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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure further have an immunoglobulin Fc Domain or an Albumin-Binding Domain to extend half-life in vivo.

[0024] In ~~detail~~one aspect, there ~~invention also is~~ provided 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:

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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~In another aspect, there is provide~~s~~d ~~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~~disclosure exhibit unexpectedly enhanced functional activities as further described below.

[0033] The diabodies of the ~~invention~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure are preferably capable of inhibiting the growth of a MOLM-13 tumor xenograft in a mouse. Preferably the diabodies of the ~~invention~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure are preferably capable of inhibiting the growth of an RS4-11 tumor xenograft in a mouse. Preferably the diabodies of the ~~invention~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure 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~~disclosure may exhibit one, two, three, more than three or all of the functional attributes described herein. Thus the diabodies of the ~~invention~~disclosure may exhibit any combination of the functional attributes described herein.

[0045] In one aspect, there is provided any of the above-described ~~The~~ diabodies ~~of the invention may be~~ for use as a pharmaceutical. ~~Preferably~~In one aspect, there is provided any of the above-described ~~the~~ diabodies ~~are~~ for use in the treatment of a disease or condition associated with or characterized by the expression of CD123. The ~~invention~~disclosure also relates to the use of diabodies of the ~~invention~~disclosure in the manufacture of a pharmaceutical composition, preferably for the treatment of a disease or condition associated with or characterized by the expression of 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~~In one aspect, there is provide~~s~~d a pharmaceutical composition comprising any of the above-described diabodies and a physiologically acceptable carrier.

[0048a] In one aspect, there is provided use of the above-described pharmaceutical composition in the manufacture of a medicament for treatment of a disease or condition associated with or characterized by the expression of CD123.

[0049] The ~~invention~~disclosure additionally provides a use of the above-described pharmaceutical composition in the treatment of a disease or condition associated with or characterized by the expression of CD123.

[0050] The ~~invention~~disclosure 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 mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, and Burkitt's lymphoma).

[0051] The ~~invention~~disclosure 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

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-coli~~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 sub-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**).

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 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~~13, 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 manufacture of a medicament for 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-