

Title of the Invention:**Bi-Specific Monovalent Diabodies That Are Capable Of Binding CD123 And CD3, And Uses Thereof****Cross-Reference to Related Applications**

[0001] This Application claims priority to United States Patent Applications No. 61/869,510 (filed on August 23, 2013; pending), 61/907,749 (filed on November 22, 2013; pending), and 61/990,475 (filed on May 8, 2014; pending), and European Patent Application No. 13198784 (filed on December 20, 2013), each of which applications is herein incorporated by reference in its entirety.

Reference to Sequence Listing:

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

Background of the Invention:**Field of the Invention:**

[0003] The present invention is directed to CD123 x CD3 bi-specific monovalent diabodies that are capable of simultaneous binding to CD123 and CD3, and to the uses of such molecules in the treatment of hematologic malignancies.

Description of Related Art:**I. CD123**

[0004] CD123 (interleukin 3 receptor alpha, IL-3Ra) is a 40 kDa molecule and is part of the interleukin 3 receptor complex (Stomski, F.C. *et al.* (1996) “*Human Interleukin-3 (IL-3) Induces Disulfide-Linked IL-3 Receptor Alpha- And Beta-Chain Heterodimerization, Which Is Required For Receptor Activation But Not High-Affinity Binding,*” *Mol. Cell. Biol.* 16(6):3035-3046). Interleukin 3 (IL-3) drives early differentiation of multipotent stem cells into cells of the erythroid, myeloid and lymphoid progenitors. CD123 is expressed on CD34+ committed progenitors

(Taussig, D.C. *et al.* (2005) “*Hematopoietic Stem Cells Express Multiple Myeloid Markers: Implications For The Origin And Targeted Therapy Of Acute Myeloid Leukemia*,” *Blood* 106:4086-4092), but not by CD34+/CD38- normal hematopoietic stem cells. CD123 is expressed by basophils, mast cells, plasmacytoid dendritic cells, some expression by monocytes, macrophages and eosinophils, and low or no expression by neutrophils and megakaryocytes. Some non-hematopoietic tissues (placenta, Leydig cells of the testis, certain brain cell elements and some endothelial cells) express CD123; however expression is mostly cytoplasmic.

[0005] CD123 is reported to be expressed by leukemic blasts and leukemia stem cells (LSC) (Jordan, C.T. *et al.* (2000) “*The Interleukin-3 Receptor Alpha Chain Is A Unique Marker For Human Acute Myelogenous Leukemia Stem Cells*,” *Leukemia* 14:1777-1784; Jin, W. *et al.* (2009) “*Regulation Of Th17 Cell Differentiation And EAE Induction By MAP3K NIK*,” *Blood* 113:6603-6610) (**Figure 1**). In human normal precursor populations, CD123 is expressed by a subset of hematopoietic progenitor cells (HPC) but not by normal hematopoietic stem cells (HSC). CD123 is also expressed by plasmacytoid dendritic cells (pDC) and basophils, and, to a lesser extent, monocytes and eosinophils (Lopez, A.F. *et al.* (1989) “*Reciprocal Inhibition Of Binding Between Interleukin 3 And Granulocyte-Macrophage Colony-Stimulating Factor To Human Eosinophils*,” *Proc. Natl. Acad. Sci. (U.S.A.)* 86:7022-7026; Sun, Q. *et al.* (1996) “*Monoclonal Antibody 7G3 Recognizes The N-Terminal Domain Of The Human Interleukin-3 (IL-3) Receptor Alpha Chain And Functions As A Specific IL-3 Receptor Antagonist*,” *Blood* 87:83-92; Muñoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies*,” *Haematologica* 86(12):1261-1269; Masten, B.J. *et al.* (2006) “*Characterization Of Myeloid And Plasmacytoid Dendritic Cells In Human Lung*,” *J. Immunol.* 177:7784-7793; Korpelainen, E.I. *et al.* (1995) “*Interferon-Gamma Upregulates Interleukin-3 (IL-3) Receptor Expression In Human Endothelial Cells And Synergizes With IL-3 In Stimulating Major Histocompatibility Complex Class II Expression And Cytokine Production*,” *Blood* 86:176-182).

[0006] CD123 has been reported to be overexpressed on malignant cells in a wide range of hematologic malignancies including acute myeloid leukemia (AML) and

myelodysplastic syndrome (MDS) (Muñoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies,*” *Haematologica* 86(12):1261-1269). Overexpression of CD123 is associated with poorer prognosis in AML (Tettamanti, M.S. *et al.* (2013) “*Targeting Of Acute Myeloid Leukaemia By Cytokine-Induced Killer Cells Redirected With A Novel CD123-Specific Chimeric Antigen Receptor,*” *Br. J. Haematol.* 161:389-401).

[0007] AML and MDS are thought to arise in and be perpetuated by a small population of leukemic stem cells (LSCs), which are generally dormant (*i.e.*, not rapidly dividing cells) and therefore resist cell death (apoptosis) and conventional chemotherapeutic agents. LSCs are characterized by high levels of CD123 expression, which is not present in the corresponding normal hematopoietic stem cell population in normal human bone marrow (Jin, W. *et al.* (2009) “*Regulation Of Th17 Cell Differentiation And EAE Induction By MAP3K NIK,*” *Blood* 113:6603-6610; Jordan, C.T. *et al.* (2000) “*The Interleukin-3 Receptor Alpha Chain Is A Unique Marker For Human Acute Myelogenous Leukemia Stem Cells,*” *Leukemia* 14:1777-1784). CD123 is expressed in 45%-95% of AML, 85% of Hairy cell leukemia (HCL), and 40% of acute B lymphoblastic leukemia (B-ALL). CD123 expression is also associated with multiple other malignancies/pre-malignancies: chronic myeloid leukemia (CML) progenitor cells (including blast crisis CML); Hodgkin’s Reed Sternberg (RS) cells; transformed non-Hodgkin’s lymphoma (NHL); some chronic lymphocytic leukemia (CLL) (CD11c+); a subset of acute T lymphoblastic leukemia (T-ALL) (16%, most immature, mostly adult), plasmacytoid dendritic cell (pDC) (DC2) malignancies and CD34+/CD38- myelodysplastic syndrome (MDS) marrow cell malignancies.

[0008] AML is a clonal disease characterized by the proliferation and accumulation of transformed myeloid progenitor cells in the bone marrow, which ultimately leads to hematopoietic failure. The incidence of AML increases with age, and older patients typically have worse treatment outcomes than do younger patients (Robak, T. *et al.* (2009) “*Current And Emerging Therapies For Acute Myeloid Leukemia,*” *Clin. Ther.* 2:2349-2370). Unfortunately, at present, most adults with AML die from their disease.

[0009] Treatment for AML initially focuses in the induction of remission (induction therapy). Once remission is achieved, treatment shifts to focus on securing such remission (post-remission or consolidation therapy) and, in some instances, maintenance therapy. The standard remission induction paradigm for AML is chemotherapy with an anthracycline/cytarabine combination, followed by either consolidation chemotherapy (usually with higher doses of the same drugs as were used during the induction period) or human stem cell transplantation, depending on the patient's ability to tolerate intensive treatment and the likelihood of cure with chemotherapy alone (see, *e.g.*, Roboz, G.J. (2012) “*Current Treatment Of Acute Myeloid Leukemia*,” *Curr. Opin. Oncol.* 24:711-719).

[0010] Agents frequently used in induction therapy include cytarabine and the anthracyclines. Cytarabine, also known as AraC, kills cancer cells (and other rapidly dividing normal cells) by interfering with DNA synthesis. Side effects associated with AraC treatment include decreased resistance to infection, a result of decreased white blood cell production; bleeding, as a result of decreased platelet production; and anemia, due to a potential reduction in red blood cells. Other side effects include nausea and vomiting. Anthracyclines (*e.g.*, daunorubicin, doxorubicin, and idarubicin) have several modes of action including inhibition of DNA and RNA synthesis, disruption of higher order structures of DNA, and production of cell damaging free oxygen radicals. The most consequential adverse effect of anthracyclines is cardiotoxicity, which considerably limits administered life-time dose and to some extent their usefulness.

[0011] Thus, unfortunately, despite substantial progress in the treatment of newly diagnosed AML, 20% to 40% of patients do not achieve remission with the standard induction chemotherapy, and 50% to 70% of patients entering a first complete remission are expected to relapse within 3 years. The optimum strategy at the time of relapse, or for patients with the resistant disease, remains uncertain. Stem cell transplantation has been established as the most effective form of anti-leukemic therapy in patients with AML in first or subsequent remission (Roboz, G.J. (2012) “*Current Treatment Of Acute Myeloid Leukemia*,” *Curr. Opin. Oncol.* 24:711-719).

II. CD3

[0012] 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). 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. 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).

III. Bi-Specific Diabodies

[0013] The ability of an intact, unmodified antibody (*e.g.*, an IgG) to bind an epitope of an antigen depends upon the presence of variable domains on the immunoglobulin light and heavy chains (*i.e.*, the VL and VH domains, respectively). The design of a diabody is based on the single chain Fv construct (scFv) (see, *e.g.*, Holliger *et al.* (1993) “*‘Diabodies’: Small Bivalent And Bispecific Antibody Fragments*,” Proc. Natl. Acad. Sci. (U.S.A.) 90:6444-6448; US 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).

[0014] 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] Natural antibodies are capable of binding to only one epitope species (*i.e.*, mono-specific), although they can bind multiple copies of that species (*i.e.*, exhibiting bi-valency or multi-valency). The art has noted the capability to produce diabodies that differ from such natural antibodies in being capable of binding two or more different epitope species (*i.e.*, exhibiting bi-specificity or multispecificity in addition to bi-valency or multi-valency) (see, *e.g.*, Holliger *et al.* (1993) “‘Diabodies’: Small Bivalent And Bispecific Antibody Fragments,” Proc. Natl. Acad. Sci. (U.S.A.) 90:6444-6448; US 2004/0058400 (Hollinger *et al.*); US 2004/0220388 (Mertens *et al.*); Alt *et al.* (1999) FEBS Lett. 454(1-2):90-94; Lu, D. *et al.* (2005) “A Fully Human Recombinant IgG-Like Bispecific Antibody To Both The Epidermal Growth Factor Receptor And The Insulin-Like Growth Factor Receptor For Enhanced Antitumor

Activity,” J. Biol. Chem. 280(20):19665-19672; WO 02/02781 (Mertens *et al.*); Mertens, N. *et al.*, “*New Recombinant Bi- and Trispecific Antibody Derivatives*,” In: NOVEL FRONTIERS IN THE PRODUCTION OF COMPOUNDS FOR BIOMEDICAL USE, A. VanBroekhoven *et al.* (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands (2001), pages 195-208; Wu, A. *et al.* (2001) “*Multimerization Of A Chimeric Anti-CD20 Single Chain Fv-Fv Fusion Protein Is Mediated Through Variable Domain Exchange*,” Protein Engineering 14(2):1025-1033; Asano *et al.* (2004) “*A Diabody For Cancer Immunotherapy And Its Functional Enhancement By Fusion Of Human Fc 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).

[0016] The provision of non-monospecific diabodies provides a significant advantage: the capacity to co-ligate and co-localize cells that express different epitopes. Bi-specific diabodies thus have wide-ranging applications including therapy and immunodiagnosis. Bi-specificity 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).

[0017] Diabody epitope binding domains may also be directed to a surface determinant of any immune effector cell such as CD3, CD16, CD32, or CD64, which

are expressed on T lymphocytes, natural killer (NK) cells or other mononuclear cells. In many studies, diabody binding to effector cell determinants, *e.g.*, Fc γ receptors (Fc γ R), 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-Fc γ R 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).

[0018] However, the above advantages come at a salient cost. The formation of such non-monospecific diabodies requires the successful assembly of two or more distinct and different polypeptides (*i.e.*, such formation requires that the diabodies be formed through the heterodimerization of different polypeptide chain species). This fact is in contrast to mono-specific diabodies, which are formed through the homodimerization of identical polypeptide chains. Because at least two dissimilar polypeptides (*i.e.*, two polypeptide species) must be provided in order to form a non-monospecific diabody, and because homodimerization of such polypeptides leads to inactive molecules (Takemura, S. *et al.* (2000) "*Construction Of A Diabody (Small Recombinant Bispecific Antibody) Using A Refolding System*," Protein Eng. 13(8):583-588), the production of such polypeptides must be accomplished in such a way as to prevent covalent bonding between polypeptides of the same species (*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).

[0019] 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).

[0020] In the face of this challenge, the art has succeeded in developing stable, covalently bonded heterodimeric non-monospecific diabodies (see, *e.g.*, WO 2006/113665; WO/2008/157379; WO 2010/080538; WO 2012/018687; WO/2012/162068; Johnson, S. *et al.* (2010) “*Effector Cell Recruitment With Novel Fv-Based Dual-Affinity Re-Targeting Protein Leads To Potent Tumor Cytolysis And In Vivo B-Cell Depletion*,” *J. Molec. Biol.* 399(3):436-449; Veri, M.C. *et al.* (2010) “*Therapeutic Control Of B Cell Activation Via Recruitment Of Fcγ Receptor IIb (CD32B) Inhibitory Function With A Novel Bispecific Antibody Scaffold*,” *Arthritis Rheum.* 62(7):1933-1943; Moore, P.A. *et al.* (2011) “*Application Of Dual Affinity Retargeting Molecules To Achieve Optimal Redirected T-Cell Killing Of B-Cell Lymphoma*,” *Blood* 117(17):4542-4551). Such approaches involve engineering one or more cysteine residues into each of the employed polypeptide species. For example, the addition of a cysteine residue to the C-terminus of such constructs has been shown to allow disulfide bonding between the polypeptide chains, stabilizing the

resulting heterodimer without interfering with the binding characteristics of the bivalent molecule.

[0021] Notwithstanding such success, the production of stable, functional heterodimeric, non-monospecific 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 CD123 x CD3 diabodies.

Summary of the Invention:

[0022] The present invention is directed to CD123 x CD3 bi-specific diabodies that are capable of simultaneous binding to CD123 and CD3, and to the uses of such molecules in the treatment of disease, in particular hematologic malignancies.

[0023] The CD123 x CD3 bi-specific diabodies of the invention comprise at least two different polypeptide chains that associate with one another in a heterodimeric manner to form one binding site specific for an epitope of CD123 and one binding site specific for an epitope of CD3. A CD123 x CD3 diabody of the invention is thus monovalent in that it is capable of binding to only one copy of an epitope of CD123 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 CD123 and to the epitope of CD3. The individual polypeptide chains of the diabodies are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. In particular embodiments, the diabodies of the present invention further have an immunoglobulin Fc Domain or an Albumin-Binding Domain to extend half-life in vivo.

[0024] In detail, the invention also provides a sequence-optimized CD123 x CD3 bi-specific monovalent diabody capable of specific binding to an epitope of CD123 and to an epitope of CD3, wherein the diabody comprises a first polypeptide chain and a second polypeptide chain, covalently bonded to one another, wherein:

- A. the first polypeptide chain comprises, in the N-terminal to C-terminal direction:
- i. a Domain 1, comprising:
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:21**); and
 - (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}) (**SEQ ID NO:26**);

wherein the sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);
 - ii. a Domain 2, wherein the Domain 2 is an E-coil Domain (**SEQ ID NO:34**) or a K-coil Domain (**SEQ ID NO:35**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:30**); and
- B. the second polypeptide chain comprises, in the N-terminal to C-terminal direction:
- i. a Domain 1, comprising:
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}) (**SEQ ID NO:25**); and
 - (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:22**);

wherein the sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);
 - ii. a Domain 2, wherein the Domain 2 is a K-coil Domain (**SEQ ID NO:35**) or an E-coil Domain (**SEQ ID NO:34**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:30**); and wherein the Domain 2 of the first and the second polypeptide chains are not both E-coil Domains or both K-coil Domains;

and wherein:

- (a) said VL Domain of said first polypeptide chain and said VH Domain of said second polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD3; and
- (b) said VL Domain of said second polypeptide chain and said VH Domain of said first polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD123.

[0025] The invention also provides a non-sequence-optimized CD123 x CD3 bi-specific monovalent diabody capable of specific binding to an epitope of CD123 and to an epitope of CD3, wherein the diabody comprises a first polypeptide chain and a second polypeptide chain, covalently bonded to one another, wherein:

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

- i. a Domain 1, comprising:
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:23**); and
 - (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}) (**SEQ ID NO:28**);

wherein the sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);

- ii. a Domain 2, wherein the Domain 2 is an E-coil Domain (**SEQ ID NO:34**) or a K-coil Domain (**SEQ ID NO:35**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:30**); and

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

- i. a Domain 1, comprising:
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}) (**SEQ ID NO:27**); and

- (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:24**);

wherein the sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);

- ii. a Domain 2, wherein the Domain 2 is a K-coil Domain (**SEQ ID NO:35**) or an E-coil Domain (**SEQ ID NO:34**), wherein the Domain 2 is separated from the Domain 1 by a peptide linker (**SEQ ID NO:30**); and wherein the Domain 2 of the first and the second polypeptide chains are not both E-coil Domains or both K-coil Domains

and wherein:

- (a) said VL Domain of said first polypeptide chain and said VH Domain of said second polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD3; and
- (b) said VL Domain of said second polypeptide chain and said VH Domain of said first polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD123.

[0026] The invention additionally provides the embodiment of the above-described bi-specific monovalent diabodies, wherein the first or second polypeptide chain additionally comprises an Albumin-Binding Domain (**SEQ ID NO:36**) linked, C-terminally to Domain 2 or N-terminally to Domain 1, via a peptide linker (**SEQ ID NO:31**).

[0027] The invention additionally provides the embodiment of the above-described bi-specific monovalent diabodies wherein the first or second polypeptide chain additionally comprises a Domain 3 comprising a CH2 and CH3 Domain of an immunoglobulin IgG Fc Domain (**SEQ ID NO:37**), wherein the Domain 3 is linked, N-terminally, to the Domain 1A via a peptide linker (**SEQ ID NO:33**).

[0028] The invention additionally provides the embodiment of the above-described bi-specific monovalent diabodies wherein the first or second polypeptide chain additionally comprises a Domain 3 comprising a CH2 and CH3 Domain of an

immunoglobulin IgG Fc Domain (**SEQ ID NO:37**), wherein the Domain 3 is linked, C-terminally, to the Domain 2 via a peptide linker (**SEQ ID NO:32**).

[0029] The invention additionally provides the embodiment of any of the above-described bi-specific monovalent diabodies wherein the Domain 2 of the first polypeptide chain is a K-coil Domain (**SEQ ID NO:35**) and the Domain 2 of the second polypeptide chain is an E-coil Domain (**SEQ ID NO:34**).

[0030] The invention additionally provides the embodiment of any of the above-described bi-specific monovalent diabodies wherein the Domain 2 of the first polypeptide chain is an E-coil Domain (**SEQ ID NO:34**) and the Domain 2 of the second polypeptide chain is a K-coil Domain (**SEQ ID NO:35**).

[0031] The invention additionally provides the embodiment of a bi-specific monovalent diabody capable of specific binding to an epitope of CD123 and to an epitope of CD3, wherein the diabody comprises a first polypeptide chain and a second polypeptide chain, covalently bonded to one another, wherein: said bi-specific diabody comprises:

- A. a first polypeptide chain having the amino acid sequence of **SEQ ID NO:1**; and
- B. a second polypeptide chain having the amino acid sequence of **SEQ ID NO:3**;

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

[0032] The diabodies of the invention exhibit unexpectedly enhanced functional activities as further described below.

[0033] The diabodies of the invention are preferably capable of cross-reacting with both human and primate CD123 and CD3 proteins, preferably cynomolgus monkey CD123 and CD3 proteins.

[0034] The diabodies of the invention are preferably capable of depleting, in an in vitro cell-based assay, plasmacytoid dendritic cells (pDC) from a culture of primary PBMCs with an IC₅₀ of about 1 ng/ml or less, about 0.8 ng/ml or less, about 0.6

ng/ml or less, about 0.4 ng/ml or less, about 0.2 ng/ml or less, about 0.1 ng/ml or less, about 0.05 ng/ml or less, about 0.04 ng/ml or less, about 0.03 ng/ml or less, about 0.02 ng/ml or less or about 0.01ng/ml or less. Preferably, the IC₅₀ is about 0.01ng/ml or less. In the above-described assay, the culture of primary PBMCs may be from cynomolgus monkey in which case said depletion is of cynomolgus monkey plasmacytoid dendritic cells (pDC). Optionally the diabodies of the invention may be capable of depleting plasmacytoid dendritic cells (pDC) from a primary culture of PBMCs as described above wherein the assay is conducted by or in accordance with the protocol of Example 14, as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0035] The diabodies of the invention preferably exhibit cytotoxicity in an in vitro Kasumi-3 assay with an EC₅₀ of about 0.05 ng/mL or less. Preferably, the EC₅₀ is about 0.04 ng/mL or less, about 0.03 ng/mL or less, about 0.02 ng/mL or less, or about 0.01 ng/mL or less. Optionally the diabodies of the invention may exhibit cytotoxicity as described above wherein the assay is conducted by or in accordance with the protocol of Example 3 as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0036] The diabodies of the invention preferably exhibit cytotoxicity in an in vitro Molm-13 assay with an EC₅₀ of about 5 ng/mL or less. Preferably, the EC₅₀ is about 3 ng/mL or less, about 2 ng/mL or less, about 1 ng/mL or less, about 0.75 ng/mL or less, or about 0.2 ng/mL or less. Optionally the diabodies of the invention may exhibit cytotoxicity as described above wherein the assay is conducted by or in accordance with the protocol of Example 3 as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0037] The diabodies of the invention are preferably capable of inhibiting the growth of a MOLM-13 tumor xenograft in a mouse. Preferably the diabodies of the invention may be capable of inhibiting the growth of a MOLM-13 tumor xenograft in

a mouse at a concentration of at least about 20 µg/kg, at least about 4 µg/kg, at least about 0.8 µg/kg, at least about 0.6 µg/kg or at least about 0.4 µg/kg. Preferred antibodies of the invention will inhibit growth of a MOLM-13 tumor xenograft in a mouse by at least 25%, but possibly by at least about 40% or more, by at least about 50% or more, by at least about 60% or more, by at least about 70% or more, by at least about 80% or more, by at least about 90% or more, or even by completely inhibiting MOLM-13 tumor growth after some period of time or by causing tumor regression or disappearance. This inhibition will take place for at least an NSG mouse strain. Optionally, the diabodies of the invention may be capable of inhibiting the growth of a MOLM-13 tumor xenograft in a mouse in the above-described manner by or in accordance with the protocol of Example 6 as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0038] The diabodies of the invention are preferably capable of inhibiting the growth of an RS4-11 tumor xenograft in a mouse. Preferably the diabodies of the invention may be capable of inhibiting the growth of a RS4-11 tumor xenograft in a mouse at a concentration of at least about 0.5 mg/kg, at least about 0.2 mg/kg, at least about 0.1 mg/kg, at least about 0.02 mg/kg or at least about 0.004 mg/kg. Preferred antibodies of the invention will inhibit growth of a RS4-11 tumor xenograft in a mouse by at least about 25%, but possibly at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or even by completely inhibiting RS4-11 tumor growth after some period of time or by causing tumor regression or disappearance. This inhibition will take place for at least an NSG mouse strain. Optionally, the diabodies of the invention may be capable of inhibiting the growth of a RS4-11 tumor xenograft in a mouse in the above-described manner by or in accordance with the protocol of Example 6 as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0039] The diabodies of the invention are preferably capable of depleting leukemic blast cells in vitro in a primary culture of AML bone marrow cells. Preferably the diabodies of the invention may be capable of depleting leukemic blast cells in vitro in

a primary culture of AML bone marrow cells at concentrations of at least about 0.01 ng/ml, at least about 0.02 ng/ml, at least about 0.04 ng/ml, at least about 0.06 ng/ml, at least about 0.08 ng/ml or at least about 0.1 ng/ml. Preferably, the diabodies of the invention may be capable of depleting leukemic blast cells *in vitro* in a primary culture of AML bone marrow cells to less than 20% of the total population of primary leukemic blast cells at diabody concentrations of at least about 0.01 ng/ml, at least about 0.02 ng/ml, at least about 0.04 ng/ml, at least about 0.06 ng/ml, at least about 0.08 ng/ml or at least about 0.1 ng/ml, optionally following incubation of the primary culture with diabody for about 120 hours. Preferably leukemic blast cells are depleted *in vitro* in a primary culture of AML bone marrow cells to less than 20% of the total population of primary leukemic blast cells at diabody concentrations of about 0.01 ng/ml or 0.1 ng/ml following incubation of the primary culture with diabody for about 120 hours.

[0040] The diabodies of the invention are preferably capable of inducing an expansion of a T cell population *in vitro* in a primary culture of AML bone marrow cells. Preferably, such expansion may be to about 70% or more of the maximum T cell population which can be expanded in the assay. Preferably the diabodies of the invention may be capable of inducing an expansion of a T cell population *in vitro* in a primary culture of AML bone marrow cells to about 70% or more of the maximum T cell population which can be expanded in the assay at diabody concentrations of at least about 0.01 ng/ml, at least about 0.02 ng/ml, at least about 0.04 ng/ml, at least about 0.06 ng/ml, at least about 0.08 ng/ml or at least about 0.1 ng/ml, optionally following incubation of the primary culture with diabody for about 120 hours. Preferably, a T cell population is expanded *in vitro* in a primary culture of AML bone marrow cells to about 70% or more of the maximum T cell population which can be expanded in the assay at diabody concentrations of about 0.01 ng/ml or about 0.1 ng/ml following incubation of the primary culture with diabody for about 120 hours.

[0041] The diabodies of the invention are preferably capable of inducing an activation of a T cell population *in vitro* in a primary culture of AML bone marrow cells. Such activation may occur at diabody concentrations of at least about 0.01 ng/ml, at least about 0.02 ng/ml, at least about 0.04 ng/ml, at least about 0.06 ng/ml,

at least about 0.08 ng/ml or at least about 0.1 ng/ml, optionally following incubation of the primary culture with diabody for about 72 hours. Such activation may be measured by the expression of a T cell activation marker such as CD25. Preferably, activation of a T cell population in vitro in a primary culture of AML bone marrow cells as measured by expression of CD25 may occur at diabody concentrations of about 0.01 ng/ml or about 0.1 ng/ml following incubation of the primary culture with diabody for about 72 hours.

[0042] The diabodies of the invention are preferably capable of depleting leukemic blast cells in vitro in a primary culture of AML bone marrow cells to less than 20% of the total population of primary leukemic blast cells and at the same time inducing an expansion of the T cell population in vitro in the primary culture of AML bone marrow cells to about 70% or more of the maximum T cell population which can be expanded in the assay at diabody concentrations of at least about 0.01 ng/ml, at least about 0.02 ng/ml, at least about 0.04 ng/ml, at least about 0.06 ng/ml, at least about 0.08 ng/ml or at least about 0.1 ng/ml, optionally following incubation of the primary culture with diabody for about 120 hours. Preferably, the diabody concentrations are about 0.01 ng/ml or about 0.1 ng/ml and the primary culture is incubated with diabody for about 120 hours.

[0043] The diabodies of the invention may be capable of depleting leukemic blast cells in vitro in a primary culture of AML bone marrow cells and/or inducing an expansion of a T cell population in vitro in a primary culture of AML bone marrow cells and/or inducing an activation of a T cell population in vitro in a primary culture of AML bone marrow cells in the above-described manner by or in accordance with the protocol of Example 8 as herein described, or by modification of such assay as would be understood by those of ordinary skill, or by other means known to those of ordinary skill.

[0044] For the avoidance of any doubt, the diabodies of the invention may exhibit one, two, three, more than three or all of the functional attributes described herein. Thus the diabodies of the invention may exhibit any combination of the functional attributes described herein.

[0045] The diabodies of the invention 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 CD123. The invention also relates to the use of diabodies of the invention in the manufacture of a pharmaceutical composition, preferably for the treatment of a disease or condition associated with or characterized by the expression of CD123 as further defined herein.

[0046] The disease or condition associated with or characterized by the expression of CD123 may be cancer. 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), 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.

[0047] The disease or condition associated with or characterized by the expression of CD123 may be an inflammatory condition. For example, the inflammatory condition may be selected from the group consisting of: Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.

[0048] The invention additionally provides a pharmaceutical composition comprising any of the above-described diabodies and a physiologically acceptable carrier.

[0049] The invention 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 CD123.

[0050] The invention is particularly directed to the embodiment of such use, wherein the disease or condition associated with or characterized by the expression of CD123 is cancer (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), 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).

[0051] The invention is also particularly directed to the embodiment of such use, wherein the disease or condition associated with or characterized by the expression of CD123 is an inflammatory condition (especially an inflammatory condition selected from the group consisting of: Autoimmune Lupus (SLE), allergy, asthma, and rheumatoid arthritis).

[0052] 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:

[0053] **Figure 1** shows that CD123 was known to be expressed on leukemic stem cells.

[0054] **Figure 2** illustrates the structures of the first and second polypeptide chains of a two chain CD123 x CD3 bi-specific monovalent diabody of the present invention.

[0055] **Figures 3A and 3B** illustrate the structures of two versions of the first, second and third polypeptide chains of a three chain CD123 x CD3 bi-specific monovalent Fc diabody of the present invention (Version 1, **Figure 3A**; Version 2, **Figure 3B**).

[0056] **Figure 4 (Panels A-E)** shows the ability of different CD123 x CD3 bi-specific diabodies to mediate T cell redirected killing of target cells displaying varying amount of CD123. The Figure provides dose-response curves indicating that the sequence-optimized CD123 x CD3 bi-specific diabody ("DART-A") having an Albumin-Binding Domain (DART-A with ABD "w/ABD") exhibited greater

cytotoxicity than a control bi-specific diabody (Control DART) or a non-sequence-optimized CD123 x CD3 bi-specific diabody (“DART-B”) in multiple target cell types: RS4-11 (**Panel A**); TF-1 (**Panel B**); Molm-13 (**Panel C**); Kasumi-3 (**Panel D**); and THP-1 (**Panel E**) at an E:T (effector : target) ratio of 10:1.

[0057] **Figure 5 (Panels A-D)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A), sequence-optimized CD123 x CD3 bi-specific diabody having an Albumin-Binding Domain (DART-A with ABD “w/ABD”) and sequence-optimized CD123 x CD3 bi-specific diabody having an immunoglobulin IgG Fc Domain (DART-A with Fc “w/Fc”) to mediate T cell activation during redirected killing of target cells. The Figure presents dose-response curves showing the cytotoxicity mediated by DART-A, DART-A w/ABD and DART-A w/Fc in Kasumi-3 (**Panel A**) and THP-1 (**Panel B**) cells and purified CD8 T cells at an E:T (effector cell : target cell) ratio of 10:1 (18 hour incubation). Panels C and D show dose-response curves of T cell activation using the marker CD25 on CD8 T cells in the presence (**Panel D**) and absence (**Panel C**) of target cells.

[0058] **Figure 6 (Panels A-B)** shows Granzyme B and Perforin levels in CD4 and CD8 T cells after treatment with the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) (**Panel A**) or a control bi-specific diabody (Control DART) (**Panel B**) in the presence of Kasumi-3 target cells and resting T cells at an E:T ratio of 10:1.

[0059] **Figure 7 (Panels A-B)** shows the *in vivo* antitumor activity of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) at nanogram per kilogram dosing levels. MOLM-13 cells (intermediate CD123 expression) were co-mixed with T cells and implanted subcutaneously (T:E 1:1) in NSG mice. Intravenous treatment was once daily for 8 days (QDx8) starting at implantation. Various concentrations of DART-A were compared to a control bi-specific diabody (Control DART). **Panel A** shows the Molm-13 cells alone or with T cells, and the effect of various doses of DART-A on tumor volume even to times beyond 30 days. **Panel B** shows the effect of increasing doses of DART-A on tumor volume seen in NSG mice receiving MOLM-13 cells and T cells (T:E 1:1) for a time course of 0-18 days.

[0060] **Figure 8** shows the *in vivo* antitumor activity of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) on RS4-11 cells (ALL with monocytic features). Cells were co-mixed with T cells and implanted subcutaneously (T:E 1:1) in NSG mice. Intravenous treatment was once daily for 4 days (QDx4) starting at implantation. Various concentrations of DART-A were compared to a control bi-specific diabody (Control DART).

[0061] **Figure 9 (Panels A-B)** shows CD123+ blasts in bone marrow mononucleocytes (BM MNC) and peripheral blood mononucleocytes (PBMCs) from AML patient 1 (**Panel A**) compared to Kasumi-3 AML cell line (**Panel B**).

[0062] **Figure 10 (Panels A-C)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate blast reduction in primary AML at 120h (**Panel A**), drive T cell expansion in primary AML at 120h (**Panel B**) and induce T cell activation in AML at 48h and 72 h (**Panel C**).

[0063] **Figure 11 (Panels A-H)** shows the identification of the CD123+ blast population in a primary sample of ALL PBMCs. **Panels A and E** show the forward and side scatter of the input population of normal PBMC (**Panel A**) and ALL PBMCs (**Panel E**). **Panels B and F** show the identification of the lymphocyte population as primarily B cells (**Panel B**) and leukemic blast cells (**Panel F**). **Panels C and G** show identification of the population of lymphocytes that are CD123+. **Panels D and H** show the identification of CD19+ cells and CD123+ cells.

[0064] **Figure 12 (Panels A-B)** shows the identification of the CD4 and CD8 populations of T cells in a primary sample of ALL PBMCs. **Panel A** shows the forward and side scatter of the input ALL PBMCs. **Panel B** shows the CD4 or CD8 populations of T cells present in the samples. The numbers indicate that CD4 T cells represent approximately 0.5% of the total cells and CD8 T cells represent approximately 0.4% of the total cells present in the ALL PBMC sample.

[0065] **Figure 13 (Panels A-H)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate ALL blast depletion with autologous CTL. **Panels A and E** show the forward and side scatter of the input

population of normal PBMC (**Panel A**) and ALL PBMCs (**Panel E**). The PBMCs were untreated (**Panels B and F**), treated with a control bi-specific diabody (Control DART) (**Panels C and G**) or treated with DART-A (**Panels D and H**) and incubated for 7 days followed by staining for CD34 and CD19.

[0066] **Figure 14 (Panels A-L)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate T cell expansion (**Panels A, B, C, G, H and I**) and activation (**Panels D, E, F, J, K and L**) in normal PBMC (**Panels A-F**) and ALL PBMC (**Panels G-L**). The cells were untreated (**Panels A, D, G and J**), or treated with a control bi-specific diabody (Control DART) (**Panels B, E, H and K**) or DART-A (**Panels C, F, I and L**) for 7 days.

[0067] **Figure 15 (Panels A-C)** shows the identification of the AML blast population and T cells in a primary AML sample. **Panel A** shows the forward and side scatter of the input AML PBMCs. **Panel B** shows the identification of the AML blast population in the AML sample. **Panel C** shows the identification of the T cell population in the AML sample.

[0068] **Figure 16 (Panels A-C)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate AML blast depletion with autologous CTL and T cell expansion. Primary AML PBMCs from patient 2 were incubated with PBS, a control bi-specific diabody (Control DART) or DART-A for 144h. Blast cells (**Panel A**), CD4 T cells (**Panel B**) and CD8 T cells (**Panel C**) were counted.

[0069] **Figure 17 (Panels A-D)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate T cell activation in AML. CD25 (**Panel A**) and Ki-67 (**Panel B**) expression was determined for the CD4 and CD8 T cells from AML patient 2 following incubation with a control bi-specific diabody (Control DART) or DART-A with autologous PBMCs. The level of perforin (**Panel C**) and granzyme B (**Panel D**) was determined for the CD4 and CD8 T cells from AML patient 2 following incubation with Control DART or DART-A with autologous PBMCs.

[0070] **Figure 18 (Panels A-D)** shows that the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) is capable of cross-reacting with both human and primate CD123 and CD3 proteins. The panels show BIACORE™ sensogram traces of the results of analyses conducted to assess the ability of DART-A to bind to human (**Panels A and C**) and non-human primate (**Panels B and D**) CD3 (**Panels A and B**) and CD123 (**Panels C and D**) proteins. The KD values are provided.

[0071] **Figure 19 (Panels A-B)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate autologous monocyte depletion *in vitro* with human and cynomolgus monkey PBMCs. The Panels present the results of dose-response curves of DART-A-mediated cytotoxicity with primary human PBMCs (**Panel A**) or cynomolgus monkey PBMCs (**Panel B**).

[0072] **Figure 20 (Panels A-N)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate the depletion of pDC in cynomolgus monkeys without systemic cytokine induction. **Panels A-D** show control results obtained at 4h and 4d with vehicle and carrier. **Panels E-H** show control results obtained at 4h and 4d with a control bi-specific diabody (Control DART). **Panels I-N** show results obtained at 4h and 4d at 10 ng/kg/d and at 4d with 30 ng/kg/d of DART-A.

[0073] **Figure 21 (Panels A-D)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to mediate dose-dependent depletion of pDC in cynomolgus monkeys. Cynomolgus monkeys were dosed with DART-A at 0.1, 1, 10, 30 100, 300, or 1000 ng/kg. PBMCs were evaluated at the indicated time and total B cells (**Panel A**), monocytes (**Panel B**), NK cells (**Panel C**) and pDC (**Panel D**) were counted.

[0074] **Figure 22 (Panels A-D)** shows the ability of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) to intermittently modulate T cells in cynomolgus monkeys. Cynomolgus monkeys were dosed with DART-A at at 0.1, 1, 10, 30 100, 300, or 1000 ng/kg. PBMCs were evaluated at the indicated time and total T cells (**Panel A**), CD4 T cells (**Panel B**), CD69 cells (**Panel C**) and CD8 T cells (**Panel D**) were counted.

[0075] **Figure 23** shows the SDS-PAGE analysis of purified DART-A protein under reducing (left) and non-reducing (right) conditions.

[0076] **Figures 24A-24B** show the physicochemical characterization of purified DART-A. **Figure 24A**: SEC profile of DART-A protein on a calibrated TSK G3000SWxL column. **Figure 24B**: Mass spectrum of DART-A protein.

[0077] **Figures 25A-25D** show SPR analysis of DART-A binding to immobilized human or cynomolgus monkey CD123 and CD3. Dashed lines represent the global fit to a 1:1 Langmuir model of the experimental binding curves obtained at DART-A concentrations of 0, 6.25, 12.5, 25, 50 or 100nM (continuous lines). The data are representative of three independent experiments.

[0078] **Figures 26A-26E** show that DART-A was capable of simultaneously binding both CD3 and CD123. **Figure 26A-26B** provides the results of a bifunctional ELISA and demonstrates simultaneous engagement of both target antigens of DART-A. ELISA plates were coated with human CD123 (**Figure 26A**) or cynomolgus monkey CD123 (**Figure 26B**). Titrating DART-A and Control DART concentrations were followed by detection with human CD3-biotin. **Figures 26C-26E** demonstrate cell-surface binding of DART-A on CD123+ Molm-13 target cell (**Figure 26C**), human T cells (**Figure 26D**) and cynomolgus T cells (**Figure 26E**). Binding was detected by FACS analysis using a monoclonal antibody specific to E-coil and K-coil region of the DART-A or Control DART molecule.

[0079] **Figures 27A-27H** show the ability of DART-A to mediate redirected target cell killing by human or monkey effector cells against CD123+ Kasumi-3 leukemic cell lines, demonstrate the ability of the molecules to bind to subsets of normal circulating leukocytes, including pDCs and monocytes and demonstrate the ability of the molecules to deplete CD14⁻CD123^{high} cells (pDC and basophils) without affecting monocytes (CD14⁺ cells). **Figure 27A** shows the relative anti-CD123-PE binding sites on U937 and Kasumi-3 leukemic cell lines as determined by QFACS analysis. **Figure 27B** shows the relatively low percent cytotoxicity mediated by DART-A or Control DART on an AML cell line (U937 cells) which, as shown in **Figure 27A** have relatively few CD123 binding sites). **Figure 27C** shows the percent cytotoxicity

mediated by DART-A or Control DART in the presence of purified human T cells (as effector cells) on an AML cell line (Kasumi-3 cells) which, as shown in **Figure 27A** have a substantial number of CD123 binding sites. In **Figures 27B-27C**, the E:T ratio is 10:1. **Figure 27D** shows the percent cytotoxicity mediated by DART-A or Control DART in the presence of purified cynomolgus monkey PBMCs (as effector cells) on Kasumi-3 cells (the E:T ratio is 15:1), and demonstrates that DART-A can bind cynomolgus monkey T cells. **Figure 27E** shows the relative anti-CD123-PE binding sites on Kasumi-3 cells, human monocytes, human plasmacytoid dendritic cells ("pDC"), cynomolgus monkey monocytes and cynomolgus monkey plasmacytoid dendritic cells as determined by QFACS analysis. **Figure 27F** shows the ability of DART-A to deplete CD14⁻ CD123^{lo} cells. **Figure 27G** shows the ability of DART-A to deplete human CD14⁻ CD123^{Hi} cells. **Figure 27H** shows the ability of DART-A to deplete cynomolgus monkey CD14⁻ CD123^{Hi} cells. Cytotoxicity was determined by LDH release, with EC50 values determined using GraphPad PRISM® software.

[0080] **Figure 28** shows the use of a two-compartment model to estimate pharmacokinetic parameters of DART-A. The data show the end of infusion (EOI) serum concentrations of DART-A in cynomolgus monkeys after receiving a 96-hour infusion at 100 ng/kg/day 300 ng/kg/day, 600 ng/kg/day, and 1000 ng/kg/day Dose. Each point represents an individual animal; horizontal lines represent the mean value for the dose group.

[0081] **Figures 29A-29C** show the effect of DART-A infusions on the production of the cytokine, IL-6. Serum IL-6 levels (mean ± SEM) in monkeys infused with DART-A are shown by treatment group. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of either vehicle (Group 1) (**Figure 29A**) or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Groups 2-5) (**Figure 29B**) or as a 7-day/week infusion for 4 weeks starting on Days 8 (Group 6) (**Figure 29C**). Treatment intervals are indicated by the filled gray bars.

[0082] **Figures 30A-30F** show the effect of DART-A infusions on the depletion of CD14-/CD123+ cells (**Figures 30A-30C**) and CD303+ cells (**Figures 30D-30F**). The

mean $\pm$ SEM of the circulating levels of CD14-/CD123+ (**Figures 30A-30C**) or CD303+ (**Figures 30D-30F**) by Study Day and by group is shown. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of either vehicle (Group 1) (**Figures 30A and 30D**) or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Groups 2-5) (**Figures 30A and 30E**) or as a 7-day/week infusion for 4 weeks starting on Days 8 (Group 6) (**Figures 30C and 30F**). Treatment intervals are indicated by the filled gray bars.

[0083] **Figures 31A-31I** show the observed changes in T cell populations (**Figures 31A-31C**), CD4+ cell populations (**Figures 31D-31F**) and CD8+ cell populations (**Figures 31G-31I**) receiving DART-A administered as 4-day infusions starting on Days 8, 15, 22, and 29. Legend: CD25+ (gray squares); CD69+ (gray triangles), PD-1+ (white triangles); Tim-3+ (white squares). T cells were enumerated via the CD4 and CD8 markers, rather than the canonical CD3, to eliminate possible interference the DART-A. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of either vehicle (Group 1) or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Group 5) or as a 7-day/week infusion for 4 weeks starting on Days 8 (Group 6). Treatment intervals are indicated by the filled gray bars. The mean $\pm$ SEM of the absolute number of total circulating T cells by Study Day and group is shown (**Figures 31A-31C**). Relative values (mean percent $\pm$ SEM) of CD25+, CD69+, PD-1+ and Tim-3+ of CD4 (**Figures 31D-31E**) or CD8 T cells (**Figures 31F-31H**) by Study Day and by group is shown.

[0084] **Figures 32A-32F** show the observed changes in T CD4+ cell populations (**Figures 32A-32C**) and CD8+ cell populations (**Figures 32D-32F**) during and after a continuous 7-day infusion of DART-A. The mean $\pm$ SEM percent of CD25+, CD69+, PD-1+ and Tim-3+ on CD4 (**Figures 32A-32C**) or CD8 (**Figures 32D-32F**) T cells by Study Day for Groups 2, 3 and 4 are shown. Treatment intervals are indicated by the filled gray bars. Legend: CD25+ (gray squares); CD69+ (gray triangles), PD-1+ (white triangles); Tim-3+ (white squares).

[0085] **Figures 33A-33F** show the observed changes in T CD4⁺ cell populations (**Figures 33A-33C**) and CD8⁺ cell populations (**Figures 33D-33F**) during and after a continuous 7-day infusion of DART-A. The mean $\pm$ SEM percent of CD4⁺ Naïve (CD95⁻/CD28⁺), CMT (CD95⁺/CD28⁺), and EMT (CD95⁺/CD28⁻) T cells in CD4⁺ population (**Figures 33A-33C**) or CD8 population (**Figures 33D-33F**) by Study Day for Groups 2, 3 and 4 are shown. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Groups 2-4). Treatment intervals are indicated by the filled gray bars. Legend: Naïve (white triangles); CMT (black triangles), EMT (gray squares).

Figure 34 shows DART-A-mediated cytotoxicity against Kasumi-3 cells with PBMCs from either naïve monkeys or monkeys treated with multiple infusions of DART-A.

[0086] **Figures 35A-35F** show that DART-A exposure increased the relative frequency of central memory CD4 cells and effector memory CD8⁺ cells at the expense of the corresponding naïve T cell population. The mean $\pm$ SEM percent of CD4⁺ Naïve (CD95⁻/CD28⁺), CMT (CD95⁺/CD28⁺), and EMT (CD95⁺/CD28⁻) T cells in CD4⁺ population (**Figures 35A-35C**) or in CD8⁺ population (**Figures 35D-35F**) by Study Day and by Group is shown. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of either vehicle (Group 1) or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Group 5) or as a 7-day/week infusion for 4 weeks starting on Days 8 (Group 6). Treatment intervals are indicated by the filled gray bars. Legend: Naïve (white triangles); CMT (black triangles), EMT (gray squares).

[0087] **Figures 36A-36F** show the effect of DART-A on red cell parameters in monkeys that had received infusions of the molecules. Circulating RBCs (**Figures 36A-36C**) or reticulocytes (**Figures 36D-36F**) levels (mean $\pm$ SEM) in samples collected at the indicated time points from monkeys treated with DART-A are shown.

[0088] **Figures 37A-37B** show that the frequency (mean percent $\pm$ SEM) of CD123⁺ cells (**Figure 37A**) or HSC (CD34⁺/CD38⁻/CD45⁻/CD90⁺ cells) (**Figure**

37B) within the Lin⁻ cell population in bone marrow samples collected at the indicated time points from monkeys treated with DART-A. Cynomolgus monkeys were treated with vehicle control on Day 1, followed by 4 weekly infusions of either vehicle (Group 1) or DART-A administered as 4-day weekly infusions starting on Days 8, 15, 22, and 29 (Groups 2-5) or as a 7-day/week infusion for 4 weeks starting on Days 8 (Group 6).

Detailed Description of the Invention:

[0089] The present invention is directed to sequence-optimized CD123 x CD3 bi-specific monovalent diabodies that are capable of simultaneous binding to CD123 and CD3, and to the uses of such molecules in the treatment of hematologic malignancies. Although non-optimized CD123 x CD3 bi-specific 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 CD123 x CD3 bi-specific diabodies by modifying or refining their sequences.

[0090] The preferred CD123 x CD3 bi-specific diabodies of the present invention are composed of at least two polypeptide chains that associate with one another to form one binding site specific for an epitope of CD123 and one binding site specific for an epitope of CD3 (**Figure 2**). The individual polypeptide chains of the diabody are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. Each polypeptide chain contains an Antigen Binding Domain of a Light Chain Variable Domain, an Antigen Binding Domain of a Heavy Chain Variable Domain and a heterodimerization domain. An intervening linker peptide (Linker 1) separates the Antigen Binding Domain of the Light Chain Variable Domain from the Antigen Binding Domain of the Heavy Chain Variable Domain. The Antigen Binding Domain of the Light Chain Variable Domain of the first polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the second polypeptide chain in order to form a first functional antigen binding site that is specific for the first antigen (*i.e.*, either CD123

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 CD123 or CD3, depending upon the identity of the first antigen). Thus, the selection of the Antigen Binding Domain of the Light Chain Variable Domain and the Antigen Binding Domain of the Heavy Chain Variable Domain of the first and second polypeptide chains are coordinated, such that the two polypeptide chains collectively comprise Antigen Binding Domains of light and Heavy Chain Variable Domains capable of binding to CD123 and CD3.

[0091] The formation of heterodimers of the first and second polypeptide chains can be driven by the heterodimerization domains. Such domains include GVEPKSC (**SEQ ID NO:50**) (or VEPKSC; **SEQ ID NO:51**) on one polypeptide chain and GFNRGEC (**SEQ ID NO:52**) (or FNRGEC; **SEQ ID NO:53**) on the other polypeptide chain (US2007/0004909). Alternatively, such domains can be engineered to contain coils of opposing charges. The heterodimerization domain of one of the polypeptide chains comprises a sequence of at least six, at least seven or at least eight positively charged amino acids, and the heterodimerization domain of the other of the polypeptide chains comprises a sequence of at least six, at least seven or at least eight negatively charged amino acids. For example, the first or the second heterodimerization domain will preferably comprise a sequence of eight positively charged amino acids and the other of the heterodimerization domains will preferably comprise a sequence of eight negatively charged amino acids. The positively charged amino acid may be lysine, arginine, histidine, *etc.* and/or the negatively charged amino acid may be glutamic acid, aspartic acid, *etc.* The positively charged amino acid is preferably lysine and/or the negatively charged amino acid is preferably glutamic acid.

[0092] The CD123 x CD3 bi-specific diabodies of the present invention are engineered so that such first and second polypeptide chains covalently bond to one another via cysteine residues along their length. Such cysteine residues may be introduced into the intervening linker that separates the VL and VH domains of the polypeptides. Alternatively, and more preferably, a second peptide (Linker 2) is

introduced into each polypeptide chain, for example, at the amino-terminus of the polypeptide chains or a position that places Linker 2 between the heterodimerization domain and the Antigen Binding Domain of the Light Chain Variable Domain or Heavy Chain Variable Domain.

[0093] In particular embodiments, the sequence-optimized CD123 x CD3 bi-specific monovalent diabodies of the present invention further have an immunoglobulin Fc Domain or an Albumin-Binding Domain to extend half-life *in vivo*.

[0094] The CD123 x CD3 bi-specific monovalent diabodies of the present invention that comprise an immunoglobulin Fc Domain (*i.e.*, CD123 x CD3 bi-specific monovalent Fc diabodies) are composed of a first polypeptide chain, a second polypeptide chain and a third polypeptide chain. The first and second polypeptide chains associate with one another to form one binding site specific for an epitope of CD123 and one binding site specific for an epitope of CD3. The first polypeptide chain and the third polypeptide chain associate with one another to form an immunoglobulin Fc Domain (**Figure 3A** and **Figure 3B**). The first and second polypeptide chains of the bi-specific monovalent Fc diabody are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain.

[0095] The first and third polypeptide chains are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within each polypeptide chain. The first and second polypeptide chains each contain an Antigen Binding Domain of a Light Chain Variable Domain, an Antigen Binding Domain of a Heavy Chain Variable Domain and a heterodimerization domain. An intervening linker peptide (Linker 1) separates the Antigen Binding Domain of the Light Chain Variable Domain from the Antigen Binding Domain of the Heavy Chain Variable Domain. The Antigen Binding Domain of the Light Chain Variable Domain of the first polypeptide chain interacts with the Antigen Binding Domain of the Heavy Chain Variable Domain of the second polypeptide chain in order to form a first functional antigen binding site that is specific for the first antigen (*i.e.*, either CD123 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 CD123, 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 CD123 and CD3. The first and third polypeptide chains each contain some or all of the CH2 Domain and/or some or all of the CH3 Domain of a complete immunoglobulin Fc Domain and a cysteine-containing peptide. The some or all of the CH2 Domain and/or the some or all of the CH3 Domain associate to form the immunoglobulin Fc Domain of the bi-specific monovalent Fc diabodies of the present invention. The first and third polypeptide chains of the bi-specific monovalent Fc diabodies of the present invention are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within the cysteine-containing peptide of the polypeptide chains.

I. The Sequence-Optimized CD123 x CD3 Bi-Specific Diabody, “DART-A”

[0096] The invention provides a sequence-optimized bi-specific diabody capable of simultaneously and specifically binding to an epitope of CD123 and to an epitope of CD3 (a “**CD123 x CD3**” bi-specific diabody or DART-A). As discussed below, DART-A was found to exhibit enhanced functional activity relative to other non-sequence-optimized CD123 x CD3 bi-specific diabodies of similar composition, and is thus termed a “sequence-optimized” CD123 x CD3 bi-specific diabody.

[0097] The sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) comprises a first polypeptide chain and a second polypeptide chain. The first polypeptide chain of the bi-specific diabody will comprise, in the N-terminal to C-terminal direction, an N-terminus, a Light Chain Variable Domain (VL Domain) of a monoclonal antibody capable of binding to CD3 (VL_{CD3}), an intervening linker peptide (Linker 1), a Heavy Chain Variable Domain (VH Domain) of a monoclonal

antibody capable of binding to CD123 (VH_{CD123}), and a C-terminus. A preferred sequence for such a VL_{CD3} Domain is **SEQ ID NO:21**:

QAVVTQEPSTLVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGG
TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
KLTVLG

[0098] The Antigen Binding Domain of VL_{CD3} comprises CDR1 **SEQ ID NO:38**: RSSTGAVTTSNYAN, CDR2 **SEQ ID NO:39**: GTNKRAP, and CDR3 **SEQ ID NO:40**: ALWYSNLWV.

[0099] A preferred sequence for such Linker 1 is **SEQ ID NO:29**: GGGSGGGG. A preferred sequence for such a VH_{CD123} Domain is **SEQ ID NO:26**:

EVQLVQSGAELKKPGASVKVSCASGYTFTDYMKWVRQAPGQGLEWIGDII
PSNGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSEDVAVYYCARSHLLRA
SWFAYWGQGTLLVTVSS

[00100] The Antigen Binding Domain of VH_{CD123} comprises CDR1 **SEQ ID NO:47**: DYMK, CDR2 **SEQ ID NO:48**: DIIPSNATFYNQKFKG, and CDR3 **SEQ ID NO:49**: SHLLRAS.

[00101] The second polypeptide chain will comprise, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}), an intervening linker peptide (*e.g.*, Linker 1), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}), and a C-terminus. A preferred sequence for such a VL_{CD123} Domain is **SEQ ID NO:25**:

DFVMTQSPDSLAVSLGERVTMSCKSSQSLNLSGNQKNYLTWYQQKPGQPPL
LIYWASTRESGVDPDRFSGSGSGTDFTLTISLQAEDVAVYYCQNDYSYPYTF
GGTKLEIK

[00102] The Antigen Binding Domain of VL_{CD123} comprises CDR1 **SEQ ID NO:44**: KSSQSLNLSGNQKNYLT, CDR2 **SEQ ID NO:45**: WASTRES, and CDR3 **SEQ ID NO:46**: QNDYSYPYT.

[00103] A preferred sequence for such a VH_{CD3} Domain is **SEQ ID NO:22**:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIR
SKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNF
GNSYVSWFAYWGQGTLLVTVSS

[00104] The Antigen Binding Domain of VH_{CD3} comprises CDR1 **SEQ ID NO:41:** TYAMN, CDR2 **SEQ ID NO:42:** RIRSKYNNYATYYADSVKD, and CDR3 **SEQ ID NO:43:** HGNFGNSYVSWFAY.

[00105] The sequence-optimized CD123 x CD3 bi-specific diabodies of the present invention are engineered so that such first and second polypeptides covalently bond to one another via cysteine residues along their length. Such cysteine residues may be introduced into the intervening linker (*e.g.*, Linker 1) that separates the VL and VH domains of the polypeptides. Alternatively, and more preferably, a second peptide (Linker 2) is introduced into each polypeptide chain, for example, at a position N-terminal to the VL domain or C-terminal to the VH domain of such polypeptide chain. A preferred sequence for such Linker 2 is **SEQ ID NO:30:** GGC GGG.

[00106] The formation of heterodimers can be driven by further engineering such polypeptide chains to contain polypeptide coils of opposing charge. Thus, in a preferred embodiment, one of the polypeptide chains will be engineered to contain an “E-coil” domain (**SEQ ID NO:34:** EVAALEKEVAALEKEVAALEKEVAALEK) whose residues will form a negative charge at pH 7, while the other of the two polypeptide chains will be engineered to contain an “K-coil” domain (**SEQ ID NO:35:** KVAALKEKVAALKEKVAALKEKVAALKE) whose residues will form a positive charge at pH 7. The presence of such charged domains promotes association between the first and second polypeptides, and thus fosters heterodimerization.

[00107] It is immaterial which coil is provided to the first or second polypeptide chains. However, a preferred sequence-optimized CD123 x CD3 bi-specific diabody of the present invention (“DART-A”) has a first polypeptide chain having the sequence (**SEQ ID NO:1**):

QAVVTQEPSTLVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGG
TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
KLTVLGGGGSGGGGEVQLVQSGAELKKPGASVKVSKASGYTFTDYMKWVR
QAPGQGLEWIGDIIPSNGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSED
TAVYYCARSHLLRASWFAYWGQGTTLTVSSGGCGGGEVAALEKEVAALEKEV
AALEKEVAALEK

[00108] DART-A Chain 1 is composed of: **SEQ ID NO:21 — SEQ ID NO:29 — SEQ ID NO:26 — SEQ ID NO:30 — SEQ ID NO:34**. A DART-A Chain 1 encoding polynucleotide is **SEQ ID NO:2**:

```
caggctgtggtgactcaggagccttcactgaccgtgtccccaggcgggaactg
tgaccttgacatgcagatccagcacaggcgcagtgaccacatctaactacgc
caattgggtgcagcagaagccaggacaggcaccaagggggcctgatcgggggt
acaaacaaaagggctccctggacccctgcacgggttttctggaagtctgctgg
gcggaaaggccgctctgactattaccggggcacaggccgaggacgaagccga
ttactattgtgctctgtggtatagcaatctgtgggtgttcgggggtggcaca
aaactgactgtgctgggaggggggtggatccggcggcggaggcgagggtgcagc
tggtgcagtcgggggtgagctgaagaaaccggagcttccgtgaagggtgtc
ttgcaaagccagtggtacaccttcacagactactatatgaagtgggtcagg
caggctccaggacagggactggaatggatcggcgatatcattccttccaacg
gggccactttctacaatcagaagtttaaaggcagggtgactattaccgtgga
caaatcaacaagcactgcttatatggagctgagctccctgcgctctgaagat
acagccgtgtactattgtgctcggtcacacctgctgagagccagctggtttg
cttattggggacagggcaccctggtgacagtgtcttccggaggatgtggcgg
tggagaagtggccgcactggagaaagaggttgctgctttggagaaggaggtc
gctgcacttgaaaaggaggtcgcagccctggagaaa
```

[00109] The second polypeptide chain of DART-A has the sequence (**SEQ ID NO:3**):

```
DFVMTQSPDSLAVSLGERVTMSCKSSQSLNLSGNQKNYLTWYQQKPGQPPKL
LIYWASTRESGVPDFRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPYTF
GQGTKLEIKGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMN
WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNS
LKTEDTAVYYCVRHGNFGNSYVSWFAYWGQGLTVTVSSGGCGGGKVAALKEK
VAALKEKVAALKEKVAALKE
```

[00110] DART-A Chain 2 is composed of: **SEQ ID NO:25 — SEQ ID NO:29 — SEQ ID NO:22 — SEQ ID NO:30 — SEQ ID NO:35**. A DART-A Chain 2 encoding polynucleotide is **SEQ ID NO:4**:

```
gacttcgtgatgacacagtcctcctgatagtcctggccgtgagtcctgggggagc
gggtgactatgtcttgcaagagctcccagtcactgctgaacagcggaaatca
gaaaaactatctgacctggtaccagcagaagccaggccagccccctaaactg
ctgatctattgggcttccaccagggaatctggcgtgcccagacagattcagcg
gcagcggcagcggcacagattttaccctgacaatttctagtcctgcaggccga
ggacgtggctgtgtactattgtcagaatgattacagctatccctacactttc
ggccaggggaccaagctggaaattaaaggaggcggatccggcggcggaggcg
aggtgcagctggtggagtcctgggggaggcttggtccagcctggagggtccct
gagactctcctgtgcagcctctggattcaccttcagcacatacgtatgaat
tgggtccgcccaggctccagggaaggggctggagtggttggaaggatcaggt
ccaagtacaacaattatgcaacctactatgccgactctgtgaaggatagatt
caccatctcaagagatgattcaaagaactcactgtatctgcaaatgaacagc
ctgaaaaccgaggacacggccgtgtattactgtgtgagacacggtaacttcg
```

```

gcaattcttacgtgtcttgggtttgcttattggggacaggggacactggtgac
tgtgtcttccggaggatgtggcggtggaaaagtggccgcactgaaggagaaa
gttgctgctttgaaagagaaggtcgccgcacttaaggaaaaggtcgcagccc
tgaaagag

```

[00111] As discussed below, the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) was found to have the ability to simultaneously bind CD123 and CD3 as arrayed by human and monkey cells. Provision of DART-A was found to cause T cell activation, to mediate blast reduction, to drive T cell expansion, to induce T cell activation and to cause the redirected killing of target cancer cells.

II. Comparative Non-Sequence-Optimized CD123 x CD3 Bi-Specific Diabody, "DART-B"

[00112] DART-B is a non-sequence-optimized CD123 x CD3 bi-specific diabody having a gross structure that is similar to that of DART-A. The first polypeptide chain of DART-B will comprise, 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}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}), an intervening Linker 2, an E-coil Domain, and a C-terminus. The VL_{CD3} Domain of the first polypeptide chain of DART-B has the sequence (SEQ ID NO:23):

```

DIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSK
VASGVPIRFSGSGSGTSLTSSMEAEADAATYYCQWSSNPLTFGAGTKLE
LK

```

The VH_{CD123} Domain of the first polypeptide chain of DART-B has the sequence (SEQ ID NO:28):

```

QVQLVQSGAELKKPGASVKVSKASGYTFDYYMKWVRQAPGQGLEWIGDII
PSNGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSEDVAVYYCARSHLLRA
SWFAYWGQGLTVTVSS

```

[00113] Thus, DART-B Chain 1 is composed of: SEQ ID NO:23 — SEQ ID NO:29 — SEQ ID NO:28 — SEQ ID NO:30 — SEQ ID NO:34. The sequence of the first polypeptide chain of DART-B is (SEQ ID NO:5):

```

DIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSK
VASGVPIRFSGSGSGTSLTSSMEAEADAATYYCQWSSNPLTFGAGTKLE
LKGSGSGGGQVQLVQSGAELKKPGASVKVSKASGYTFDYYMKWVRQAPG
QGLEWIGDIIPSNAGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSEDVAVY

```

YCARSHLLRASWFAYWGQGLTVTVSSGGCGGGEVAALEKEVAALEKEVAALE
KEVAALEK

[00114] A DART-B Chain 1 encoding polynucleotide is **SEQ ID NO:6**:

gacattcagctgacccagctctccagcaatcatgtctgcatctccaggggaga
aggtcaccatgacctgcagagccagttcaagtgttaagttacatgaactggta
ccagcagaagtcaggcacctcccccaaaagatggatttatgacacatccaaa
gtggcttctgaggtcccttatcgcttcagtgaggcagtggtctgggacctcat
actctctcacaatcagcagcatggaggctgaagatgctgccacttattactg
ccaacagtggagtagtaacccgctcacgttcggtgctgggaccaagctggag
ctgaaaggaggcggtatccggcgggcggaggccaggtgcagctggtgcagtcg
gggctgagctgaagaaacccggagcttccgtgaagggtgtcttgcaaagccag
tggctacaccttcacagactactatatgaagtgggtcaggcaggctccagga
cagggactggaatggatcggcgatatcattccttccaacggggccactttct
acaatcagaagtttaaaggcagggtgactattaccgtggacaaatcaacaag
cactgcttatatggagctgagctccctgcgctctgaagatacagccgtgtac
tattgtgctcggtcacacctgctgagagccagctggtttgcttattggggac
agggcaccctggtgacagtgcttccggaggatgtggcgggtggagaagtggc
cgactggagaaagaggttgctgctttggagaaggaggtcgctgcacttgaa
aaggaggtcgagccctggagaaa

[00115] The second polypeptide chain of DART-B will comprise, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}), an intervening linker peptide (Linker 1) and a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}), an intervening Linker 2, a K-coil Domain, and a C-terminus.

[00116] The VL_{CD123} Domain of the second polypeptide chain of DART-B has the sequence (**SEQ ID NO:27**):

DFVMTQSPDSLAVSLGERVTMSCKSSQSLNLSGNQKNYLTWYQQKPGQPPKL
LIYWASTRESGVDPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPYTF
GGTKLEIK

The VH_{CD3} Domain of the second polypeptide chain of DART-B has the sequence (**SEQ ID NO:24**):

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYIN
PSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHY
CLDYWGQGTTLTVSS

[00117] Thus, DART-B Chain 2 is composed of: **SEQ ID NO:27 — SEQ ID NO:29 — SEQ ID NO:24 — SEQ ID NO:30 — SEQ ID NO:35**. The sequence of the second polypeptide chain of DART-B is (**SEQ ID NO:7**):

```
DFVMTQSPDSLAVSLGERVTMSCKSSQSLNSGNQKNYLTWYQQKPGQPPKL
LIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPYTF
GQGTKLEIKGGGSGGGGDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMH
WVKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLT
SEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGCGGGKVAALKEKVAALKE
KVAALKEKVAALKE
```

[00118] A DART-B Chain 2 encoding polynucleotide is **SEQ ID NO:8**:

```
gacttcgtgatgacacagtctcctgatagtcctggccgtgagtcctgggggagc
gggtgactatgtccttgcaagagctcccagtcactgctgaacagcggaaatca
gaaaaactatctgacctggtaccagcagaagccaggccagccccctaaactg
ctgatctattgggcttcaccagggaatctggcgtgcccagacagattcagcg
gcagcggcagcggcacagatctttaccctgacaatttctagtcctgcaggccga
ggacgtggctgtgtactattgtcagaatgattacagctatccctacactttc
ggccaggggaccaagctggaaattaaaggaggcggatccggcggcggaggcg
atatcaaactgcagcagtcaggggctgaactggcaagacctggggcctcagt
gaagatgtcctgcaagacttctggctacacctttactaggtacacgatgcac
tgggtaaaacagaggcctggacagggctctggaatggattggatacattaatc
ctagccgtggttatactaattacaatcagaagttcaaggacaaggccacatt
gactacagacaaatcctccagcacagcctacatgcaactgagcagcctgaca
tctgaggactctgcagtcctattactgtgcaagatattatgatgatcattact
gccttgactactgggggccaaggcaccactctcacagtcctcctccggaggatg
tggcgggtggaaaagtggccgcactgaaggagaaagttgctgctttgaaagag
aaggtcgccgcacttaaggaaaaggtcgagccctgaaagag
```

III. Modified Variants of Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A)

A. Sequence-Optimized CD123 x CD3 Bi-Specific Diabody Having An Albumin-Binding Domain (DART-A with ABD “w/ABD”)

[00119] In a second embodiment of the invention, the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) will comprise one or more Albumin-Binding Domain (“ABD”) (DART-A with ABD “w/ABD”) on one or both of the polypeptide chains of the diabody.

[00120] As disclosed in WO 2012/018687, in order to improve the *in vivo* pharmacokinetic properties of diabodies, the 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.

[00121] 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.

[00122] Thus, the first polypeptide chain of such a sequence-optimized CD123 x CD3 bi-specific diabody having an Albumin-Binding Domain contains a third linker (Linker 3), which separates the E-coil (or K-coil) of such polypeptide chain from the Albumin-Binding Domain. A preferred sequence for such Linker 3 is **SEQ ID NO:31**: GGGG. A preferred Albumin-Binding Domain (**ABD**) has the sequence (**SEQ ID NO:36**): LAEAKVLANRELDKYGVSDYYKNLIDNAKSAEGVKALIDEILAALP.

[00123] Thus, a preferred first chain of a sequence-optimized CD123 x CD3 bi-specific diabody having an Albumin-Binding Domain has the sequence (**SEQ ID NO:9**):

QAVVTQEPSTLVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGG
TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
KLTVLGGGGSGGGGEVQLVQSGAELKKPGASVKVSCKASGYTFTDYMKWVR
QAPGQGLEWIGDIIPSNGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSED
TAVYYCARSHLLRASWFAYWGQGLTVTVSSGGCGGGEVAALEKEVAALEKEV
AALEKEVAALEKGGGSLAEAKVLANRELDKYGVSDYYKNLIDNAKSAEGVKA
LIDEILAALP

[00124] A sequence-optimized CD123 x CD3 diabody having an Albumin-Binding Domain is composed of: **SEQ ID NO:21** — **SEQ ID NO:29** — **SEQ ID NO:26** — **SEQ ID NO:30** — **SEQ ID NO:34** — **SEQ ID NO:31** — **SEQ ID NO:36**. A

polynucleotide encoding such a sequence-optimized CD123 x CD3 diabody having an Albumin-Binding Domain derivative is **SEQ ID NO:10**:

```
caggctgtggtgactcaggagccttcactgaccgtgtccccaggcggaactg
tgaccctgacatgcagatccagcacaggcgagtgaccacatctaactacgc
caattgggtgcagcagaagccaggacaggcaccaaggggctgatcggggtg
acaaacaaaagggtccctggaccctgcacggttttctggaagtctgctgg
gcggaaggccgctctgactattaccggggcacaggccgaggacgaagccga
ttactattgtgctctgtggtatagcaatctgtgggtgttcgggggtggcaca
aaactgactgtgctgggaggggtggatccggcggcgaggcgaggtgcagc
tgggtgcagtcgggggtgagctgaagaaacccggagcttccgtgaaggtgtc
ttgcaaagccagtggtacaccttcacagactactatatgaagtgggtcagg
caggctccaggacagggactggaatggatcggcgatatcattccttccaacg
gggccactttctacaatcagaagtttaaaggcagggtgactattaccgtgga
caaatcaacaagcactgcttatatggagctgagctccctgcgctctgaagat
acagccgtgtactattgtgctcggtcacacctgctgagagccagctggtttg
cttattggggacagggcaccttggtgacagtgtcttccggaggatgtggcgg
tggagaagtggccgactggagaaagaggttgctgctttggagaaggaggtc
gctgcacttgaaaaggaggtcgcagccctggagaaaggcgggcgggtctctgg
ccgaagcaaaaagtgtggtgccaaccgcgaactggataaatatggcgtgagcga
ttattataagaacctgattgacaacgcaaatccgcggaaggcgtgaaagca
ctgattgatgaaattctggccgcctgcct
```

[00125] The second polypeptide chain of such a sequence-optimized CD123 x CD3 diabody having an Albumin-Binding Domain has the sequence described above (**SEQ ID NO:3**) and is encoded by a polynucleotide having the sequence of **SEQ ID NO:4**.

B. Sequence-Optimized CD123 x CD3 Bi-Specific Diabodies Having An IgG Fc Domain (DART-A with Fc “w/Fc”)

[00126] In a third embodiment, the invention provides a sequence-optimized CD123 x CD3 bi-specific diabody composed of three polypeptide chains and possessing an IgG Fc Domain (DART-A with Fc “w/Fc” Version 1 and Version 2) (**Figure 3A-3B**).

[00127] In order to form such IgG Fc Domain, the first and third polypeptide chain of the diabodies contain, in the N-terminal to C-terminal direction, a cysteine-containing peptide, (most preferably, Peptide 1 having the amino acid sequence (**SEQ ID NO:55**): DKTHTCPPCP), some or all of the CH2 Domain and/or some or all of the CH3 Domain of a complete immunoglobulin Fc Domain, and a C-terminus. The some or all of the CH2 Domain and/or the some or all of the CH3 Domain associate to form the immunoglobulin Fc Domain of the bi-specific monovalent Fc Domain-containing diabodies of the present invention. The first and second polypeptide

chains of the bi-specific monovalent Fc diabodies of the present invention are covalently bonded to one another, for example by disulfide bonding of cysteine residues located within the cysteine-containing peptide of the polypeptide chains.

[00128] The CH2 and/or CH3 Domains of the first and third polypeptides need not be identical, and advantageously are modified to foster complexing between the two polypeptides. For example, an amino acid substitution (preferably a substitution with an amino acid comprising a bulky side group forming a 'knob', *e.g.*, tryptophan) can be introduced into the CH2 or CH3 Domain such that steric interference will prevent interaction with a similarly mutated domain and will obligate the mutated domain to pair with a domain into which a complementary, or accommodating mutation has been engineered, *i.e.*, 'the hole' (*e.g.*, a substitution with glycine). Such sets of mutations can be engineered into any pair of polypeptides comprising the bi-specific monovalent Fc diabody molecule, and further, engineered into any portion of the polypeptides chains of said pair. Methods of protein engineering to favor heterodimerization over homodimerization are well known in the art, in particular with respect to the engineering of immunoglobulin-like molecules, and are encompassed herein (see *e.g.*, Ridgway *et al.* (1996) "'Knobs-Into-Holes' Engineering Of Antibody CH3 Domains For Heavy Chain Heterodimerization," Protein Engr. 9:617-621, Atwell *et al.* (1997) "Stable Heterodimers From Remodeling The Domain Interface Of A Homodimer Using A Phage Display Library," J. Mol. Biol. 270: 26-35, and Xie *et al.* (2005) "A New Format Of Bispecific Antibody: Highly Efficient Heterodimerization, Expression And Tumor Cell Lysis," J. Immunol. Methods 296:95-101; each of which is hereby incorporated herein by reference in its entirety). Preferably the 'knob' is engineered into the CH2-CH3 Domains of the first polypeptide chain and the 'hole' is engineered into the CH2-CH3 Domains of the third polypeptide chain. Thus, the 'knob' will help in preventing the first polypeptide chain from homodimerizing via its CH2 and/or CH3 Domains. As the third polypeptide chain preferably contains the 'hole' substitution it will heterodimerize with the first polypeptide chain as well as homodimerize with itself. A preferred knob is created by modifying 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.

[00129] A preferred sequence for the CH2 and CH3 Domains of an antibody Fc Domain present in the first polypeptide chain is (**SEQ ID NO:56**):

```
APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQ
PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMEALHNHYTQ
KSLSLSPGK
```

[00130] A preferred sequence for the CH2 and CH3 Domains of an antibody Fc Domain present in the third polypeptide chain is (**SEQ ID NO:11**):

```
APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQ
PENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQGNVVFSCSVMEALHNRYTQ
KSLSLSPGK
```

C. DART-A w/Fc Version 1 Construct

[00131] In order to illustrate such Fc diabodies, the invention provides a DART-A w/Fc version 1 construct. The first polypeptide of the DART-A w/Fc version 1 construct comprises, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}), a Linker 2, an E-coil Domain, a Linker 5, Peptide 1, a polypeptide that contains the CH2 and CH3 Domains of an Fc Domain and a C-terminus. A preferred Linker 5 has the sequence (**SEQ ID NO:32**): GGG. A preferred polypeptide that contains the CH2 and CH3 Domains of an Fc Domain has the sequence (**SEQ ID NO:37**):

APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
 EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
 TISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQ
 PENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVVFSCSVMHEALHNHYTQ
 KSLSLSPGK

[00132] Thus, the first polypeptide of such a DART-A w/Fc version 1 construct is composed of: **SEQ ID NO:25 — SEQ ID NO:29 — SEQ ID NO:22 — SEQ ID NO:30 — SEQ ID NO:34 — SEQ ID NO:32 — SEQ ID NO:55 — SEQ ID NO:37.**

[00133] A preferred sequence of the first polypeptide of such a DART-A w/Fc version 1 construct has the sequence (**SEQ ID NO:13**):

DFVMTQSPDSLAVSLGERVTMSCKSSQSLNLSGNQKNYLTWYQQKPGQPPKLL
 LIYWASTRESGVDPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPYTF
 GQGTKLEIKGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFTSTYAMN
 WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNS
 LKTEDTAVYYCVRHGNFGNSYVSWFAYWGQGLTVTVSSGGCGGGEVAALEKE
 VAALEKEVAALEKEVAALEKGGGDKTHTCPPCPAPEAAGGPSVFLFPPKPKD
 TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
 VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP
 SREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFF
 LYSKLTVDKSRWQQGNVVFSCSVMHEALHNHYTQKSLSLSPGK

[00134] A preferred polynucleotide encoding such a polypeptide is (**SEQ ID NO:14**):

gacttcgtgatgacacagtctcctgatagctctggccgtgagtctgggggagc
 ggggtgactatgtcttgcaagagctcccagtcactgctgaacagcggaatca
 gaaaaactatctgacctggtaccagcagaagccaggccagccccctaaactg
 ctgatctattgggcttccaccagggaatctggcgtgcccagacagattcagcg
 gcagcggcagcggcacagattttaccctgacaatttctagctctgcaggccga
 ggacgtggctgtgtactattgtcagaatgattacagctatccctacactttc
 ggccaggggaccaagctggaaattaaaggaggcggtatccggcgggcgaggcg
 aggtgcagctggtggagtctgggggaggcttggtccagcctggagggtccct
 gagactctcctgtgcagcctctggattcaccttcagcacatacgtatgaat
 tgggtccgccaggctccagggaaggggctggagtgggttggaaggatcaggt
 ccaagtacaacaattatgcaacctactatgccgactctgtgaaggatagatt
 caccatctcaagagatgattcaaagaactcactgtatctgcaaatgaacagc
 ctgaaaaccgaggacacggccgtgtattactgtgtgagacacggtaacttcg
 gcaattcttacgtgtcttggtttgcttattggggacaggggacactggtgac
 tgtgtcttccggaggatgtggcggtggagaagtggccgactggagaaagag
 gttgctgctttggagaaggaggtcgctgcacttgaaaaggaggtcgagccc
 tggagaaaggcgggcggggacaaaactcacacatgcccaccgtgcccagcacc
 tgaagccgcggggggaccgtcagctcttctcttccccccaaaacccaaggac
 accctcatgatctcccgaccctgaggtcacatgcgtggtggtggacgtga
 gccacgaagaccctgaggtcaagttcaactggtacgtggacggcggtgaggt

gcataatgccaaagacaaagccgcgggaggagcagtacaacagcacgtaccgt
 gtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagt
 acaagtgaaggtctccaacaaagccctcccagccccatcgagaaaaccat
 ctccaaagccaaagggcagccccgagaaccacaggtgtacacctgccccca
 tcccgggaggagatgaccaagaaccaggtcagcctgtggtgcctggtcaaag
 gcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccgga
 gaacaactacaagaccacgcctcccgtgctggactccgacggctccttcttc
 ctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtct
 tctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagag
 cctctccctgtctccgggtaaa

[00135] The second chain of such a DART-A w/Fc version 1 construct will comprise, 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}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}), a Linker 2, a K-coil Domain, and a C-terminus. Thus, the second polypeptide of such a DART-A w/Fc version 1 construct is composed of: **SEQ ID NO:21 — SEQ ID NO:29 — SEQ ID NO:26 — SEQ ID NO:30 — SEQ ID NO:35.**

Such a polypeptide has the sequence (**SEQ ID NO:15**):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGG
 TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
 KLTVLGGGSGGGGEVQLVQSGAELKKPGASVKVSCKASGYTFTDYMKWVR
 QAPGQGLEWIGDIIPSNGATFYNQKFKGRVTITVDKSTSTAYMELSSLRSED
 TAVYYCARSHLLRASWFAYWGQGLTVTVSSGGCGGGKVAALKEKVAALKEKV
 AALKEKVAALKE

[00136] A preferred polynucleotide encoding such a polypeptide has the sequence (**SEQ ID NO:16**):

caggctgtggtgactcaggagccttactgaccgtgtccccaggcggaaactg
 tgacctgacatgcagatccagcacaggcgcagtgaccacatctaactacgc
 caattgggtgcagcagaagccaggacaggcaccaaggggcctgatcgggggt
 acaaacaaaagggtccctggacccctgcacggttttctggaagtctgctgg
 gcggaaaggccgctctgactattaccggggcacaggccgaggacgaagccga
 ttactattgtgctctgtggtatagcaatctgtgggtgttcgggggtggcaca
 aaactgactgtgctgggagggggtggatccggcggcggaggcgaggtgcagc
 tgggtgcagtcgggggtgagctgaagaaaccggagcttccgtgaaggtgtc
 ttgcaaagccagtggttacaccttcacagactactatatgaagtgggtcagg
 caggctccaggacagggactggaatggatcgggcatatcattccttccaacg
 gggccactttctacaatcagaagtttaaaggcagggtgactattaccgtgga
 caaatcaacaagcactgcttatatggagctgagctccctgcgctctgaagat
 acagccgtgtactattgtgctcggtcacacctgctgagagccagctggtttg
 ctattggggacagggcaccctggtgacagtgcttccggaggatgtggcgg
 tggaaaagtggccgcactgaaggagaaagtgtgctgctttgaaagagaaggtc
 gccgcacttaaggaaaaggtcgcagccctgaaagag

[00137] The third polypeptide chain of such a DART-A w/Fc version 1 will comprise the CH2 and CH3 Domains of an IgG Fc Domain. A preferred polypeptide that is composed of Peptide 1 (SEQ ID NO:55) and the CH2 and CH3 Domains of an Fc Domain (SEQ ID NO:11) and has the sequence of SEQ ID NO:54:

```
DKTHTCPCPCPAPEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVVS
NKALPAPIEKTIISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQGNVFSQVSM
HEALHNRYSYTKSLSLSLSPGK
```

[00138] A preferred polynucleotide that encodes such a polypeptide has the sequence (SEQ ID NO:12):

```
gacaaaactcacacatgccaccgtgccagcacctgaagccgcgggggggac
cgtcagtccttctcttccccccaaaacccaaggacaccctcatgatctcccg
gacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgag
gtcaagttcaactggtacgtggacggcggtggaggtgcataatgccaaagacaa
agccgcggggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcac
cgtcctgcaccaggactggctgaatggcaaggagtacaagtgaaggtctcc
aacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggc
agccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgac
caagaaccaggtcagcctgagttgcgagtcgcaaggcttctatcccagcgac
atcgccgtggagtgggagagcaatgggcagccggagaacaactacaagacca
cgctcccggtgctggactccgacggctccttcttctcctcgtcagcaagctcac
cgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatg
catgaggctctgcacaaccgctacacgcagaagagcctctccctgtctccgg
gtaaa
```

D. DART-A w/Fc Version 2 Construct

[00139] As a second example of such a DART-A w/Fc diabody, the invention provides a three chain diabody, “DART-A w/Fc Diabody Version 2” (**Figure 3B**).

[00140] The first polypeptide of such a DART-A w/Fc version 2 construct comprises, in the N-terminal to C-terminal direction, an N-terminus, a peptide linker (Peptide 1), a polypeptide that contains the CH2 and CH3 Domains of an Fc Domain linked (via a Linker 4) to the VL domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}), a Linker 2, a K-coil Domain, and a C-terminus.

[00141] A preferred polypeptide that contains the CH2 and CH3 Domains of an Fc Domain has the sequence (SEQ ID NO:37):

```
APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQ
PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHEALHNHYTQ
KSLSLSPGK
```

[00142] "Linker 4" will preferably comprise the amino acid sequence (SEQ ID NO:57): APSSS. A preferred "Linker 4" has the sequence (SEQ ID NO:33): APSSSPME. Thus, the first polypeptide of such a DART-A w/Fc version 2 construct is composed of: SEQ ID NO:55 — SEQ ID NO:37 — SEQ ID NO:33 — SEQ ID NO:25 — SEQ ID NO:29 — SEQ ID NO:22 — SEQ ID NO:30 — SEQ ID NO:35. A polypeptide having such a sequence is (SEQ ID NO:17):

```
DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTIISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVM
HEALHNHYTQKSLSLSPGKAPSSSPMEDFVMTQSPDSLAVSLGERVTMSCKS
QSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCQNDYSYPYTFGGQGTKLEIKGGGSGGGGGEVQLVESG
GGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKYNNYAT
YYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVSWF
AYWGQGTLLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKE
```

[00143] A preferred polynucleotide encoding such a polypeptide has the sequence (SEQ ID NO:18):

```
gacaaaactcacacatgccaccgtgccagcacctgaagccgcggggggac
cgtcagtccttctcttccccccaaaacccaaggacaccctcatgatctcccg
gacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgag
gtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaaagaaa
agccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcac
cgtcctgcaccaggactggctgaatggcaaggagtacaagtgaaggtctcc
aacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggc
agccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgac
caagaaccaggtcagcctgtggtgcctggtcaaaggcttctatcccagcgac
atcgccgtggagtgaggagagcaatgggcagccggagagaacaactacaagacca
cgctcccgtgctggactccgacggctccttcttctcttacagcaagctcac
cgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatg
catgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgg
gtaaagccccttccagctcccctatggaagacttcgtgatgacacagctctcc
tgatagtctggccgtgagctctgggggagcgggtgactatgtcttgcaagagc
tcccagtcactgctgaacagcggaaatcagaaaaactatctgacctggtacc
agcagaagccaggccagccccctaactgctgatctattgggcttccaccag
```

ggaatctggcgtgcccgcagagattcagcggcagcggcagcggcacagatttt
 accctgacaattttctagtctgcaggccgaggacgtggctgtgtactattgtc
 agaatgattacagctatccctacacttttcggccaggggaccaagctggaaat
 taaaggaggcggatccggcggcggaggcgaggtgcagctgggtggagtctggg
 ggaggcttggtccagcctggagggtccctgagactctcctgtgcagcctctg
 gattcaccttcagcacatacgtatgaattgggtccgccaggctccaggga
 ggggctggagtgggttggaaggatcaggtccaagtacaacaattatgcaacc
 tactatgccgactctgtgaaggatagattcaccatctcaagagatgattcaa
 agaactcactgtatctgcaaataaacagcctgaaaaccgaggacacggccgt
 gtattactgtgtgagacacggtaacttcggcaattcttacgtgtcttggttt
 gcttattggggacaggggacactgggtgactgtgtcttccggaggatgtggcg
 gtggaaaagtggccgcactgaaggagaaagttgctgctttgaaagagaaggt
 cgccgcacttaaggaaaaggtcgcagccctgaaagag

[00144] The second polypeptide chain of such a DART-A w/Fc version 2 construct comprises, in the N-terminal to C-terminal direction, the VL domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}), an intervening linker peptide (Linker 1) and a VH domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}). This portion of the molecule is linked (via Linker 2) to an E-coil Domain. Thus, the third polypeptide of such a DART-A w/Fc version 2 construct is composed of: **SEQ ID NO:21 — SEQ ID NO:29 — SEQ ID NO:26 — SEQ ID NO:30 — SEQ ID NO:34**. A polypeptide having such a sequence is (**SEQ ID NO:1**), and is preferably encoded by a polynucleotide having the sequence of **SEQ ID NO:2**.

[00145] The third polypeptide chain will comprise the CH2 and CH3 Domains of an IgG Fc Domain. A preferred polypeptide is composed of Peptide 1 (**SEQ ID NO:55**) and the CH2 and CH3 Domains of an Fc Domain (**SEQ ID NO:11**) and has the sequence of **SEQ ID NO:54**.

[00146] In order to assess the activity of the above-mentioned CD123 x CD3 bi-specific diabodies (DART-A, DART-A w/ABD, DART-A w/Fc, DART-B), a control bi-specific diabody (Control DART) was produced. The Control DART is capable of simultaneously binding to FITC and CD3. Its two polypeptide chains have the following respective sequences:

Control DART Chain 1 (SEQ ID NO:19):

DVVMQTQTPFSLPVSLGDQASISCRSSQSLVHSNGNTYLRWYLQKPGQSPKVL
 IYKVSNRFGVDPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPWTFG
 GGTKLEIKGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNW
 VRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSL

KTEDTAVYYCVRHGNFGNSYVSWFAYWGQGTTLVTVSSGGCGGGEVAALEKEV
AALEKEVAALEKEVAALEK

Control DART Chain 2 (SEQ ID NO:20):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQKPGQAPRGLIGG
TNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFGGGT
KLTVLGGGGSGGGGEVKLDETGGGLVQPGRPMKLSCVASGFTFSDYWMNWVR
QSPEKGLEWVAQIRNKPYNYETYYSDSVKGRFTISRDDSKSSVYLQMNLRV
EDMGIYYCTGSYYGMDYWGQGTSTVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKE

IV. Pharmaceutical Compositions

[00147] 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 sequence-optimized CD123 x CD3 bi-specific 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 sequence-optimized CD123 x CD3 bi-specific diabody of the invention and a pharmaceutically acceptable carrier.

[00148] The invention also encompasses pharmaceutical compositions comprising sequence-optimized CD123 x CD3 bi-specific diabodies of the invention, and a second therapeutic antibody (*e.g.*, tumor specific monoclonal antibody) that is specific for a particular cancer antigen, and a pharmaceutically acceptable carrier.

[00149] 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.

[00150] 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.

[00151] 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.*

[00152] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with sequence-optimized CD123 x CD3 bi-specific diabodies of the invention 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.

[00153] The present invention provides kits that can be used in the above methods. A kit can comprise sequence-optimized CD123 x CD3 bi-specific diabodies 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.

V. Methods of Administration

[00154] 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.

[00155] 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.*

[00156] 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 sequence-optimized CD123 x CD3 bi-specific 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.

[00157] The invention also provides that the sequence-optimized CD123 x CD3 bi-specific diabodies 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 sequence-optimized CD123 x CD3 bi-specific 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 sequence-optimized CD123 x CD3 bi-specific 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.

[00158] The lyophilized sequence-optimized CD123 x CD3 bi-specific diabodies of the invention should be stored at between 2 and 8°C in their original container and the

molecules should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, sequence-optimized CD123 x CD3 bi-specific 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 sequence-optimized CD123 x CD3 bi-specific 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.

[00159] 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.

[00160] For sequence-optimized CD123 x CD3 bi-specific 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.

[00161] In another embodiment, the patient is administered a treatment regimen comprising one or more doses of such prophylactically or therapeutically effective amount of the sequence-optimized CD123 x CD3 bi-specific 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 sequence-optimized CD123 x CD3 bi-specific 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 sequence-optimized CD123 x CD3 bi-specific 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.

[00162] 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 sequence-optimized CD123 x CD3 bi-specific diabodies encompassed by the invention is achieved.

[00163] **Table 1** provides 5 examples of different dosing regimens described above for a typical course of treatment.

Table 1						
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

[00164] The dosage and frequency of administration of sequence-optimized CD123 x CD3 bi-specific diabodies of the invention may be reduced or altered by enhancing

uptake and tissue penetration of the sequence-optimized CD123 x CD3 bi-specific diabodies by modifications such as, for example, lipidation.

[00165] The dosage of the sequence-optimized CD123 x CD3 bi-specific diabodies of the invention administered to a patient may be calculated for use as a single agent therapy. Alternatively, the sequence-optimized CD123 x CD3 bi-specific 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.

[00166] 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.

[00167] 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; see generally *ibid.*).

[00168] 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 sequence-optimized CD123 x CD3 bi-specific 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).

[00169] 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.

[00170] Where the composition of the invention is a nucleic acid encoding a sequence-optimized CD123 x CD3 bi-specific diabody of the invention, the nucleic acid can be administered *in vivo* to promote expression of its encoded sequence-optimized CD123 x CD3 bi-specific 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.

[00171] Treatment of a subject with a therapeutically or prophylactically effective amount of sequence-optimized CD123 x CD3 bi-specific diabodies of the invention can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with sequence-optimized CD123 x CD3 bi-specific diabodies of the invention one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. 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.

VI. Uses of the Compositions of the Invention

[00172] The sequence-optimized CD123 x CD3 bi-specific diabodies of the present invention have the ability to treat any disease or condition associated with or characterized by the expression of CD123. 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), 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 (see **Example 2**); Autoimmune Lupus (SLE), allergy, asthma and

rheumatoid arthritis. The bi-specific diabodies of the present invention may additionally be used in the manufacture of medicaments for the treatment of the above-described conditions.

[00173] 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

Construction Of CD123 x CD3 Bi-Specific Diabodies And Control Protein

[00174] Table 2 contains a list of bi-specific diabodies that were expressed and purified. Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) and non-sequence-optimized CD123 x CD3 bi-specific diabody (DART-B) are capable of simultaneously binding to CD123 and CD3. The control bi-specific diabody (Control DART) is capable of simultaneously binding to FITC and CD3. The bi-specific diabodies are heterodimers or heterotrimers of the recited amino acid sequences. Methods for forming bi-specific diabodies are provided in WO 2006/113665, WO 2008/157379, WO 2010/080538, WO 2012/018687, WO 2012/162068 and WO 2012/162067.

Table 2		
Bi-Specific Diabodies	Polypeptide Chain Amino Acid Sequences	Nucleic Acid Encoding Sequences
Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) (Binds to CD3 at epitope 1)	SEQ ID NO:1 SEQ ID NO:3	SEQ ID NO:2 SEQ ID NO:4
Non-Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-B) (Binds to CD3 at epitope 2)	SEQ ID NO:5 SEQ ID NO:7	SEQ ID NO:6 SEQ ID NO:8
Sequence-Optimized CD123 x CD3 Bi-Specific Diabody Having an Albumin-Binding Domain (DART-A w/ABD) (Binds to CD3 at epitope 1) Comprises an Albumin-Binding Domain (ABD) for extension of half- life <i>in vivo</i>	SEQ ID NO:9 SEQ ID NO:3	SEQ ID NO:10 SEQ ID NO:4

Table 2		
Bi-Specific Diabodies	Polypeptide Chain Amino Acid Sequences	Nucleic Acid Encoding Sequences
Sequence-Optimized CD123 x CD3 Bi-Specific Diabody Having an IgG Fc Domain Version 1 (DART-A w/Fc version 1) (Binds to CD3 at epitope 1) Comprises an Fc Domain for extension of half-life <i>in vivo</i>	SEQ ID NO:54 SEQ ID NO:13 SEQ ID NO:15	SEQ ID NO:12 SEQ ID NO:14 SEQ ID NO:16
Sequence-Optimized CD123 x CD3 Bi-Specific Diabody Having an IgG Fc Domain Version 2 (DART-A w/Fc version 2) (Binds to CD3 at epitope 1) Comprises an Fc Domain for extension of half-life <i>in vivo</i>	SEQ ID NO:54 SEQ ID NO:17 SEQ ID NO:1	SEQ ID NO:12 SEQ ID NO:18 SEQ ID NO:2
Control Bi-Specific Diabody (Control DART (or Control DART) (Binds to CD3 at epitope 1) (Binds to an irrelevant target - FITC)	SEQ ID NO:19 SEQ ID NO:20	

Example 2

Antibody Labeling Of Target Cells For Quantitative FACS (QFACS)

[00175] A total of 10^6 target cells were harvested from the culture, resuspended in 10% human AB serum in FACS buffer (PBS + 1% BSA+ 0.1% NaAzide) and incubated for 5 min for blocking Fc receptors. Antibody labeling of microspheres with different antibody binding capacities (Quantum™ Simply Cellular® (QSC), Bangs Laboratories, Inc., Fishers, IN) and target cells were labeled with anti-CD123 PE antibody (BD Biosciences) according to the manufacturer's instructions. Briefly, one drop of each QSC microsphere was added to a 5 mL polypropylene tube and PE labeled-anti-CD123 antibody was added at 1 μ g/mL concentration to both target cells and microspheres. Tubes were incubated in the dark for 30 minutes at 4 °C. Cells and microspheres were washed by adding 2mL FACS buffer and centrifuging at 2500 x G for 5 minutes. One drop of the blank microsphere population was added after washing. Microspheres were analyzed first on the flow cytometer to set the test-specific instrument settings (PMT voltages and compensation). Using the same instrument settings, geometric mean fluorescence values of microspheres and target

cells were recorded. A standard curve of antibody binding sites on microsphere populations was generated from geometric mean fluorescence of microsphere populations. Antibody binding sites on target cells were calculated based on geometric mean fluorescence of target cells using the standard curve generated for microspheres in QuickCal spreadsheet (Bangs Laboratories).

[00176] To determine suitable target cell lines for evaluating CD123 x CD3 bi-specific diabodies, CD123 surface expression levels on target lines Kasumi-3 (AML), Molm13 (AML), THP-1 (AML), TF-1 (Erythroleukemia), and RS4-11 (ALL) were evaluated by quantitative FACS (QFACS). Absolute numbers of CD123 antibody binding sites on the cell surface were calculated using a QFACS kit. As shown in **Table 3**, the absolute number of CD123 antibody binding sites on cell lines were in the order of Kasumi-3 (high) > Molm13 (medium) > THP-1 (medium) > TF-1 (medium low) > RS4-11 (low). The three highest expressing cell lines were the AML cell lines: Kasumi-3, MOLM13, and THP-1. The non-AML cell lines: TF-1 and RS4-11 had medium-low / low expression of CD123, respectively.

Table 3	
Target Cell Line	CD123 Surface Expression (Antibody Binding Sites)
Kasumi-3	118620
Molm13	27311
THP-1	58316
TF-1	14163
RS4-11	957
A498	Negative
HT29	Negative

Example 3

CTL Cytotoxicity Assay (LDH Release Assay)

[00177] Adherent target tumor cells were detached with 0.25% Trypsin-EDTA solution and collected by centrifugation at 1000 rpm for 5 min. Suspension target cell lines were harvested from the culture, washed with assay medium. The cell concentration and viability were measured by Trypan Blue exclusion using a Beckman Coulter Vi-Cell counter. The target cells were diluted to 4×10^5 cells/mL in

the assay medium. 50 μ L of the diluted cell suspension was added to a 96-well U-bottom cell culture treated plate (BD Falcon Cat#353077).

[00178] Three sets of controls to measure target maximal release (**MR**), antibody independent cellular cytotoxicity (**AICC**) and target cell spontaneous release (**SR**) were set up as follows:

- 1) **MR**: 200 μ L assay medium without CD123 x CD3 bi-specific diabodies and 50 μ L target cells; detergent added at the end of the experiment to determine the maximal LDH release.
- 2) **AICC**: 50 μ L assay medium without CD123 x CD3 bi-specific diabodies, 50 μ L target cells and 100 μ L T cells.
- 3) **SR**: 150 μ L medium without CD123 x CD3 bi-specific diabodies and 50 μ L target cells.

[00179] CD123 x CD3 bi-specific diabodies (DART-A, DART-A w/ABD and DART-B) and controls were initially diluted to a concentration of 4 μ g/mL, and serial dilutions were then prepared down to a final concentration of 0.00004 ng/mL (*i.e.*, 40 fg/mL). 50 μ L of dilutions were added to the plate containing 50 μ L target cells/well.

[00180] Purified T cells were washed once with assay medium and resuspended in assay medium at cell density of 2×10^6 cells/mL. 2×10^5 T cells in 100 μ L were added to each well, for a final effector-to-target cell (E:T) ratio of 10:1. Plates were incubated for approximately 18hr at 37°C in 5% CO₂.

[00181] Following incubation, 25 μ L of 10x lysis solution (Promega # G182A) or 1 mg/mL digitonin was added to the maximum release control wells, mixed by pipetting 3 times and plates were incubated for 10 min to completely lyse the target cells. The plates were centrifuged at 1200 rpm for 5 minutes and 50 μ L of supernatant were transferred from each assay plate well to a flat bottom ELISA plate and 50 μ L of LDH substrate solution (Promega #G1780) was added to each well. Plates were incubated for 10-20 min at room temperature (RT) in the dark, then 50 μ L of Stop solution was added. The optical density (O.D.) was measured at 490 nm within 1 hour on a Victor2 Multilabel plate reader (Perkin Elmer #1420-014). The percent cytotoxicity

was calculated as described below and dose-response curves were generated using GraphPad PRISM5® software.

[00182] Specific cell lysis was calculated from O.D. data using the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times (\text{OD of Sample} - \text{OD of AICC}) / (\text{OD of MR} - \text{OD of SR})$$

Redirected Killing of Target Cell Lines with Different Levels of CD123 Surface Levels:

[00183] The CD123 x CD3 bi-specific diabodies exhibited a potent redirected killing ability with concentrations required to achieve 50% of maximal activity (EC50s) in sub-ng/mL range, regardless of CD3 epitope binding specificity (DART-A versus DART-B) in target cell lines with high CD123 expression, Kasumi-3 (EC50=0.01 ng/mL) (**Figure 4 Panel D**), medium CD123-expression, Molm13 (EC50=0.18 ng/mL) and THP-1 (EC50=0.24 ng/mL) (**Figure 4, Panel C and E**, respectively) and medium low or low CD123 expression, TF-1 (EC50=0.46 ng/mL) and RS4-11 (EC50=0.5 ng/mL) (**Figure 4, Panel B and A**, respectively). Similarly, CD123 x CD3 bi-specific molecules mediated redirected killing was also observed with multiple target cell lines with T cells from different donors and no redirected killing activity was observed in cell lines that do not express CD123. Results are summarized in **Table 4**.

Table 4			
Target cell line	CD123 surface expression (antibody binding sites)	EC50 of Sequence-optimized CD123 x CD3 bi-specific diabodies (ng/mL) E:T=10:1	Max % killing
Kasumi-3	118620	0.01	94
Molm13	27311	0.18	43
THP-1	58316	0.24	40
TF-1	14163	0.46	46
RS4-11	957	0.5	60
A498	Negative	No activity	No activity
HT29	Negative	No activity	No activity

[00184] Should it be necessary to replicate this example it will be appreciated that one of skill in the art may, within reasonable and acceptable limits, vary the above-

described protocol in a manner appropriate for replicating the described results. Thus, the exemplified protocol is not intended to be adhered to in a precisely rigid manner.

Example 4

T Cell Activation During Redirected Killing By Sequence-Optimized CD123 x CD3 Bi-Specific Diabodies (DART-A, DART-A w/ABD and DART-A w/Fc)

[00185] The sequence-optimized CD123 x CD3 bi-specific diabodies exhibited a potent redirected killing ability regardless of the presence or absence of half-life extension technology (DART-A versus DART-A w/ABD versus DART-A w/Fc) in target cell lines with high CD123 expression, Kasumi-3, and medium, THP-1, CD123-expression, (**Figure 5, Panels A and B**, respectively) To characterize T cell activation during sequence-optimized CD123 x CD3 bi-specific diabody mediated redirected killing process, T cells from redirected killing assays were stained for T cell activation marker CD25 and analyzed by FACS. As shown in **Figure 5, Panel D**, CD25 was up-regulated in CD8 T cells in a dose-dependent manner indicating that sequence-optimized CD123 x CD3 bi-specific diabodies induce T cell activation in the process of redirected killing. Conversely, in the absence of target cells there is no activation of CD8 T cells (**Figure 5, Panel C**) indicating the sequence-optimized CD123 x CD3 bi-specific diabodies do not activate T cells in the absence of target cells. Likewise, CD8 T cells are not activated when incubated with target cells and a control bi-specific diabody (Control DART) (**Figure 5, Panel D**) indicating the requirement of cross-linking the T cell and target cell with the sequence-optimized CD123 x CD3 bi-specific diabodies.

Example 5

Intracellular Staining for Granzyme B and Perforin

[00186] To determine the intracellular levels of granzyme B and perforin in T cells, a CTL assay was setup as described above. After approximately 18 h, cells from the assay plate were stained with anti-CD4 and anti-CD8 antibodies by incubating for 30 minutes at 4°C. Following surface staining, cells were incubated in 100 µL fixation and permeabilization buffer (BD BioSciences) for 20 min at 4°C. Cells were washed with permeabilization/wash buffer (BD BioSciences) and incubated in 50 µL of granzyme B and a perforin antibody mixture (prepared in 1X permeabilization/wash

buffer) at 4°C for 30 minutes. Then cells were washed with 250 µL permeabilization/wash buffer and resuspended in permeabilization/wash buffer for FACS acquisition.

Upregulation Of Granzyme B And Perforin By Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) In T Cells During Redirected Killing

[00187] To investigate the possible mechanism for sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) mediated cytotoxicity by T cells, intracellular granzyme B and perforin levels were measured in T cells after the redirected killing. Dose-dependent upregulation of granzyme B and perforin levels in both CD8 and CD4 T cells was observed following incubation of T cells and Kasumi-3 cells with DART-A (**Figure 6, Panel A**). Interestingly, the upregulation was almost two-fold higher in CD8 T cells compared to CD4 T cells (**Figure 6, Panel A**). When the assay was performed in the presence of granzyme B and perforin inhibitors no cell killing was observed. There was no upregulation of granzyme B or perforin in CD8 or CD4 T cells when T cells were incubated with Kasumi-3 target cells and a control bi-specific diabody (Control DART) (**Figure 5, Panel B**). These data indicate that DART-A mediated target cell killing may be mediated through granzyme B and perforin mechanisms.

Example 6

***in vivo* Antitumor Activity Of Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A)**

Isolation of PBMCs and T Cells from Human Whole Blood

[00188] PBMCs from healthy human donors were isolated from whole blood by using Ficoll gradient centrifugation. In brief, whole blood was diluted 1:1 with sterile PBS. Thirty-five mL of the diluted blood was layered onto 15 mL Ficoll-PaqueTM Plus in 50-mL tubes and the tubes were centrifuged at 1400 rpm for 20 min with the brake off. The buffy coat layer between the two phases was collected into a 50 mL tube and washed with 45 mL PBS by centrifuging the tubes at 600 x g (1620 rpm) for 5 min. The supernatant was discarded and the cell pellet was washed once with PBS and viable cell count was determined by Trypan Blue dye exclusion. The PBMCs were resuspended to a final concentration of 2.5×10^6 cells/mL in complete medium

(RPMI 1640, 10%FBS, 2 mM Glutamine, 10mM HEPES, 100 μ /100 μ /mL penicillin/Streptomycin (P/S).

[00189] T cell isolation: Untouched T cells were isolated by negative selection from PBMCs from human whole blood using Dynabeads Untouched Human T Cell isolation kit (Life Technologies) according to manufacturer's instructions. After the isolation, T cells were cultured overnight in RPMI medium with 10% FBS, 1% penicillin/Streptomycin.

Tumor Model

[00190] Human T cells and tumor cells (Molm13 or RS4-11) were combined at a ratio of 1:5 (1×10^6 and 5×10^6 , respectively) and suspended in 200 μ L of sterile saline and injected subcutaneously (SC) on Study Day 0 (SD0). Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) or a control bi-specific diabody (Control DART) were administered intravenously (IV) via tail vein injections in 100 μ L as outlined in **Table 5** (MOLM13) and **Table 6** (RS4-11).

Table 5 Study Design for MOLM13 Model			
Treatment Group	Dose (mg/kg)	Schedule	Number of Animals
Vehicle Control (MOLM-13 cells alone implanted or + T cells)	-	SD0, 1, 2, 3	8
DART-A	0.5	SD0, 1, 2, 3	8
DART-A	0.2	SD0, 1, 2, 3	8
DART-A	0.1	SD0, 1, 2, 3	8
DART-A	0.02	SD0, 1, 2, 3	8
DART-A	0.004	SD0, 1, 2, 3	8
DART-A	0.0008	SD0, 1, 2, 3	8
DART-A	0.00016	SD0, 1, 2, 3	8

Table 6 Study Design for RS4-11 Model			
Treatment Group	Dose (mg/kg)	Schedule	Number of Animals
Vehicle Control (RS4-11 cells alone implanted)	-	SD0, 1, 2, 3	8
Vehicle Control (RS4-11 + T cells implanted)	-	SD0, 1, 2, 3	8
Control DART	0.2	SD0, 1, 2, 3	8
DART-A	0.5	SD0, 1, 2, 3	8
DART-A	0.2	SD0, 1, 2, 3	8
DART-A	0.1	SD0, 1, 2, 3	8
DART-A	0.02	SD0, 1, 2, 3	8
DART-A	0.004	SD0, 1, 2, 3	8

Data Collection and Statistical Analysis:

[00191] Animal weights - Individual animal weights were recorded twice weekly until study completion beginning at the time of tumor cell injection.

[00192] Moribundity/Mortality - Animals were observed twice weekly for general moribundity and daily for mortality. Animal deaths were assessed as drug-related or technical based on factors including gross observation and weight loss; animal deaths were recorded daily.

[00193] Tumor volume - Individual tumor volumes were recorded twice weekly beginning within one week of tumor implantation and continuing until study completion.

$$\text{Tumor Volume (mm}^3\text{)} = \frac{\text{Length (mm)} \times \text{width}^2}{2}$$

[00194] Animals experiencing technical or drug-related deaths were censored from the data calculations.

[00195] Tumor growth inhibition - Tumor growth inhibition (TGI) values were calculated for each group containing treated animals using the formula:

$$1 - \frac{\text{Mean Final Tumor Volume (Treated)} - \text{Mean Initial Tumor Volume (Treated)}}{\text{Mean Final Tumor Volume (Control)} - \text{Mean Initial Tumor Volume (Control)}} \times 100$$

[00196] Animals experiencing a partial or complete response, or animals experiencing technical or drug-related deaths were censored from the TGI calculations. The National Cancer Institute criteria for compound activity is TGI>58% (Corbett *et al.* (2004) *Anticancer Drug Development Guide*; Totowa, NJ: Humana 99-123).

[00197] **Partial/Complete Tumor Response** - Individual mice possessing tumors measuring less than 1mm³ on Day 1 were classified as having partial response (PR) and a percent tumor regression (%TR) value was determined using the formula:

$$1 - \frac{\text{Final Tumor Volume (mm}^3\text{)}}{\text{Initial Tumor Volume (mm}^3\text{)}} \times 100\%$$

[00198] Individual mice lacking palpable tumors were classified as undergoing a complete response (CR).

[00199] **Tumor Volume Statistics** – Statistical analyses were carried out between treated and control groups comparing tumor volumes. For these analyses, a two-way analyses of variance followed by a Bonferroni post-test were employed. All analyses were performed using GraphPad PRISM® software (version 5.02). Weight and tumor data from individual animals experiencing technical or drug-related deaths were censored from analysis. However, tumor data from animals reporting partial or complete responses were included in these calculations.

MOLM13 Results

[00200] The AML cell line MOLM13 was pre-mixed with activated T cells and implanted SC in NOD/SCID gamma (NSG) knockout mice (N = 8/group) on SD0 as detailed above. The MOLM13 tumors in the vehicle-treated group (MOLM13 cells alone or plus T cells) demonstrated a relatively aggressive growth profile *in vivo* (**Figure 7, Panels A and B**). At SD8, the average volume of the tumors in the vehicle-treated group was 129.8 ± 29.5 mm³ and by SD15 the tumors had reached an average volume of 786.4 ± 156.7 mm³. By the end of the experiment on SD18, the tumors had reached an average volume of 1398.8 ± 236.9 mm³.

[00201] Treatment with DART-A was initiated on the same day the tumor cell/T cell mixture was implanted [(SD0)] and proceeded subsequently with daily injections for an additional 7 days for a total of 8 daily injections. The animals were treated with DART-A at 9 dose levels (0.5, 0.2, 0.1, 0.02, and 0.004 mg/kg and 20, 4, 0.8 and 0.16 µg/kg). Results are shown in **Figure 7, Panel A** (0.5, 0.2, 0.1, 0.02, and 0.004 mg/kg) **and Figure 7, Panel B** (20, 4, 0.8 and 0.16 µg/kg). By Study Day 11, the growth of the MOLM13 tumors was significantly inhibited at the 0.16, 0.5, 0.2, 0.1, 0.02, and 0.004 mg/kg dose levels ($p < 0.001$). Moreover, the treatment of the MOLM13 tumor-bearing mice at the 20 and 4 µg/kg dose levels resulted in 8/8 and 7/8 CRs, respectively. By the end of the experiment on SD18, the average volume of the tumors treated with DART-A at the 0.8 – 20 µg/kg) ranged from $713.6.0 \pm 267.4$ to 0 mm³, all of which were significantly smaller than the tumors in the vehicle-treated control group. The TGI values were 100, 94, and 49% for the 20, 4, and 0.8 µg/kg dose groups, respectively. In comparison to the vehicle-treated MOLM13 tumor cell group, the groups that received DART-A at the 20 and 4 µg/kg dose level reached statistical significance by SD15 while the group treated with 0.8 µg/kg reached significance on SD18.

RS4-11 Results

[00202] The ALL cell line RS4-11 was pre-mixed with activated T cells and implanted SC in NOD/SCID gamma knockout mice (N = 8/group) on SD0 as detailed above. The RS4-11 tumors in the vehicle-treated group (RS4-11 cells alone or plus T cells) demonstrated a relatively aggressive growth profile in vivo (**Figure 8**).

[00203] Treatment with DART-A was initiated on the same day the tumor cell/T cell mixture was implanted [(SD0)] and proceeded subsequently with daily injections for an additional 3 days for a total of 4 daily injections. The animals were treated with DART-A at 5 dose levels (0.5, 0.2, 0.1, 0.02, and 0.004 mg/kg). Results are shown in **Figure 8**.

[00204] Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) effectively inhibited the growth of both MOLM13 AML and RS4-11ALL tumors implanted SC in NOD/SCID mice in the context of the Winn model when dosing was

initiated on the day of implantation and continued for 3 or more consecutive days. Based on the criteria established by the National Cancer Institute, DART-A at the 0.1 mg/kg dose level and higher (TGI >58) is considered active in the RS4-11 model and an DART-A dose of 0.004 mg/kg and higher was active in the MOLM13 model. The lower DART-A doses associated with the inhibition of tumor growth in the MOLM13 model compared with the RS4-11 model are consistent with the *in vitro* data demonstrating that MOLM13 cells have a higher level of CD123 expression than RS4-11 cells, which correlated with increased sensitivity to DART-A mediated cytotoxicity in vitro in MOLM13 cells.

[00205] Should it be necessary to replicate this example it will be appreciated that one of skill in the art may, within reasonable and acceptable limits, vary the above-described protocol in a manner appropriate for replicating the described results. Thus, the exemplified protocol is not intended to be adhered to in a precisely rigid manner.

Example 7
CD123 Surface Expression On Leukemic Blast Cells And Stem Cells In Primary Tissue Sample From AML Patient 1

[00206] To define the CD123 expression pattern in AML patient 1 primary samples, cryopreserved primary AML patient bone marrow and PBMC samples were evaluated for CD123 surface expression on leukemic blast cells.

[00207] AML Bone Marrow Sample – Clinical Report

Age: 42

Gender: Female

AML Subtype: M2

Cancer cell percentage based on morphology: 67.5%

Bone marrow immunophenotyping:

CD15=19%, CD33=98.5%, CD38=28.8%, CD45=81.8%, CD64=39.7%,

CD117=42.9%, HLA-DR=17%, CD2=1.8%, CD5=0.53%, CD7=0.2%,

CD10=0.41%, CD19=1.1%, CD20=1.4%, CD22=0.71% CD34=0.82%

CD123 Expression in Leukemic Blast Cells in Bone Marrow Mononucleocytes (BM MNC)

[00208] A total of 0.5×10^6 bone marrow mononucleocytes (BM MNC) and peripheral blood mononucleocytes (PBMC)) from AML patient 1 were evaluated for CD123 expression. The Kasumi-3 cell line was included as a control. Leukemic blast cells were identified using the myeloid marker CD33. As shown in **Figure 9, Panel A**, 87% of the cells from AML bone marrow from patient 1 expressed CD123 and CD33. CD123 expression levels were slightly lower than the CD123 high-expressing Kasumi-3 AML cell line (**Figure 9, Panel B**).

Example 8**Autologous CTL Killing Assay Using AML Patient Primary Specimens**

[00209] Cryopreserved primary AML specimen (bone marrow mononucleocytes (BMNC) and peripheral blood mononucleocytes (PBMC)) from AML patient 1 were thawed in RPMI 1640 with 10% FBS and allowed to recover overnight at 37°C in 5% CO₂. Cells were washed with assay medium (RPMI 1640+10%FBS) and viable cell count was determined by Trypan Blue exclusion. 150,000 cells / well in 150 µL assay medium were added to 96-well U-bottom plate (BD Biosciences). Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) was diluted to 0.1, and 0.01 ng/mL and 50 µL of each dilution was added to each well (final volume = 200 µL). Control bi-specific diabody (Control DART) was diluted to 0.1 ng/mL and 50 µL of each dilution was added to each well (final volume = 200 µL). A separate assay plate was set up for each time point (48, 72, 120 and 144 hours) and plates were incubated at 37 °C in a 5% CO₂ incubator. At each time point, cells were stained with CD4, CD8, CD25, CD45, CD33, and CD123 antibodies. Labeled cells were analyzed in FACS Calibur flow cytometer equipped with CellQuest Pro acquisition software, Version 5.2.1 (BD Biosciences). Data analysis was performed using Flowjo v9.3.3 software (Treestar, Inc). T cell expansion was measured by gating on CD4+ and CD8+ populations and activation was determined by measuring CD25 mean fluorescent intensity (MFI) on the CD4+ and CD8+ -gated populations. Leukemic blast cell population was identified by CD45+CD33+ gating.

Autologous Tumor Cell Depletion, T Cell Expansion And Activation By Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) In Primary Specimens From AML Patient 1

[00210] To determine the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) mediated activity in AML patient 1, patient samples were incubated with 0.1 ng/mL or 0.01 ng/mL of DART-A and percentages of leukemic blast cells and T cells were measured at different time points following the treatment. Leukemic blast cells were identified by CD45+/CD33+ gating. Incubation of primary AML bone marrow samples with DART-A resulted in depletion of the leukemic cell population over time (**Figure 10, Panel A**), accompanied by a concomitant expansion of the residual T cells (**Figure 10, Panel B**) and the induction of T cell activation markers (**Figure 10, Panel C**). In DART-A treated samples, T cells were expanded from around 7 % to around 80% by 120 hours. T cell activation measured by CD25 expression on CD4 and CD8 cells peaked at 72 h and decreased by the 120 h timepoint.

[00211] Should it be necessary to replicate this example it will be appreciated that one of skill in the art may, within reasonable and acceptable limits, vary the above-described protocol in a manner appropriate for replicating the described results. Thus, the exemplified protocol is not intended to be adhered to in a precisely rigid manner.

Example 9
CD123 Surface Expression On Leukemic Blast Cells And Stem Cells In Primary Tissue Sample From ALL Patient

[00212] To define the CD123 expression pattern in ALL patient primary samples, cryopreserved primary ALL patient PBMC sample was evaluated for CD123 surface expression on leukemic blast cells.

CD123 Expression in Leukemic Blast Cells in Peripheral Blood Mononucleocytes (PBMC)

[00213] A total of 0.5×10^6 peripheral blood mononucleocytes (PBMC)) from a healthy donor and an ALL patient were evaluated for CD123 expression. As shown in **Figure 11, Panels E-H**, the vast majority of the cells from ALL bone marrow

expressed CD123. Conversely, as expected in the normal donor B cells are CD123 negative and pDCs and monocytes are CD123 positive (**Figure 11, Panel D**).

[00214] The T cell population was identified in the ALL patient sample by staining the cells for CD4 and CD8. As shown in **Figure 12, Panel B**, only a small fraction of the total PBMCs in the ALL patient sample are T cells (approximately 0.5% are CD4 T cells and approximately 0.4% are CD8 T cells).

Example 10

Autologous CTL Killing Assay Using ALL Patient Primary Specimens

[00215] Cryopreserved primary ALL specimen (peripheral blood mononucleocytes (PBMC)) were thawed in RPMI 11640 with 10% FBS and allowed to recover overnight at 37°C in 5% CO₂. Cells were washed with assay medium (RPMI 1640+10%FBS) and viable cell count was determined by Trypan Blue exclusion. 150,000 cells / well in 150 µL assay medium were added to 96-well U-bottom plate (BD Biosciences). Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) was diluted to 10, 1 ng/mL and 50 µL of each dilution was added to each well (final volume = 200 µL). A separate assay plate was set up for each time point (48, 72, 120 and 144 hours) and plates were incubated at 37 °C in a 5% CO₂ incubator. At each time point, cells were stained with CD4, CD8, CD25, CD45, CD33, and CD123 antibodies. Labeled cells were analyzed in FACS Calibur flow cytometer equipped with CellQuest Pro acquisition software, Version 5.2.1 (BD Biosciences). Data analysis was performed using Flowjo v9.3.3 software (Treestar, Inc). T cell expansion was measured by gating on CD4⁺ and CD8⁺ populations and activation was determined by measuring CD25 MFI on the CD4⁺ and CD8⁺ -gated populations. Leukemic blast cell population was identified by CD45⁺CD33⁺ gating.

Autologous Tumor Cell Depletion, T Cell Expansion And Activation By Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) In Primary Specimens From ALL Patients

[00216] To determine the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) mediated activity in ALL patient primary patient samples, patient samples were incubated with 1 ng/mL of DART-A and percentages of leukemic blast cells and T cells were measured at different time points following the treatment. Leukemic

blast cells were identified by CD45⁺/CD33⁺ gating. Incubation of primary ALL bone marrow samples with DART-A resulted in depletion of the leukemic cell population over time compared to untreated control or Control DART (**Figure 13, Panel H versus Panels F and G**). When the T cells were counted (CD8 and CD4 staining) and activation (CD25 staining) were assayed, the T cells expanded and were activated in the DART-A sample (**Figure 14, Panels I and L**, respectively) compared to untreated or Control DART samples (**Figure 14, Panels H, G, K and J**, respectively).

Example 11

CD123 Surface Expression On Leukemic Blast Cells And Stem Cells In Primary Tissue Sample From AML Patient 2

[00217] To define the CD123 expression pattern in AML patient 2 primary samples, cryopreserved primary AML patient bone marrow and PBMC samples were evaluated for CD123 surface expression on leukemic blast cells.

CD123 Expression in Leukemic Blast Cells in Bone Marrow Mononucleocytes (BMNC)

[00218] A total of 0.5×10^6 bone marrow mononucleocytes (BM MNC) and peripheral blood mononucleocytes (PBMC)) from an AML patient 2 were evaluated for leukemic blast cell identification. Leukemic blast cells were identified using the myeloid markers CD33 and CD45. As shown in **Figure 15, Panel B**, 94% of the cells from AML bone marrow are leukemic blast cells. The T cell population was identified by CD3 expression. As shown in **Figure 15, Panel C**, approximately 15% of the cell from the AML bone marrow and PBMC sample are T cells.

Example 12

Autologous CTL Killing Assay Using AML Patient 2 Primary Specimens

[00219] Cryopreserved primary AML specimen (bone marrow mononucleocytes (BM MNC) and peripheral blood mononucleocytes (PBMC)) from AML patient 2 were thawed in RPMI 1640 with 10% FBS and allowed to recover overnight at 37°C in 5% CO₂. Cells were washed with assay medium (RPMI 1640+10%FBS) and viable cell count was determined by Trypan Blue exclusion. 150,000 cells / well in 150 µL assay medium were added to 96-well U-bottom plate (BD Biosciences). Sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) and control bi-

specific diabody (Control DART) were diluted to 0.1, and 0.01 ng/mL and 50 μ L of each dilution was added to each well (final volume = 200 μ L). A separate assay plate was set up for each time point (48, 72, 120 and 144 hours) and plates were incubated at 37 °C in a 5% CO₂ incubator. At each time point, cells were stained with CD4, CD8, CD25, CD45, CD33, and CD123 antibodies. Labeled cells were analyzed in FACS Calibur flow cytometer equipped with CellQuest Pro acquisition software, Version 5.2.1 (BD Biosciences). Data analysis was performed using Flowjo v9.3.3 software (Treestar, Inc). T cell expansion was measured by gating on CD4⁺ and CD8⁺ populations and activation was determined by measuring CD25 MFI on the CD4⁺ and CD8⁺ -gated populations. Leukemic blast cell population was identified by CD45⁺CD33⁺ gating.

Autologous Tumor Cell Depletion, T Cell Expansion and Activation in Primary Specimens from AML Patient 2

[00220] To determine the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) mediated activity in AML patient primary patient 2 samples, patient samples were incubated with 0.1 or 0.01 ng/mL of DART-A and percentages of leukemic blast cells and T cells were measured at different time points following the treatment. Incubation of primary AML bone marrow samples with DART-A resulted in depletion of the leukemic cell population over time (**Figure 16, Panel A**), accompanied by a concomitant expansion of the residual T cells (both CD4 and CD8) (**Figure 16, Panel B** and **Figure 16, Panel C**, respectively). To determine if the T cells were activated, cells were stained for CD25 or Ki-67, both markers of T cell activation. As shown in **Figure 17, Panels A and B**, incubation of primary AML bone marrow samples with DART-A resulted in T cell activation. These data represent the 144h time point.

Intracellular Staining for Granzyme B and Perforin

[00221] To determine the intracellular levels of granzyme B and Perforin in T cells, CTL assay was setup. After approximately 18 h, cells from the assay plate were stained with anti-CD4 and anti-CD8 antibodies by incubating for 30 minutes at 4 °C. Following surface staining, cells were incubated in 100 μ L Fixation and Permeabilization buffer for 20 min at 4 °C. Cells were washed with

permeabilization/wash buffer and incubated in 50 µl of granzyme B and perforin antibody mixture prepared in 1X Perm/Wash buffer at 4 C for 30 minutes. Then cells were washed with 250 µl Perm/Wash buffer and resuspended in Perm/Wash buffer for FACS acquisition.

Upregulation Of Granzyme B And Perforin By Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) In T Cells During Redirected Killing.

[00222] To investigate the possible mechanism for sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) mediated cytotoxicity by T cells, intracellular granzyme B and perforin levels were measured in T cells after the redirected killing. There was no upregulation of granzyme B and perforin when T cells were incubated with control bi-specific diabody (Control DART). Upregulation of granzyme B and perforin levels in both CD8 and CD4 T cells was observed with sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) (**Figure 17, Panels C and D**). Interestingly, the upregulation was almost two-fold higher in CD8 T cells compared to CD4 T cells (**Figure 17, Panel C** and **Figure 17, Panel D**). These data indicate that DART-A-mediated target cell killing was mediated through granzyme B and perforin pathway.

Example 13

Sequence-Optimized CD123 x CD3 Bi-Specific Diabody Cross-React With Non-Human Primate CD123 And CD3 Proteins.

[00223] In order to quantitate the extent of binding between sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) and human or cynomolgus monkey CD3, BIACORE™ analyses were conducted. 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. Recombinant human or cynomolgus monkey CD3 was directly immobilized to a support. Purified human or cynomolgus monkey CD123 was captured and immobilized to a support. The time course of dissociation was measured and a bivalent fit of the data conducted. Binding constants and affinity were obtained using a 1:1 binding fit. The results of the BIACORE™ analyses comparing binding to

human versus cynomolgus monkey CD123 and CD3 proteins are shown in **Figure 18**. Binding affinities to the cynomolgus monkey CD123 (**Figure 18D**) and CD3 (**Figure 18B**) proteins is comparable to binding affinities for human CD123 (**Figure 18C**) and CD3 (**Figure 18A**) proteins.

Example 14

Autologous Monocyte Depletion In Vitro With Human And Cynomolgus Monkey PBMCs

[00224] PBMCs from human or cynomolgus monkey whole blood samples were added to U-bottom plates at cell density of 200,000 cells/well in 150 μ L of assay medium. Dilutions of sequence-optimized CD123 x CD3 bi-specific diabodies (DART-A or DART-A w/ABD) were prepared in assay medium. 50 μ L of each DART-A or DART-A w/ABD dilution was added to the plate containing PBMCs in duplicate wells. The plates were incubated for ~ 18 - 24 h at 37°C. Supernatants were used to determine the cytotoxicity as described above. As shown in **Figure 19 (Panels A and B)**, depletion of pDCs cells was observed in both human (**Figure 19, Panel A**) and cynomolgus monkey PBMCs (**Figure 19, Panel B**). These results indicate that circulating pDC can be used as a pharmacodynamic marker for preclinical toxicology studies in cynomolgus monkeys.

[00225] Should it be necessary to replicate this example it will be appreciated that one of skill in the art may, within reasonable and acceptable limits, vary the above-described protocol in a manner appropriate for replicating the described results. Thus, the exemplified protocol is not intended to be adhered to in a precisely rigid manner.

Example 15

Plasmacytoid Dendritic Cell Depletion In Cynomolgus Monkeys Treated With Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A)

[00226] As part of a dose-range finding toxicology study, cynomolgus monkeys were administered sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) as 4-day infusions at doses of 0.1, 1, 10, 30 100, 300, or 1000 ng/kg . The Control DART was administered at 100 ng/kg. To identify pDCs and monocyte populations in cynomolgus monkey PBMCs, cells were labeled with CD14-FITC antibody. Monocytes were identified as the CD14⁺ population and pDCs were identified as the

CD14⁻CD123⁺ population. As shown in **Figure 20 Panels K and L**, the pDCs were depleted as early as 4 days post infusion with as little as 10 ng/kg DART-A. No pDC depletion was seen in the control bi-specific diabody-(Control DART) treated monkeys or the vehicle + carrier-treated monkeys at the 4d time point (**Figure 20, Panels G, H, C and D**, respectively). Cytokine levels of interferon-gamma, TNF-alpha, IL6, IL5, IL4 and IL2 were determined at 4 hours after infusion. There was little to no elevation in cytokine levels at the DART-A treated animals compared to Control DART or vehicle-treated animals.

[00227] **Figure 21** and **Figure 22** provide the results of the FACS analysis for B cells (CD20⁺) (**Figure 21, Panel A**), monocytes (CD14⁺) (**Figure 21, Panel B**), NK cells (CD159⁺CD16⁺) (**Figure 21, Panel C**), pDC (CD123^{HI}, CD14⁻) (**Figure 21, Panel D**), and T cells (total, CD4⁺, and CD8⁺) (**Figure 22, Panel A, Figure 22, Panel B**, and **Figure 22, Panel D**, respectively).

[00228] Treatment of monkeys with Control DART had no noticeable effects on T or B lymphocytes, NK cells, monocytes and pDCs. Treatment of monkeys with DART-A at doses of 10 ng/kg/d or higher resulted in the abrogation of pDCs (**Figure 21, Panel D**). The depletion of pDC was complete and durable, returning to pre-dose levels several weeks after completion of dosing. Circulating levels of T lymphocytes decreased upon DART-A administration, but returned to pre-dose level by the end of each weekly cycle, suggesting changes in trafficking rather than true depletion. Both CD4 and CD8 T lymphocytes followed the same pattern. The T-lymphocyte activation marker, CD69 (**Figure 22, Panel C**), was only marginally positive among circulating cells and did not track with DART-A dosing. B lymphocytes, monocytes and NK cells fluctuated over the course of DART-A dosing with substantial variability observed among monkeys. A trend toward increased circulating levels of B lymphocytes and monocytes was observed in both monkeys at the highest doses.

[00229] In summary, the above results demonstrate the therapeutic efficacy of the sequence-optimized CD123 x CD3 bi-specific diabody (DART-A). The sequence-optimized CD123 x CD3 bi-specific diabody (DART-A) may be employed as a therapeutic agent for the treatment of multiple diseases and conditions, including:

AML, ABL (ALL), CLL, MDS, pDCL, mantel cell leukemia, hairy cell leukemia, Richter transformation of CLL, Blastic crisis of CML, BLL (subset are CD123+) (see **Example 2**); Autoimmune Lupus (SLE), allergy (basophils are CD123+), asthma, *etc.*

Example 16

Comparative Properties of Sequence-Optimized CD123 x CD3 Bi-Specific Diabody (DART-A) and Non-Sequence-Optimized CD123 xCD3 Bi-Specific Diabody (DART-B)

Unexpected Advantage and Attributes of the Sequence-Optimized CD123 x CD3 Bi-Specific Diabodies

[00230] As discussed above, DART-A and DART-B are similarly designed and the first polypeptide of both constructs comprise, 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}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}), a Linker 2, an E-coil Domain, and a C-terminus. Likewise, the second polypeptide of both constructs comprise, in the N-terminal to C-terminal direction, an N-terminus, a VL domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}), an intervening linker peptide (Linker 1), a VH domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}), a Linker 2, a K-coil Domain and a C-terminus.

[00231] As indicated in Example 1, both CD123 x CD3 bi-specific diabodies were found to be capable of simultaneously binding to CD3 and CD123. Additionally, as disclosed in **Example 3** and in **Figure 4, Panels C and D**, the two CD123 x CD3 bi-specific diabodies exhibited a potent redirected killing ability with concentrations required to achieve 50% of maximal activity (EC50s) in sub-ng/mL range, regardless of CD3 epitope binding specificity (DART-A versus DART-B) in target cell lines with high CD123 expression. Thus, slight variations in the specific sequences of the CD123 x CD3 bi-specific diabodies do not completely abrogate biological activity.

[00232] However, in all cell lines tested, DART-A was found to be more active and more potent at redirected killing than DART-B (see, *e.g.*, **Figure 4, Panels A, C, and D**). Thus DART-A exhibited an unexpected advantage over similar DART-B.

Example 17
Non-Human Primate Pharmacology of DART-A for the Treatment of Hematological Malignancies

[00233] The interleukin 3 (IL-3) receptor alpha chain, CD123, is overexpressed on malignant cells in a wide range of hematological malignancies (Munoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies*,” *Haematologica* 86:1261-1269; Testa, U. *et al.* (2014) “*CD123 Is A Membrane Biomarker And A Therapeutic Target In Hematologic Malignancies*,” *Biomark. Res.* 2:4) and is associated with poor prognosis (Vergez, F. *et al.* (2011) “*High Levels Of CD34+CD38low/-CD123+ Blasts Are Predictive Of An Adverse Outcome In Acute Myeloid Leukemia: A Groupe Ouest-Est Des Leucemies Aigues Et Maladies Du Sang (GOELAMS) Study*,” *Haematologica* 96:1792-1798). Moreover, CD123 has been reported to be expressed by leukemia stem cells (LSC) (Jordan, C.T. *et al.* (2000) “*The Interleukin-3 Receptor Alpha Chain Is A Unique Marker For Human Acute Myelogenous Leukemia Stem Cells*,” *Leukemia* 14:1777-1784; Jin, L. *et al.* (2009) “*Monoclonal Antibody-Mediated Targeting Of CD123, IL-3 Receptor Alpha Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells*,” *Cell Stem Cell* 5:31-42), which is an attractive feature that enables targeting the root cause of such diseases. Consistent with this conclusion, CD123 also takes part in an IL-3 autocrine loop that sustains leukemogenesis, as shown by the ability of a CD123-blocking monoclonal antibody to reduce leukemic stem cell engraftment and improve survival in a mouse model of acute myelogenous leukemia (AML) (Jin, L. *et al.* (2009) “*Monoclonal Antibody-Mediated Targeting Of CD123, IL-3 Receptor Alpha Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells*,” *Cell Stem Cell* 5:31-42). In a phase 1 study in high-risk AML patients, however, the monoclonal antibody exhibited no anti-leukemic activity (Roberts, A. W. *et al.* (2010) “*A Phase I Study Of Anti-CD123 Monoclonal Antibody (mAb) CSL360 Targeting Leukemia Stem Cells (LSC) In AML*,” *J. Clin. Oncol.* 28(Suppl):e13012). Thus, alternate CD123-targeting approaches, including depleting strategies are desired. Although CD123 is expressed by a subset of normal hematopoietic progenitor cells (HPC), hematopoietic stem cells (HSC) express little to no CD123 (Jordan, C.T. *et al.* (2000) “*The Interleukin-3 Receptor Alpha Chain Is A Unique Marker For Human Acute Myelogenous Leukemia Stem Cells*,” *Leukemia* 14:1777-1784; Jin, W. *et al.* (2009) “*Regulation Of Th17 Cell*

Differentiation And EAE Induction By MAP3K NIK,” Blood 113:6603-6610), indicating that CD123 cell-depleting strategies allow reconstitution via normal hematopoiesis.

[00234] Enabling a patient's own T lymphocytes to target leukemic cells represents a promising immunotherapeutic strategy for the treatment of hematological malignancies. The therapeutic potential of this approach has been attempted using blinatumomab (a bi-specific antibody-based BiTE having the ability to bond CD3 and the B cell CD19 antigen) in patients with B cell lymphomas and B-precursor acute lymphoblastic leukemia (Klinger, M. *et al.* (2012) “*Immunopharmacologic Response Of Patients With B-Lineage Acute Lymphoblastic Leukemia To Continuous Infusion Of T Cell-Engaging CD19/CD3-Bispecific BiTE Antibody Blinatumomab,*” *Blood* 119:6226-6233; Topp, M.S. *et al.* (2012) “*Long-Term Follow-Up Of Hematologic Relapse-Free Survival In A Phase 2 Study Of Blinatumomab In Patients With MRD In B-Lineage ALL,*” *Blood* 120:5185-5187; 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.* 29:2493-2498).

[00235] The CD123 x CD3 bi-specific diabody molecules of the present invention, such as DART-A, comprise an alternate bi-specific, antibody-based modality that offers improved stability and more robust manufacturability properties (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.* 399:436-449; Moore, P.A. *et al.* (2011) “*Application Of Dual Affinity Retargeting Molecules To Achieve Optimal Redirected T-Cell Killing Of B-Cell Lymphoma,*” *Blood* 117:4542-4551).

[00236] In order to demonstrate the superiority and effectiveness of the CD123 x CD3 bi-specific diabody molecules of the present invention, the biological activity of the above-described DART-A in *in vitro* and preclinical models of leukemia was confirmed, and its pharmacokinetics, pharmacodynamics and safety pharmacology in

cynomolgus macaques (*Macaca fascicularis*) was assessed relative to either the above-described Control DART (bi-specific for CD3 and fluorescein) or a “Control DART-2” that was bi-specific for CD123 and fluorescein).

**Amino Acid Sequence of First Polypeptide Chain of “Control DART-2”
(CD123VL — Linker — 4-4420VH — Linker — E-coil; linkers are underlined)
(SEQ ID NO:58):**

```
DFVMTQSPDS LAVSLGERVT MSCKSSQSLN NSGNQKNYLT WYQQKPGQPP
KLLIYWASTR ESGVPDRFSG SSGTDFTLT ISSLQAEDVA VYYCQNDYSY
PYTFGQGTKL EIKGGGSGGG GEVKLDETGG GLVQPGRPML LSCVASGFTF
SDYWMNWVRQ SPEKGLEWVA QIRNKPYNIE TYSDSVKGR FTISRDDSKS
SVYLMNNLR VEDMGIYYCT GSYGMDYWG QGTSVTVSSG GCGGGEVAAL
EKEVAALKE VAALEKEVAA LEK
```

**Amino Acid Sequence of Second Polypeptide Chain of “Control DART-2”
(4420VL — Linker— CD123VH — Linker — K-coil) (SEQ ID NO:59):**

```
DVVMQTTPFS LPVSLGDQAS ISCRSSQSLV HSNQNTYLRW YLQKPGQSPK
VLIYKVSNER SGVPDRFSGS SSGTDFTLKI SRVEAEDLGV YFCSQSTHVP
WTFGGGTKLE IKGGGSGGG EVQLVQSGAE LKKPGASVKV SCKASGYTFT
DYMKWVRQA PGQGLEWIGD IIPSNQATFY NQKFKGRVTI TVDKSTSTAY
MELSSLRSED TAVYYCARSH LLRASWFAYW GQGLTVTVSS GGCGGGKVAA
LKEKVAALKE KVAALKEKVA ALKE
```

Bifunctional ELISA

[00237] A MaxiSorp ELISA plate (Nunc) coated overnight with the soluble human or cynomolgus IL3R-alpha (0.5 µg/mL) in bicarbonate buffer was blocked with 0.5% BSA; 0.1% Tween-20 in PBS (PBST/BSA) for 30 minutes at room temperature. DART-A molecules were applied, followed by the sequential addition of human CD3εδ-biotin and Streptavidin HRP (Jackson ImmunoResearch). HRP activity was detected by conversion of tetramethylbenzidine (BioFX) as substrate for 5 min; the reaction was terminated with 40µL/well of 1% H₂SO₄ and the absorbance read at 450 nm.

Surface Plasmon Resonance Analysis

[00238] The ability of DART-A to bind to human and cynomolgus monkey CD3 or CD123 proteins was analyzed using a BIAcore 3000 biosensor (GE, Healthcare) as described by 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. 399:436-449) 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 117:4542-4551). Briefly, the carboxyl groups on the CM5 sensor chip were activated with an injection of 0.2M N-ethyl-N-(3diethylamino-propyl) carbodiimide and 0.05M N-hydroxy-succinimide. Soluble CD3 or CD123 (1µg/ml) was injected over the activated CM5 surface in 10mM sodium-acetate, pH 5.0, at flow rate 5 µL/min, followed by 1 M ethanolamine for deactivation. Binding experiments were performed in 10mM HEPES, pH 7.4, 150mM NaCl, 3mM EDTA and 0.005% P20 surfactant. Regeneration of the immobilized receptor surfaces was performed by pulse injection of 10mM glycine, pH 1.5. KD values were determined by a global fit of binding curves to the Langmuir 1:1 binding model (BIAevaluation software v4.1).

Cell Killing Assay

[00239] Cell lines used for cell killing assays were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). PBMCs were isolated from healthy donor blood using the Ficoll-Paque Plus kit (GE Healthcare); T cells were purified with a negative selection kit (Life Technologies). CD123 cell-surface density was determined using Quantum Simply Cellular beads (Bangs Laboratories, Inc., Fishers, IN). Cytotoxicity assays were performed as described by Moore, P.A. *et al.* (2011) (“*Application Of Dual Affinity Retargeting Molecules To Achieve Optimal Redirected T-Cell Killing Of B-Cell Lymphoma*,” Blood 117:4542-4551). Briefly, target cell lines (10^5 cells/mL) were treated with serial dilutions of DART-A or Control DART proteins in the presence of T cells at the indicated effector cells:target cells ratios and incubated at 37°C overnight. Cell killing was determined as the release of lactate dehydrogenase (LDH, Promega) in culture supernatant. For flow-based killing, target cells were labeled with CMTMR (Life Technologies) and cell killing was monitored using a FACSCalibur flow cytometer. Data were analyzed by using PRISM® 5 software (GraphPad) and presented as percent cytotoxicity.

Cynomolgus Monkey Pharmacology

[00240] Non-human primate experiments were performed at Charles River Laboratories (Reno, NV), according to the guidelines of the local Institutional Animal Care and Use Committee (IACUC). Purpose-bred, naïve cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin (age range 2.5-9 years, weight range of 2.7-5 kg) were provided with vehicle or DART-A via intravenous infusion through femoral and jugular ports using battery-powered programmable infusion pumps (CADD-Legacy®, SIMS Deltec, Inc., St. Paul, MN). Peripheral blood or bone marrow samples were collected in anticoagulant containing tubes at the indicated time points. Cell-surface phenotype analyses were performed with an LSR Fortessa analyzer (BD Biosciences) equipped with 488nm, 640nm and 405nm lasers and the following antibodies: CD4-V450, CD8-V450, CD123-PE-Cy7, CD45-PerCP, CD4-APC-H7, CD8-FITC, CD25-PE-Cy7, CD69-PerCP, PD-1-PE, TIM3-APC, CD3-Pacific Blue, CD95-APC, CD28-BV421, CD16-FITC, CD3-Alexa488, CD38-PE, CD123-PE-Cy7, CD117-PerCP-Cy5.5, CD34-APC, CD90-BV421, CD45RA-APC -H7 and CD33-APC (BD Biosciences). The absolute number of cells was determined using TruCOUNT (BD Biosciences). Serum levels of IL-2, IL-4, IL-5, IL-6, TNF- α , and IFN- γ cytokines were measured with the Non-Human Primate Th1/Th2 Cytokine Cytometric Bead Array Kit (BD Bioscience). The concentration of DART-A in monkey serum samples was measured using a sandwich immunoassay with electrochemiluminescence detection (MesoScale Diagnostics, MSD, Rockville, MD). Briefly, the assay plate (MSD) was coated with recombinant human IL-3 Ra (R&D System) and blocked with 5 % BSA. Calibration standards or diluted test samples were applied, followed by the addition of a biotinylated monoclonal antibody exhibiting specific binding for the above-described E-coil (**SEQ ID NO:34**) and K-coil (**SEQ ID NO:35**) domains of the molecule. A SULFO-TAG™ labeled streptavidin conjugate (MSD) was added and the formation of complexes was analyzed in an MSD SECTOR® imager. DART-A concentrations were determined from standard curves generated by fitting light intensity data in a five-parameter logistic model.

[00241] Physicochemical characterization of the purified DART-A demonstrated a homogeneous heterodimer with a molecular mass of 58.9 kDa (**Figure 23; Figures 24A-24B**), which was stable at 2–8°C for up to 12 months in PBS. SPR analysis demonstrated nearly identical binding affinities of DART-A to the corresponding soluble human and cynomolgus monkey CD3 and CD123 antigens (**Figures 25A-25D and Table 7**). Furthermore, DART-A simultaneously bound both antigens in an ELISA format that employed human or monkey CD123 for capture and human CD3 for detection (**Figures 26A-26B**), and demonstrated similar binding to human and monkey T lymphocytes (**Figures 26C-26E**). The data in **Table 7** are averages of 3 independent experiments each performed in duplicates.

Table 7 Equilibrium Dissociation Constants (K_D) for the Binding of DART-A to Human and Cynomolgus Monkey CD3 and CD123			
Antigens	k_a ($\pm$ SD) ($M^{-1} s^{-1}$)	k_d ($\pm$ SD) (s^{-1})	K_D ($\pm$ SD) (nM)
Human CD3 ϵ/δ	$5.7 (\pm 0.6) \times 10^5$	$5.0 (\pm 0.9) \times 10^{-3}$	9.0 ± 2.3
Cynomolgus CD3 ϵ/δ	$5.5 (\pm 0.5) \times 10^5$	$5.0 (\pm 0.9) \times 10^{-3}$	9.2 ± 2.3
Human CD123-His	$1.6 (\pm 0.4) \times 10^6$	$1.9 (\pm 0.4) \times 10^{-4}$	0.13 ± 0.01
Cynomolgus CD123-His	$1.5 (\pm 0.3) \times 10^6$	$4.0 (\pm 0.7) \times 10^{-4}$	0.27 ± 0.02

DART-A Mediates Redirected Killing by Human or Cynomolgus Monkey T Lymphocytes

[00242] DART-A mediated redirected target cell killing by human or monkey effector cells against CD123+ Kasumi-3 leukemic cell lines (**Figure 27A-27D**), which was accompanied by induction of activation markers. No activity was observed against CD123-negative targets (U937 cells) or with Control DART, indicating that T cell activation is strictly dependent upon target cell engagement and that monovalent engagement of CD3 by DART-A was insufficient to trigger T cell activation. Since CD123 is expressed by subsets of normal circulating leukocytes, including pDCs and monocytes (**Figure 27E**), the effect of DART-A were further investigated in normal human and monkey's PBMCs.

[00243] A graded effect was observed among human PBMC, with a dose-dependent rapid depletion of CD14⁻CD123^{high} cells (pDC and basophils) observed as early as 3 hours following initiation of treatment, while monocytes (CD14⁺ cells) remained unaffected at this time point (**Figures 27F-27G**). CD14⁻CD123^{high} cells depletion increased over time across all DART-A molecule concentrations, while monocytes were slightly decreased by 6 hours and depleted only after 18 hours and at the concentrations higher than 1 ng/mL. Incubation of monkey PBMCs with DART-A resulted in a comparable dose-dependent depletion of CD14⁻CD123^{high} cells (**Figure 27H**), further supporting the relevance of this species for DART-A pharmacology (CD14⁺ monkey cells express little to no CD123 and were not depleted).

Pharmacokinetics of DART-A in Cynomolgus Monkeys

[00244] 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, consistent with published information (Munoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies*,” *Haematologica* 86:1261-1269; Korpelainen, E.I. *et al.* (1996) “*IL-3 Receptor Expression, Regulation And Function In Cells Of The Vasculature*,” *Immunol. Cell Biol.* 74:1-7).

[00245] The study conducted in accordance with the present invention included 6 treatment groups consisting of 8 cynomolgus monkeys per group (4 males, 4 females) (**Table 8**). All groups received vehicle control for the first infusion; then vehicle or DART-A were administered intravenously for 4 weekly cycles. Group 1 animals received vehicle control for all 4 subsequent infusions, whereas Groups 2-5 received weekly escalating doses of DART-A for 4 days a week for all subsequent infusions. Group 6 animals were treated with 7-day uninterrupted weekly escalating doses of DART-A for all infusions. The 4-day-on/3-day-off and 7-day-on schedules were designed to distinguish between durable from transient effects associated with DART-A administration. Two males and 2 females per group were sacrificed at the end of the treatment phase (Day 36), while the remaining monkeys were necropsied after a 4-

week recovery (Day 65). A subset of monkeys developed anti-drug antibodies (ADA) directed against the humanized Fv of both CD3 and CD123 and the data points following the appearance of ADA were excluded from the PK analysis. All monkeys were exposed to DART-A during the study period.

Table 8							
Infusion No.	Study Days	Vehicle	DART-A Infusion				
			(4-day-on/3-day-off) ng/kg/day [ng/kg/4 days]				(7-day-on) ng/kg/day [ng/kg/7days]
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
1	1	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
2	8	Vehicle	100 [400]	100 [400]	100 [400]	100 [400]	100 [700]
3	15	Vehicle	100 [400]	300 [1200]	300 [1200]	300 [1200]	300 [2100]
4	22	Vehicle	100 [400]	300 [1200]	600 [2400]	600 [2400]	600 [4200]
5	29	Vehicle	100 [400]	300 [1200]	600 [2400]	1000 [4000]	1000 [7000]
Recovery	36-65						

[00246] A two-compartment model was used to estimate PK parameters (**Table 9** and **Figure 28**). $T_{1/2\alpha}$ was short (4-5min), reflecting rapid binding to circulating targets; $T_{1/2\beta}$ was also rapid, as expected for a molecule of this size, which is subject to renal clearance. Analysis of serum samples collected at the end of each infusion from group 6 monkeys showed a dose-dependent increase in DART-A C_{max} . In Table 9, Vehicle was PBS, pH 6.0, containing 0.1 mg/mL recombinant human albumin, 0.1 mg/mL PS-80, and 0.24 % benzyl alcohol was used for all vehicle infusions during the first 4 days of each infusion week followed the same formulation without benzyl alcohol for the remaining 3 days of each weekly infusion. DART-A was administered for the indicated times as a continuous IV infusion of a solution of PBS, pH 6.0, containing 0.1 mg/mL recombinant human albumin, 0.1 mg/mL PS-80, and 0.24 % benzyl alcohol at the required concentration.

Table 9 Two-Compartment Analysis of PK Parameters of DART-A in Cynomolgus Monkeys		
Attribute	300 ng/kg/d (mean $\pm$ SD)	600 ng/kg/d (mean $\pm$ SD)
C_{max} (pg/mL)	77.4 $\pm$ 9.4	113.8 $\pm$ 33.5
AUC (h*pg/mL)	7465 $\pm$ 913	11188 $\pm$ 3282
V_{ss} (L/kg)	1.078 $\pm$ 0.511	2.098 $\pm$ 1.846
t_{1/2}, alpha (h)	0.07 $\pm$ 0.018	0.067 $\pm$ 0.023
t_{1/2}, beta (h)	13.79 $\pm$ 4.928	21.828 $\pm$ 18.779
MRT (h)	6.73 $\pm$ 3.327	9.604 $\pm$ 8.891

Cytokine Release in DART-A-Treated Cynomolgus Monkeys

[00247] Given the T cell activation properties of DART-A, an increase in circulating cytokines accompanying the infusion was anticipated and a low starting dose was therefore used as a “desensitization” strategy, based on previous experience with similar compounds (see, e.g., 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. 29:2493-2498; Bargou, R. *et al.* (2008) “*Tumor Regression In Cancer Patients By Very Low Doses Of A T Cell-Engaging Antibody*,” Science 321:974-977). Of the cytokine tested, IL-6 demonstrated the largest changes upon infusion, albeit transient in nature, of minimal magnitude and with large inter-animal and inter-group variations (**Figures 29A-29C**). Small, transient increases in IL-6 were also observed after vehicle infusions (all Group 1 and all Day 1 infusions), indicating a sensitivity of this cytokine to manipulative stress. Nonetheless, DART-A-dependent increases (<80pg/mL) in serum IL-6 were seen in some monkeys following the first DART-A infusion (100ng/kg/day), which returned to baseline by 72 hours. Interestingly, the magnitude of IL-6 release decreased with each successive DART-A infusion, even when the dose level was increased to up to 1000 ng/kg/day. Minimal and transient DART-A-related increases in serum TNF- α (<10pg/mL) were also observed; as with IL-6, the largest magnitude in TNF- α release was observed following the first infusion. There were no DART-A-related changes in the levels of IL-5, IL-4, IL-2, or IFN- γ throughout the study when compared with controls. In conclusion, cytokine

release in response to treatment of monkeys with DART-A was minimal, transient and represented a first-dose effect manageable via intra-subject dose escalation.

DART-A-Mediated Depletion of Circulating CD14⁻/CD123⁺ Leukocytes *in vivo*

[00248] The circulating absolute levels of CD14⁻/CD123⁺ cells were measured throughout the study as a pharmacodynamic endpoint. While the number of CD123⁺ cells in control Group 1 remained stable over time, DART-A treatment was associated with extensive depletion of circulating CD14⁻/CD123⁺ cells (94-100% from prestudy baseline) observable from the first time point measured (72 hours) following the start of the first DART-A infusion (100 ng/kg/day) in all animals across all active treatment groups (**Figures 30A-30C**). The depletion was durable, as it persisted during the 3-day weekly dosing holiday in Group 2-5, returning to baseline levels only during the prolonged recovery period. To eliminate the possibility of DART-A masking or modulating CD123 (an unlikely scenario, given the low circulating DART-A levels), pDCs were enumerated by the orthogonal marker, CD303. Consistent with the CD123 data, CD303⁺ pDC were similarly depleted in monkeys treated with DART-A (**Figures 30D-30F**).

Circulating T-Lymphocyte Levels, Activation and Subset Analysis

[00249] In contrast to the persistent depletion of circulating CD123⁺ cells, DART-A administered on the 4-day-on/3-day-off schedule (Groups 2-5) were associated with weekly fluctuations in circulating T cells, while administration as continuous 7-day infusions resulted in similarly decreased circulating T cell levels following the first administration that slowly recovered without fluctuation even during the dosing period (**Figures 31A-31C**). The difference between the two dosing strategies indicates that the effect of DART-A on T lymphocytes is consistent with trafficking and/or margination, rather than depletion. Following cessation of dosing, T cells rebounded to levels approximately 2-fold higher than baseline for the duration of the recovery period. Infusion of DART-A was associated with an exposure-dependent, progressive increased frequency of T cells expressing the late activation marker, PD-1, particularly in CD4⁺ cells, with dose Group 6 displaying the highest overall levels (**Figures 31D-31I** and **Figures 32A-32F** and **Figures 33A-33F**). Tim-3, a marker

associated with T cell exhaustion, was not detected on CD4+ T cells and only at low frequency among CD8+ cells (5.5-9.7%) and comprising 20.5-35.5% of the CD8+/PD-1+ double-positive cells. There was no consistent change in the early T cell activation marker, CD69, and only modest variations in CD25 expression among circulating cells.

[00250] To rule out exhaustion after *in vivo* exposure, the *ex vivo* cytotoxic potential of effector cells isolated from cynomolgus monkeys receiving multiple infusions of DART-A was compared to that of cells from naïve monkeys. As shown in **Figure 34**, PBMC isolated from DART-A-treated monkeys show cytotoxicity comparable to that of cells isolated from naïve monkeys, indicating that *in vivo* exposure to DART-A does not negatively impact the ability of T cells to kill target cells.

[00251] DART-A exposure increased the relative frequency of central memory CD4 cells and effector memory CD8+ cells at the expense of the corresponding naïve T cell population (**Figures 35A-35F** and **Figures 32A-32F** and **Figures 33A-33F**), indicating that DART-A exposure promoted expansion and/or mobilization of these cells.

Effects on Hematopoiesis and Bone Marrow Precursors

[00252] DART-A was well tolerated in monkeys at all doses tested; however, reversible reductions in red cell parameters were observed at the highest doses (**Figures 36A-36C**). Frequent blood sampling could have been a potential contributing factor, since vehicle-treated animals showed a modest decline in red cell mass. Reticulocyte response was observed in all animals; at the highest exposure (Group 6), however, the response appeared slightly less robust for similar decrease in red cell mass (**Figures 36D-36F**). Morphological analysis of bone marrow smears throughout the study was unremarkable. Flow cytometry analysis, however, revealed that the frequency of CD123+ cells within the immature lineage-negative (Lin-) bone marrow populations decreased in DART-A-treated animals at the end of the dosing period, returning to baseline levels by the end of the recovery time (**Figure 37A-37B**). HSC (defined as Lin-/CD34+/CD38-/CD45RA-/CD90+ cells (Pang, W.W. *et al.* (2011) “*Human Bone Marrow Hematopoietic Stem Cells Are Increased In Frequency*

And Myeloid-Biased With Age,” Proc. Natl. Acad. Sci. (U.S.A.) 108:20012-20017)) showed large inter-group variability; Group 4-6 DART-A-treated monkeys show some apparent reduction compared to the corresponding pre-dose levels, however, no decrease was seen in all treated groups compared to vehicle-treated animals. These data indicate that HSC are less susceptible to targeting by DART-A and are consistent with the observed reversibility of the negative effects of DART-A treatment on hematopoiesis.

[00253] As demonstrated above, with respect to infusions for 4 weeks on a 4-day-on/3-day-off weekly schedule or a 7-day-on schedule at starting doses of 100ng/kg/day that were escalated stepwise weekly to 300, 600, and 1,000ng/kg/day, the administration of DART-A to cynomolgus monkeys was well tolerated. Depletion of circulating CD123+ cells, including pDCs, was observed after the start of the first administration and persisted throughout the study at all doses and schedules. Reversible reduction in bone marrow CD123+ precursor was also observed. Cytokine release, as significant safety concern with CD3-targeted therapies, appeared manageable and consistent with a first-dose effect. Modest reversible anemia was noted at the highest doses, but no other (on- or off-target) adverse events were noted.

[00254] The cynomolgus monkey is an appropriate animal model for the pharmacological assessment of DART-A, given the high homology between the orthologs and the ability of DART-A to bind with similar affinity to the antigens and mediate redirected T cell killing in both species. Furthermore, both antigens are concordantly expressed in monkeys and humans, including similar expression by hematopoietic precursors and in the cytoplasm of the endothelium of multiple tissues. Minor exceptions are the expression in testicular Leydig cells in humans but not monkeys and low-to-absent CD123 in monkey monocytes compared to humans.

[00255] A primary concern associated with therapeutic strategies that involve T cell activation includes the release of cytokines and off-target cytotoxic effects. A recent study with a CD3xCD123 bi-specific scFv immunofusion construct with bivalent CD3 recognition demonstrated anti-leukemic activity *in vitro*, but caused non-specific activation of T cells and IFN- γ secretion (Kuo, S.R. *et al.* (2012) “*Engineering A*

CD123xCD3 Bispecific scFv Immunofusion For The Treatment Of Leukemia And Elimination Of Leukemia Stem Cells,” Protein Eng. Des. Sel. 25:561-569). The monovalent nature of each binding arms and the highly homogeneous monomeric form of DART-A ensure that T cell activation depends exclusively upon target cell engagement: no T cell activation was observed in the absence of target cells or by using a Control DART molecule that included only the CD3-targeting arm. Furthermore, high doses (up to 100ug/kg/day) of the Control DART molecule did not trigger cytokine release in cynomolgus monkeys.

[00256] The DART-A molecule starting dose of 100ng/kg/day was well tolerated, with minimal cytokine release. Cytokine storm, however, did occur with a high starting dose (5µg/kg/day); however, such dose could be reached safely via stepwise weekly dose escalations, indicating that DART-A-mediated cytokine release appears to be primarily a first-dose effect. Depletion of the CD123+ target cells, thereby eliminating a source of CD3 ligation, may explain the first-dose effect: nearly complete CD123+ cell depletion was observed at doses as low as 3-10ng/kg/day, indicating that cytokine release *in vivo* follows a shifted dose-response compared to cytotoxicity. Dose-response profiles for cytotoxicity and cytokine release by human T cells were also consistent with this observation.

[00257] T cell desensitization, in which DART-A-induced PD1 upregulation may play a role, appears to also contribute to limit cytokine release after the first infusion of DART-A. Recent studies show that increased PD-1 expression after antigen-induced arrest of T cells at inflammation sites contributes, through interactions with PD-L1, to terminating the stop signal, thus releasing and desensitizing the cells (Honda, T. *et al.* (2014) “*Tuning Of Antigen Sensitivity By T Cell Receptor-Dependent Negative Feedback Controls T Cell Effector Function In Inflamed Tissues,*” Immunity 40:235-247; Wei, F. *et al.* (2013) “*Strength Of PD-1 Signaling Differentially Affects T-Cell Effector Functions,*” Proc. Natl. Acad. Sci. (U.S.A.) 110:E2480-E2489). The PD-1 countering of TCR signaling strength is not uniform: while proliferation and cytokine production appear most sensitive to PD-1 inhibition, cytotoxicity is the least affected (Wei, F. *et al.* (2013) “*Strength Of PD-1 Signaling Differentially Affects T-Cell Effector Functions,*” Proc. Natl. Acad. Sci. (U.S.A.) 110:E2480-E2489).

Consistently, the *ex vivo* cytotoxic potential of T cells from monkeys exposed to multiple infusions of DART-A was comparable to that of T cells from naïve monkeys, despite increased PD-1 levels in the former. Furthermore, PD-1 upregulation was not accompanied by TIM3 expression, a hallmark of T cell exhaustion, as shown for T cells exposed to protracted stimulation with CD3 antibodies or chronic infections (Gebel, H.M. *et al.* (1989) “*T Cells From Patients Successfully Treated With OKT3 Do Not React With The T-Cell Receptor Antibody*,” *Hum. Immunol.* 26:123-129; Wherry, E.J. (2011) “*T Cell Exhaustion*,” *Nat. Immunol.* 12:492-499).

[00258] The depletion of circulating CD123+ cells in DART-A-treated monkeys was rapid and persisted during the weekly dosing holidays in the 4-day-on/3-day-off schedule, consistent with target cell elimination. In contrast, the transient fluctuations in circulating T cells were likely the result of trafficking from/to tissues and lymphoid organs as a function of DART-A. DART-A exposure promotes the expansion and/or mobilization of antigen experienced T lymphocytes, cells that preferentially home to tissues and more readily exert cytotoxic effector function (Mirenda, V. *et al.* (2007) “*Physiologic And Aberrant Regulation Of Memory T-Cell Trafficking By The Costimulatory Molecule CD28*,” *Blood* 109:2968-2977; Marelli-Berg, F.M. *et al.* (2010) “*Memory T-Cell Trafficking: New Directions For Busy Commuters*,” *Immunology* 130:158-165).

[00259] Depletion of CD123+ normal cells may carry potential liabilities. pDCs and basophils express high levels of CD123, compared to lower levels in monocytes and eosinophils (Lopez, A.F. *et al.* (1989) “*Reciprocal Inhibition Of Binding Between Interleukin 3 And Granulocyte-Macrophage Colony-Stimulating Factor To Human Eosinophils*,” *Proc. Natl. Acad. Sci. (U.S.A.)* 86:7022-7026; Munoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies*,” *Haematologica* 86:1261-1269; Masten, B.J. *et al.* (2006) “*Characterization Of Myeloid And Plasmacytoid Dendritic Cells In Human Lung*,” *J. Immunol.* 177:7784-7793; Korpelainen, E.I. *et al.* (1995) “*Interferon-Gamma Upregulates Interleukin-3 (IL-3) Receptor Expression In Human Endothelial Cells And Synergizes With IL-3 In Stimulating Major Histocompatibility Complex Class II Expression And Cytokine Production*,” *Blood* 86:176-182). pDCs have been shown

to play a role in the control of certain viruses in mouse or monkey models of infection, although they do not appear critical for controlling the immune response to flu (Colonna, M. *et al.* (1997) “*Specificity And Function Of Immunoglobulin Superfamily NK Cell Inhibitory And Stimulatory Receptors*,” *Immunol. Rev.* 155:127-133; Smit, J.J. *et al.* (2006) “*Plasmacytoid Dendritic Cells Inhibit Pulmonary Immunopathology And Promote Clearance Of Respiratory Syncytial Virus*,” *J. Exp. Med.* 203:1153-1159). In tumor models, pDCs may promote tumor growth and metastasis, while pDC depletion resulted in tumor inhibition (Sawant, A. *et al.* (2012) “*Depletion Of Plasmacytoid Dendritic Cells Inhibits Tumor Growth And Prevents Bone Metastasis Of Breast Cancer Cells*,” *J. Immunol.* 189:4258-4265). Transient, modest, dose-independent facial swelling was observed in some monkeys treated with DART-A; however, no increased histamine levels were observed in these monkeys or when human basophils were lysed via DART-A-mediated T cell killing. Monocyte depletion may carry increased risks of infection; the consequence of pDC, basophil or eosinophils depletion in humans should thus be monitored.

[00260] Committed hematopoietic precursors that express CD123, such as the common myeloid precursor (CMP) (Jordan, C.T. *et al.* (2000) “*The Interleukin-3 Receptor Alpha Chain Is A Unique Marker For Human Acute Myelogenous Leukemia Stem Cells*,” *Leukemia* 14:1777-1784; Rieger, M.A. *et al.* (2012) “*Hematopoiesis*,” *Cold Spring Harb. Perspect. Biol.* 4:a008250), may be targeted by DART-A, a possible explanation for the modest anemia observed following administration of DART-A at the highest dose. The erythropoietic reticulocyte response appeared to function at all DART-A dose levels; however, for commensurate drops in red cell parameters, animals subjected to the greatest DART-A exposure (Group 6, 7-day-on infusion) showed a reduced reticulocyte response, suggesting a possible cytotoxic activity on precursors (e.g., CMP). The effect was reversible following cessation of DART-A treatment, consistent with repopulation from spared CD123^{low}/negative HSC.

[00261] Alternate approaches for depletion of CD123⁺ cells include a second-generation CD123-specific Fc-enhanced monoclonal antibody (Jin, L. *et al.* (2009) “*Monoclonal Antibody-Mediated Targeting Of CD123, IL-3 Receptor Alpha Chain*,

*Eliminates Human Acute Myeloid Leukemic Stem Cells,” Cell Stem Cell 5:31-42; Roberts, A. W. et al. (2010) “A Phase I Study Of Anti-CD123 Monoclonal Antibody (mAb) CSL360 Targeting Leukemia Stem Cells (LSC) In AML,” J. Clin. Oncol. 28(Suppl):e13012), IL-3 bound diphtheria toxin (Frankel, A. et al. (2008) “Phase I Clinical Study Of Diphtheria Toxin-Interleukin 3 Fusion Protein In Patients With Acute Myeloid Leukemia And Myelodysplasia,” Leuk. Lymphoma 49:543-553), cytokine-induced killer (CIK) cells expressing CD123-specific chimeric antigen receptors (CAR) (Tettamanti, S. et al. (2013) “Targeting Of Acute Myeloid Leukaemia By Cytokine-Induced Killer Cells Redirected With A Novel CD123-Specific Chimeric Antigen Receptor,” Br. J. Haematol. 161:389-401) and CD123 CAR T cells (Gill, S. et al. (2014) “Efficacy Against Human Acute Myeloid Leukemia And Myeloablation Of Normal Hematopoiesis In A Mouse Model Using Chimeric Antigen Receptor-Modified T Cells,” Blood 123(15): 2343-2354; Mardiros, A. et al. (2013) “T Cells Expressing CD123-Specific Chimeric Antigen Receptors Exhibit Specific Cytolytic Effector Functions And Antitumor Effects Against Human Acute Myeloid Leukemia,” Blood 122:3138-3148). CAR T cells exhibited potent leukemic blast cell killing *in vitro* and anti-leukemic activity in a xenogeneic model of disseminated AML (Mardiros, A. et al. (2013) “T Cells Expressing CD123-Specific Chimeric Antigen Receptors Exhibit Specific Cytolytic Effector Functions And Antitumor Effects Against Human Acute Myeloid Leukemia,” Blood 122:3138-3148). A recent study reported ablation of normal hematopoiesis in NSG mice engrafted with human CD34+ cells following CD123 CAR T cell transfer (Gill, S. et al. (2014) “Efficacy Against Human Acute Myeloid Leukemia And Myeloablation Of Normal Hematopoiesis In A Mouse Model Using Chimeric Antigen Receptor-Modified T Cells,” Blood 123(15): 2343-2354), although others have not observed similar effects *in vitro* or *in vivo* (Tettamanti, S. et al. (2013) “Targeting Of Acute Myeloid Leukaemia By Cytokine-Induced Killer Cells Redirected With A Novel CD123-Specific Chimeric Antigen Receptor,” Br. J. Haematol. 161:389-401; Pizzitola, I. et al. (2014) “Chimeric Antigen Receptors Against CD33/CD123 Antigens Efficiently Target Primary Acute Myeloid Leukemia Cells *in vivo*,” Leukemia doi:10.1038/leu.2014.62). In the above-discussed experiments, depletion of CD123+ bone marrow precursor populations was observed, but reversed during recovery; furthermore, depletion of this minority population*

resulted in no changes in bone marrow cellularity or erythroid to myeloid cell (E:M) ratio at all DART-A dose levels tested. These differences underscore the potential advantages of DART-A over cell therapies, as it provides a titratable system that relies on autologous T cells in contrast to “supercharged” ex vivo transduced cells that may be more difficult to control. CD123 is overexpressed in several hematologic malignancies, including AML, hairy cell leukemia, blastic plasmacytoid dendritic cell neoplasms (BPDCNs), a subset of B-precursor acute lymphoblastic leukemia (B-ALL) and chronic lymphocytic leukemia, Hodgkin’s disease Reed-Stenberg cells, as well as in myelodysplastic syndrome and systemic mastocytosis (Kharfan-Dabaja, M.A. *et al.* (2013) “*Diagnostic And Therapeutic Advances In Blastic Plasmacytoid Dendritic Cell Neoplasm: A Focus On Hematopoietic Cell Transplantation,*” *Biol. Blood Marrow Transplant.* 19:1006-1012; Florian, S. *et al.* (2006) “*Detection Of Molecular Targets On The Surface Of CD34+/CD38-- Stem Cells In Various Myeloid Malignancies,*” *Leuk. Lymphoma* 47:207-222; Munoz, L. *et al.* (2001) “*Interleukin-3 Receptor Alpha Chain (CD123) Is Widely Expressed In Hematologic Malignancies,*” *Haematologica* 86:1261-1269; Fromm, J.R. (2011) “*Flow Cytometric Analysis Of CD123 Is Useful For Immunophenotyping Classical Hodgkin Lymphoma,*” *Cytometry B Clin. Cytom.* 80:91-99). The predictable pharmacodynamic activity and manageable safety profile observed in non-human primates further supports the clinical utility and efficacy of DART-A as immunotherapy for these disorders.

[00262] In sum, DART-A is an antibody-based molecule engaging the CD3 ϵ subunit of the TCR to redirect T lymphocytes against cells expressing CD123, an antigen up-regulated in several hematological malignancies. DART-A binds to both human and cynomolgus monkey’s antigens with similar affinities and redirects T cells from both species to kill CD123+ cells. Monkeys infused 4 or 7 days a week with weekly escalating doses of DART-A showed depletion of circulating CD123+ cells 72h after treatment initiation that persisted throughout the 4 weeks of treatment, irrespective of dosing schedules. A decrease in circulating T cells also occurred, but recovered to baseline before the subsequent infusion in monkeys on the 4-day dose schedule, consistent with DART-A-mediated mobilization. DART-A administration increased circulating PD1+, but not TIM-3+, T cells; furthermore, *ex vivo* analysis of T cells from treated monkeys exhibited unaltered redirected target cell lysis, indicating no

exhaustion. Toxicity was limited to a minimal transient release of cytokines following the DART-A first infusion, but not after subsequent administrations even when the dose was escalated, and a minimal reversible decrease in red cell mass with concomitant reduction in CD123+ bone marrow progenitors. Clinical testing of DART-A in hematological malignancies appears warranted.

[00263] 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 sequence-optimized diabody capable of specific binding to an epitope of CD123 and to an epitope of CD3, wherein the diabody comprises a first polypeptide chain and a second polypeptide chain, covalently bonded to one another, wherein:

- A. the first polypeptide chain comprises, in the N-terminal to C-terminal direction:
 - i. a Domain 1, comprising
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD3 (VL_{CD3}) (**SEQ ID NO:21**); and
 - (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD123 (VH_{CD123}) (**SEQ ID NO:26**),wherein said sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);
 - ii. a Domain 2, wherein said Domain 2 is an E-coil Domain (**SEQ ID NO:34**) or a K-coil Domain (**SEQ ID NO:35**), wherein said Domain 2 is separated from said Domain 1 by a peptide linker (**SEQ ID NO:30**); and
- B. the second polypeptide chain comprises, in the N-terminal to C-terminal direction:
 - i. a Domain 1, comprising
 - (1) a sub-Domain (1A), which comprises a VL Domain of a monoclonal antibody capable of binding to CD123 (VL_{CD123}) (**SEQ ID NO:25**); and
 - (2) a sub-Domain (1B), which comprises a VH Domain of a monoclonal antibody capable of binding to CD3 (VH_{CD3}) (**SEQ ID NO:22**),wherein said sub-Domains 1A and 1B are separated from one another by a peptide linker (**SEQ ID NO:29**);

- ii. a Domain 2, wherein said Domain 2 is a K-coil Domain (**SEQ ID NO:35**) or an E-coil Domain (**SEQ ID NO:34**), wherein said Domain 2 is separated from said Domain 1 by a peptide linker (**SEQ ID NO:30**); and wherein said Domain 2 of said first and said second polypeptide chains are not both E-coil Domains or both K-coil Domains

and wherein:

- (a) said VL Domain of said first polypeptide chain and said VH Domain of said second polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD3; and
- (b) said VL Domain of said second polypeptide chain and said VH Domain of said first polypeptide chain form an Antigen Binding Domain capable of specifically binding to an epitope of CD123.

Claim 2. The diabody of claim 1, wherein said first polypeptide chain additionally comprises an Albumin-Binding Domain (**SEQ ID NO:36**) linked to said Domain 2 via a peptide linker (**SEQ ID NO:31**).

Claim 3. The diabody of claim 1, wherein said second polypeptide chain additionally comprises a Domain 3 comprising a CH2 and CH3 Domain of an immunoglobulin Fc Domain (**SEQ ID NO:37**), wherein said Domain 3 is linked to said Domain 1A via a peptide linker (**SEQ ID NO:33**).

Claim 4. The diabody of claim 1, wherein said second polypeptide chain additionally comprises a Domain 3 comprising a CH2 and CH3 Domain of an immunoglobulin Fc Domain (**SEQ ID NO:37**), wherein said Domain 3 is linked to said Domain 2 via a peptide linker (**SEQ ID NO:32**).

- Claim 5. The diabody of any of claims 1 to 4, wherein said Domain 2 of said first polypeptide chain is a K-coil Domain (**SEQ ID NO:35**) and said Domain 2 of said second polypeptide chain is an E-coil Domain (**SEQ ID NO:34**).
- Claim 6. The diabody of any of claims 1 to 4, wherein said Domain 2 of said first polypeptide chain is an E-coil Domain (**SEQ ID NO:34**) and said Domain 2 of said second polypeptide chain is a K-coil Domain (**SEQ ID NO:35**).
- Claim 7. The diabody of any preceding claim, wherein the diabody is capable of cross-reacting with both human and primate CD123 and CD3 proteins.
- Claim 8. A bi-specific diabody capable of specific binding to an epitope of CD123 and to an epitope of CD3, wherein the diabody comprises a first polypeptide chain and a second polypeptide chain, covalently bonded to one another, wherein: said bi-specific diabody comprises:
- A. a first polypeptide chain having the amino acid sequence of **SEQ ID NO:1**; and
 - B. a second polypeptide chain having the amino acid sequence of **SEQ ID NO:3**;
- wherein said first and said second polypeptide chains are covalently bonded to one another by a disulfide bond.
- Claim 9. The diabody of any preceding claim for use as a pharmaceutical.
- Claim 10. The diabody of claim 8 for use in the treatment of a disease or condition associated with or characterized by the expression of CD123.
- Claim 11. The diabody of claim 10, wherein said disease or condition associated with or characterized by the expression of CD123 is cancer.
- Claim 12. The diabody of claim 11, wherein said 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), 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.

- Claim 13. The diabody of claim 10, wherein said disease or condition associated with or characterized by the expression of CD123 is an inflammatory condition.
- Claim 14. The diabody of claim 11, wherein said wherein said inflammatory condition is selected from the group consisting of: Autoimmune Lupus (SLE), allergy and asthma, rheumatoid arthritis.
- Claim 15. A pharmaceutical composition comprising the diabody of any of claims 1 to 8 and a physiologically acceptable carrier.
- Claim 16. Use of the pharmaceutical composition of claim 15 in the treatment of a disease or condition associated with or characterized by the expression of CD123.
- Claim 17. The use of claim 16, wherein said disease or condition associated with or characterized by the expression of CD123 is cancer.
- Claim 18. The use of claim 17, wherein said 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), 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.

Claim 19. The use of claim 16, wherein said disease or condition associated with or characterized by the expression of CD123 is an inflammatory condition.

Claim 20. The use of claim 19, wherein said inflammatory condition is selected from the group consisting of: Autoimmune Lupus (SLE), allergy and asthma.