcgggtataatccgcactgaagagcagactgaccatcagcaa
 ggatacatccaagaaccagggtgttctgaccatgaccaacatggaccctgtc

gatacagccacctactattgtgctcgcatggagttgtggctcctactacttcg
 actattggggacaaggcacaaccgtgactgtctcatccgcctccaccaagg
 caaagtggccgcatgtaaggagaaagttgctgctttgaaagagaaggtcgcc
 gcacttaaggaaaaggctcgagccctgaaagag

[00127] The third polypeptide chain of **DART-A** comprises, in the N-terminal to C-terminal direction, an N-terminus, a peptide (**Linker 3**; DKTHTCPPCP (**SEQ ID NO:4**)), a polypeptide that contains the “hole-bearing” CH2 and CH3 Domains (Fc Domain sequence, **SEQ ID NO:16**) and a C-terminus.

[00128] Thus, the third polypeptide of **DART-A** is composed of: **SEQ ID NO:4** — **SEQ ID NO:16**.

[00129] The amino acid sequence of the third polypeptide of **DART-A** is (**SEQ ID NO:39**):

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
 VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
 NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFS
 CSVM
 HEALHNRYTQKSLSLSPGK

[00130] A preferred polynucleotide that encodes such a polypeptide has the sequence (**SEQ ID NO:40**):

gacaaaactcacacatgcccaccgtgcccagcacctgaagccgcgggggggac
 cgtcagtccttctcttccccccaaaacccaaggacaccctcatgatctcccg
 gacccttgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgag
 gtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaaagacaa
 agccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcac
 cgtcctgcaccaggactggctgaatggcaaggagtacaagtgaaggtctcc
 aacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggc
 agccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgac
 caagaaccaggtcagcctgagttgcgagcgtcaaaggcttctatcccagcgac
 atcgccgtggagtgaggagagcaatgggcagccggagaacaactacaagacca
 cgctcccgtgctggactccgacggctccttcttctcctcgtcagcaagctcac
 cgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatg
 catgaggctctgcacaaccgctacacgcagaagagcctctccctgtctccgg
 gtaaa

D. Control DARTS

[00131] In order to more meaningfully demonstrate the properties of the CD19 x CD3 bi-specific monovalent diabodies of the present invention, two Control DARTS were constructed. The first control DART (“**Control DART 1**”) is capable of binding

to fluorescein and CD3. The second control DART (“**Control DART 2**”) is capable of binding to fluorescein and CD19.

[00132] The anti-fluorescein antibody used to form **Control DART 1** and **2** was antibody 4-4-20 (Gruber, M. *et al.* (1994) “*Efficient Tumor Cell Lysis Mediated By A Bispecific Single Chain Antibody Expressed In Escherichia coli*,” J. Immunol. 152(11):5368-5374; Bedzyk, W.D. *et al.* (1989) “*Comparison Of Variable Region Primary Structures Within An Anti-Fluorescein Idiotypic Family*,” J. Biol. Chem. 264(3): 1565-1569) were 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:

Amino Acid Sequence Of The Variable Light Chain Domain Of Anti-Fluorescein Antibody 4-4-20 (**SEQ ID NO:41**):

DVVMQTPTFS LPVSLGDQAS ISCRSSQSLV HSNGNTYLRW YLQKPGQSPK
 VLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHVP
WTFGGGTKLE IK

Amino Acid Sequence Of The Variable Heavy Chain Domain Of Anti-Fluorescein Antibody 4-4-20 (**SEQ ID NO:42**):

EVKLDETGGG LVQPGRPMKL SCVASGFTFS DYWMNWVRQS PEKGLEWVAQ
IRNKPYNYET YYSDSVKGRF TISRDDSKSS VYLQMNNLRV EDMGIYYCTG
SYYGMDYWGQ GTSVTVSS

1. **Control DART 1 (Fluorescein x CD3)**

[00133] The first polypeptide chain of **Control DART 1** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to fluorescein (VL₄₋₄₋₂₀) (**SEQ ID NO:41**), an intervening linker peptide (**Linker 1**; GGGSGGGG (**SEQ ID NO:1**)), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:29**), an intervening linker peptide (**Linker 2**; GGCGGG (**SEQ ID NO:3**)), a Heterodimer-Promoting (E-coil) Domain (EVAALEK-EVAALEK-EVAALEK-EVAALEK (**SEQ ID NO:10**)), an intervening linker peptide (**Spacer-Linker 3**; GGGDKTHTCPPCP (**SEQ ID NO:5**)), a polypeptide that contains the “knob-bearing” CH2 and CH3 Domains (Fc Domain sequence, **SEQ ID NO:15**) and a C-terminus.

[00134] Thus, the first polypeptide chain of **Control DART 1** is composed of: **SEQ ID NO:41 — SEQ ID NO:1 — SEQ ID NO:29 — SEQ ID NO:3 — SEQ ID NO:10 — SEQ ID NO:5 — SEQ ID NO:15.**

[00135] The amino acid sequence of the first polypeptide chain of **Control DART 1** is (**SEQ ID NO:43**):

DVVMQTPTFSLPVSLGDQASISCRSSQSLVHSNGNTYLRWYLQKPGQSPKVLIIYK
VSNRFGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPWTFGGGTKLE
IKGGSGGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGL
EWVGRIRSKYNNYATYYADSVKGRFTISRDDSKNSLYLQMNLSLKTEDTAVYYCVR
HGNFGNSYVSWFAYWGQGLTVTVSSASTKGEVAACEKEVAALEKEVAALEKEVAA
LEKGGGDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVDSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAV
EWESNGQPENNYKTTTPVLDSDGSFFLYSLKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK

[00136] The second polypeptide chain of **Control DART 1** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:25**), an intervening linker peptide (**Linker 1**; GGGSGGGG (**SEQ ID NO:1**)), a VH domain of a monoclonal antibody capable of binding to fluorescein (VH_{Fluor}) (**SEQ ID NO:42**), an intervening linker peptide (**Linker 2**; GGCGGG (**SEQ ID NO:3**)), a Heterodimer-Promoting (K-coil) Domain (KVAAALKE-KVAAALKE-KVAAALKE-KVAAALKE (**SEQ ID NO:11**) and a C-terminus.

[00137] Thus, the second polypeptide chain of **Control DART 1** is composed of: **SEQ ID NO:25 — SEQ ID NO:1 — SEQ ID NO:42 — SEQ ID NO:3 — SEQ ID NO:11.**

[00138] The amino acid sequence of the second polypeptide chain of **Control DART 1** is (**SEQ ID NO:44**):

QAVVTQEPSTLTVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGGTNK
RAPWTPARFSGSLGGAALTITGAQAEDEADYICALWYSNLWVFGGGTKLTVLG
GGGSGGGGGEVKLDETGGGLVQPGRPMLSCVASGFTFSDYWMNWVRQSPEKGLEW
VAQIRNKPYNYETYYSDSVKGRFTISRDDSKSSVYLQMNLRVEDMGIYYCTGSY
YGMDYWGQGTSTVTVSSASTKGKVAACKEKVAALKEKVAALKEKVAALKE

[00139] The third polypeptide chain of **Control DART 1** comprises, in the N-terminal to C-terminal direction, an N-terminus, a peptide (**Linker 3**; DKTHTCPPCP (SEQ ID NO:4)), a polypeptide that contains the “hole-bearing” CH2 and CH3 Domains (Fc Domain sequence, SEQ ID NO:16) and a C-terminus.

[00140] Thus, the third polypeptide chain of **Control DART 1** is composed of: SEQ ID NO:4 — SEQ ID NO:16.

[00141] The amino acid sequence of the third polypeptide chain of **Control DART 1** is SEQ ID NO:39):

```
DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
NKALPAPIEKTIISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFCFSVM
HEALHNRYTQKSLSLSPGK
```

2. Control DART 2 (Fluorescein x CD19)

[00142] The first polypeptide chain of **Control DART 2** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD19 (VL_{CD19}) (SEQ ID NO:17), an intervening linker peptide (**Linker 1**; GGGSGGGG (SEQ ID NO:1)), a VH domain of a monoclonal antibody capable of binding to fluorescein (VH_{Fluor}) (SEQ ID NO:42), an intervening linker peptide (**Linker 2**; GGCGGG (SEQ ID NO:3)), a Heterodimer-Promoting (E-coil) Domain (EVAALEK-EVAALEK-EVAALEK-EVAALEK (SEQ ID NO:10)), an intervening linker peptide (**Spacer-Linker 3**; GGGDKTHTCPPCP (SEQ ID NO:5)), a polypeptide that contains the “knob-bearing” CH2 and CH3 Domains (Fc Domain sequence, SEQ ID NO:15) and a C-terminus.

[00143] Thus, the first polypeptide chain of **Control DART 2** is composed of: SEQ ID NO:17 — SEQ ID NO:1 — SEQ ID NO:42 — SEQ ID NO:3 — SEQ ID NO:10 — SEQ ID NO:5 — SEQ ID NO:15.

[00144] The amino acid sequence of the first polypeptide chain of **Control DART 2** is (SEQ ID NO:45):

```
ENVLTQSPATLSVTPGEKATITCRASQSVSYMHYQQKPGQAPRLLIYDASNRAS
GVPSRFSGSGSGTDHTLTISILEAEDAATYYCFQGSVPFTFGQGTKLEIKGGGS
```

GGGGEVKLDETGGGLVQPGRPMKLSCVASGFTFSDYWMNWVRQSPEKGLEWVAQI
 RNKPYNYYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRVEDMGIIYYCTGSYYGMD
 YWGQGTSTVTVSSASTKGEEVAACEKEVAALEKEVAALEKEVAALEKGGGDKTHTCP
 PCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
 EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
 KAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYK
 TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHEALHNHYTQKSLSLSPGK

[00145] The second polypeptide chain of **Control DART 2** comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to fluorescein (VL_{Fluor}) (**SEQ ID NO:41**), an intervening linker peptide (**Linker 1**; GGGSGGGG (**SEQ ID NO:1**)), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:29**), an intervening linker peptide (**Linker 2**; GGCGGG (**SEQ ID NO:3**)), a Heterodimer-Promoting (K-coil) Domain (KVAAALKE-KVAAALKE-KVAAALKE-KVAAALKE (**SEQ ID NO:11**) and a C-terminus.

[00146] Thus, the second polypeptide chain of **Control DART 2** is composed of: **SEQ ID NO:41** — **SEQ ID NO:1** — **SEQ ID NO:29** — **SEQ ID NO:3** — **SEQ ID NO:11**.

[00147] The amino acid sequence of the second polypeptide chain of **Control DART 2** is (**SEQ ID NO:46**):

QAVVTQEPSTLVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGGTNK
 RAPWTPARFSGSLGGAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLG
 GGGSGGGGEVKLDETGGGLVQPGRPMKLSCVASGFTFSDYWMNWVRQSPEKGLEW
 VAQIRNKPYNYYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRVEDMGIIYYCTGSY
 YGMDYWGQGTSTVTVSSASTKGKVAACKEKVAALKEKVAALKEKVAALKE

[00148] The third polypeptide chain of **Control DART 2** comprises, in the N-terminal to C-terminal direction, an N-terminus, a peptide (**Linker 3**; DKHTTCPPCP (**SEQ ID NO:4**)), a polypeptide that contains the “hole-bearing” CH2 and CH3 Domains (Fc Domain sequence, **SEQ ID NO:16**) and a C-terminus.

[00149] Thus, the third polypeptide chain of **Control DART 2** is composed of: **SEQ ID NO:4** — **SEQ ID NO:16**.

[00150] The amino acid sequence of the third polypeptide of **Control DART 2** is **SEQ ID NO:39**):

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFS
CSVM
HEALHNRYTQKSLSLSPGK

E. Pharmaceutical Compositions

[00151] 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 CD19 x CD3 bi-specific monovalent diabodies 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 CD19 x CD3 bi-specific monovalent diabody of the invention and a pharmaceutically acceptable carrier.

[00152] The invention also encompasses pharmaceutical compositions comprising a CD19 x CD3 bi-specific monovalent diabody of the invention (and particularly, a CD19 x CD3 bi-specific monovalent Fc diabody), and a second therapeutic antibody (*e.g.*, tumor specific monoclonal antibody) that is specific for a particular cancer antigen, and a pharmaceutically acceptable carrier.

[00153] 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.

[00154] 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.

[00155] 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.*

[00156] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with a CD19 x CD3 bi-specific monovalent diabody of the present invention (and particularly, a CD19 x CD3 bi-specific monovalent Fc diabody) alone or with such pharmaceutically acceptable carrier. Additionally, one or more other prophylactic or therapeutic agents useful for the treatment of a disease can also be included in the pharmaceutical pack or kit. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more

of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00157] The present invention provides kits that can be used in the above methods. A kit can comprise CD19 x CD3 bi-specific monovalent diabodies, and more preferably, a CD19 x CD3 bi-specific monovalent Fc diabody, of the 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.

F. Methods of Administration

[00158] 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.

[00159] 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.*

[00160] 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 CD19 x CD3 bi-specific monovalent diabodies or CD19 x CD3 bi-specific monovalent Fc diabodies of the invention are administered intramuscularly, intravenously, or subcutaneously. The compositions may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. See, *e.g.*, U.S. Patent Nos. 6,019,968; 5,985,320; 5,985,309; 5,934,272; 5,874,064; 5,855,913; 5,290,540; and 4,880,078; and PCT Publication Nos. WO 92/19244; WO 97/32572; WO 97/44013; WO 98/31346; and WO 99/66903, each of which is incorporated herein by reference in its entirety.

[00161] The invention also provides that the CD19 x CD3 bi-specific monovalent diabodies (and particularly, the CD19 x CD3 bi-specific monovalent Fc diabody) of the invention are packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of the molecule. In one embodiment, the CD19 x CD3 bi-specific monovalent diabodies of the invention are supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, the CD19 x CD3 bi-specific monovalent diabodies of the invention are supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of at least 5 µg, more preferably at least 10 µg, at least 15 µg, at least 25 µg, at least 50 µg, at least 100 µg, or at least 200 µg.

[00162] The lyophilized CD19 x CD3 bi-specific monovalent diabodies or CD19 x CD3 bi-specific monovalent Fc diabodies 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, CD19 x CD3 bi-specific monovalent diabodies of the invention are supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the molecule, fusion protein, or conjugated molecule. Preferably, the liquid form of the CD19 x CD3 bi-specific monovalent diabodies of the invention 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.

[00163] 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.

[00164] For CD19 x CD3 bi-specific monovalent diabodies, or CD19 x CD3 bi-specific monovalent Fc 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.

[00165] In another embodiment, the patient is administered a treatment regimen comprising one or more doses of such prophylactically or therapeutically effective amount of the CD19 x CD3 bi-specific monovalent diabodies (and particularly, the CD19 x CD3 bi-specific monovalent Fc diabodies) encompassed by the 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 CD19 x CD3 bi-specific monovalent diabodies encompassed by the 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 CD19 x CD3 bi-specific monovalent diabodies (and particularly, a CD19 x CD3 bi-specific monovalent Fc diabodies) encompassed by the invention 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.

[00166] 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 CD19 x CD3 bi-specific monovalent diabodies (and particularly, the CD19 x CD3 bi-specific monovalent Fc diabodies) encompassed by the invention is achieved.

[00167] **Table 2** provides 5 examples of different dosing regimens described above for a typical course of treatment.

Table 2						
Regimen	Day	Diabody Dosage (ng diabody per kg subject weight per day)				
1	1, 2, 3, 4	100	100	100	100	100
	5, 6, 7	none	none	none	none	none
2	1, 2, 3, 4	300	500	700	900	1,000
	5, 6, 7	none	none	none	none	none
3	1, 2, 3, 4	300	500	700	900	1,000
	5, 6, 7	none	none	none	none	none
4	1, 2, 3, 4	300	500	700	900	1,000
	5, 6, 7	none	none	none	none	none

[00168] The dosage and frequency of administration of the CD19 x CD3 bi-specific monovalent diabodies or CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention may be reduced or altered by enhancing uptake and tissue penetration of the CD19 x CD3 bi-specific monovalent diabodies by modifications such as, for example, lipidation.

[00169] The dosage of the CD19 x CD3 bi-specific monovalent diabodies or CD19 x CD3 bi-specific monovalent Fc diabodies of the invention administered to a patient may be calculated for use as a single agent therapy. Alternatively, the CD19 x CD3 bi-specific monovalent diabodies or CD19 x CD3 bi-specific monovalent Fc diabodies of the invention are used in combination with other therapeutic compositions and the dosage administered to a patient are lower than when said molecules are used as a single agent therapy.

[00170] 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.

[00171] The compositions of the invention can be delivered in a vesicle, in particular a liposome (*See Langer (1990) "New Methods Of Drug Delivery," Science 249:1527-1533*); Treat *et al.*, in LIPOSOMES IN THE THERAPY OF INFECTIOUS DISEASE AND

CANCER, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp. 3 17-327).

[00172] The compositions of the invention can be delivered in a controlled-release or sustained-release system. Any technique known to one of skill in the art can be used to produce sustained-release formulations comprising one or more CD19 x CD3 bi-specific monovalent diabodies of the invention. *See, e.g.*, U.S. Patent No. 4,526,938; PCT publication WO 91/05548; PCT publication WO 96/20698; Ning *et al.* (1996) “*Intratumoral Radioimmunotherapy Of A Human Colon Cancer Xenograft Using A Sustained-Release Gel*,” *Radiotherapy & Oncology* 39:179-189, Song *et al.* (1995) “*Antibody Mediated Lung Targeting Of Long-Circulating Emulsions*,” *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek *et al.* (1997) “*Biodegradable Polymeric Carriers For A bFGF Antibody For Cardiovascular Application*,” *Pro. Int’l. Symp. Control. Rel. Bioact. Mater.* 24:853-854; and Lam *et al.* (1997) “*Microencapsulation Of Recombinant Humanized Monoclonal Antibody For Local Delivery*,” *Proc. Int’l. Symp. Control Rel. Bioact. Mater.* 24:759-760, each of which is incorporated herein by reference in its entirety. In one embodiment, a pump may be used in a controlled-release system (*See Langer, supra*; Sefton, (1987) “*Implantable Pumps*,” *CRC Crit. Rev. Biomed. Eng.* 14:201-240; Buchwald *et al.* (1980) “*Long-Term, Continuous Intravenous Heparin Administration By An Implantable Infusion Pump In Ambulatory Patients With Recurrent Venous Thrombosis*,” *Surgery* 88:507-516; and Saudek *et al.* (1989) “*A Preliminary Trial Of The Programmable Implantable Medication System For Insulin Delivery*,” *N. Engl. J. Med.* 321:574-579). In another embodiment, polymeric materials can be used to achieve controlled-release of the molecules (*see e.g.*, MEDICAL APPLICATIONS OF CONTROLLED RELEASE, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); CONTROLLED DRUG BIOAVAILABILITY, DRUG PRODUCT DESIGN AND PERFORMANCE, Smolen and Ball (eds.), Wiley, New York (1984); Levy *et al.* (1985) “*Inhibition Of Calcification Of Bioprosthetic Heart Valves By Local Controlled-Release Diphosphonate*,” *Science* 228:190-192; During *et al.* (1989) “*Controlled Release Of Dopamine From A Polymeric Brain Implant: In Vivo Characterization*,” *Ann. Neurol.* 25:351-356; Howard *et al.* (1989) “*Intracerebral Drug Delivery In Rats With Lesion-Induced Memory Deficits*,” *J. Neurosurg.* 7(1):105-112); U.S. Patent No.

5,679,377; U.S. Patent No. 5,916,597; U.S. Patent No. 5,912,015; U.S. Patent No. 5,989,463; U.S. Patent No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253). Examples of polymers used in sustained-release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. 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).

[00173] Controlled-release systems are discussed in the review by Langer (1990, “*New Methods Of Drug Delivery*,” *Science* 249:1527-1533). Any technique known to one of skill in the art can be used to produce sustained-release formulations comprising one or more therapeutic agents of the invention. See, *e.g.*, U.S. Patent No. 4,526,938; International Publication Nos. WO 91/05548 and WO 96/20698; Ning *et al.* (1996) “*Intratumoral Radioimmunotherapy Of A Human Colon Cancer Xenograft Using A Sustained-Release Gel*,” *Radiotherapy & Oncology* 39:179-189, Song *et al.* (1995) “*Antibody Mediated Lung Targeting Of Long-Circulating Emulsions*,” *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek *et al.* (1997) “*Biodegradable Polymeric Carriers For A bFGF Antibody For Cardiovascular Application*,” *Pro. Int’l. Symp. Control. Rel. Bioact. Mater.*

24:853-854; and Lam *et al.* (1997) "Microencapsulation Of Recombinant Humanized Monoclonal Antibody For Local Delivery," Proc. Int'l. Symp. Control Rel. Bioact. Mater. 24:759-760, each of which is incorporated herein by reference in its entirety.

[00174] Where the composition of the invention is a nucleic acid encoding a CD19 x CD3 bi-specific monovalent diabody of the invention, the nucleic acid can be administered *in vivo* to promote expression of its encoded CD19 x CD3 bi-specific monovalent diabody or CD19 x CD3 bi-specific monovalent Fc diabody, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (See U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (See *e.g.*, Joliot *et al.* (1991) "Antennapedia Homeobox Peptide Regulates Neural Morphogenesis," Proc. Natl. Acad. Sci. (U.S.A.) 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination.

[00175] Treatment of a subject with a therapeutically or prophylactically effective amount of a CD19 x CD3 bi-specific monovalent diabody (and particularly, a CD19 x CD3 bi-specific monovalent Fc diabody) of the invention can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with 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.

G. Uses of the Compositions of the Invention

[00176] The CD19 x CD3 bi-specific monovalent diabodies of the present invention (and particularly, the CD19 x CD3 bi-specific monovalent Fc diabodies of the present invention) have the ability to treat any disease or condition associated with or characterized by the expression of CD19. Thus, without limitation, such molecules may be employed in the diagnosis or treatment of acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), including blastic crisis of CML and Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter's syndrome or Richter's transformation of CLL, hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), including mantle cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, and Burkitt's lymphoma; Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis. The bi-specific monovalent diabodies of the present invention may additionally be used in the manufacture of medicaments for the treatment of the above-described conditions.

Examples

[00177] Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

Example 1 Quantitation of CD19 Cell Surface Expression

[00178] To identify suitable target cell lines for evaluating the biological activity of CD19 x CD3 bispecific diabodies, CD19 cell surface expression levels were first confirmed using quantitative FACS (QFACS) on a panel of human B-cell lymphoma/leukemia cell lines, including Nalm-6 (acute lymphoblastic leukemia), Raji (Burkitt's lymphoma), Daudi (Burkitt's lymphoma), HBL-2 (mantle cell lymphoma), MEC-1 (chronic lymphocytic leukemia), and Jeko-1 (mantle cell

lymphoma), MOLM-13 (acute myeloid leukemia cell line), JIMT1 (breast cancer) and Colo205 (colon cancer). Absolute numbers of CD19 antibody binding sites on the surface were calculated using a QFACS kit. As shown in **Table 3**, the absolute numbers of CD19 binding sites on the cell lines were in the order of Raji (high) > Nalm-6 (medium) > Daudi (medium) > HBL2 (medium) > MEC-1 (low) > Jeko-1 (low). As expected MOLM-3, JIMT-1 and Colo205 lacked CD19 expression, which is consistent with CD19 being a B-cell specific antigen.

Table 3	
Target Cell Line	CD19 Surface Expression (Antibody Binding Sites)
Raji	670,322
Nalm-6	442,730
Daudi	369,860
HBL-2	334,725
MEC-1	177,025
Jeko-1	124,396

Example 2

Binding Affinities

[00179] In order to quantitate the extent of binding between a CD19 x CD3 bi-specific monovalent diabody and human or cynomolgus monkey CD3, BIACORE™ analyses were conducted using the illustrative CD19 x CD3 bi-specific monovalent Fc diabody, **DART-A**. BIACORE™ analyses measure the dissociation off-rate, k_d . The binding affinity (KD) between an antibody and its target is a function of the kinetic constants for association (on rate, k_a) and dissociation (off-rate, k_d) according to the formula: $KD = [k_d]/[k_a]$. The BIACORE™ analysis uses surface plasmon resonance to directly measure these kinetic parameters.

[00180] The results of binding of **DART-A** (0-100 nM) to soluble human and cynomolgus monkey CD3 and CD19, analyzed by surface plasmon resonance (SPR) technology (BIAcore), are shown in **Table 4**.

[00181] The binding affinity of **DART-A** is similar for human and cynomolgus monkey CD3 (KD = 21.2 nM and 21.9 nM, respectively). However, **DART-A** has an approximate 10 fold lower affinity for cynomolgus monkey CD19 than for human

CD19 (KD = 20.3 nM and 2.0 nM, respectively) due to an increased dissociation rate constant (kd) from cynomolgus monkey CD19. The cynomolgus monkey remains a relevant species for toxicology evaluations, although the difference in CD19 binding affinity should be considered.

Table 4			
Antigens	ka (± SD) (M-1s-1)	kd (± SD) (s-1)	KD (± SD) (nM)
Human CD3ε/δ	2.2 × 10 ⁵	4.5 (± 0.1) × 10 ⁻³	21.2 (± 1.7)
Cynomolgus CD3ε/δ	2.0 (± 0.1) × 10 ⁵	4.3 (± 0.2) × 10 ⁻³	21.9 (± 1.2)
Human CD19-His	2.8 (± 0.3) × 10 ⁵	5.6 (± 0.6) × 10 ⁻⁴	2.0 (± 0.2)
Cynomolgus CD19-His	2.4 (± 0.1) × 10 ⁵	4.7 (± 1.1) × 10 ⁻³	20.3 (± 1.3)

Example 3

Cell Binding Characteristics

[00182] **DART-A** binding to mouse, rat, rabbit, cynomolgus monkey, and human blood leukocytes (purified from whole blood) was evaluated *in vitro* by flow cytometry. Leukocytes were stained with **DART-A**, as well as **Control DART 1** and **Control DART 2**, at concentrations of 25 or 100 nM for approximately 1 hour. **Control DART 2** contains the same CD19 binding component as **DART-A**, while **Control DART 1** contains the same CD3 binding component as **DART-A**. Following incubation, DART proteins bound to leukocytes were detected with an anti-E-coil/K-coil (EK) mAb, which recognizes the EK coil heterodimerization region of the DART proteins. No **DART-A** binding was observed on mouse, rat, or rabbit blood leukocytes. Likewise, neither Control DART diabody showed any binding to mouse, rat, or rabbit leukocytes. As expected, both concentrations of **DART-A** tested showed specific binding to both human and cynomolgus monkey blood leukocytes.

[00183] A bifunctional ELISA assay was used to demonstrate simultaneous engagement of both target antigens by **DART-A**. ELISA plates were coated with soluble human CD3ε/δ heterodimer and incubated at 4C overnight and then blocked with BSA. Various concentrations of **DART-A** were added followed by addition of soluble human CD19-biotin. The plates were washed and the immune complex was detected with chemoluminescent substrate conjugated to streptavidin. As shown in **Figure 3**, **DART-A** is capable of simultaneous binding to both CD19 and CD3.

[00184] The bi-specific binding of **DART-A** was further evaluated in both cynomolgus monkey and human blood leukocytes (purified from whole blood) by flow cytometry. Leukocytes were stained with **DART-A**, **Control DART 1** or **Control DART 2** at concentrations ranging from 0.005 to 80 nM for approximately 1 hour. Following incubation, binding to leukocytes was detected using a biotinylated anti-E-coil/K-coil (EK) mAb, which recognizes the EK coil heterodimerization region of the DART proteins and streptavidin-PE. Since **DART-A** binds to CD3, a combination of CD4 and CD8 was used to define the T cell population. Similarly, CD20 was used as a B cell marker instead of CD19. Therefore, in this study, the CD4⁺ and CD8⁺ gated events represent the T cell population and CD20⁺ gated events represent the B cell population. **DART-A** demonstrated bi-specific binding to both human and cynomolgus monkey B cells and T cells. In contrast, the Control DART diabodies showed only mono-specific binding to B cells (**Control DART 2**) or T cells (**Control DART 1**) consistent with their corresponding binding specificity. The **DART-A** and control DART diabodies binding titration curves for human and cynomolgus monkey B and T cells are provided in **Figures 4A-4B** and **Figures 5A-5B**.

Example 4

DART-A-Mediated Redirected Killing of Target Tumor Cells Using Human T Cells

[00185] The ability of **DART-A** to mediate redirected target cell killing was evaluated on 3 target B lymphoma cell lines, Raji/GF (Burkitt's lymphoma), HBL-2 (mantle cell lymphoma), and Jeko-1 (mantle cell lymphoma), displaying a range of CD19 expression, using purified human primary T cells as effector cells. Raji/GF cells showed the highest levels of CD19 expression, followed by medium levels of CD19 expression in HBL-2 cells, and lower CD19 expression levels in Jeko-1 cells. Target tumor cell killing was measured using a lactate dehydrogenase (LDH) release assay in which the enzymatic activity of LDH released from cells upon cell death is quantitatively measured, or by a luciferase assay in which luciferase relative light unit (RLU) is the read-out to indicate relative viability of Raji/GF target cells, which are engineered to express both the green fluorescent protein (GFP) and luciferase reporter genes.

[00186] **DART-A** exhibited potent redirected cell killing in all 3 cell lines when purified T cells from multiple independent human donors were used at an effector to target (E:T) cell ratio of 10:1 (**Figures 6A-6C**). The average effective concentration at 50% of maximal activity (EC₅₀) values were 1.05×10^{-1} pM for HBL-2 cells; 3.07×10^{-1} pM for Raji/GF cells; and 1.46×10^{-1} pM for Jeko-1 cells (**Table 5**). **Control DART 1** did not mediate T cell redirected killing of target cells. Most importantly, no cell killing activity was observed in cell lines (Molm-13 and Colo205) that do not express CD19 (**Figures 6D-6F**).

Table 5 Summary of EC₅₀ Values from Representative DART-A-Mediated Redirected Killing of Target Raji/GF Cells at Varying E:T Cell Ratios						
	E:T = 10:1		E:T = 5:1		E:T = 2.5:1	
Cytotoxicity	EC ₅₀ (pM)	E _{max} (%)	EC ₅₀ (pM)	E _{max} (%)	EC ₅₀ (pM)	E _{max} (%)
24 hours	0.14	63	0.67	24	N/A	4
48 hours	0.0441	73	0.38	48	1.08	11

[00187] To evaluate the activity of **DART-A** in the presence of a lower number of effector cells, redirected cell killing was tested with lower E:T cell ratios of 5:1, 2.5:1, and 1:1 in Raji/GF cells following incubation with **DART-A** for 24 to 96 hours. Cytotoxic activity observed at the 5:1 ratio was about half the maximal activity observed at the 10:1 ratio; lower, specific activity was also observed with an E:T cell ratio of 2.5:1 at both the 24 and 48 hour time points (**Figures 7A-7D** and **Table 5**). Following incubation for 72 or 96 hours, cytotoxicity of Raji/GF cells was significantly increased at the 2.5:1 and 1:1 ratios (see **Figures 8A-8D** and **Table 6**). It is noteworthy that efficient cell lysis at lower E:T cell ratios increased in Raji/GF cells over time, suggesting serial target cell killing by **DART-A**-activated T cells. The kinetics suggest that time is the limiting factor for low T cell numbers to efficiently eliminate larger numbers of target cells. In conclusion, data from multiple experiments using human T cells from different donors indicate that **DART-A** redirected cell killing activity is dependent on both the E:T cell ratio and time, particularly at lower E:T cell ratios.

Table 6 Summary of EC50 Values from Representative DART-A-Mediated Redirected Killing of Target Raji/GF Cells at Lower E:T Cell Ratios				
	E:T = 2.5:1		E:T = 1:1	
Cytotoxicity	EC50 (pM)	E _{max} (%)	EC50 (pM)	E _{max} (%)
72 hours	1.54	64	2.79	33
96 hours	0.422	87	0.440	56

Example 5

DART-A-Mediated Autologous B Cell Depletion in Human and Cynomolgus Monkey PBMCs

[00188] Since normal B cells express CD19 and since **DART-A** was shown to cross-react with cynomolgus monkey CD19 and CD3 (**Example 2, Figures 4A-4B and Figures 5A-5B**), **DART-A**-mediated cytotoxicity was investigated in both human and cynomolgus monkey peripheral blood mononuclear cells (PBMCs). There was dose-dependent CD20+ B cell depletion observed in both human and cynomolgus monkey PBMCs after treatment with **DART-A** (**Figures 9A-9B**). As expected, no B cell depletion was observed after treatment with **Control DART 1**.

[00189] To further confirm **DART-A** activity in PBMCs, multiple independent human or cynomolgus monkey PBMC donors with varying T cell (effector) to B cell (target) E:T cell ratios were used to evaluate potency. In summary (**Table 7**), the EC50 values for human B cell depletion ranged from 1.84 to 6.27 pM; while the EC50 values for cynomolgus monkey B cell depletion were between 106.8 to 859.1 pM. The potency of **DART-A** for autologous human B cell depletion appeared greater than that for cynomolgus monkey B cell depletion (approximately 100-fold greater by average; range: 19 to 312-fold). However, it is noteworthy that the E:T cell ratio in cynomolgus monkey PBMCs (about 4:1 on average) was lower than that in human PBMCs (about 8:1 on average), which likely contributed to the greater difference in EC50 values given that **DART-A** redirected killing activity is dependent on the E:T cell ratio. Potential differences in CD19 expression levels between human and cynomolgus monkey B cells may be another variable factor contributing to the apparent difference in potency.

Table 7					
Summary of EC50 Values for Autologous B Cell Depletion Following Treatment of Human or Cynomolgus Monkey PBMCs with DART-A					
	EC50 Values (pM)			EC50 Values (pM)	
	DART-A	Control DART		DART-A	Control DART
Human 5 (E:T= 8:1)	6.27	No activity	Cyno 5 (E:T= 3:1)	166.2	No activity
Human 6 (E:T= 8:1)	2.75	No activity	Cyno 6 (E:T= 2:1)	859.1	No activity
Human 7 (E:T= 5:1)	5.52	No activity	Cyno 7 (E:T= 3:1)	106.8	No activity
Human 8 (E:T= 9:1)	1.84	No activity	Cyno 8 (E:T= 7:1)	239.1	No activity
Mean	4.20	Not applicable	Mean	342.8	Not applicable

[00190] It is important to note that **DART-A** mediated efficient cytotoxicity against Raji/GF target cells when cynomolgus monkey PBMCs were used as effector cells with an EC50 of 1.30×10^{-2} pM (**Figure 10**) similar to that observed when human T cells were used as effector cells (average EC50 = 3.07×10^{-1} pM), indicating that human and cynomolgus monkey T cells have comparable activity against the same target cell line. These data further support the use of the cynomolgus monkey as a relevant species for the evaluation of **DART-A** in non-clinical toxicology studies.

Example 6

Evaluation of DART-A-Mediated Cytokine Release Using Human or Cynomolgus Monkey Effector Cells

[00191] **DART-A**-mediated depletion of the B cell population in human and cynomolgus monkey PBMCs was associated with cytokine release in both human and cynomolgus monkey PBMCs. As summarized below in **Table 8**, the EC50 values for cytokine release are equivalent to or higher than for human or cynomolgus monkey autologous B cell depletion, indicating that **DART-A** is more potent in mediating B cell depletion than inducing cytokine release *in vitro*. The cytokine release profiles between human and cynomolgus monkeys *in vitro* have some similarities and differences. IFN- γ and TNF- α were the predominant cytokines released in both species following treatment with **DART-A**. However, IL-6 was the third most predominant cytokine produced in humans based on Emax values, while IL-2 was produced in substantial amounts only in cynomolgus monkey PBMCs. Comparison

of the mean EC50 values for the most sensitive cytokines produced in PBMCs following incubation with **DART-A** (IFN- γ , TNF- α , and IL-6 in humans) with the mean EC50 values for autologous B cell depletion, revealed there is at least a 6-fold apparent safety margin as reflected in the difference between B cell depletion and cytokine production in humans (**Table 9**).

Table 8 Summary of EC50 Values for Autologous B Cell Depletion and Cytokine Production Following Treatment of Human or Cynomolgus Monkey PBMCs with DART-A	
Human PBMCs	
Assay (number of donors tested)	Mean EC50 (pM)
Human B Cell Depletion (n = 4)	4.20
Human Cytokine Release (n = 2)	
IFN- γ	25.21
TNF- α	31.83
IL-10	70.11
IL-6	23.55
IL-4	Not applicable*
IL-2	56.77
Cynomolgus Monkey PBMCs	
Cynomolgus Monkey B Cell Depletion (n = 4)	342.8
Cynomolgus Monkey Cytokine Release (n = 2)	
IFN- γ	925.24
TNF- α	262.22
IL-6	306.68
IL-5	486.22
IL-4	1718.63
IL-2	2930.19
* below limit of detection	

Table 9			
Summary of EC50 Values for Cytotoxicity vs Cytokine Production Following <i>in vitro</i> Evaluation of DART-A in Human Cells			
CTL Assay ¹		Human PBMCs ²	
Assay (number of donors)	Mean EC50 (pM)	Assay (number of donors)	Mean EC50 (pM)
Cytotoxicity		Autologous B cell Depletion	
HBL-2 (n = 5)	0.11	PBMCs (n = 7)	4.20
Raji/GF (n = 5)	0.31		
Jeko-1 (n = 3)	0.15		
Cytokine Release (n = 2)		Cytokine Release (n = 2)	
IFN- γ	5.51	IFN- γ	25.21
TNF- α	3.71	TNF- α	31.83
IL-2	6.32	IL-6	23.55
EC50 fold difference (Indicative Safety Margin)	12-58	EC50 fold difference (Indicative Safety Margin)	6-8
1 Activity evaluated using CTL assay with CD19-expressing tumor cells (HBL-2, Raji/GF, or Jeko-1) in the presence of purified human T cells (E:T = 10:1). Cytokine release was evaluated using Raji/GF tumor cells.			
2 Activity evaluated in normal human PBMCs.			

[00192] **DART-A**-mediated cytokine release by T cells in the presence of target cells was also investigated. **DART-A** dose-dependent cytokine production of TNF- α , IFN- γ , and IL-2 from human T cells was observed with the highest levels (Emax) observed for IL-2 (> 7000 pg/mL) after co-incubation with Raji/GF target cells (**Figures 11A-11C**; **Table 10**). Substantial levels of TNF- α and IFN- γ (~2000-4000 pg/mL) were also produced. As expected, incubation with a Control DART diabody did not result in any detectable cytokine production (**Figures 11A-11C**). Considering the mean EC50 value for the most sensitive cytokine produced by purified human T cells (TNF- α) following incubation with **DART-A** in the presence of CD19-expressing target cells (Raji/GF) compared with the mean EC50 value for target cell cytotoxicity in the least sensitive target cell line (Raji/GF), there is at least a 12-fold apparent safety margin between target cell killing and cytokine production (see **Table 9** above).

Table 10 EC50 Values for DART-A-Mediated Cytokine Production by Human T Cells in the Presence of Raji/GF Target Cells from Two Independent Donors at E:T = 10:1				
	D47067		D44284	
Cytokine	EC50 (pM)	E _{max} (pg/mL)	EC50 (pM)	E _{max} (pg/mL)
IFN- γ	8.58	3885	2.44	2219
TNF- α	6.79	3722	0.626	2361
IL-2	11.31	8496	1.33	7492

Example 7

Evaluation of DART-A-Mediated Human and Cynomolgus Monkey T Cell Activation

[00193] The effect on T cells during **DART-A**-mediated redirected target cell (Raji/GF cells) lysis was characterized by evaluating expression of the T cell activation markers CD69 and CD25. Both CD25 and CD69 were upregulated in human and cynomolgus monkey CD4⁺ and CD8⁺ T cells in a dose-dependent manner (**Figures 12A-12D** and **Figures 13A-13D**), indicating that **DART-A**-mediated redirected cell killing is associated with a concomitant activation of T cells. No T cell activation was observed in the presence of CD19-negative JIMT-1 cells (**Figures 14A-14D**). These data suggest that T cell activation is dependent upon target cell co-engagement. Further supporting this conclusion, the **Control DART 1** did not mediate induction of T cell activation markers (**Figures 12A-12D** and **Figures 13A-13D**).

Example 8

Evaluation of CD19 x CD3 Mechanism of Action

[00194] To confirm that the anticipated mechanism for CD19 x CD3-mediated redirected cell killing by T cells is mediated by granzyme B and perforin, intracellular levels of granzyme B and perforin were measured in T cells following exposure to **DART-A** or **Control DART 1** in a CTL assay. Dose-dependent upregulation of granzyme B and perforin levels in both CD8⁺ and CD4⁺ T cells was observed following 24 hours incubation of human T cells with **DART-A** in the presence of Raji/GF target cells at an E:T cell ration of 10:1 (**Figures 15A-15B**). In contrast, no upregulation of granzyme B or perforin was observed when human T cells were incubated with Raji/GF target cells and **Control DART 1**. These data support that

DART-A-mediated target cell killing may be mediated through the granzyme B and perforin pathway. Of note, the upregulation of granzyme B (**Figure 15A**) and perforin (**Figure 15B**) was higher in CD8⁺ T cells compared with CD4⁺ T cells, consistent with the expected higher CTL potency of CD8⁺ T cells.

[00195] The expansion of T cells during effector:target cell co-engagement by CD9 x CD3 was also evaluated, given that proliferation concomitant with T cell activation has been previously reported. CFSE-labeled human T cells were co-cultured with HBL-2 target cells at an E:T cell ratio of 10:1 in the presence of **DART-A** or **Control DART 1** at a concentration of 200 ng/mL. Proliferation of CFSE-labeled T cells was monitored by levels of CFSE dilution over time by FACS analysis (**Figures 16A-16B**). **Figure 16A** shows the CFSE-staining profiles in the presence of **DART-A** or **Control DART1** and target cells. The addition of **DART-A** led to proliferation (CFSE dilution) with about 75% of cells proliferating after 72 hours incubation (**Figure 16B**). In contract, no proliferation of CFSE-labeled T cells was observed in the presence of **Control DART 1**.

Example 9 Efficacy in Co-Mix Xenografts

[00196] The inhibition of human B cell lymphoma tumor growth by **DART-A** was evaluated in female NOD/SCID mice (n = 8/group) injected with HBL-2 (human mantle cell lymphoma) or Raji (Burkitt's lymphoma) tumor cells co-mixed with activated human T cells. In both studies, human T cells and tumor cells (HBL-2 or Raji) were combined at a ratio of 1:5 (1×10^6 and 5×10^6 cells, respectively) and injected SC into mice on Day 0. Vehicle control (tumor cells alone), **DART-A**, or **Control DART 1** were subsequently administered IV on Days 0, 1, 2, and 3.

[00197] As shown in **Figure 17**, the growth of HBL-2 tumor cells was completely inhibited following IV treatment with **DART-A** at dose levels $\geq 0.2 \mu\text{g/kg}$. Although treatment with $0.02 \mu\text{g/kg}$ **DART-A** appeared to delay the growth of the HBL-2 tumors, the response was not statistically significant. The HBL-2 tumors in the vehicle and control DART groups reached an average tumor volume of approximately 1000 mm^3 by Day 13.

[00198] As shown in **Figure 18**, no significant growth of Raji tumor cells was observed through the end of study (Day 28) in mice treated with **DART-A** at all doses evaluated (0.8 to 100 $\mu\text{g/kg}$). Treatment with 0.8 $\mu\text{g/kg}$ **DART-A** resulted in a complete response in 7/8 mice. The Raji tumors in the vehicle and **Control DART 1** groups demonstrated tumor growth *in vivo* with average tumor volumes reaching $2449.4 \pm 421.1 \text{ mm}^3$ and $2968.2 \pm 200.0 \text{ mm}^3$, respectively, by the end of the study (Day 28).

Example 10

Efficacy in Established Tumor Models

[00199] The anti-tumor activity of **DART-A** was evaluated in an HBL-2 (human mantle cell lymphoma) xenograft model in female NSG B2m^{-/-} mice (n = 8/group). Mice were implanted with HBL-2 tumor cells (5×10^6 cells) intradermally (ID) on Day 0 followed by intraperitoneal (IP) injection of PBMCs (5×10^7 cells) on Day 4. Treatment with vehicle, **Control DART 1**, or **DART-A** administered IV was initiated on Day 17 when the average tumor volumes for the groups receiving vehicle, **Control DART 1**, or **DART-A** were 438.4 ± 80.2 , 433.7 ± 63.8 , and $531.7 \pm 122.5 \text{ mm}^3$, respectively. On Day 21 (2nd dose administration), the average tumor volumes had increased to 1306.1 ± 240.4 , 1185.6 ± 138.7 , and $958.5 \pm 216.1 \text{ mm}^3$ in the groups treated with vehicle, **Control DART 1**, and **DART-A**, respectively. On Day 24 (3rd dose administration), the average tumor volume in the vehicle and **Control DART 1** groups had increased to 2401.3 ± 397.4 and $2623.7 \pm 351.5 \text{ mm}^3$, respectively. In contrast, the group that received **DART-A** had decreased in volume by 45% to $527.3 \pm 148.3 \text{ mm}^3$. The tumors in the **DART-A** treated group continued to decrease in size ultimately reaching a final volume of $111.4 \pm 38.9 \text{ mm}^3$ on Day 42, an 88.3% decrease from the maximum noted tumor volume (**Figure 19A**). In summary, treatment with 0.5 mg/kg of **DART-A** resulted in the shrinkage of large established HBL-2 mantle cell lymphoma tumors. No relapse was noted up to Day 42 when the study was terminated.

[00200] Another group of tumor-bearing mice with an average tumor size of 541 mm^3 (n = 4 mice) at Study Day 14 were scheduled to be treated with 500 $\mu\text{g/kg}$ **DART-A**. At the time of the first dose (Study Day 17), the tumors had reached an

average volume of $2279 \pm 61 \text{ mm}^3$ (**Figure 19B**). However, prior to the third (Study Day 24) and fourth (Study Day 28) doses, the tumor volumes were 1469 ± 162 and $848 \pm 74 \text{ mm}^3$, respectively, representing a 36% and 63%, respectively, decrease in tumor volume with respect to the peak volume recorded on Study Day 21 (**Figure 19B**). The tumors continued to decrease in size to the extent that on Day 42, the average tumor volume was $248 \pm 52 \text{ mm}^3$, an 89% decrease in the tumor volume (**Figure 19B**).

Example 11

Toxicokinetic Study of DART-A in Cynomolgus Monkeys

[00201] A non-GLP (Good Laboratory Practice) toxicology study that was conducted in cynomolgus monkeys to evaluate different doses and regimens of **DART-A**. Phase A was 1st conducted to evaluate 2-hour IV infusions of intra-animal escalating doses of **DART-A** each successive week for 4 weeks as summarized in **Table 11** (Phase A). Half of the Phase A cynomolgus monkeys in each group (1M/1F) were sacrificed on Day 36 or Day 64. Phase B of the study was subsequently conducted to evaluate 2-hour IV infusions of **DART-A** administered as fixed doses in each group (**Table 11**, Phase B). For Groups 3, 4, and 5 the same dose (5, 50, or 500 ng/kg, respectively) was administered each successive week for a total of 3 doses per animal. For Group 6, 500 ng/kg was administered on Study Days 1, 4, 8, 11, and 15 as a 2-hour IV infusion for a total of 5 doses per animal. All Phase B animals were sacrificed on Day 18.

Table 11								
Experimental Design (Phase A)								
Group No.	Male	Female	Test Material	Dose	Study Dosing Day	Dose Level (ng/kg)	Dose Volume (mL/kg)	Dose Concentration (ng/mL)
1	2	2	Vehicle	1	1	0	0.3	0
			DART-A	2	8	500	0.3	1700
				3	15	5000	0.3	16,700
				4	22	50,000	0.3	166,700
				5	29	50,000	0.3	166,700
2	2	2	DART-A	1	1	0	0.3	0
				2	8	2000	0.3	7000
				3	15	10,000	0.3	33,300
				4	22	100,000	0.3	333,300
				5	29	100,000	0.3	333,300

Table 11								
Experimental Design (Phase B)								
Group No.	Male	Female	Test Material	Dose	Study Dosing Day	Actual Dose Level * (ng/kg)	Dose Volume (mL/kg)	Actual Dose Concentration (ng/mL)
3	1	1	DART-A	1	1	5	0.3	15.0
				2	8	5	0.3	15.0
				3	15	5	0.3	15.0
4	1	1	DART-A	1	1	50	0.3	150.0
				2	8	50	0.3	150.0
				3	15	50	0.3	150.0
5	1	1	DART-A	1	1	500	0.3	1500.0
				2	8	500	0.3	1500.0
				3	15	500	0.3	1500.0
6	1	1	DART-A	1	1	500	0.3	1500.0
				2	4	500	0.3	1500.0
				3	8	500	0.3	1500.0
				4	11	500	0.3	1500.0
				5	15	500	0.3	1500.0
* Actual dose level administered based on analysis of the dosing formulation concentrations is shown. It is noted that the actual doses were 10-fold less than the protocol-specified dose levels for Phase B.								

1. A Toxicokinetic Study of DART-A in Cynomolgus Monkeys (Phase A)

[00202] A non-GLP, pilot, exploratory, dose-escalation study of **DART-A** was performed in cynomolgus monkeys. In Phase A of the study, two groups of cynomolgus monkeys (n = 4/group; 2M/2F) were administered intra-escalating doses of vehicle (Day 1) or **DART-A** as 2-hour IV infusions on Days 1, 8, 15, 22, and 29. Cynomolgus monkeys in Group 1 received vehicle control → 500 → 5000 → 50,000 → 50,000 ng/kg **DART-A**. Cynomolgus monkeys in Group 2 received vehicle control → 2000 → 10,000 → 100,000 → 100,000 ng/kg **DART-A**. Half of the Phase A cynomolgus monkeys in each group (1M/1F) were sacrificed on Day 36 or Day 64.

[00203] There was no **DART-A**-related mortality during Phase A of the study, and there were no **DART-A**-related macroscopic or organ weight changes. However, there were **DART-A**-related effects at dose levels ≥ 500 ng/kg as detailed below. These effects were consistent with the pharmacology of the test article and were not considered toxicologically significant.

[00204] In Phase A on Day 36, **DART-A**-related microscopic findings were limited to the lymph nodes (inguinal, mandibular, and mesenteric), spleen, and gut-associated lymphoid tissue (GALT) for all Group 1 and 2 animals with no recovery of

microscopic findings by Day 64. On Day 36, microscopic findings in the lymph nodes and spleen were similar and consisted of a moderate to marked decrease in the number and size of lymphoid follicular germinal centers identified; in the GALT there was a minimal decrease in the number and size of lymphoid follicular germinal centers. By Day 64, test article-related findings were still present in the lymph nodes, spleen, and GALT; in the lymph nodes and spleen, microscopic findings consisted of mild to marked decreased numbers and size of lymphoid follicular germinal centers and in the GALT there was a minimal decrease in the number and size of lymphoid follicular germinal centers.

[00205] Evaluation of bone marrow (sternum), lymph nodes (inguinal, mandibular, and mesenteric), and spleen by immunohistochemistry (IHC) revealed greatly reduced numbers of CD20-positive cells in Phase A animals on Day 36 with only partial recovery of CD20 staining by Day 64. On Day 36, there was, in general, no CD20-positive staining noted in the bone marrow or lymph nodes of Phase A animals with the exception of one animal from Group 1 in which very rare, mild to moderate, CD20-positive staining was present in the germinal centers and lymphocyte corona, respectively, of the inguinal lymph node only. In the spleen, CD20-positive cells were identified in the red pulp only of 3 animals (1 animal from Group 1; 2 animals from Group 2) with the frequency of CD20-positive cells being very rare to rare and minimal or mild to moderate intensity.

[00206] In recovery animals (Day 64), very rare to occasional, moderately stained CD20-positive cells were noted in the bone marrow of Group 1 animals only. In the lymph nodes (inguinal, mandibular, and mesenteric), CD20-positive staining was only identified in one animal from Group 1; the frequency of CD20-positive cells was generally greatly reduced in all lymph nodes, while the staining intensity of positive cells was moderate to marked. CD20-positive cells were identified in the spleen of all Group 1 and 2 animals. In Group 1 animals, CD20-positive cells were identified in the lymphoid follicles, periarteriolar lymphoid sheaths (PALS), and red pulp in one animal and in the PALS and red pulp in another animal. In Group 2 animals, very rare to rare CD20-positive cells were identified in the red pulp only with mild staining intensity.

[00207] Evaluation of FACS-based B cell depletion, cytokines, and ADA in the Phase A portion of this study is summarized below.

a. FACS Evaluation for B, T, NK Cells, and Monocytes

[00208] Whole blood samples were collected for flow cytometric evaluation of a variety of lymphocyte subsets and monocyte cell populations. Phase A animals were assessed at Week -1; prior to dosing on Days 1, 8, 15, 22, and 29; 24 hours post-start of infusion (SOI) on Days 2, 9, 16, 23, and 30; 72 hours post-SOI on Days 4, 11, 18, 25, and 32; Day 35; and on Days 43, 50, 57, and 63 (only applied to the half of the Phase A cynomolgus monkeys from each group with recovery sacrifice on Day 64).

[00209] Dose-dependent reductions in the absolute counts of B lymphocytes (CD20+) were present beginning at Day 9 (24 hours post-SOI) for both groups (Groups 1 and 2). The B lymphocyte populations for the 500 ng/kg/dose animals (Group 1) trended toward pre-study levels on Days 11 and 15, whereas the values for the 2000 ng/kg/dose animals (Group 2) remained reduced. The B lymphocytes were virtually depleted at Day 16 (24 hours post-SOI) following the second **DART-A** dose administration for both dose groups and remained depleted for all subsequent time points (**Figures 20A-20B**). The B lymphocyte depletion was an anticipated pharmacologic effect of **DART-A**.

[00210] Other immune cell populations, including monocytes, natural killer (NK) cells, and T cells, were also evaluated by flow cytometry.

[00211] Transient, dose-dependent reductions in the number of CD14+ monocytes were present beginning on Day 9, 24 hours post-SOI, for the majority of animals dosed at 2000 ng/kg (Group 2). Reductions in the number of CD14+ monocytes were also present at Day 16 and Day 23, 24 hours post-SOI, for the majority of animals dosed at 5000 ng/kg and 50,000 ng/kg, respectively (Group 1) and for all animals dosed at 10,000 ng/kg and 100,000 ng/kg, respectively (Group 2). The CD14+ monocyte populations recovered quickly after the end of each infusion and generally reached or exceeded pre-study baseline values by the next pre-dose time point before reducing again 24 hours after subsequent doses. At the end of the dosing phase, the

CD14⁺ monocytes fluctuated with values that trended toward pre-study levels and maintained values that were at or above pre-study baseline levels.

[00212] Transient, dose-dependent reductions were also observed for the total T lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, T regulatory lymphocytes (CD4⁺/CD25⁺/FoxP3⁺), CD159a⁺ NK cells, CD16⁺ NK cells, and CD16⁺/CD159a⁺ NK cells, with the greatest reductions observed for the 2000 ng/kg/dose animals (Group 2) at Day 9 (24 hours post-SOI) and following the 5000 ng/kg/dose in Group 1 animals on Day 16 (24 hours post-SOI). The T lymphocyte and NK cell subsets recovered quickly after the end of each infusion and generally reached or exceeded pre-study baseline levels before the next dose on Days 15 and 22 before reducing again 24 hours following each dose. No further reductions were present for Group 1 or 2 at Day 30 (24 hours post-SOI) following the doses administered on Day 29.

[00213] The CD4⁺ and CD8⁺ T lymphocyte populations were also assessed for up-regulation of the activation markers PD-1, TIM-3, CD25, and CD69 to determine the activation status of the cells after **DART-A** dose administration. Variable increases in the frequencies of PD-1⁺, CD69⁺, and CD25⁺ in CD4⁺ T lymphocytes and of PD-1⁺ and CD69⁺ in CD8⁺ T lymphocytes were present after dosing, indicating an increase in the frequencies of activated CD4⁺ and CD8⁺ T lymphocytes in the whole blood after dosing. The frequency of CD69⁺, PD-1⁺, and CD25⁺ (to a lesser degree, occurring in some but not all animals) in CD4⁺ T lymphocytes and PD-1⁺ and CD69⁺ in CD8⁺ T lymphocytes increased following the 500 ng/kg (Group 1) and 2000 ng/kg (Group 2) doses beginning on Day 9 (24 hours post-SOI). These values generally remained above baseline levels and were highly variable for all subsequent time points. The relative percentages of CD25⁺/CD69⁺, TIM-3⁺, and PD-1⁺/TIM3⁺ lymphocyte populations were generally < 1% of the total CD4⁺ and CD8⁺ T lymphocyte populations, making any **DART-A**-related changes difficult to assess. Collectively, these data suggest that **DART-A** administration resulted in an upward trend in PD-1 and CD69 expression on both CD4⁺ and CD8⁺ T lymphocytes together with a more modest increase in CD25⁺ in CD4⁺ T lymphocytes in some, but not all, animals.

b. Cytokine Evaluation

[00214] Serum IL-2, IL-4, IL-5, IL-6, IL-10, TNF- α , and IFN- γ levels were determined (EMD Millipore assay) pre-dose and at 4 and 24 hours following the start of each infusion. Transient, **DART-A** dose-correlated increases in IL-10 levels (up to 1918 pg/mL) were observed 4 hours following the start of infusion when cynomolgus monkeys were administered escalating doses ranging from 500 to 50,000 ng/kg in Group 1 and from 2000 to 100,000 ng/kg in Group 2 by IV infusion over a 2-hour period for the first 3 doses; levels generally returned to baseline within 24 hours of the start of infusion. There was generally no IL-10 detected 4 hours following the start of the last **DART-A** infusion in either group (50,000 ng/kg in Group 1 or 100,000 ng/kg in Group 2) with one exception (Animal 2502). Animal 2502 experienced a transient IL-10 increase to 332 pg/mL following the last dose (100,000 ng/kg on Day 29), but this increase was lower in magnitude than the IL-10 increase following the first 100 μ g/kg dose on Day 22 (570 pg/mL). One cynomolgus monkey (Animal 1001) at the 50,000 ng/kg dose level, who experienced the highest IL 10 levels (1918 pg/mL on Day 22), also experienced concurrent transient increases in IL-2 (32 pg/mL), IL 5 (45 pg/mL), and IL-6 (357 pg/mL). Transient increases in IL-6 levels were observed in the majority of cynomolgus monkeys 4 hours following infusion of **DART-A**; however, the increases were generally similar to or of a lower magnitude (with the exception of Animal 1001 described above) compared with the levels observed following infusion of vehicle control on Day 1 (up to 190 pg/mL) with no clear dose-response. There were no apparent clinical observations associated with increased cytokine levels.

[00215] There were no consistent changes in levels of IL-5, IL-4, IL-2, IFN- γ , or TNF- α following infusion of **DART-A**. Levels of IL-5 were variable with about half the animals in each group experiencing small and transient increases (~10-20 pg/mL; except Animal 1001 discussed above) following **DART-A** infusion on Days 15 or 22. Levels of IFN- γ were also variable across the study with small (< ~10 pg/mL) and transient increases following infusion in some animals.

2. A Toxicokinetic Study of DART-A in Cynomolgus Monkeys (Phase B)

[00216] In Phase B of the study, 4 groups of cynomolgus monkeys (n = 2/group; 1M/1F) were administered fixed doses of **DART-A** as 2-hour IV infusions with 3 doses administered on Days 1, 8, and 15 for Group 3 (5 ng/kg), Group 4 (50 ng/kg), and Group 5 (500 ng/kg); and 5 doses administered on Days 1, 4, 8, 11, and 15 for Group 6 (500 ng/kg). All Phase B animals were euthanized on Day 18.

[00217] There was no **DART-A**-related mortality during Phase B of the study, and there were no **DART-A**-related macroscopic or organ weight changes.

[00218] On Day 18, **DART-A**-related microscopic findings were limited to the lymph nodes (inguinal, mandibular, and mesenteric) and spleen of Groups 4, 5, and 6 animals and consisted of a minimal to mild decrease in the number and size of lymphoid follicular germinal centers identified with no apparent change in the size and/or cell density of the lymphoid tissue comprising the PALS in the spleen. There were no reported microscopic findings noted in Group 3 (5 ng/kg).

[00219] Additional formalin-fixed paraffin-embedded sections of bone marrow (sternum), lymph nodes (inguinal, mandibular, and mesenteric), and spleen were evaluated by IHC for cell populations that express CD20 (Mordenti, J. *et al.* (1991) "*Interspecies Scaling Of Clearance And Volume Of Distribution Data For Five Therapeutic Proteins*," Pharm. Res. 8(11):1351-1359). There was no appreciable difference in the distribution and/or staining intensity of CD20-positive cells in the bone marrow (sternum), lymph nodes (inguinal, mandibular, and mesenteric), or spleen in Groups 3, 4, 5, or 6. In all tissues, positive staining was associated with the cell membrane. In general, in the bone marrow occasional CD20-positive cells were located throughout the bone marrow stroma and staining intensity of positive cells was moderate to marked. In the lymph nodes, CD20-positive cells were primarily associated with the germinal centers and lymphocyte corona of follicles with fewer CD20-positive cells located within the paracortex, medullary cords, and medullary and subcapsular sinuses. Staining intensity in the germinal centers, lymphocyte corona, medullary cords, and medullary and subcapsular sinuses was moderate to marked. Staining intensity in the paracortex was of mild to marked. In the spleen,

CD20-positive cells were primarily associated with the germinal centers, follicular mantle, and marginal zone of lymphoid follicles with fewer CD20-positive cells within PALS and red pulp and staining was of moderate to marked intensity.

[00220] Evaluation of FACS-based B cell depletion, cytokines, and ADA in the Phase B portion of this study is summarized below.

a. FACS Evaluation for B Cells, T Cells, NK Cells, and Monocytes

[00221] Whole blood samples were collected for flow cytometric evaluation of a variety of lymphocyte subsets and monocyte cell populations. Phase B animals were assessed at Week -1; prior to dosing on Days 1, 4, (Group 6 only) 8, 11 (Group 6 only), and 15; 24 hours post-SOI on Days 2, 9, and 16; and at 72 hours post-SOI on Days 4 and Day 11 (Group 3-5 only).

[00222] Marked dose-dependent reductions in the total number of B lymphocytes (CD20+) were present 24 hours after the start of infusion for doses administered on Days 1, 8, and 15. The greatest reductions for the 5 ng/kg (Group 3), 50 ng/kg (Group 4), and 500 ng/kg (Groups 5 and 6) treatment groups were present at Day 2 (24 hours post-SOI). The B lymphocyte populations recovered quickly after each infusion in Groups 3 and 4 and presented with fluctuating values near pre-study baseline levels at all other time points. Groups 5 and 6 presented with minimal recovery between doses and maintained generally reduced values at all time points after Day 2 (**Figures 21A-21D**). Sustained B lymphocyte depletion was an anticipated pharmacologic effect of **DART-A** at higher doses.

[00223] Other immune cell populations, including monocytes, NK cells, and T cells, were also evaluated by flow cytometry.

[00224] Transient, dose-dependent reductions were present for total CD14+ monocytes beginning on Day 2 (24 hours post-SOI) for most animals in all dose groups (Groups 3-6). Additional changes in CD14+ monocytes were also present for most animals in all dose groups on Days 9 and 16 and values were highly variable.

[00225] Transient, dose-dependent reductions in the number of total T lymphocytes, T regulatory lymphocytes (CD4+/CD25+/FoxP3+), CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cell subsets were present, with the greatest reductions for all groups (Group 3-6) generally present on Day 2 (24 hours post-SOI). The T lymphocyte subsets recovered quickly after each infusion and all doses generally reached or exceeded pre-study levels before the next infusion in all groups. Additional reductions for the CD4+, CD8+, and T regulatory lymphocytes were also present at Days 9 and 16 (24 hours post-SOI) for the 50 ng/kg (Group 4), 500 ng/kg (Group 5), and multi-dosed 500 ng/kg (Group 6) dosed animals; however, the reductions present at these time points were of a lower magnitude compared with those present at Day 2 following the first **DART-A** dose. The NK cell subsets generally trended toward recovery after the first day of infusion and presented with variable values across all remaining time points.

[00226] The CD4+ and CD8+ T lymphocyte populations were assessed for up-regulation of the activation markers PD-1, TIM-3, CD25, and CD69 to determine the activation status of the cells after **DART-A** dose administration. The relative percentages of CD25+/CD69+ (for CD4+ T lymphocytes only), TIM-3+, and PD-1+/TIM3+ in the CD4+ and CD8+ T lymphocyte populations were generally < 1% of the total CD4+ and CD8+ T lymphocyte populations, making any **DART-A**-related changes difficult to assess. Increases in the frequencies of PD-1+ and CD69+ in CD4+ and CD8+ T lymphocytes were present across multiple time points for the 500 ng/kg/dose animals in Groups 5 and 6 beginning on Day 2 (24 hours post-SOI). Additionally, more modest increases in the frequency of CD25+ in CD4+ and CD8+ T lymphocytes were present for the 500 ng/kg/dose animals (Groups 5 and 6) beginning on Day 2 (24 hours post-SOI), as well as increases in the relative percentages of CD25+/CD69+ in CD8+ T lymphocytes for the multi-dose 500 ng/kg/dose animals (Group 6). Increases of a magnitude comparable to those present on Day 2 were also generally present on Days 9 and 16 (24 hours post-SOI) following subsequent doses. Collectively, these data indicate that **DART-A** administration resulted in an upward trend in PD-1, CD69, and CD25 expression on CD4+ and CD8+ T lymphocytes.

b. Cytokine Evaluation

[00227] Serum IL-2, IL-4, IL-5, IL-6, IL-10, TNF- α , and IFN- γ levels were determined (EMD Millipore assay) prior to dosing on Days -7, 8, and 15; and at 4 and 24 hours post-start of infusions on Days 1, 8, and 15. Transient and dose-correlated increases in IL-10 levels (up to 173 pg/mL) were observed 4 hours following the start of the 1st **DART-A** infusion when cynomolgus monkeys were administered **DART-A** doses of 5, 50, or 500 ng/kg (Groups 3-6); levels generally returned to baseline within 24 hours of the start of infusion. Increases in IL-10 appeared to be more pronounced in all groups following the 1st dose of **DART-A** compared with IL-10 increases following the 2nd and 3rd doses (< 22 pg/mL in all animals with the exception of Animal 5001 described below), suggesting either “desensitization” of cytokine release and/or rapid reduction/elimination of the target cell population responsible for T cell activation following the 1st dose. Transient increases in IL-6 levels (up to 162 pg/mL) were variably observed in some animals across all dose groups 4 hours following the start of each **DART-A** infusion with levels generally returning to at or near baseline levels within 24 hours. However, based on the data generated from vehicle administration in Phase A in which increases in IL-6 levels up to 190 pg/mL were observed, the levels of IL-6 fluctuation in Phase B ($\leq$ 162 pg/mL) may not necessarily reflect drug-related responses. There were no consistent changes observed in levels of IL 5, IL-4, IL-2, IFN γ , or TNF- α throughout the study.

[00228] One cynomolgus monkey (Animal 5001) experienced elevated levels of IFN- γ , TNF- α , IL-2, IL-4, IL 5, and IL-10 on Day 8 prior to the 2nd **DART-A** infusion. Cytokine levels further increased (IL-4, IL-5, IL-6, IL-10, IFN- γ , and TNF- α) or remained stable (IL-2) at 4 hours following the 2nd infusion on Day 8. Cytokine levels began to decline by 24 hours following the 2nd dose with variable increases in some cytokines following the 3rd **DART-A** infusion on Day 15. Although all cytokine levels declined from their peak values, cytokine levels remained elevated above baseline levels at the last time point evaluated (Day 16) in this cynomolgus monkey. There were no apparent clinical observations associated with increased cytokine levels.

Example 12

Ig-Like Half-Life in Human FcRn Transgenic Mice

[00229] A human FcRn transgenic mouse pharmacokinetic (PK) study was performed using the human FcRn transgenic mouse strain B6.Cg-*Fcgrt*^{tm1Dcr} (CAG-FCGRT)276Dcr/DcrJ mouse (Stock Number 004919), available from the Jackson Laboratories, Bar Harbor, Maine, US, to evaluate **DART-A** and **Control DART 1** (**Figure 22**). The terminal half-life was 85.6 and 92.1 hours, respectively. Three human mAbs were also evaluated in same test system for comparison and had terminal half-lives ranging from 58.7 to 102.7 hours.

Example 13

Efficacy in Disseminated Raji Cell Leukemia/Lymphoma Models in Human PBMC-Reconstituted Mice

[00230] Disseminated tumor models were established using luciferase-transduced Raji lymphoma cells (Raji.luc) in female NSG B2m^{-/-} mice reconstituted with human PBMCs in which the mice were treated with vehicle, **Control DART 1** or **DART-A** either one day post-tumor cell injection (the “early treatment” or leukemia paradigm) or after at least two weeks of growth in vivo (the “established tumor” or lymphoma model).

Early Treatment Model

[00231] Following human PBMC reconstitution on Study Day -14, Raji.luc lymphoma cells were injected on Study Day 0 and treatment was initiated the following day (Study Day 1). Vehicle control, 0.5 mg/kg **Control DART 1** or 0.5 mg/kg **DART-A** were injected IV once every 3-4 days for a total of 12 doses (i.e., Study Days 1, 4, 6, 8, 11, 13, 15, 18, 20, 22, 25, and 27). Mice were followed for survival through Study Day 54 or Study Day 56.

[00232] In the groups receiving vehicle or **Control DART 1**, diffuse disseminated tumor cell profiles rapidly coalesced and expanded in the majority of mice by Weeks 2 and 3. Animal deaths in these two control groups were noted as early as two weeks post-tumor cell implant. All mice in the vehicle or **Control DART 1** groups died or were humanely sacrificed (i.e., if the animals became moribund or lost more than 15%

of their body weight) by Study Days 40 or 53, respectively. The survival curves are depicted in **Figure 23**. In contrast, the group treated with **DART-A** showed no tumor cell growth at any time point and no animal deaths were observed through the end of the study. The survival curve is depicted in **Figure 23**.

[00233] A dose-range finding study was conducted in a follow-up experiment under the same experimental conditions described above, except 0.16 µg/kg, 0.8 µg/kg, 4 µg/kg, 20 µg/kg, 0.1 mg/kg or 0.5 mg/kg **DART-A** were injected IV once every 3-4 days for a total of 12 doses (*i.e.*, Study Days 1, 4, 6, 8, 11, 13, 15, 18, 20, 22, 25, and 27). Vehicle and **Control DART 1** were administered as described above on the same Study Days. A rapid progression of Raji.luc tumor growth was observed in mice treated with vehicle or **Control DART 1**. **DART-A** demonstrated efficacy at all doses tested with the majority of mice being alive (>8%) and tumor free at the end of the study (Day 56) at doses as low as 20 µg/kg (**Figure 24**).

Established Tumor Model

[00234] The ability of CD19 x CD3 to control Raji.luc cell tumor burden when treatment was delayed, was tested in a separate set of experiments. In the established tumor model, human-PBMC-reconstituted mice were inoculated IV with Raji.luc cells of Study Day 0 (approximately one week after PBMC injection) and allowed to grow for 14 days prior to treatment initiation.

[00235] Mice were randomized on Study Day 12 into 3 groups based on tumor burden and subsequently treated with vehicle, 0.1 mg/kg **DART-A** or 0.5 mg/kg **DART-A** starting on Study Day 14 (2 weeks after Raji.luc cell inoculation) for a total of 10 doses (*i.e.*, Study Days 14, 15, 16, 19, 21, 23, 30, 33, 35 and 37). In the vehicle control group, the initial foci markedly increased in size by the end of the first week of treatment and 5/7 mice died within 2 weeks of treatment initiation (**Figure 25**). In contrast, the tumor burden in the majority of animals in both **DART-A** treatment groups was dramatically reduced or cleared during the first week of treatment. Only one animal in each **DART-A** treatment group, each with a relatively high tumor burden present at randomization, died during the first week of treatment (**Figure 25**).

By Study Day 50, only 2 mice in the 0.1 mg/kg **DART-A** treatment group had peripheral tumors remaining.

[00236] A dose-range finding study in the delayed treatment model was conducted under the general experimental conditions described above. In this experiment, human PBMC-reconstituted mice were inoculated IV with Raji.luc cells on Study Day 0 (5 days after PBMC injection) and allowed to grow to Study Day 14 prior to randomization. Treatment was initiated on Study Day 15 with vehicle, 0.1 mg/kg **Control DART 1** or 0.16 µg/kg, 0.8 µg/kg, 4 µg/kg, 20 µg/kg, 0.1 mg/kg or 0.5 mg/kg **DART-A** for a total of 9 doses (i.e., Study Days 15, 17, 21, 23, 25, 28, 30, 32 and 35).

[00237] In the vehicle control group, the tumor burden increased progressively and all mice died or were sacrificed by the end of the second week of treatment (**Figure 26**). Mice treated with 0.1 mg/kg **Control DART 1** showed a slight delay of tumor progression compared with vehicle-treated mice (**Figure 26**). In contrast, the tumor burden in the animals treated with **DART-A** showed a generally dose-dependent reduction over time (**Figure 26**).

Example 14

Pharmacokinetics of **DART-A** in Cynomolgus Monkeys

[00238] The cynomolgus monkey was selected as an appropriate pharmacological model for **DART-A** analysis based on the equivalent distribution of both target antigens in this species compared to humans based on immunohistochemistry with the precursor mAbs.

[00239] The study conducted in accordance with the present invention included 5 treatment groups, consisting of 10 cynomolgus monkeys per group (5 males, 5 females). Groups were treated by IV infusion over 2 hours with vehicle control for the first infusion on Day 1, followed by treatment with vehicle control (Group 1) or **DART-A** (Groups 2-5) once weekly for four weeks. While Group 1 animals continued receiving vehicle control for all 4 subsequent infusions, animals of Groups 2-5 received **DART-A** at 0.2 µg/kg, 2 µg/kg, 5 µg/kg or 10 µg/kg for all subsequent infusions on Days 8, 15, 22 and 29 (**Table 12**). Three males and 3 females were

euthanized and necropsied on Day 37, while the remaining recovery group monkeys (2 males and 2 females) were euthanized and necropsied on Day 121 after a 12-week recover period.

Table 12							
Study Infusion No.	DART-A Infusion No. (Groups 2-5 only)	Study Day	DART-A ($\mu\text{g/kg}$) Administered as a 2-hour IV Infusion				
			Group 1	Group 2	Group 3	Group 4	Group 5
1	NA	1	0	0	0	0	0
2	1	8	0	0.2	2	5	10
3	2	15	0	0.2	2	5	10
4	3	22	0	0.2	2	5	10
5	4	29	0	0.2	2	5	10

[00240] Concentration analysis of **DART-A** in serum collected at various time points was conducted with ELISA. **DART-A** serum concentration-time profile for a representative animal from Group 5 is shown on **Figure 27**. PK parameters (two-compartment model) were estimated following the 0.2, 2, 5 and 10 $\mu\text{g/kg}$ doses (**Table 13**). In general, dose-proportional increases in maximum serum concentrations (C_{max}) and AUC_{inf} were observed across the dose range evaluated. Although differences existed for several pairwise dose comparisons of primary PK parameters, overall, there were no consistent trends in changes in clearance (CL), intercompartmental clearance (CLD_2) and volumes of distribution (V_1 and V_2) for **DART-A** across the evaluable doses suggesting linear PK. The CL values across the animals of Groups 2-5 are lower than the glomerular filtration rate (GFR) for cynomolgus monkeys ($\sim 125 \text{ mL/h/kg}$), which indicates that virtually no elimination occurs by renal filtration, as would be expected for a protein of this molecular weight (109.3 kDa). Mean initial volumes of distribution (V_1) for animals of Groups 2, 3, 4, and 5, is similar to or slightly higher than the plasma volume in cynomolgus monkeys ($\sim 45 \text{ mL/kg}$), suggesting little depletion of **DART-A** in the central compartment by binding to target cells. The mean beta-phase half-lives and MRT showed an increasing trend with increasing dose with the mean beta half-life ($t_{1/2,\beta}$) was 161 hours (6.7 days) and mean residence time (MRT) was 191 hours (8 days). Of note only three animals from the GLP study ($n = 1$ in 2 $\mu\text{g/kg}$ dose group; $n = 2$ in 5 $\mu\text{g/kg}$ dose group) had aberrant PK profiles following the 2nd and subsequent **DART-A** infusions ($n = 2$) or following the fourth **DART-A** infusion ($n = 1$) and were

subsequently confirmed positive for ADA. Thus, serum concentration data following the affected infusion cycles were excluded from PK modeling for these 3 animals.

Table 13 Two-Compartment Analysis of PK Parameters of DART-A in Cynomolgus Monkeys				
Attribute	0.2 µg/kg/d (mean ± SD)	2 µg/kg/d (mean ± SD)	5 µg/kg/d (mean ± SD)	10 µg/kg/d (mean ± SD)
C _{max} (ng/mL)	3.18 ± 0.46	31.9 ± 3.0	82.9 ± 5.6	166.6 ± 11.0
AUC _{inf} (h*ng/mL)	155 ± 37	2258 ± 634	6714 ± 1846	13756 ± 2534
CL (mL/h/kg)	0.95 ± 0.17	0.80 ± 0.22	0.73 ± 0.24	0.70 ± 0.13
CLD ₂ (mL/h/kg)	0.87 ± 0.15	1.65 ± 0.36	1.70 ± 0.74	1.40 ± 0.40
V ₁ (mL/kg)	43.4 ± 5.8	50.9 ± 5.1	52.3 ± 3.5	54.5 ± 4.5
V ₂ (mL/kg)	65.3 ± 17.5	77.9 ± 17.9	93.4 ± 33.3	105.3 ± 33.0
V _{ss} (mL/kg)	108.7 ± 16.6	128.8 ± 17.2	145.8 ± 34.6	159.8 ± 36.0
t _{1/2} (h)	14.0 ± 1.5	11.0 ± 2.2	12.3 ± 3.8	15.0 ± 4.4
t _{1/2,β} (h)	119.1 ± 27.6	143.4 ± 43.4	184.9 ± 72.0	205.6 ± 66.9
MRT (h)	116.3 ± 20.3	173.0 ± 54.5	221.2 ± 86.5	235.9 ± 72.0

Cytokine Release in DART-A-Treated Cynomolgus Monkeys

[00241] Serum samples to evaluate cytokine levels were collected prior to infusion and at 2 and 22 hours following the end of infusion on Days 1, 8, 15, 22 and 29. Cytokines evaluated were TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10.

[00242] An increase in the levels of IL-6, IL-10, IFN-γ and TNF-α was observed following the first **DART-A** infusion (DAY 8), with respect to the greatest increase in cytokine levels occurring 2 hours following the end of infusion. A **DART-A** dose-dependent increase was observed for IL-10 and IFN-γ at dose levels >2 µg/kg, while an increase in IL-6 was observed at dose levels ≥5 µg/kg. Changes in TNF-α levels were small and were observed only at the highest dose level (10 µg/kg). Changes in cytokine levels were transient and returned to at or near baseline within 24 hours of the start of the infusion, and there were no clinical observations associated with these increased cytokine levels. Subsequent infusions of **DART-A** on Days 15, 22 and 29 resulted in smaller cytokine level increases, generally comparable to those observed following control article infusion. Mean levels of IL-2, IL-4 and IL-5 showed variable and isolated changes in individual animals, but remained <20 pg/mL across all groups throughout the duration of the study and were not considered toxicologically significant.

[00243] Cytokine levels in animals receiving 0.2 µg/kg **DART-A** were comparable to the variations observed following control article infusion (Group 1).

DART-A-Mediated Depletion of Circulating B-Cells in Cynomolgus Monkeys

[00244] The circulating absolute levels of B cells, T cells, including T cell subsets (CD4 vs CD8) and activation markers (CD25, CD69, PD-1 and TIM-3), and NK cells, were measured throughout the study as a pharmacodynamic endpoint. Samples were collected prior to infusion and at 2, 22 and 70 hours following the end of infusion on Days 1, 8, 22 and 29, prior to terminal necropsy on Day 37, and then weekly or biweekly during the recovery period on Days 36, 43, 50, 57, 64, 78, 92, 106 and 119.

[00245] **Figure 28** shows that **DART-A** treatment resulted in a dose-dependent depletion of CD20+ B cells, the intended target population. Complete or near complete depletion of CD20+ cells was observed by 24 hours following the start of the first infusion of **DART-A** at dose levels ≥ 2 µg/kg. The depletion of CD20+ B cells was durable throughout the treatment period and was sustained for 3-5 weeks after final dose. There was a dose-dependent return to pre-dose levels of CD20+ B cells during the last several weeks of the 12-week recovery period.

Example 15

Autologous Depletion of Malignant B Cells in CLL Primary Patient Samples

[00246] To evaluate **DART-A**-mediated cytolytic activity in CLL primary patient samples, patient PBMCs were incubated with **DART-A** or **Control DART 1**. Due to the limited patient sample availability, only 2 concentrations (50 ng/mL and 200 ng/mL) were tested. The percentages of CLL malignant B cells (CD19+/CD5+ or CD20+/CD5+) and T cells (CD19-/CD5+ or CD4+ and CD8+) were measured by flow cytometry prior to treatment and following treatment for 3 and 6 days. **DART-A** at the concentrations tested partially blocks staining of anti-CD19 antibody, therefore, it was not appropriate to use CD19+/CD5+ to gate malignant B cells after treatment with **DART-A**. Thus, malignant B cell depletion was determined by gating on CD20+/CD5+ cells following **DART-A** treatment.

[00247] The E:T ratio determined from CLL malignant B cell and T cell frequencies was generally $\leq 1:10$. In the representative CLL donor experiment shown, the E:T cell

ratio was approximately 1:23 prior to treatment based on CD19-/CD5+ T cells comprising only 4% of the total PBMCs and about 90% of PBMCs identified as CD19+/CD5+ malignant B cells. **Figures 29A-29I** and **Table 14** show the effects of **DART-A** treatment compared with CLL patient sample. Incubation of **DART-A** with CLL PBMCs resulted in a time-dependent depletion of malignant B cells at both **DART-A** concentrations evaluated, accompanied by a concomitant increase in T cells (CD4 and CD8) observed on Day 6. In addition, the T cell activation marker CD25 was upregulated following **DART-A** treatment for 6 days, indicating the **DART-A** induces T cell activation in the process of redirected killing of malignant B cells. In contrast, no B cell killing or upregulation of CD25 was observed with **Control DART 1** through Day 6.

[00248] Of note, **DART-A** mediated the same level of killing at both concentrations evaluated, but the level of killing increased over time with approximately 40% and 85% of malignant B cells being killed on Day 3 and Day 6, respectively, suggesting that time is the critical factor for the efficient elimination of target cells under low T cell to target ratios when sufficient **DART-A** is present.

Table 14					
Percentages of Malignant B Cells (CD20+/CD5+), T cells (CD4, CD8+) and Activated T Cells (CD25+/CD4+ and CD25+/CD8+) in CLL PBMCs (E:T = 1:23) after DART-A or Control DART 1 Treatment for 3 and 6 Days					
Time Point	Percentages (%)				
	CD20+/CD5+	CD4+	CD8+	CD25+/CD4+	CD25+/CD8+
Day 0					
Pretreatment	70.7	n.d. ¹		CD4+ and CD8+ T cell subsets were not measured on Day 0. CD19-/CD5+ was used to define the T cell population on Day 0 (4%) as described above.	
Day 3					
DART-A (50ng/mL)	40.5	1.01	1.78	Overall T cell percentages were too low (<5%) to evaluate T cell activation marker CD25 on Day 0 or Day 3.	
Control DART 1 (50 ng/mL)	73.5	3.08	1.81		
DART-A (200 ng/mL)	42.3	1.61	1.68		
Control DART 1 (200 ng/mL)	72.9	3.23	1.62		
Day 6					
DART-A (50ng/mL)	11.3	57.4	22.2	87.7	82.8
Control DART 1 (50 ng/mL)	74.9	3.54	2.26	1.4	20.6
DART-A (200 ng/mL)	12.3	65.2	20.9	91.6	90.7
Control DART 1 (200 ng/mL)	73.4	2.79	1.82	1.4	14.3

[00249] 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 CD19 x CD3 bi-specific Fc diabody capable of specific binding to CD19 and to CD3, wherein the diabody comprises a first, a second and a third polypeptide chain, wherein said polypeptide chains form a covalently bonded complex, and wherein:

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

A. a Domain IA, comprising

(1) a sub-Domain (IA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and

(2) a sub-Domain (IA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});

wherein said sub-Domains IA1 and IA2 are separated from one another by a polypeptide linker, and are either

(a) VL_{CD19} and VH_{CD3}; or

(b) VL_{CD3} and VH_{CD19};

B. a Domain IB, comprising a charged Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**, wherein said Domain IB is separated from said Domain IA by a polypeptide linker;

C. a Domain IC, comprising a CH2-CH3 Domain of an antibody; and

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

A. a Domain IIA, comprising

(1) a sub-Domain (IIA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and

- (2) a sub-Domain (IIA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});
- wherein said sub-Domains IIA1 and IIA2 are separated from one another by a polypeptide linker, and are:
- (a) VL_{CD19} and VH_{CD3}, if said sub-Domains IA1 and IA2 are VL_{CD3} and VH_{CD19}; or
 - (b) VL_{CD3} and VH_{CD19}, if said sub-Domains IA1 and IA2 are VL_{CD19} and VH_{CD3};
- B. a Domain IIB, comprising a charged Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**, wherein said Domain IIB is separated from said Domain IIA by a polypeptide linker, and wherein said charged Heterodimer-Promoting Domain of said Domain IB and said charged Heterodimer-Promoting Domain of said Domain IIB have opposite charges; and
- III. said third polypeptide chain comprises, in the N-terminal to C-terminal direction a Domain IIIC that comprises a CH2-CH3 Domain of an antibody;
- wherein said VL_{CD19} and said VH_{CD19} domains form a monovalent CD19 binding domain, and said VL_{CD3} and VH_{CD3} domains form a monovalent CD3 binding domain; and said CH2-CH3 Domains of said first and third polypeptide chains form an Fc domain capable of binding to an Fc receptor, thereby forming said CD19 x CD3 bi-specific Fc diabody.

Claim 2. The CD19 x CD3 bi-specific Fc diabody of claim 1, wherein:

- (A) said Domains IB and IIB each comprise a cysteine residue that covalently bonds said first polypeptide chain to said second polypeptide chain via a disulfide bond; and

(B) said Domains IC and IIIC each comprise a cysteine residue that covalently bonds said first polypeptide chain to said third polypeptide chain via a disulfide bond.

Claim 3. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-2, wherein said VL_{CD19} comprises the amino acid sequence of **SEQ ID NO:17** and said VH_{CD19} comprises the amino acid sequence of **SEQ ID NO:21**.

Claim 4. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-3, wherein said VL_{CD3} comprises the amino acid sequence of **SEQ ID NO:25** and said VH_{CD3} comprises the amino acid sequence of **SEQ ID NO:29**.

Claim 5. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-4, wherein said CH2-CH3 Domain of said Domain IC comprises the amino acid sequence of **SEQ ID NO:15** and said CH2-CH3 Domain of said Domain IIIC comprises the amino acid sequence of **SEQ ID NO:16**.

Claim 6. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-5, wherein said polypeptide linker that separates said Domain IB from said Domain IA comprises the amino acid sequence of **SEQ ID NO: 2**, and said polypeptide linker that separates said Domain IIB from said Domain IIA comprises the amino acid sequence of **SEQ ID NO: 2**.

Claim 7. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-5, wherein said polypeptide linker that separates said Domain IB from said Domain IA comprises the amino acid sequence of **SEQ ID NO: 3**, and said polypeptide linker that separates said Domain IIB from said Domain IIA comprises the amino acid sequence of **SEQ ID NO: 3**.

- Claim 8. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-6, wherein:
- (A) said first polypeptide chain comprises the amino acid sequence of **SEQ ID NO:35**;
 - (B) said second polypeptide chain comprises the amino acid sequence of **SEQ ID NO:37**; and
 - (C) said third polypeptide chain comprises the amino acid sequence of **SEQ ID NO:39**.
- Claim 9. The CD19 x CD3 bi-specific Fc diabody of any one of claims 1-8, which is capable of cross-reacting with both human and primate CD19 and CD3.
- Claim 10. A covalently associated polypeptide complex, wherein said polypeptide complex comprises a first polypeptide chain and a second polypeptide chain, wherein:
- (A) said first polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:2** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**; and
 - (B) said second polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:2** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**;
- wherein said Heterodimer-Promoting Domain of said first polypeptide chain and said Heterodimer-Promoting Domain of said second polypeptide chain are covalently bonded to one another via a disulfide bond.
- Claim 11. A covalently associated polypeptide complex, wherein said polypeptide complex comprises a first polypeptide chain and a second polypeptide chain, wherein:

- (A) said first polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:3** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:12**; and
- (B) said second polypeptide chain comprises a polypeptide comprising the amino acid sequence of **SEQ ID NO:3** linked to a Heterodimer-Promoting Domain comprising the amino acid sequence of **SEQ ID NO:13**;

wherein said Heterodimer-Promoting Domain of said first polypeptide chain and said Heterodimer-Promoting Domain of said second polypeptide chain are covalently bonded to one another via a disulfide bond.

- Claim 12. A pharmaceutical composition comprising the CD19 x CD3 bi-specific Fc diabody of any one of claims 1-9 and a physiologically acceptable carrier.
- Claim 13. A pharmaceutical composition comprising the covalently associated polypeptide complex of any one of claims 10-11 and a physiologically acceptable carrier.
- Claim 14. Use of the CD19 x CD3 bi-specific Fc diabody of any one of claims 1-9, or the pharmaceutical composition of claim 12, in the manufacture of a medicament for the treatment of a disease or condition associated with or characterized by the expression of CD19, wherein said disease or condition is cancer.
- Claim 15. The use of claim 14, wherein said cancer is selected from the group consisting of: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blastic crisis of CML, Abelson oncogene associated with CML (Bcr-ABL translocation), myelodysplastic syndrome (MDS), acute B lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), Richter's syndrome, Richter's transformation of CLL,

hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin lymphomas (NHL), mantel cell leukemia (MCL), small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, and Burkitt's lymphoma.

Claim 16. A polynucleotide encoding a polypeptide comprising the amino acid sequences of **SEQ ID NO:35, SEQ ID NO: 37, and SEQ ID NO:39.**

Claim 17. An expression vector comprising the polynucleotide of claim 16.

Claim 18. The polynucleotide of claim 16, which comprises the sequences of **SEQ ID NO:36, SEQ ID NO:38, and SEQ ID NO:40.**