

FORMULATIONS FOR PROTEIN THERAPEUTICS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 63/121,633, filed December 4, 2020, and 62/960,562, filed January 13, 2020, each of which is incorporated by reference herein in its entirety for all purposes.

FIELD OF THE DISCLOSURE

[0002] The disclosure relates to formulations for protein therapeutics. Specifically, the disclosure relates to compositions comprising a bispecific or multispecific protein, a buffer, an excipient, and a surfactant. More specifically, the disclosure relates to formulations for a bispecific or multispecific protein comprising a CD123 binding domain and a CD3 binding domain. The disclosure also relates to clinical methods, including dosing regimens, for administration of the protein therapeutics to a subject in need thereof.

SEQUENCE LISTING

[0003] This application contains a Sequence Listing which has been submitted electronically and is hereby incorporated by reference in its entirety. The Sequence Listing was recorded January 13, 2021, is named APVO_061_02SeqList_ST25.txt, and is about 103 kilobytes in size.

BACKGROUND

[0004] One of the key steps in the development of a successful protein therapeutic is development of a formulation that maintains the physical and chemical integrity of the protein during long-term storage, handling by healthcare professionals, and administration. Protein therapeutics to be administered by the intravenous (i.v.) route are often stored frozen, in a concentrated solution, and diluted in the clinic before use. Moreover, protein therapeutics such as bispecific antibodies and multispecific antibodies, especially those comprising one or more scFv domains, can be prone to formation of aggregates.

[0005] Developing a protein formulation, particularly for administration by the intravenous route, requires careful consideration of many factors, including the properties of the protein, the composition of the formulation, the choice of diluent,

storage temperature, infusion rate, exposure to light, etc. There is a need in the art for stable formulations for protein therapeutics, particularly for administration by the intravenous route.

[0006] There is also a need for dosing strategies to mitigate the risk associated with the effects of cytokine release in patients treated with bispecific and multispecific therapeutics that act by T-cell engagement (i.e., T-cell engagers). This class of therapeutics includes bispecific therapeutics that target CD123 and CD3. mAb14045 (Xencor), a CD123 x CD3 bispecific antibody molecule being evaluated in patients with relapsed or refractory acute myeloid leukemia and other CD123-expressing hematologic malignancies, was placed on a partial clinical hold by the FDA in 2019 due to the deaths of two patients in a Phase I trial, including one death caused by cytokine release syndrome (CRS).

[0007] Dosing strategies designed to reduce the likelihood of severe effects of cytokine release, including cytokine release syndrome, may fail to be therapeutically effective. Accordingly, a need remains for methods to deliver a therapeutically effective dose of a T-cell engager (such as a CD123 x CD3 therapeutic) to a patient in a manner that mitigates risk of toxicity, including cytokine toxicity.

BRIEF SUMMARY

[0008] Described herein are compositions comprising protein therapeutics, including multispecific polypeptides and fusion proteins, for intravenous administration. The compositions may comprise, for example, a multispecific protein, a buffer, an excipient, and a surfactant. In some embodiments, the compositions may comprise a multispecific protein, a succinate buffer, sucrose, and polysorbate 80. In some embodiments, the composition is for intravenous or subcutaneous administration.

[0009] The disclosure provides multispecific polypeptides that are formulated with a succinate buffer and sucrose. In some embodiments, the multispecific polypeptide comprises two or more scFv binding domains. In some embodiments, the multispecific polypeptide forms a homodimer. In other embodiments, the multispecific polypeptide forms a heterodimer. In some embodiments, the multispecific polypeptide is in a format selected from the group consisting of scFv-Fc-scFv (e.g., ADAPTIR®), quadromas, K λ -bodies, dAbs, diabodies, TandAbs, nanobodies, DOCK-AND-LOCKS® (DNLs®), CrossMab Fabs, CrossMab VH-VLs, strand-exchange engineered domain bodies

(SEEDbodies), Affibodies, Fynomers, Kunitz Domains, Albu-dabs, two engineered Fv fragments with exchanged VHs (e.g., a dual-affinity re-targeting molecules (D.A.R.T.s)), scFv x scFv (e.g., BiTE), DVD-IG, Covx-bodies, peptibodies, scFv-Igs, SVD-Igs, dAb-Igs, Knobs-in-Holes, IgG1 antibodies comprising matched mutations in the CH3 domain (e.g., DuoBody antibodies) and triomAbs.

[0010] In some embodiments, the disclosure provides a composition comprising a multispecific protein, a buffer, an excipient and a surfactant, wherein the multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof. In some embodiments, each polypeptide comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 31.

[0011] In some embodiments, the composition comprises from about 1 mM to about 10 mM succinate, or a pharmaceutically acceptable salt or acid thereof. In some embodiments, the composition comprises about 5 mM succinate, or a pharmaceutically acceptable salt or acid thereof.

[0012] In some embodiments, the excipient comprises or consists of a sugar, such as sucrose. In some embodiments, the composition may comprise about 1% weight/volume (w/v) to about 12% w/v of the sugar. In some embodiments, the composition comprises about 6.5% (w/v) of the sugar.

[0013] In some embodiments, the surfactant comprises or consists of polysorbate 80. In some embodiments, the composition comprises about 0.02% w/v polysorbate 80.

[0014] In some embodiments, the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein. For example, the composition may comprise from about 1 mg/ml to about 5 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80. In some embodiments, the composition has a pH from about 4.0 to about 5.5. In some embodiments, the composition has a pH of about 4.8.

[0015] In some embodiments, the immunoglobulin constant region is a human Fc domain. In some embodiments, the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD.

[0016] In some embodiments, the first binding domain is a CD3 binding domain and the second binding domain is a tumor antigen binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the CD3 binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain. In some embodiments, the first domain is a tumor antigen binding domain, and the second binding domain is a CD3 binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the CD3 binding domain. In some embodiments, the tumor antigen binding domain binds to CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0017] In some embodiments, the first binding domain is a 4-1-BB binding domain and the second binding domain is a tumor antigen binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the 4-1-BB binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain. In some embodiments, the first binding domain is a tumor antigen binding domain, and the second binding domain is a 4-1-BB binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the 4-1-BB binding domain. In some embodiments, the tumor antigen binding domain binds to CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0018] In some embodiments, at least one of the first binding domain and the second binding domain comprises (i) an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, at least one of the first binding domain and the second binding domain is a single chain variable fragment (scFv). In some embodiments, the light chain variable region of the scFv is carboxy-terminal to the heavy chain variable region of the scFv. In some embodiments, the light chain variable region of the scFv is amino-terminal to the heavy chain variable region of the scFv. In some embodiments, the scFv comprises a linker polypeptide. The linker polypeptide may be, for example, between the light chain variable region and the heavy chain variable region of the scFv. In some embodiments,

the linker polypeptide comprises a Gly₄Ser (SEQ ID NO: 128) linker, such as (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129).

[0019] In some embodiments, a tumor antigen binding domain is an anti-CD123 scFv comprising a HCDR1 that comprises SEQ ID NO: 10, a HCDR2 that comprises SEQ ID NO: 11, and a HCDR3 that comprises SEQ ID NO: 12; and a LCDR1 that comprises SEQ ID NO: 13, a LCDR2 that comprises SEQ ID NO: 14, and a LCDR3 that comprises SEQ ID NO: 15. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv comprising a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv, and wherein the scFv comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18.

[0020] In some embodiments, a CD3 binding domain is an anti-CD3 scFv comprising a HCDR1 that comprises SEQ ID NO: 19, a HCDR2 that comprises SEQ ID NO: 20, and a HCDR3 that comprises SEQ ID NO: 21; and a LCDR1 that comprises SEQ ID NO: 22, a LCDR2 that comprises SEQ ID NO: 23, and a LCDR3 that comprises SEQ ID NO: 24. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

[0021] In some embodiments, the immunoglobulin constant region comprises one or more mutations to reduce/prevent FcγR binding, ADCC activity, and/or CDC activity. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, E318A, K320A and K322A, according to the EU numbering system. In some embodiments, the immunoglobulin comprises the sequence of SEQ ID NO: 131, or a sequence at least 90% or at least 95% identical thereto.

[0022] In some embodiments, the hinge region is derived from an immunoglobulin hinge region.

[0023] In some embodiments, each polypeptide comprises an Fc-binding domain linker between the immunoglobulin constant region, and the second binding domain. In some embodiments, the Fc-binding domain linker comprises a Gly₄Ser (SEQ ID NO: 128) sequence. In some embodiments, the Fc-binding domain linker comprises the formula (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129).

[0024] In some embodiments, the composition substantially prevents degradation of the multispecific protein. In some embodiments, the composition is substantially stable for at least 1 year at 4°C. In some embodiments, the composition is substantially resistant to formation of aggregates of multispecific protein.

[0025] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a first binding domain that specifically binds to CD123, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain that specifically binds to CD3; and (b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0026] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises a first binding domain that specifically binds to CD123 and a second binding domain that specifically binds to CD3; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0027] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ

ID NO: 23, and LCDR3 of SEQ ID NO: 24; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0028] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

[0029] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 136; and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 134; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 383 or 387; and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 384; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0030] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the first binding domain comprises SEQ ID NO: 18; and (ii) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises SEQ ID NO: 27; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0031] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein is a dimer of two

identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15, (ii) a hinge region of SEQ ID NO: 47, (iii) an immunoglobulin constant region of SEQ ID NO: 33, (iv) a Fc-binding domain linker of SEQ ID NO: 132, and (v) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0032] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15, (ii) a hinge region of SEQ ID NO: 47, (iii) an immunoglobulin constant region of SEQ ID NO: 33, (iv) a Fc-binding domain linker of SEQ ID NO: 132, and (v) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

[0033] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein comprises or consists of SEQ ID NO: 31;

wherein the composition comprises about 2 mg/ml of the fusion protein; and wherein the composition has a pH of about 4.8.

[0034] The disclosure further provides a method for inhibiting the growth of psoriatic plaques in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a composition of the disclosure.

[0035] The disclosure further provides a method for treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a composition of the disclosure. The cancer may be, for example, a hematological malignancy. For instance, the cancer may be acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm, B-cell acute lymphoblastic leukemia (ALL), or chronic myeloid leukemia (CML).

[0036] Also provided are uses of the compositions of the disclosure for treating cancer in a subject. Also provided are uses of the compositions of the disclosure in the manufacture of a medicament for treating cancer. For instance, compositions of the disclosure may be used for treatment of acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). In some embodiments, compositions of the disclosure may be used for treatment of high-risk or high-grade MDS. A composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain may be administered to a subject by IV infusion at a weekly dose of about 0.3, about 1, about 3, about 6, about 9, about 12, about 18, about 20, about 24, about 30, about 36, about 50, about 48, about 60, about 75, or about 100 μ g. To reduce the risk of an adverse event, the first dose may be administered by IV to the patient over several hours, e.g., about 20-24 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours. The composition can also be administered to a subject by continuous IV infusion, e.g., continuous IV infusion up to about 72 hours in duration.

[0037] A method for treatment of a patient in need thereof may include administering multispecific protein comprising a CD123 binding domain and a CD3 binding domain to a patient intravenously such that the dosage is increased each week for at least the first two or first three doses. For instance, a composition may be

delivered to the patient by IV infusion according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; and week 4 dosage and subsequent week dosages: 12 µg. In some embodiments, a composition may be administered to a patient intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; and week 4 dosage and subsequent week dosages: 18 µg. In some embodiments, the highest dose administered to the patient is about 24 µg, about 36 µg, about 48 µg, about 60 µg, or about 100 µg. In some embodiments, the highest dose administered to the patient is in the range of about 100 µg to about 130 µg.

[0038] In some embodiments, a composition may be administered to a patient in a treatment cycle lasting about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, or more. In some embodiments, the composition may be administered to the patient over more than one treatment cycle, such as 2, 3, 4, 5, 6, 7, 8, or more treatment cycles. In some embodiments the treatment cycle lasts 4 weeks, and may be repeated for up to 6 cycles. In some embodiments, the treatment cycle may be repeated for up to 36 cycles.

[0039] In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage: 6 µg; and week 2 and subsequent week dosages: 9 µg, and in some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule week 1 dosage: 9 µg; and week 2 and subsequent week dosages: 12 µg. In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage 12 µg, and week 2 and subsequent week dosages: 18 µg.

[0040] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 18 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 9 µg is administered on day 15, and 9 µg is administered on day 22. In some embodiments, 9 µg is administered on day 1, 12 µg is administered on day 8, 12 µg is administered on day

15, and 12 µg is administered on day 22. In some embodiments, 12 µg is administered on day 1, 18 µg is administered on day 8, 18 µg is administered on day 15, and 18 µg is administered on day 22. In some embodiments, a patient treated according to the methods of the disclosure exhibits a decrease in bone marrow blast percentage, and in some embodiments, a patient exhibits a decrease in absolute blast counts in the blood. In some embodiments, the treatment results in reduction in patient blast levels in the blood by at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 45%, at least 50%, or more, compared to the patient's levels immediately before the treatment.

[0041] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a 28-day cycle. In some embodiments, the composition is administered to the patient once, twice, three times, or four times each week during the 28-day cycle. In some embodiments, the dosage is increased over the course of the 28-day cycle. In some embodiments, the dosage is decreased over the course of the 28-day cycle. In some embodiments, the dosage is increased each week during the 28-day cycle. In some embodiments, the dosage is decreased each week during the 28-day cycle.

[0042] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22 of a first 28-day cycle, and on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the dose administered on day 22 of the first 28-day cycle is the same as the dose administered on days 1, 8, 15, and 22 of the at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0043] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, and 22 of a first 28-day cycle. In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain

to the patient on days 1, 2, 3, 4, 8, 11, 15, and 22 of a first 28-day cycle, and then subsequently on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0044] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, 18, 22, and 25 of a first 28-day cycle. In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, 18, 22, and 25 of a first 28-day cycle, and then subsequently on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0045] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 μ g of the multispecific protein is administered on day 1, 9 μ g of the multispecific protein is administered on day 2, 12 μ g of the multispecific protein is administered on day 3, 18 μ g of the multispecific protein is administered on day 4, 18 μ g of the multispecific protein is administered on day 8, 18 μ g of the multispecific protein is administered on day 11, 36 μ g of the multispecific protein is administered on day 15, and 36 μ g of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 36 μ g of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0046] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 μ g of the multispecific protein is administered on day 1, 12 μ g of

the multispecific protein is administered on day 2, 18 µg of the multispecific protein is administered on day 3, 24 µg of the multispecific protein is administered on day 4, 24 µg of the multispecific protein is administered on day 8, 24 µg of the multispecific protein is administered on day 11, 48 µg of the multispecific protein is administered on day 15, and 48 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 48 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0047] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 36 µg of the multispecific protein is administered on day 8, 36 µg of the multispecific protein is administered on day 11, 60 µg of the multispecific protein is administered on day 15, and 60 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 60 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0048] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 48 µg of the multispecific protein is administered on day 8, 48 µg of the multispecific protein is administered on day 11, 100 µg of the multispecific protein is administered on day 15, and 100 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein

100 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0049] In some embodiments, the highest dose administered to the patient in any one of the aforementioned schemes is increased by about 5% to about 40%, such as about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, or about 40%. In these embodiments, this increased dose may first be administered to the patient on the first day that the highest dose was previously administered in the aforementioned schemes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] **FIG. 1A** and **FIG. 1B** are schematics showing the structures of exemplary therapeutic proteins for use with the compositions and methods of the disclosure. **FIG. 1A** shows a homodimeric protein comprising two identical polypeptides each comprising a CD3 binding domain and an Fc domain. **FIG. 1B** shows a homodimeric protein comprising two identical polypeptides each comprising a tumor binding domain (e.g., a CD123 binding domain), an Fc domain, and a CD3 binding domain. An exemplary CD123 x CD3 bispecific therapeutic protein is referred to herein as TRI130.

[0051] **FIG. 2** is a schematic that shows the design of a Phase 1/1b dose escalation clinical study, wherein TRI130 is administered to patients with relapsed or refractory acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS).

[0052] **FIG. 3A-3D** show percentage of blasts in bone marrow aspirates, plotted over time for patients in the Phase 1/1b study described in **FIG. 2** and Example 3. Data is graphed for patients in cohorts receiving a highest dose of ≥ 12 µg. N = 14 patients evaluable for changes from baseline. Cohorts 6a and 6b tested different step dosing regimens, as shown in Table 10.

[0053] **FIG. 4A-4D** show serum concentrations of interleukin-6 (IL-6, **FIG. 4A**), interleukin-10 (IL-10, **FIG. 4B**), interferon-gamma (IFN-γ, **FIG. 4C**) and tumor necrosis factor-alpha (TNF-α, **FIG. 4D**) in patient samples from scheduled blood collections (pre-dose, about 15-30 minutes post-dose, and about 20-26 hours post-dose; N=26) in the Phase 1/1b study described in **FIG. 2** and Example 3. The scheduled blood

collections were collected at the first administration of the highest planned dose for each patient. Peak cytokine levels observed at unscheduled collections during IRR/CRS events are shown for comparison (N=6 events in 4 patients).

DETAILED DESCRIPTION

[0054] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited herein, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated documents or portions of documents define a term that contradicts that term's definition in the application, the definition that appears in this application controls. However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as an acknowledgment, or any form of suggestion, that they constitute valid prior art or form part of the common general knowledge in any country in the world.

[0055] In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components unless otherwise indicated. The use of the alternative (e.g., "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include" and "comprise" are used synonymously. In addition, it should be understood that the polypeptides comprising the various combinations of the components (e.g., domains or regions) and substituents described herein, are disclosed by the present application to the same extent as if each polypeptide was set forth individually. Thus, selection of particular components of individual polypeptides is within the scope of the present disclosure.

Definitions

[0056] The term "about" when immediately preceding a numerical value means $\pm$ up to 10% of the numerical value. For example, "about 40" means $\pm$ up to 10% of 40 (i.e., from 36 to 44), for example $\pm$ up to 10%, $\pm$ up to 9%, $\pm$ up to 8%, $\pm$ up to 7%, $\pm$

up to 6%, $\pm$ up to 5%, $\pm$ up to 4%, $\pm$ up to 3%, $\pm$ up to 2%, $\pm$ up to 1%, $\pm$ up to less than 1%, or any other value or range of values therein.

[0057] As used herein, substantially has its ordinary meaning as used in the art. For example, “substantially” may mean “significantly,” “considerably,” “largely,” “mostly,” or “essentially.” In some embodiments, “substantially” may refer to at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%.

[0058] The term “CD123” may refer to any isoform of CD123, also known as Cluster of Differentiation 123, Interleukin-3 receptor alpha chain, and IL3RA. CD123 associates with the beta chain of the interleukin-3 receptor to form the receptor. CD123 is a type I transmembrane glycoprotein, with an extracellular domain comprising a predicted Ig-like domain and two FnIII domains. The CD123-binding domains of the disclosure bind to the extracellular domain of CD123. CD123 is also known as the alpha chain of the human interleukin-3 (IL-3) receptor. CD123 is a type I transmembrane glycoprotein and is a member of the cytokine receptor superfamily. The interleukin-3 receptor is a heterodimer formed by CD123 and the beta chain (CD131). IL-3 binds to CD123, and signal transduction is provided by CD131. IL-3 regulates the function and production of hematopoietic and immune cells and stimulates endothelial cell proliferation (Testa et al., *Biomark Res.* 2:4 (2014)).

[0059] CD123 is overexpressed in many hematologic malignancies, including a subset of acute myeloid leukemia (AML), B-lymphoid leukemia, blastic plasmacytoid dendritic neoplasms (BPDCN) and hairy cell leukemia. While most AML patients respond well to initial therapies, the majority of AML patients are ultimately diagnosed with relapsed or refractory disease (Ramos et al., *J. Clin. Med.* 4:665-695 (2015)). There is a need for molecules targeting CD123 with increased efficiency and potency and reduced adverse effects and that may be used to treat disorders associated with dysregulation of CD123.

[0060] “CD3” is known in the art as a multi-protein complex of six chains (see, e.g., Abbas and Lichtman, 2003; Janeway et al., p. 172 and 178, 1999), which are subunits of the T-cell receptor complex. In mammals, the CD3 subunits of the T-cell receptor complex are a CD3 γ chain, a CD3 δ chain, two CD3 ϵ chains, and a homodimer of CD3 ζ chains. The CD3 γ , CD3 δ , and CD3 ϵ chains are highly related cell surface proteins of the immunoglobulin superfamily containing a single immunoglobulin domain. The

transmembrane regions of the CD3 γ , CD3 δ , and CD3 ϵ chains are negatively charged, which is a characteristic that allows these chains to associate with the positively charged T-cell receptor chains. The intracellular tails of the CD3 γ , CD3 δ , and CD3 ϵ chains each contain a single conserved motif known as an immunoreceptor tyrosine-based activation motif or ITAM, whereas each CD3 ζ chain has three. It is believed the ITAMs are important for the signaling capacity of a TCR complex. CD3 as used in the present disclosure can be from various animal species, including human, monkey, mouse, rat, or other mammals.

[0061] “Cytokine release” or “cytokine storm” or “infusion reaction” refers to the release of cytokines from T-cells. When cytokines are released into the circulation, systemic symptoms such as fever, nausea, chills, hypotension, tachycardia, asthenia, headache, rash, scratchy throat, and dyspnea can result. Some patients may experience severe, life-threatening reactions that result from massive release of cytokines. “Reduced” cytokine release refers to the reduction in the release of at least one cytokine (e.g., IFN- γ , TNF- α , IL-6, IL-2, IL-8, IL-10, IL-17, GM-CSF, IL-4, IL-12, IL-13 or IL-1 β) following administration of a bispecific molecule as disclosed herein, as compared to the OKT3 antibody (which binds CD3) or other CD3 binding bispecific molecule. Reduced cytokine release can be measured using *in vitro* assays or *in vivo* assays.

[0062] As used herein, the term “step dosing” or “stepped dosing” or similar terms refers to a dosing regimen wherein a multispecific polypeptide as described herein is administered to a patient on at least a first day and a second day, wherein the dose administered to the patient is either kept constant or increased between the first day and the second day. For example, in some step dosing regimens, a patient may be administered a first, a second, a third, and a fourth dose, wherein each dose is administered on different day, and wherein the second dose is greater than the first dose. The third dose may be greater than the second dose, or may be the same as the second dose. The fourth dose may be greater than the third dose, or may be the same as the third dose. In some embodiments, if the patient has an adverse response to a particular dose, the subsequent dose may be reduced.

[0063] As used herein, the term “binding domain” or “binding region” refers to the domain, region, portion, or site of a protein, polypeptide, oligopeptide, peptide, antibody, or binding domain derived from an antibody, receptor or ligand that possesses the ability to specifically recognize and bind to a target molecule, such as

an antigen, ligand, receptor, substrate, or inhibitor. Exemplary binding domains include, antibodies and antibody-like proteins or domains, antibody heavy and light chain variable regions, and single-chain antibody variable regions (e.g., domain antibodies, sFv, scFv, scFab), receptor ectodomains and ligands (e.g., cytokines, chemokines). In certain embodiments, the binding domain comprises or consists of an antigen binding site (e.g., comprising a variable heavy chain sequence and variable light chain sequence or three light chain complementary determining regions (CDRs) and three heavy chain CDRs from an antibody placed into alternative framework regions (FRs) (e.g., human FRs optionally comprising one or more amino acid substitutions). A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, including Western blot, ELISA, phage display library screening, and BIACORE® interaction analysis.

[0064] A binding domain or protein comprising a binding domain “specifically binds” a target if it binds the target with an affinity or K_a (i.e., an equilibrium association constant of a particular binding interaction with units of $1/M$) equal to or greater than $10^5 M^{-1}$, while not significantly binding other components present in a test sample. Binding domains can be classified as “high affinity” binding domains and “low affinity” binding domains. “High affinity” binding domains refer to those binding domains with a K_a of at least $10^7 M^{-1}$, at least $10^8 M^{-1}$, at least $10^9 M^{-1}$, at least $10^{10} M^{-1}$, at least $10^{11} M^{-1}$, at least $10^{12} M^{-1}$, or at least $10^{13} M^{-1}$. “Low affinity” binding domains refer to those binding domains with a K_a of up to $10^7 M^{-1}$, up to $10^6 M^{-1}$, up to $10^5 M^{-1}$. Alternatively, affinity can be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (e.g., $10^{-5} M$ to 10^{-13} , or about 500 nM, about 300 nM, about 250 nM, about 200 nM, about 150 nM, about 100 nM, about 50 nM, about 25 nM, about 10 nM, or about 5 nM). Affinities of binding domain polypeptides and single chain polypeptides according to the present disclosure can be readily determined using conventional techniques (see, e.g., Scatchard et al. (1949) Ann. N.Y. Acad. Sci. 51:660; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

[0065] As used herein, a “conservative substitution” is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well-known in the art (see, e.g., PCT Application Publication No. WO 97/09433, page 10, published March 13, 1997; Lehninger, Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge,

MA (1990), p. 8). In certain embodiments, a conservative substitution includes a leucine to serine substitution.

[0066] As used herein, the term "derivative" refers to a modification of one or more amino acid residues of a peptide by chemical or biological means, either with or without an enzyme, *e.g.*, by glycosylation, alkylation, acylation, ester formation, or amide formation.

[0067] As used herein, a polypeptide or amino acid sequence "derived from" a designated polypeptide or protein refers to the origin of the polypeptide. In certain embodiments, the polypeptide or amino acid sequence which is derived from a particular sequence (sometimes referred to as the "starting" or "parent" or "parental" sequence) and has an amino acid sequence that is essentially identical to the parent sequence or a portion thereof, wherein the portion consists of at least 10-20 amino acids, at least 20-30 amino acids, or at least 30-50 amino acids, or at least 50-150 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the parent sequence. For example, a binding domain can be derived from an antibody, *e.g.*, a Fab, F(ab')₂, Fab', scFv, single domain antibody (sdAb), etc.

[0068] Polypeptides derived from another polypeptide can have one or more mutations or alterations relative to the parent polypeptide, *e.g.*, one or more amino acid residues which have been substituted with another amino acid residue or which has one or more amino acid insertions or deletions. In such embodiments, polypeptides derived from a parent polypeptide and comprising one or more mutations or alteration are referred to as "variants." As used herein, the term "variant" or "variants" refers to a polynucleotide or polypeptide with a sequence differing from that of a reference polynucleotide or polypeptide but retaining essential properties thereof. Generally, variant polynucleotide or polypeptide sequences are overall closely similar, and, in many regions, identical to the reference polynucleotide or polypeptide. For instance, a variant polynucleotide or polypeptide may exhibit at least about 70%, at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity compared to the active portion or full length reference polynucleotide or polypeptide. The polypeptide can comprise an amino acid sequence which is not naturally occurring. Such variations necessarily have less than 100% sequence identity or similarity with the parent polypeptide. In one embodiment, the variant will have an amino acid sequence from

about 60% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the parent polypeptide. In another embodiment, the variant will have an amino acid sequence from about 75% to less than 100%, from about 80% to less than 100%, from about 85% to less than 100%, from about 90% to less than 100%, from about 95% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the parent polypeptide.

[0069] As used herein, the term “sequence identity” refers to a relationship between two or more polynucleotide sequences or between two or more polypeptide sequences. When a position in one sequence is occupied by the same nucleic acid base or amino acid residue in the corresponding position of the comparator sequence, the sequences are said to be “identical” at that position. The percentage sequence identity is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of identical positions. The number of identical positions is then divided by the total number of positions in the comparison window and multiplied by 100 to yield the percentage of sequence identity. Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window. The comparison window for polynucleotide sequences can be, for instance, at least about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900 or about 1000 or more nucleic acids in length. The comparison window for polypeptide sequences can be, for instance, at least about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 300 or more amino acids in length. In order to optimally align sequences for comparison, the portion of a polynucleotide or polypeptide sequence in the comparison window can comprise additions or deletions termed gaps while the reference sequence is kept constant. An optimal alignment is that alignment which, even with gaps, produces the greatest possible number of “identical” positions between the reference and comparator sequences. Percentage “sequence identity” between two sequences can be determined using the version of the program “BLAST 2 Sequences” which was available from the National Center for Biotechnology Information as of September 1, 2004, which program incorporates the programs

BLASTN (for nucleotide sequence comparison) and BLASTP (for polypeptide sequence comparison), which programs are based on the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90(12):5873-5877, 1993). When utilizing "BLAST 2 Sequences," parameters that were default parameters as of September 1, 2004, can be used for word size (3), open gap penalty (11), extension gap penalty (1), gap dropoff (50), expect value (10) and any other required parameter including but not limited to matrix option. Two nucleotide or amino acid sequences are considered to have "substantially similar sequence identity" or "substantial sequence identity" if the two sequences have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity relative to each other.

[0070] As used herein, unless otherwise provided, a position of an amino acid residue in a variable region of an immunoglobulin molecule is numbered according to the IMGT numbering convention (Brochet, X, *et al*, Nucl. Acids Res. (2008) 36, W503-508), and a position of an amino acid residue in a constant region of an immunoglobulin molecule is numbered according to EU nomenclature (Ward *et al.*, 1995 *Therap. Immunol.* 2:77-94). Other numbering conventions are known in the art (e.g., the Kabat numbering convention (Kabat, Sequences of Proteins of Immunological Interest, 5th ed. Bethesda, MD: Public Health Service, National Institutes of Health (1991)).

[0071] As used herein, the term "dimer" refers to a biological entity that consists of two subunits associated with each other via one or more forms of intramolecular forces, including covalent bonds (e.g., disulfide bonds) and other interactions (e.g., electrostatic interactions, salt bridges, hydrogen bonding, and hydrophobic interactions), and is stable under appropriate conditions (e.g., under physiological conditions, in an aqueous solution suitable for expressing, purifying, and/or storing recombinant proteins, or under conditions for non-denaturing and/or non-reducing electrophoresis). A "heterodimer" or "heterodimeric protein," as used herein, refers to a dimer formed from two different polypeptides. A heterodimer does not include an antibody formed from four polypeptides (*i.e.*, two light chains and two heavy chains). A "homodimer" or "homodimeric protein," as used herein, refers to a dimer formed from two identical polypeptides. All disclosure of the polypeptide, including characteristics and activities (such as binding and RTCC) should be understood to include the polypeptide in its dimer form as well as other multimeric forms.

[0072] When a polypeptide of the disclosure is in dimeric form (i.e., a dimeric protein), it contains two binding sites at the amino-terminus and two binding sites at the carboxyl terminus. The binding domains are thus considered bivalent (i.e., two binding portions at each terminus) when the single chain polypeptides are dimerized.

[0073] An “immunoglobulin constant region” or “constant region” is a term defined herein to refer to a peptide or polypeptide sequence that corresponds to or is derived from part or all of one or more constant domains of an immunoglobulin. In certain embodiments, the constant region comprises IgG CH2 and CH3 domains, e.g., IgG1 CH2 and CH3 domains. In certain embodiments, the constant region does not comprise a CH1 domain. In certain embodiments, the constant domains making up the constant region are human. In some embodiments, the constant region of a fusion protein of this disclosure lacks or has minimal effector functions while retaining the ability to bind some Fc receptors such as the neonatal Fc receptor (FcRn) and retaining a relatively long half-life *in vivo*. For example, the constant region of a fusion protein of this disclosure do not result in, or substantially reduce the induction of antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), complement activation, and/or complement-dependent cytotoxicity (CDC). In other variations, a fusion protein of this disclosure comprises constant domains that retain one or more effector functions, such as of one or both of ADCC and CDC. In certain embodiments, a binding domain of this disclosure is fused to a human IgG1 constant region, wherein the IgG1 constant region has one or more of the following amino acids mutated: leucine at position 234 (L234), leucine at position 235 (L235), glycine at position 237 (G237), glutamate at position 318 (E318), lysine at position 320 (K320), lysine at position 322 (K322), or any combination thereof (numbering according to EU). For example, any one or more of these amino acids can be changed to alanine. In a further embodiment, an IgG1 Fc domain has each of L234, L235, G237, E318, K320, and K322 (according to EU numbering) mutated to an alanine (i.e., L234A, L235A, G237A, E318A, K320A, and K322A, respectively), and optionally an N297A mutation as well (i.e., essentially eliminating glycosylation of the CH2 domain).

[0074] The terms “light chain variable region” (also referred to as “light chain variable domain” or “V_L”) and “heavy chain variable region” (also referred to as “heavy chain variable domain” or “V_H”) refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of

discrete, well-defined sub-regions known as “complementarity determining regions” (CDRs) and “framework regions” (FRs). In one embodiment, the FRs are humanized. The term “CL” refers to an “immunoglobulin light chain constant region” or a “light chain constant region,” i.e., a constant region from an antibody light chain. The term “CH” refers to an “immunoglobulin heavy chain constant region” or a “heavy chain constant region,” which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A “Fab” (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 domain of the heavy chain linked to the light chain via an inter-chain disulfide bond.

[0075] As used herein, the term “linker” generally refers to a short polypeptide sequence connecting two sub-domains of a polypeptide. Non-limiting examples of linkers include flexible linkers comprising glycine-serine repeats, and linkers derived from (a) an interdomain region of a transmembrane protein (e.g., a type I transmembrane protein); or (b) an immunoglobulin hinge. In some embodiments, a linker provides a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that comprises the same light and heavy chain variable regions. In certain embodiments, a linker is comprised of five to about 35 amino acids, for instance, about 15 to about 25 amino acids. As used herein, the phrase a “linker between CH3 and CH1 or CL” refers to one or more amino acid residues (e.g., about 2-12, about 2-10, about 4-10, about 5-10, about 6-10, about 7-10, about 8-10, about 9-10, about 8-12, about 9-12, or about 10-12) between the C-terminus of a CH3 domain (e.g., a wild type CH3 or a mutated CH3) and the N-terminus of a CH1 domain or CL domain (e.g., Cκ).

[0076] In some embodiments, depending on context, a linker may refer to (1) a polypeptide region between V_H and V_L regions in a single-chain Fv (scFv) or (2) a polypeptide region between a first binding domain and a second binding domain in a multispecific polypeptide comprising two binding domains. In the later example, wherein a linker connects two or more binding domains, such a linker is referred to herein as a “Fc-binding domain linker.” In some embodiments, a Fc-binding domain linker may directly link or connect two or more binding domains, resulting in a construct comprising the following structure: binding domain – Fc-binding domain linker – binding domain. In some embodiments, the multispecific polypeptides described

herein comprise, in order from amino-terminus to carboxyl-terminus (i) a first binding domain, (ii) a Fc-binding domain linker, and (iii) a second binding domain. In some embodiments, a multispecific polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a second binding domain, (ii) a Fc-binding domain linker, and (iii) a first binding domain. In some embodiments, a Fc-binding domain linker may link or connect two or more binding domains by linking at least one binding domain to a non-binding domain polypeptide, such as an immunoglobulin Fc domain (*i.e.*, a polypeptide comprising the structure: Ig hinge – Ig constant region). In such embodiments, the resulting constructs may comprise the following structure: binding domain – Fc domain – Fc-binding domain linker – binding domain. In some embodiments, the multispecific polypeptides described herein comprise, in order from amino-terminus to carboxyl-terminus: (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) a Fc-binding domain linker, and (v) a second binding domain. In some embodiments, a multispecific polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a second binding domain, (ii) a Fc-binding domain linker, (iii) an immunoglobulin constant region, (iv) a hinge region, and (v) a first binding domain. A polypeptide region between an immunoglobulin constant region and a second binding domain in a multispecific polypeptide comprising two binding domains (*e.g.*, a Fc-binding domain linker) may also be referred to as a “carboxyl-terminus linker” or an “amino-terminus linker” depending on the orientation of the domains within the multispecific polypeptide. Non-limiting examples of linkers are provided in Table 1.

[0077] In some embodiments, a “hinge” or a “hinge region” refers to a polypeptide derived from an immunoglobulin hinge region and located between a binding domain and an immunoglobulin constant region in a polypeptide described herein. A “wild-type immunoglobulin hinge region” refers to a naturally occurring upper and middle hinge amino acid sequences interposed between and connecting the CH1 and CH2 domains (for IgG, IgA, and IgD) or interposed between and connecting the CH1 and CH3 domains (for IgE and IgM) found in the heavy chain of an antibody. In certain embodiments, a wild type immunoglobulin hinge region sequence is human, and can comprise a human IgG hinge region (*e.g.*, and IgG1, IgG2, IgG3, or IgG4 hinge region).

[0078] An “altered immunoglobulin hinge region” or “variant immunoglobulin hinge region” refers to a hinge region polypeptide with one or more mutations, substitutions, insertions, or deletions compared to a corresponding parental wild-type immunoglobulin hinge region. In certain embodiments, an altered immunoglobulin

hinge region is at least about 70% identical to a wild-type immunoglobulin hinge region (e.g., at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% identical). In certain embodiments, an altered immunoglobulin hinge region is a fragment of a wild type immunoglobulin hinge region that has a length of about 5 amino acids (e.g., about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, or more amino acids) up to about 120 amino acids (for instance, having a length of about 10 to about 40 amino acids or about 15 to about 30 amino acids or about 15 to about 20 amino acids or about 20 to about 25 amino acids). Typically, an altered immunoglobulin hinge region that is a fragment of a wild type immunoglobulin hinge region comprises an IgG core hinge region (e.g., a polypeptide comprising the sequence C-X-X-C, wherein X is any amino acid (SEQ ID NO: 390)) as disclosed in U.S. Patent Application Publication Nos. 2013/0129723 and 2013/0095097. Non-limiting examples of hinges are provided in Table 2.

[0079] As used herein, the term “humanized” refers to a process of making an antibody or immunoglobulin binding proteins and polypeptides derived from a non-human species (e.g., mouse or rat) less immunogenic to humans, while still retaining antigen-binding properties of the original antibody, using genetic engineering techniques. In some embodiments, the binding domain(s) of an antibody or immunoglobulin binding proteins and polypeptides (e.g., light and heavy chain variable regions, Fab, scFv) are humanized. Non-human binding domains can be humanized using techniques known as CDR grafting (Jones *et al.*, Nature 321:522 (1986)) and variants thereof, including “reshaping” (Verhoeyen, *et al.*, 1988 Science 239:1534-1536; Riechmann, *et al.*, 1988 Nature 332:323-337; Tempest, *et al.*, Bio/Technol 1991 9:266-271), “hyperchimerization” (Queen, *et al.*, 1989 Proc Natl Acad Sci USA 86:10029-10033; Co, *et al.*, 1991 Proc Natl Acad Sci USA 88:2869-2873; Co, *et al.*, 1992 J Immunol 148:1149-1154), and “veneering” (Mark, *et al.*, “Derivation of therapeutically active humanized and veneered anti-CD18 antibodies.” In: Metcalf BW, Dalton BJ, eds. Cellular adhesion: molecular definition to therapeutic potential. New York: Plenum Press, 1994: 291-312). If derived from a non-human source, other regions of the antibody or immunoglobulin binding proteins and polypeptides, such as the hinge region and constant region domains, can also be humanized.

[0080] An “immunoglobulin dimerization domain” or “immunoglobulin heterodimerization domain”, as used herein, refers to an immunoglobulin domain of a polypeptide chain that preferentially interacts or associates with a different immunoglobulin domain of a second polypeptide chain, wherein the interaction of the different immunoglobulin heterodimerization domains substantially contributes to or efficiently promotes heterodimerization of the first and second polypeptide chains (*i.e.*, the formation of a dimer between two different polypeptide chains, which is also referred to as a “heterodimer”). The interactions between immunoglobulin heterodimerization domains “substantially contributes to or efficiently promotes” the heterodimerization of first and second polypeptide chains if there is a statistically significant reduction in the dimerization between the first and second polypeptide chains in the absence of the immunoglobulin heterodimerization domain of the first polypeptide chain and/or the immunoglobulin heterodimerization domain of the second polypeptide chain. In certain embodiments, when the first and second polypeptide chains are co-expressed, at least 60%, at least about 60% to about 70%, at least about 70% to about 80%, at least 80% to about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% of the first and second polypeptide chains form heterodimers with each other. Representative immunoglobulin heterodimerization domains include an immunoglobulin CH1 domain, an immunoglobulin CL domain (*e.g.*, C κ or C λ isotypes), or derivatives thereof, including wild type immunoglobulin CH1 and CL domains and altered (or mutated) immunoglobulin CH1 and CL domains, as provided therein.

[0081] The terms patient and subject are used interchangeably herein. As used herein, the term “patient in need” or “subject in need” refers to a patient or subject at risk of, or suffering from, a disease, disorder or condition that is amenable to treatment or amelioration with a binding protein or multispecific polypeptide or a composition thereof provided herein. “Patient” and “subject” are used interchangeably herein.

[0082] As used herein, the term “pharmaceutically acceptable” refers to molecular entities and compositions that do not generally produce allergic or other serious adverse reactions when administered using routes well known in the art. Molecular entities and compositions 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 are considered to be “pharmaceutically acceptable.”

[0083] As used herein, the terms "nucleic acid," "nucleic acid molecule," or "polynucleotide" refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka *et al.* (1985) *J. Biol. Chem.* 260:2605-2608; Cassol *et al.* (1992); Rossolini *et al.* (1994) *Mol. Cell. Probes* 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene. As used herein, the terms "nucleic acid," "nucleic acid molecule," or "polynucleotide" are intended to include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs thereof.

[0084] The term "expression" refers to the biosynthesis of a product encoded by a nucleic acid. For example, in the case of nucleic acid segment encoding a polypeptide of interest, expression involves transcription of the nucleic acid segment into mRNA and the translation of mRNA into one or more polypeptides.

[0085] The terms "expression unit" and "expression cassette" are used interchangeably herein and denote a nucleic acid segment encoding a polypeptide of interest and capable of providing expression of the nucleic acid segment in a host cell. An expression unit typically comprises a transcription promoter, an open reading frame encoding the polypeptide of interest, and a transcription terminator, all in operable configuration. In addition to a transcriptional promoter and terminator, an expression unit can further include other nucleic acid segments such as, e.g., an enhancer or a polyadenylation signal.

[0086] The term "expression vector," as used herein, refers to a nucleic acid molecule, linear or circular, comprising one or more expression units. In addition to one or more expression units, an expression vector can also include additional nucleic acid segments such as, for example, one or more origins of replication or one or more

selectable markers. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both.

[0087] As used herein, a “polypeptide,” “polypeptide chain,” or “protein” refers to a contiguous arrangement of covalently linked amino acids. Polypeptides can form one or more intrachain disulfide bonds. With regard to polypeptides as described herein, reference to modifications or alterations of amino acid residues corresponding to those specified by SEQ ID NO includes post-translational modifications of such residues. The terms polypeptide and protein also encompass embodiments where two polypeptide chains link together in a non-linear fashion, such as via an interchain disulfide bond. For example, a native immunoglobulin molecule is comprised of two heavy chain polypeptides and two light chain polypeptides.

[0088] As used herein, a “multispecific polypeptide” refers to a polypeptide comprising two or more binding domains each capable of specifically binding to a target antigen. For example, the polypeptides described herein may comprise 2, 3, 4, or more binding domains and may be able to bind 2, 3, 4, or more target antigens. In some embodiments, a multispecific polypeptide is a bispecific polypeptide. Herein, a “bispecific polypeptide” comprises two binding domains and capable of binding to two distinct target antigens. In some embodiments, the bispecific polypeptides described herein comprise a first binding domain that specifically binds to a cell surface antigen expressed on a target cell. In some embodiments, the bispecific polypeptides described herein comprise a binding domain that specifically binds to a cell surface antigen expressed on an effector cell. A binding domain may be derived from an antibody (e.g., a variable heavy chain and/or variable light chain, scFv), a ligand, or a receptor.

[0089] Multispecific polypeptides are disclosed, for instance, in PCT Publication Nos. WO 2007/146968; WO 2010/040105; WO 2010/003108; WO 2016/094873; WO 2017/053469; U.S. Patent Application Publication No. 2006/0051844; and U.S. Patent Nos. 7,166,707; and 8,409,577, which are each incorporated herein by reference in their entirety. In certain embodiments, the multispecific polypeptides described herein are bispecific polypeptides and may comprise an scFv-Fc-scFv structure, also referred to herein as an ADAPTIR™ polypeptide. The structure of a polypeptide comprising such a structure comprises, from N-terminus to C-terminus: a first scFv binding domain – an immunoglobulin (Ig) hinge region – an Ig constant region – a second scFv binding domain.

[0001] A protein or polypeptide may be an antibody or an antigen-binding fragment of an antibody. In some embodiments, a protein may be a recombinant multispecific protein. In other embodiments, a multispecific protein may be produced by chemically linking two different monoclonal antibodies or by fusing two hybridoma cell lines to produce a hybrid-hybridoma. Other multivalent formats that can be used include, for example, quadromas, K λ -bodies, dAbs, diabodies, TandAbs, nanobodies, Small Modular ImmunoPharmaceuticals (SMIPs™), DOCK-AND-LOCKS® (DNLs®), CrossMab Fabs, CrossMab VH-VLs, strand-exchange engineered domain bodies (SEEDbodies), Affibodies, Fynomers, Kunitz Domains, Albu-dabs, two engineered Fv fragments with exchanged VHs (e.g., a dual-affinity re-targeting molecules (D.A.R.T.s)), scFv x scFv (e.g., BiTE), DVD-IG, Covx-bodies, peptibodies, scFv-Igs, SVD-Igs, dAb-Igs, Knobs-in-Holes, IgG1 antibodies comprising matched mutations in the CH3 domain (e.g., DuoBody antibodies) and triomAbs. Exemplary bispecific formats are discussed in Garber et al., *Nature Reviews Drug Discovery* 13:799-801 (2014), which is herein incorporated by reference in its entirety. Additional exemplary bispecific formats are discussed in Liu et al. *Front. Immunol.* 8:38 doi: 10.2289/fimmu.2017.00038, and Brinkmann and Kontermann, *MABS* 9: 2, 182–212 (2017), each of which is herein incorporated by reference in its entirety. In certain embodiments, a bispecific antibody can be a F(ab')₂ fragment. A F(ab')₂ fragment contains the two antigen-binding arms of a tetrameric antibody molecule linked by disulfide bonds in the hinge region.

[0090] As will be appreciated by one of skill in the art, proteins and polypeptides are defined herein in terms of the amino acid sequences of the individual polypeptide chains, which are indicated by the SEQ ID NOs referenced throughout this disclosure. For example, in some embodiments an scFv-Fc-scFv protein or polypeptide described herein is comprised of two scFv-Fc-scFv polypeptide chains associated by interchain bonds (e.g., interchain disulfide bonds) to form a dimeric scFv-Fc-scFv protein (e.g., a homodimeric or heterodimeric scFv-Fc-scFv protein). In such embodiments, the scFv-Fc-scFv protein is defined by the amino acid sequences of the individual scFv-Fc-scFv polypeptide chains. Polypeptides and proteins can also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents can be added to a protein or polypeptide by the cell in which the protein is produced, and will vary with the type of cell. Proteins and polypeptides are

defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

[0091] The terms “light chain variable region” (also referred to as “light chain variable domain” or “VL” or V_L) and “heavy chain variable region” (also referred to as “heavy chain variable domain” or “VH” or V_H) refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of discrete, well-defined sub-regions known as “complementarity determining regions” (CDRs) and “framework regions” (FRs), generally comprising in order FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 from amino-terminus to carboxyl-terminus. In one embodiment, the FRs are humanized. The term “CL” refers to an “immunoglobulin light chain constant region” or a “light chain constant region,” i.e., a constant region from an antibody light chain. The term “CH” refers to an “immunoglobulin heavy chain constant region” or a “heavy chain constant region,” which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A “Fab” (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 domain of the heavy chain linked to the light chain via an inter-chain disulfide bond.

[0092] The terms “amino-terminal” and “carboxyl-terminal” are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl-terminus of the reference sequence, but is not necessarily at the carboxyl-terminus of the complete polypeptide.

[0093] As used herein, the term “transformation,” “transfection,” and “transduction” refer to the transfer of nucleic acid (i.e., a nucleotide polymer) into a cell. As used herein, the term “genetic transformation” refers to the transfer and incorporation of DNA, especially recombinant DNA, into a cell. The transferred nucleic acid can be introduced into a cell via an expression vector.

[0094] “Antibody-dependent cell-mediated cytotoxicity” and “ADCC,” as used herein, refer to a cell-mediated process in which nonspecific cytotoxic cells that express FcγRs (e.g., monocytic cells such as natural killer (NK) cells and macrophages) recognize bound antibody (or other protein capable of binding FcγRs)

on a target cell and subsequently cause lysis of the target cell. In principle, any effector cell with an activating Fc γ R can be triggered to mediate ADCC. The primary cells for mediating ADCC are NK cells, which express only Fc γ RIII, whereas monocytes, depending on their state of activation, localization, or differentiation, can express Fc γ RI, Fc γ RII, and Fc γ RIII. For a review of Fc γ R expression on hematopoietic cells, see, *e.g.*, Ravetch et al., 1991, *Annu. Rev. Immunol.*, 9:457-92.

[0095] The term “having ADCC activity,” as used herein in reference to a polypeptide or protein, means that the polypeptide or protein, for example, one comprising an Fc domain (*e.g.*, an immunoglobulin hinge region and an immunoglobulin constant region having CH2 and CH3 domains) such as derived from IgG (*e.g.*, IgG1), is capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) through binding of a cytolytic Fc receptor (*e.g.*, Fc γ RIII) on a cytolytic immune effector cell expressing the Fc receptor (*e.g.*, an NK cell). In some embodiments, a multispecific polypeptide or protein comprising an Fc domain may lack effector function (*e.g.*, null ADCC activity) as the result of mutations in the CH2 and/or CH3 domain.

[0096] “Complement-dependent cytotoxicity” and “CDC,” as used herein, refer to a process in which components in normal serum (“complement”), together with an antibody or other C1q-complement-binding protein bound to a target antigen, exhibit lysis of a target cell expressing the target antigen. Complement consists of a group of serum proteins that act in concert and in an orderly sequence to exert their effect.

[0097] The terms “classical complement pathway” and “classical complement system,” as used herein, are synonymous and refer to a particular pathway for the activation of complement. The classical pathway requires antigen-antibody complexes for initiation and involves the activation, in an orderly fashion, of nine major protein components designated C1 through C9. For several steps in the activation process, the product is an enzyme that catalyzes the subsequent step. This cascade provides amplification and activation of large amounts of complement by a relatively small initial signal.

[0098] The term “having CDC activity,” as used herein in reference to a polypeptide or protein, means that the polypeptide or protein, for example, one comprising an Fc domain (*e.g.*, an immunoglobulin hinge region and an immunoglobulin constant region having CH2 and CH3 domains) such as derived from IgG (*e.g.*, IgG1) is capable of

mediating complement-dependent cytotoxicity (CDC) through binding of C1q complement protein and activation of the classical complement system. In some embodiments, a multispecific polypeptide or protein may lack effector function (e.g., null CDC activity) as the result of one or more mutations in the CH2 and/or CH3 domains.

[0099] "Enhanced effector cell activation" as used herein, refers to the increase, prolonging, and/or potentiation of an effector cell response by the polypeptides or proteins described herein. In some embodiments, enhanced effector cell activation refers to an increase in the cytotoxic activity of an effector cell. In some embodiments, enhanced effector cell activation refers to an increase in cytokine production, cell proliferation, or a change in cell-surface molecule expression such that the ability of the effector cell to lyse a target cell is enhanced.

[0100] As used herein, the term "effector cell" refers to a cell of the immune system that is capable of lysing or killing a target cell, such as a tumor cell. Herein, an effector cell may refer to a lymphocyte, such as a T cell, a natural killer (NK) cell, or an NKT cell, a monocyte, a macrophage, a dendritic cell, or a granulocyte. In particular embodiments, the term effector cell refers to a T cell, an NK cell, or an NKT cell.

[0101] As used herein, the terms "treatment," "treating," or "ameliorating" refers to either a therapeutic treatment or prophylactic/preventative treatment. A treatment is therapeutic if at least one symptom of disease in an individual receiving treatment improves or a treatment can delay worsening of a progressive disease in an individual, or prevent onset of additional associated diseases.

[0102] As used herein, the term "therapeutically effective amount (or dose)" or "effective amount (or dose)" of a polypeptide or protein described herein or a composition thereof refers to that amount of the compound sufficient to result in amelioration of one or more symptoms of the disease being treated in a statistically significant manner or a statistically significant improvement in organ function. When referring to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone. When referring to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered serially or simultaneously (in the same formulation or concurrently in separate formulations).

Pharmaceutical Compositions

[0103] Described herein are stable pharmaceutical formulations of protein therapeutics, such as multispecific polypeptides, that prevent denaturation and/or prevent or substantially reduce the formation of aggregates, especially upon freezing. In addition to a therapeutic protein, the pharmaceutical compositions described herein may further comprise one or more of a buffer, an excipient, and a surfactant. In some embodiments, the compositions comprise, consist of, or consist essentially of a buffer, an excipient and a surfactant, wherein the multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0104] In some embodiments, the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein. In some embodiments, the composition comprises from about 1 mg/ml to about 5 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80.

[0105] In some embodiments, wherein the composition substantially prevents degradation of the multispecific protein. In some embodiments, the composition slows or reduces the degradation of the multispecific polypeptide as compared to an identical multispecific polypeptide stored in histidine buffer under identical storage conditions. In some embodiments, the composition is substantially stable for at least 1 year at 4°C. In some embodiments, the composition is substantially resistant to formation of aggregates of multispecific protein.

[0106] In some embodiments, the composition is capable of withstanding freeze to thaw conditions. In some embodiments, the composition slows or reduces degradation of the multispecific polypeptide in freeze to thaw conditions as compared to a multispecific polypeptide stored in a histidine buffer under identical freeze to thaw conditions.

[0107] In other embodiments, a CD123 x CD3 targeting multispecific polypeptide undergoes little to no degradation after lyophilization when formulated as disclosed herein. For instance, a CD123 x CD3 targeting multispecific polypeptide may be

formulated in a succinate and sucrose formulation that exhibits reduced degradation after lyophilization as compared to an identical polypeptide formulated with a histidine buffer. Also provided herein is a lyophilized anti-CD123 x anti-CD3 multispecific polypeptide, including but not limited to TRI130 and TRI129, formulated in about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80. In some embodiments, the composition is lyophilized.

Buffer

[0108] As used herein, the term “buffer” or “buffering agent” refers to one or more components that when added to an aqueous solution is able to protect the solution against variations in pH when adding acid or alkali, or upon dilution with a solvent.

[0109] In some embodiments, the buffer comprises, consists of, or consists essentially of any pharmaceutically acceptable buffer. For example, the buffer may be potassium phosphate, acetic acid/sodium acetate, citric acid/sodium citrate, succinic acid/sodium succinate, tartaric acid/sodium tartrate, histidine/histidine HCl, glycine, Tris, glutamate, acetate, mixtures thereof, or pharmaceutically acceptable salts or acids thereof. In particular embodiments, the buffer comprises, consists of, or consists essentially of succinate or a pharmaceutically acceptable salt or acid thereof.

[0110] In some embodiments, the concentration of the buffer in the composition is from about 1 mM to about 500 mM, from about 1mM to about 100 mM, from about 1mM to about 50 mM, from about 1 to about 10 mM, from about 5 mM to about 50 mM, or from about 5mM to about 20 mM from about 5 mM to about 10mM. In some embodiments, the composition comprises from about 1 mM to about 10 mM succinate or a pharmaceutically acceptable salt or acid thereof. In some embodiments, the composition comprises about 5 mM succinate or a pharmaceutically acceptable salt or acid thereof.

[0111] In some embodiments, the pH of the composition is 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, 5.75, 6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5, 9.75, 10.0, 10.25, 10.5, 10.75, 11.0, 11.25 or 11.5. In some embodiments, the pH of the composition is about 3.0 to about 6.0. In some embodiments, the composition has a pH from about 4.0 to about 5.5. In some embodiments, the pH of the composition is about 4.8.

Excipient

[0112] As referred to herein, an excipient is a pharmacologically inactive substance formulated alongside the active pharmaceutical ingredient of a composition. Excipients might aid in lubricity, flowability, disintegration, or taste and may confer some form of antimicrobial function.

[0113] Exemplary excipients which may be used in the compositions disclosed herein include pharmaceutical binders, diluents, release retarding excipients, lubricant, glidants, gas generating agents, coating systems, solvents, and coloring agents. Suitable excipients include the substances mentioned as excipients in the Handbook of Pharmaceutical Excipients, Third Edition, Edited by A. H. Kibbe, American Pharmaceutical Association and Pharmaceutical Press (2000), and Tables 3-5 in E. T. Cole et al., Advanced Drug Delivery Reviews 60 (2008), 747-756. For example, an excipient may be selected from the group consisting of polypropylene glycol; polyethylene glycol, polyoxyethylene castor oil derivatives, polyoxyethylene glycerol oxystearate, saturated polyglycolized glycerides, polyethylene polypropylene glycol, Vitamin E, and Vitamin E TPGS (d-alpha - tocopheryl polyethylene glycol 1000 succinate).

[0114] In some embodiments, the composition comprises from about 1% weight/volume (w/v) to about 20% w/v, about 1% w/v to about 10% w/v, about 5% w/v to about 15% w/v, or about 10% w/v of the excipient. In some embodiments, the composition comprises from about 1% w/v to about 12% w/v of the excipient, such as about 6.5% w/v of the excipient.

[0115] In some embodiments, the excipient the excipient comprises, consists of, or consists essentially of a sugar. In some embodiments, the composition comprises from about 1% w/v to about 12% w/v of the sugar. In some embodiments, the composition comprises about 4% to about 8% w/v of the sugar. In some embodiments, the composition comprises about 6.5% w/v of the sugar. In some embodiments, the sugar is sucrose.

Surfactant

[0116] As described herein, a "surfactant" is a surface active molecule containing both a hydrophobic portion (e.g., alkyl chain) and a hydrophilic portion (e.g., carboxyl and carboxylate groups).

[0117] Surfactants suitable for use in the compositions described herein include, but are not limited to, polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g.

poloxamer 188); sorbitan esters and derivatives; Triton; sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetadine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauramidopropyl-cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropylbetaine (e.g., lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate); and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethylene glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g., Pluronic, PF68 etc.). In particular embodiments, the surfactant comprises or consists of polysorbate 80.

[0118] In some embodiments, the composition comprises from about 0.001% w/v to about 1% w/v, about 0.01% w/v to about 0.5% w/v, or about 0.01% w/v to about 0.1% w/v of the surfactant. In some embodiments, the composition comprises about 0.02% w/v of the surfactant.

[0119] In some embodiments, the composition comprises from about 0.001% w/v to about 1% w/v, about 0.01% w/v to about 0.5% w/v, or about 0.01% w/v to about 0.1% w/v of polysorbate 80. In some embodiments, the composition comprises about 0.02% w/v of polysorbate 80.

Therapeutic Proteins

[0120] The compositions described herein may be used in connection with many different protein therapeutics as described herein.

Binding domains

[0121] In some embodiments, the therapeutic proteins comprise a binding domain. The binding domain may provide for specific binding to at least one cell-surface molecule (e.g., a cell-surface receptor). The binding domain can be in the form of an antibody, or fragment thereof, or a fusion protein of any of a variety of different formats (e.g., the fusion protein can be in the form of a bispecific or multispecific molecule). In other embodiments, the binding domain can comprise, for example, a particular cytokine or a molecule that targets the binding domain polypeptide to, for example, a particular cell type, a toxin, an additional cell receptor, or an antibody.

[0122] In some embodiments, a binding domain described herein is derived from an antibody and comprises a variable heavy chain (V_H) and a variable light chain (V_L).

For example, an scFv comprising a V_H and V_L chain. These binding domains and variable chains may be arranged in any order that still retains some binding to the target(s). In some embodiments, a binding domain comprises (i) an immunoglobulin heavy chain variable region (V_H) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (V_L) comprising LCDR1, LCDR2, and LCDR3.

[0123] In some embodiments, the polypeptides and proteins described herein comprise binding domains that are scFvs. In such embodiments, the binding domains may be referred to as scFv domains. In some embodiments, a binding domain is a single-chain Fv fragment (scFv) that comprises V_H and V_L regions specific for a target of interest. In certain embodiments, the V_H and V_L regions are human or humanized. In some variations, a binding domain is a single-chain Fv (scFv) comprising V_L and V_H regions joined by a peptide linker.

[0124] In certain embodiments, the binding domains of the polypeptides described herein comprise (i) an immunoglobulin light chain variable region (V_L) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (V_H) comprising CDRs HCDR1, HCDR2, and HCDR3. In some embodiments, amino acid sequences provided for polypeptide constructs do not include the human immunoglobulin leader sequences. CDR sequences and amino acid substitution positions shown are those defined using the IMGT criteria (Brochet et al, Nucl. Acids Res. (2008) 36, W503-508).

[0125] In certain embodiments, a binding domain V_L and/or V_H region of the present disclosure is derived from a V_L and/or V_H of a parent V_L and/or V_H region (e.g., 1618/1619 as described in PCT Application Publication No. WO 2016/185016) and optionally contains about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (e.g., conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the V_L and/or V_H sequence of a known monoclonal antibody. The insertion(s), deletion(s) or substitution(s) can be anywhere in the V_L and/or V_H region, including at the amino- or carboxyl-terminus or both ends of this region, provided that each CDR comprises zero changes or at most one, two, or three changes. In some embodiments, the binding domain containing the modified

V_L and/or V_H region can still specifically bind its target with an affinity similar to or greater than the parent binding domain.

[0126] The use of peptide linkers for joining V_L and V_H regions is well-known in the art, and a large number of publications exist within this particular field. In some embodiments, a peptide linker is a 15mer consisting of three repeats of a Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 128) amino acid sequence ((Gly₄Ser)₃) (SEQ ID NO: 59). Other linkers have been used, and phage display technology, as well as selective infective phage technology, has been used to diversify and select appropriate linker sequences (Tang *et al.*, *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke *et al.*, *Protein Eng.* 11, 405-410, 1998). In certain embodiments, the V_L and V_H regions are joined by a peptide linker having an amino acid sequence comprising the formula (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129). For instance, in some embodiments, the linker comprises (Gly₄Ser)₄ (SEQ ID NO: 61). Other suitable linkers can be obtained by optimizing a simple linker through random mutagenesis. In some embodiments, the V_H region of the scFv described herein may be positioned N-terminally to a linker sequence. In some embodiments, the V_L region of the scFvs described herein may be positioned C-terminally to the linker sequence.

[0127] In some embodiments, the binding domain may bind to a tumor antigen, such as CD123, PSMA, CD19, CD33, 5T4, or HER2. In some embodiments, the binding domain may be a CD3 binding domain. In some embodiments, the binding domain may bind to 4-1-BB. In some embodiments, the binding domain may bind to OX40. In some embodiments, a formulated multispecific protein binds to both 4-1BB and OX40.

Hinge

[0128] In addition to a binding domain, the therapeutic polypeptides may further comprise a hinge region. In some embodiments, the hinge is an altered immunoglobulin hinge in which one or more cysteine residues in a wild type immunoglobulin hinge region are substituted with one or more other amino acid residues (*e.g.*, serine or alanine). Exemplary altered immunoglobulin hinges, carboxyl-terminus linkers, and amino-terminus linkers include an immunoglobulin human IgG1 hinge region having one, two or three cysteine residues found in a wild type human IgG1 hinge substituted by one, two or three different amino acid residues (*e.g.*, serine or alanine). An altered immunoglobulin hinge can additionally have a proline

substituted with another amino acid (e.g., serine or alanine). For example, the above-described altered human IgG1 hinge can additionally have a proline located carboxyl-terminal to the three cysteines of wild type human IgG1 hinge region substituted by another amino acid residue (e.g., serine, alanine). In one embodiment, the prolines of the core hinge region are not substituted. In certain embodiments, a hinge, a carboxyl-terminus linker, or an amino-terminus linker polypeptide comprises or is a sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a wild type immunoglobulin hinge region, such as a wild type human IgG1 hinge, a wild type human IgG2 hinge, or a wild type human IgG4 hinge.

Immunoglobulin Constant Domain

[0129] The therapeutic proteins may also comprise an immunoglobulin constant (Fc) domain (also referred to herein as a constant region, Fc domain, Fc region, and the like). In some embodiments, the constant region comprises IgG CH2 and CH3 domains, e.g., IgG1 CH2 and CH3 domains. In some embodiments, the constant region does not comprise a CH1 domain. In some embodiments, the immunoglobulin constant region is a human Fc domain. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to reduce or prevent binding to FcγR1, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb. In some embodiments, the constant domains making up the constant region are human or derived from human sequences. In some embodiments, the Fc domain comprises one or more mutations the Fc region to reduce or prevent complement fixation and interaction with Fcγ receptors. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce Fc-mediated T-cell activation. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce CDC activity. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce ADCC activity.

[0130] In some embodiments, the Fc region comprises one or more mutations at positions 234, 235, 237 and 322 of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations at positions 234, 235, 237, 318, 320 and 322 of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations L234A, L235A, G237A and K322A of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations L234A, L235A, G237A, E318A, K320A, and K322A of the CH2 domain, according to the EU numbering system. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions E233P, L234A, L235A, G237A, and K322A and a deletion of G236, according to the EU numbering system. In some embodiments, the Fc domain is derived from human IgG1. In some embodiments, the two or more mutations in the IgG1 Fc domain prevent or substantially reduce signaling through Fc-mediated cross-linking.

[0131] In some embodiments, the immunoglobulin constant region comprises an amino acid sequence of any one of SEQ ID NO:32-35, or a variant thereof. The inclusion of an immunoglobulin constant region slows clearance of the polypeptides and proteins of the present disclosure from circulation after administration to a subject. By mutations or other alterations, an immunoglobulin constant region further enables relatively easy modulation of polypeptide effector functions (*e.g.*, ADCC, ADCP, CDC, complement fixation, and binding to Fc receptors), which can either be increased or decreased depending on the disease being treated, as known in the art and described herein. In certain embodiments, the polypeptides and proteins described herein comprise an immunoglobulin constant region capable of mediating one or more of these effector functions. In other embodiments, one or more of these effector functions are reduced or absent in an immunoglobulin constant region of a polypeptide or protein described in the present disclosure, as compared to a corresponding wild-type immunoglobulin constant region.

[0132] An immunoglobulin constant region present in the polypeptides and proteins of the present disclosure can comprise or can be derived from part or all of: a CH2 domain, a CH3 domain, a CH4 domain, or any combination thereof. For example, an immunoglobulin constant region can comprise a CH2 domain, a CH3 domain, both CH2 and CH3 domains, both CH3 and CH4 domains, two CH3 domains, a CH4 domain, two CH4 domains, and a CH2 domain and part of a CH3 domain. In certain

embodiments, the polypeptides or proteins described herein do not comprise a CH1 domain.

[0133] A polypeptide or protein described herein may comprise a wild type immunoglobulin CH2 domain or an altered immunoglobulin CH2 domain from certain immunoglobulin classes or subclasses (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, or IgD) and from various species (including human, mouse, rat, and other mammals). In certain embodiments, a CH2 domain of a polypeptide or a protein described herein is a wild type human immunoglobulin CH2 domain, such as wild type CH2 domains of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, or IgD, as set forth in SEQ ID NOs: 115, 199-201 and 195-197, respectively, of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, the CH2 domain is a wild type human IgG1 CH2 domain as set forth in SEQ ID NO: 115 of U.S. Patent Application Publication No. US 2013/0129723 (said sequence incorporated by reference herein).

[0134] In certain embodiments, an altered CH2 region in a polypeptide or a protein of the present disclosure comprises or is a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to a wild type immunoglobulin CH2 region, such as the CH2 region of wild type human IgG1, IgG2, or IgG4, or mouse IgG2a (e.g.,IGHG2c).

[0135] An altered immunoglobulin CH2 region in a polypeptide or protein of the present disclosure can be derived from a CH2 region of various immunoglobulin isotypes, such as IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, and IgD, from various species (including human, mouse, rat, and other mammals). In certain embodiments, an altered immunoglobulin CH2 region in a fusion protein of the present disclosure can be derived from a CH2 region of human IgG1, IgG2 or IgG4, or mouse IgG2a (e.g., IGHG2c), whose sequences are set forth in SEQ ID NOs: 115, 199, 201, and 320 of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, an altered CH2 domain of a polypeptide or a protein described herein is an altered human IgG1 CH2 domain with mutations known in the art that enhance or reduce immunological activities (*i.e.*, effector functions) such as ADCC, ADCP, CDC, complement fixation, Fc receptor binding, or any combination thereof.

[0136] In certain embodiments, a CH2 domain of a polypeptide or a protein described herein is an altered immunoglobulin CH2 region (e.g., an altered human IgG1 CH2 domain) that comprises one or more amino acid deletions or substitutions. In some embodiments, the CH2 domain comprises an amino acid substitution at the asparagine of position 297 (e.g., asparagine to alanine). Such an amino acid substitution reduces or eliminates glycosylation at this site and abrogates efficient Fc binding to FcγR and C1q. The sequence of an altered human IgG1 CH2 domain with an Asn to Ala substitution at position 297 is set forth in SEQ ID NO: 324 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein). In some embodiments, the altered CH2 domain comprises at least one substitution or deletion at positions 234 to 238. For example, an immunoglobulin CH2 region can comprise a substitution at position 234, 235, 236, 237 or 238; positions 234 and 235; positions 234 and 236; positions 234 and 237; positions 234 and 238; positions 234-236; positions 234, 235 and 237; positions 234, 236 and 238; positions 234, 235, 237, and 238; positions 236-238; or any other combination of two, three, four, or five amino acids at positions 234-238. In some embodiments, an altered CH2 region comprises one or more (e.g., two, three, four or five) amino acid deletions at positions 234-238, for instance, at one of position 236 or position 237 while the other position is substituted. In certain embodiments, the amino acid residues at one or more of positions 234-238 has been replaced with one or more alanine residues. In further embodiments, only one of the amino acid residues at positions 234-238 have been deleted while one or more of the remaining amino acids at positions 234-238 can be substituted with another amino acid (e.g., alanine or serine).

[0137] In some embodiments, the above-noted mutation(s) decrease or eliminate the ADCC activity or Fc receptor-binding capability of a polypeptide that comprises the altered CH2 domain.

[0138] In certain other embodiments, a CH2 domain of a polypeptide or a protein described herein is an altered immunoglobulin CH2 region (e.g., an altered human IgG1 CH2 domain) that comprises one or more amino acid substitutions at positions 253, 310, 318, 320, 322, and 331. For example, an immunoglobulin CH2 region can comprise a substitution at position 253, 310, 318, 320, 322, or 331, positions 318 and 320, positions 318 and 322, positions 318, 320 and 322, or any other combination of two, three, four, five or six amino acids at positions 253, 310, 318, 320, 322, and 331.

In such embodiments, the above-noted mutation(s) decrease or eliminate the CDC activity of a polypeptide comprising the altered CH2 domain.

[0139] In certain other embodiments, in addition to the amino acid substitution at position 297, an altered CH2 region of a polypeptide or a protein described herein (e.g., an altered human IgG1 CH2 domain) can further comprise one or more (e.g., two, three, four, or five) additional substitutions at positions 234-238. For example, an immunoglobulin CH2 region can comprise a substitution at positions 234 and 297, positions 234, 235, and 297, positions 234, 236 and 297, positions 234-236 and 297, positions 234, 235, 237 and 297, positions 234, 236, 238 and 297, positions 234, 235, 237, 238 and 297, positions 236-238 and 297, or any combination of two, three, four, or five amino acids at positions 234-238 in addition to position 297. In addition or alternatively, an altered CH2 region can comprise one or more (e.g., two, three, four or five) amino acid deletions at positions 234-238, such as at position 236 or position 237. The additional mutation(s) decreases or eliminates the ADCC activity or Fc receptor-binding capability of a polypeptide comprising the altered CH2 domain. In certain embodiments, the amino acid residues at one or more of positions 234-238 have been replaced with one or more alanine residues. In further embodiments, only one of the amino acid residues at positions 234-238 has been deleted while one or more of the remaining amino acids at positions 234-238 can be substituted with another amino acid (e.g., alanine or serine).

[0140] In certain embodiments, in addition to one or more (e.g., 2, 3, 4, or 5) amino acid substitutions at positions 234-238, a mutated CH2 region of a polypeptide or a protein described herein (e.g., an altered human IgG1 CH2 domain) in a fusion protein of the present disclosure can contain one or more (e.g., 2, 3, 4, 5, or 6) additional amino acid substitutions (e.g., substituted with alanine) at one or more positions involved in complement fixation (e.g., at positions I253, H310, E318, K320, K322, or P331). Examples of mutated immunoglobulin CH2 regions include human IgG1, IgG2, IgG4 and mouse IgG2a CH2 regions with alanine substitutions at positions 234, 235, 237 (if present), 318, 320 and 322. An exemplary mutated immunoglobulin CH2 region is mouse IGHG2c CH2 region with alanine substitutions at L234, L235, G237, E318, K320, and K322.

[0141] In still further embodiments, in addition to the amino acid substitution at position 297 and the additional deletion(s) or substitution(s) at positions 234-238, an altered CH2 region of a polypeptide or a protein described herein (e.g., an altered

human IgG1 CH2 domain) can further comprise one or more (e.g., two, three, four, five, or six) additional substitutions at positions 253, 310, 318, 320, 322, and 331. For example, an immunoglobulin CH2 region can comprise a (1) substitution at position 297, (2) one or more substitutions or deletions or a combination thereof at positions 234-238, and one or more (e.g., 2, 3, 4, 5, or 6) amino acid substitutions at positions I253, H310, E318, K320, K322, and P331, such as one, two, three substitutions at positions E318, K320 and K322. The amino acids at the above-noted positions can be substituted by alanine or serine.

[0142] In certain embodiments, an immunoglobulin CH2 region of a polypeptide or a protein described herein comprises: (i) an amino acid substitution at the asparagines of position 297 and one amino acid substitution at position 234, 235, 236 or 237; (ii) an amino acid substitution at the asparagine of position 297 and amino acid substitutions at two of positions 234-237; (iii) an amino acid substitution at the asparagine of position 297 and amino acid substitutions at three of positions 234-237; (iv) an amino acid substitution at the asparagine of position 297, amino acid substitutions at positions 234, 235 and 237, and an amino acid deletion at position 236; (v) amino acid substitutions at three of positions 234-237 and amino acid substitutions at positions 318, 320 and 322; or (vi) amino acid substitutions at three of positions 234-237, an amino acid deletion at position 236, and amino acid substitutions at positions 318, 320 and 322.

[0143] Exemplary altered immunoglobulin CH2 regions with amino acid substitutions at the asparagine of position 297 include: human IgG1 CH2 region with alanine substitutions at L234, L235, G237 and N297 and a deletion at G236 (SEQ ID NO: 325 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG2 CH2 region with alanine substitutions at V234, G236, and N297 (SEQ ID NO: 326 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at F234, L235, G237 and N297 and a deletion of G236 (SEQ ID NO: 322 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at F234 and N297 (SEQ ID NO: 343 of U.S. Patent Application Publication No. US 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at L235 and N297 (SEQ ID NO: 344 of U.S. Patent Application Publication No. 2013/0129723, said sequence

incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at G236 and N297 (SEQ ID NO: 345 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and human IgG4 CH2 region with alanine substitutions at G237 and N297 (SEQ ID NO: 346 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein). These CH2 regions can be used in a polypeptide of the present disclosure.

[0144] In certain embodiments, in addition to the amino acid substitutions described above, an altered CH2 region of a polypeptide or a protein described herein (e.g., an altered human IgG1 CH2 domain) can contain one or more additional amino acid substitutions at one or more positions other than the above-noted positions. Such amino acid substitutions can be conservative or non-conservative amino acid substitutions. For example, in certain embodiments, P233 can be changed to E233 in an altered IgG2 CH2 region (see, e.g., SEQ ID NO: 326 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein). In addition or alternatively, in certain embodiments, the altered CH2 region can contain one or more amino acid insertions, deletions, or both. The insertion(s), deletion(s) or substitution(s) can be anywhere in an immunoglobulin CH2 region, such as at the N- or C-terminus of a wild type immunoglobulin CH2 region resulting from linking the CH2 region with another region (e.g., a binding domain or an immunoglobulin heterodimerization domain) via a hinge.

[0145] In certain embodiments, an altered CH2 domain of a polypeptide or protein described herein is a human IgG1 CH2 domain with alanine substitutions at positions 235, 318, 320, and 322 (i.e., a human IgG1 CH2 domain with L235A, E318A, K320A and K322A substitutions) (SEQ ID NO: 595 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and optionally an N297 mutation (e.g., to alanine). In certain other embodiments, an altered CH2 domain is a human IgG1 CH2 domain with alanine substitutions at positions 234, 235, 237, 318, 320 and 322 (i.e., a human IgG1 CH2 domain with L234A, L235A, G237A, E318A, K320A and K322A substitutions) (SEQ ID NO: 596 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and optionally an N297 mutation (e.g., to alanine).

[0146] In some embodiments, an immunoglobulin constant region of a polypeptide or a protein described herein comprises a human IgG1 CH2 domain comprising the

substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system.

[0147] The CH3 domain that can form an immunoglobulin constant region of a polypeptide or a protein described herein can be a wild type immunoglobulin CH3 domain or an altered immunoglobulin CH3 domain thereof from certain immunoglobulin classes or subclasses (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, IgM) of various species (including human, mouse, rat, and other mammals). In certain embodiments, a CH3 domain of a polypeptide described herein is a wild type human immunoglobulin CH3 domain, such as wild type CH3 domains of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM as set forth in SEQ ID NOs: 116, 208-210, 204-207, and 212, respectively of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, the CH3 domain is a wild type human IgG1 CH3 domain as set forth in SEQ ID NO: 116 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein).

[0148] In certain embodiments, a CH3 domain of a polypeptide described herein is an altered human immunoglobulin CH3 domain, such as an altered CH3 domain based on or derived from a wild-type CH3 domain of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM antibodies. For example, an altered CH3 domain can be a human IgG1 CH3 domain with one or two mutations at positions H433 and N434 (positions are numbered according to EU numbering). The mutations in such positions can be involved in complement fixation. In certain other embodiments, an altered CH3 domain of a polypeptide described herein can be a human IgG1 CH3 domain but with one or two amino acid substitutions at position F405 or Y407. The amino acids at such positions are involved in interacting with another CH3 domain. In certain embodiments, an altered CH3 domain of polypeptide described herein can be an altered human IgG1 CH3 domain with its last lysine deleted. The sequence of this altered CH3 domain is set forth in SEQ ID NO: 761 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein).

[0149] In certain embodiments, a polypeptide or a protein described herein comprises a CH3 domain that comprises so called "knobs-into-holes" mutations (see, Marvin and Zhu, *Acta Pharmacologica Sinica* 26:649-58, 2005; Ridgway *et al.*, *Protein Engineering* 9:617-21, 1966). More specifically, mutations can be introduced into each of the CH3 domains of each polypeptide chain so that the steric complementarity

required for CH3/CH3 association obligates these two CH3 domains to pair with each other. For example, a CH3 domain in one single chain polypeptide of a polypeptide heterodimer can contain a T366W mutation (a “knob” mutation, which substitutes a small amino acid with a larger one), and a CH3 domain in the other single chain polypeptide of the polypeptide heterodimer can contain a Y407A mutation (a “hole” mutation, which substitutes a large amino acid with a smaller one). Other exemplary knobs-into-holes mutations include (1) a T366Y mutation in one CH3 domain and a Y407T in the other CH3 domain, and (2) a T366W mutation in one CH3 domain and T366S, L368A and Y407V mutations in the other CH3 domain.

[0150] The CH4 domain that can form an immunoglobulin constant region a polypeptide or a protein described herein can be a wild type immunoglobulin CH4 domain or an altered immunoglobulin CH4 domain thereof from IgE or IgM molecules. In certain embodiments, the CH4 domain of a polypeptide described herein is a wild type human immunoglobulin CH4 domain, such as wild type CH4 domains of human IgE and IgM molecules as set forth in SEQ ID NOs: 213 and 214, respectively, of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, a CH4 domain of a polypeptide described herein is an altered human immunoglobulin CH4 domain, such as an altered CH4 domain based on or derived from a CH4 domain of human IgE or IgM molecules, which have mutations that increase or decrease an immunological activity known to be associated with an IgE or IgM Fc region.

[0151] In certain embodiments, an immunoglobulin constant region of a polypeptide or a protein described herein comprises a combination of CH2, CH3 or CH4 domains (*i.e.*, more than one constant region domain selected from CH2, CH3 and CH4). For example, the immunoglobulin constant region can comprise CH2 and CH3 domains or CH3 and CH4 domains. In certain other embodiments, the immunoglobulin constant region can comprise two CH3 domains and no CH2 or CH4 domains (*i.e.*, only two or more CH3). The multiple constant region domains that form an immunoglobulin constant region of the polypeptides described herein can be based on or derived from the same immunoglobulin molecule, or the same class or subclass immunoglobulin molecules. In certain embodiments, the immunoglobulin constant region is an IgG CH2-CH3 (*e.g.*, IgG1 CH2-CH3, IgG2 CH2-CH3, and IgG4 CH2-CH3) and can be a human (*e.g.*, human IgG1, IgG2, and IgG4) CH2CH3. For example, in certain embodiments, the immunoglobulin constant region of a polypeptide described

herein comprises (1) wild type human IgG1 CH2 and CH3 domains, (2) human IgG1 CH2 with N297A substitution (*i.e.*, CH2(N297A)) and wild type human IgG1 CH3, or (3) human IgG1 CH2(N297A) and an altered human IgG1 CH3 with the last lysine deleted. Alternatively, the multiple constant region domains of a polypeptide or a protein described herein can be based on or derived from different immunoglobulin molecules, or different classes or subclasses immunoglobulin molecules. For example, in certain embodiments, an immunoglobulin constant region comprises both human IgM CH3 domain and human IgG1 CH3 domain. The multiple constant region domains that form an immunoglobulin constant region of a polypeptide described herein can be directly linked together or can be linked to each other via one or more (*e.g.*, about 2-10) amino acids.

[0152] Exemplary immunoglobulin constant regions that can be used in a polypeptide or a protein described herein are set forth in SEQ ID NOs: 305-309, 321, 323, 341, 342, and 762 of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). Further exemplary immunoglobulin constant regions that can be used in a polypeptide or a protein described herein are provided in the table below.

Table 1: Exemplary immunoglobulin constant regions

Name	DNA Sequence	DNA SEQ ID NO:	AA Sequence	AA SEQ ID NO:
SS-Fc domain	TCGAGTGAGCCCAATCTTCTGACAAAAC TCACACATGCCCACCGTGCCACGACCTG AAGCCGCGGGTGCACCGTCAGTCTTCCTC TTCCCCCAAAACCAAGGACACCCTCAT GATCTCCCGGACCCCTGAGGTCACATGCG TGGTGGTGGACGTGAGCCACGAAGACCCT GAGGTCAAGTTCAACTGGTACGTGGACGG CGTGGAGGTGCATAATGCCAAGACAAAGC CGCGGGAGGAGCAGTACAACAGCACGTAC CGTGTGGTCAGCGTCCTCACCCTCCTGCA CCAGGACTGGCTGAATGGCAAGGAATACA AGTGCGCGGTCTCCAACAAAGCCCTCCCA GCCCCATCGAGAAAACCATCTCCAAAGC CAAAGGGCAGCCCCGAGAACCACAGGTGT ACACCCTGCCCCCATCCCGGGATGAGCTG ACCAAGAACCAGGTCAGCCTGACCTGCCT GGTCAAAGGCTTCTATCCAAGCGACATCG CCGTGGAGTGGGAGAGCAATGGGCAGCCG GAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCCTCT ACAGCAAGCTCACCGTGGACAAGAGCAGG TGGCAGCAGGGGAACGTCTTCTCATGCTC CGTGATGCATGAGGCTCTGCACAACCACT	32	SSEPKSSDKTHTCPPC PAPEAAGAPSVFLFPP KPKDTLMISRTPEVTC VVVDVSHEDPEVKFNW YVDGVEVHNAKTKPRE EQYNSTYRVVSVLTVL HQDWLNGKEYKCAVSN KALPAPIEKTISKAKG QPREPQVYTLPPSRDE LTKNQVSLTCLVKGFY PSDIAVEWESNGQPEN NYKTTTPVLDSDGSFF LYSKLTVDKSRWQQGN VFSCSVMHEALHNNHYT QKSLSLSPG	33

	ACACGCAGAAGAGCCTCTCCCTGTCTCCG GGT			
Delta SS-Fc domain	GAGCCCAAATCTTCTGACAAACTCACAC ATGCCACACCGTGCCAGCACCTGAAGCCG CGGGTGACACCGTCAGTCTTCTCTTCCCC CCAAAACCCAAGGACACCTCATGATCTC CCGGACCCCTGAGGTCACATGCGTGGTGG TGGACGTGAGCCACGAAGACCCTGAGGTC AAGTTCAACTGGTACGTGGACGGCGTGGA GGTGATAATGCCAAGACAAAGCCGCGGG AGGAGCAGTACAACAGCACGTACCGTGTG GTCAGCGTCCTCACCGTCCTGCACAGGA CTGGCTGAATGGCAAGGAATACAAGTGCG CGGTCTCCAACAAAGCCCTCCCAGCCCCC ATCGAGAAAACCATCTCCAAGCCAAAGG GCAGCCCCGAGAACCACAGGTGTACACCC TGCCCCCATCCCGGGATGAGCTGACCAAG AACCAGGTGAGCCTGACCTGCCTGGTCAA AGGCTTCTATCCAAGCGACATCGCCGTGG AGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACGCCTCCCGTGCTGGA CTCCGACGGCTCCTTCTTCTCTACAGCA AGCTCACCGTGGACAAGAGCAGGTGGCAG CAGGGGAACGTCTTCTCATGCTCCGTGAT GCATGAGGCTCTGCACAACCACTACACGC AGAAGAGCCTCTCCCTGTCTCCGGGT	34	EPKSSDKTHTCPPCPA PEAAGAPSVFLFPPKP KDTLMISRTPEVTCVV VDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQ DWLNGKEYKCAVSNKA LPAPIEKTISKAKGQP REPQVYTLPPSRDELT KNQVSLTCLVKGFYPS DIAVEWESNGQPENNY KTTTPVLDSDGSFFLY SKLTVDKSRWQQGNVF SCSVMEALHNHYTQK SLSLSPG	35

[0153] In certain embodiments, the immunoglobulin constant regions of each polypeptide chain of a homodimeric or heterodimeric protein described herein are identical to each other. In certain other embodiments, the immunoglobulin constant region of one polypeptide chain of a heterodimeric protein is different from the immunoglobulin constant region of the other polypeptide chain of the heterodimer. For example, one immunoglobulin constant region of a heterodimeric protein can contain a CH3 domain with a “knob” mutation, whereas the other immunoglobulin constant region of the heterodimeric protein can contain a CH3 domain with a “hole” mutation.

Fc-Binding Domain Linker

[0154] In some embodiments, the polypeptide may further comprise a Fc-binding domain linker. In some embodiments, the Fc-binding domain linker can be used to link the immunoglobulin constant region to a C-terminal binding domain (e.g., a CD3 binding domain). In some embodiments, the Fc-binding domain linker can be used as a hinge domain and/or incorporated into an scFv. In some embodiments, the Fc-binding domain linker is a Gly₄Ser linker (SEQ ID NO: 128). In some embodiments, the Fc-binding domain linker is a 20mer consisting of four repeats of a Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 128) amino acid sequence ((Gly₄Ser)₄) (SEQ ID NO:61). In some embodiments, the Fc-binding domain linker comprises an amino acid sequence

selected from any one of SEQ ID NOs 50-70. Other linkers have been used, and phage display technology, as well as selective infective phage technology, has been used to diversify and select appropriate linker sequences (Tang *et al.*, *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke *et al.*, *Protein Eng.* 11, 405-410, 1998). In certain embodiments, the V_L and V_H regions are joined by a peptide linker having an amino acid sequence comprising the formula (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129). Other suitable linkers can be obtained by optimizing a simple linker through random mutagenesis. In some embodiments, bispecific molecules do not comprise a hinge region or a constant region.

[0155] In certain embodiments, a Fc-binding domain linker is a flexible linker sequence comprising glycine-serine (e.g., Gly₄Ser, SEQ ID NO: 128) repeats. In certain embodiments, the linker comprises three Gly₄Ser repeats (SEQ ID NO: 59) followed by a proline residue. In certain embodiments the proline residue is followed by an amino acid selected from the group consisting of glycine, arginine and serine. In some embodiments, a Fc-binding domain linker comprises or consists of a sequence selected from SEQ ID NO: 50-70.

[0156] Some exemplary hinge, Fc-binding domain linker sequences suitable for use in accordance with the present disclosure are shown in Table 2. Additional exemplary hinge and linker regions are set forth in SEQ ID NOs: 241-244, 601, 78, 763-791, 228, 379-434, 618-749 of U.S. 2013/0129723 (said sequences incorporated by reference herein).

Table 2: Exemplary hinges and linkers

Name	Amino Acid Sequence	SEQ ID NO
sss(s)-hIgG1 hinge	EPKSSDKTHTSPSS	SEQ ID NO:36
csc(s)-hIgG1 hinge	EPKSCDKTHTSPPCS	SEQ ID NO:37
ssc(s)-hIgG1 hinge	EPKSSDKTHTSPPCS	SEQ ID NO:38
scc(s)-hIgG1 hinge	EPKSSDKTHTCPPCS	SEQ ID NO:39
css(s)-hIgG1 hinge	EPKSCDKTHTSPSS	SEQ ID NO:40

Name	Amino Acid Sequence	SEQ ID NO
scs(s)-hIgG1 hinge	EPKSSDKTHTCPPSS	SEQ ID NO:41
ccc(s)-hIgG1 hinge	EPKSCDKTHTSPPCS	SEQ ID NO:42
ccc(p)-hIgG1 hinge	EPKSCDKTHTSPPCP	SEQ ID NO:43
sss(p)-hIgG1 hinge	EPKSSDKTHTSPPSP	SEQ ID NO:44
csc(p)-hIgG1 hinge	EPKSCDKTHTSPPCP	SEQ ID NO:45
ssc(p)-hIgG1 hinge	EPKSSDKTHTSPPCP	SEQ ID NO:46
scc(p)-hIgG1 hinge	EPKSSDKTHTCPPCP	SEQ ID NO:47
css(p)-hIgG1 hinge	EPKSCDKTHTSPPSP	SEQ ID NO:48
scs(p)-hIgG1 hinge	EPKSSDKTHTCPPSP	SEQ ID NO:49
Scppcp	SCPPCP	SEQ ID NO:50
STD1	NYGGGGSGGGGSGGG GSGNS	SEQ ID NO:51
STD2	NYGGGGSGGGGSGGG GSGNYGGGGSGGGGS GGGGSGNS	SEQ ID NO:52
H1	NS	--
H2	GGGGSGNS	SEQ ID NO:53
H3	NYGGGGSGNS	SEQ ID NO:54
H4	GGGGSGGGGSGNS	SEQ ID NO:55
H5	NYGGGGSGGGGSGNS	SEQ ID NO:56
H6	GGGGSGGGGSGGGGS GNS	SEQ ID NO:57
H7	GCPPCPNS	SEQ ID NO:58
(G ₄ S) ₃	GGGGSGGGGSGGGGS	SEQ ID NO:59
H105	SGGGSGGGGSGGGG S	SEQ ID NO:60
(G ₄ S) ₄	GGGGSGGGGSGGGGS GGGS	SEQ ID NO:61

Name	Amino Acid Sequence	SEQ ID NO
(G ₄ S) ₅	GGGGSGGGGSGGGGS GGGGSGGGGS	SEQ ID NO: 62
H94	SGGGGSGGGGSGGGG SPNS	SEQ ID NO:68
H111	SGGGGSGGGGSGGGG SPGS	SEQ ID NO:69
H114	GGGGSGGGGSGGGGS PS	SEQ ID NO:70

[0157] In addition to the aforementioned domains, the therapeutic polypeptides can further comprise immunoglobulin dimerization/heterodimerization domains, junctional amino acids, tags, additional binding domains, etc. In some embodiments, the polypeptides and proteins described herein are conjugated to a drug or a toxic moiety.

Bispecific/Multispecific Proteins

[0158] In some embodiments, a therapeutic protein may be a bispecific or multispecific protein. Non-limiting examples of bispecific molecules include an scFv-Fc-scFv molecule, an scFv-Ig molecule and an scFv-scFv molecule. In some embodiments, the bispecific molecules described herein comprise or consist of a first binding domain scFv linked to a second binding domain scFv and do not include other sequences such as an immunoglobulin constant region. In some embodiments, a therapeutic protein may be a bispecific or multispecific protein that comprises, from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus, (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain.

[0159] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a CD3 binding domain, a hinge region, an immunoglobulin constant region, and a tumor antigen binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0160] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a tumor antigen binding domain, a hinge region, an immunoglobulin constant region, and a CD3 binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0161] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, the 4-1-BB binding domain, a hinge region, an immunoglobulin constant region, and a tumor antigen binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0162] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a tumor antigen binding domain, a hinge region, an immunoglobulin constant region, and a 4-1-BB binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

Homodimers/Heterodimers

[0163] In some embodiments, a therapeutic protein may be a homodimer or a heterodimer. In some embodiments, a therapeutic protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, and (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain. In some embodiments, the bispecific or multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus: (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain. In other embodiments, the bispecific proteins described herein are diabodies.

[0164] In certain embodiments, a hinge present in a polypeptide that forms a heterodimer with another polypeptide chain can be an immunoglobulin hinge, such as a wild-type immunoglobulin hinge region or an altered immunoglobulin hinge region thereof. In certain embodiments, a hinge of one polypeptide chain of a heterodimeric protein is identical to a corresponding hinge of the other polypeptide chain of the heterodimer. In certain other embodiments, a hinge of one chain is different from that of the other chain (in their length or sequence). The different hinges in the different chains allow different manipulation of the binding affinities of the binding domains to which the hinges are connected, so that the heterodimer is able to preferentially bind to the target of one binding domain over the target of the other binding domain.

[0165] In other embodiments, the polypeptides and proteins described herein include a heterodimerization domain that is capable of heterodimerization with a

different heterodimerization domain in a second, non-identical polypeptide chain. In certain variations, the second polypeptide chain for heterodimerization includes a second binding domain. Accordingly, in certain embodiments of the present disclosure, two non-identical polypeptide chains, one comprising the polypeptide comprising a first binding domain and the second optionally comprising a second binding domain, dimerize to form a heterodimeric binding protein. Dimerization/heterodimerization domains can be used where it is desired to form heterodimers from two non-identical polypeptide chains, where one or both polypeptide chains comprise a binding domain. In certain embodiments, one polypeptide chain member of certain heterodimers described herein does not contain a binding domain. Examples of types of heterodimers include those described in U.S. Patent Application Publication Nos. 2013/0095097 and 2013/0129723, and International PCT Publication No. WO 2016/094873.

[0166] In certain embodiments, the first and second polypeptide chains dimerize via the inclusion of an “immunoglobulin dimerization domain” or “immunoglobulin heterodimerization domain.” An “immunoglobulin dimerization domain” or “immunoglobulin heterodimerization domain” refers herein to an immunoglobulin domain of a first polypeptide chain that preferentially interacts or associates with a different immunoglobulin domain of a second polypeptide chain, wherein the interaction of the different immunoglobulin domains substantially contributes to or efficiently promotes heterodimerization of the first and second polypeptide chains (*i.e.*, the formation of a dimer between two different polypeptide chains, which is also referred to as a “heterodimer”). The immunoglobulin heterodimerization domains in the polypeptide chains of a heterodimer are different from each other and thus can be differentially modified to facilitate heterodimerization of both chains and to minimize homodimerization of either chain. Immunoglobulin heterodimerization domains provided herein allow for efficient heterodimerization between different polypeptides and facilitate purification of the resulting heterodimeric protein.

[0167] As provided herein, immunoglobulin heterodimerization domains useful for promoting heterodimerization of two different polypeptide chains according to the present disclosure include wild-type and altered immunoglobulin CH1 and CL domains, for instance, human CH1 and CL domains. In certain embodiments, an immunoglobulin heterodimerization domain is a wild-type CH1 domain, such as a wild type IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM CH1 domain, for example,

as set forth in SEQ ID NOs: 114, 186-192 and 194, respectively, of U.S. Patent Application Publication No. 2013/0129723 or SEQ ID NO: 114 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein). In further embodiments, a cysteine residue of a wild-type CH1 domain (e.g., a human CH1) involved in forming a disulfide bond with a wild type immunoglobulin CL domain (e.g., a human CL) is deleted or substituted in the altered immunoglobulin CH1 domain such that a disulfide bond is not formed between the altered CH1 domain and the wild-type CL domain.

[0168] Polypeptides and proteins described herein may be made using scaffolding as generally disclosed in U.S. Patent Application Publication Nos. 2013/0129723 and 2013/0095097, which are each incorporated herein by reference in their entirety. The polypeptides described herein may comprise two non-identical polypeptide chains, each polypeptide chain comprising an immunoglobulin heterodimerization domain. The interfacing immunoglobulin heterodimerization domains are different. In one embodiment, the immunoglobulin heterodimerization domain comprises a CH1 domain or a derivative thereof. In another embodiment, the immunoglobulin heterodimerization domain comprises a CL domain or a derivative thereof. In one embodiment, the CL domain is a C κ or C λ isotype or a derivative thereof.

Exemplary protein therapeutics: Anti-CD123 x Anti-CD3 Polypeptides and Dimers thereof

[0169] An exemplary protein therapeutic may bind both CD123-expressing cells and the T-cell receptor complex on T-cells to induce target-dependent T-cell cytotoxicity, activation and proliferation.

[0170] Thus, in certain embodiments, the therapeutic protein used in connection with the methods and compositions described herein is a bispecific single chain molecule comprising a CD123 binding domain and a CD3 binding domain. In some embodiments, a CD123 and/or a CD3 binding domain is derived from an antibody and comprises a variable heavy chain (VH) and a variable light chain (VL). For example, the CD123 and/or CD3 binding domains may be an scFv that comprises a VH and VL. These binding domains and variable chains may be arranged in any order that still retains some binding to the target(s). For example, the variable domains may be arranged in the order such as (VH CD123)-(VL CD123)-(VH CD3)-(VL CD3); (VL

CD123)-(VH CD123)-(VH CD3)-(VL CD3); (VH CD123)-(VL CD123)-(VL CD3)-(VH CD3); (VL CD123)-(VH CD123)-(VL CD3)-(VH CD3); (VH CD3)-(VL CD3)-(VH CD123)-(VL CD123); (VL CD3)-(VH CD3)-(VL CD123)-(VH CD123); (VH CD3)-(VL CD3)-(VL CD123)-(VH CD123); or (VL CD3)-(VH CD3)-(VH CD123)-(VL CD123). The pairs of VH regions and VL regions in the binding domain binding to CD3 may be in the format of a single chain antibody (scFv). The VH and VL regions may be arranged in the order VH-VL or VL-VH. In some embodiments, the scFv may bind to CD123 more effectively than the antibody comprising the same VH and VL region sequences in the same orientation. In certain embodiments, the scFv may bind more effectively to CD123 in the VL-VH orientation than in the VH-VL orientation, or vice versa. The VH-region may be positioned N-terminally to a linker sequence. The VL region may be positioned C-terminally to the linker sequence. The domain arrangement in the CD3 binding domain of the bispecific single chain molecule may be VH-VL, with the CD3 binding domain located C-terminally to the CD123-binding domain. A bispecific molecule may comprise an scFv binding to CD123 linked to an scFv binding to CD3. These scFvs may be linked with a short peptide. In some embodiments, bispecific single chain molecules do not comprise a hinge region or a constant region (see, for example, US 2013/0295121, WO 2010/037836, WO 2004/106381 and WO 2011/121110; each incorporated herein by reference in its entirety).

[0171] The CD123-bispecific binding construct may comprise one or more sequences shown in Table 3, Table 4, and/or Table 5.

Table 3: Binding Polypeptide Sequences and Components

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 variable light chain domain	gacatcgtgatgaccagctctccagactccctggctgtgtctctgggcgag agggccaccatcaactgcaagtcagccacagtgtttatacagctcaa caataagaactacttagcttggtaccagcagaaaccaggacagcctccta agctgctcatttactgggcatctaccgggaatccgggtccctgaccgat tcagtggcagcgggtctgggacagatttcactctcaccatcagcagcctgc aggctgaagatgtggcagtttattactgtcagcaatattatagtactctcc gaccactttcggcggagggaaccaaggtggagatcaaa	divmtqspdslavslger atincksshsvlyssnnk nylawyqqkpgppkll iywastresgvpdrfsgs gsgtdftltisslqaedva vyycqqyystppttfggg tkveik	SEQ ID NO:133 (SEQ ID NO:134)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 variable heavy chain domain	gagggtcagctgttggagctctgggggaggcttggtacagcctggggggc cctgagactctcctgtgcagcctctggattcaccttttagcagctatggcatg agctgggtccgccaggctccaggggaaggggctggaggggtctcagcta ttagtggttagtggtgtagcacatactacgagactccgtgaaggccgg ttcaccatctccagagacaattccaagaacacgctgtatctgcaaatgaa cagcctgagagccgaggacacggcgtatattactgtgcgaaagaaaag ttacgatattttgactgggtatccgatgcttttgatatctggggccaaggga caatggtcaccgtctcttca	evqllesggglvqpggslr lscaasgftfssygmswv rqapgkglegvsaisgsg gstyyadsvkgrftisrdn skntlylqmnsraedta vyycakeklryfdwlsda fdiwgqgtmvtvss	SEQ ID NO:135 (SEQ ID NO:136)
OMT1 CDR L1	cacagtgtttatacagctccaacaataagaactac	HSVLYSSNNKNY	SEQ ID NO:137 (SEQ ID NO:138)
OMT1 CDR L2	tgggcatct	WAS	SEQ ID NO:139 (SEQ ID NO:140)
OMT1 CDR L3	cagcaatattatagtagtctctccgaccact	QQYYSTPPTT	SEQ ID NO:141 (SEQ ID NO:142)
OMT1 CDR H1	ggattcaccttttagcagctatggc	GFTFSSYG	SEQ ID NO:143 (SEQ ID NO:144)
OMT1 CDR H2	attagtggtagtggtgtagcaca	ISGSGGST	SEQ ID NO:145 (SEQ ID NO:146)
OMT1 CDR H3	gcgaaagaaaagttagcatattttgactgggtatccgatgcttttgatc	AKEKLRYFDWLSDA FDI	SEQ ID NO:147 (SEQ ID NO:148)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOS: nucleotide (amino acid)
MT1 VHVL x TSC456 scFv-Fc- scFv TRI129	atggaagcaccagcgcagcttcttctcctgctactctggctccagat accaccggtaggtgcagctgttgagcttggggaggccttggtacagcc tggggggctccctgagactctctgtgcagcctctggattcaccttagcagc tatggcatgagctgggtccgccaggctccagggaagggtggaggggg tctcagctattagtgttagtggtgtagcacatactacgcagactccgtga agggccgggtcaccatctccagagacaattccaagaacacgcgtgatctg caaatgaacagcctgagagccgaggacacggcgtatattactgtgcga aagaaaagttagcatattttgactggttatccgatgcttttgatatctgggg ccaagggaacaatggtcaccgtctcttcagggtggaggcgggtcaggcggag gtggatccggcgggtggcgggtccgggtggcggcggtatcgacatcgtgatg accagctctccagactccctggctgtgtctctcgggcgagaggccaccatc aactgcaagtcaggccacagtgttttatcacagctccaacaataagaacta cttagcttggtaccagcagaaccaggacagcctcctaagctgtcattta ctgggcatcatcccggaatccgggttccctgaccgattcagtggcagcg ggctcgggacagatttactctcaccatcagcagcctgcaggctgaagat gtggcagttattactgtcagcaatattatagtactcctccgaccatttcg gcggagggaccaaggtggagatcaaatcctcgagtgagccaaatctctt gacaaaactcacacatgcccaccgtgccagcacctgaagccgagggtg caccgtcagttcttctcttcccccaaaaccaaggacacctcatgatct cccggacccttgaggtcacatgcgtgggtgggacgtgagccacaaga cctgaggtcgaagttcaactggtagctggacggcgtggagggtgcataatg ccaagacaaaggccggaggagcagtagtaacaacagcagcagctaccgtgtg tcagcgtctctaccgtctgcaccaggactggctgaatggcaaggaatac aagtgcgcggttctcaacaaagcctcccagccccatcgagaaaacca tctccaaagccaaaggcgagccccgagaaccacaggtgtacacctgcc ccatcccgggatgagctgaccaagaaccaggtcagcctgactgcctgg tcaaaggcttctatccaagcgacatcgccgtggagtgaggagcaatgg gcagccggagaaacaactacaagaccacgctcccgtgctggactccgac ggctccttctctctacagcaagctcaccgtggacaagagcagggtggca gcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaacc actacacgcagaagagcctctccctgtctccgggttcggaggagggggt tcaggtgggggagggttctcggcggcgggggaagcccttcacaggtgcaact ggtcgagatggcaccgaggttaaaaaaacagggtcctccgttaaggtta gctgcaaaagccttggtacacattttccaggagtacaatgcactgggtga ggcaggctcctggacagggactcgatggatcgggtatatcaaccatct agcgctataccaattacaacaaaagtttaaggaccgagttaccattac cgctgacaaatccaccagtagccttatatggagctgtcatctttaggtc cgaggacactgctgtttattactgcgtcgtcctcaggttcaactatgactat aatggttttccctactggggtcagggaacccgtggtgactgtcttcttggcg gtggaggcagcgggtgggggtgggtctggaggcgggtggcagtgccggcg aggctctgatattcagatgactcagctctctagcactctcagcgccagcgt gggggactcgtgtgacaatgactgtctcgcgtagcagtagtgttctcat gaattggtatcagcagaaagccggggaagcacctaagcgtggatctat gactcttcaagctggcaagtggtgtccctcacgggttctctggctcaggtt ctggtactgactatactttgactatctcctccctcagcccgatgatttcgct acctattattgtcagcagtgtaggcgtaaccacccactttcggaggcgggt accaaatggagatcaagagggtcataa	evqllesggglvpqgslr lscaasgftfssygmwv rqapgkglegvsaisgs gstyyadsvkgrftisrdn skntlylqmnsfraedta vyycakeklryfdwlsda fdiwigqgtmvtvssggg gsgggsgggsgggsgggs divmtqspdslavslger atinksshslyssnnk nylawyqqkpgqpkkll iywastresgvpdfrgsgs gsdgtflltisslqaedva vyyccqyystpptftggg tkveikssepksdkth tcppcpapeaagapsvf lfppkpkdltmistrpev tcvvvdvshedpevkfn wyvdgvevhnaktkpr eeqynstyrvsvltvlh qdwlngkeykcavsnk alpapiektiskakqpr epqvyltppsrldetknq vsltlvkgfypsdiave wesngqpennyktp vldsdsfflyskltvdksr wqqgnvfscsvmheal hnhytqkslspsggg gsgggsgggsgspsqv lvqsgpevkpgssvkv sckasgytfrstwmhw rqapgqfseirgwinps saytnynqkfkdvrtita dkststaymelslrsed tavyycarpqvhydyng fpywqgtltvtssggg gsgggsgggsgggsgggs diqmtqspstlsasvdr vtmtcsasssvymnw yqqkpgkapkrwiys sklasgvpsrfsgsgsgtd ytltisslqpddfatyycq qtrsnrnpptfgggtkvei krs	SEQ ID NO:309 (SEQ ID NO:310)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 VLVH x TSC456 scFv-Fc- scFv TRI130	atggaagcaccagcgcagcttctctctcctgctactctggctcccagat accaccggtgacatcgtgatgaccagctcctcagactccctggctgtgtct ctggcgagaggccaccatcaactgcaagtcagccacagtggtttata cagctccaacaataagaactacttagcttggtaccagcagaaccaggga cagcctcctaagctgctcattactgggcatctaccgggaatccggggtc cctgaccgattcagtgaggcgggtctgggacagatttactctcaccatc agcagctgcaggctgaagatgtggcagtttattactgtcagcaattattat agtactcctccgaccatttcggcggaggaccaaaggtggagatcaaaag gtggaggcgggttcaggcggagggtgatccggcgggtggcggctccgggtg cggcggatctgaggtgcagctgttgaggtctggggaggccttggtacagc ctggggggctcctgagactctcctgtgcagcctctggattcacctttagcag ctatggcatgagctgggtccgagggctccagggaaggggctggagggg gtctcagctattagtggttagtggttagcacatactacgcagactccgtg aaggccgggttcaccatctccagagacaattccaagaacacgctgtatct gcaaatgaacagcctgagagccgaggacacggcgtatattactgtgcg aaagaaaagttagcatatttgactggttatccgatgcttttgatatctggg gccaaggggacaatgggtaccgtctcctcagtgagccaaatctctgac aaaactcacacatgccaccgtgccagcacctgaagccgggggtgcac cgtcagcttctcttcccccaaaacccaaggacaccctcatgatctccc ggaccctgagggtcacatgcgtgggtggagctgagccacgaagaccct gagggtcaagttcaactggtacgtggacggcgtggagggtgcataatgcaa gacaaagccgaggaggagcagtagacaacagcacgtaccgtgtggtcag cgtcctcaccgtcctgaccaggactggctgaatggcaaggaaatacaagt gcgcggtctccaacaaagccctcccagccccatcgagaaaaccatctcc aaagccaaagggcagccccgagaaccacaggtgtacaccctgccccat ccgggatgagctgacaaagaaaccaggtcagcctgacctgctggtcaa aggcttctatccaagcgacatcgctggagtgaggagcaatgggcag ccggagaacaactacaagaccacgcctccgtgctggactccgacggctc cttcttctctacagcaagctcaccgtggacaagagcaggtggcagcagg ggaacgtcttctcatgctcgtgatgcatgaggctctgcacaaccactaca cgcagaagagcctctcctgctcgggttccggaggagggggttcaggt ggggggagggttctggcggcgggggaagcccttcacaggtgcaactgggtc agagtgagcccgagggttaaaaaaccagggtcctcctgtaaggttagctgc aaagcctctggctacacattttccaggagtacaatgcactgggtgaggca ggctcctggacagggactcagtggtatcggttatatcaaccatctagcg cctataccaattacaacaaaagttaaggaccgagttaccattaccgtg acaaatccaccagtacagcttataggagctgtcatctcttaggtccgagg acactgctgtttattactgcgtcgtcctcaggttactatgactataatggt tttccctactggggtcagggaacccctgggtgactgtctcttctggcgggtggag gcagcgggtgggggtgggtctggaggcgggtggcagtgccggcggaggctc tgatattcagatgactcagctcctagcactctcagcgcagcgtggggga tcgtgtgacaatgacttgcctcgtagcagtagtgtgtcttacatgaattgg tatcagcagaagcccgggaaagcacctaagcgtggtatctgactcttc caagctggcaagtggtgtcccctcacgggttctctggctcaggttctggtact gactatactttgactatctcctccctcagcccgatgatttcgctacctatta ttgtcagcagtgaggcgttaaccaccactttcggaggcgggtaccaaag tggagatcaagaggtcataa	divmtqspdsavlslger atincksshsvlyssnnk nylawyqqkpgqppkl iywastresgvpdrfsgs gsgrtdftltisslqaedva vyycqqyytppptfagg tkveikgggsgggsgg ggsgggsgsevqllesggg lvqpggslrlscaasgftfs sygmswvrqapgkgle gvsaisgsgstyadsv kgrftisrdnsntlylqm nslraedtavycakekl ryfdwlsdafdiwqgt mvtvssepksdktht cppeapeaagapsvfl fppkpkdtlmisrtpvt cvvvdvshedpevkfn wyvdgvevhnaktkpr eeqynstyrvvsvltvlh qdwlngkeykcavsnk alpapiektiskakqpr epqvylppsrldeltnkq vsltlvkgfypsdiave wesngqpennykttpp vldsdgsfflyskltvdksr wqqgnvfscsvmheal hnhytqkslslspgsgg gsggsgsgsgsgpsqvq lvqsgpevkkpgssvk sckasgytfsrstmhww rqapggglewigyinp saytnynqkfkdrtvita dkststaymelslrsed tavvyrcarpqvhydyng fpywqgtlvtvssggg gsggsgsgsgsgsggs diqmtqspstlsasvgr vtmtcsasssvsymnw yqqkpgkapkriwids sklasgvpsrfsrgsgtd ytltisslqpdffatyycc qwsrnpptfagggtkvei krs	SEQ ID NO:311 (SEQ ID NO:312)
Cris7 and DRA222 VH CDR1 (Kabat)		RSTMH	(SEQ ID NO:345)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
Cris7 and DRA222 VH CDR2 (Kabat)		YINPSSAYTNYNQKF K	(SEQ ID NO:346)
Cris7 and DRA222 VH CDR3 (Kabat)		QVHYDYNGFPY	(SEQ ID NO:347)
Cris7 and DRA222 VL CDR1 (Kabat)		SASSSVSYMN	(SEQ ID NO:348)
Cris7 and DRA222 VL CDR2 (Kabat)		DSSKLAS	(SEQ ID NO:349)
Cris7 and DRA222 VL CDR3 (Kabat)		QQWSRNPPT	(SEQ ID NO:350)
Cris7 and DRA222 VH CDR1 (IMGT)		GYTFTRST	(SEQ ID NO:351)
Cris7 and DRA222 VH CDR2 (IMGT)		INPSSAYT	(SEQ ID NO:352)
Cris7 and DRA222 VH CDR3 (IMGT)		QQWSRNPPT	(SEQ ID NO:353)
Cris7 and DRA222 VL CDR1 (IMGT)		ASSSVSY	(SEQ ID NO:354)
Cris7 and DRA222 VL CDR2 (IMGT)		DSS	(SEQ ID NO:355)
Cris7 and DRA222 VL CDR3 (IMGT)		QQWSRNPPT	(SEQ ID NO:356)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
Cris-7 variable light chain sequence		QVVLTSQSPAIMSA FPGEKVTMTCSAS SSVSYMNWYQQK SGTSPKRWIYDSS KLASGVPARFSGS GSGTSYSLTISSME TEDAATYYCQQW SRNPPTFGGGTKL QITR	(SEQ ID NO: 341)
Cris-7 variable heavy chain sequence		QVQLQQSGAELA RPGASVKMSCKA SGYTFTRSTMHW VKQRPQGQGLEWI GYINPSSAYTNYN QKFKDKATLTADK SSSTAYMQLSSLTS EDSAVYYCASPQV HYDYNGFPYWGQ GTLVTVSA	(SEQ ID NO: 342)
HuM291 variable light chain sequence		DIQMTQSPSSLSA SVGDRVITITCSASS SVSYMNWYQQK PGKAPKRLIYDTSK LASGVPSRFSGSG SGTDFTLTISLQP EDFATYYCQQWS SNPPTFGGGTKVE IK	(SEQ ID NO: 343)
HuM291 variable heavy chain sequence		QVQLVQSGAEVK KPGASVKVSKKAS GYTFISYTMHWV RQAPGQGLEWM GYINPRSGYTHYN QKLKDKATLTADK SASTAYMELSSLRS EDTAVYYCARSAY YDYDGFAYWGQG TLVTVSS	(SEQ ID NO: 344)
I2C VH CDR1 (Kabat)		KYAMN	(SEQ ID NO:357)
I2C VH CDR2 (Kabat)		RIRSKYNNYATYYAD SVKD	(SEQ ID NO:358)
I2C VH CDR3 (Kabat)		HGNFGNSYISYWAY	(SEQ ID NO:359)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
I2C VL CDR1 (Kabat)		GSSTGAVTSGNYPN	(SEQ ID NO:360)
I2C VL CDR2 (Kabat)		GTKFLAP	(SEQ ID NO:361)
I2C VL CDR3 (Kabat)		VLWYSNRWV	(SEQ ID NO:362)
I2C VH CDR1 (IMGT)		GFTFNKYA	(SEQ ID NO:363)
I2C VH CDR2 (IMGT)		IRSKYNNYAT	(SEQ ID NO:364)
I2C VH CDR3 (IMGT)		VRHGNFGNSYISYW AY	(SEQ ID NO:365)
I2C VL CDR1 (IMGT)		TGAVTSGNY	(SEQ ID NO:366)
I2C VL CDR2 (IMGT)		GTK	(SEQ ID NO:367)
I2C VL CDR3 (IMGT)		VLWYSNRWV	(SEQ ID NO:368)
HuM291 VH CDR1 (Kabat)		SYTMH	(SEQ ID NO:369)
HuM291 VH CDR2 (Kabat)		YINPRSGYTHYNQKL KD	(SEQ ID NO:370)
HuM291 VH CDR3 (Kabat)		SAYYDYDGFAY	(SEQ ID NO:371)
HuM291 VL CDR1 (Kabat)		SASSSVSYMN	(SEQ ID NO:372)
HuM291 VL CDR2 (Kabat)		DTSKLAS	(SEQ ID NO:373)
HuM291 VL CDR3 (Kabat)		QQWSSNPPT	(SEQ ID NO:374)
HuM291 VH CDR1 (IMGT)		GYTFISYT	(SEQ ID NO:375)
HuM291 VH CDR2 (IMGT)		INPRSGYT	(SEQ ID NO:376)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
HuM291 VH CDR3 (IMGT)		ARSAYDYDGFAY	(SEQ ID NO:377)
HuM291 VL CDR1 (IMGT)		ASSSVSY	(SEQ ID NO:378)
HuM291 VL CDR2 (IMGT)		DTS	(SEQ ID NO:379)
HuM291 VL CDR3 (IMGT)		QQWSSNPPT	(SEQ ID NO:380)
TSC455 (anti-CD3) TSC394 F87Y scFv		QVQLVQSGPEVKKP GSSVKVSCKASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVITIT ADKSTSTAYMELSSL RSEDNAVYYCARPQ VHYDYNQFPYWGQ GTLVTVSSGGGGSG GGGSGGGGGSGGGG SDIQMTQSPSTLSAS VGDRVMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTEY TLTISSLQPDFFATYY CQQWSRNPPTFGG GTKVEIKRSSS	(SEQ ID NO:381)
TSC456 (anti-CD3) TSC394 E86D F87Y scFv		QVQLVQSGPEVKKP GSSVKVSCKASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVITIT ADKSTSTAYMELSSL RSEDNAVYYCARPQ VHYDYNQFPYWGQ GTLVTVSSGGGGSG GGGSGGGGGSGGGG SDIQMTQSPSTLSAS VGDRVMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTD YTLTISSLQPDFFATY YCQQWSRNPPTFG GGTKVEIKRSSS	(SEQ ID NO:382)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
TSC455 and TSC456 variable heavy domain		QVQLVQSGPEVKKP GSSVKVSCASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVTIT ADKSTSTAYMELSSL RSED TAVYYCARPQ VHYDYN G F P Y W G Q GTLVTVSS	(SEQ ID NO:383)
TSC455 variable light domain		DIQMTQSPSTLSAS VGDRVMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFGSGSGTEY TLTISSLQPD F ATYY CQQWSRNPPTFGG GTKVEIKRS	(SEQ ID NO:384)
TSC456 variable light domain		DIQMTQSPSTLSAS VGDRVMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFGSGSGTD YTLTISSLQPD F ATY YCQQWSRNPPTFG GGTKVEIKRS	(SEQ ID NO:385)
DRA222 (anti-CD3) scFv		QVQLVESGGGVVQ PGRSLRLSCKASGYT FTRSTMHWVRQAP QGLEWIGYINPSS AYTNYNQKFKDRFTI SADKSKSTAF L QMD SLRPEDTGVYFCARP QVHYDYN G F P Y W G QGTPVTVSSGGGGGS GGGGSGGGGSAQD IQMTQSPSSLSASV GDRVMTCSASSSV SYMNWYQQKPGK APKRWIYDSSKLAS GVPARFSGSGSGTD YTLTISSLQPED F ATY YCQQWSRNPPTFG GGTKLQITSSS	(SEQ ID NO:386)

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
DRA222 variable heavy domain		QVQLVESGGGVVQ PGRSLRLSCKASGYT FTRSTMHWVRQAP GQGLEWIGYINPSS AYTNYNQKFKDRFTI SADKSKSTAFQMD SLRPEDTGVYFCARP QVHYDYNGFPYWG QGTPVTSS	(SEQ ID NO:387)
DRA222 variable light domain		DIQMTQSPSSLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPARFSGSGSGTD YTLTISSLPEDFATY YCQQWSRNPPTFG GGTKLQITS	(SEQ ID NO:388)

Table 4: Composition of Humanized Constructs

Construct ID	scFv Orientation	Nucleotide SEQ ID NO	Amino acid SEQ ID NO
TRI129	VHVL	309	310
TRI130	VLVH	311	312

Table 5: Amino acid sequences of exemplary binding protein construct

Construct name	Sequence	SEQ ID NO
TRI130 (CD123 binding domain in bold, CD3 binding domain in italics) (CDR sequences)	MEAPAQLLFLLLWLPDTTGDIVMTQSPDSLAVSLGERATINCKS SHSVLYSSNNKNY LAWYQQKPGQPPKLLIYWASTRESGVPDR FSGSGSGTDFTLTISSLQAEDVAVYYC QQYYSTPPTTF GGGGTKV EIKGGGGSGGGGSGGGGSGGGGSEVQLLES GGGLVQPGGSL RLSCAASGFTFSSYGMSWVRQAPGKGLEGVSAISGSGGSTYY ADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKEKLRY FDWLSDAFDI WGQGTMTVSSSEPCKSSDKTHTCPPCPAPEAAG APSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAV	337

are single-underlined)	SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG <u>SGGGGSGG</u> <u>GGSGGGGSPSQVQLVQSGPEVKKPGSSVKVSCKASGYTFSRS</u> <u>TMHWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRVITADKST</u> STAYMELSSLRSEDTAVYYCARPQVHYDYNQFPYWGQGLVTV SSGGGGSGGGGSGGGGSGGGGSDIQMTQSPSTLSASVGDRV TMTCSA <u>SSSVSYMNWYQQKPGKAPKRWIYDSSKLASGVPSRFS</u> GSGSGTDYTLTISSLQPDDFATYYC <u>QQWSRNPPTFGGGTKVEIK</u> RS	
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[0172] In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (VL) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (VH) comprising CDRs HCDR1, HCDR2, and HCDR3 with HCDR1 comprising an amino acid sequence as set forth in SEQ ID NO:144, with HCDR2 comprising an amino acid sequence as set forth in SEQ ID NO:146 and with HCDR3 comprising an amino acid sequence as set forth in SEQ ID NO:148. In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (VL) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (VH) comprising CDRs HCDR1, HCDR2, and HCDR3. In some such embodiments, (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:138 or a sequence that differs from SEQ ID NO:138 by at least one amino acid substitution; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:140 or a sequence that differs from SEQ ID NO:140 by at least one amino acid substitution; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:142 or a sequence that differs from SEQ ID NO:142 by at least one amino acid substitution; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:144 or a sequence that differs from SEQ ID NO:144 by at least one amino acid substitution; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:146 or a sequence that differs from SEQ ID NO:146 by at least one amino acid substitution; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:148 or a sequence that differs from SEQ ID NO:148 by at least one

amino acid substitution. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution. In some embodiments, an LCDR1, LCDR2, LCDR3, HCDR1, HCDR2, and/or HCDR3 differs from a recited sequence by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In certain embodiments, a CDR of the present disclosure contains about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (e.g., conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the CDR sequence of a known monoclonal antibody. For instance, the disclosure includes a recombinant polypeptide comprising (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:138 or a sequence that differs from SEQ ID NO:138 by one or two amino acid substitutions; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:140 or a sequence that differs from SEQ ID NO:140 by one or two amino acid substitutions; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:142 or a sequence that differs from SEQ ID NO:142 by one or two amino acid substitutions; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:144 or a sequence that differs from SEQ ID NO:144 by one or two amino acid substitutions; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:146 or a sequence that differs from SEQ ID NO:146 by one or two amino acid substitutions; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:148 or a sequence that differs from SEQ ID NO:148 by one or two amino acid substitutions. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution.

[0173] In related embodiments, a recombinant polypeptide of the disclosure comprises or is a sequence that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of a light chain variable region (V_L) (e.g., SEQ ID NO:134) or to a heavy chain variable region (V_H) (e.g., SEQ ID NO:136), or both. In one embodiment, the CD123-binding domain of the recombinant polypeptide is an scFv comprising a variable heavy chain comprising SEQ ID NO:136 and a variable light chain comprising SEQ ID NO:134 in the VHVL orientation. In another embodiment, the CD123-binding domain of the recombinant polypeptide is an scFv comprising a variable light chain

comprising SEQ ID NO:134 and a variable heavy chain comprising SEQ ID NO:136 in the VLVH orientation. For instance, in certain embodiments, the polypeptide of the disclosure comprises an amino acid sequence of SEQ ID NO:337. The instant disclosure includes a recombinant polypeptide that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of SEQ ID NO:337.

[0174] In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (V_L) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (V_H) comprising CDRs HCDR1, HCDR2, and HCDR3. In some such embodiments, (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:154 or a sequence that differs from SEQ ID NO:154 by at least one amino acid substitution; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:156 or a sequence that differs from SEQ ID NO:156 by at least one amino acid substitution; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:158 or a sequence that differs from SEQ ID NO:158 by at least one amino acid substitution; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:160 or a sequence that differs from SEQ ID NO:160 by at least one amino acid substitution; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:162 or a sequence that differs from SEQ ID NO:162 by at least one amino acid substitution; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:164 or a sequence that differs from SEQ ID NO:164 by at least one amino acid substitution. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution. In some embodiments, an LCDR1, LCDR2, LCDR3, HCDR1, HCDR2, and/or HCDR3 differs from a recited sequence by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In certain embodiments, a CDR of the present disclosure contains about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the CDR sequence of a known monoclonal antibody.

[0175] In related embodiments, a CD123-binding domain comprises or is a sequence that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of a light chain variable region (V_L) (e.g., SEQ ID NO:17) or to a heavy chain variable region (V_H) (e.g., SEQ ID NO: 16), or both.

[0176] In certain embodiments, a CD123-binding domain comprises humanized immunoglobulin V_L and/or V_H regions. Techniques for humanizing immunoglobulin V_L and V_H regions are known in the art and are discussed, for example, in U.S. Patent Application Publication No. 2006/0153837. In certain embodiments, a CD123-binding domain comprises human immunoglobulin V_L and/or V_H regions.

[0177] Essentially, humanization by CDR grafting involves recombining only the CDRs of a non-human antibody onto a human variable region framework and a human constant region. Theoretically, this should substantially reduce or eliminate immunogenicity (except if allotypic or idiotypic differences exist). However, it has been reported that some framework residues of the original antibody also may need to be preserved (Reichmann *et al.*, *Nature*, 332:323 (1988); Queen *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:10,029 (1989)).

[0178] The framework residues that need to be preserved are amenable to identification through computer modeling. Alternatively, critical framework residues can potentially be identified by comparing known antigen-binding site structures (Padlan, *Molec. Immunol.*, 31(3):169-217 (1994), incorporated herein by reference).

[0179] The residues that potentially affect antigen binding fall into several groups. The first group comprises residues that are contiguous with the antigen site surface, which could therefore make direct contact with antigens. These residues include the amino-terminal residues and those adjacent to the CDRs. The second group includes residues that could alter the structure or relative alignment of the CDRs, either by contacting the CDRs or another peptide chain in the antibody. The third group comprises amino acids with buried side chains that could influence the structural integrity of the variable domains. The residues in these groups are usually found in the same positions (Padlan, 1994, *supra*) although their positions as identified may differ depending on the numbering system (see Kabat *et al.*, "Sequences of proteins of

immunological interest, 5th ed., Pub. No. 91-3242, U.S. Dept. Health & Human Services, NIH, Bethesda, Md., 1991).

[0180] Knowledge about humanized antibodies in the art is applicable to the polypeptides according to the disclosure, even if these polypeptides are not antibodies.

[0181] In some embodiments, an anti-CD123 scFv comprises a HCDR1 that comprises SEQ ID NO: 10, a HCDR2 that comprises SEQ ID NO: 11, and a HCDR3 that comprises SEQ ID NO: 12; and a LCDR1 that comprises SEQ ID NO: 13, a LCDR2 that comprises SEQ ID NO: 14, and a LCDR3 that comprises SEQ ID NO: 15. In some embodiments, the anti-CD123 scFv comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134. In some embodiments, the anti-CD123 scFv comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 16. In some embodiments, the anti-CD123 scFv comprises a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 17. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv, and wherein the scFv comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18.

[0182] In some embodiments, the disclosure relates to CD123-binding domains wherein (i) the immunoglobulin light chain variable region comprises an amino acid sequence that is at least 88%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:134 and the immunoglobulin heavy chain variable region comprises an amino acid sequence that is at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:136.

[0183] In further embodiments, each CDR comprises no more than one, two, or three substitutions, insertions or deletions, as compared to that from a monoclonal antibody or fragment or derivative thereof that specifically binds to a target of interest (*e.g.*, CD123).

[0184] In certain embodiments, a CD123-binding domain does not inhibit IL-3 binding to CD123.

[0185] In certain embodiments, a CD123-binding molecule or protein can comprise a T-cell binding domain for recruitment of T-cells to target cells expressing CD123. In

certain embodiments, a CD123-binding protein as described herein can comprise (i) a binding domain that specifically binds a TCR complex or a component thereof (e.g., TCR α , TCR β , CD3 γ , CD3 δ , and CD3 ϵ) and (ii) another binding domain that specifically binds to CD123. A CD123-binding protein can utilize essentially any binding domain that binds a T-cell, e.g., an antibody derived binding domain. Exemplary anti-CD3 antibodies from which the CD3 binding domain can be derived include the CRIS-7 monoclonal antibody (Reinherz, E. L. *et al.* (eds.), *Leukocyte typing II.*, Springer Verlag, New York, (1986); V_L and V_H amino acid sequences respectively shown in SEQ ID NO: 341 (QVVLTSQSPAIMSAFPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDSSKLA SGVPARFSGSGSGTSYSLTISMETEDAATYYCQQWSRNPPTFGGGTKLQITR) and SEQ ID NO: 342 (QVQLQQSGAELARPGASVKMSCKASGYTFTRSTMHWWKQRPGQGLEWIGYINP SSAYTNYNQKFKDKATLTADKSSSTAYMQLSSLTSEDSAVYYCASPQVHYDYNGF PYWGQGTLVTVSA)); HuM291 (Chau *et al.* (2001) *Transplantation* 71:941-950; V_L and V_H amino acid sequences respectively shown in SEQ ID NO: 343 (DIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQKPGKAPKRLIYDTSKLAS GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQWSSNPPTFGGGTKVEIK) and SEQ ID NO: 344 (QVQLVQSGAEVKKPGASVKVSKASGYTFISYTMHWVRQAPGQGLEWMGYINPR SGYTHYNQKLKDKATLTADKSASTAYMELSSLRSEDVAVYYCARSAYDYDGFAY WGQGTLVTVSS)); BC3 monoclonal antibody (Anasetti *et al.* (1990) *J. Exp. Med.* 172:1691); OKT3 monoclonal antibody (Ortho multicenter Transplant Study Group (1985) *N. Engl. J. Med.* 313:337) and derivatives thereof such as OKT3 ala-ala (also referred to as OKT3 AA-FL or OKT3 FL), a humanized, Fc variant with alanine substitutions at positions 234 and 235 (Herold *et al.* (2003) *J. Clin. Invest.* 11:409); visilizumab (Carpenter *et al.* (2002) *Blood* 99:2712), G19-4 monoclonal antibody (Ledbetter *et al.*, 1986, *J. Immunol.* 136:3945), 145-2C11 monoclonal antibody (Hirsch *et al.* (1988) *J. Immunol.* 140: 3766) and I2C monoclonal antibody (see, e.g., US 2011/0293619 and US20120244162). For example, a CD3 binding domain may comprise a CD3 binding domain disclosed in U.S. Patent Application Publication No. 2012/0244162, including a CD3 binding domain comprising a V_L region selected from SEQ ID NO: 17, 21, 35, 39, 53, 57, 71, 75, 89, 83, 107, 111, 125, 129, 143, 147, 161, 165, 179 and 183 of US 2012/0244162 and/or a V_H region selected from SEQ ID

NO:15, 19, 33, 37, 51, 55, 69, 73, 87, 91, 105, 109, 123, 127, 141, 145, 159, 163, 177 and 181 of US 2012/0244162. In some embodiments, a CD3 binding domain comprises an amino acid sequence selected from SEQ ID NO: 23, 25, 41, 43, 59, 61, 77, 79, 95, 97, 113, 115, 131, 133, 149, 151, 167, 169, 185, and 187 of US 2012/0244162. In some embodiments, a CD3 binding domain is one described in WO2004/106380, WO2005/040220A1, US 2014/0099318 or derived from a CD3 binding domain thereof. An exemplary anti-TCR antibody is the BMA031 monoclonal antibody (Borst *et al.* (1990) *Human Immunology* 29:175-188). The CD3 binding domain may be derived from any of the antibodies or sequences described in WO 2013/158856 (incorporated herein by reference in its entirety).

[0186] In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs:348, 349 and 350, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 345, 346 and 347, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NO:354, SEQ ID NO:355, and SEQ ID NO:356, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NO: 351, SEQ ID NO:352, and SEQ ID NO:353, respectively. In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 182, 183 and 184, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 351, 352 and 353, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 357, 359 and 359, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 359, 367 and 368, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 363, 364 and 365, respectively. In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1,

LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 372, 373 and 374, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 378, 379 and 380, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 375, 376 and 377, respectively. In some embodiments, the second binding domains comprising the CDR sequences recited in this paragraph are humanized.

[0187] In some embodiments of a CD123-binding protein comprising a second binding domain that specifically binds CD3 ϵ , the second binding domain competes for binding to CD3 ϵ with the CRIS-7, HuM291 or I2C monoclonal antibody. In some embodiments, the CD3-binding domain comprises an immunoglobulin light chain variable region (V_L) and an immunoglobulin heavy chain variable region (V_H) derived from the CRIS-7, HuM291 or I2C monoclonal antibody (e.g., the V_L and V_H of the second binding domain can be humanized variable regions comprising, respectively, the light chain CDRs and the heavy chain CDRs of the monoclonal antibody). A second binding domain may comprise the light chain variable region, the heavy chain variable region, or both, of the DRA222, TSC455, or TSC456 CD3-binding domains. The amino acid sequences of DRA222, TSC455, and TSC456 are provided in Table 4. The DRA222 binding domains are also described in WO 2013/158856. TSC455 may also be referred to as TSC394 F87Y. TSC455 may also be referred to as TSC394 E86D F87Y or TSC394 DY.

[0188] In some embodiments, the second binding domain specifically binds CD3 and comprises an immunoglobulin light chain variable region and an immunoglobulin heavy chain variable region; wherein the immunoglobulin light chain variable region comprises an amino acid sequence that is at least about 93% identical, at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:384; or at least about 94% identical, at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:385; and wherein the immunoglobulin heavy chain variable region comprises an amino acid sequence that is at least about 82% identical, at least about 85% identical, at least about 87% identical, at least about 90% identical, at least about 92% identical,

at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:383.

[0189] In some embodiments, the second binding domain is a CD3 binding domain that comprises a HCDR1 that comprises SEQ ID NO: 19, a HCDR2 that comprises SEQ ID NO: 20, and a HCDR3 that comprises SEQ ID NO: 21; and a LCDR1 that comprises SEQ ID NO: 22, a LCDR2 that comprises SEQ ID NO: 23, and a LCDR3 that comprises SEQ ID NO: 24. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384. In some embodiments, the CD3 binding domain comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 25. In some embodiments, the CD3 binding domain comprises a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 26. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

[0190] In some embodiments, a CD123-binding polypeptide or protein further comprising a CD3-binding domain may have a low level of high molecular weight aggregates produced during recombinant expression of the polypeptide or protein. A CD123-binding polypeptide or protein further comprising a CD3-binding domain may exhibit a relatively long stability in human serum, depending on the CD3-binding domain present in the polypeptide or protein.

[0191] In certain variations, the CD3-binding domain and comprises one or more of the CD3-binding sequences (e.g., CDRs or variable regions) disclosed in US 2013/0129730, US 2011/0293619, US 7,635,472, WO 2010/037836, WO 2004/106381, or WO 2011/121110; each incorporated herein by reference in its entirety. In some embodiments, a CD3-binding domain comprises one or more of the sequences shown in Table 6.

Table 6: Exemplary CD3-binding domain light chain CDRs

LCDR1	LCDR2	LCDR3
GSSTGAVTSGYYPN (SEQ ID NO:110)	GTKFLAP (SEQ ID NO:113)	ALWYSNRWW (SEQ ID NO:116)
RSSTGAVTSGYYPN (SEQ ID NO:111)	ATDMRPS (SEQ ID NO:114)	ALWYSNRWW (SEQ ID NO:117)

GSSTGAVTSGNYPN (SEQ ID NO:112)	GTKFLAP (SEQ ID NO:115)	VLWYSNRWW (SEQ ID NO:118)
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[0192] In various embodiments, a CD3-binding domain comprises one or more of the sequences shown in Table 7.

Table 7: Exemplary CD3-binding domain heavy chain CDRs

HCDR1	HCDR2	HCDR3
IYAMN (SEQ ID NO:119)	RIRSKYNNYATYYADSVKS (SEQ ID NO:122)	HGNFGNSYVSFFAY (SEQ ID NO:125)
KYAMN (SEQ ID NO:120)	RIRSKYNNYATYYADSVKD (SEQ ID NO:123)	HGNFGNSYISYWAY (SEQ ID NO:126)
SYAMN (SEQ ID NO:121)	RIRSKYNNYATYYADSVKG (SEQ ID NO:124)	HGNFGNSYLSFWAY (SEQ ID NO:127)

[0193] In some embodiments, a therapeutic protein comprises, in order from amino terminus to carboxyl terminus a first binding domain, a hinge region, an immunoglobulin constant region, and a second binding domain. In some embodiments, the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD. In some embodiments, the first binding domain comprises: an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, the HCDR1 comprises SEQ ID NO: 10, the HCDR2 comprises SEQ ID NO: 11, and the HCDR3 comprises SEQ ID NO: 12. In some embodiments, the LCDR1 comprises SEQ ID NO: 13, the LCDR2 comprises SEQ ID NO: 14, and the LCDR3 comprises SEQ ID NO: 15. In some embodiments, the HCDR1 comprises SEQ ID NO: 10, the HCDR2 comprises SEQ ID NO: 11, and the HCDR3 comprises SEQ ID NO: 12; and the LCDR1 comprises SEQ ID NO: 13, the LCDR2 comprises SEQ ID NO: 14, and the LCDR3 comprises SEQ ID NO: 15. In some embodiments, the first binding domain comprises a sequence at least 95% identical to SEQ ID NO: 18. In some embodiments, the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, the HCDR1 comprises SEQ ID NO: 19, the HCDR2 comprises SEQ ID NO: 20, and the HCDR3

comprises SEQ ID NO: 21. In some embodiments, the LCDR1 comprises SEQ ID NO: 22, the LCDR2 comprises SEQ ID NO: 23, and the LCDR3 comprises SEQ ID NO: 24. In some embodiments, the HCDR1 comprises SEQ ID NO: 19, the HCDR2 comprises SEQ ID NO: 20, and the HCDR3 comprises SEQ ID NO: 21; and the LCDR1 comprises SEQ ID NO: 22, the LCDR2 comprises SEQ ID NO: 23, and the LCDR3 comprises SEQ ID NO: 24. In some embodiments, the second binding domain comprises a sequence at least 95% or 100% identical to SEQ ID NO: 27. In some embodiments, the therapeutic protein comprises the sequence of SEQ ID NO: 31.

[0194] The structural format of multispecific anti-CD123 and anti-CD3 molecules disclosed herein induces potent tumor cell lysis but reduced cytokine release compared to multispecific anti-CD123 and anti-CD3 molecules in alternative structural formats. Without being bound by any theory, the polypeptide structural format disclosed herein (e.g., in order from amino terminus to carboxyl terminus): (a) a first binding domain that is a CD123-binding domain; (b) a hinge region; (c) an immunoglobulin constant region; and (d) a second binding domain that is a human or humanized binding domain that specifically binds a T-cell, CD3, CD3 ϵ or a T-cell receptor (TCR) complex) induces a moderate level of T-cell Receptor (TCR) stimulation, compared to other T-cell engagers. It has been extensively documented that the strength or magnitude of the TCR signal regulates the outcome of T-cell activation. TCR stimulation triggers a number of cellular events that include initiation of effector function (e.g., cytolytic granzymes), and cytokine secretion and cell division (Corse, Gottschalk and Allison. J Immunol 2011, 186:5039-5045). These distinct cellular events can proceed with different kinetics and reach variable maximum levels, depending on the intensity of the TCR stimulus and additional factors. The multispecific structural format disclosed herein is sufficiently potent to cause lysis of tumor cells over multiple days and to induce multiple rounds of T-cell division but moderate enough to limit the amount of cytokine secretion.

[0195] In some embodiments, the multispecific polypeptide comprising a CD123-binding domain and a CD3-binding domain when bound to a CD3 protein on a T cell induces reduced cytokine release from said T cell as compared to an OKT3 antibody control. In some embodiments, the multispecific polypeptide comprising a CD123-binding domain and CD3-binding domain induces reduced cytokine release from said T cell as compared to a multispecific polypeptide comprising an CD3-binding domain derived from OKT3 or I2C. In some embodiments, the multispecific polypeptide

comprising a CD123-binding domain (e.g., a CD123-binding domain comprising an amino acid sequence at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to SEQ ID NO: 312 and/or SEQ ID NO:337) and a CD3-binding domain and in the scFv-Fc-scFv format induces reduced cytokine release in a non-human primate or human as compared to a bispecific polypeptide comprising a CD123-binding domain and I2C derived CD3-binding domain in a bispecific T-cell engager (scFv-scFv) format or dual affinity re-targeting format.

[0196] Also provided herein are pharmaceutical compositions comprising the therapeutic proteins described herein. In some embodiments, the compositions comprise 1-20 mg/m, 2.5-12 mg/ml, or 5-10 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise from about 2.5 mg/ml to about 12 mg/ml, or from about 5 mg/ml to about 10 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, or about 12 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise about 5 mg/ml of a therapeutic protein.

Methods of Use

[0197] The present disclosure provides methods for treating a subject with a disease or disorder, the methods comprising administering a therapeutically effective amount of at least one composition of the disclosure to the subject.

[0198] In some embodiments, the disease or disorder may be cancer. The cancer may be selected from, for example, acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm, B-cell acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML).

[0199] In some embodiments, the disease or disorder may be an inflammatory disease or disorder. In embodiments, the inflammatory disease or disorder may be an autoimmune disease or disorder. In some embodiments, the autoimmune disease or disorder is selected from irritable bowel syndrome, inflammatory bowel disease (e.g. Crohn's disease or ulcerative colitis), psoriasis, rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, asthma, multiple sclerosis, dermatomyositis, polymyositis, pernicious anaemia, primary biliary cirrhosis, acute disseminated encephalomyelitis (ADEM), Addison's disease,

ankylosing spondylitis, antiphospholipid antibody syndrome (aPL), autoimmune hepatitis, diabetes mellitus type 1, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's disease, idiopathic thrombocytopenic purpura, pemphigus vulgaris, Sjögren's syndrome, temporal arteritis, autoimmune hemolytic anemia, bullous pemphigoid, vasculitis, celiac disease, endometriosis, hidradenitis suppurativa, interstitial cystitis, morphea, scleroderma, narcolepsy, neuromyotonia, vitiligo, autoimmune inner ear disease and myasthenia gravis. In some embodiments, the inflammatory disease or disorder is psoriasis.

[0200] In some embodiments, the inflammatory disease or disorder may be a "neuroimmune disease" such as neuropathic pain, osteoarthritis, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease.

[0201] In some embodiments, the inflammatory disease or disorder may be an adverse transplant associated event, i.e. transplant rejection, allograft disease or graft-versus-host disease.

[0202] In some embodiments, for treatment methods and uses described herein, a protein or polypeptide described herein is delivered in a manner consistent with conventional methodologies associated with management of the disease or disorder for which treatment is sought. In accordance with the disclosure herein, a therapeutically effective amount of the protein or polypeptide is administered to a subject in need of such treatment for a time and under conditions sufficient to prevent or treat the disease or disorder.

[0203] Subjects for administration of a protein of the present disclosure include patients at high risk for developing a particular disorder as well as patients presenting with an existing such disorder. Typically, the subject has been diagnosed as having the disorder for which treatment is sought. Further, subjects can be monitored during the course of treatment for any change in the disorder (e.g., for an increase or decrease in clinical symptoms of the disorder). Also, in some variations, the subject does not suffer from another disorder requiring treatment.

[0204] In prophylactic applications, pharmaceutical compositions or medicaments comprising a protein of the present disclosure are administered to a patient susceptible to, or otherwise at risk of, a particular disorder in an amount sufficient to eliminate or reduce the risk or delay the onset of the disorder. In therapeutic applications, compositions or medicaments comprising a protein of the present disclosure are administered to a patient suspected of, or already suffering from such a disorder in an

amount sufficient to cure, or at least partially arrest, the symptoms of the disorder and its complications. An amount adequate to accomplish this is referred to as a therapeutically effective dose or amount. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient response (e.g., inhibition of inappropriate angiogenesis activity) has been achieved. Typically, the response is monitored and repeated dosages are given if the desired response starts to fade.

[0205] To identify subject patients for treatment according to the methods of the disclosure, accepted screening methods can be employed to determine risk factors associated with specific disorders or to determine the status of an existing disorder identified in a subject. Such methods can include, for example, determining whether an individual has relatives who have been diagnosed with a particular disorder. Screening methods can also include, for example, conventional work-ups to determine familial status for a particular disorder known to have a heritable component. For example, various cancers are also known to have certain inheritable components. Inheritable components of cancers include, for example, mutations in multiple genes that are transforming (e.g., Ras, Raf, EGFR, cMet, and others), the presence or absence of certain HLA and killer inhibitory receptor (KIR) molecules, or mechanisms by which cancer cells are able to modulate immune suppression of cells like NK cells and T-cells, either directly or indirectly (see, e.g., Ljunggren and Malmberg, *Nature Rev. Immunol.* 7:329-339, 2007; Boyton and Altmann, *Clin. Exp. Immunol.* 149:1-8, 2007). Toward this end, nucleotide probes can be routinely employed to identify individuals carrying genetic markers associated with a particular disorder of interest. In addition, a wide variety of immunological methods are known in the art that are useful to identify markers for specific disorder. For example, various ELISA immunoassay methods are available and well-known in the art that employ monoclonal antibody probes to detect antigens associated with specific tumors. Screening can be implemented as indicated by known patient symptomology, age factors, related risk factors, etc. These methods allow the clinician to routinely select patients in need of the methods described herein for treatment.

[0206] For administration, the pharmaceutical compositions of the disclosure may comprise: (i) therapeutic protein/polypeptide; and (ii) a pharmaceutically acceptable carrier, diluent or excipient. In some embodiments, the pharmaceutical composition

may comprise (i) a therapeutic protein/peptide, (ii) a buffer, (iii) an excipient, and (iv) a surfactant.

[0207] A pharmaceutical composition comprising a polypeptide or protein described herein may be formulated in a dosage form selected from the group consisting of: an oral unit dosage form, an intravenous unit dosage form, an intranasal unit dosage form, a suppository unit dosage form, an intradermal unit dosage form, an intramuscular unit dosage form, an intraperitoneal unit dosage form, a subcutaneous unit dosage form, an epidural unit dosage form, a sublingual unit dosage form, and an intracerebral unit dosage form. The oral unit dosage form may be selected from the group consisting of: tablets, pills, pellets, capsules, powders, lozenges, granules, solutions, suspensions, emulsions, syrups, elixirs, sustained-release formulations, aerosols, and sprays.

[0208] A pharmaceutical composition comprising polypeptide or protein described herein may be administered to a subject in a therapeutically effective amount. According to the methods of the present disclosure, polypeptide or protein described herein can be administered to subjects by a variety of administration modes, including, for example, by intramuscular, subcutaneous, intravenous, intra-atrial, intra-articular, parenteral, intranasal, intrapulmonary, transdermal, intrapleural, intrathecal, and oral routes of administration. For prevention and treatment purposes, an antagonist can be administered to a subject in a single bolus delivery, via continuous delivery (e.g., continuous transdermal delivery) over an extended time period, or in a repeated administration protocol (e.g., on an hourly, daily, weekly, or monthly basis).

[0209] Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by determining effective dosages and administration protocols that significantly reduce the occurrence or severity of the subject disorder in model subjects. Effective doses of the compositions of the present disclosure vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, whether treatment is prophylactic or therapeutic, as well as the specific activity of the composition itself and its ability to elicit the desired response in the individual. Usually, the patient is a human, but in some diseases, the patient can be a nonhuman mammal. Typically, dosage regimens are adjusted to provide an optimum therapeutic response, *i.e.*, to optimize safety and efficacy.

[0210] Also provided herein are uses of the compositions of the disclosure in the manufacture of a medicament for treating cancer. For instance, compositions of the disclosure may be used for treatment of acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Also provided are methods comprising administering a composition comprising a multispecific polypeptide comprising a CD123 binding domain and a CD3 binding domain to a patient by IV infusion at a weekly dose of about 0.3, about 1, about 3, about 6, about 9, about 12, about 18, about 20, about 24, about 30, about 36, about 50, about 48, about 60, about 75, or about 100 μ g. Typically a patient is treated once or twice a week for 4 to 6 weeks. The patient may receive the same dosage each week or the dosage may be increased, for instance, each week.

[0211] In some embodiments, the dosage is increased each week, with the first dosage being less than what a patient would be expected to tolerate. This type of step-up treatment regimen reduces the risk that the patient will develop an infusion related reaction or cytokine release syndrome. In some embodiments, a multispecific protein comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129) may be administered to a patient intravenously such that the dosage is increased each week for at least the first two or first three doses. For instance, a composition of the disclosure may be administered by IV infusion according to the following weekly treatment schedule: week 1 dosage: 6 μ g; week 2 dosage: 9 μ g; week 3 dosage: 12 μ g; and week 4 dosage and subsequent week dosages: 12 μ g. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 μ g; week 2 dosage: 9 μ g; week 3 dosage: 12 μ g; and week 4 dosage and subsequent week dosages: 18 μ g. In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage: 6 μ g; and week 2 and subsequent week dosages: 9 μ g, and in some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage: 9 μ g; and week 2 and subsequent week dosages: 12 μ g. In other embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage 12 μ g, and week 2 and subsequent week dosages: 18 μ g.

[0212] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week

1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 12 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 18 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 12 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 24 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 36 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 48 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 60 µg.

[0213] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 9 µg; day 3: 12 µg; day 4: 18 µg; day 8: 18 µg; day 11: 18 µg; day 15: 36 µg; day 22: 36 µg; followed by weekly doses of 36 µg.

[0214] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 18 µg; day 4: 24 µg; day 8: 24 µg; day 11: 24 µg; day 15: 48 µg; day 22: 48 µg; followed by weekly doses of 48 µg.

[0215] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 24 µg; day 4: 36 µg; day 8: 36 µg; day 11: 36 µg; day 15: 60 µg; day 22: 60 µg, followed by weekly doses of 60 µg.

[0216] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 24 µg; day 4: 36 µg; day 8: 48 µg; day 11: 48 µg; day 15: 100 µg; day 22: 100 µg, followed by weekly doses of 100 µg.

[0217] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 18 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 9 µg is administered on day 15, and 9 µg is administered on day 22. In some embodiments, 9 µg is administered on day 1, 12 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22. In some embodiments, 12 µg is administered on day 1, 18 µg is administered on day 8, 18 µg is administered on day 15, and 18 µg is administered on day 22.

[0218] In some embodiments, a patient treated according to the methods of the disclosure exhibits a decrease in bone marrow blast percentage, and in some embodiments, a patient exhibits a decrease in absolute blast counts in the blood. In some embodiments, the treatment results in reduction in patient blast levels by at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or more, compared to the patient's levels immediately before the treatment.

[0219] In some embodiments, a patient treated according to the methods of the disclosure exhibits a complete remission (CR). As used herein, complete remission refers to a reduction in bone marrow blasts to less than about 5%, absence of circulating blasts and blasts with Auer rods, absence of extramedullary disease, and absolute neutrophil count (ANC) $\geq 1.0 \times 10^9/\text{L}$ (1,000/ μL) and PLT $\geq 100 \times 10^9/\text{L}$ (100,000/ μL). In some embodiments, a patient treated according to the methods of the disclosure exhibits a CR without Minimal Residual Disease (CR_{MRD}). As used herein, CR_{MRD} refers to CR with negativity for a genetic marker by quantitative reverse transcription polymerase chain reaction (RT-qPCR) or CR with negativity by

multiparameter flow cytometry. In some embodiments, a patient treated according to the methods of the disclosure exhibits a CR with Incomplete Hematologic Recovery (CR_i). CR_i includes all the criteria of CR, described above, except for residual neutropenia (ANC < $1.0 \times 10^9/L$ [$1,000/\mu L$]) or thrombocytopenia (PLT < $100 \times 10^9/L$ [$100,000/\mu L$]).

[0220] In some embodiments, a patient treated according to the methods of the disclosure exhibits a Morphologic Leukemia-Free State (MLFS). As used herein, MLFS refers to bone marrow blasts < 5% (i.e., marrow should not be merely “aplastic;” at least 200 cells should be enumerated or cellularity should be at least 10%); absence of blasts with Auer rods; and absence of extramedullary disease. No hematologic recovery is required.

[0221] In some embodiments, a patient treated according to the methods of the disclosure exhibits a Partial Remission (PR). As used herein, a PR includes all hematologic criteria of CR, described above, plus a decrease of bone marrow blast percentage to 5 to 25%, and at least 50% decrease of pretreatment bone marrow blast percentage.

[0222] In some embodiments, a patient treated according to the methods of the disclosure exhibits Stable Disease (SD), characterized by an absence of CR_{MRD}, CR, CR_i, PR, and MLFS, but without progressive disease (i.e., increase in bone marrow blast percentage and/or increase of absolute blast counts in the blood).

[0223] A patient treated with the CD123 x CD3 targeting multispecific polypeptide (e.g., TRI130 or TRI129) at either the same dosage each week or with a step-up treatment regimen may also have infusion times (i.e., the length of the infusion) modified to further reduce the likelihood of an infusion reaction or cytokine release syndrome. To reduce the risk of an adverse event, the first dose is administered by IV to the patient over several hours, e.g., 20-24 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours, wherein each of the first,

second, third, and fourth dose are the same. The composition can also be administered to a subject by continuous IV infusion, e.g., continuous IV infusion up to about 72 hours in duration.

[0224] A patient treated with the CD123 x CD3 targeting multispecific polypeptide (e.g., TRI130 or TRI129) may also be treated with one or more additional therapeutic agents. The one or more additional therapeutic agents may be administered at or around the same time as the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents are administered before (i.e., as a “premedication”) administration of the multispecific polypeptide, such as about 1-3 hours before administration thereof. In some embodiments, the one or more additional therapeutic agents are administered after administration of one or more doses of the multispecific polypeptide.

[0225] In some embodiments, the one or more additional therapeutic agents are diphenhydramine, acetaminophen, and/or dexamethasone. In some embodiments, the one or more additional therapeutic agents may be administered intravenously or orally. In some embodiments, dexamethasone may be administered at a dose of about 10 to about 20 mg. In some embodiments, methylprednisolone may be administered at a dose of about 1 mg/kg. In some embodiments, acetaminophen may be administered at a dose of about 650 or about 1,000 mg. In some embodiments, the acetaminophen may be administered three times a day for 1 day, with the first dose administered 1 to 3 hours before administration of the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents may comprise an antihistamine such as diphenhydramine. Diphenhydramine may be administered at a dose of about 50 mg. In some embodiments, the one or more therapeutic agents may comprise allopurinol. In some embodiments, allopurinol is administered at least 2 days prior to administration of the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents may comprise tocilizumab.

[0226] In some embodiments, a method for treating a disorder characterized by overexpression of CD123 in a patient in need thereof comprises administering to the patient an effective amount of a pharmaceutical composition comprising a recombinant polypeptide comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129) at any of the doses or regimens described herein. In some embodiments, a method for treating a disorder characterized by overexpression

of CD123 in a patient in need thereof comprises administering to the patient an effective amount of a pharmaceutical composition comprising a recombinant polypeptide comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129); wherein the administration of the pharmaceutical composition induces reduced cytokine levels in the subject as compared to administration of (a) a dual affinity re-targeting antibody comprising the CD123 binding domain and the CD3 binding domain of the recombinant polypeptide; or (b) a bispecific T-cell engager molecule comprising the CD123 binding domain and the CD3 binding domain of the recombinant polypeptide. In some embodiments, the disorder is cancer, such as AML or MDS. In some embodiments, the subject was previously treated with a different CD123-binding molecule, and wherein the subject experienced an adverse event after the previous treatment. In some embodiments, the adverse event was excessive cytokine release. In some embodiments, the cytokine levels were levels of IFN- γ , TNF- α , IL-6, IL-2, IL-8, IL-10, IL-17, GM-CSF, IL-4, IL-12, IL-13 or IL-1 β , or any combination thereof. In some embodiments, the cytokine levels were levels of IFN- γ , IL-2, TNF- α and IL-10. In some embodiments, cytokine levels are measured in an *in vitro* activated T cell assay.

[0227] Pharmaceutical compositions comprising the proteins and polypeptides described herein can be supplied as a kit comprising a container that comprises the pharmaceutical composition as described herein. A pharmaceutical composition can be provided, for example, in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Such a kit can further comprise written information on indications and usage of the pharmaceutical composition. In some embodiments, the kit comprises the pharmaceutical composition and an IV stabilizing solution (0.1 M succinate buffer, and 0.08 % weight/volume polysorbate 80, at pH 6.0 or similar solution that is designed to prevent or reduce the likelihood that the multispecific polypeptides will adhere to plastic tubing and bags).

[0228] The disclosure will be further clarified by the following examples, which are intended to be purely exemplary of the disclosure and in no way limiting.

EXAMPLES

[0229] The invention is further described in detail by reference to the following examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

[0230] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLE 1: Formulation of Anti-CD123 x Anti-CD3 Bispecific Proteins

[0231] It is critical for therapeutic protein drugs to maintain their product quality during long term storage at distribution centers, shipping, and storage and use as the site of dose administration. Proteins may be exposed to a variety of temperatures following production and formulation, including frozen (-80 °C, -20 °C), refrigerated (4 °C) and room temperature (25 °C). Conditions impacting protein drug stability include pH, protein concentration and the concentration of salts and excipients that may be included in the formulation.

[0232] To evaluate the 5 mM succinate, 6.5% sucrose, 0.02% weight/volume polysorbate-80 (abbreviated SSuT), pH 4.8 formulation for anti-CD123 x anti-CD3 bispecifics (anti-CD123 – hinge region – CH2-CH3 – anti-CD3), Chinese Hamster Ovary (CHO) cells were stably transfected with DNA plasmids encoding either TRI-129 or TRI-130 along with a selection marker. Following transfection, these cells were grown under selective pressure to assure stable integration of the bispecific protein-encoding gene into the CHO cell genome and to kill cells that did not contain the selection marker.

[0233] The TRI-129 and TRI-130 CHO pools were initially grown in shake flasks, then transferred to 10L bioreactors and cultured in defined, animal component-free media. After approximately two weeks of culture, the cell culture supernatant was

clarified using a combination of depth and sterile filtration, then purified using a combination of affinity and mixed mode column chromatography. The two-step purified protein was diafiltered into succinate buffer using a Tangential Flow Filtration (TFF) apparatus, then spiked with the appropriate volumes of concentrated stock solutions of sucrose and polysorbate-80 to achieve the defined composition. The TRI-129 and TRI-130 ADAPTIR proteins were evaluated in the preferred formulation buffer at both 2 mg/mL and 10 mg/mL. The stability in SSuT was compared to protein in Dulbecco's phosphate-buffered saline (dPBS).

[0234] In one experiment, TRI-129 and TRI-130 were formulated either in SSuT or PBS and samples were stored at 4 °C and 25 °C. The purity of the sample was determined at the beginning of the study and after 9 days of storage using an analytical Size Exclusion Chromatography (SEC) assay. The change in the %purity of the drug product after 9 days is reported in Table 8 below. The negative values for the samples in dPBS indicate that the purity of the sample is declining and is indicative of product peak area redistributing from the main peak to aggregate/higher molecular weight species (HMW). After 9 days, both TRI-129 and TRI-130 in SSuT show much smaller changes in purity as compared to the samples in dPBS, indicating that the formulation has significant stabilizing properties.

Table 8. Comparison of PBS versus SSuT, stability of TRI-129 and TRI-130 at 2 mg/mL after 9 days

Construct ID	dPBS Change in % MP 9 days @ 4 °C	dPBS Change in % MP 9 days @ 25 °C	SSuT Change in % MP 9 days @ 4 °C	SSuT Change in % MP 9 days @ 25 °C
TRI129	-1.2	-4.1	-0.4	-0.6
TRI130	-1.7	-3.4	0.0	-0.3

[0235] It is common for protein therapeutics to be submitted to one or more freeze-thaw cycles from either -80 or -20 °C to 4 °C or room temperature during production and/or storage. It is important that the selected bulk drug substance and drug product formulations stabilize the protein and prevent drastic changes in drug quality. TRI-129 and TRI-130 formulated in SSuT were examined for their ability to resist cryoaggregation, which is the formation of aggregation caused by freezing and thawing protein solutions. The stability of TRI-129 and TRI-130 were investigated at

2 and 10 mg/mL. The purity of the samples was assessed by analytical SEC, then 200 μ L were placed at -80 or -20 °C for several hours to allow for sufficient time for the samples to completely freeze. The samples were removed from the freezers and allowed to completely thaw at room temperature. The samples were submitted to a total of three freeze-thaw cycles at -80 or -20 °C, then retested by SEC to determine the change in %MP. As show in Table 9 below, both TRI-129 and TRI-130 showed minimal change in %MP at either -80 or -20 °C. The higher protein concentration samples at 10 mg/mL showed slightly greater changes in %MP than the samples at 2 mg/mL.

Table 9. Analysis of Freeze-Thaw stability of TRI-129 and TRI-130 in SSuT formulation buffer, at 2 and 10 mg/mL.

Construct ID	2 mg/mL Change in % MP -80 °C FT x 3	2 mg/mL Change in % MP -20 °C FT x 3,	10 mg/mL Change in % MP -80 °C FT x 3,	10 mg/mL Change in % MP -20 °C FT x 3
TRI129	-0.1	-0.1	-0.4	-0.4
TRI130	0.0	0.0	-0.1	-0.1

EXAMPLE 2: Determination of the No Observed Adverse Effect Level (NOAEL) and the Minimum Anticipated Biological Effect Level (MABEL) for TRI130

The No Observed Adverse Effect Level (NOAEL)

[0236] A 28-day repeat-dose toxicology study with a 5-week recovery period was conducted in non-human primates (NHP). Animals in four study groups received weekly doses of vehicle, or 0.5, 2.5, or 10 mg/kg of TRI130. Parameters measured included safety pharmacology, laboratory evaluations, and necropsy with full histopathology. There were no clinical adverse findings, no changes in animal weights or organ weights, and no abnormal macroscopic or microscopic findings in histopathology related to TRI130. Minimal cytokines were detected after dosing and were attenuated after the second dose compared to the first dose. The expected pharmacodynamic effect of the anti-CD3 binding domain of the molecule was observed with transient redistribution of T cells. The elimination half-life was approximately 73 hours at the high dose. The no observed adverse effect level

(NOAEL) was 10 mg/kg in NHP, which translates into a human equivalent dose (HED) of approximately 3.2 mg/kg.

Minimum Anticipated Biological Effect Level (MABEL)

[0237] TRI130 mobilizes T cells to lyse tumor cells expressing the target antigen CD123, and the maximum activity occurs at very low levels of TCR occupancy (data not shown). To calculate the minimum anticipated biologic effect level (MABEL), in vitro activity assays were used in place of in vitro receptor occupancy.

[0238] The MABEL was determined using the effective concentration necessary to elicit 10% activity (EC10) (Muller et al., 2009) in a human T-cell activation in vitro assay. While the potency of TRI130 in these T-cell activation assays may vary depending on the degree of activation of T cells and the effector-to-target (E:T) cell ratio, the activation assay represents a more sensitive assay for calculation of MABEL, compared to the redirected T cell cytotoxicity (RTCC) assay. Both RTCC and T-cell activation were evaluated as possible assays to predict the MABEL for the starting clinical dose. The RTCC assay, using purified T cells at a 10:1 E:T ratio with CD123+ KG1a tumor cell line had an average EC10 of 5.8 pM, assessed from 5 donors (a range of 4.4 to 6.7 pM across the donors evaluated). In contrast, the T-cell activation assay was a more sensitive assay to estimate the MABEL. To measure T-cell activation induced in vitro, T cells were isolated from peripheral blood mononuclear cells and incubated with TRI130 in the presence of CD123+ tumor cells (MOLM-13). Upregulation of CD69 and CD25 on T cells was monitored at 20 hours using multi-color flow cytometry, after gating on live CD4+ and CD8+ T cells. These assays assessed three donors, for activation of both CD4 and CD8 T cells. The average EC10 calculation for these assays was 1.2 pM for CD4 T cells (range 0.7 to 1.6 pM) and 1.3 pM for CD8 T cells (range 0.9 to 2.0 pM). This assay represents a more conservative estimate of MABEL and was used to estimate the starting dose for patients. For TRI130, 0.7 pM (0.113 ng/ml) was the most conservative approach to MABEL, based on the CD4+ T-cell response from the donors.

[0239] Clearance and volume estimates for Group 4 in the single dose NHP study were determined using a WinNonlin (v6.4) precompiled 2 compartment model for intravenous (IV) dosing. Allometric scaling was used to predict human clearance and volume parameter estimates that could be used to simulate dosing strategies that would result in a Cmax below the EC10 (MABEL) value of 0.7 pM, the lowest EC10

determined from an individual donor used in the activation assay. With this modeling, a dose of about 0.005 µg/kg would have a C_{max} below the MABEL concentration of 0.113 ng/mL (0.7 pM).

[0240] A flat or fixed dose, instead of a weight-based dose is being utilized in this study. Several studies have shown that flat dosing compared to weight-based dosing performed similarly across a number of monoclonal antibodies and that the PK variability introduced by either dosing regimen is moderate relative to the variability generally observed in pharmacodynamics, efficacy, and safety (Wang et al., 2009). Assuming a 60-kg patient and a MABEL dose of 0.005 µg/kg, the starting dose for the study is 0.3 µg.

EXAMPLE 3: Administration of TRI130, a formulated anti-CD123 x anti-CD3 therapeutic candidate, to patients

[0241] Formulated TRI130 (5 mM succinate, 6.5% sucrose, 0.02% weight/volume polysorbate-80, pH 4.8) is being administered to patients in an ongoing Phase 1/1b open-label, dose-escalation study of patients with relapsed or refractory acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). The study is being conducted in two parts. The first part is a Phase 1 open-label, dose escalation study to determine the recommended dose for Phase 1b. Phase 1b is an open-label expansion study to assess the clinical activity and safety of the drug at the recommended dose. The study design is outlined in **FIG. 2**. Endpoints include safety, immunogenicity, pharmacokinetics, pharmacodynamics, and clinical activity.

[0242] Table 10 is a dosing schedule that sets forth the amount that cohorts 1-10 are being dosed weekly (by IV administration) (dose escalation cohorts). In both parts of the study, patients receive drug intravenously weekly for six 28-day cycles, unless disease progression, intolerable toxicity, or withdrawal of consent occurs earlier. There is an option for longer treatment if the patient is responding.

Table 10: Dose Escalation Cohorts 1 to 10

Cohort	1st IV Dose	2nd IV Dose	3rd IV Dose	≥ 4th IV Dose	Number of Patients
1	0.3 µg	0.3 µg	0.3 µg	0.3 µg	1
2	1 µg	1 µg	1 µg	1 µg	1

3	3 µg	3 µg	3 µg	3 µg	1
4	9 µg	9 µg	9 µg	9 µg	3 + 3
5	6 µg	9 µg	12 µg	12 µg	3 + 3
6	Cohort 6 will consist of 2 dose Cohorts conducted concurrently, 6a and 6b				
6a	6 µg	9 µg	12 µg	18 µg	3 + 3
6b	6 µg	12 µg	12 µg	12 µg	3 + 3
7	6 µg	12 µg	18 µg	24 µg	3 + 3
8	6 µg	12 µg	18 µg	36 µg	3 + 3
9	6 µg	12 µg	18 µg	48 µg	3 + 3
10	6 µg	12 µg	18 µg	60 µg	3 + 3

[0243] Drug is supplied in single-use vials containing 2 mg drug product in 1 mL of liquid at a concentration of 2 mg/mL. The drug product is mixed with an IV stabilizing solution to prevent drug product from adhering to IV bags and IV tubing sets. The stabilizing solution is supplied sterile and refrigerated (2 to 8°C) in 10 mL vials comprised of 0.1 M succinate buffer, and 0.08 % weight/volume polysorbate 80, at pH 6.0.

[0244] For Cohorts 1 through 4, administration is by IV infusion over approximately 20 to 24 hours for the first dose (Cycle 1, Day 1), over 8 hours ($\pm$ 1 hour) for the second dose (Cycle 1, Day 8), over 6 hours ($\pm$ 1 hour) for the third dose (Cycle 1, Day 15) and over 4 hours ($\pm$ 1 hour) for all subsequent doses (Cycle 1, Day 22 and onwards). For Cohorts 5 and above, administration is by IV infusion over 20 to 24 hours for the first dose and every time the dose is increased. The second time the same dose is administered it is infused over 8 hours ($\pm$ 30 minutes), for the third time over 6 hours ($\pm$ 30 minutes), and for the fourth time and all further times over 4 hours ($\pm$ 30 minutes).

[0245] If necessary, to manage or prevent any adverse event, and in particular, infusion related reaction (IRR) or cytokine release syndrome (CRS), any dose infusion may be slowed and/or interrupted, with the administration time extended up to 72 hours. If an infusion is extended past 60 hours, then the patient must be observed for 12 hours after the infusion is completed. Table 10 discloses a stepped dosing regimen with the potential to reduce the likelihood of IRR and/or CRS (starting at cohort 5).

[0246] The frequency of patient dosing is weekly for up to 6 months. Dosing on a weekly schedule was selected based on the cynomolgus monkey toxicology study of escalating TRI130 doses (data not shown). The half-life of TRI130 following a single dose ranged from approximately 25 to 113 hours for individual animals dosed with

0.25 to 1 mg/kg; the longer half-life estimates were associated with animals in the high dose group (1 mg/kg).

[0247] To mitigate infusion related reactions (IRRs) and cytokine release syndrome (CRS), patients are administered the following pre-medications: diphenhydramine, acetaminophen, and dexamethasone. All premedications are administered 1 to 3 hours before the infusion is initiated. The dose of any of the premedications may be reduced, if, in the opinion of the Investigator, comorbidities require it. Dexamethasone is optional after Cycle 2, Day 15, so long as the patient has not experienced any IRRs or CRS with earlier doses. The doses of the 3 premedications are:

1. Dexamethasone 10 to 20 mg IV, or methylprednisolone 1 mg/kg IV, or equivalent; the dose is at the Investigator's discretion based on patient's comorbidities;
2. Acetaminophen 650 or 1,000 mg, or equivalent, orally (PO) three times a day for 1 day (650- or 1,000-mg dose at the Investigators discretion), with the first dose administered 1 to 3 hours before the study drug infusion; and
3. Antihistamine: diphenhydramine 50 mg PO or IV; or equivalent.

If an investigator elects to administer allopurinol for tumor lysis prophylaxis, then it must be started at least 2 days prior to the start of study drug.

Phase 1 - Dose Escalation Study

[0248] Dosing was started at the minimum anticipated biologic effect level (MABEL) in patient cohorts. Patients enrolled had either: 1) relapsed or refractory AML and refused or were not eligible for intensive chemotherapy or an allogeneic stem cell transplant, or 2) relapsed or refractory MDS and had > 5% blasts in the marrow or any circulating blasts in the peripheral blood and had failed a prior hypomethylating agent (HMA); failure is defined as intolerance to HMA, lack of response (no CR by at least 6 cycles), or IWG defined progressive disease during or after treatment with an HMA. Demographics for 32 patients enrolled in the ongoing Phase 1 dose escalation study (through Cohort 7) are shown below in Table 11. For patients enrolled through Cohort 7, the median age was 67 years, and 79% of the patients had AML. The average number of doses administered per patient was 8.5 with an average treatment duration of 54 days.

Table 11: Demographics and exposure, patients enrolled through cohort 7

<u>Characteristic</u>	<u>Statistic</u>	<u>All Patients (N=32)</u>
Age	Median (Range)	67 (18, 81)
Female	N (%)	15 (47%)
Prior Lines of Therapy	Median (Range)	3 (1, 13)
<u>Disease</u>		
MDS	N (%)	7 (22%)
AML Primary	N (%)	21 (66%)
AML Therapy Related	N (%)	4 (13%)
<u>Cytogenetic Risk</u>		
Favorable	N (%)	3 (9%)
Intermediate	N (%)	7 (22%)
Poor	N (%)	6 (19%)
Unknown	N (%)	16 (50%)

<u>Summary of Treatment Exposure</u>	<u>32 Patients Mean (SD)</u>
Doses Administered	8.5 (8.3)
Treatment Duration (days)	54 (59)

[0249] Treatment-Related Adverse Events for the 32 patients enrolled in the ongoing Phase 1 dose escalation study are shown below in Table 12. 34% of patients experienced one or more IRR/CRS events (Grade ≥ 3 reported in 16%). The most common symptoms were dyspnea, fever, hypotension, hypoxia, tachycardia, and rigors/chills. Notably, IRR/CRS was the only treatment-related serious adverse event to occur in two or more patients. 3 out of the 11 patients that experienced an IRR/CRS event received tocilizumab to treat the same.

Table 12: Treatment-Related Adverse Events

<u>Treatment-Related Adverse Events*</u>	<u>All (N=32)</u>	<u>Grade ≥ 3 (N=32)</u>
IRR/CRS**	11 (34%)	5 (16%)
Fatigue	5 (16%)	1 (3%)
Diarrhea	3 (9%)	

Fever	3 (9%)	
Anemia	2 (6%)	2 (6%)
Flushing	2 (6%)	
Hypotension	2 (6%)	
Nausea	2 (6%)	1 (3%)
Peripheral Edema	2 (6%)	
Rigors/Chills	2 (6%)	
Treatment-Related Serious Adverse Events		
IRR/CRS	8 (25%)	4 (13%)
*Treatment-related AE and SAE events occurring in 2 or more patients **Infusion Related Reaction/Cytokine Release Syndrome Toxicity grading is based on CTCAE criteria version 5.0		

[0250] The percentage of blasts in bone marrow aspirates was monitored over time for patients. As shown in **FIG. 3A-3D**, a reduction of bone marrow blasts was observed in several patients that received a highest dose ≥ 12 μg . Two patients each had reductions in bone marrow blasts from 29% to 0% (Cohort 6b, **FIG. 3C**), and from 33% to 4% (Cohort 6a, **FIG. 3B**). Absolute neutrophil and platelet counts met Complete Remission criteria. Both patients remain on study, as well as one patient with decreasing blasts in Cohort 7.

[0251] Serum cytokines were assessed at scheduled timepoints before and after administration of the highest dose in each patient, and were also evaluated at intervals during infusion related reactions or cytokine release syndrome events. As shown in **FIG. 4A-4D**, cytokines were not elevated during scheduled collections. Elevated cytokines, particularly IL-6, were observed during adverse events of IRR/CRS. Notably, in this small data set, no correlation was observed between the highest cytokine concentrations and dose level or grade of event.

[0252] Thus, in this preliminary study, administration of TRI130, through doses of 24 μg , was tolerated with a manageable safety profile. As noted above, two patients had complete remission (CR). Cytokines were not significantly elevated unless there was a concurrent adverse event of IRR/CRS. Preliminary data suggested no evidence of treatment-induced anti-drug antibodies (ADA).

Phase 1 - Additional Dose-Escalation Cohorts

[0253] After completion of a dose-limiting toxicity (DLT) observation period for Cohort 7, four additional sequential cohorts (Cohorts A, B, C, and D) will enroll patients while Cohorts ≥ 8 are treated. These cohorts will run sequentially and are independent of Cohorts 8 to 10, and will achieve a more rapid dose escalation.

[0254] Patients in Cohorts A, B, C, and D will have continuous IV dosing (20-24 hours/day) for the first 4 days of Cycle 1, then twice a week dosing during Week 2, followed by once a week dosing thereafter in Cycle 1 and all subsequent cycles. Table 14 shows the dosing schedule for Cohorts A, B, C, and D. The first week of dosing in Cohort A utilizes doses tested in Cohort 6a (Day 1 of 6 μg , Day 2 of 9 μg , Day 3 of 12 μg , and Day 4 of 18 μg). During the second week, the dose of 18 μg is administered on Day 8 and Day 11. In Week 3 the dose is escalated to 36 μg and is maintained at that level. The advantage of escalating the daily dose during the first week is that the C_{max} is gradually increased, which may lower the propensity for the development of IRR/CRS. Aggressive treatment with tocilizumab will be administered for Grade ≥ 2 IRR or CRS that does not respond within 2 hours of symptomatic treatment and dose interruption.

Table 14: Dosing for Cohorts A, B, C and D

Cohort	Week 1				Week 2		Weeks 3 & 4	$\geq$ Cycle 2	Number of Patients
	Day 1	Day 2	Day 3	Day 4	Day 8	Day 11	Days 15, & 22	Days 1, 8, 15, and 22	
A	6 μg	9 μg	12 μg	18 μg	18 μg	18 μg	36 μg	36 μg	3 + 3
B	6 μg	12 μg	18 μg	24 μg	24 μg	24 μg	48 μg	48 μg	3 + 3
C	6 μg	12 μg	24 μg	36 μg	36 μg	36 μg	60 μg	60 μg	3 + 3
D	6 μg	12 μg	24 μg	36 μg	48 μg	48 μg	100 μg	100 μg	3 + 3

[0255] For Cohorts A-D, administration is by IV infusion over 20 to 24 hours for the first dose and every time the dose is increased. The second time the same dose is

administered it is infused over 8 hours ($\pm$ 30 minutes), for the third time over 6 hours ($\pm$ 30 minutes), and for the fourth time and all further times over 4 hours ($\pm$ 30 minutes).

[0256] Premedications are administered before Cycle 1, Day 1; Cycle 1, Day 8; Cycle 1, Day 11; Cycle 1, Day 15; and Cycle 1, Day 22. For all subsequent doses, dexamethasone is optional, but acetaminophen and diphenhydramine are required.

Phase 1b - Expansion

[0257] The recommended-dose regimen will be further examined in two expansion cohorts consisting of the same type of patients: 1) Cohort 1 will consist of 24 patients with relapsed or refractory AML that are not eligible for intensive chemotherapy or an allogeneic transplant, and 2) Cohort 2 will consist of 24 patients with relapsed or refractory MDS that have $> 5\%$ blasts in the marrow or any circulating blasts in the peripheral blood and have failed a prior HMA; failure is defined as intolerance to HMA, lack of response (no CR by at least 6 cycles), or IWG-defined progressive disease during or after treatment with an HMA.

[0258] Serum samples will be collected for serial PK assessment for drug levels.