

Retargeting Molecules To Achieve Optimal Redirected T-Cell Killing Of B-Cell Lymphoma,” Blood 117(17):4542-4551). Compared with the bi-specific scFv-CD19 x CD3 BiTE, this molecule exhibited similar binding affinities but had a lower EC₅₀ for target cell lysis *in vitro* of ~0.5 to 5 pg/ml and showed consistently higher levels of maximum lysis. AFM11, a CD19 x CD3 a bi-specific Tandab has also been described. AFM11 is reported to have an EC₅₀ for target cell lysis *in vitro* of ~0.5 to 5 pg/ml (Zhukovsky *et al.* (2013) “A CD19/CD3 Bispecific Tandab, AFM11, Recruits T Cells To Potently and Safely Kill CD19+ Tumor Cells,” J Clin Oncol 31, 2013 (suppl; abstr 3068)). None of these constructs include an Fc domain.

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

Summary:

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

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

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

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

- I. the first polypeptide chain comprises, in the N-terminal to C-terminal direction:
 - A. a Domain IA, comprising
 - (1) a sub-Domain (IA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and
 - (2) a sub-Domain (IA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});wherein the sub-Domains IA1 and IA2 are separated from one another by a polypeptide linker, and are either
 - (a) VL_{CD19} and VH_{CD3}; or

- (b) VL_{CD3} and VH_{CD19} ;
 - B. a Domain IB, comprising a charged Heterodimer-Promoting Domain, wherein the Domain IB is separated from the Domain 1A by a polypeptide linker;
 - C. a Domain IC, comprising a CH2-CH3 Domain of an antibody; and
- II. the second polypeptide chain comprises, in the N-terminal to C-terminal direction:
 - A. a Domain IIA, comprising
 - (1) a sub-Domain (IIA1), which comprises a VL Domain capable of binding to either CD19 (VL_{CD19}) or CD3 (VL_{CD3}); and
 - (2) a sub-Domain (IIA2), which comprises a VH Domain capable of binding to either CD19 (VH_{CD19}) or CD3 (VH_{CD3});
 wherein the sub-Domains IIA1 and IIA2 are separated from one another by a polypeptide linker, and are:
 - (a) VL_{CD19} and VH_{CD3} , if the sub-Domains IIA1 and IIA2 are VL_{CD3} and VH_{CD19} ; or
 - (b) VL_{CD3} and VH_{CD19} , if the sub-Domains IIA1 and IIA2 are VL_{CD19} and VH_{CD3} ;
 - B. a Domain IIB, comprising a charged Heterodimer-Promoting Domain, wherein the Domain IIB is separated from the Domain IIA by a polypeptide linker, and wherein the charged Heterodimer-Promoting Domain of the Domain IB and the charged Heterodimer-Promoting Domain of the Domain IIB have opposite charges; and
- III. the third polypeptide chain comprises, in the N-terminal to C-terminal direction a Domain IIIC that comprises a CH2-CH3 Domain of an antibody; wherein the VL_{CD19} and the VH_{CD19} domains form a CD19 binding domain, and the VL_{CD3} and VH_{CD3} domains form a CD3 binding domain; and the CH2-CH3 Domains of the first and third polypeptide chains form an Fc domain capable of binding to an Fc receptor, thereby forming the CD19 x CD3 bi-specific monovalent diabody.

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

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

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

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

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

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

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

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

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

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

[0032] In another aspect, there is provided any of the above-described CD19 x CD3 bi-specific monovalent Fc diabodies for use as a pharmaceutical.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

- Claim 13. A covalently associated polypeptide complex, wherein said polypeptide complex comprises a first polypeptide chain and a second polypeptide chain, wherein:
- (A) said first polypeptide chain comprises a polypeptide having the amino acid sequence of SEQ ID NO:2 linked to a Heterodimer-Promoting Domain having the amino acid sequence of SEQ ID NO:12; and
 - (B) said second polypeptide chain comprises a polypeptide having the amino acid sequence of SEQ ID NO:2 linked to a Heterodimer-Promoting Domain having the amino acid sequence of SEQ ID NO:13;
- wherein said Heterodimer-Promoting Domains of said first polypeptide chain and said Heterodimer-Promoting Domains of said second polypeptide chain are covalently bonded to one another via a disulfide bond.
- Claim 14. A pharmaceutical composition comprising the CD19 x CD3 bi-specific monovalent Fc diabody of any one of claims 1-12 and a physiologically acceptable carrier.
- Claim 15. Use of the pharmaceutical composition of claim 14 in the manufacture of a medicament for the treatment of a disease or condition associated with or characterized by the expression of CD19.
- Claim 16. The use of claim 15, wherein said disease or condition associated with or characterized by the expression of CD19 is cancer.
- Claim 17. The use of claim 16, 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), diffuse large B cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), including Richter's