TRISPECIFIC ANTIBODY CONSTRUCTS TARGETING DLL3 AND METHODS OF USE THEREOF

CROSS-REFERENCE

[0001] This application claims priority to and the benefit of U.S. Provisional Application No. 63/730,303, filed December 10, 2024, U.S. Provisional Application No. 63/685,886, filed August 22, 2024, U.S. Provisional Application No. 63/575,343, filed April 5, 2024, and U.S. Provisional Application No. 63/560,940, filed March 4, 2024, the entire contents of which are incorporated by reference herein for all purposes.

TECHNICAL FIELD

[0002] The present disclosure generally relates to trispecific T cell engaging antibody constructs that can comprise a first binding domain capable of binding a first antigen on a cytotoxic effector cell, a second binding domain capable of binding a second antigen on a cytotoxic effector cell, and a third binding domain capable of binding a tumor-associated antigen (TAA), such as Delta-like Ligand 3 (also known and referred to herein as DLL3, or delta-like canonical Notch ligand 3), on a tumor cell. The disclosure also relates to pharmaceutical compositions comprising such constructs, as well as methods of using such constructs for the treatment of a disease such as cancer.

BACKGROUND

[0003] Cancer continues to pose a major unmet medical need, despite the considerable progress that has been made in its treatment over the past decades. While the current standard of care, as well as more recently developed anti-cancer therapies, have shown some clinical progress, various indications such as those with low T cell tumor infiltration still present a major clinical challenge.

[0004] Small cell lung cancer (SCLC), for instance, is an aggressive neuroendocrine cancer with a poor prognosis and high unmet medical need. Current standards of care, including chemotherapy, targeted therapy and immunotherapy, are associated with limited duration of response. Furthermore, SCLC is characterized by an immunosuppressive microenvironment and poor T cell infiltration which present additional treatment challenges.

[0005] DLL3 is an inhibitory Notch ligand that is aberrantly overexpressed in SCLC and other neuroendocrine tumors, but minimally expressed in normal tissues, and thus can be potentially exploited as a therapeutic target.

SUMMARY

[0006] In various embodiments, the present disclosure describes trispecific and trivalent antibody constructs that are capable of engaging two different antigens on one or more immune cell(s) (e.g., T cell(s)), as well as a TAA on a tumor cell. In certain embodiments of the present disclosure, described are trivalent and trispecific antibody constructs that comprise three binding domains, wherein a first binding domain is capable of binding a first antigen on a cytotoxic effector cell, a second binding domain is capable of binding a second antigen on a cytotoxic effector cell, and a third binding domain is capable of binding a TAA on a tumor cell.

[0007] In some embodiments, described herein is an antibody construct, comprising: (i) a first fragment antigen binding (Fab) domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first single-chain variable fragment (scFv) domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a third binding domain capable of binding DLL3 on a tumor cell, wherein the third binding domain is a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to (1) the C-terminus of the light chain of either the first Fab domain or the second Fab domain, if the third binding domain is the second Fab domain, or (2) the C-terminus of the light chain of the first Fab domain if the third binding domain is the second scFv domain.

[0008] In some embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding a TAA on a tumor cell; (iii) an scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of a Fab light chain.

[0009] In some embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain

capable of binding DLL3 on a tumor cell; (iii) an scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of a Fab light chain.

[0010] In some embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding cluster of differentiation 3 (CD3, e.g., CD3ε) on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) an scFv domain capable of binding cluster of differentiation 28 (CD28) on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the Fab light chain of the first Fab domain.

[0011] In some embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain. In some embodiments, the third binding domain is a second Fab domain. In other embodiments, the third binding domain is a second scFv domain.

[0012] In some embodiments, described herein is an antibody construct, comprising (from N- to C-terminus): (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 Fab heavy chain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer H1-H2; (iii) a first light chain (L1), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid

sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein L1 and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain; and (iv) a second light chain (L2), comprising an anti-DLL3 Fab light chain, wherein L2 and the anti-DLL3 Fab heavy chain of H2 associate to form an anti-DLL3 Fab domain.

[0013] In some embodiments, described herein is an antibody construct, comprising: (i) a Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0014] In some embodiments, described herein is an antibody construct, comprising: (i) a Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain N-terminus of the second Fc polypeptide, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0015] In some embodiments, described herein is an antibody construct, comprising: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 scFv domain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer H1-H2; and (iii) a light chain (L), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein L and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain.

[0016] In some embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding a TAA on a tumor cell, wherein the third binding domain comprises or consists of a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0017] Further described herein are pharmaceutical compositions comprising one or more of the trivalent and trispecific DLL3-targeting antibody constructs of the present disclosure, nucleic acid molecule(s) encoding the one or more (e.g., two, three, four, or more) polypeptide chains of the antibody constructs described herein, as well as methods of producing and using such antibody constructs, e.g., for the treatment of DLL3-positive cancers.

[0018] In some embodiments, the disclosure relates to a trivalent and trispecific antibody construct of the present disclosure for use in the treatment of cancer.

[0019] In some embodiments, the disclosure relates to a trivalent and trispecific antibody construct of the present disclosure for use in the manufacture of a medicament for the treatment of cancer.

[0020] In various embodiments, the cancer in the treatment of which a trivalent and trispecific antibody construct of the present disclosure can be used is a DLL3-positive cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The implementations disclosed herein are illustrated by way of example, and not by way of limitation, in the accompanying drawings. The description and drawings are only for the purpose of illustration and as an aid to understanding and are not intended as a definition of the limits of the antibody constructs, pharmaceutical compositions, and methods of the present disclosure.

[0022] **FIGS. 1A-1D** show schematic representations of the format and geometry of trivalent and trispecific antibody constructs that comprise binding domains capable of engaging CD3, CD28, and DLL3, according to embodiments of the present disclosure. **FIGS. 1E-1F** show schematic representations of bispecific control constructs used as described in this disclosure. “A” and “B” refer to two different heavy chains (can include homodimeric and heterodimeric Fc domains) and

the dark and light shadings represent heavy and light chains, respectively, according to certain embodiments of the present disclosure.

[0023] FIGS. 1G-1H show schematic representations of the format and geometry of trivalent and trispecific antibody constructs that comprise binding domains capable of engaging CD3 (e.g., via a Fab domain) and CD28 (e.g., via an scFv domain) on a cytotoxic effector cell, and a TAA on a tumor cell (e.g., via a Fab or an scFv domain), according to certain embodiments of the present disclosure.

[0024] FIGS. 2A-2C show dose-response curves of primary human T cells in co-culture with DLL3+ NCI-H82-RFP cells as well as primary human Pan-T cells in co-culture with DLL3+ NCI-H82-RFP cells following treatment with the respective anti-(DLL3xCD3xCD28) trivalent and trispecific antibody constructs and bispecific controls, respectively.

[0025] FIGS. 3A-3B show binding curves for certain trivalent and trispecific antibody constructs and bispecific control constructs to (i) wildtype Jurkat cells (e.g., T cells expressing both CD3 and CD28), (ii) CD28 knock-out (KO) Jurkat cells, and (iii) CD3 knock-out (KO) Jurkat cells, respectively.

[0026] FIG. 4A shows concentration response curves and *in vitro* cytotoxicities for certain tested constructs in various DLL3-positive cell lines in co-culture with human T cells. For this study, T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP (top left), SHP-77-RFP (top right) or COR-L279-RFP (bottom) cells such that the ratio of T cells to tumor cells was adjusted to an effector to target cell (E:T) ratio of 1:5, 1:2 and 1:2 for the H82, SHP-77 and COR-L279 cell lines, respectively. **FIG. 4B** shows concentration response curves and *in vitro* cytotoxicities for certain tested constructs in various DLL3-positive cell lines in co-culture with human T cells in a follow-on study in which human T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP (top left), SHP-77-RFP (top middle), COR-L279-RFP (top right), NCI-H69-RFP (bottom left), NCI-H524-RFP (bottom middle) and NCI-H526-RFP (bottom right) cells such that the ratio of T cells to tumor cells was adjusted to an effector to target cell (E:T) ratio of 1:5 for NCI-H82 and 1:2 for the remaining cell lines.

[0027] FIGS. 5A-5B show T cell proliferation in the presence of certain antibody constructs. **FIG. 5A** shows T cell proliferation in the presence of the tested antibody constructs and DLL3+ NCI-H82 cells, and **FIG. 5B** shows T cell proliferation in the presence of the tested antibody constructs only (i.e., no DLL3+ cells present).

[0028] FIGS. 6A-6B show upregulation of Bcl-XL expression in activated T-cells using certain trivalent and trispecific antibody constructs of the present disclosure as well as bispecific and reference constructs.

[0029] FIG. 7 shows tumor growth delay observed in an *in vivo* study using donor A-engrafted mice treated with 2.5 mg/kg of the trivalent and trispecific antibody construct v38967 when compared to a clinical benchmark (arrows indicate time points of test article administration). **FIG. 8** shows that percent survival in this *in vivo* study was significantly higher in mice receiving 2.5 mg/kg of v38967 compared to mice receiving equivalent doses of reference construct (Long-rank $p = 0.0047$). **FIG. 9** shows that body weights of mice treated with v38967 over the course of this *in vivo* study remained stable.

[0030] FIG. 10 shows tumor regression observed in donor A-engrafted mice treated with the trivalent and trispecific antibody construct v39917 compared to the reference construct (v38589) and irrelevant mAb (i.e., control antibody against an irrelevant target, v22277; arrows indicate time points of test article administration). **FIG. 11** and **FIG. 12** show the percent survival and change in body weight, respectively, over the course of this *in vivo* study.

[0031] FIG. 13A depicts curves showing the percent of T cells proliferated with the respective titrated antibody constructs tested and in either the presence or absence of target cells. Corresponding histograms of proliferation at 200 pM are displayed in **FIG. 13B**.

[0032] FIG. 14 shows tumor regression observed in donor B-engrafted mice treated with v41086, CD28-KO (v41753), and the reference construct (v38589), compared to the irrelevant mAb (v40552). Treatment with 6.6 nmol/kg and 3.3 nmol/kg of v41086 resulted in full tumor regression in 7 out of 7 mice (arrows indicate time points of test article administration). Body weights remained stable for all mice as shown in **FIG. 15**.

[0033] FIG. 16 shows tumor growth inhibition as observed in donor-engrafted mice treated with 2.85 nmol/kg of the trivalent and trispecific antibody construct v41086 when compared to treatment with the reference construct (v38985) (arrows indicate time points of test article administration).

[0034] FIG. 17 shows serum cytokine (IFNγ (or IFNg), IL-2, TNFα (or TNFa), and IL-6; in pg/mL) concentrations measured 72 hours post-treatment of humanized mice with the respective construct.

[0035] **FIG. 18** shows serum concentrations of the measured cytokines IL-6, IL-10, MCP-1 and IL-2 in cynomolgus monkeys that were given two intravenous (i.v.) doses of 10 mg/kg of the respective tested constructs.

DETAILED DESCRIPTION

[0036] In various embodiments, the present disclosure describes trivalent and trispecific antibody constructs capable of binding two different antigens on a cytotoxic effector cell (e.g., a T cell), and a TAA on a tumor cell.

[0037] In certain embodiments, the two different effector cell antigens to which a trivalent and trispecific antibody construct of the present disclosure binds are located on the same cytotoxic effector cell (e.g., a T cell).

[0038] In other embodiments, the two different effector cell antigens to which a trivalent and trispecific antibody construct of the present disclosure binds are located on different cytotoxic effector cells.

[0039] In embodiments in which the two different effector cell antigens to which a trivalent and trispecific antibody construct binds are located on the same cytotoxic effector cell (e.g., a T cell), such antibody construct comprises an anti-CD28 scFv domain linked to the C-terminus of an anti-CD3 Fab light chain (e.g., as depicted in **FIGs. 1B**, **1D**, **1G** and **1H**) of an anti-CD3 Fab domain and can cross-link the two effector cell antigens (e.g., CD3 epsilon (CD3e) and CD28) on the same cell, thereby stimulating a specific effector cell response, e.g., an anti-tumor response, in the presence of tumor cells expressing a TAA (e.g., DLL3). Such anti-tumor response may be differentiated from effector cell responses elicited by antibody constructs that merely target one effector cell antigen (e.g., CD3 or CD28, but not both, such as a bispecific anti-(CD3xTAA) or anti-(CD28xTAA) antibody construct), and/or it can be differentiated from a response elicited by trivalent and trispecific antibody constructs in which the anti-CD3 and anti-CD28 antigen binding domains are in a different configuration and proximity to each other, e.g., constructs that have formats as depicted in **FIG. 1A** or **FIG. 1C** in which the anti-CD28 scFv domain is not linked to the C-terminus of the anti-CD3 Fab light chain.

[0040] In various embodiments, the trivalent and trispecific (e.g., Het-Fab and $scFv^2$) antibody constructs described herein bind CD3 and CD28 on the same cytotoxic effector cell (e.g., T cell) and not – or at least to a significantly lower degree – on two different effector cells, thereby eliciting no (or reduced) cross-linking of two cytotoxic effector cells compared to trivalent and

trispecific antibody construct with a different format and/or geometry, e.g., constructs in which the anti-CD28 scFv and anti-CD3 Fab domains are in a different configuration to each other, in which the anti-CD28 scFv domain is attached to either the N-terminus of the anti-CD3 Fab domain (e.g., as depicted in **FIG. 1A**) or a C-terminus of the Fc domain (e.g., as depicted in **FIG. 1C**).

[0041] In various embodiments, the TAA that is targeted by a trivalent and trispecific antibody construct of this disclosure is DLL3, and thus, in various embodiments, the tumor cells that are targeted by the constructs described herein comprise DLL3-expressing tumor cells which can have the same or different tissue(s) of origin.

[0042] A trivalent and trispecific antibody construct of the present disclosure can comprise a first binding domain capable of binding a first antigen on a cytotoxic effector cell, a second binding domain capable of binding a second antigen on a cytotoxic effector cell, and a third binding domain capable of binding DLL3 on a tumor cell. In certain embodiments, described herein are trivalent and trispecific T cell engaging antibody constructs capable of co-stimulating an effector cell (e.g., a T cell) by engaging two effector cell antigens (e.g., CD3 and CD28) on the same effector cell while being capable of binding DLL3 on a tumor cell.

[0043] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises (i) a first Fab domain (i.e., the first binding domain) that is capable of binding a first antigen on a cytotoxic effector cell, (ii) a first scFv domain (i.e., the second binding domain) that is capable of binding a second antigen on a cytotoxic effector cell, and (iii) a third binding domain capable of binding DLL3, wherein the third binding domain can comprise or consist of a second Fab domain or a second scFv domain.

[0044] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises (i) a first Fab domain (i.e., the first binding domain) that is capable of binding a first antigen on a cytotoxic effector cell, (ii) a second Fab domain (i.e., the third binding domain) capable of binding DLL3 on a tumor cell, and (iii) a first scFv domain (i.e., the second binding domain) that is capable of binding a second antigen on a cytotoxic effector cell.

[0045] In other embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises (i) a first Fab domain (i.e., the first binding domain) that is capable of binding a first antigen on a cytotoxic effector cell, (ii) a first scFv domain (i.e., the second binding domain) that is capable of binding a second antigen on a cytotoxic effector cell; and (iii) a second scFv domain (i.e., the third binding domain) that is capable binding DLL3 on a tumor cell.

[0046] In certain embodiments, the first and second antigens on the cytotoxic effector cell are independently selected from CD3 and CD28. In certain embodiments, the first antigen on the cytotoxic effector cell (e.g., engaged via a Fab domain) is CD3 and the second antigen on the cytotoxic effector cell (e.g., engaged via an scFv domain) is CD28.

[0047] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises (i) a first Fab domain (i.e., the first binding domain) that is capable of binding CD3 on a cytotoxic effector cell, (ii) a first scFv domain (i.e., the second binding domain) capable of binding CD28 on a cytotoxic effector cell, and (iii) a third binding domain capable of binding DLL3, wherein the third binding domain can comprise or consist of a second Fab domain or a second scFv domain, and wherein the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0048] Any of the trivalent and trispecific antibody constructs described herein can further comprise an Fc domain (e.g., a homodimeric or a heterodimeric Fc domain) comprising a first Fc polypeptide and a second Fc polypeptide and to which the various binding domains are coupled, either directly (e.g., without a linker) or indirectly, e.g., via a linker and/or via another binding domain (e.g., in instances in which a first binding domain is coupled indirectly to an Fc polypeptide via a second binding domain, e.g., a C-terminus of the first binding domain is coupled to an N-terminus of the second binding domain, and such second binding domain is in turn coupled to the N-terminus of the Fc polypeptide, thereby indirectly coupling the first binding domain to the Fc polypeptide).

[0049] As further described herein, **FIGS. 1A-1D** and **FIGS. 1G-1H** depict certain trivalent and trispecific antibody construct formats and geometries with different relative orientations of the three binding domains, according to certain embodiments of this disclosure.

[0050] Further described herein are pharmaceutical compositions comprising one or more of the trivalent and trispecific antibody constructs disclosed herein, and a pharmaceutically acceptable carrier or excipient.

[0051] Other embodiments of this disclosure relate to a nucleic acid molecule, or a set of nucleic acid molecules, that encode the one or more (e.g., 2, 3, 4 or more) polypeptide chains (e.g., H1, H2, L1, L2, etc.) of an antibody construct described herein. Certain other embodiments of this disclosure can relate to a vector, or a set of vectors, that comprise a nucleic acid molecule or a set of nucleic acid molecules encoding an antibody construct of the present disclosure. Host cells

comprising such nucleic acid molecule, or set of nucleic acid molecules, and/or such vector, or set of vectors, for expressing a trivalent and trispecific antibody construct are also described herein.

[0052] Other embodiments of this disclosure describe methods of producing and using the trivalent and trispecific antibody constructs described herein, e.g., for the treatment of a cancer in a subject (such as a rodent or a human).

I. DEFINITIONS

[0053] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0054] The term "about," as used herein in the context of a numerical value or range, generally refers to ±10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or ±1% of the numerical value or range recited or claimed, unless otherwise specified. In various embodiments, the term "about" refers to an approximately ±10% variation from a given value or range. In other embodiments, the term "about" refers to an approximately ±5% variation from a given value or range. In yet other embodiments, the term "about" refers to an approximately ±1% variation from a given value or range. Unless otherwise recited, it is to be understood that ±10% variation is always included in any given value provided herein, whether it is specifically referred to or not.

[0055] The use of the word "a" or "an," when used herein in conjunction with the term "comprising," can mean "one," but it is also consistent with the meaning of "one or more," "at least one" and "one or more than one."

[0056] As used herein, the terms "comprising," "having," "including" and "containing," and grammatical variations thereof, are inclusive or open-ended and do not exclude additional, unrecited elements and/or method steps. The term "consisting essentially of" when used herein in connection with a construct, composition, use or method, denotes that additional features, elements and/or method steps can be present, but that these additions do not materially affect the manner in which the recited construct, composition, method or use functions. The term "consisting of," when used herein in connection with a construct, composition, use or method, excludes the presence of additional elements and/or method steps. An antibody construct, composition, use, or method described herein as comprising certain elements and/or steps can also, in certain embodiments, consist essentially of those elements and/or steps, and in other embodiments, consist of those elements and/or steps, whether or not these embodiments are specifically referred to.

[0057] The terms "subject" and "patient" can be used interchangeably herein and generally refer to an animal in need of treatment. An animal in need of treatment can be a human or a non-human animal, such as a mammal, bird, or fish. In certain embodiments, the subject is a mammal. In some embodiments, the subject is a human. In other embodiments, the subject is a rodent or a non-human primate.

[0058] An "effective amount" of a trivalent and trispecific antibody construct described herein, or a pharmaceutical composition comprising such antibody construct, in respect of a particular result to be achieved is an amount sufficient to achieve the desired result. For example, an "effective amount" of an antibody construct or pharmaceutical composition when referred to in respect of the killing of cancer cells, refers to an amount of antibody construct or composition comprising the antibody construct sufficient to produce a killing effect.

[0059] Unless specified otherwise, the terms "Fc region," "Fc" and "Fc domain" are used interchangeably herein and refer to a C-terminal region of an immunoglobulin (Ig) heavy chain that contains at least a portion of a constant region. In various embodiments, an Fc domain herein can be dimeric. Such dimeric Fc domain can comprise a first Fc polypeptide and a second Fc polypeptide, wherein each Fc polypeptide can comprise a CH2 domain and a CH3 domain. Such dimeric Fc can either be homodimeric, i.e., comprising first and second Fc polypeptides that have identical amino acid sequences, or heterodimeric, i.e., comprising first and second Fc polypeptides that have different amino acid sequences, e.g., sequences that share about 95%, 96%, 97%, 98%, or about 99% sequence identity. In some embodiments, an antibody construct of the present disclosure comprises a homodimeric Fc domain. In yet other embodiments, and as further described herein, an antibody construct comprises a heterodimeric Fc domain in which at least one of the CH2 and/or CH3 domains of the first and second Fc polypeptides have amino acid sequences that share about 99%, 98%, or about 97% sequence identity.

[0060] The term "multispecific," as used herein in the context of an antibody construct, refers to a biologically functional protein (e.g., an antibody construct as described herein) which is "at least bispecific," i.e., it comprises at least a first binding domain and a second binding domain, wherein such first and second binding domain can bind specifically two distinct epitopes, e.g., a first epitope and a second epitope. Such first and second epitopes can be located on the same antigen or on different antigens, e.g., a first epitope on cluster of differentiation 3 (CD3) or cluster of differentiation 28 (CD28) and a second epitope on a TAA. Accordingly, in some embodiments,

antibody constructs according to the present disclosure can comprise specificities for at least two different antigens or for at least three different antigens or targets. Hence, the term "multispecific" in the context of an antibody construct herein encompasses antibody constructs that are at least bispecific (i.e., comprising two binding domains with specificities for two different antigens or targets), or at least trispecific (i.e., comprising three binding domains with specificities for three different antigens or targets, e.g., CD3, CD28, and a TAA such as DLL3).

[0061] In various embodiments, an antibody construct of the present disclosure that is capable of binding CD3 refers to a construct that is able to bind an epitope on the epsilon chain of CD3, also referred to herein as "CD3ε," "CD3e" or "CD3 epsilon."

[0062] The term "trispecific," as used herein in the context of an antibody construct, refers to a biologically functional protein (e.g., an antibody construct as described herein) which is "at least trispecific," i.e., it comprises at least a first binding domain, a second binding domain and a third binding domain, wherein such first, second and third binding domains can bind specifically three distinct epitopes, e.g., a first epitope, a second epitope and a third epitope. Such first, second and third epitopes can be located on the same antigen or on different antigens, e.g., a first epitope on CD3, a second epitope on CD28, and a third epitope on a TAA (e.g., DLL3). Accordingly, in some embodiments, trispecific antibody constructs according to the present disclosure can comprise specificities for at least three different antigens or targets. Hence, the specificity in the context of an antibody construct herein describes the total number of different epitopes and/or antigens an antibody construct can specifically bind to, e.g., a monospecific antibody construct comprises one or more binding domain(s) with a specificity for one epitope or antigen, a trispecific antibody construct comprises three or more binding domains with specificities for three different epitopes and/or antigens, and so forth.

[0063] The term "trivalent," as used herein in the context of an antibody construct, refers to a biologically functional protein (e.g., an antibody construct as described herein) which is "at least trivalent," i.e., it comprises at least three binding domains, e.g., at least a first binding domain, a second binding domain and a third binding domain, wherein each of the first, second, and third binding domains is capable of specifically binding an epitope and/or antigen, e.g., CD3, CD28, and/or a TAA. The three binding domains can either have specificities for three different epitopes or antigens, or two or more of the three binding domains have a specificity for the same epitope or antigen. Hence, the valency in the context of an antibody construct herein, e.g., being mono-, bi-,

or trivalent, describes the total number of antigen binding domains of an antibody construct. Accordingly, the valency of an antibody construct is generally at least equal to its specificity, i.e., a trispecific antibody construct has to be at least trivalent. In embodiments in which the antibody construct is trivalent and trispecific, each of the three binding domains of the construct is capable of binding a different epitope or antigen, and thus the construct engages each of the three epitopes and/or antigens monovalently.

[0064] As used herein, the term "binding domain" in the context of an antibody construct described herein can generally be used interchangeably with the term "antigen binding domain," unless the binding domain has been specified to bind to a moiety other than an antigen on the surface of a target cell.

[0065] As used herein, the term "format" in the context of an antibody construct described herein generally describes attributes of the antibody construct including its antigen valency (e.g., a construct being mono- or bivalent for a given antigen), the type(s) of binding domain(s) (e.g., possessing one or more scFv domain(s), one or more Fab domain(s), etc.) present in an antibody construct, as well as the presence, absence, and/or type of an Fc domain (e.g., homodimeric, heterodimeric, containing one or more constant heavy domains, CH2, CH3, etc.). As an example, in some embodiments, an antibody construct of the present disclosure can be trivalent and trispecific in a 1+1+1 format illustrating that the construct contains three binding domains, wherein each binding domain has an affinity for a different antigen (e.g., CD3, CD28 and a TAA), i.e., the trivalent construct is monovalent (as indicated by "1") for each of the three antigens.

[0066] As used herein, the term "geometry" in the context of an antibody construct described herein generally describes the overall (e.g., 3-dimensional) structure of an antibody construct, including the relative spatial localization and arrangement and/or connectivity of the various domains of an antibody construct, e.g., the relative arrangement and connectivity of binding domains and Fc domains, as further described herein and as illustrated in, e.g., **FIGS. 1A-1D** and **FIGS. 1G-1H**, according to certain embodiments of the present disclosure.

[0067] Generally, and unless specified otherwise, an amino acid sequence of a polypeptide described herein is described and defined in the direction from N- to C-terminus. As an example, a polypeptide described as comprising an scFv domain coupled to an Fc polypeptide is defined herein as a polypeptide in which the C-terminus of the scFv domain is coupled, either with or

without a linker, to the N-terminus of the Fc polypeptide and the domain structure can be described as: scFv-Fc, or with the inclusion of a linker as: scFv-Linker$^{scFv\text{-}Fc}$-Fc.

[0068] As used herein, abbreviations such as "H1" and "H2," or "A" and "B," are generally used as generic heavy chain identifiers and broadly refer to a first heavy chain and a second heavy chain of an antibody construct, respectively, and thus are not intended to be limited to any specific heavy chain amino acid (or polynucleotide) sequence(s).

[0069] The term "amino acid modification," as used herein in the context of an amino acid sequence of a polypeptide, generally refers to an amino acid sequence of a polypeptide in which one or more amino acid substitution(s), one or more amino acid insertion(s), and/or one or more amino acid deletion(s) have been introduced relative to a corresponding unmodified (e.g., WT or reference) amino acid sequence of the polypeptide.

[0070] Descriptions of antibody constructs such as "anti-(DLL3xCD28xCD3)" and "anti-DLL3/anti-CD28/anti-CD3" can be used interchangeably herein and generally refer to an antibody construct that is at least trispecific and thus contains at least three binding domains that are capable of binding an epitope on DLL3, an epitope on CD28 and an epitope on CD3, respectively. In various embodiments, such description refers to a trivalent and trispecific antibody construct having three binding domains, one capable of binding an epitope on DLL3, one an epitope on CD28 and one an epitope on CD3, respectively, e.g., as shown in **FIGS. 1A-1D** and **FIGS. 1G-1H**.

[0071] The term "dissociation constant (K_D or K_d)" as used herein, is intended to refer to the equilibrium dissociation constant of a particular ligand-protein interaction. As used herein, ligand-protein interactions refer to, but are not limited to protein-protein interactions or antibody-antigen interactions. The K_D measures the propensity of two proteins complexed together (e.g. AB) to dissociate reversibly into constituent components (A+B), and is defined as the ratio of the rate of dissociation, also called the "off-rate (k_{off})", to the association rate, or "on-rate (k_{on})". Thus, K_D equals k_{off}/k_{on} and is expressed as a molar concentration (M). It follows that the smaller the K_D, the stronger the affinity of binding, and thus a decrease in K_D indicates an increase in affinity. Therefore, a K_D of 1 mM indicates weak binding affinity compared to a K_D of 1 nM. Affinity is sometimes measured in terms of a K_A or K_a, which is the reciprocal of the K_D or K_d. K_D values for antigen-binding constructs can be determined using methods well established in the art. One method for determining the K_D of an antigen-binding construct is by using surface plasmon

resonance (SPR), typically using a biosensor system such as a Biacore® system. Isothermal titration calorimetry (ITC) is another method that can be used to measure K_D.

[0072] Generally, it is to be understood that the positive recitation of a feature in one embodiment serves as a basis for excluding the feature in an alternative embodiment. In particular, where a list of options is presented for a given embodiment or claim, it is to be understood that one or more option can be deleted from the list and the shortened list can form an alternative embodiment, whether or not such an alternative embodiment is specifically referred to.

[0073] It is further contemplated that any embodiment discussed herein can be implemented with respect to any antibody construct, method, use, or composition disclosed herein, and vice versa. Furthermore, modifications of the specific embodiments described herein that would be apparent to those skilled in the art are intended to be included within the scope of the claims recited herein.

II. ANTIBODY CONSTRUCTS

[0074] The present disclosure describes trivalent and trispecific T cell engaging antibody constructs comprising three binding domains capable of engaging two different antigens on a cytotoxic effector cell and a TAA on a tumor cell, and which can have varying formats and structures, e.g., as shown in **FIGS. 1G-1H**, according to certain embodiments of this disclosure.

[0075] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises: (i) a first binding domain capable of binding a first antigen on a cytotoxic effector cell, (ii) a second binding domain capable of binding a second antigen on a cytotoxic effector cell, (iii) a third binding domain capable of binding DLL3 on a tumor cell, and (iv) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

[0076] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell, (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell, (iii) a third binding domain capable of binding DLL3 on a tumor cell, and (iv) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, and wherein the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain, e.g., as shown in **FIGS. 1B, 1D, 1G** and **1H**, according to certain embodiments of this disclosure.

A. Structure of Antibody Constructs and Their Biological Targets

[0077] In various embodiments, the antibody constructs described herein can have various formats and geometries, e.g., the number and type of binding domains may vary, and the binding domains

and the Fc domain can be interconnected (e.g., covalently or non-covalently) in various configurations, as further described herein.

[0078] In certain embodiments, the antibody constructs described in the present disclosure are trivalent and trispecific and thus comprise at least three antigen binding domains, with each of the three binding domains being capable of binding a different antigen. In various embodiments, the trivalent and trispecific antibody constructs of the present disclosure can have a format described as "1+1+1", indicating that each of the three binding domains binds a different antigen, and hence such constructs bind each antigen in a monovalent ("1") manner. **FIGS. 1A-1D** depict the formats and structures of certain trivalent and trispecific antibody constructs according to embodiments of the present disclosure.

[0079] Described herein are trivalent and trispecific antibody constructs comprising one or more Fab domain(s) and one or more scFv domain(s) as binding domains, as well as an Fc domain, and, optionally, one or more linkers that couple (e.g., covalently couple) at least some of these domains to each other.

[0080] In various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a first Fab domain capable of binding a first antigen on a cytotoxic effector cell, a first scFv domain capable of binding a second antigen on a cytotoxic effector cell, a third binding domain capable of binding a TAA on a tumor cell, wherein the third binding domain can comprise or consist of a second Fab domain or a second scFv domain, and an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

[0081] In some embodiments, described herein are trivalent and trispecific antibody constructs that comprise two Fab domains and one scFv domain as binding domains. Such antibody constructs can also be referred to as trivalent and trispecific heterodimeric Fab (or "Het-Fab") antibody constructs because each Fab domain binds a different antigen and as such contains different (i.e., antigen specific) variable domain sequences; optionally, such Het-Fab antibody construct can further contain one or more amino acid modifications in the CH1 and/or CL sequences of each Fab domain to facilitate correct heavy and light chain pairing, as further described herein.

[0082] In some embodiments, described herein is a trivalent and trispecific Het-Fab antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding a TAA on a tumor cell; (iii) a first scFv

domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of a Fab light chain. **FIG. 1D** shows a schematic of such an antibody construct.

[0083] In various embodiments, the TAA is DLL3.

[0084] In some embodiments, described herein is a trivalent and trispecific Het-Fab antibody construct comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of a Fab light chain.

[0085] In some embodiments, the first antigen is CD3 or CD28. In some embodiments, the second antigen is CD3 or CD28. In some embodiments, the first antigen is CD3 or CD28 and the second antigen is CD3 or CD28, and wherein the first antigen and the second antigen are different antigens. In certain embodiments, the first antigen is CD3 (e.g., engaged via the first Fab domain) and the second antigen is CD28 (e.g., engaged via the first scFv domain).

[0086] In various embodiments, described herein is a trivalent and trispecific Het-Fab antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of a Fab light chain. In some embodiments, the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain, e.g., as depicted in **FIG. 1D**.

[0087] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises or consists of one or more polypeptide chains. In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises or consists

of four polypeptide chains. Such four polypeptide chains can comprise or consist of two immunoglobulin heavy chains (e.g., H1 and H2) and two immunoglobulin light chains (e.g., L1 and L2).

[0088] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises or consists of a first immunoglobulin heavy chain (H1) comprising at least a first Fab heavy chain, a second immunoglobulin heavy chain (H2) comprising at least a second Fab heavy chain, a first immunoglobulin light chain (L1) comprising at least a first Fab light chain and a second immunoglobulin light chain (L2) comprising at least a second Fab light chain.

[0089] In some embodiments of a trivalent and trispecific Het-Fab antibody construct that is capable of engaging CD3, CD28 and DLL3 and comprises four polypeptide chains H1, H2, L1 and L2, H1 comprises, from N- to C-terminus, a first Fab heavy chain comprising a first VH sequence and a first CH1 sequence, coupled to a first Fc polypeptide comprising a first CH2 sequence and a first CH3 sequence, and wherein the first Fab heavy chain of H1 and a first Fab light chain of L1 associate to form the first Fab domain that is capable of binding CD3. In some embodiments, H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the C-terminus of the first Fab heavy chain to the N-terminus of the first Fc polypeptide. In some embodiments, such trivalent and trispecific Het-Fab antibody construct comprises a H2 comprising, from N- to C-terminus, a second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, coupled to a second Fc polypeptide comprising a second CH2 sequence and a second CH3 sequence, wherein the second Fab heavy chain of H2 and a second Fab light chain of L2 associate to form the second Fab domain that is capable of binding DLL3. In some embodiments, H2 further comprises a second linker$^{Fab\text{-}Fc}$ that couples the C-terminus of the second Fab heavy chain to the N-terminus of the second Fc polypeptide.

[0090] In some embodiments, the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ of a trivalent and trispecific Het-Fab antibody construct are polypeptide linkers, each independently comprising from about 5 to about 50, from about 5 to about 25 or from about 5 to about 15 consecutive amino acid residues. In some embodiments, the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ are each derived independently from an IgG1, IgG2 or IgG4 hinge region.

[0091] In various embodiments of a Het-Fab antibody construct of the present disclosure, the first scFv domain can be coupled to the C-terminus of a Fab light chain, e.g., the C-terminus of L1 or L2. In various embodiments, the first scFv domain is coupled to the C-terminus of the first Fab

light chain, L1, such that the two binding domains that engage CD3 and CD28 on a cytotoxic effector cell are configurated as depicted in, e.g., **FIGS. 1B**, **1D**, **1G** and **1H**.

[0092] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, L1 comprises, from N- to C-terminus, the first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to the first scFv domain comprising, from N- to C-terminus, either (i) a third VH sequence coupled to a third VL sequence or (ii) a third VL sequence coupled to a third VH sequence. In some embodiments, the first scFv domain comprises, from N- to C-terminus, the third VL sequence coupled to the third VH sequence. In some embodiments, L1 further comprises a $linker^{Fab\text{-}scFv}$ that couples the C-terminus of the first Fab light chain to the N-terminus of the first scFv domain (e.g., the N-terminus of the third VL sequence). In some embodiments, the $linker^{Fab\text{-}scFv}$ comprises the amino acid sequence $(G_4S)_x$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 119). In some of these embodiments, the $linker^{Fab\text{-}scFv}$ comprises the amino acid sequence G_4SG (SEQ ID NO: 58).

[0093] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, L2 comprises, from N- to C-terminus, the second Fab light chain comprising a second VL sequence and a second CL sequence.

[0094] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first Fab domain, wherein the first Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) a second Fab domain, wherein the second Fab domain is capable of binding DLL3 on the tumor cell; (iii) a first scFv domain, wherein the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising the first Fc polypeptide and the second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0095] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising a HCDR1 sequence comprising the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 sequence comprising the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 sequence comprising the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 sequence comprising the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 sequence

comprising the sequence RND, and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

[0096] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 5.

[0097] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising a HCDR1 sequence comprising the sequence SYGVH (SEQ ID NO: 28), a HCDR2 sequence comprising the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 sequence comprising the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L sequence comprising a LCDR1 sequence comprising the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 sequence

comprising the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 sequence comprising the sequence QQSRKVPFT (SEQ ID NO: 48).

[0098] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 16.

[0099] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that can independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8. In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fc polypeptide, the second Fc polypeptide, or both Fc polypeptides, comprise one or more amino acid substitutions in the CH2 domain compared to a corresponding wild-type IgG1 CH2 domain sequence, wherein the one or more amino acid

substitutions reduce or eliminate binding of the antibody construct to an Fcγ-receptor (FcγR). In some embodiments, the CH2 domain sequences of the first and second Fc polypeptides comprise the amino acid substitutions L234A_L235A_D265S, and wherein the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

[0100] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, the dimeric Fc domain is a heterodimeric Fc domain in which one Fc polypeptide has an amino acid sequence that differs in at least one residue from the amino acid sequence of the other Fc polypeptide. In some embodiments, one of the Fc polypeptides comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 9, and the other Fc polypeptide comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 10.

[0101] In certain embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 Fab heavy chain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein the first Fc polypeptide of H1 and the second Fc polypeptide of H2 associate to form a heterodimer H1-H2; (iii) a first light chain (L1), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13 or 107, and wherein the anti-CD3 Fab light chain of L1 and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain; and (iv) a second light chain (L2), comprising an anti-DLL3 Fab light chain, and wherein the anti-DLL3 Fab light chain of L2 and the anti-DLL3 Fab heavy chain of H2 associate to form an anti-DLL3 Fab domain.

[0102] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, one or more of the Fab domains comprise one or more amino acid modifications (e.g., relative to unmodified reference Fab domain sequences) that promote correct heavy and light chain pairing to produce the correctly paired one or more Fab domains (e.g., association of the Fab portions of

H1 with L1 and H2 with L2), respectively, and relative to the formation of incorrectly paired Fab domains (e.g., association of the Fab portions of H1 with L2 and H2 with L1). Thus, in some embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain, the second Fab domain, or both Fab domains, comprise one or more amino acid modifications that promote correct heavy and light chain pairing to produce the first and second Fab domains, respectively, and relative to the formation of incorrectly paired Fab domains. In some embodiments, the one or more amino acid modifications comprise or consist of one or more amino acid substitutions.

[0103] Amino acid modifications that can promote heavy and light chain pairing are known in the art and are described in, e.g., International Patent Publication Nos. WO 2014/082179, WO 2015/181805, and WO 2017/059551, which are incorporated herein by reference in their entireties.

[0104] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain and the second Fab domain each comprise a kappa light chain. In other embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain and the second Fab domain each comprise a lambda light chain. In yet other embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain comprises a kappa light chain and the second Fab domain comprises a lambda light chain. In yet other embodiments of a trivalent and trispecific Het-Fab antibody construct, the first Fab domain comprises a lambda light chain and the second Fab domain comprises a kappa light chain.

[0105] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct in which one Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light chain, the Fab domain comprising the kappa light chain comprises one or more, or all of the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence of the heavy chain and one or more, or all of the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the other Fab domain comprising the lambda light chain comprises one or more, or all of the amino acid substitutions 125R and 188K in the CH1 sequence of the heavy chain and one or more, or all of the amino acid substitutions 122D, 129T, 176E and 178E in the CL sequence of the lambda light chain, wherein the amino acid residues are identified according to the Kabat numbering system.

[0106] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct in which one Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light

chain, the Fab domain comprising the kappa light chain comprises one or more, or all of the amino acid substitutions 124R and 186K in the CH1 sequence of the heavy chain and one or more, or all of the amino acid substitutions 133G, 135W, 176D and 180E in the CL sequence of the kappa light chain, and the other Fab domain comprising the lambda light chain comprises one or more, or all of the amino acid substitutions 139W, 143D and 145T in the CH1 sequence of the heavy chain and one or more, or all of the amino acid substitutions 124Q and 131R in the CL sequence of the lambda light chain, wherein the amino acid residues are identified according to the Kabat numbering system.

[0107] In certain embodiments of a trivalent and trispecific Het-Fab antibody construct, the second Fab domain that is capable of binding DLL3 has a K_D value for binding DLL3 from about 0.01 nM to about 100 nM or from about 0.1 nM to about 50 nM.

[0108] In various other embodiments, described herein are trivalent and trispecific antibody constructs that comprise one Fab domain and two scFv domains as binding domains. Such antibody constructs can also be referred to as trivalent and trispecific "$scFv^2$" antibody constructs indicating that such constructs comprise two scFv binding domains.

[0109] In certain embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the first Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide. **FIG. 1B** shows a schematic of such an antibody construct according to certain embodiments of this disclosure.

[0110] In some embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the first antigen on a cytotoxic effector cell is CD3 or CD28. In some embodiments, the second antigen on a cytotoxic effector cell is CD3 or CD28. In some embodiments, the first antigen is CD3 or CD28 and the second antigen is CD3 or CD28, wherein the first antigen and the second antigen are different. In certain embodiments, the first antigen is CD3 and the second antigen is CD28.

[0111] In certain embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell;

(ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the first Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0112] In certain embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure can comprise or consist of one or more polypeptide chains. In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct herein comprises or consists of two immunoglobulin heavy chains (e.g., H1 and H2) and one immunoglobulin light chain (e.g., L). In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct comprises or consists of a first immunoglobulin heavy chain (H1) comprising at least a first Fab heavy chain, a second immunoglobulin heavy chain (H2) comprising at least a second Fab heavy chain and an immunoglobulin light chain (L) comprising at least a first Fab light chain.

[0113] In some of these embodiments in which a trivalent and trispecific $scFv^2$ antibody construct comprises three polypeptide chains H1, H2 and L, H1 comprises, from N- to C-terminus, the first Fab heavy chain comprising a first VH sequence and a CH1 sequence, coupled to the first Fc polypeptide comprising a first CH2 sequence and a first CH3 sequence, and wherein the first Fab heavy chain of H1 and the first Fab light chain of L associate to form the first Fab domain. In some embodiments, H1 further comprises a $linker^{Fab\text{-}Fc}$ that couples the C-terminus of the first Fab heavy chain to the N-terminus of the first Fc polypeptide.

[0114] In certain embodiments of a trivalent and trispecific $scFv^2$ antibody construct, H2 comprises, from N- to C-terminus, the first scFv domain, comprising, from N- to C-terminus, either (i) an anti-DLL3 VH sequence coupled to an anti-DLL3 VL sequence or (ii) an anti-DLL3 VL sequence coupled to an anti-DLL3 VH sequence, coupled to the second Fc polypeptide comprising a second CH2 sequence and a second CH3 sequence, and wherein the first Fc polypeptide of H1 and the second Fc polypeptide of H2 associate to form a heterodimer H1-H2. In some of these embodiments, the first scFv domain comprises, from N- to C-terminus, the anti-DLL3 VL sequence coupled to the anti-DLL3 VH sequence. In some embodiments, H2 further comprises a $linker^{scFv\text{-}Fc}$ that couples the C-terminus of the first scFv domain to the N-terminus of the second Fc polypeptide.

[0115] In certain embodiments of a trivalent and trispecific scFv^2 antibody construct, the linker^{Fab-Fc} and the linker$^{scFv-Fc}$ are polypeptide linkers, each independently comprising from about 5 to about 50 consecutive amino acid residues. In some embodiments, the linker^{Fab-Fc} and the linker$^{scFv-Fc}$ are each independently derived from an IgG1, IgG2, or IgG4 hinge region.

[0116] In certain embodiments of a trivalent and trispecific scFv^2 antibody construct, L comprises, from N- to C-terminus, a first Fab light chain comprising a first VL sequence and a CL sequence, coupled to the second scFv domain comprising, from N- to C-terminus, either (i) a second VH sequence coupled to a second VL sequence or (ii) a second VL sequence coupled to a second VH sequence. In some embodiments, the second scFv domain comprises, from N- to C-terminus, the second VL sequence coupled to the second VH sequence. In some embodiments, L further comprises a linker$^{Fab-scFv}$ that couples the C-terminus of the first Fab light chain to the N-terminus of the second scFv domain. In some embodiments, the linker$^{Fab-scFv}$ comprises the amino acid sequence $(G_4S)_X$, wherein x is 1, 2, 3, 4 or 5.

[0117] In certain embodiments, a trivalent and trispecific scFv^2 antibody construct comprises: (i) a first Fab domain, wherein the first Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain, wherein the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the first Fab light chain N-terminus of the second Fc polypeptide, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0118] In certain embodiments of a trivalent and trispecific scFv^2 antibody construct, the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising a HCDR1 sequence comprising the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 sequence comprising the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 sequence comprising the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 sequence comprising the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 sequence comprising the sequence RND (SEQ ID NO: 54), and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

[0119] In certain embodiments of a trivalent and trispecific scFv^2 antibody construct, the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 5. In some embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 5.

[0120] In certain embodiments of a trivalent and trispecific scFv^2 antibody construct, the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising a HCDR1 sequence comprising the sequence SYGVH (SEQ ID NO: 28), a HCDR2 sequence comprising the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 sequence comprising the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L sequence comprising a LCDR1 sequence comprising the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 sequence comprising the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 sequence comprising the sequence QQSRKVPFT (SEQ ID NO: 48).

[0121] In some embodiments, the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 97% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 16. In some embodiments, the anti-CD28 scFv domain comprises a V_H sequence comprising the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 16.

[0122] In certain embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that can independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8. In some embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the first Fc polypeptide, the second Fc polypeptide, or both Fc polypeptides, comprise one or more amino acid substitutions in the CH2 domain compared to a corresponding wild-type IgG1 CH2 domain sequence, wherein the one or more amino acid substitutions reduce or eliminate binding of the antibody construct to an FcγR. In some embodiments, the CH2 domain sequences of the first and second Fc polypeptides comprise the

amino acid substitutions L234A_L235A_D265S, and wherein the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

[0123] In certain embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the dimeric Fc domain is a heterodimeric Fc domain in which one Fc polypeptide has an amino acid sequence that differs in at least one residue from the amino acid sequence of the other Fc polypeptide. In some embodiments, one of the Fc polypeptides comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 9, and the other Fc polypeptide comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 10.

[0124] In certain embodiments, a trivalent and trispecific $scFv^2$ antibody construct comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 scFv domain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein the first Fc polypeptide of H1 and the second Fc polypeptide of H2 associate to form a heterodimer H1-H2; and (iii) a light chain (L), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein the anti-CD3 Fab light chain of L and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain.

[0125] In certain embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a third binding domain capable of binding DLL3 on a tumor cell, wherein the third binding domain is a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to: the C-terminus of the light chain of either

the first Fab domain or the second Fab domain if the third binding domain is the second Fab domain, or the C-terminus of the light chain of the first Fab domain if the third binding domain is the second scFv domain. In some embodiments, the third binding domain is a second Fab domain. In other embodiments, the third binding domain is a second scFv domain. In various embodiments, the first scFv domain is coupled to the C-terminus of the Fab light chain of the first Fab domain, independent of structure or format (e.g., scFv or Fab) of the third binding domain.

[0126] In certain embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding a TAA on a tumor cell, wherein the third binding domain is a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain. In some embodiments, the third binding domain is a second Fab domain. In other embodiments, the third binding domain is a second scFv domain.

[0127] In various embodiments, the TAA is DLL3.

[0128] In certain embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the first scFv domain has a K_D value for binding DLL3 from about 0.01 nM to about 100 nM or from about 0.1 nM to about 50 nM.

[0129] In certain embodiments of a trivalent and trispecific antibody construct (e.g., a Het-Fab or $scFv^2$ antibody construct), the first Fc polypeptide and the second Fc polypeptide comprise IgG1 or IgG4 CH3 sequences comprising one or more amino acid modifications compared to wildtype CH3 sequences that promote preferential pairing of the first and second Fc polypeptide to form a heterodimeric Fc domain compared to the formation of a corresponding homodimeric Fc domain. In some embodiments, the CH3 sequence of one of the Fc polypeptides comprises a set of amino acid substitutions selected from the group consisting of: L351Y_F405A_Y407V, T350V_L351Y_F405A_Y407V and T350V_L351Y_S400E_ F405A_Y407V, and the CH3 sequence of the other Fc polypeptide comprises a set of amino acid substitutions selected from the group consisting of: T366L_K392M_T394W, T366L_K392L_T394W, T350V_T366L_K392L_T394W, T350V_T366L_K392M_T394W and T350V_T366L_N390R_K392M_T394W, and wherein the

numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system. In some embodiments of a trivalent and trispecific Het-Fab antibody construct (e.g., a Het-Fab or $scFv^2$ antibody construct), the first or second Fc polypeptide comprises the amino acid substitutions T350V_L351Y_F405A_Y407V, and the other Fc polypeptide comprises the amino acid substitutions T350V_T366L_K392L_T394W, and wherein the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

[0130] In certain embodiments of a trivalent and trispecific antibody construct (e.g., a Het-Fab or $scFv^2$ antibody construct), the first and second effector cell antigens that the antibody construct binds to can either be located on the same cytotoxic effector cell or on different cytotoxic effector cells. In some embodiments, the one or more cytotoxic effector cell(s) comprise one or more T cell(s). In various embodiments, the first and second effector cell antigens that the antibody construct binds to are located on the same cytotoxic effector cell. In other embodiments, the first and second effector cell antigens that the antibody construct binds to are located on different cytotoxic effector cells.

B. Domains of an Antibody Construct

[0131] As further described herein, a trivalent and trispecific antibody construct of the present disclosure can comprise one or more antibody (or immunoglobulin ("Ig")) domains. In various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a plurality of (i.e., two or more) antibody domains. Such plurality of antibody domains can comprise (i) one or more immunoglobulin Fc domain(s), wherein an Fc domain can comprise a first Fc polypeptide and a second Fc polypeptide, and can be either homodimeric or heterodimeric, (ii) one or more Fab domain(s), wherein a Fab domain can comprise a Fab heavy chain polypeptide comprising a heavy variable domain (V_H) sequence and a heavy constant domain (C_{H1}) sequence and a Fab light chain polypeptide comprising a light variable domain (V_L) sequence and light constant domain (C_L) sequence, and/or (iii) one or more scFv domains, wherein an scFv domain can comprise an scFv V_H sequence coupled to an scFv V_L sequence (from either N- to C-terminus or C- to N-terminus). The various domains an antibody construct can comprise are further described herein.

[0132] An Ig structural unit as known in the art is typically composed of two pairs of polypeptide chains, each pair having one "light" chain (about 25 kilodalton (kD)) and one "heavy" chain (about 50-70 kD). Light chains can be classified as either kappa or lambda. The "class" of an Ig refers to

the type of constant domain possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG and IgM, and several of these can be further divided into subclasses (isotypes), for example, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins (Igs) are called alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ), respectively.

[0133] In various embodiments, a trivalent and trispecific antibody construct described herein is based on an IgG class immunoglobulin, for example, an IgG1, IgG2, IgG3 or IgG4 immunoglobulin. In some embodiments, an antibody construct described herein is based on an IgG1, IgG2 or IgG4 immunoglobulin. In certain embodiments, an antibody construct described herein is based on an IgG1 immunoglobulin. In the context of the present disclosure, when an antibody construct is based on a specified Ig isotype, it refers to an antibody construct that comprises either all or a portion of the constant region (i.e., a portion of an Fc domain) of the specified Ig isotype. It is to be understood that an antibody construct can also comprise hybrids of isotypes and/or subclasses, according to certain embodiments of this disclosure.

[0134] Generally, in antibodies, the N-terminal domain of each polypeptide chain usually defines a variable region (e.g., V_H or V_L) of about 100 to 110 or more amino acids in length that is primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these domains in the light and heavy chain, respectively. As described herein, in various embodiments, a trivalent and trispecific antibody construct of the present disclosure can comprise two or more variable domain sequences as part of its antigen binding domains. In various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises two variable domain sequence per binding domain (e.g., per Fab and/or scFv), and thus by being trivalent (i.e., containing three binding domains) and trispecific (i.e., each of the three binding domains targets a different antigen) can comprise a total of six variable domain sequences, e.g., three V_H domain sequences and three V_L domain sequences.

[0135] In some embodiments, two or more of such variable domains are coupled to one another in tandem and in a single polypeptide chain format, e.g., as described for scFv-type binding domains which contain (from either N- or C-terminus) a V_H domain coupled to a V_L domain. The two or more variable domains can be coupled to each other in tandem either directly or via a linker.

[0136] Accordingly, an antibody construct of the present disclosure that is derived from an Ig molecule (i.e., an antibody construct that comprises at least one Ig domain, wherein the one Ig

domain can comprise a wildtype amino acid sequence and/or an amino acid sequence that contains one or more amino acid modifications relative to wildtype) can comprise different Ig domains within its heavy and light chain(s). Heavy chain domains can include the Fc domain (or Fc region), e.g., comprising a CH2 domain and CH3 domain, a hinge domain (or hinge region), and a heavy chain Fab domain comprising a variable heavy domain (V_H) and a constant heavy domain (C_{H1}), and light chain domains can include the variable light domain (V_L) and the light constant domain (C_L). In some embodiments, and as further described herein, a light chain of an antibody construct can further comprise an scFv domain coupled to, e.g., C-terminus of the CL constant domain of the light chain. In some embodiments, and according to certain nomenclatures, the "Fc domain" or "Fc region" can include the CH2 and CH3 domains as well as a hinge domain (or hinge region).

[0137] In some embodiments, an antibody construct described herein can further comprise another domain or moiety that may not be derived from an Ig molecule. Such a non-Ig domain can be referred to as a moiety. Such moiety can be a detectable label (e.g., a radiolabel or fluorescent label), a low molecular weight (e.g., <750 Da) drug molecule, another peptide (e.g., signal peptide(s)) or polypeptide molecule, or combinations thereof.

B.1 Complementarity Determining Regions (CDRs) and Binding Domains

[0138] In each of the V_H and V_L domains of an antibody construct herein are three loops which are hypervariable in sequence and form an antigen-binding site. Each of these loops is referred to as a "hypervariable region" or "HVR," or "complementarity determining region" or "CDR." The terms hypervariable region (HVR) and complementarity determining region (CDR) are used herein interchangeably in reference to the portions of the variable domain (e.g., V_H or V_L) that form the antigen-binding site. With the exception of CDR1 in V_H, CDRs generally comprise the amino acid residues that form the hypervariable loops. The V_H and V_L domains consist of relatively invariant stretches called framework regions (FRs) of between about 15 to 30 amino acids in length separated by the shorter CDRs, which are each typically between about 5 and 15 amino acids in length, although can occasionally be longer or shorter. The three CDRs and four FRs that make up each V_H and V_L domain are generally arranged from N- to C-terminus as follows: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4.

[0139] Several different definitions and numbering conventions of the CDR regions in Ig molecules are in common use, including those described by Kabat *et al.* (1983, *Sequences of Proteins of Immunological Interest*, NIH Publication No. 369-847, Bethesda, MD), by Chothia *et*

al. (1987, *J Mol Biol*, 196:901-917), as well as the IMGT, AbM and Contact definitions. These different definitions include overlapping or subsets of amino acid residues when compared against each other. By way of example, CDR definitions according to Kabat, Chothia, IMGT, AbM and Contact are provided in **TABLE 1** below.

[0140] Accordingly, as can be readily apparent to one skilled in the art, the exact numbering and placement of CDRs can differ based on the numbering system employed. However, it is to be understood that the disclosure herein of a variable heavy domain (V_H) includes the disclosure of the associated (inherent) heavy chain CDRs (HCDRs or HCDR1-3) as defined by any of the known numbering systems. Similarly, disclosure herein of a variable light domain (V_L) includes the disclosure of the associated (inherent) light chain CDRs (LCDRs or LCDR1-3) as defined by any of the known numbering systems. In case a set of CDR sequences (e.g., the 3 HCDRs and the 3 LCDRs) for a binding domain against a certain target is given or claimed using a specific numbering system (e.g., Kabat or IMGT), it is to be understood that such claim also encompasses the corresponding sets of CDR sequences as determined using any other of the known numbering systems.

[0141] One skilled in the art can appreciate that a limited number of amino acid substitutions can be introduced into the CDR sequences or to the V_H or V_L sequences of known antibodies without the antibody losing its ability to bind its target, e.g., a reduction in binding affinity of at least about 10-fold, 100-fold, or 1000-fold or more. Candidate amino acid substitutions can be identified by computer modeling or by techniques such as alanine scanning, with the resulting variants being tested for binding activity (e.g., expressed as binding affinity, e.g., given as the measured K_D value) by standard techniques. As an example, in certain embodiments, the CD3 binding domain(s) of antibody constructs described herein can comprise a set of CDRs (i.e., heavy chain CDR1, CDR2 and CDR3 (HCDR1-3), and light chain CDR1, CDR2 and CDR3 (LCDR1-3) that have 90% or greater, 95% or greater, 98% or greater, 99% or greater, or 100% sequence identity to the amino acid sequences set forth in SEQ ID NOs: 49-55, respectively, wherein the binding domain retains or substantially retains the ability to bind CD3. In this context, the term "substantially" refers to a change in binding affinity of less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1%. However, in certain other instances, one or skill in the art can also appreciate that introduction of one or more amino acid modifications into a set of six CDRs for a particular binding domain can result in substantial changes in one or more of the following

property parameters: antigen binding affinity, stability of the binding domain, and expression or production yields.

TABLE 1: Common CDR Definitions[1]

Definition	Heavy Chain			Light Chain		
	CDR1[2]	CDR2	CDR3	CDR1	CDR2	CDR3
Kabat	H31-H35B	H50-H65	H95-H102	L24-L34	L50-L56	L89-L97
Chothia	H26-H32, H33 or H34	H52-H56	H95-H102	L24-L34	L50-L56	L89-L97
IMGT	H26-H33, H34, H35, H35A or H35B	H51-H57	H93-102	L27-L32	L50-L52	L89-L97
AbM	H26-H35B	H50-H58	H95-H102	L24-L34	L50-L56	L89-L97
Contact	H30-H35B	H47-H58	H95-H101	L30-L36	L46-L55	L89-L96

[1] Either the Kabat or Chothia numbering system can be used for HCDR2, HCDR3 and the light chain CDRs for all definitions except Contact, which uses Chothia numbering.

[2] Using Kabat numbering. The position in the Kabat numbering scheme that demarcates the end of the Chothia and IMGT CDR-H1 loop varies depending on the length of the loop due to the placement of insertions outside of those CDR definitions at positions 35A and 35B in Kabat. The IMGT and Chothia CDR-H1 loop can be unambiguously defined using Chothia numbering. CDR-H1 definitions using Chothia numbering are: Kabat H31-H35, Chothia H26-H32, AbM H26-H35, IMGT H26-H33, Contact H30-H35.

[0142] In some embodiments, the antibody constructs described herein comprise at least one Ig domain from a mammalian Ig, such as a bovine Ig, a human Ig, a camelid Ig, a rat Ig, or a mouse Ig. In some embodiments, an antibody construct herein can be a chimeric construct comprising two or more Ig domains, in which at least one domain is from a first mammalian Ig, for example a human Ig, and at least a second domain is from a second mammalian Ig, for example, a mouse or rat Ig. In other embodiments, an antibody construct can be derived from Igs that are from different species, for example, an antibody construct can be chimeric or humanized. A “chimeric antibody construct” refers to an antibody that typically comprises at least one variable domain from a rodent antibody (usually a murine antibody) and at least one constant domain from a human antibody. A “humanized antibody construct” is a type of chimeric antibody that contains minimal sequence derived from a non-human antibody. In some embodiments, an antibody construct herein can comprise at least one Ig constant domain from a human Ig. In various embodiments, all domains of an antibody construct described herein can be (or be derived from) from a human Ig.

[0143] In some embodiments, and as further described herein, modifications (e.g., to the amino acid sequence) to one or more domains of an antibody construct can be made to further refine the properties and performance of the antibody construct (e.g., antigen affinity, stability, and/or

pharmacokinetics, tumor cell killing, etc.). For example, framework region (FR) residues of a human Ig can be replaced by corresponding non-human residues, or the humanized antibodies can comprise residues that are not found in either the recipient antibody or the donor antibody. In general, a variable domain in a humanized antibody or a humanized antibody domain comprises all or substantially all of the hypervariable regions from a non-human Ig and all or substantially all of the FRs from a human Ig sequence. As further described herein, modifications in the Fc domain can enable preferential pairing of the Fc polypeptides to form a heterodimeric Fc domain rather than a homodimeric Fc domain, and/or alter interaction of the Fc domain with one or more FcγRs.

[0144] In some embodiments, the present disclosure relates to antibody constructs that can have certain valencies, e.g., can be trivalent. Hence, in various embodiments, an antibody construct herein comprises three antigen binding domains, i.e., is at least trivalent. In various embodiments of this disclosure, an antibody construct can be trispecific and trivalent, and thus such antibody construct can comprise three binding domains, wherein each of the three binding domains has a unique binding specificity for a different epitope (located on either the same antigen and/or on different antigens). In some of these embodiments, a trispecific and trivalent antibody construct of the present disclosure comprises three binding domains, e.g., one or more Fab domain(s) and/or one or more scFv domain(s), each capable of binding an epitope on one of three different antigens (e.g., CD3, CD28, and a TAA such as DLL3).

[0145] In some embodiments, an antibody construct of the present disclosure can comprise (i) one or more Fab domain(s), (ii) one or more scFv domain(s), and (iii) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

[0146] In various embodiments, a trivalent and trispecific antibody construct as described herein comprises (i) a first Fab domain capable of binding a first antigen, (ii) a first scFv domain capable of binding a second antigen, (iii) a third binding domain capable of binding a third antigen, wherein the third binding domain comprises or consists of a second Fab domain or a second scFv domain, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

[0147] In some embodiments, a trivalent and trispecific antibody construct as described herein comprises (i) a first Fab domain capable of binding a first antigen, (ii) a second Fab domain capable of binding a second antigen, (iii) a first scFv domain capable of binding a third antigen, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide. In other

embodiments, a trivalent and trispecific antibody construct as described herein comprises (i) a Fab domain capable of binding a first antigen, (ii) a first scFv domain capable of binding a second antigen, (iii) a second scFv domain capable of binding a third antigen, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

[0148] Generally, a "Fab domain," as used herein (also commonly known as an abbreviation of "fragment, antigen binding"), comprises a constant region comprising the constant domain (C_L) of the light chain and the first constant domain (C_{H1}) of the heavy chain, and a variable region comprising the variable domains V_L and V_H on the light and heavy chains, respectively, which comprise the CDRs as described herein. In some embodiments, a Fab domain can be a single chain Fab. A single chain Fab can be a Fab molecule in which the Fab light chain and the Fab heavy constant chain are connected by a peptide linker to form a single polypeptide chain. In such embodiments, typically, the C-terminus of the Fab light chain is connected to the N-terminus of the Fab heavy chain in the single-chain Fab molecule, however, other formats are also encompassed herein. In various embodiments herein, however, a Fab domain of an antibody construct is formed by two separately expressed polypeptide chains, i.e., a Fab light chain and a Fab heavy chain. The heavy chain and light chain parts of the Fab domain can, however, be interconnected by covalent bonds, such as disulfide bonds.

[0149] An "scFv domain," as used herein (also commonly known as an abbreviation of "single-chain fragment, variable"), generally comprises a heavy chain variable domain (V_H) and a light chain variable domain (V_L) in a single polypeptide chain format. The scFv can optionally comprise a peptide linker between the V_H and V_L domains which can assist the scFv in forming a functional structure for antigen binding. Hence, in various embodiments, an scFv domain herein can include a V_L domain that is coupled via its C-terminus to the N-terminus of a V_H domain by a $linker^{scFv}$, i.e., an scFv domain can have the domain structure (from N- to C-terminus): V_L-$linker^{scFv}$-V_H, or alternatively, an scFv can comprise a V_H connected by its C-terminus to the N-terminus of a V_L by a $linker^{scFv}$, i.e., having the domain structure (from N- to C-terminus): V_H-$linker^{scFv}$-V_L.

B.2 Binding Domains against Antigens on Cytotoxic Effector Cells

[0150] As further described herein, in various embodiments, an antibody construct of the present disclosure can comprise at least two binding domains capable of binding to one or more molecule(s), e.g., a polypeptide(s), on the surface of one or more cytotoxic effector cell(s). Such one or more cytotoxic effector cell(s) can be one or more immune cell(s). Such one or more

immune cell(s) can comprise a T cell, a macrophage, a dendritic cell, a neutrophil, a B-cell, an NK cell, or a combination thereof.

[0151] In various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises at least one binding domain capable of binding a first antigen on a cytotoxic effector cell, and at least one binding domain capable of binding a second antigen on a cytotoxic effector cell.

[0152] In various embodiments, the cytotoxic effector cell is one cell, i.e., the first and the second antigens that an antibody construct of this disclosure can bind to are located on the surface of the same cell. The binding of an antibody construct to two different antigens located on one cytotoxic effector cell can also be referred to as "cis"-binding. In other embodiments, the cytotoxic effector cell is more than one cell, i.e., the first and the second antigens that an antibody constructs of this disclosure can bind to are located on the surfaces of different cells. The binding of an antibody construct to two different antigens located on two different cytotoxic effector cells can also be referred to as "trans"-binding or cross-linking of effector cells (e.g., T cells).

[0153] In some embodiments of a trivalent and trispecific DLL3-engaging antibody construct herein, the first antigen on a cytotoxic effector cell is CD3, and the second antigen on a cytotoxic effector cell is CD28. In some of these embodiments, both CD3 and CD28 can be engaged by a trivalent and trispecific antibody construct when located on the surface of the same cytotoxic effector cell (i.e., the antibody construct is capable of cis-binding CD3 and CD28 on the same effector cell). Trivalent and trispecific antibody constructs of the present disclosure that are capable of CD3 and CD28 cis-binding comprise a light chain comprising, from N- to C-terminus, an anti-CD3 VL sequence, a CL sequence, an anti-CD28 VL sequence, and an anti-CD28 VH sequence. Such light chain can further comprise one or more linkers, as described herein. Antibody constructs comprising such light chains are depicted in, e.g., **FIGS. 1B, 1D, 1G** and **1H.** In some embodiments, such antibody constructs nearly exclusively engage CD3 and CD28 in cis (i.e., on the same effector cell). Hence, the type of anti-CD3 (Fab) and anti-CD28 (scFv) binding domains as well as their relative orientation and configuration can drive cis-binding of the antibody construct.

[0154] In other embodiments, both antigens CD3 and CD28 can be engaged by a trivalent and trispecific antibody construct when located on the surfaces of different cytotoxic effector cells (i.e., the antibody construct is capable of trans-binding CD3 and CD28 on two effector cells). Antibody

constructs capable of engaging CD3 and CD28 in trans can have a format and structure as depicted, e.g., **FIGS. 1A** and **1C**.

[0155] In some embodiments, the present disclosure relates to trivalent and trispecific antibody constructs comprising a first binding domain capable of binding CD3, a second binding domain capable of binding CD28, and a third binding domain capable of binding a DLL3. Hence, the antibody constructs described herein can also be referred to as "T cell engagers," "TCEs" or "T cell engager molecules," describing the ability of the construct to bind both, antigens on one or more effector cell(s) (e.g., T cell(s)) as well as DLL3 on a tumor cell. In some embodiments, the engagement of a trivalent and trispecific antibody construct with two different effector cell antigens on an effector cell and DLL3 on a tumor cell can be – at least temporarily – simultaneous, and thereby establish a TCR-independent immune synapse, and direct T cell-mediated cytotoxic activity to a tumor environment which contains tumor cells expressing DLL3. In various embodiments, and as further described herein, a trivalent and trispecific antibody construct may cause a significantly reduced immune cell (e.g., T cell) activation in the absence of DLL3, e.g., when the immune synapse cannot be fully formed due to an absence of DLL3, and relative to immune cell activation in the presence of DLL3.

[0156] In various embodiments, a first antigen binding domain capable of binding CD3 on a cytotoxic effector cell can be a Fab domain or an scFv domain. In these embodiments, a second antigen binding domain capable of binding CD28 on a cytotoxic effector cell can also be a Fab domain or an scFv domain.

[0157] In some embodiments, a first binding domain capable of binding CD3 on a cytotoxic effector cell is a Fab domain, and a second binding domain capable of binding CD28 on a cytotoxic effector cell is an scFv domain.

[0158] In various embodiments of a trivalent and trispecific antibody construct herein, the Fab domain that is capable of binding CD3 can comprise a heavy chain constant domain (C_{H1}) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, such C_{H1} domain sequence comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, such C_{H1} domain sequence comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid

sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, such C_{H1} domain sequence comprises or consists of an amino acid sequence having at least about 97% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, such C_{H1} domain sequence comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, such C_{H1} domain sequence comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4.

[0159] In various embodiments of a trivalent and trispecific antibody construct, the Fab domain that is capable of binding CD3 can comprise a light chain constant domain (C_L) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, such C_L domain sequence comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, such C_L domain sequence comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, such C_L domain sequence comprises or consists of an amino acid sequence having at least about 97% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, such C_L domain sequence comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, such C_L domain sequence comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7.

B.2.1 Binding Domains Against CD3

[0160] As described herein, in some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a binding domain capable of binding CD3 (e.g., CD3ε) on a cytotoxic effector cell (e.g., T cell).

[0161] In various embodiments, such anti-CD3 binding domain (e.g., an scFv domain or a Fab domain) of an antibody construct herein can have an affinity for CD3 (given as a K_D value for binding CD3) that is not more than about 1 nM, 5 nM, 10 nM, 20 nM, or not more than about 30 nM. In various embodiments, an anti-CD3 binding domain herein has a K_D value for binding CD3

that is from about 20 nM to about 80 nM, from about 30 nM to about 60 nM, or from about 40 nM to about 50 nM. In some embodiments, an anti-CD3 binding domain herein has a K_D value for binding CD3 that is not more than about 30 nM, 40 nM, 50 nM, or about 60 nM. In various embodiments, an antibody construct herein comprises an anti-CD3 binding domain that has a K_D value for binding CD3 from about 20 nM to about 40 nM, e.g., of about 30 nM.

[0162] In some embodiments, the anti-CD3 binding domain is capable of binding CD3 with a K_D value from about 20 nM to about 200 nM, from about 30 nM to about 150 nM, from about 40 nM to about 100 nM, or from 50 nM to about 80 nM.

[0163] In various embodiments, an antibody construct herein comprises a binding domain capable of binding CD3 on a T cell, wherein such binding domain has a K_D value for binding CD3 from about 20 nM to about 40 nM, e.g., of about 30 nM, and comprises the CDR sequences of the V_H sequence as set forth in SEQ ID NOS: 49, 50 and 52 or SEQ ID NOS: 49, 51 and 52, and the CDR sequences of the V_L sequence as set forth in SEQ ID NO: 53-55.

[0164] In various embodiments, an antibody construct herein comprises a binding domain capable of binding CD3 on a T cell, wherein such binding domain has a K_D value for binding CD3 from about 20 nM to about 40 nM, e.g., of about 30 nM, and comprises a V_H domain comprising a HCDR1 sequence comprising the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 sequence comprising the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 sequence comprising the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 sequence comprising the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 sequence comprising the sequence RND (SEQ ID NO: 54), and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

[0165] In some embodiments, the anti-CD3 binding domain comprises a V_H domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 5. In certain embodiments, the anti-CD3 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5.

[0166] In some embodiments, the CDRs of an anti-CD3 paratope used in an antibody construct of the present disclosure comprise one or more amino acid modifications in one or more of the CDR sequences set forth in SEQ ID NOs: 49, 50 and 52-55 or SEQ ID NOS: 49 and 51-55, wherein at least about 80%, 90%, or 95% binding affinity to CD3 is retained compared to the paratope without such amino acid modifications.

[0167] In various embodiments of a trivalent and trispecific antibody construct, the anti-CD3 binding domain is a Fab domain as described herein.

[0168] The V_H domain of an anti-CD3 Fab domain of an antibody construct herein can comprise or consist of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the V_H domain of such Fab domain comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the V_H domain of such Fab domain comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the V_H domain of such Fab domain comprises or consists of an amino acid sequence having at least about 97% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the V_H domain of such Fab domain comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In yet other embodiments, the V_H domain of such Fab domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

[0169] The V_L domain of an anti-CD3 Fab domain of an antibody construct herein, which can be part of a light chain that pairs with an anti-CD3 Fab domain sequence of a heavy chain to form the Fab domain, can comprise or consist of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. In some embodiments, the V_L domain of such scFv or Fab domain comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. In some embodiments, the V_L domain of such scFv or Fab domain comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. In some embodiments, the V_L domain of such scFv or Fab domain comprises or consists of an amino acid sequence having at least about 97%

sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. In some embodiments, the V_L domain of such scFv or Fab domain comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. In various embodiments, the V_L domain of such scFv or Fab domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 5.

[0170] In embodiments in which the anti-CD3 domain is a Fab domain, which further comprises a C_{H1} domain and a C_L domain in its heavy and light chains, respectively, the C_{H1} domain of an anti-CD3 Fab domain of an antibody construct herein can comprise or consist of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, the C_{H1} domain of such Fab domain comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, the C_{H1} domain of such Fab domain comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, the C_{H1} domain of such Fab domain comprises or consists of an amino acid sequence having at least about 97% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, the C_{H1} domain of such Fab domain comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. In some embodiments, the C_{H1} domain of such Fab domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4.

[0171] Moreover, the C_L domain of an anti-CD3 Fab domain of an antibody construct herein can comprise or consist of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the C_L domain of such Fab domain comprises or consists of an amino acid sequence having at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the C_L domain of such Fab domain comprises or consists of an amino acid sequence having at least about 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the C_L domain of such Fab domain comprises or consists of an amino acid sequence having at least about 97% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID

NO: 7. In some embodiments, the C_L domain of such Fab domain comprises or consists of an amino acid sequence having at least about 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In yet other embodiments, the C_L domain of such Fab domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7.

[0172] In some embodiments, an anti-CD3 binding domain of an antibody construct is a Fab domain. In some embodiments, such an anti-CD3 Fab domain comprises a heavy chain, or a portion thereof (e.g., in cases in which the heavy chain further comprises an Fc portion, etc.), and a light chain. The heavy chain of the anti-CD3 Fab domain can comprise or consist of a V_H domain coupled to a C_{H1} domain, from N- to C-terminus. In various embodiments, the anti-CD3 Fab domain comprises (i) a V_H domain comprising or consisting of an amino acid sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, (ii) a V_L domain comprising or consisting of an amino acid sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 5, (iii) a C_{H1} domain comprising or consisting of an amino acid sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4, and (iv) C_L domain comprising or consisting of an amino acid sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 7.

[0173] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 3, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 6.

[0174] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 4, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 7.

[0175] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1, a C_{H1} domain comprising or

consisting of the amino acid sequence set forth in SEQ ID NO: 4, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 7.

[0176] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 3, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 7.

[0177] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 3, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 6.

[0178] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 2, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 4, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 7.

[0179] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 2, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 3, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 7.

[0180] In certain embodiments, the anti-CD3 Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 2, a C_{H1} domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 3, a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5, and a C_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 6.

[0181] As further described herein, in some embodiments, the binding affinity of a trivalent and trispecific antibody construct of the present disclosure for one or more specific targets (e.g., CD3) can – at least in part – be dependent on its format and/or geometry. As an example, the relative

orientation and vicinity of an anti-CD3 binding domain in an antibody construct can affect the binding domain's ability to interact with the target epitope it is binding, e.g., through steric hindrance, conformational changes occurring when the construct interacts with one or more of its targets (e.g., conditional degrees of freedom for a binding domain such as its steric flexibility), etc.

[0182] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a first Fab domain capable of binding CD3 and comprising the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 2, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 5, wherein the CDR sequences are determined using any of the numbering system known in the art.

[0183] In further embodiments, the CD3 binding affinity of an anti-CD3 binding domain of an antibody construct herein can be engineered and altered (e.g., increased/decreased relative to unmodified domains), e.g., by using one or more amino acid modifications relative to one or more reference sequence(s). In some embodiments, a trispecific and trivalent antibody construct of the present disclosure can comprise a variant anti-CD3 binding domain that comprises one or more amino acid modifications in its VH and/or VL domain(s) compared to the anti-CD3 binding domains described herein which comprise a VH sequence set forth in, e.g., SEQ ID NOs: 1 or 2 and a VL sequence set forth in, e.g., SEQ ID NO: 5. Such one or more amino acid modifications can reduce or increase the binding affinity of the variant anti-CD3 binding domain to CD3 when compared to the binding affinity of a corresponding anti-CD3 binding domain that does not comprise such one or more amino acid modifications.

[0184] In some embodiments, the one or more amino acid modifications used to alter the binding affinity of an anti-CD3 binding domain can include one or more amino acid substitution(s), one or more amino acid addition(s), and/or one or more amino acid deletion(s). In certain embodiments, the one or more amino acid modifications used to alter the binding affinity of an anti-CD3 binding domain comprise or consist of one or more amino acid substitution(s) relative to an unmodified binding domain sequence (e.g., an anti-CD3 VH or VL sequence).

[0185] In some embodiments, the anti-CD3 affinity of an affinity-altered CD3 binding domain can be about ±2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold higher or lower than a corresponding parental anti-CD3 binding domain.

[0186] In some embodiments, other anti-CD3 binding domains, such as those known in the art (e.g., SP34, OKT3, etc.), as well as variants thereof, can also be used in the trivalent and trispecific antibody constructs described herein.

[0187] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises an anti-CD3 binding domain comprising a VH sequence comprising the VHCDRs1-3 as set forth in SEQ ID NOs: 84-86, and a VL sequence comprising the VLCDRs1-3 as set forth in SEQ ID NOs: 87-89.

[0188] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises an anti-CD3 binding domain comprising a VH sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 82, and a VL sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 83. In some embodiments, such anti-CD3 binding domain comprises a VH sequence comprising the sequence set forth in SEQ ID NO: 82, and a VL sequence comprising the sequence set forth in SEQ ID NO: 83.

B.2.2 Binding Domains Against CD28

[0189] As further described herein, in various embodiments, a trivalent and trispecific antibody construct of the present disclosure (e.g., a Het-Fab or $scFv^2$ antibody construct) comprises a binding domain capable of binding CD28.

[0190] In various embodiments, such anti-CD28 binding domain (e.g., an scFv domain or a Fab domain) of an antibody construct herein can have an affinity for CD28 (given as a K_D value for binding CD28) that is from about 10 nM to about 500 nM or from about 20 nM to about 250 nM.

[0191] In some embodiments, an antibody construct herein comprises a binding domain (e.g., an scFv domain or a Fab domain) capable of binding CD28 on a T cell, wherein such binding domain has an affinity for CD28 that is from about 15 nM to about 35 nM or from about 5 nM to about 50 nM and comprises an anti-CD28 V_H sequence comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and an anti-CD28 V_L sequence comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48).

[0192] In such embodiments, an antibody construct herein comprises a binding domain (e.g., an scFv domain or a Fab domain) capable of binding CD28 on a T cell, wherein such binding domain comprises a V_H domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 binding domain comprises a V_H domain comprising or consisting of the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the sequence set forth in SEQ ID NO: 16.

[0193] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a first scFv domain capable of binding CD28 and comprising the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 15, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 16, wherein the CDR sequences are determined using any of the numbering system known in the art.

[0194] In other embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises an anti-CD28 binding domain comprising (i) a V_H domain that comprises a sequence having one or more amino acid substitution(s) compared to the sequence set forth in SEQ ID NO: 15, and/or (ii) a V_L domain that comprises a sequence having one or more amino acid substitution(s) compared to the sequence set forth in SEQ ID NO: 16, wherein the position of such amino acid substitution can be provided herein according to the IMGT numbering system.

[0195] In some embodiments, such one or more amino acid substitutions in either the anti-CD28 V_H domain and/or the anti-CD28 V_L domain can reduce the binding affinity of the corresponding anti-CD28 binding domain to CD28 by about 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 2.0-fold, 3.0-fold, 3.3-fold, 3.5-fold, 3.7-fold, 3.9-fold, 5.0-fold, 5.2-fold, 5.5-fold, 6.0-fold, 7.0-fold, 8.0-fold, 8.5-fold, 9.0-fold, 10-fold, 20-fold, or 25-fold, or from about 1.5-fold to about 25-fold, from about 2.0-fold to about 20-fold, from about 3.0-fold to about 20-fold, or from about 5.0-fold to about 10-fold, when compared to the binding affinity of an anti-CD28 binding domain that does not contain a V_H and/or V_L sequence with such amino acid substitutions (e.g., a binding domain comprising the V_H and V_L sequence set forth in SEQ ID NOs: 15 and 16, respectively).

[0196] Hence, in some embodiments, an anti-CD28 binding domain (e.g., an scFv domain or a Fab domain) of an antibody construct herein can have an affinity for CD28 (given as a K_D value

for binding CD28) that is from about 10 nM to about 500 nM, from about 20 nM to about 600 nM, from about 20 nM to about 250 nM, from about 20 nM to about 150 nM, from about 20 nM to about 100 nM, or from about 20 nM to about 50 nM.

[0197] In various embodiments, the anti-CD28 binding domain of an antibody construct herein comprises or consists of an scFv domain.

[0198] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution P1058A relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 21.

[0199] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution G1064S relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 24.

[0200] In some embodiments, an anti-CD28 binding domain herein comprises a V_L domain comprising the amino acid substitution V1035G relative to the amino acid sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 V_L domain comprises the amino acid sequence set forth in SEQ ID NO: 17.

[0201] In some embodiments, an anti-CD28 binding domain herein comprises a V_L domain comprising the amino acid substitution D1068E relative to the amino acid sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 V_L domain comprises the amino acid sequence set forth in SEQ ID NO: 26.

[0202] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution E1080K relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 20.

[0203] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution Y1110S relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 18.

[0204] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution N1111aA relative to the amino acid sequence set forth in

SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 25.

[0205] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution Y1112S relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 76.

[0206] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution L1112aN relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 23.

[0207] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution Y1113S relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 27.

[0208] In some embodiments, an anti-CD28 binding domain herein comprises a V_H domain comprising the amino acid substitution Y1037A relative to the amino acid sequence set forth in SEQ ID NO: 15. In some of these embodiments, the anti-CD28 V_H domain comprises the amino acid sequence set forth in SEQ ID NO: 22.

[0209] In some embodiments, an anti-CD28 binding domain herein comprises a V_L domain comprising the amino acid substitution Y1031A relative to the amino acid sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 V_L domain comprises the amino acid sequence set forth in SEQ ID NO: 77.

[0210] In some embodiments, an anti-CD28 binding domain herein comprises a V_L domain comprising the amino acid substitution N1066A relative to the amino acid sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 V_L domain comprises the amino acid sequence set forth in SEQ ID NO: 19.

[0211] In some embodiments, an antibody construct comprising an anti-CD28 binding domain comprising one or more of the V_H and/or V_L domain substitutions described herein can exhibit a reduced non-specific anti-tumor activity *in vitro* and/or *in vivo*. In one such embodiment, an antibody construct comprising a mutated (e.g., relative to the huTN228 wildtype sequence) anti-CD28 binding domain can induce less non-specific (e.g., in the absence of the TAA) immune cell

activity, e.g., having reduced non-specific cytokine production by the immune cells. In certain embodiments, an antibody construct comprising an anti-CD28 binding domain that carries the N1066A substitution in the V_L domain relative to huTN228 wildtype can induce a reduced non-specific T cell activity, e.g., cytokine production, e.g., in the absence of the TAA. In another embodiment, an antibody construct comprising an anti-CD28 binding domain that carries the Y1031A substitution in the V_L domain relative to huTN228 wildtype can induce a reduced non-specific T cell activity, e.g., cytokine production. In some embodiments, non-specific T cell activity, e.g., cytokine production such as production of TNFα, IL-2, etc., can be reduced by about 10-fold, 20-fold, 30-fold, 50-fold, 60-fold, 70-fold, or about 100-fold.

[0212] In various embodiments, an antibody construct herein comprises a binding domain capable of binding CD28 on a T cell, wherein such binding domain has a K_D value for binding CD28 from about 20 nM to about 600 nM and comprises a V_H domain comprising a HCDR1 having the sequence SX_1GVH (SEQ ID NO: 30), a HCDR2 having the sequence $VIWX_2GGX_3TNFNSALMS$ (SEQ ID NO: 34), and a HCDR3 having the sequence $DRAX_4GX_5YX_6X_7AMDY$ (SEQ ID NO: 40) and a V_L sequence comprising a LCDR1 having the sequence $RASESVEYYX_8TSLMQ$ (SEQ ID NO: 43), a LCDR2 having the sequence $AASX_9VX_{10}S$ (SEQ ID NO: 47), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), and wherein X_1 = Y, A; X_2 = P, A; X_3 = G, S; X_4 = S, Y; X_5 = N, A; X_6 = L, N; X_7 = S, Y; X_8 = G, V; X_9 = N, A; and X_{10} = E, D.

[0213] In some embodiments, an anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 16. In some of these embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0214] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 21, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0215] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 24, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0216] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 17.

[0217] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 26.

[0218] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 20, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0219] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 18, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0220] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 25, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0221] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 76, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0222] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID

NO: 23, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0223] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 27, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0224] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 22, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0225] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 77.

[0226] In some embodiments, the anti-CD28 binding domain of an antibody construct herein comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 29.

[0227] In various embodiments of a trivalent and trispecific antibody construct, the anti-CD28 binding domain comprises or consists of an scFv domain. As further described herein, such anti-CD28 binding domain can have the domain structure, from N- to C-terminus, of: VL-$Linker^{scFv}$-VH or VH-$Linker^{scFv}$-VL.

[0228] In embodiments in which the anti-CD28 binding domain of an antibody construct is an scFv domain, such anti-CD28 scFv domain can comprise, from either N- to C-terminus of C- to N-terminus, a V_H domain comprising the amino acid sequence set forth in any one of SEQ ID NOs: 15, 18, 20, 21, 22, 23, 24, 25, 27, or 76, coupled to a V_L domain comprising the amino acid sequence set forth in any one of SEQ ID NOs: 16, 17, 19, 26, or 77, via a $linker^{scFv}$ which sequence is $(G_nS)_m$, wherein n, m can independently be 1, 2, 3, 4 or 5, and as set forth in SEQ ID NO: 69. In some of these instances, the $linker^{scFv}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 59. And in some embodiments, the anti-CD28 scFv domain has a domain structure,

from N- to C-terminus, of: V_H-linker^{scFv}-V_L. In other embodiments, the anti-CD28 scFv domain has a domain structure, from N- to C-terminus, of: V_L-linker^{scFv}-V_H.

[0229] In certain embodiments, described herein is an antibody construct comprising a binding domain capable of binding CD28, wherein the binding domain comprises a V_H sequence comprising a HCDR1 having the sequence SX_1GVH (SEQ ID NO: 30), a HCDR2 having the sequence $VIWX_2GGX_3TNFNSALMS$ (SEQ ID NO: 34), and a HCDR3 having the sequence $DRAX_4GX_5YX_6X_7AMDY$ (SEQ ID NO: 40), and a V_L sequence comprising a LCDR1 having the sequence $RASESVEYYX_8TSLMQ$ (SEQ ID NO: 43), a LCDR2 having the sequence $AASX_9VX_{10}S$ (SEQ ID NO: 47), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), and comprises one or more of the following amino acid substitutions at the positions as identified in the CDR sequences: X_1: Y to A, X_2: P to A, X_3: G to S, X_4: S to Y, X_5: N to A, X_6: L to N, X_7: S to Y, X_8: G to V, X_9: N to A, or X_{10}: E to D.

[0230] In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_1: Y to A. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_2: P to A. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_3: G to S. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_4: S to Y. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_5: N to A. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_6: L to N. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_7: S to Y. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_8: G to V. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_9: N to A. In some embodiments, the antibody construct comprises an anti-CD28 binding domain comprising the substitution X_{10}: E to D.

[0231] In some embodiments, such antibody construct can have a binding affinity for CD28 that is reduced by about 1.5-fold to about 25-fold, by about 2.0-fold to about 20-fold, by about 3.0-fold to about 20-fold, or by about 5.0-fold to about 10-fold, when compared to the binding affinity of an antibody construct comprising an anti-CD28 binding domain that does not contain the one or more amino acid substitutions in one or more of the CDR sequences.

[0232] In some embodiments, such antibody construct can comprise an anti-CD28 binding domain that comprises the CDRs of a V_H domain as set forth in SEQ ID NOS: 28, 31, and 35, and the CDRs of the V_L sequence set forth in SEQ ID NOS: 41, 44, and 48.

[0233] In some embodiments, such antibody construct can comprise an anti-CD28 binding domain that comprises an anti-CD28 V_H sequence comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and an anti-CD28 V_L sequence comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48).

[0234] As further described herein, in various embodiments, an anti-CD28 binding domain of a trivalent and trispecific antibody construct is an scFv domain. Such anti-CD28 scFv domain can be coupled to a Fab light chain sequence, e.g., to the C-terminus of a Fab light chain sequence.

[0235] In some embodiments, other anti-CD28 binding domains, such as those known in the art (e.g., CD28.3, TGN1412, etc.), as well as variants thereof, can also be used in the trivalent and trispecific antibody constructs described herein.

B.3 Binding Domains Against DLL3

[0236] As described herein, in various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a binding domain capable of binding DLL3. In various embodiments, an antibody construct herein can be trivalent and trispecific and comprises a first binding domain capable of binding a first antigen on a cytotoxic effector cell, a second binding domain capable of binding a second antigen on a cytotoxic effector cell, and a third binding domain, wherein such third binding domain is capable of binding DLL3.

[0237] Generally, the anti-DLL3 binding domain of an antibody construct herein can comprise or consist of an scFv domain or a Fab domain. In various embodiments of a trivalent and trispecific Het-Fab antibody construct, the anti-DLL3 binding domain comprises a Fab domain. In other embodiments of a trivalent and trispecific Het-Fab antibody construct, the anti-DLL3 binding domain comprises an scFv domain.

[0238] The anti-DLL3 binding domains that can be part of a trivalent and trispecific antibody construct described herein can encompass binding domains capable of recognizing and binding any one of the epitopes, portions, domains or motifs located on, e.g., mouse, cynomolgus and/or

human DLL3. In various embodiments, an anti-DLL3 binding domain of a trivalent and trispecific antibody construct described herein can bind to an epitope of human DLL3, which can include an N-terminal ECD domain, EGF1-6, and/or the membrane proximal peptide.

[0239] Anti-DLL3 binding domains against one or more of these human DLL3 epitopes are known in the art, and any such binding domain which is capable of binding DLL3 can generally be used in the trivalent and trispecific antibody constructs described herein.

[0240] In some embodiments, an anti-DLL3 binding domain, e.g., an anti-DLL3 VH and/or an anti-DLL3 VL sequence, of a trivalent and trispecific antibody construct of this disclosure can be one that is known in the art, or a variant thereof, e.g., an anti-DLL3 binding domain that is described in any one of International Patent Publication Nos. WO 2013/126746, WO 2016/138038, WO 2017/021349, WO 2021/200898, WO 2019/234220, WO 2021/173307, WO 2021/226204 and WO 2021/155380, which are incorporated herein by reference in their entireties.

[0241] In various embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that has a K_D value for binding DLL3 from about 0.01 nM to about 100 nM or from about 0.1 nM to about 50 nM. In some embodiments, a trivalent and trispecific antibody construct has a binding affinity (e.g., provided as a K_D value) for DLL3 of at least about 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 3 nM, 2 nM, 1 nM, 0.5 nM, or at least about 0.2 nM. In some embodiments, a trivalent and trispecific antibody construct herein has a binding affinity (e.g., provided as a K_D value) for DLL3 of from about 40 nM to about 0.1 nM, from about 20 nM to about 0.2 nM, from about 10 nM to about 0.2 nM, or from about 5 nM to about 0.2 nM, e.g., using SPR or other methods known in the art.

[0242] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH sequence comprising the HCDR1 sequence as set forth in SEQ ID NO: 90, the HCDR2 sequence as set forth in SEQ ID NO: 91 and the HCDR3 sequence as set forth in SEQ ID NO: 92, and a VL sequence comprising the LCDR1 sequence as set forth in SEQ ID NO: 93, the LCDR2 sequence as set forth in SEQ ID NO: 94 and the LCDR3 sequence as set forth in SEQ ID NO: 95.

[0243] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-

DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH sequence comprising the amino acid sequence set forth in SEQ ID NO: 99, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 100.

[0244] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises a third binding domain (e.g., a Fab domain or an scFv domain) capable of binding DLL3 on a tumor cell comprising the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 99, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 100, wherein the CDR sequences are determined using any of the numbering system known in the art.

[0245] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH sequence comprising the HCDR1 sequence as set forth in SEQ ID NO: 90, the HCDR2 sequence as set forth in SEQ ID NO: 96 and the HCDR3 sequence as set forth in SEQ ID NO: 92, and a VL sequence comprising the LCDR1 sequence as set forth in SEQ ID NO: 93, the LCDR2 sequence as set forth in SEQ ID NO: 94 and the LCDR3 sequence as set forth in SEQ ID NO: 95.

[0246] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH sequence comprising the amino acid sequence set forth in SEQ ID NO: 101, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 102.

[0247] In certain embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH sequence comprising the HCDR1 sequence as set forth in SEQ ID NO: 90, the HCDR2 sequence as set forth in SEQ ID NO: 96 and the HCDR3 sequence as set forth in SEQ ID NO: 92, and a VL sequence comprising the LCDR1 sequence as set forth in SEQ ID NO: 97, the LCDR2 sequence as set forth in SEQ ID NO: 98 and the LCDR3 sequence as set forth in SEQ ID NO: 95.

[0248] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure, e.g., a trivalent and trispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., a Fab domain or an scFv domain, respectively) that comprises a VH

sequence comprising the amino acid sequence set forth in SEQ ID NO: 103, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 104.

C. Fc Domains

[0249] As described herein, a trivalent and trispecific antibody construct of this disclosure can comprise an Fc domain (or Fc region, or Fc, also commonly known as an abbreviation of "fragment, crystallizable") comprising a first Fc polypeptide and a second Fc polypeptide. In various embodiments, the Fc domain is a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein the first Fc polypeptide and the second Fc polypeptide share at least about 90%, 95%, 97%, or at least about 99% amino acid sequence identity. In some embodiments, each Fc polypeptide comprises one or more asymmetric amino acid substitutions that can promote preferential pairing of the Fc polypeptides to form the heterodimeric Fc domain (e.g., H1-H2) compared to formation of a respective homodimeric Fc domain (e.g., H1-H1 or H2-H2).

[0250] The term "Fc domain," as used herein, includes native (or wildtype) sequence Fc domains as well as variant Fc domains comprising one or more amino acid modifications relative to a corresponding native or wildtype Fc domain. Unless otherwise specified herein, numbering of amino acid residues in the Fc domain or constant region is according to the EU numbering system, also called the EU index, as described, e.g., in Kabat *et al.,* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). An "Fc polypeptide" of a dimeric (e.g., heterodimeric) Fc domain refers to one of the two polypeptide chains (e.g., a first and second Fc polypeptide) forming the dimeric (e.g., heterodimeric) Fc domain. In some embodiments, Fc polypeptides can comprise a C-terminal constant region of an Ig heavy chain that is capable of stable self-association. In various embodiments, and as further described herein, an Fc polypeptide (e.g., a first or a second Fc polypeptide) comprises at least one of a CH2 domain and/or a CH3 domain. In certain embodiments, an Fc polypeptide of an antibody construct described herein comprises a CH2 domain and a CH3 domain.

[0251] As disclosed herein, a trivalent and trispecific antibody construct can comprise an Fc domain, wherein such Fc domain can be a heterodimeric Fc domain. The Fc domain, e.g., heterodimeric Fc domain, of an antibody construct, unless otherwise specified, comprises a first Fc polypeptide and a second Fc polypeptide. Generally, each Fc polypeptide of a (e.g.,

heterodimeric) Fc domain can comprise a CH2 domain, a CH3 domain, or, as described in various embodiments herein, both a CH2 domain and a CH3 domain.

[0252] In certain embodiments, an antibody construct herein comprises an Fc domain based on a human IgG Fc domain. In some embodiments, an antibody construct comprises an Fc domain based on a human IgG1 or IgG4 Fc domain. In various embodiments, an antibody construct comprises a heterodimeric IgG Fc domain comprising two different Fc polypeptides, e.g., a first Fc polypeptide and a second polypeptide, wherein the first and second Fc polypeptides have different amino acid sequences, e.g., amino acid sequences that have about 90%, 95%, 97%, or 99% sequence identity when compared and aligned to one another, e.g., as further described herein. In some embodiments, the differences in the amino acid sequences of a first and second Fc polypeptide can be due to asymmetric amino acid substitutions that can be introduced into each Fc polypeptide chain to promote preferential paring of the heavy chains to form the heterodimeric Fc domain, compared to a corresponding homodimeric Fc domain.

[0253] In various embodiments, a trivalent and trispecific antibody construct herein comprises an Fc domain that is a modified IgG Fc domain, and in which at least the CH3 domain of at least one Fc polypeptide comprises one or more amino acid modifications compared to a respective wildtype CH3 domain. In some embodiments, an antibody construct herein comprises an Fc domain that is a modified IgG Fc domain in which at least the CH2 domain of at least one Fc polypeptide comprises one or more amino acid modifications compared to a respective wildtype CH2 domain. In some embodiments, an antibody construct comprises an Fc domain that is a modified IgG Fc domain in which both the CH3 domain and the CH2 domain of at least one Fc polypeptide comprises one or more amino acid modifications compared to respective wildtype CH3 and CH2 domains. In various embodiments, both Fc polypeptides of a heterodimeric Fc domain can comprise one or more amino acid modifications in their CH3 domains. In some embodiments, both Fc polypeptides of a heterodimeric Fc domain can comprise one or more amino acid modifications in their CH2 domains. In yet other embodiments, both Fc polypeptides of a heterodimeric Fc domain can comprise one or more amino acid modifications in their CH2 domains and CH3 domains.

C.1 Modified Fc Domains

[0254] In some embodiments, the present disclosure relates to trivalent and trispecific antibody constructs that can comprise a heterodimeric Ig (e.g., IgG) Fc domain comprising a modified

heterodimeric CH3 domain, wherein the modified heterodimeric CH3 domain comprises one or more asymmetric amino acid modifications, i.e., one or both the first and the second Fc polypeptides each comprise one or more amino acid modifications in their CH3 domain sequences compared to respective wildtype sequences. As used herein, the term "asymmetric amino acid modification" generally refers to a modification in which an amino acid at a specific position on the first Fc polypeptide is different to the amino acid at the corresponding position on the second Fc polypeptide. These asymmetric amino acid modifications can comprise modifications of only one of the two amino acids at the corresponding position on each Fc polypeptide, or they can comprise modifications of both amino acids at the corresponding positions on each of the first and second Fc polypeptides. In various embodiments, an "asymmetric amino acid modification" is an asymmetric amino acid substitution.

[0255] In some embodiments, an antibody construct herein comprises a heterodimeric Fc domain comprising a modified CH3 domain (i.e., a heterodimeric CH3 domain consisting of the two CH3 domains of the first and second Fc polypeptides), wherein the modified CH3 domain comprises one or more asymmetric amino acid modifications that promote formation of the heterodimeric Fc domain (e.g., pairing of a first Fc polypeptide with a second Fc polypeptide) over formation of a corresponding homodimeric Fc domain (e.g., pairing of a first Fc polypeptide with another first Fc polypeptide). Amino acid modifications that can be made to the CH3 domain of an Fc domain in order to promote formation of a heterodimeric Fc domain are known in the art and include, for example, those described in International Publication No. WO 96/027011 ("knobs into holes"), Gunasekaran *et al.*, 2010, *J Biol Chem*, 285, 19637-46 ("electrostatic steering"), Davis *et al.*, 2010, *Prot Eng Des Sel*, 23(4):195-202 (strand exchange engineered domain (SEED) technology) and Labrijn *et al.*, 2013, *Proc Natl Acad Sci USA*, 110(13):5145-50 (Fab-arm exchange). Other examples include approaches combining positive and negative design strategies to produce stable asymmetrically modified Fc regions as described in International Patent Publication Nos. WO 2012/058768 and WO 2013/063702.

[0256] In certain embodiments, an antibody construct described herein comprises a heterodimeric Fc domain comprising a modified heterodimeric CH3 domain in which at least one, or both of the Fc polypeptide chains comprise one or more amino acid modifications, as described in International Publication No. WO 2012/058768 or International Patent Publication No. WO 2013/063702.

[0257] In some embodiments, an antibody construct described herein comprises a heterodimeric human IgG1 Fc domain having a modified CH3 domain. **TABLE 2** herein provides the amino acid sequence of a human IgG1 Fc domain sequence (e.g., a sequence that a first and/or a second Fc polypeptide can be derived from), corresponding to amino acids 231 to 447 of a full-length human IgG1 heavy chain (e.g., one that comprises V_H, C_{H1}, Hinge, CH2 and CH3 domains), and identified by SEQ ID NO: 78. The CH2 domain is typically defined as comprising amino acids 231-340 of the full-length human IgG1 heavy chain and the CH3 domain is typically defined as comprising amino acids 341-447 of the full-length human IgG1 heavy chain.

[0258] As described herein, an antibody construct can comprise a heterodimeric Fc domain having a modified CH3 domain comprising one or more asymmetric amino acid modifications that promote formation of the heterodimeric Fc domain over formation of a homodimeric Fc domain, and in which the modified CH3 domain comprises a first Fc polypeptide including amino acid modifications at positions F405 and Y407, relative to SEQ ID NO: 78, and a second Fc polypeptide including amino acid modifications at positions T366 and T394, relative to SEQ ID NO: 78. In various embodiments, the one or more amino acid modifications comprise one or more amino acid substitutions. Hence, in some embodiments, the amino acid modification at position F405 of the first Fc polypeptide of the modified CH3 domain is F405A, F405I, F405M, F405S, F405T or F405V. In some embodiments, the amino acid modification at position Y407 of the first Fc polypeptide of the modified CH3 domain is Y407I or Y407V. In some embodiments, the amino acid modification at position T366 of the second Fc polypeptide of the modified CH3 domain is T366I, T366L or T366M. In some embodiments, the amino acid modification at position T394 of the second Fc polypeptide of the modified CH3 domain is T394W. In some embodiments, the modified CH3 domain of a first Fc polypeptide further includes an amino acid modification at position L351, relative to SEQ ID NO: 78. In some embodiments, the amino acid modification at position L351 in the first Fc polypeptide of the modified CH3 domain is L351Y. In some embodiments, the second Fc polypeptide of the modified CH3 domain further includes an amino acid modification at position K392, relative to SEQ ID NO: 78. In some embodiments, the amino acid modification at position K392 in the second Fc polypeptide of the modified CH3 domain is K392F, K392L or K392M. In some embodiments, one or both of the first and second Fc polypeptides of the modified CH3 domain further comprises the amino acid modification T350V.

[0259] In certain embodiments, an antibody construct herein comprises a heterodimeric Fc domain having a modified CH3 domain comprising one or more asymmetric amino acid modifications that promote formation of the heterodimeric Fc domain over formation of a homodimeric Fc domain, and in which the modified CH3 domain comprises a first Fc polypeptide including the amino acid modification F405A, F405I, F405M, F405S, F405T or F405V together with the amino acid modification Y407I or Y407V, and relative to SEQ ID NO: 78, and a second Fc polypeptide including the amino acid modification T366I, T366L or T366M, together with the amino acid modification T394W, and relative to SEQ ID NO: 78. In some embodiments, the first Fc polypeptide of the modified CH3 domain further includes the amino acid modification L351Y. In some embodiments, the second Fc polypeptide of the modified CH3 domain further includes the amino acid modification K392F, K392L or K392M. In some embodiments, one or both of the first and second Fc polypeptides having a modified CH3 domain further comprises the amino acid modification T350V.

TABLE 2: Exemplary Human IgG1 Fc Domain Sequences and Variants Thereof

Human IgG1 Fc Domain sequence of amino acid residues 231-447 (EU-numbering)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 78)	
Variant #	**Polypeptide Chain***	**Mutations**
1	A	L351Y_F405A_Y407V
	B	T366L_K392M_T394W
2	A	L351Y_F405A_Y407V
	B	T366L_K392L_T394W
3	A	T350V_L351Y_F405A_Y407V
	B	T350V_T366L_K392L_T394W
4	A	T350V_L351Y_F405A_Y407V
	B	T350V_T366L_K392M_T394W
5	A	T350V_L351Y_S400E_F405A_Y407V
	B	T350V_T366L_N390R_K392M_T394W

* "A" corresponds to a first Fc polypeptide chain and "B" to corresponds to a second Fc polypeptide chain.

[0260] In certain embodiments, an antibody construct herein comprises a heterodimeric Fc domain comprising a modified CH3 domain having a first Fc polypeptide that comprises amino acid modifications at positions F405 and Y407, and optionally further comprises an amino acid modification at position L351, and a second Fc polypeptide that comprises amino acid

modifications at positions T366 and T394, and optionally further comprises an amino acid modification at position K392, as described above, and the first Fc polypeptide further comprises an amino acid modification at one or both of positions S400 or Q347 and/or the second Fc polypeptide further comprises an amino acid modification at one or both of positions K360 or N390, wherein the amino acid modification at position S400 is S400E, S400D, S400R or S400K; the amino acid modification at position Q347 is Q347R, Q347E or Q347K; the amino acid modification at position K360 is K360D or K360E, and the amino acid modification at position N390 is N390R, N390K or N390D, relative to SEQ ID NO: 78.

[0261] In some embodiments, an antibody construct comprises a heterodimeric Fc domain comprising a modified CH3 domain comprising the modifications of any one of Variant 1, Variant 2, Variant 3, Variant 4 or Variant 5, as shown in **TABLE 2**.

[0262] In various embodiments, an antibody construct of the present disclosure can comprise a heterodimeric Fc domain comprising a first Fc polypeptide (A) and a second Fc polypeptide (B), wherein the first Fc polypeptide (A) and the second Fc polypeptide (B) comprise the amino acid substitutions in their CH3 domains according to variant #1 as shown in **TABLE 2**. In other embodiments, an antibody construct of the present disclosure can comprise a heterodimeric Fc domain comprising a first Fc polypeptide (A) and a second Fc polypeptide (B), wherein the first Fc polypeptide (A) and the second Fc polypeptide (B) comprise the amino acid substitutions in their CH3 domains according to variant #2 as shown in **TABLE 2**. In some embodiments, an antibody construct of the present disclosure can comprise a heterodimeric Fc domain comprising a first Fc polypeptide (A) and a second Fc polypeptide (B), wherein the first Fc polypeptide (A) and the second Fc polypeptide (B) comprise the amino acid substitutions in their CH3 domains according to variant #3 as shown in **TABLE 2**. In some embodiments, an antibody construct of the present disclosure can comprise a heterodimeric Fc domain comprising a first Fc polypeptide (A) and a second Fc polypeptide (B), wherein the first Fc polypeptide (A) and the second Fc polypeptide (B) comprise the amino acid substitutions in their CH3 domains according to variant #4 as shown in **TABLE 2**. In yet other embodiments, an antibody construct of the present disclosure can comprise a heterodimeric Fc domain comprising a first Fc polypeptide (A) and a second Fc polypeptide (B), wherein the first Fc polypeptide (A) and the second Fc polypeptide (B) comprise the amino acid substitutions in their CH3 domains according to variant #5 as shown in **TABLE 2**.

[0263] In certain embodiments, the CH3 domain of a first Fc polypeptide of an antibody construct herein has an amino acid sequence that is at least about 80%, about 85%, about 90%, about 95%, about 97%, or at least about 99% identical to the amino acid sequence set forth in SEQ ID NO: 9. In certain embodiments, the CH3 domain of a second Fc polypeptide of an antibody construct herein has an amino acid sequence that is at least about 80%, about 85%, about 90%, about 95%, about 97%, or at least about 99% identical to the amino acid sequence set forth SEQ ID NO: 10. In some embodiments, the CH3 domain of a first Fc polypeptide of an antibody construct herein has the amino acid sequence set forth in SEQ ID NO: 9 and a second Fc polypeptide of the antibody construct herein has the amino acid sequence set forth SEQ ID NO: 10.

[0264] In certain embodiments, an antibody construct herein comprises a heterodimeric Fc domain based on an IgG Fc domain having a modified CH2 domain (i.e., a heterodimeric CH2 domain consisting of the two CH2 domain sequences of the respective first and second Fc polypeptides). In some embodiments, an antibody construct comprises a heterodimeric Fc domain based on an IgG Fc domain having a modified CH2 domain, wherein the modification(s) of the CH2 domain result(s) in altered (e.g., reduced or abated) binding to one or more Fc receptors (FcRs) such as receptors of the FcγRI, FcγRII and FcγRIII subclasses.

[0265] Several amino acid modifications to the CH2 domain of the first and/or second Fc polypeptide(s) of an Fc domain that selectively alter the affinity of such Fc domain for different FcγRs are known in the art. Amino acid modifications that result in increased binding and amino acid modifications that result in decreased binding can both be useful in certain indications. For example, increasing binding affinity of an Fc for FcγRIIIa (an activating receptor) can result in increased antibody dependent cell-mediated cytotoxicity (ADCC), which in turn can result in increased lysis of the target cell. Decreased binding to FcγRIIb (an inhibitory receptor) likewise can be beneficial in some circumstances. In certain indications, a decrease in, or elimination of, ADCC and complement-mediated cytotoxicity (CDC) can be desirable. In such embodiments, modified CH2 domains comprising amino acid modifications that result in increased binding to FcγRIIb or amino acid modifications that can decrease or eliminate binding of the Fc region to all of the FcγRs ("knock-out" variants) can be useful.

[0266] Non-limiting examples of amino acid modifications to the CH2 domain that alter binding of the Fc domain by FcγRs include, but are not limited to, the following: S298A/E333A/K334A and S298A/E333A/K334A/K326A (increased affinity for FcγRIIIa) (Lu, *et al.*, 2011, *J Immunol*

Methods, 365(1-2):132-41); F243L/R292P/Y300L/V305I/P396L (increased affinity for FcγRIIIa) (Stavenhagen, *et al.,* 2007, *Cancer Res,* 67(18):8882-90); F243L/R292P/Y300L/L235V/P396L (increased affinity for FcγRIIIa) (Nordstrom JL, *et al.,* 2011, *Breast Cancer Res*, 13(6):R123); F243L (increased affinity for FcγRIIIa) (Stewart, *et al.*, 2011, *Protein Eng Des Sel.,* 24(9):671-8); S298A/E333A/K334A (increased affinity for FcγRIIIa) (Shields, *et al.,* 2001, *J Biol Chem,* 276(9):6591-604); S239D/I332E/A330L and S239D/I332E (increased affinity for FcγRIIIa) (Lazar, *et al.*, 2006, *Proc Natl Acad Sci USA*, 103(11):4005-10), and S239D/S267E and S267E/L328F (increased affinity for FcγRIIb) (Chu, *et al.,* 2008, *Mol Immunol,* 45(15):3926-33). Additional modifications that affect Fc domain binding to FcγRs are described in *Therapeutic Antibody Engineering* (Strohl & Strohl, Woodhead Publishing series in Biomedicine No 11, ISBN 1 907568 37 9, Oct 2012, page 283).

[0267] In various embodiments, an antibody construct of the present disclosure comprises a heterodimeric Fc domain based on an IgG Fc domain having a modified CH2 domain, in which one or both of the CH2 sequences (i.e., of the first/second Fc polypeptide) of the modified dimeric CH2 domain comprise one or more amino acid modifications that can result in decreased or eliminated binding of the Fc domain to one or more, or all of the FcγRs (i.e., a "knock-out" or "KO" variant).

[0268] Various publications describe strategies that have been used to engineer antibodies to produce "knock-out" Fc variants (see, for example, Strohl, 2009, *Curr Opin Biotech* 20:685-691, and Strohl & Strohl, "*Antibody Fc engineering for optimal antibody performance*" In Therapeutic Antibody Engineering, Cambridge: Woodhead Publishing, 2012, pp 225-249). These strategies include reduction of effector function through modification of glycosylation, use of IgG2/IgG4 scaffolds, or the introduction of mutations in the hinge or CH2 domain of the Fc (see also, U.S. Patent Publication No. 2011/0212087, International Patent Publication No. WO 2006/105338, U.S. Patent Publication No. 2012/0225058, U.S. Patent Publication No. 2012/0251531, and Strop *et al.,* 2012, *J. Mol. Biol.,* 420: 204-219).

[0269] In some embodiments, an Fc domain of an antibody construct can comprise one or more of known amino acid modifications to reduce FcγR and/or complement binding of the Fc domain. In some embodiments, such modifications can include those identified in **TABLE 3**.

TABLE 3: Modifications to Reduce Fcγ Receptor or Complement Binding

Company	Mutations
GSK	N297A
Ortho Biotech	L234A/L235A
Protein Design labs	IgG2 V234A/G237A
Wellcome Labs	IgG4 L235A/G237A/E318A
GSK	IgG4 S228P/L236E
Merck	IgG2 H268Q/V309L/A330S/A331S
Bristol-Myers	C220S/C226S/C229S/P238S
Seattle Genetics	C226S/C229S/E3233P/L235V/L235A
Medimmune	L234F/L235E/P331S

[0270] Additional examples herein include Fc domains engineered to include the amino acid modifications L235A/L236A/D265S, e.g., based on the sequence set forth in SEQ ID NO: 78. In addition, asymmetric amino acid modifications in the CH2 domain that decrease binding of the Fc to all FcγRs are described in International Patent Publication No. WO 2014/190441.

[0271] In certain embodiments, the CH2 domain of a first and a second Fc polypeptide herein comprises or consists of an amino acid sequence having at least about 80%, about 85%, about 90%, about 95%, about 97%, or at least about 99% sequence identity to the sequence set forth in SEQ ID NO: 8. In some embodiments, the CH2 domain of a first and/or a second Fc polypeptide herein comprises or consists of the sequence set forth in SEQ ID NO: 8.

[0272] In certain embodiments, an antibody construct herein comprises a heterodimeric Fc domain in which native glycosylation has been modified. As is known in the art, glycosylation of an Fc can be modified to increase or decrease effector function. For example, mutation of the conserved asparagine residue at position 297 to alanine, glutamine, lysine, or histidine (i.e., N297A, Q, K or H) results in an aglycoslated Fc that lacks all effector function (Bolt *et al.*, 1993, *Eur. J. Immunol.,* 23:403-411; Tao & Morrison, 1989, *J. Immunol.,* 143:2595-2601). Conversely, removal of fucose from heavy chain N297-linked oligosaccharides has been shown to enhance ADCC, based on improved binding to FcγRIIIa (see, for example, Shields *et al.*, 2002, *J Biol Chem.,* 277:26733-26740, and Niwa *et al.,* 2005, *J. Immunol. Methods,* 306:151-160). Such low fucose antibody constructs can be produced, for example in knockout Chinese hamster ovary (CHO) cells lacking fucosyltransferase (FUT8) (Yamane-Ohnuki *et al.*, 2004, *Biotechnol. Bioeng.*, 87:614-622), in the variant CHO cell line, Lec 13, that has a reduced ability to attach fucose to N297-linked carbohydrates (International Publication No. WO 03/035835), or in other cells that generate

afucosylated antibodies (see, for example, Li *et al.*, 2006, *Nat Biotechnol*, 24:210-215; Shields *et al.*, 2002, *ibid*, and Shinkawa *et al.*, 2003, *J. Biol. Chem.*, 278:3466-3473). In addition, International Patent Publication No. WO 2009/135181 describes the addition of fucose analogs to culture medium during antibody production to inhibit incorporation of fucose into the carbohydrate on the antibody.

D. Linkers

[0273] In various embodiments of this disclosure, a trivalent and trispecific antibody construct described herein can comprise one or more linkers. In some embodiments, such one or more linkers are peptide (also referred to herein as "peptitic") linkers comprising or consisting of an amino acid sequence of about 1, 2, 3, 5, 10, 15, 20, 25, 30, 40, or about 50 consecutive amino acid residues in length. The one or more peptide linker of an antibody construct can comprise or consist of an amino acid sequence from 1 to about 50, from 2 to about 40, from 3 to about 30, or from 5 to about 25 consecutive amino acid residues in length.

[0274] Such peptide linkers can couple, or link, two or more polypeptide sequences and/or domains of an antibody construct to each other. In various embodiments, a linker herein can couple a first polypeptide chain, e.g., a heavy chain constant domain (C_{H1}), to an Fc polypeptide. Thus, a linker can be used to couple one domain of an antibody construct to another domain, from N- to C-terminus, e.g., a Fab domain to an Fc domain, e.g., a $linker^{Fab\text{-}Fc}$, a V_H domain to a V_L domain, e.g., $linker^{scFv}$, and so forth. In embodiments in which an antibody construct comprises two scFv domains (e.g., an $scFv^2$ antibody construct), both such scFv domains of an antibody construct can contain a $linker^{scFv}$ with identical amino acid sequence, and such antibody construct can be described as comprising a $linker^{scFv}$, instead of specifying that it contains a $linker^{scFv1}$ and a $linker^{scFv2}$. In other embodiments, however, in which the $linker^{scFv}$ of both scFv domains have different amino acid sequences, such antibody construct can be described as comprising a $linker^{scFv1}$ and a $linker^{scFv2}$.

[0275] In embodiments in which a linker couples, e.g., a heavy chain variable domain (V_H) to, e.g., a light chain variable domain (V_L), the linker can be of sufficient length to allow both domains to elicit their biological function. In addition to providing a spacing function, a linker herein (e.g., a peptide linker) can provide flexibility or rigidity suitable for properly orienting the one or more domains of an antibody construct, both within the antibody construct itself and between the antibody construct and its target(s).

[0276] Further, a linker herein (e.g., a peptide linker) can support (i) expression of a full-length fusion protein, e.g., a full-length polypeptide chain H1, L1, H2, and/or L2 etc. of an antibody construct, and (ii) provide increased stability of the purified protein both *in vitro* and *in vivo*, e.g., following administration to a subject in need thereof, such as a human. The one or more linkers used in antibody constructs herein are generally non-immunogenic or poorly immunogenic in mammalian subjects that a construct may be administered to. In certain embodiments, one or more of the linkers used in an antibody construct herein can comprise part or all of a human Ig hinge region, a stalk region of C-type lectins, a family of type II membrane proteins, or combinations thereof. In certain embodiments, one or more of the linker used in an antibody construct herein can comprise part or all of a human Ig hinge region, such as an IgG1 hinge region, such as a $linker^{Fab\text{-}Fc}$ or a $linker^{scFv\text{-}Fc}$.

[0277] In certain embodiments, each linker used in an antibody construct herein can comprise or consist of an amino acid sequence having a length of 2 to about 50 amino acids. In some embodiments, each linker used in an antibody construct herein can comprise or consist of an amino acid sequence having a length from about 3 to about 40 amino acids, from about 10 to about 50 amino acids, from about 2 to about 40 amino acids, from about 5 to about 30 amino acids, from about 5 to about 25 amino acids, from about 4 to about 30 amino acids, from about 10 to about 30 amino acids, or from about 15 to about 25 amino acids. In some embodiments, the one or more linkers of an antibody construct can each comprise an amino acid sequence comprising or consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive amino acids.

[0278] In certain embodiments, a linker (e.g., a $linker^{scFv}$, $linker^{Fab\text{-}scFv}$, $linker^{Fab\text{-}Fc}$, etc.) of an antibody construct herein comprises of consists of the amino acid sequence $(EAAAK)_n$ wherein n is an integer from 1 to 5 (SEQ ID NO: 60). In some embodiments, a linker comprises or consists of the sequence EAAAK (SEQ ID NO: 61). In some embodiments, a linker comprises or consists of the sequence EAAAKEAAAK (SEQ ID NO: 62). In some embodiments, a linker comprises a polyproline linker, e.g., having an amino acid sequence of PPP (SEQ ID NO: 63) or PPPP (SEQ ID NO: 64). In certain embodiments, a linker is a glycine (G)-proline (P) polypeptide linker, e.g., comprising or consisting of one or more of GPPPG (SEQ ID NO: 65), GGPPPGG (SEQ ID NO: 66), GPPPPG (SEQ ID NO: 67), or GGPPPPGG (SEQ ID NO: 68). In some embodiments, a linker

herein is a $(G_nS)_m$ linker, wherein n and m are independently integers from 1 to 5 (SEQ ID NO: 69). In certain embodiments, a linker comprises or consists of an amino acid sequence of $(G_3S)_n(G_4S)_1$ (SEQ ID NO: 70), $(G_3S)_1(G_4S)_n$ (SEQ ID NO: 71), $(G_3S)_n(G_4S)_n$ (SEQ ID NO: 72), wherein each n is an integer from 1 to 5, or $(G_4S)_x$ (SEQ ID NO: 119) wherein each x is an integer from 1 to 5. In certain embodiments, a linker herein is suitable for connecting two different domains of an antibody construct and comprises a sequence comprising glycine-serine linkers, for example, but not limited to, $(G_mS)_n$-GG (SEQ ID NO: 73), $(SG_n)_m$ (SEQ ID NO: 74), or $(SEG_n)_m$ (SEQ ID NO: 75), wherein m and n are independently integers from 1 to 20.

[0279] In some embodiments, an antibody construct described herein comprises any one or more of the linkers described herein. In some embodiments, an antibody construct comprises a plurality of linkers, e.g., 1, 2, 3, 4, or 5 linkers, which can include one or more $linker^{Fab\text{-}Fc}$, one or more $linker^{Fab\text{-}scFv}$, and/or one or more $linker^{scFv}$.

[0280] In certain embodiments, a $linker^{scFv}$ of an antibody construct herein comprises or consists of the amino acid sequence $(G_nS)_m$ linker, wherein n and m are independently integers from 1 to 5 (SEQ ID NO: 69). In such embodiments, n and m can both be 4, and thus the one or more $linker^{scFv}$ of an antibody construct can comprise or consist of the sequence $(G_4S)_4$ (SEQ ID NO: 59). In some embodiments, the one or more $linker^{scFv}$ of an antibody construct (e.g., a $linker^{scFv1}$ and a $linker^{scFv2}$) can comprise or consist of an amino acid sequence having about 80%, 90%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 59.

[0281] In some embodiments, an antibody construct of the present disclosure can comprise a $linker^{Fab\text{-}scFv}$ that couples the C-terminus of a Fab light chain to the N-terminus of an scFv domain. In some embodiments, such $linker^{Fab\text{-}scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 58 (GGGGSG), or a sequence having about 40%, 60%, or 80% sequence identity thereto.

[0282] In certain embodiments, one or more of the linkers that an antibody construct comprises can be an amino acid sequence obtained, derived, or designed from an antibody hinge region sequence. In some embodiments, such linker can have at least one cysteine capable of participating in at least one disulfide bond under physiological conditions or other standard peptide conditions (e.g., peptide purification conditions, conditions for peptide storage, etc.). In certain embodiments, a linker corresponding to, or similar to, an Ig hinge region peptide retains a cysteine that corresponds to the hinge cysteine disposed toward the amino (or N-) terminus of that hinge region.

In further embodiments, a linker is derived from an IgG1 hinge region and can be modified to remove any one or more of the cysteine residues. In some embodiments, a linker comprises an IgG1 hinge region that has one cysteine or two cysteines that correspond to wildtype hinge cysteine residues.

[0283] In certain embodiments, a linker of an antibody construct described herein can comprise an "altered wildtype Ig hinge region" or an "altered Ig hinge region". Such altered hinge regions can refer to (a) a wild type Ig hinge region with up to 30 percent amino acid changes (or up to 25 percent, 20 percent, 15 percent, 10 percent, or 5 percent amino acid substitutions, insertions or deletions), (b) a portion of a wild type Ig hinge region that is at least 10 amino acids (e.g., at least 12, 13, 14 or 15 amino acids) in length with up to 30 percent amino acid changes (or up to 25 percent, 20 percent, 15 percent, 10 percent, or 5 percent amino acid substitutions or deletions), (c) a portion of a wild type Ig hinge region that comprises the core hinge region, which portion can be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15, or at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids in length, or (d) a combination of any of (a)-(c). In certain embodiments, one or more cysteine residues in a wildtype Ig hinge region, such as an IgG1 hinge region comprising the upper and core regions, can be substituted by one or more other amino acid residues (e.g., one or more serine residues). An altered Ig hinge region can alternatively or additionally have a proline residue of a wildtype Ig hinge region, such as an IgG1 hinge region, substituted by another amino acid residue (e.g., a serine residue).

[0284] In some embodiments, an antibody construct of this disclosure comprises a linker comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 56. In some embodiments, an antibody construct comprises a linker comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 57. In some embodiments, an antibody construct comprises a linker comprising or consisting of an amino acid sequence having at least about 80%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 58.

[0285] In certain embodiments, a trivalent and trispecific Het-Fab antibody construct of this disclosure can comprise a first linker$^{Fab\text{-}Fc}$ that couples one Fab heavy chain to one Fc polypeptide and a second linker$^{Fab\text{-}Fc}$ that couples the other Fab heavy chain to the other Fc polypeptide. In some embodiments, the first linker$^{Fab\text{-}Fc}$ can comprise or consist of the amino acid sequence set

forth in SEQ ID NO: 56, and the second linker$^{Fab\text{-}Fc}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 120.

[0286] In various embodiments, an antibody construct of this disclosure comprises a linker that couples a Fab domain to a first Fc polypeptide, linker$^{Fab\text{-}Fc}$, wherein the linker$^{Fab\text{-}Fc}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 56, and another linker coupling a first or second scFv domain to a first or second Fc polypeptide, linker$^{scFv\text{-}Fc}$, wherein the linker$^{scFv\text{-}Fc}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 57.

E. Certain Embodiments of Antibody Constructs

[0287] The present disclosure describes trivalent and trispecific antibody constructs that can have different formats and/or geometries, e.g., spatial molecular structures. The trivalent and trispecific antibody constructs described herein have the ability to engage three different antigens, two different antigens on one or more cytotoxic effector cell(s) (e.g., T cell(s)) and DLL3 on a tumor cell, wherein each of these antigens is engaged in a monovalent manner.

[0288] In various embodiments, the present disclosure relates to trivalent and trispecific Het-Fab antibody constructs. Such Het-Fab antibody constructs can comprise three antigen binding domains, two of which are Fab domains. Since both Fab domains have binding specificities for different antigens, the two Fab domains comprise different heavy and light chain amino acid sequences (e.g., different sequences in the corresponding VH/VL pairs) they can be described as heterodimeric-Fabs or Het-Fabs (e.g., compared to Fabs that share heavy and light chains with identical amino acid sequences and target the same antigen, e.g., homodimeric-Fabs).

[0289] In certain embodiments, the trivalent and trispecific Het-Fab antibody constructs described herein are capable of binding DLL3 on a target cell (e.g., a tumor cell).

[0290] In various embodiments, described herein is a trivalent and trispecific Het-Fab antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) an scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of a Fab light chain. In some embodiments, the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0291] In some embodiments herein, a trivalent and trispecific Het-Fab antibody construct is capable of monovalently engaging the following three antigens: CD3, CD28 and DLL3.

[0292] In some of these embodiments, a trivalent and trispecific Het-Fab antibody construct comprises: (i) the first Fab domain, wherein the first Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) the second Fab domain, wherein the second Fab domain is capable of binding DLL3 on the tumor cell; (iii) the scFv domain, wherein the scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (iv) the dimeric Fc domain comprising the first Fc polypeptide and the second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0293] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure that is capable of binding CD3, CD28 and DLL3 comprises or consists of four polypeptide chains, two immunoglobulin heavy chains (e.g., H1 and H2) and two immunoglobulin light chains (e.g., L1 and L2). In some embodiments, the four polypeptide chains associate, either covalently and/or non-covalently, to form the trivalent and trispecific Het-Fab antibody construct.

[0294] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a heterodimeric Fc domain. In such embodiments, H1 and/or H2 can comprise one or more amino acid modifications that promote formation of the heterodimer H1-H2 compared to formation of respective homodimers H1-H1 or H2-H2. In some of these embodiments, and as further described herein, H1 and H2 each comprise a CH3 sequence, wherein each CH3 sequence comprises one or more amino acid substitutions, relative to a respective wildtype CH3 sequence, that promote heterodimer H1-H2 formation compared to homodimer formation.

[0295] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, the CH3 sequence of one of the Fc polypeptides comprises a set of amino acid substitutions selected from the group consisting of: L351Y_F405A_Y407V, T350V_L351Y_F405A_Y407V and T350V_L351Y_S400E_F405A_Y407V, and the CH3 sequence of the other Fc polypeptide comprises a set of amino acid substitutions selected from the group consisting of: T366L_K392M_T394W, T366L_K392L_T394W, T350V_T366L_K392L_T394W,

T350V_T366L_K392M_T394W and T350V_T366L_N390R_K392M_T394W, and wherein the numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system.

[0296] In some embodiments of a trivalent and trispecific Het-Fab antibody construct, H1, H2, L1 and/or L2 each comprise one or more amino acid modifications that promote correct heavy and light chain pairing to form the desired H1-L1 and H2-L2 heterodimer pairs, when compared to the formation of incorrectly paired heterodimers, e.g., H1L2 and H2L1. In some embodiments, H1, H2, L1 and/or L2 each comprise one or more amino acid substitutions in their CH1 and CL sequences, respectively, that promote correct heavy and light chain pairing, and relative to unmodified parental and/or wildtype CH1 and CL sequences.

[0297] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises one Fab domain comprising one or more of the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence of the heavy chain and one or more of the amino acid substitutions 121K, 124R and 178R in the CL sequence of the light chain, and another Fab domain comprising one or more of the amino acid substitutions 125R and 188K in the CH1 sequence of the heavy chain and one or more of the amino acid substitutions 122D, 129T, 176E and 178E in the CL sequence of the light chain, wherein these amino acid substitutions are according to the Kabat numbering scheme and relative to unmodified parental and/or wildtype CH1 and CL sequences and promote correct heavy and light chain pairing for the formation of the desired H1-L1 and H2-L2 heterodimers, compared to formation of the incorrectly paired heterodimers H1L2 and H2L1.

[0298] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 Fab heavy chain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer; (iii) a first light chain (L1), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein L1 and the anti-CD3 Fab heavy chain of H1 associate to

form an anti-CD3 Fab domain; and (iv) a second light chain (L2), comprising an anti-DLL3 Fab light chain, and wherein L2 and the anti-DLL3 Fab heavy chain of H2 associate to form an anti-DLL3 Fab domain.

[0299] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 Fab heavy chain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer; (iii) a first light chain (L1), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having the sequence set forth in SEQ ID NO: 13, and wherein L1 and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain; and (iv) a second light chain (L2), comprising an anti-DLL3 Fab light chain, and wherein L2 and the anti-DLL3 Fab heavy chain of H2 associate to form an anti-DLL3 Fab domain.

[0300] In certain embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first Fab domain that binds CD3 comprising a first Fab heavy chain comprising a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 49, a HCDR2 sequence as set forth in SEQ ID NO: 51, and a HCDR3 sequence as set forth in SEQ ID NO: 52, and a first Fab light chain comprising a V_L domain comprising a LCDR1 sequence as set forth in SEQ ID NO: 53, a LCDR2 sequence as set forth in SEQ ID NO: 54, and a LCDR3 sequence as set forth in SEQ ID NO: 55, as identified according to IMGT numbering; (ii) an scFv domain that binds CD28 comprising a VL sequence comprising a LCDR1 sequence as set forth in SEQ ID NO: 41, a LCDR2 sequence as set forth in SEQ ID NO: 44, and a LCDR3 sequence as set forth in SEQ ID NO: 48, and a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 28, a HCDR2 sequence as set forth in SEQ ID NO: 31, and a HCDR3 sequence as set forth in SEQ ID NO: 35, as identified according to Kabat numbering; (iii) a second Fab domain that binds DLL3 comprising a second Fab heavy chain comprising a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 90, a HCDR2 sequence as set forth in SEQ ID NO: 91, and a HCDR3 sequence as set forth in SEQ ID NO: 92, and a second Fab light chain comprising a VL domain comprising a LCDR1 sequence as set forth in SEQ ID NO: 93, a LCDR2 sequence as set forth in

SEQ ID NO: 94, and a LCDR3 sequence as set forth in SEQ ID NO: 95, as identified according to Kabat numbering; and (iv) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein (1) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via the C-terminus of the first Fab heavy chain, (2) the scFv domain is coupled to the C-terminus of the first Fab light chain, and (3) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide via the C-terminus of the second Fab heavy chain.

[0301] In certain embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises: (i) a first Fab domain that binds CD3 comprising a first Fab heavy chain comprising a VH sequence comprising the amino acid sequence set forth in SEQ ID NO: 2, coupled to a first CH1 sequence, and a first Fab light chain comprising a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 5, coupled to a first CL sequence; (ii) an scFv domain that binds CD28 comprising a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 16, coupled to a VH sequence comprising the amino acid sequence set forth in SEQ ID NO: 15; (iii) a second Fab domain that binds DLL3 comprising a second Fab heavy chain comprising a VH sequence comprising the amino acid sequence set forth in SEQ ID NO: 99, coupled to a second CH1 sequence, and a second Fab light chain comprising a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 100, coupled to a first CL sequence; and (iv) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein (1) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via the C-terminus of the first Fab heavy chain, (2) the scFv domain is coupled to the C-terminus of the first Fab light chain, and (3) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide via the C-terminus of the second Fab heavy chain.

[0302] In various embodiments, the present disclosure relates to trivalent and trispecific $scFv^2$ antibody constructs. Such $scFv^2$ antibody constructs can comprise three antigen binding domains, two of which are scFv domains, thus the descriptor "$scFv^2$".

[0303] In certain embodiments, the trivalent and trispecific $scFv^2$ antibody constructs described herein are capable of binding DLL3 on a target cell (e.g., a tumor cell).

[0304] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) a Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain

comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0305] In some embodiments herein, a trivalent and trispecific $scFv^2$ antibody construct is capable of monovalently engaging the following three antigens: CD3, CD28 and DLL3.

[0306] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) the Fab domain, wherein the Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) the first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) the second scFv domain, wherein the second scFv domain is capable of binding DLL3 on a tumor cell; and (iv) the dimeric Fc domain comprising the first Fc polypeptide and the second Fc polypeptide, wherein: (a) the Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain N-terminus of the second Fc polypeptide, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

[0307] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure that is capable of binding CD3, CD28 and DLL3 comprises or consists of three polypeptide chains, two immunoglobulin heavy chains (e.g., H1 and H2) and one immunoglobulin light chain (e.g., L). In some embodiments, the three polypeptide chains associate, either covalently and/or non-covalently, to form the trivalent and trispecific $scFv^2$ antibody construct.

[0308] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises a heterodimeric Fc domain. In such embodiments, H1 and/or H2 can comprise one or more amino acid modifications that promote formation of the heterodimer H1-H2 compared to formation of respective homodimers H1-H1 or H2-H2. In some of these embodiments, and as further described herein, H1 and H2 each comprise a CH3 sequence, wherein each CH3 sequence comprises one or more amino acid substitutions, relative to a respective wildtype CH3 sequence, that promote heterodimer H1-H2 formation compared to homodimer formation.

[0309] In some embodiments of a trivalent and trispecific $scFv^2$ antibody construct, the CH3 sequence of one of the Fc polypeptides comprises a set of amino acid substitutions selected from the group consisting of: L351Y_F405A_Y407V, T350V_L351Y_F405A_Y407V and

T350V_L351Y_S400E_F405A_Y407V, and the CH3 sequence of the other Fc polypeptide comprises a set of amino acid substitutions selected from the group consisting of: T366L_K392M_T394W, T366L_K392L_T394W, T350V_T366L_K392L_T394W, T350V_T366L_K392M_T394W and T350V_T366L_N390R_K392M_T394W, and wherein the numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system.

[0310] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 scFv domain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer H1-H2; and (iii) a light chain (L), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein L and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain.

[0311] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 scFv domain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having the sequence set forth in SEQ ID NO: 12, and wherein H1 and H2 associate to form a heterodimer H1-H2; and (iii) a light chain (L), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having the sequence set forth in SEQ ID NO: 13, and wherein L and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain.

[0312] In certain embodiments, described herein is an antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding a TAA on a tumor cell, wherein the third binding domain comprises or consists of a second Fab

domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain. In some embodiments, the third binding domain comprises or consists of a second Fab domain. In other embodiments, the third binding domain comprises or consists of a second scFv domain. The dimeric Fc domain can be a heterodimeric Fc domain, as further described herein. **FIGS. 1G** (e.g., third binding domain is a second scFv domain) and **1H** (e.g., third binding domain is a second Fab domain) show the format and geometry of such trivalent and trispecific antibody constructs, according to certain embodiments of this disclosure.

[0313] In some embodiments, such trivalent and trispecific antibody construct comprises a light chain having the following domain structure, from N- to C-terminus, of VL-CL-scFv, wherein the scFv domain can comprise, from N- to C-terminus, a VH coupled to a VL sequence, or a VL sequence coupled to a VH sequence. Thus, in certain embodiments, such Fab light chain has the domain structure, from N- to C-terminus, of $(VL\text{-}CL)_{Fab}$-$(VL\text{-}VH)_{scFv}$. In other embodiments, the light chain has the domain structure, from N- to C-terminus, of $(VL\text{-}CL)_{Fab}$-$(VH\text{-}VL)_{scFv}$.

[0314] In certain embodiments, described herein is a trivalent and trispecific antibody construct comprising a light chain having the following domain structure, from N- to C-terminus, of $(VL\text{-}CL)_{Fab}$-$(VL\text{-}VH)_{scFv}$. In some embodiments, such light chain can further comprise one or more linkers, as further described herein. In some embodiments, the light chain comprises a peptide $linker^{Fab\text{-}scFv}$ between the Fab portion and the scFv portion which couples the Fab portion to the scFv portion to yield the light chain with the domain structure $(VL\text{-}CL)_{Fab}$-$Linker^{Fab\text{-}scFv}$-$(VL\text{-}VH)_{scFv}$. The $linker^{Fab\text{-}scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 58. As further described herein, the scFv domain of the light chain can also comprise a $linker^{scFv}$ that coupled the VL sequence to the VH sequence. Thus, in certain embodiments, the trivalent and trispecific antibody construct comprises a light chain with the domain structure $(VL\text{-}CL)_{Fab}$-$Linker^{Fab\text{-}scFv}$-$(VL\text{-}Linker^{scFv}\text{-}VH)_{scFv}$. The $linker^{scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 59.

[0315] In some embodiments, described herein is a trivalent and trispecific antibody construct comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell and comprising a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO:

49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell and comprising a V_H domain comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system.; (iii) a third binding domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain. In some embodiments, the third binding domain is a second Fab domain. In other embodiments, the third binding domain is a second scFv domain.

[0316] In some embodiments, described herein is a trivalent and trispecific antibody construct comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell and comprising a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell and comprising a V_H domain comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system.; (iii) a third binding domain capable of binding DLL3 on a tumor

cell and comprising a V_H domain comprising a HCDR1 having the sequence HYYVH (SEQ ID NO: 90), a HCDR2 having the sequence IIDPGGGTTSYAQKFLG (SEQ ID NO: 91) and a HCDR3 having the sequence GERVTGNYFYYGMDV (SEQ ID NO: 92), and a VL domain comprising a LCDR1 having the sequence VANQGISNYLV (SEQ ID NO: 93), a LCDR2 having the sequence SVNSLYS (SEQ ID NO: 94) and a LCDR3 having the sequence LQHDSYPYT (SEQ ID NO: 95), according to the Kabat numbering system; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain. In some embodiments, the third binding domain is a second Fab domain. In other embodiments, the third binding domain is a second scFv domain.

[0317] In certain embodiments, described herein is a trivalent and trispecific antibody construct comprising a light chain comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 13. In some embodiments, the light chain of a trivalent and trispecific antibody construct can comprise an amino acid sequence having the amino acid sequence set forth in SEQ ID NO: 13.

[0318] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first immunoglobulin (Ig) heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 108.

[0319] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID

NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 108.

[0320] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first immunoglobulin (Ig) heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 115, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 116.

[0321] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 115, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 116.

[0322] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first immunoglobulin (Ig) heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 117, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 118.

[0323] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 117, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in

SEQ ID NO: 106, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 118.

[0324] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 109, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 110, and a light chain (L) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 111.

[0325] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 109, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 110, and a light chain (L) comprising the amino acid sequence set forth in SEQ ID NO: 111.

[0326] In some embodiments, a trivalent and trispecific $scFv^2$ antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 112, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 113, and a light chain (L) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 114.

[0327] In some embodiments, a trivalent and trispecific Het-Fab antibody construct of the present disclosure comprises a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 112, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 113, and a light chain (L) comprising the amino acid sequence set forth in SEQ ID NO: 114.

[0328] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure can further comprise a C-terminal lysine (K) residue in the sequence of one or both of its heavy chain(s).

F. Certain Properties of Costimulatory DLL3-Targeting Antibody Constructs

[0329] The trivalent and trispecific antibody constructs of the present disclosure (e.g., Het-Fab as well as $scFv^2$ antibody constructs as described herein) can have several specific properties due to their specific combination of format, geometry and antigen affinities. In some embodiments, a trivalent and trispecific antibody construct herein comprises a light chain comprising a Fab portion targeting CD3 and an scFv portion targeting CD28 and thus can possess certain properties that can be unique to a construct with that specified light chain format and geometry.

[0330] In some embodiments, the engagement of a herein disclosed trivalent and trispecific antibody construct with two different antigens, CD3 and CD28, on one or more T cell(s) and with DLL3 on a tumor cell, e.g., in a tumor (micro)environment, can be – at least temporarily – simultaneous, thereby establishing a TCR-independent immune synapse, and directing T cell-mediated cytotoxic activity to a tumor environment which contains tumor cells expressing DLL3. In various embodiments, and as further described herein, a trivalent and trispecific antibody construct may cause a significantly reduced immune cell (e.g., T cell) activation in the absence of DLL3, e.g., when the immune synapse cannot be fully formed due to an absence of DLL3. Such property can be advantageous over those of conventional constructs as it allows, for example, activation of a subject's immune system in a more TAA (e.g., DLL3)-dependent manner, and thus may cause less off-target effects in the subject compared to conventional constructs or antibodies that act in a less DLL3-dependent manner.

[0331] In some embodiments, the antibody constructs described herein can exhibit an enhanced anti-tumor activity in tumors that have a relatively low T cell infiltration when compared to conventional constructs that target only one immune cell antigen (e.g., CD3 or CD28, but not both), at least in part, due to their co-stimulatory activity by being able to engage both CD3 and CD28 on the same immune cell (e.g., T cell).

[0332] In various embodiments, binding of a trivalent and trispecific antibody construct of the present disclosure to CD3 comprises or consists of binding of such construct to CD3e, i.e., the epsilon subunit of the T cell receptor complex.

[0333] Generally, and as it may be appreciated by a person of ordinary skill in the art, natural T cell activation can require both TCR (e.g., involving the CD3 complex) and CD28 co-stimulation. The antibody constructs of the present disclosure have been specifically designed, e.g., through their format, geometry and antigen affinities, to provide both CD3 and CD28 co-stimulation. Furthermore, and according to various embodiments of the present disclosure, the anti-CD3 and anti-CD28 binding affinities of the anti-CD3 and anti-CD28 binding domains (e.g., scFv(s), Fab(s), etc.) of antibody constructs described herein, in combination with their relative positioning within the construct, have been specifically selected and engineered to generate signals for immune cell (e.g., T cell) activation with appropriate strength to reduce both T cell anergy on the one side and T cell overreaction and dysfunction on the other side of the spectrum, and to provide an improved ratio of anti-tumor, on-target to healthy tissue, off-target activity. In various embodiments, such improved on-target-to-off-target activities can be achieved by optimizing the format and geometry of the antibody constructs in a way such that engagement of all three antigens, namely CD3, CD28 and DLL3, at the same time allows for the most potent anti-tumor activity, compared to instances in which, e.g., only one of the immune cell antigens CD3 and/or CD28 are engaged and bound by the construct.

[0334] As further described herein, an antibody construct of the present disclosure can be trivalent and trispecific and bind each antigen, e.g., CD3, CD28, DLL3, monovalently via one of its three antigen binding domains.

[0335] In some embodiments, a trivalent and trispecific antibody construct has a melting temperature at Tm1, Tm2, and/or Tm3 that is within 10 °C, 5 °C, 2 °C, or within 1 °C degree of that of a bivalent and monospecific (e.g., monospecific for either CD3, CD28 or DLL3) IgG1 monoclonal antibody, as determined using, e.g., differential scanning calorimetry (DSC) or differential scanning fluorometry (DSF). In some embodiments, such bivalent and monospecific IgG1 monoclonal antibody can be any conventional IgG1 antibody capable of binding a specific antigen. In certain embodiments, such monoclonal, monospecific and bivalent IgG1 reference antibody is one that comprises two Fab domains as described herein in the context of trispecific antibody constructs. In some embodiments, the bivalent and monospecific IgG1 monoclonal antibody comprises two anti-CD3 Fab domains as described herein, two anti-CD28 Fab domains as described herein, or two anti-DLL3 Fab domains as described herein.

[0336] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure binds a cytotoxic effector cell (e.g., a T cell) that expresses CD3 and CD28 with an affinity (i.e., a K_D value) from about 5 nM to about 100 pM, from about 1 nM to about 100 pM, from about 1 nM to about 250 pM, from about 1 nM to about 500 pM, or from about 1 nM to about 750 pM. In certain embodiments, such antibody construct binds the cytotoxic effector cell that expresses CD3 and CD28 with an affinity that is about 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or about 200-fold higher, and/or from about 2-fold to about 200-fold, from about 2-fold to about 150-fold, from about 2-fold to about 100-fold, or from about 20-fold to about 200-fold higher than that of a corresponding bispecific anti-CD3xDLL3 and/or anti-CD28xDLL3 antibody construct. As described herein, K_D values can be determined using methods known in the art, e.g., surface plasmon resonance (SPR).

[0337] In some embodiments, using the engineered anti-CD28 binding domains with reduced CD28 affinity, e.g., compared to the parental huTN228 paratope, can result in trivalent and trispecific antibody constructs that may induce less CD28-mediated toxicities.

[0338] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure exhibits an IC_{50} value from about 50 pM to about 0.01 pM, from about 25 pM to about 0.01 pM, from about from about 10 pM to about 0.05 pM, from about 10 pM to about 0.1 pM, from about 10 pM to about 1 pM, from about 5 pM to about 1 pM for killing TAA-expressing tumor cells that express at least about 100,000 DLL3/cell by TDCC in the presence of the cytotoxic effector cell and using an E:T ratio of 2:1 and an incubation period of 72 hours. In some of such embodiments, the antibody construct achieves a maximum killing of DLL3-expressing tumor cells of at least about 60%, 65%, 70%, 75%, or 80%, 85%, or 90%, 100%, or from about 60% to about 100%, from about 70% to about 90%, or from about 75% to about 85%.

[0339] Furthermore, in various embodiments, the trivalent and trispecific antibody constructs of the present disclosure may provide a (e.g., strictly) target cell (e.g., tumor cell) dependent cytotoxicity profile, e.g., as shown herein when cytokine release is significantly reduced in the presence of only isolated T cells compared to conditions in which the T cells are in co-culture with DLL3-expressing tumor cells.

[0340] In some embodiments, the trivalent and trispecific antibody constructs disclosed herein can have a thermal stability when measured at 40 °C and over a time period of about 2, 3, 5, 7, 10, or 14 days of at least about 90%, 95%, 97%, 98% or 99%, i.e., 90%, 95%, 97%, 98% or 99% of intact

construct is when measured using, e.g., size-exclusion chromatography, or other methods known in the art. In certain embodiments, such constructs can comprise one Fab domain capable of binding CD3, and two scFv domains, wherein one such scFv domain is capable of binding either CD28, and one scFv domain is capable of binding DLL3. In other embodiments, such constructs can comprise two Fab domains, one of which is capable of binding CD3 and the other is capable of binding DLL3, and one scFv domain capable of binding CD28. In some embodiments, the stability of a trispecific and trivalent antibody construct over a period of 14 days at 40 °C is at least about 97% or 98% percent (i.e., at least about 97% or 98% of construct is intact as measured, e.g., using size-exclusion chromatography). In some embodiments, the concentration of the construct in such stability experiment is about 1 mg/mL.

[0341] In various embodiments, and in order to address certain treatment challenges with known and conventional therapeutic modalities, e.g., durability of response and sustained T cell activation, the herein disclosed trivalent and trispecific co-stimulatory T cell engager antibody constructs have been rationally designed to, e.g., optimally engage CD3 and CD28 and redirect and enhance cytotoxic T cell responses to DLL3-expressing tumor cells while maintaining an improved safety profile when compared to conventional therapeutic modalities.

[0342] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure can mediate a sustained T cell-mediated cytotoxicity toward tumor cells over multiple cycles of T cell stimulation. In some embodiments, such trispecific antibody construct can be used to stimulate a T cell population about 4, 5 or 6 times over a 14-day period (e.g., first day of stimulation is on day 0) and achieve anti-tumor cell cytotoxicity of at least about 70%, 75%, 80%, 85%, or 90% and T cell viability of at least about 60%, 65%, 70%, 75%, or 80% as measured at certain time points over the 14-day period.

[0343] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure can exhibit conditional binding to CD28, i.e., binds CD28 with a significantly lower dissociation constant (i.e., higher affinity) when co-engaged with CD3, and compared to binding of CD28 alone, e.g., in the absence of CD3 (e.g., when using a CD3 knock-out cell line). In some embodiments, a trivalent and trispecific antibody construct of the present disclosure binds CD28 with an at least about 2-fold, 3-fold, 5-fold, 7-fold, 8-fold, or at least about 10-fold increased affinity (e.g., expressed in change in K_D) when co-engaged with CD3, and when compared to the binding of CD28 in the absence of CD3.

[0344] In some embodiments, a trivalent and trispecific antibody construct of the present disclosure has the ability to bind CD3 and CD28 on the same T cell, which can also be referred to herein as "cis" (on the same cell) T cell binding. Hence, in various embodiments, a trivalent and trispecific antibody construct that comprises a light chain comprising a Fab portion and an scFv portion does not bridge two T cells by, e.g., binding CD3 on one T cell and CD28 on another T cell, which can also be referred to herein as "trans" (on different cells) T cell binding.

III. SEQUENCE IDENTITY OF AMINO ACID AND NUCLEIC ACID SEQUENCES

[0345] As described in other parts of this disclosure, certain embodiments herein relate to an isolated polypeptide or a set of isolated polypeptides (e.g., polypeptide chains H1, H2, L1, L2 or H1, H2 and L etc., or portions, e.g., domains, thereof) of a trivalent and trispecific antibody construct, as well as to a polynucleotide or a set of polynucleotides encoding the one or more polypeptide chains of an antibody construct described herein. A polynucleotide in this context can encode all or part of an antibody construct, such as one or more polypeptide chains (e.g., H1, H2, L1, L2 etc.) of an antibody construct.

[0346] In some embodiments, described herein is a nucleic acid molecule or a set of nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form any of the trivalent and trispecific antibody constructs disclosed herein.

[0347] The terms "nucleic acid," "nucleic acid molecule" and "polynucleotide" are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogues thereof. Non-limiting examples of polynucleotides include a gene, a gene fragment, messenger RNA (mRNA), cDNA, recombinant polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

[0348] In some embodiments, described herein is a vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules that encode the one or more polypeptide chains (e.g., one or more of H1, H2, L1, L2 etc.) of an antibody construct disclosed herein.

[0349] A polynucleotide that "encodes" a given polypeptide is a polynucleotide that is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence can be located 3' to the coding sequence.

[0350] In certain embodiments, the present disclosure relates to polynucleotide and/or polypeptide sequences that are identical or substantially identical to another polynucleotide and/or polypeptide sequence. The term "identical," in the context of two or more polynucleotide or polypeptide sequences, refers to two or more sequences or subsequences that are the same, i.e., have the identical sequence of nucleotide or amino acid monomers (i.e., 100% sequence identity), respectively. Polypeptide or polynucleotide sequences herein share "sequence identity" if they have a percentage or a certain number of amino acid residues or nucleotides, respectively, that are at least about 80%, about 85%, about 90%, about 95%, about 97%, or at least about 99% identity over a specified region when compared and aligned for maximum correspondence over a comparison window or over a designated region as measured using one of the commonly used sequence comparison algorithms as known to persons of ordinary skill in the art or by manual alignment and visual inspection. This definition also refers to the complement of a test polynucleotide sequence. The identity can exist over a region that is at least about 50 amino acids or nucleotides in length, or over a region that is from about 75 to about 100 amino acids or nucleotides in length, or, where not specified, across the entire sequence of a polypeptide or polynucleotide. For sequence comparison, typically test sequences are compared to a designated reference sequence. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent (%) sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0351] The term "comparison window," as used herein, refers to a segment of a sequence comprising contiguous amino acid or nucleotide positions which can be from about 20 to about 1000 contiguous amino acid or nucleotide positions, for example from about 50 to about 600 or from about 100 to about 300 or from about 150 to about 200 contiguous amino acid or nucleotide positions over which a test sequence can be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Longer segments up to and including the full-length sequence may also be used as a comparison window in certain embodiments. Methods of alignment of sequences for comparison are known to those of ordinary skill in the art. Optimal alignment of sequences for comparison can be conducted, for example, by

the local homology algorithm of Smith & Waterman, 1970, *Adv. Appl. Math.,* 2:482c; by the homology alignment algorithm of Needleman & Wunsch, 1970, *J. Mol. Biol.,* 48:443; by the search for similarity method of Pearson & Lipman, 1988, *Proc. Natl. Acad. Sci. USA,* 85:2444, or by computerized implementations of these algorithms (for example, GAP, BESTFIT, FASTA or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI), or by manual alignment and visual inspection (see, for example, Ausubel *et al.,* Current Protocols in Molecular Biology, (1995 supplement), Cold Spring Harbor Laboratory Press). Examples of available algorithms suitable for determining percent sequence identity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.,* 1997, *Nuc. Acids Res.,* 25:3389-3402, and Altschul *et al.,* 1990, *J. Mol. Biol.,* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the website for the National Center for Biotechnology Information (NCBI).

[0352] Certain embodiments described herein relate to variant sequences (e.g., variant V_H domains, variant Fc polypeptides, etc.) that comprise one or more amino acid modification, e.g., one or more amino acid insertions, one or more amino acid deletions, and/or one or more amino acid substitutions, when compared to, e.g., a reference such as a wildtype sequence (e.g., a sequence that is disclosed in a known reference). In certain embodiments, the one or more amino acid modification of a variant sequence comprises one or more amino acid substitutions when compared to a reference such as a wildtype sequence. In some embodiments, the one or more amino acid substitutions are one or more non-conservative substitutions. In other embodiments, the one or more amino acid substitutions are one or more conservative substitutions.

[0353] In general, a "conservative substitution," as used herein, is considered to be a substitution of one amino acid with another amino acid having similar physical, chemical and/or structural properties. Common conservative substitutions are listed under Column 1 of **TABLE 4**.

TABLE 4: Conservative Amino Acid Substitutions

Original Amino Acid	**Column 1**	**Column 2**
Ala (A)	Gly, Ile, Leu, Met, Norleucine, Val	Cys, Gly, Ile, Leu, Met, Norleucine, Phe, Trp, Tyr, Val
Arg (R)	His, Lys	His, Lys
Asn (N)	Cys, Gln, Ser, Thr	Asp, Cys, Gln, Glu, Ser, Thr
Asp (D)	Glu	Asn, Cys, Gln, Glu, Ser, Thr
Cys (C)	Asn, Gln, Ser, Thr	Asn, Asp, Gln, Glu, Ser, Thr
Gln (Q)	Asn, Cys, Ser, Thr	Asn, Asp, Cys, Glu, Ser, Thr

Original Amino Acid	Column 1	Column 2
Glu (E)	Asp	Asp, Asn, Cys, Gln, Ser, Thr
Gly (G)	Pro	Ala, Ile, Leu, Met, Norleucine, Pro, Val
His (H)	Arg, Lys	Arg, Lys, Phe, Trp, Tyr
Ile (I)	Ala, Gly, Leu, Met, Norleucine, Val	Ala, Cys, Gly, Leu, Met, Norleucine, Phe, Trp, Tyr, Val
Leu (L)	Ala, Gly, Ile, Met, Norleucine, Val	Ala, Cys, Gly, Ile, Met, Norleucine, Phe, Trp, Tyr, Val
Lys (K)	Arg, His	Arg, His
Met (M)	Ala, Gly, Ile, Leu, Norleucine, Val	Ala, Cys, Gly, Ile, Leu, Norleucine, Phe, Trp, Tyr, Val
Phe (F)	Tyr, Trp	Ala, Cys, Gly, His, Ile, Leu, Met, Norleucine, Trp, Tyr, Val
Pro (P)	Gly	Gly
Ser (S)	Asn, Cys, Gln, Thr	Asp, Asn, Cys, Gln, Glu, Thr
Thr (T)	Asn, Cys, Gln, Ser	Asp, Asn, Cys, Gln, Glu, Ser
Trp (W)	Phe, Tyr	Ala, Cys, Gly, His, Ile, Leu, Met, Norleucine, Phe, Tyr, Val
Tyr (Y)	Phe, Trp	Ala, Cys, Gly, His, Ile, Leu, Met, Norleucine, Phe, Trp, Val
Val (V)	Ala, Gly, Ile, Leu, Met, Norleucine	Ala, Cys, Gly, Ile, Leu, Met, Norleucine, Phe, Trp, Tyr

[0354] One skilled in the art will appreciate that the main factors in determining what constitutes a conservative substitution are usually the size of the amino acid side chain and its physical/chemical properties, but that certain environments allow for substitution of a given amino acid with a broader range of amino acids than those listed in Column 1 of **TABLE 4**. These additional amino acids tend to either have similar properties to the amino acid being substituted but to vary more widely in size or be of similar size but vary more widely in physical/chemical properties. This broader range of conservative substitutions is listed under Column 2 of **TABLE 4**. The skilled person can readily ascertain the most appropriate group of substituents to select from in view of the particular protein environment in which the amino acid substitution is being made.

IV. PHARMACEUTICAL COMPOSITIONS

[0355] In certain embodiments, the present disclosure relates to pharmaceutical compositions that can comprise one or more of the trivalent and trispecific antibody constructs described herein. In

various embodiments, a pharmaceutical composition herein can further comprise a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, or other materials well known to those skilled in the art. Such materials are generally non-toxic and do not interfere with the efficacy of the active ingredient (i.e., antibody construct). The precise nature of a carrier or other material can depend on the route of administration. Hence, a pharmaceutical composition herein can be formulated for various used and administration routes, e.g., for oral, intravenous, cutaneous, subcutaneous, nasal, intramuscular, or intraperitoneal administration routes.

[0356] A pharmaceutical composition for oral administration can be in tablet, capsule, powder, or liquid form. A tablet can include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil, or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol can be included.

[0357] For intravenous, cutaneous, or subcutaneous injection, or injection at the site of affliction (e.g., at a tumor site), the active ingredient (i.e., antibody construct) can be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives can be included, as required.

[0358] For antibody constructs according to the present disclosure that are administered to a subject, administration is preferably in a “therapeutically effective amount” that is sufficient to show benefit to the individual, as further described herein. The actual amount administered, and rate and time-course of administration, can depend on the nature and severity of the disease (e.g., cancer) being treated. Prescription of treatment, e.g., decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

[0359] In some embodiments, a pharmaceutical composition can comprise a second active ingredient (e.g., another protein or small molecule) in addition to an antibody construct described herein.

[0360] Hence, also described herein is a pharmaceutical composition comprising any one or more of the trivalent and trispecific antibody construct(s) disclosed herein, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

V. KITS

[0361] The present disclosure also describes kits comprising one or more of the trivalent and trispecific antibody constructs described herein, or a pharmaceutical composition as described herein and that comprises such antibody construct(s), as well as instructions for use. Thus, in certain embodiments, described herein are kits comprising vectors for expressing an antibody construct described herein and instructions for use. In certain embodiments, described herein are kits comprising host cells comprising a vector for expressing an antibody construct and instructions for use. In some embodiments, the present disclosure relates to kits comprising a purified antibody construct and instructions for use. The purified antibody construct can be lyophilized or provided in a dry form, such as a powder or granules, and the kit can additionally contain a suitable solvent for reconstitution of the lyophilized or dried component(s).

[0362] A kit can further comprise a container and a label and/or package insert on or associated with the container. The label or package insert contains instructions customarily included in commercial packages of therapeutic products, providing information or instructions about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products (e.g., an antibody construct described herein). The label or package insert can further include a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, for use or sale for human or animal administration. The container can hold a composition comprising an antibody construct of this disclosure. In some embodiments, the container can have a sterile access port. For example, the container can be an intravenous solution bag or a vial having a stopper that can be pierced by a hypodermic injection needle.

[0363] In addition to the container containing a composition comprising an antibody construct, the kit can further comprise one or more additional containers comprising other components of the kit. For example, a pharmaceutically acceptable buffer (such as bacteriostatic water for injection)

(BWFI), phosphate-buffered saline, Ringer's solution, or dextrose solution), or other buffers or diluents can be included in such kit.

[0364] Suitable containers can include, for example, bottles, vials, syringes, intravenous solution bags, and the like. The containers can be formed from a variety of materials such as glass or plastic. If appropriate, one or more components (e.g., an antibody construct) of the kit can be lyophilized or provided in a dry form, such as a powder or granules, and the kit can additionally contain a suitable solvent for reconstitution of the lyophilized or dried component(s).

[0365] A kit herein can further include other materials desirable from a commercial or user standpoint, such as filters, needles, and syringes.

VI. METHODS

[0366] Further described herein are methods of producing and using the trivalent and trispecific antibody constructs of the present disclosure.

A. Methods of Producing an Antibody Construct

[0367] In some embodiments, the present disclosure relates to methods for preparing the trivalent and trispecific antibody constructs described herein. In various embodiments, an antibody construct of the present disclosure can be produced using standard recombinant methods known in the art (see, for example, U.S. Patent No. 4,816,567 and "*Antibodies: A Laboratory Manual*," 2nd Edition, Ed. Greenfield, Cold Spring Harbor Laboratory Press, New York, 2014).

[0368] For recombinant production of an antibody construct described herein, a polynucleotide or set of polynucleotides encoding the antibody construct can be generated and inserted into one or more vectors for further cloning and/or expression in a host cell. Polynucleotide(s) encoding the antibody construct can be produced by standard methods known in the art (see, for example, Ausubel *et al., Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994 & update, and "*Antibodies: A Laboratory Manual*," 2nd Edition, Ed. Greenfield, Cold Spring Harbor Laboratory Press, New York, 2014). As would be appreciated by one of skill in the art, the number of polynucleotides required for expression of the antibody construct may be dependent on the format and/or geometry of the antibody construct, including, for example, the number of polypeptide chains that the antibody construct is comprised of. For example, when an antibody construct comprises three polypeptide chains (e.g., H1, H2 and L), three polynucleotides each encoding one polypeptide chain can be used, and when an antibody construct comprises four polypeptide chains (e.g., H1, H2, L1 and L2), four polynucleotides each encoding one polypeptide

chain can be used. In embodiments in which two or more polynucleotides are used, such two or more polynucleotides can be incorporated into one vector or into more than one vector (e.g., two or three separate vectors).

[0369] Generally, for expression, the polynucleotide or set of polynucleotides encoding an antibody construct herein can be incorporated into an expression vector together with one or more regulatory elements, such as transcriptional elements, which can be used for efficient transcription of the polynucleotide(s). Examples of such regulatory elements include, but are not limited to, promoters, enhancers, terminators, and polyadenylation signals. One skilled in the art will appreciate that the choice of regulatory elements can be dependent on the host cell selected for expression of the polypeptides of the antibody construct and that such regulatory elements can be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes. The expression vector can optionally further contain heterologous nucleic acid sequences that facilitate expression or purification of the expressed protein. Examples include, but are not limited to, signal peptides and affinity tags such as metal-affinity tags, histidine tags, avidin/streptavidin encoding sequences, glutathione-S-transferase (GST) encoding sequences and biotin encoding sequences. The expression vector can be an extrachromosomal vector or an integrating vector. Hence, in some embodiments, the amino acid sequences of the polypeptide chains of an expressed antibody construct described herein, e.g., chains H1, H2, L1 and/or L2 etc., can comprise a signal peptide sequence. Such signal peptide sequences may vary depending on the expression system and conditions used for producing an antibody construct. Exemplary signal peptide sequences can comprise the amino acid sequence METDTLLLWVLLLWVPGSTG (SEQ ID NO: 79) or MRPTWAWWLFLVLLLALWAPARG (SEQ ID NO: 80), e.g., for H1, H2, etc., or MRPTWAWWLFLVLLLALWAPARG (SEQ ID NO: 80) or MGWSCIILFLVATATGVHS (SEQ ID NO: 81), e.g., for L1, L2, etc. In certain embodiments, one or more heavy chains (e.g., H1, H2, etc.) of an antibody construct described herein can comprise a C-terminal lysine residue following expression of the polypeptide chains inside the cell. In various embodiments, such C-terminal lysine residue may be enzymatically cleaved from the polypeptide chains prior to further processing (e.g., purification, formulation, etc.) and prior to use of the corresponding antibody construct, e.g., prior to administration of the construct to a subject in need thereof.

[0370] Certain embodiments for producing an antibody construct of the present disclosure relate to vectors (such as expression vectors) comprising one or more polynucleotides encoding at least

a portion of an antibody construct described herein. The polynucleotide(s) can be comprised by a single vector or by more than one vector. In some embodiments, the polynucleotides are comprised by a multi-cistronic vector. Expression vectors that can be used to express polynucleotides include but are not limited to pTT5 and pUC15 cells comprising vectors encoding an antibody construct.

[0371] Suitable host cells for cloning or expression of the antibody construct polypeptides include various prokaryotic or eukaryotic cells as known in the art. Eukaryotic host cells include, for example, mammalian cells, plant cells, insect cells and yeast cells (such as *Saccharomyces* or *Pichia* cells). Prokaryotic host cells include, for example, *E. coli, A. salmonicida* or *B. subtilis* cells. In certain embodiments, an antibody construct can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed or desired for the indented purpose of the antibody construct, as described for example in U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523, and in Charlton, *Methods in Molecular Biology,* Vol. 248, pp. 245-254, B.K.C. Lo, ed., Humana Press, Totowa, N.J., 2003. Eukaryotic microbes such as filamentous fungi or yeast are suitable expression host cells in certain embodiments, in particular fungi and yeast strains whose glycosylation pathways have been "humanized" resulting in the production of an antibody with a partially or fully human glycosylation pattern (see, for example, Gerngross, 2004, *Nat. Biotech.* 22:1409-1414, and Li *et al.*, 2006, *Nat. Biotech.* 24:210-215).

[0372] Suitable host cells for the expression of glycosylated antibody constructs are, in various embodiments, eukaryotic cells. For example, U.S. Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978 and 6,417,429 describe PLANTIBODIES™ technology for producing antibodies and portions thereof (e.g., scFv(s), Fab(s), etc.) in transgenic plants. Mammalian cell lines adapted to grow in suspension are particularly useful for the expression of antibody constructs described herein. Examples include, but are not limited to, monkey kidney CV1 line transformed by SV40 (COS-7), human embryonic kidney (HEK) line 293 or 293 cells (see, for example, Graham *et al.,* 1977, *J. Gen Virol.,* 36:59), baby hamster kidney cells (BHK), mouse sertoli TM4 cells (see, for example, Mather, 1980, *Biol Reprod,* 23:243-251); monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma (HeLa) cells, canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumour (MMT 060562), TRI cells (see, for example, Mather *et al.,* 1982, *Annals N.Y. Acad Sci,* 383:44-68), MRC 5 cells, FS4 cells, Chinese hamster ovary (CHO) cells (including $DHFR^-$ CHO cells, see Urlaub *et al.,* 1980, *Proc Natl Acad Sci USA*, 77:4216), and myeloma cell

lines (such as Y0, NS0 and Sp2/0). Exemplary mammalian host cell lines suitable for production of antibodies are reviewed in Yazaki & Wu, *Methods in Molecular Biology*, Vol. 248, pp. 255-268 (B.K.C. Lo, ed., Humana Press, Totowa, N.J., 2003).

[0373] In certain embodiments, the host cell used to produce a trivalent and trispecific antibody construct herein is a transient or stable higher eukaryotic cell line, such as a mammalian cell line. In some embodiments, the host cell is a mammalian HEK293T, CHO, HeLa, NS0 or COS cell. In some embodiments, the host cell is a stable cell line that allows for mature glycosylation of the antibody construct.

[0374] The host cells comprising the expression vector(s) encoding the antibody construct can be cultured using routine methods to produce the antibody construct. Alternatively, in some embodiments, host cells comprising the expression vector(s) encoding the antibody construct can be used therapeutically or prophylactically to deliver the antibody construct to a subject, or polynucleotides or expression vectors can be administered to a cell from a subject *ex vivo* and the cell then returned to the body of the subject.

[0375] In some embodiments, a host cell comprises (for example, has been transformed with) a vector comprising a polynucleotide that encodes a V_L and a V_H of a binding domain of an antibody construct described herein. In some embodiments, a host cell comprises (for example, has been transformed with) a vector comprising a polynucleotide that encodes a full-length polypeptide chain of an antibody construct described herein, e.g., H1, H2, L1 and/or L2 as described herein. In another example, a host cell comprises a first vector comprising a polynucleotide that encodes the V_L of a binding domain and a second vector comprising a polynucleotide that encodes the corresponding V_H of the binding domain. In various embodiments, the host cell is eukaryotic, for example, a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., Y0, NS0, Sp20 cell). In certain embodiments, the host cell is Expi293™ (Thermo Fisher, Waltham, MA). In certain embodiments, the host cell used herein is CHO-S cells (National Research Council Canada) or HEK293 cells.

[0376] Certain embodiments of the present disclosure relate to a method of making an antibody construct comprising culturing a host cell into which one or more polynucleotides encoding the antibody construct, or one or more expression vectors encoding the antibody construct, have been introduced, under conditions suitable for expression of the antibody construct. Such method can further comprise recovering the antibody construct from the host cell (or from host cell culture

medium). In some embodiments, such method can further comprise purifying the antibody construct.

[0377] Cell culture media that can be used include, but are not limited to, DMEM (Thermo Fisher, Waltham, MA), Opti-MEM™ (Thermo Fisher, Waltham, MA), Opti-MEM™ I Reduced Serum Medium (Thermo Fisher, Waltham, MA), RPMI-1640 medium, Expi293™ Expression Medium (Thermo Fisher, Waltham, MA), and FreeStyle CHO expression medium (Thermo Fisher Scientific, Waltham, MA). The cell culture medium can be supplemented with serum, e.g., fetal bovine serum (FBS), amino acids, e.g., L-glutamine, antibiotics, e.g., penicillin, and streptomycin, and/or antimycotics, e.g., amphotericin, or any other supplements routinely used in the to support cell culture.

[0378] In various embodiments, an antibody construct of the present disclosure is purified after expression. Proteins, such as an antibody construct of the present disclosure, can be isolated or purified in a variety of ways known to those skilled in the art (see, for example, *Protein Purification: Principles and Practice*, 3rd Ed., Scopes, Springer-Verlag, NY, 1994). Standard purification methods that can be used for the antibody constructs disclosed herein include chromatographic techniques, including ion exchange, hydrophobic interaction, affinity, sizing or gel filtration, and reverse-phase, carried out at atmospheric pressure or at high pressure using systems such as FPLC and HPLC. Additional purification methods include electrophoretic, immunological, precipitation, dialysis and chromatofocusing techniques. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, can also be used. As is well known in the art, a variety of natural proteins bind Fc domains and other structural elements of an antibody construct, and, in some embodiments, these proteins can be used for purification of an antibody construct. For example, the bacterial proteins A and G can bind to the Fc domain of some antibody constructs. Likewise, the bacterial protein L can bind to the Fab domain of some antibody constructs. Purification can also be enabled by a particular fusion partner. For example, antibody constructs can be purified using glutathione resin if a GST fusion is employed, Ni^{+2} affinity chromatography if a His-tag is employed or immobilized anti-flag antibody if a flag-tag is used. The degree of purification necessary may vary depending on the use of the antibody constructs. Hence, in some embodiments, no purification may be necessary.

[0379] In certain embodiments, an antibody construct of this disclosure is substantially pure. The term “substantially pure” (or “substantially purified”) when used in reference to an antibody

construct described herein, refers to an antibody construct as substantially or essentially free of components that normally accompany or interact with the protein as found in its naturally occurring environment, such as a native cell, or a host cell in the case of a recombinantly produced antibody construct. In certain embodiments, an antibody construct that is substantially pure is an antibody construct purified to have less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, or less than about 2% (by dry weight) of other contaminating protein species.

[0380] Assessment of antibody construct purity and/or homogeneity can be performed by any method known in the art, including, but not limited to, non-reducing/reducing CE-SDS, non-reducing/reducing SDS-PAGE, Ultra-high performance liquid chromatography-size exclusion chromatography (UPLC-SEC), High Performance Liquid Chromatography (HPLC), mass spectrometry, multi angle light scattering (MALS), and dynamic light scattering (DLS).

[0381] In certain embodiments, an antibody construct described herein can comprise one or more post-translational modifications. Such post-translational modifications can occur *in vivo*, or they be conducted *in vitro* after isolation of the antibody construct from the host cell.

[0382] Post-translational modifications can include various modifications as are known in the art (see, for example, *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; *Post-Translational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12, 1983; Seifter *et al.*, 1990, *Meth. Enzymol.*, 182:626-646, and Rattan *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 663:48-62). In those embodiments in which an antibody construct comprises one or more post-translational modifications, the antibody construct can comprise the same type of modification at one or several sites (e.g., amino acid residues), or it can comprise different modifications at different sites.

[0383] Examples of post-translational modifications can include glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, formylation, oxidation, reduction, proteolytic cleavage or specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease or $NaBH_4$.

[0384] Other examples of post-translational modifications can include, for example, addition or removal of N-linked or O-linked carbohydrate chains, chemical modifications of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, and addition or deletion of an N-terminal methionine residue

resulting from prokaryotic host cell expression. Post-translational modifications can also include modification with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein. Examples of suitable enzyme labels include, but are not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase and acetylcholinesterase. Examples of suitable prosthetic group complexes include, but are not limited to, streptavidin/biotin and avidin/biotin. Examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin. An example of a luminescent material is luminol, examples of bioluminescent materials include luciferase, luciferin and aequorin, and examples of suitable radioactive materials include iodine, carbon, sulfur, tritium, indium, technetium, thallium, gallium, palladium, molybdenum, xenon, and fluorine.

[0385] Additional examples of post-translational modifications can include acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, pegylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0386] In some embodiments, described herein is a method of producing a trivalent and trispecific antibody construct of the present disclosure, the method comprising: (a) obtaining a host cell culture comprising at least one host cell comprising one or more nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture. In some embodiments, such method can further comprise, subsequent to step (b), purifying the antibody construct.

B. Methods of Using an Antibody Construct of the Present Disclosure

[0387] In certain embodiments, the present disclosure relates to methods of using a trivalent and trispecific antibody construct of the present disclosure. In some embodiments, described herein

are methods of using an antibody construct described herein for the treatment of a disease or condition in a subject in need thereof.

[0388] Such method can comprise administering a trivalent and trispecific antibody construct, or a pharmaceutical composition comprising such antibody construct, to a subject in need thereof. In certain embodiments, the subject is a mammal. In some embodiments, the subject is a human.

[0389] In some embodiments, the present disclosure relates to a method of treating a cancer in a subject in need thereof, the method comprising administering to the subject a trivalent and trispecific antibody construct of the present disclosure, or a pharmaceutical composition comprising such antibody construct. Cancers that can be treated using the methods and antibody constructs disclosed herein can include, but are not limited to, hematologic neoplasms (including leukemias, myelomas and lymphomas), carcinomas (including adenocarcinomas and squamous cell carcinomas), melanomas and sarcomas. Carcinomas and sarcomas are also frequently referred to as “solid tumors”. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is leukemia. In certain embodiments, the cancer is lymphoma. In certain embodiments, the cancer is a DLL3-positive (or DLL3-expressing) cancer.

[0390] When used in a method described herein, an antibody construct of this disclosure can exert either a cytotoxic or cytostatic effect that can result in one or more of a reduction in the size of a tumor, the slowing or prevention of an increase in the size of a tumor, an increase in the disease-free survival time between the disappearance or removal of a tumor and its reappearance, prevention of an initial or subsequent occurrence of a tumor (e.g., metastasis), an increase in the time to progression, reduction of one or more adverse symptom(s) associated with a tumor, an increase in the overall survival time of a subject having a tumor, or a combination of the above.

[0391] The methods described herein can comprise administering a trivalent and trispecific antibody construct to a subject in need thereof. An antibody construct can be administered to a subject by any appropriate route of administration. As will be appreciated by the person of skill in the art, the route and/or mode of administration can vary depending upon the desired therapeutic results. In various embodiments, antibody constructs of this disclosure can be administered by systemic administration or local administration. Local administration can be at the site of a tumor or into a tumor draining lymph node. Generally, the antibody constructs can be administered by parenteral administration, for example, by intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, or spinal administration, such as by injection or infusion.

[0392] A treatment (e.g., of a cancer in a subject) can be achieved by administration of a therapeutically effective amount of a trivalent and trispecific antibody construct to a subject in need thereof. A "therapeutically effective amount," as used herein, generally refers to an amount of an antibody construct described herein that is effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount can vary according to factors such as the disease state, age, sex, and weight of the subject. A therapeutically effective amount is also one in which any potential toxic or detrimental effects of the antibody constructs are outweighed by the therapeutically beneficial effects. "Sufficient amount" generally refers to an amount sufficient to produce a desired effect, e.g., an amount sufficient to generate an anti-tumor immune response to a target (e.g., tumor) cell or tissue, e.g., by engaging an immune cell (e.g., T cell) using a trivalent and trispecific antibody construct described herein.

[0393] A suitable dosage of a trivalent and trispecific antibody construct described herein can be determined by a skilled medical practitioner. The selected dosage level may depend upon a variety of pharmacokinetic factors including the activity (e.g., antigen affinity(ies)) of the particular antibody construct employed, the route of administration, the time of administration, the rate of excretion of the construct, the duration of the treatment, other drugs, compounds and/or materials used in combination with the antibody construct, e.g., anti-cancer agents, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors well known in the medical arts.

[0394] In some embodiments, a method of treating a disease (e.g., a cancer) in a subject comprises administering a second active ingredient (e.g., another protein or small molecule) in addition to an antibody construct described herein. Such second active ingredient can be administered simultaneously or sequentially with an antibody construct dependent upon the condition to be treated.

[0395] In some embodiments, the present disclosure relates to a method of eliciting an anti-tumor immune response in a cell population comprising immune cells expressing CD3 and CD28 and tumor cells expressing DLL3, the method comprising contacting the cell population with an effective amount of a trivalent and trispecific antibody construct of the present disclosure. In some embodiments, such trivalent and trispecific antibody construct binds CD3 and CD28 on one or more cytotoxic effector cell, such as an immune cell, and DLL3 on a tumor cell, thereby forming a TCR-independent immune synapse, and the antibody construct is either an $scFv^2$ antibody

construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a first scFv domain capable of binding CD28 on an immune cell, (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, or the antibody construct is a Het-Fab antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on an immune cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0396] In some embodiments, the present disclosure relates to a method of inhibiting the proliferation of tumor cells expressing DLL3 in a cell population comprising the tumor cells and immune cells expressing CD3 and CD28, the method comprising contacting the cell population with an effective amount of a trivalent and trispecific antibody construct of the present disclosure. In some embodiments, such trivalent and trispecific antibody construct binds CD3 and CD28 on one or more immune cell(s) and DLL3 on a tumor cell, thereby forming a TCR-independent immune synapse, and the antibody construct is either an $scFv^2$ antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a first scFv domain capable of binding CD28 on an immune cell, (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, or the antibody construct is a Het-Fab antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on an immune cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide

and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0397] In some embodiments, the present disclosure relates to a method of killing tumor cells expressing DLL3, such method comprising contacting a cell population comprising the tumor cells and immune cells expressing CD3 and CD28 with an effective amount of a trivalent and trispecific antibody construct of the present disclosure. In various embodiments, such trivalent and trispecific antibody construct binds CD3 and CD28 on one or more immune cell(s) and DLL3 on a tumor cell, thereby forming a TCR-independent immune synapse, and the antibody construct is either an $scFv^2$ antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a first scFv domain capable of binding CD28 on an immune cell, (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, or the antibody construct is a Het-Fab antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on an immune cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0398] In any of the methods described herein, the immune cell is a T cell, and an immune cell population can comprise or consist of one or more type(s) of T cells.

[0399] As described herein, in various embodiments, an antibody construct herein can be administered to a subject in need thereof, for example, a subject having cancer, in order to modulate an immune response in the subject. The immune response that can be modulated using an antibody construct of this disclosure can be an anti-tumor immune response in the subject, e.g., in various embodiments, such modulated immune response can occur locally at a tumor site. Thus,

in certain embodiments, an antibody construct described herein can initiate and/or upregulate a local immune response, e.g., an anti-tumor response of a subject's immune system in order to elicit a localized cytotoxic effect against the tumor at the tumor site.

[0400] In various embodiments, an antibody construct described herein, e.g., a trivalent and trispecific antibody construct capable of monovalent binding of CD3 (e.g., via one Fab domain), monovalent binding of CD28 (e.g., via one scFv domain), and monovalent binding of DLL3 (e.g., via an scFv or Fab domain), can have a broader therapeutic window, compared to comparable conventional molecules, and can allow administration of higher doses of the herein described constructs, leading potentially to increased anti-tumor effects without inducing, or inducing lower grades of, side effects and/or off-target effects. Such broader therapeutic window can be due to certain properties of the antibody constructs described herein, including higher ratios of anti-tumor activity compared to cytokine induction, i.e., higher tumor cell killing activities can be achieved at lower cytokine induction levels.

[0401] In some embodiments, the present disclosure relates to a method of inhibiting the growth of a DLL3-expressing tumor and/or reducing the volume of the tumor in a subject in need thereof, the method comprising administering to the subject an effective amount of a trivalent and trispecific antibody construct of the present disclosure. In various embodiments, such *in vivo* anti-tumor effect is elicited by the trivalent and trispecific antibody construct that binds (e.g., simultaneously) CD3 and CD28 on one or more immune cell(s) and DLL3 on a tumor cell, thereby forming a TCR-independent immune synapse, and the antibody construct is either an $scFv^2$ antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a first scFv domain capable of binding CD28 on an immune cell, (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the C-terminus of the Fab light chain, and (c) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, or the antibody construct is a Het-Fab antibody construct as described herein, comprising: (i) a first Fab domain capable of binding CD3 on an immune cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on an immune cell; and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the

first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0402] In various embodiments, and as further described herein, inhibition of tumor growth and/or a reduction in tumor volume in a subject can be elicited by simultaneous binding of the antibody construct to CD3 and CD28 on one or more immune cell(s) and to DLL3 on a tumor cell and formation of a TCR-independent artificial immune synapse within a tumor environment in the subject, thereby eliciting an anti-tumor cytotoxic effect mediated by the activated immune cell and directed against the tumor cell. The immune cell can be a T cell.

[0403] In various embodiments of the methods described herein, the trivalent and trispecific antibody constructs binds CD3 and CD28 on the same immune cell (e.g., T cell). In other embodiments, the trivalent and trispecific antibody constructs binds CD3 on a first immune cell (e.g., T cell) and CD28 on a second immune cell (e.g., T cell), wherein the first and the second immune cells are different cells.

[0404] In various embodiments of the methods described herein, the subject is a rodent, a non-human primate, or a human.

[0405] In further embodiments, and in relation to a method described herein, administration of a sufficient amount of a trivalent and trispecific antibody construct to a subject in need thereof can provide one or more of the following to activate or upregulate an immune response in the subject: (i) modulation of T-cell receptor signaling, (ii) modulation of T-cell activation, (iii) modulation of pro-inflammatory cytokines, (iv) modulation of interferon-γ (IFNγ) production by T cells, (v) modulation of T-cell suppression, (vi) modulation of M2-type tumor associated macrophages (TAM) or myeloid-derived suppressor cell (MDSC) survival and/or differentiation, and/or (vii) modulation of cytotoxic or cytostatic effects on cells, e.g., cancer cells.

[0406] In some embodiments, the present disclosure relates to methods of modulating an immune response in a cell population or in a subject using one or more of the trivalent and trispecific antibody construct(s) of the present disclosure, wherein such modulation can comprise one or more of (i) immune cell activation, (ii) stimulation of T-cell receptor signaling, (iii) stimulation of antibody-dependent cellular cytotoxicity (ADCC), (iv) T cell-dependent cytotoxicity (TDCC), (v) cell-dependent cytotoxicity (CDC), (vi) antibody-dependent cellular phagocytosis (ADCP), and combinations of the above. As described herein, in certain embodiments, an antibody construct of

the present disclosure activates T effector cells. In some embodiments, and as demonstrated herein, an antibody construct increases production of one or more cytokines and/or signalling molecules, e.g., compared to baseline or untreated conditions, such as GM-CSF, TNFα, a MIP-1, IFN-γ, IL-2, IL-6, IL-10, IL-12, IL-17, IL-21 and/or C-X-C motif ligand 13 (CXCL13) by T effector cells.

[0407] As described herein, in various embodiments, a trivalent and trispecific antibody construct of the present disclosure comprises an Fc domain comprising a first and a second Fc polypeptide, wherein one or more of the Fc polypeptides can comprise a modified CH2 domain (e.g., compared to a WT domain) that comprises one or more amino acid modifications that can result in a decrease or elimination of binding of the Fc domain to one or more, or to all of the FcγRs (also referred to herein as an Fc "knock-out" or "KO" variant).

C. Experimental Methods

[0408] In some embodiments, the present disclosure relates to experimental methods for analyzing and/or detecting a trivalent and trispecific antibody construct of the present disclosure. Such methods can be used, for example, to assess *in vitro* and/or *in vivo* properties of such antibody construct such as its pharmacokinetic (PK) and pharmacodynamic (PD) properties. Other properties and characteristics of an antibody construct can be evaluated such as its stability under certain conditions (e.g., temperature, pH, etc.), its solubility, or its behaviour in the presence of certain other chemical components such as other proteins or cells.

[0409] Specific binding of a trivalent and trispecific antibody construct described herein to an antigen (e.g., CD3, CD28, TAA) can be measured, for example, through an enzyme-linked immunosorbent assay (ELISA), a surface plasmon resonance (SPR) technique (employing, for example, a BIAcore instrument) (Liljeblad *et al*., 2000, *Glyco J,* 17:323-329), or a traditional binding assay (Heeley, 2002, *Endocr Res*, 28:217-229). In certain embodiments, specific binding is defined as the extent of binding to an unrelated protein being less than about 10% of the binding to the target antigen (e.g., CD3, CD28, TAA, etc.) as measured by SPR, for example.

[0410] Additional experimental methods are described in, e.g., **EXAMPLES 1-28** herein.

VII. CERTAIN EMBODIMENTS OF THE DISCLOSURE

[0411] Certain embodiments of the present disclosure relate to embodiments 1-146 below, as well as to any combination of one or more of embodiments 1-146.

[0412] Embodiment 1. An antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a

second antigen on a cytotoxic effector cell; (iii) a third binding domain capable of binding DLL3 on a tumor cell, wherein the third binding domain is a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to: the C-terminus of the light chain of either the first Fab domain or the second Fab domain, if the third binding domain is the second Fab domain, or the C-terminus of the light chain of the first Fab domain, if the third binding domain is the second scFv domain.

[0413] Embodiment 2. The antibody construct of embodiment 1, wherein the third binding domain is the second Fab domain.

[0414] Embodiment 3. An antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding a tumor-associated antigen (TAA) on a tumor cell; (iii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain or the second Fab domain.

[0415] Embodiment 4. The antibody construct of embodiment 3, wherein the TAA is DLL3.

[0416] Embodiment 5. An antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain or the second Fab domain.

[0417] Embodiment 6. The antibody construct of any one of embodiments 1-5, wherein the first antigen is CD3 or CD28.

[0418] Embodiment 7. The antibody construct of any one of embodiments 1-6, wherein the second antigen is CD3 or CD28.

[0419] Embodiment 8. The antibody construct of any one of embodiments 1-7, wherein the first antigen is CD3 or CD28 and the second antigen is CD3 or CD28, and wherein the first antigen and the second antigen are different.

[0420] Embodiment 9. The antibody construct of any one of embodiments 1-8, wherein the first antigen is CD3 and the second antigen is CD28.

[0421] Embodiment 10. An antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on a tumor cell; (iii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain or the second Fab domain.

[0422] Embodiment 11. The antibody construct of any one of embodiments 1-10, wherein the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0423] Embodiment 12. The antibody construct of any one of embodiments 1-11, wherein the antibody construct comprises a first immunoglobulin heavy chain (H1) comprising at least a first Fab heavy chain, a second immunoglobulin heavy chain (H2) comprising at least a second Fab heavy chain, a first immunoglobulin light chain (L1) comprising at least a first Fab light chain, and a second immunoglobulin light chain (L2) comprising at least a second Fab light chain.

[0424] Embodiment 13. The antibody construct of embodiment 12, wherein H1 comprises, from N- to C-terminus, the first Fab heavy chain comprising a first VH sequence and a first CH1 sequence, coupled to the first Fc polypeptide, and wherein the first Fab heavy chain of H1 and the first Fab light chain of L1 associate to form the first Fab domain.

[0425] Embodiment 14. The antibody construct of embodiment 13, wherein H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the C-terminus of the first Fab heavy chain to the N-terminus of the first Fc polypeptide.

[0426] Embodiment 15. The antibody construct of any one of embodiment 12-14, wherein H2 comprises, from N- to C-terminus, the second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, coupled to the second Fc polypeptide, and wherein the second Fab heavy chain of H2 and the second Fab light chain of L2 associate to form the second Fab domain.

[0427] Embodiment 16. The antibody construct of embodiment 15, wherein H2 further comprises a second linker$^{Fab\text{-}Fc}$ that couples the C-terminus of the second Fab heavy chain to the N-terminus of the second Fc polypeptide.

[0428] Embodiment 17. The antibody construct of any one of embodiments 14 or 16, wherein the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ are polypeptide linkers, each independently comprising from about 5 to about 50 consecutive amino acid residues.

[0429] Embodiment 18. The antibody construct of embodiment 17, wherein the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ are each derived independently from an IgG1, IgG2 or IgG4 hinge region.

[0430] Embodiment 19. The antibody construct of any one of embodiments 12-18, wherein L1 further comprises the first scFv domain, and wherein the N-terminus of the first scFv domain is coupled to the C-terminus of a first constant domain (CL) sequence of L1.

[0431] Embodiment 20. The antibody construct of embodiment 19, wherein L1 comprises, from N- to C-terminus, the first Fab light chain comprising a first VL sequence and the first CL sequence, and the first scFv domain comprising, from N- to C-terminus, either (i) a third VH sequence coupled to a third VL sequence or (ii) a third VL sequence coupled to a third VH sequence.

[0432] Embodiment 21. The antibody construct of embodiment 20, wherein the first scFv domain comprises, from N- to C-terminus, the third VL sequence coupled to the third VH sequence.

[0433] Embodiment 22. The antibody construct of any one of embodiments 19-21, wherein L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the C-terminus of the first Fab light chain to the N-terminus of the first scFv domain.

[0434] Embodiment 23. The antibody construct of embodiment 22, wherein the linker$^{Fab\text{-}scFv}$ comprises the amino acid sequence $(G_4S)_X$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 119).

[0435] Embodiment 24. The antibody construct of any one of embodiments 12-23, wherein L2 comprises the second Fab light chain comprising a second VL sequence and a second CL sequence.

[0436] Embodiment 25. The antibody construct of any one of embodiments 1-24, comprising: (i) the first Fab domain, wherein the first Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) the second Fab domain, wherein the second Fab domain is capable of binding DLL3 on the tumor cell; (iii) the first scFv domain, wherein the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (iv) the dimeric Fc domain comprising the first Fc

polypeptide and the second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0437] Embodiment 26. The antibody construct of any one of embodiments 1-25, wherein the first antigen and the second antigen are located on the same cell or on different cells.

[0438] Embodiment 27. The antibody construct of any one of embodiments 1-26, wherein the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising a HCDR1 sequence comprising the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 sequence comprising the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 sequence comprising the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 sequence comprising the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 sequence comprising the sequence RND (SEQ ID NO: 54), and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

[0439] Embodiment 28. The antibody construct of any one of embodiments 1-27, wherein the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5.

[0440] Embodiment 29. The antibody construct of any one of embodiments 1-28, wherein the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising a HCDR1 sequence comprising the sequence SYGVH (SEQ ID NO: 28), a HCDR2 sequence comprising the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 sequence comprising the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L sequence comprising a LCDR1 sequence comprising the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 sequence comprising the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 sequence comprising the sequence QQSRKVPFT (SEQ ID NO: 48).

[0441] Embodiment 30. The antibody construct of any one of embodiments 1-29, wherein the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the

sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16.

[0442] Embodiment 31. The antibody construct of any one of embodiments 1-30, wherein the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that can independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8.

[0443] Embodiment 32. The antibody construct of any one of embodiments 1-31, wherein the dimeric Fc domain is a heterodimeric Fc domain in which one Fc polypeptide has an amino acid sequence that differs in at least one residue from the amino acid sequence of the other Fc polypeptide.

[0444] Embodiment 33. The antibody construct of any one of embodiments 1-32, wherein one of the Fc polypeptides comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 9, and the other Fc polypeptide comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 10.

[0445] Embodiment 34. The antibody construct of any one of embodiments 1-33, wherein the first Fab domain, the second Fab domain, or both Fab domains, comprise one or more amino acid modifications that promote correct heavy and light chain pairing to produce the first and second Fab domains, respectively, relative to the formation of incorrectly paired Fab domains.

[0446] Embodiment 35. The antibody construct of any one of embodiments 1-34, wherein (i) both Fab domains each comprise a kappa light chain; (ii) both Fab domains each comprise a lambda light chain; or (iii) one Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

[0447] Embodiment 36. The antibody construct of embodiment 34 or embodiment 35, wherein one Fab domain comprises a kappa light chain and comprises one or more of the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence and one or more of the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the other Fab domain comprises a lambda light chain and comprises one or more of the amino acid substitutions 125R and 188K in the CH1 sequence and one or more of the amino acid substitutions 122D, 129T,

176E and 178E in the CL sequence of the lambda light chain, and wherein the amino acid residues are identified according to the Kabat numbering system.

[0448] Embodiment 37. The antibody construct of embodiment 34 or embodiment 35, wherein one Fab domain comprises a kappa light chain and comprises one or more of the amino acid substitutions 124R and 186K in the CH1 sequence and one or more of the amino acid substitutions 133G, 135W, 176D and 180E in the CL sequence of the kappa light chain, and the other Fab domain comprises a lambda light chain and comprises one or more of the amino acid substitutions 139W, 143D and 145T in the CH1 sequence and one or more of the amino acid substitutions 124Q and 131R in the CL sequence of the lambda light chain, and wherein the amino acid residues are identified according to the Kabat numbering system.

[0449] Embodiment 38. An antibody construct, comprising: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 Fab heavy chain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein the first Fc polypeptide of H1 and the second Fc polypeptide of H2 associate to form a heterodimer; (iii) a first light chain (L1), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein the anti-CD3 Fab light chain of L1 and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain; and (iv) a second light chain (L2), comprising an anti-DLL3 Fab light chain, and wherein L2 and the anti-DLL3 Fab heavy chain of H2 associate to form an anti-DLL3 Fab domain.

[0450] Embodiment 39. The antibody construct of any one of embodiments 1-38, wherein the second Fab domain has a K_D value for binding DLL3 from about 0.01 nM to about 100 nM or from about 0.1 nM to about 50 nM.

[0451] Embodiment 40. The antibody construct of any one of embodiments 1-26, comprising a first immunoglobulin (Ig) heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising an amino acid sequence

having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 108.

[0452] Embodiment 41. The antibody construct of embodiment 40, comprising a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 108.

[0453] Embodiment 42. The antibody construct of any one of embodiments 1-26, comprising a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 115, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 116.

[0454] Embodiment 43. The antibody construct of embodiment 42, comprising a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 115, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 116.

[0455] Embodiment 44. The antibody construct of any one of embodiments 1-26, comprising a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 117, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth

in SEQ ID NO: 106, a first light chain (L1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 118.

[0456] Embodiment 45. The antibody construct of embodiment 44, comprising a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 117, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 106, a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107, and a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 118.

[0457] Embodiment 46. The antibody construct of embodiment 1, wherein the third binding domain comprises or consists of the second scFv domain.

[0458] Embodiment 47. An antibody construct, comprising: (i) a first Fab domain capable of binding a first antigen on a cytotoxic effector cell; (ii) a first scFv domain capable of binding a second antigen on a cytotoxic effector cell; (iii) a second scFv domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0459] Embodiment 48. The antibody construct of embodiment 46 or embodiment 47, wherein the first antigen is CD3 or CD28.

[0460] Embodiment 49. The antibody construct of any one of embodiments 46-48, wherein the second antigen is CD3 or CD28.

[0461] Embodiment 50. The antibody construct of any one of embodiments 46-49, wherein the first antigen is CD3 or CD28 and the second antigen is CD3 or CD28, and wherein the first antigen and the second antigen are different.

[0462] Embodiment 51. The antibody construct of any one of embodiments 46-50, wherein the first antigen is CD3 and the second antigen is CD28.

[0463] Embodiment 52. The antibody construct of any one of embodiments 46-51, wherein the antibody construct comprises a first immunoglobulin heavy chain (H1) comprising at least a first

Fab heavy chain, a second immunoglobulin heavy chain (H2) and an immunoglobulin light chain (L) comprising at least a Fab light chain.

[0464] Embodiment 53. The antibody construct of embodiment 52, wherein H1 comprises, from N- to C-terminus, the first Fab heavy chain comprising a first VH sequence and a CH1 sequence, coupled to the first Fc polypeptide, and wherein the first Fab heavy chain of H1 and the Fab light chain of L associate to form the first Fab domain.

[0465] Embodiment 54. The antibody construct of embodiment 53, wherein H1 further comprises a linker$^{Fab\text{-}Fc}$ that couples the C-terminus of the first Fab heavy chain to the N-terminus of the first Fc polypeptide.

[0466] Embodiment 55. The antibody construct of any one of embodiment 52-54, wherein H2 comprises, from N- to C-terminus, the second scFv domain, comprising either (i) an anti-DLL3 VH sequence coupled to an anti-DLL3 VL sequence or (ii) an anti-DLL3 VL sequence coupled to an anti-DLL3 VH sequence, coupled to the second Fc polypeptide.

[0467] Embodiment 56. The antibody construct of embodiment 55, wherein the second scFv domain comprises, from N- to C-terminus, the anti-DLL3 VL sequence coupled to the anti-DLL3 VH sequence.

[0468] Embodiment 57. The antibody construct of any one of embodiments 55-56, wherein H2 further comprises a linker$^{scFv\text{-}Fc}$ that couples the C-terminus of the second scFv domain to the N-terminus of the second Fc polypeptide.

[0469] Embodiment 58. The antibody construct of any one of embodiments 54 or 57, wherein the linker$^{Fab\text{-}Fc}$ and the linker$^{scFv\text{-}Fc}$ are polypeptide linkers, each independently comprising from about 5 to about 50 consecutive amino acid residues.

[0470] Embodiment 59. The antibody construct of embodiment 58, wherein the linker$^{Fab\text{-}Fc}$ and the linker$^{scFv\text{-}Fc}$ are each independently derived from an IgG1, IgG2, or IgG4 hinge region.

[0471] Embodiment 60. The antibody construct of any one of embodiments 52-59, wherein L comprises, from N- to C-terminus, (i) the Fab light chain comprising a first VL sequence and a CL sequence, and (ii) the first scFv domain, wherein the first scFv domain comprises, from N- to C-terminus, either a second VH sequence coupled to a second VL sequence or a second VL sequence coupled to a second VH sequence.

[0472] Embodiment 61. The antibody construct of embodiment 60, wherein the first scFv domain comprises, from N- to C-terminus, the second VL sequence coupled to the second VH sequence.

[0473] Embodiment 62. The antibody construct of any one of embodiments 60-61, wherein L further comprises a linker$^{Fab\text{-}scFv}$ that couples the C-terminus of the Fab light chain to the N-terminus of the first scFv domain.

[0474] Embodiment 63. The antibody construct of embodiment 62, wherein the linker$^{Fab\text{-}scFv}$ comprises the amino acid sequence $(G_4S)_X$, wherein x is 1, 2, 3, 4 or 5.

[0475] Embodiment 64. The antibody construct of any one of embodiments 46-63, comprising: (i) the first Fab domain, wherein the first Fab domain is capable of binding CD3 on a cytotoxic effector cell; (ii) the first scFv domain, wherein the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; (iii) the second scFv domain capable of binding DLL3 on a tumor cell; and (iv) the dimeric Fc domain comprising the first Fc polypeptide and the second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0476] Embodiment 65. The antibody construct of any one of embodiments 46-64, wherein the first antigen and the second antigen are located on the same cell or on different cells.

[0477] Embodiment 66. The antibody construct of any one of embodiments 46-65, wherein the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising a HCDR1 sequence comprising the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 sequence comprising the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 sequence comprising the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 sequence comprising the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 sequence comprising the sequence RND (SEQ ID NO: 54), and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

[0478] Embodiment 67. The antibody construct of any one of embodiments 46-66, wherein the first Fab domain is capable of binding CD3 and comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5.

[0479] Embodiment 68. The antibody construct of any one of embodiments 46-67, wherein the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising a HCDR1

sequence comprising the sequence SYGVH (SEQ ID NO: 28), a HCDR2 sequence comprising the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 sequence comprising the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L sequence comprising a LCDR1 sequence comprising the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 sequence comprising the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 sequence comprising the sequence QQSRKVPFT (SEQ ID NO: 48).

[0480] Embodiment 69. The antibody construct of any one of embodiments 46-68, wherein the first scFv domain is capable of binding CD28 and comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16.

[0481] Embodiment 70. The antibody construct of any one of embodiments 46-69, wherein the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that can independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8.

[0482] Embodiment 71. The antibody construct of any one of embodiments 46-70, wherein the dimeric Fc domain is a heterodimeric Fc domain in which one Fc polypeptide has an amino acid sequence that differs in at least one residue from the amino acid sequence of the other Fc polypeptide.

[0483] Embodiment 72. The antibody construct of any one of embodiments 46-71, wherein one of the Fc polypeptides comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 9, and the other Fc polypeptide comprises a CH3 domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 10.

[0484] Embodiment 73. An antibody construct, comprising: (i) a first heavy chain (H1), comprising an anti-CD3 Fab heavy chain coupled to a first Fc polypeptide, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 11; (ii) a second heavy chain (H2), comprising an anti-DLL3 scFv domain coupled to a second Fc polypeptide, wherein the second Fc polypeptide comprises

an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 12, and wherein the first Fc polypeptide of H1 and the second Fc polypeptide of H2 associate to form a heterodimer H1-H2; and (iii) a light chain (L), comprising an anti-CD3 Fab light chain coupled to an anti-CD28 scFv domain, and comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 13, and wherein the anti-CD3 Fab light chain of L and the anti-CD3 Fab heavy chain of H1 associate to form an anti-CD3 Fab domain.

[0485] Embodiment 74. The antibody construct of any one of embodiments 46-73, wherein the first scFv domain has a K_D value for binding DLL3 from about 0.01 nM to about 100 nM or from about 0.1 nM to about 50 nM.

[0486] Embodiment 75. The antibody construct of any one of embodiments 46-74, comprising a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 109, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 110, and a light chain (L) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 111.

[0487] Embodiment 76. The antibody construct of embodiment 75, comprising a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 109, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 110, and a light chain (L) comprising the amino acid sequence set forth in SEQ ID NO: 111.

[0488] Embodiment 77. The antibody construct of any one of embodiments 46-74, comprising a first Ig heavy chain (H1) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 112, a second Ig heavy chain (H2) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 113, and a light chain (L) comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 114.

[0489] Embodiment 78. The antibody construct of embodiment 77, comprising a first Ig heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 112, a second Ig heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 113, and a light chain (L) comprising the amino acid sequence set forth in SEQ ID NO: 114.

[0490] Embodiment 79. An antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding a TAA on a tumor cell, wherein the third binding domain is a second Fab domain or a second scFv domain; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0491] Embodiment 80. The antibody construct of any one of embodiments 1-79, wherein the cytotoxic effector cell comprises a T cell.

[0492] Embodiment 81. An antibody construct, comprising: (i) a first Fab domain that binds CD3 comprising a first Fab heavy chain comprising a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 49, a HCDR2 sequence as set forth in SEQ ID NO: 51, and a HCDR3 sequence as set forth in SEQ ID NO: 52, and a first Fab light chain comprising a V_L domain comprising a LCDR1 sequence as set forth in SEQ ID NO: 53, a LCDR2 sequence as set forth in SEQ ID NO: 54, and a LCDR3 sequence as set forth in SEQ ID NO: 55, as identified according to IMGT numbering; (ii) an scFv domain that binds CD28 comprising a VL sequence comprising a LCDR1 sequence as set forth in SEQ ID NO: 41, a LCDR2 sequence as set forth in SEQ ID NO: 44, and a LCDR3 sequence as set forth in SEQ ID NO: 48, and a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 28, a HCDR2 sequence as set forth in SEQ ID NO: 31, and a HCDR3 sequence as set forth in SEQ ID NO: 35, as identified according to Kabat numbering; (iii) a second Fab domain that binds DLL3 comprising a second Fab heavy chain comprising a VH sequence comprising a HCDR1 sequence as set forth in SEQ ID NO: 90, a HCDR2 sequence as set forth in SEQ ID NO: 91, and a HCDR3 sequence as set forth in SEQ ID NO: 92, and a second Fab light chain comprising a VL domain comprising a LCDR1 sequence as set forth in SEQ ID NO: 93, a LCDR2 sequence as set forth in SEQ ID NO: 94, and a LCDR3 sequence as set forth in SEQ ID NO: 95, as identified according to Kabat numbering; and (iv) an

Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein (1) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via the C-terminus of the first Fab heavy chain, (2) the scFv domain is coupled to the C-terminus of the first Fab light chain, and (3) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide via the C-terminus of the second Fab heavy chain.

[0493] Embodiment 82. The antibody construct of any one of embodiments 1-81, wherein the first Fc polypeptide and/or the second Fc polypeptide comprise IgG1 or IgG4 CH2 sequences comprising one or more amino acid modifications compared to wildtype CH2 sequences that decrease or eliminate binding of the Fc domain to one or more FcγRs.

[0494] Embodiment 83. The antibody construct of any one of embodiments 1-82, wherein the Fc domain is capable of engaging with a neonatal Fc receptor (FcRn).

[0495] Embodiment 84. The antibody construct of any one of embodiments 1-83, wherein the first Fc polypeptide and the second Fc polypeptide each comprise an IgG1 or IgG4 CH3 sequence comprising one or more amino acid modifications compared to wildtype CH3 sequences that promote preferential pairing of the first and second Fc polypeptide to form a heterodimeric Fc domain compared to the formation of a corresponding homodimeric Fc domain.

[0496] Embodiment 85. The antibody construct of embodiment 84, wherein the CH3 sequence of one of the Fc polypeptides comprises a set of amino acid substitutions selected from the group consisting of: L351Y_F405A_Y407V, T350V_L351Y_F405A_Y407V and T350V_L351Y_S400E_F405A_Y407V, and the CH3 sequence of the other Fc polypeptide comprises a set of amino acid substitutions selected from the group consisting of: T366L_K392M_T394W, T366L_K392L_T394W, T350V_T366L_K392L_T394W, T350V_T366L_K392M_T394W and T350V_T366L_N390R_K392M_T394W, and wherein the numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system.

[0497] Embodiment 86. The antibody construct of any one of embodiments 1-85, comprising (i) an anti-DLL3 VH sequence comprising or consisting of an amino acid sequence having about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in any one of SEQ ID NOs: 99, 101 or 103, and (ii) an anti-DLL3 VL sequence comprising or consisting of an amino acid sequence having about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in any one of SEQ ID NOs: 100, 102 or 104.

[0498] Embodiment 87. A pharmaceutical composition comprising the antibody construct of any one of embodiments 1-86, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

[0499] Embodiment 88. A nucleic acid molecule or a set of nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct of any one of embodiments 1-86.

[0500] Embodiment 89. A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 88.

[0501] Embodiment 90. A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 88, or the vector or set of vectors of embodiment 89.

[0502] Embodiment 91. A method of producing an antibody construct of any one of embodiments 1-86, the method comprising: (a) obtaining a host cell culture comprising at least one host cell comprising one or more nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture.

[0503] Embodiment 92. The method of embodiment 91, further comprising, subsequent to step (b), purifying the antibody construct.

[0504] Embodiment 93. A method of eliciting an anti-tumor immune response in a cell population comprising immune cells and tumor cells, the method comprising contacting the cell population with an effective amount of the antibody construct of any one of embodiments 1-86, wherein the immune cells express the first and second antigens and the tumor cells express DLL3.

[0505] Embodiment 94. A method of inhibiting the proliferation of tumor cells, the method comprising contacting a cell population comprising the tumor cells and immune cells with an effective amount of the antibody construct of any one of embodiments 1-86, wherein the immune cells express the first and second antigens and the tumor cells express DLL3.

[0506] Embodiment 95. A method of killing tumor cells, the method comprising contacting a cell population comprising the tumor cells and immune cells with an effective amount of the antibody construct of any one of embodiments 1-86, wherein the immune cells express the first and second antigens and the tumor cells express DLL3.

[0507] Embodiment 96. The method of any one of embodiments 93-95, wherein the immune cells comprise T cells.

[0508] Embodiment 97. The method of any one of embodiments 93-96, wherein the antibody construct binds (i) CD3 and CD28 on either one T cell or two different T cells and (ii) DLL3 on a tumor cell.

[0509] Embodiment 98. The method of embodiment 97, wherein the binding of the antibody construct to CD3 and CD28 on one or more T cell(s) and DLL3 on a tumor cell forms a TCR-independent artificial immune synapse between the one or more T cell(s) and the tumor cell, thereby eliciting a cytotoxic immune response of the one or more T cell(s) against the tumor cell.

[0510] Embodiment 99. The method of any one of embodiments 93-98, wherein the cell population is within a subject.

[0511] Embodiment 100. A method for treating a cancer in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of embodiments 1-86.

[0512] Embodiment 101. The method of embodiment 100, wherein a cytotoxic immune response against the cancer is elicited in the subject, thereby treating the cancer in the subject.

[0513] Embodiment 102. The method of embodiment 100 or embodiment 101, wherein the cancer is a DLL3-expressing cancer.

[0514] Embodiment 103. An antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a second Fab domain capable of binding DLL3 on the tumor cell; (iii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain constant domain (CL) of the first Fab domain, wherein the antibody construct is capable of binding CD3 and CD28 on the same cytotoxic effector cell, as measured in a cell-cell bridging assay with recorded double positive events of less than about 5, less than about 3, or less than about 2.

[0515] Embodiment 104. An antibody construct, comprising: (i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell; (iii) a third binding domain capable of binding DLL3 on a tumor cell; and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the third

binding domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

[0516] Embodiment 105. The antibody construct of embodiment 104, wherein the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.

[0517] Embodiment 106. The antibody construct of embodiment 104 or embodiment 105, wherein the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.

[0518] Embodiment 107. The antibody construct of any one of embodiments 104-106, wherein the first Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 2, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5.

[0519] Embodiment 108. The antibody construct of any one of embodiments 104-107, wherein the first Fab domain comprises a V_H domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 2, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 5.

[0520] Embodiment 109. The antibody construct of any one of embodiments 104-108, wherein the first scFv domain comprises a V_H domain comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2

having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system.

[0521] Embodiment 110. The antibody construct of any one of embodiments 104-109, wherein the first scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16.

[0522] Embodiment 111. The antibody construct of any one of embodiments 104-110, wherein the first scFv domain comprises a V_H sequence comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 16.

[0523] Embodiment 112. The antibody construct of any one of embodiments 104-111, wherein the third binding domain is a second Fab domain or a second scFv domain.

[0524] Embodiment 113. The antibody construct of any one of embodiments 104-112, wherein third binding domain comprises a V_H domain comprising a HCDR1 having the sequence HYYVH (SEQ ID NO: 90), a HCDR2 having the sequence IIDPGGGTTSYAQKFLG (SEQ ID NO: 91) and a HCDR3 having the sequence GERVTGNYFYYGMDV (SEQ ID NO: 92), and a VL domain comprising a LCDR1 having the sequence VANQGISNYLV (SEQ ID NO: 93), a LCDR2 having the sequence SVNSLYS (SEQ ID NO: 94) and a LCDR3 having the sequence LQHDSYPYT (SEQ ID NO: 95), according to the Kabat numbering system.

[0525] Embodiment 114. The antibody construct of any one of embodiments 104-113, wherein the third binding domain comprises a V_H sequence comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 99, and a V_L domain comprising or consisting of the amino acid sequence the sequence set forth in SEQ ID NO: 100.

[0526] Embodiment 115. The antibody construct of embodiment 104, wherein (i) the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system, (ii) the first scFv domain comprises a V_H domain comprising a HCDR1 having the sequence SYGVH

(SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system, and (iii) the third binding domain comprises a V_H domain comprising a HCDR1 having the sequence HYYVH (SEQ ID NO: 90), a HCDR2 having the sequence IIDPGGGTTSYAQKFLG (SEQ ID NO: 91) and a HCDR3 having the sequence GERVTGNYFYYGMDV (SEQ ID NO: 92), and a VL domain comprising a LCDR1 having the sequence VANQGISNYLV (SEQ ID NO: 93), a LCDR2 having the sequence SVNSLYS (SEQ ID NO: 94) and a LCDR3 having the sequence LQHDSYPYT (SEQ ID NO: 95), according to the Kabat numbering system.

[0527] Embodiment 116. The antibody construct of embodiment 104, wherein (i) the first Fab domain comprises the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 2, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 5, (ii) the first scFv domain comprises the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 15, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 16, and (iii) the third binding domain comprises the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 99, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 100.

[0528] Embodiment 117. The antibody construct of embodiment 104, wherein (i) the first Fab domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 2, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5, (ii) the first scFv domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16, and (iii) the third binding domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 99, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 100.

[0529] Embodiment 118. The antibody construct of any one of embodiments 104-117, wherein the third binding domain is a second Fab domain.

[0530] Embodiment 119. The antibody construct of embodiment 118, wherein (i) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via a first linker$^{Fab\text{-}Fc}$ and (ii) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide via a second linker$^{Fab\text{-}Fc}$.

[0531] Embodiment 120. The antibody construct of embodiment 119, wherein the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consist of the amino acid sequence set forth in SEQ ID NOs: 56 or 120.

[0532] Embodiment 121. The antibody construct of any one of embodiments 118-120, wherein one Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

[0533] Embodiment 122. The antibody construct of embodiment 121, wherein the Fab domain comprising the kappa light chain comprises one or more of the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence and one or more of the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the Fab domain comprising the lambda light chain comprises one or more of the amino acid substitutions 125R and 188K in the CH1 sequence and one or more of the amino acid substitutions 122D, 129T, 176E and 178E in the CL sequence of the lambda light chain, and wherein the amino acid residues are identified according to the Kabat numbering system.

[0534] Embodiment 123. The antibody construct of embodiment 122, wherein the Fab domain comprising the kappa light chain comprises the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence and the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the Fab domain comprising the lambda light chain comprises the amino acid substitutions 125R and 188K in the CH1 sequence the amino acid substitutions 122D, 129T, 176E and 178E in the CL sequence of the lambda light chain.

[0535] Embodiment 124. The antibody construct of any one of embodiments 104-117, wherein the third binding domain is a second scFv domain.

[0536] Embodiment 125. The antibody construct of embodiment 124, wherein (i) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via a first linker$^{Fab\text{-}Fc}$ and (ii) the

second scFv domain is coupled to the N-terminus of the second Fc polypeptide via a first linker$^{scFv-Fc}$.

[0537] Embodiment 126. The antibody construct of embodiment 125, wherein the first linker^{Fab-Fc} and the first linker$^{scFv-Fc}$ independently comprise or consist of the amino acid sequence set forth in SEQ ID NOs: 56, 120 or 57.

[0538] Embodiment 127. The antibody construct of any one of embodiments 104-126, wherein the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8.

[0539] Embodiment 128. The antibody construct of embodiment 127, wherein the first Fc polypeptide and the second Fc polypeptide each comprise the amino acid substitutions L234A_L235A_D265S, and wherein the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

[0540] Embodiment 129. The antibody construct of any one of embodiments 104-128, wherein the first Fc polypeptide and the second Fc polypeptide each comprise an IgG1 CH3 domain, and wherein each CH3 domain comprises one or more amino acid substitutions compared to a corresponding wild-type IgG1 CH3 domain sequence, wherein the one or more amino acid substitutions promote preferential pairing of the first and second Fc polypeptides to form a heterodimeric Fc domain.

[0541] Embodiment 130. The antibody construct of embodiment 129, wherein the first or the second Fc polypeptide comprises the amino acid substitutions T350V_L351Y_F405A_Y407V, and the other Fc polypeptide comprises the amino acid substitutions T350V_T366L_K392L_T394W, and the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

[0542] Embodiment 131. The antibody construct of any one of embodiments 104-130, wherein the C-terminus of the light chain of the first Fab domain is coupled to the N-terminus of the first scFv domain via a linker$^{Fab-scFv}$ comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 58.

[0543] Embodiment 132. The antibody construct of any one of embodiments 104-131, wherein the first scFv domain has the domain structure, from N- to C-terminus, of: VL-Linker^{scFv}-VH, and

wherein the Linker^{scFv} comprises or consists of the amino acid sequence set forth in SEQ ID NO: 59.

[0544] Embodiment 133. The antibody construct of any one of embodiments 104-123, comprising: (i) a first heavy chain (H1) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 105; (ii) a second heavy chain (H2) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 106; (iii) a first light chain (L1) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 107; and (iv) a second light chain (L2) comprising or consisting of an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 108.

[0545] Embodiment 134. The antibody construct of embodiment 133, comprising: (i) a first heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105; (ii) a second heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 106; (iii) a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107; and (iv) a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 108.

[0546] Embodiment 135. The antibody construct of any one of embodiments 104-134, wherein the cytotoxic effector cell is a T cell, and wherein the antibody construct binds CD3 and CD28 on the same T cell as compared to binding CD3 and CD28 on different T cells, as measured in a cell-cell bridging assay with recorded double positive events of less than about 5, less than about 3, less than about 2, or less than about 1.

[0547] Embodiment 136. A pharmaceutical composition comprising the antibody construct of any one of embodiments 104-135, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

[0548] Embodiment 137. A nucleic acid molecule or a set of nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct of any one of embodiments 104-135.

[0549] Embodiment 138. A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 137.

[0550] Embodiment 139. A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 137, or the vector or set of vectors of embodiment 138.

[0551] Embodiment 140. A method of producing an antibody construct of any one of embodiments 104-135, the method comprising: (a) obtaining a host cell culture comprising at least one host cell comprising one or more nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture.

[0552] Embodiment 141. A method of eliciting an anti-tumor immune response in a cell population comprising T cells and tumor cells, the method comprising contacting the cell population with an effective amount of the antibody construct of any one of embodiments 104-135, wherein the T cells express CD3 and CD28 and the tumor cells express DLL3.

[0553] Embodiment 142. A method of treating a cancer in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of embodiments 104-135.

[0554] Embodiment 143. An antibody construct of any one of embodiments 1-86 or 104-135 for use in the treatment of cancer.

[0555] Embodiment 144. Use of an antibody construct of any one of embodiments 1-86 or 104-135 in the manufacture of a medicament for the treatment of cancer.

[0556] Embodiment 145. In a method comprising treating a subject having cancer by administering to the subject an antibody construct targeting DLL3, the improvement comprising said antibody construct comprising an anti-CD3 Fab domain and an anti-CD28 scFv domain and the anti-CD28 scFv domain is coupled to the C-terminus of the anti-CD3 Fab light chain, wherein said antibody construct induces less T cell-T cell cross-linking when compared to an antibody construct in which the anti-CD28 scFv domain is not coupled to the C-terminus of the anti-CD3 Fab light chain, as measured in a T cell-T cell bridging assay.

[0557] Embodiment 146. A method of treating a subject having cancer by administering to the subject an antibody construct targeting DLL3, comprising: (a) means for binding DLL3; and (b) an anti-CD3 Fab domain and an anti-CD28 scFv domain, wherein the anti-CD28 scFv domain is coupled to the C-terminus of the anti-CD3 Fab light chain, wherein said antibody construct induces less T cell-T cell cross-linking when compared to an antibody construct in which the anti-CD28

scFv domain is not coupled to the C-terminus of the anti-CD3 Fab light chain, as measured in a T cell-T cell bridging assay.

EXAMPLES

[0558] The following Examples are provided for illustrative purposes and are not intended to limit the scope of the invention in any way.

[0559] The practice of the present disclosure can employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992).

[0560] Unless stated otherwise in the examples section, the abbreviation "ND" or "nd" with respect to certain data point refers to "not determined" (e.g., when a curve-fit is not feasible).

EXAMPLE 1: DESIGN, STRUCTURE AND PREPARATION OF TRIVALENT AND TRISPECIFIC ANTI-(DLL3xCD28xCD3) ANTIBODY CONSTRUCTS

[0561] Several trivalent and trispecific anti-(DLL3xCD28xCD3) antibody constructs were developed to engage and activate T-cells leading to lysis of DLL3-expressing tumor cells. The trispecific antibody constructs were prepared in different formats and geometries, and bispecific anti-(DLL3xCD3) and anti-(DLL3xCD28) control constructs were also prepared as shown and described in **TABLES 5-7** below. These antibody constructs were prepared to examine the impact of antibody format, geometry and anti-CD28 and anti-CD3 paratope affinity on the potency of these T cell engager constructs, e.g., their capability of targeting and killing DLL3-expressing tumor cells.

Design of Trivalent and Trispecific Antibody Constructs Targeting DLL3, CD28 and CD3

[0562] Several trivalent and trispecific antibody constructs were prepared in a format in which the DLL3 antigen-binding arm was an scFv domain or a Fab domain, the CD3 antigen-binding domain

was either an scFv domain or a Fab domain, and the CD28 antigen-binding domain was either an scFv domain or a Fab domain.

[0563] Bispecific control antibody constructs were prepared in a format in which DLL3 antigen-binding domain was an scFv domain or a Fab domain and the CD3 or CD28 antigen-binding domain was a Fab domain.

[0564] These trispecific and bispecific antibody constructs comprised a human IgG1 heterodimeric Fc domain which further comprised sets of CH3 domain amino acid substitutions promoting the formation of a heterodimeric Fc domain (see, e.g., "Het-Fc" in **TABLE 6**). These sets of amino acid substitutions are referred to herein as (i) Het FcA having the amino acid substitutions T350V/L351Y/F405A/Y407V in the first Fc polypeptide chain (A) and (ii) Het FcB having the amino acid substitutions T350V/T366L/K392L/T394W in the second Fc polypeptide chain (B). These antibody construct variants in **TABLE 6** herein noted as having "FcKO" comprised the following CH2 amino acid substitutions which knock out, e.g., measurably reduce or ablate, FcγR binding: L234A, L235A and D265S. Amino acid residues in the Fc domain are identified according to the EU index.

[0565] The trivalent and trispecific antibody constructs targeting DLL3, CD28 and CD3 as well as the bispecific control constructs against DLL3, CD28 and/or CD3 were designed, expressed, and characterized as described in International Patent Publication No. WO 2015/109131. Briefly, genes encoding the heavy and light chains of the antibody constructs were constructed via gene synthesis using codons optimized for human/mammalian expression. The anti-CD3 scFv domain and Fab domain sequences were generated from the anti-CD3 monoclonal antibody C3E6 (*see*, e.g., U.S. Pat. Publ. No. 2019/0359712). The anti-CD28 scFv domain and Fab domain sequences were generated from the anti-CD28 monoclonal antibody huTN228 as described herein.

[0566] In trivalent and trispecific antibody constructs as well as in bispecific control constructs containing an anti-CD3 Fab domain or an anti-CD28 Fab domain, the V_H and V_L sequences were fused to human IgG1 C_{H1} and C_L sequences, respectively. In some trispecific antibody constructs of this disclosure, an anti-CD3 scFv domain or an anti-CD28 scFv domain was fused to the N- or C-terminus of one of the Fc polypeptides of the heterodimeric Fc domain. In other trispecific antibody constructs of this disclosure, an anti-CD3 scFv domain was fused to the N-terminus of the V_H domain of the anti-CD28 Fab domain. In certain other trispecific antibody constructs of this disclosure, an anti-CD28 scFv domain was fused to the N-terminus of the V_H domain of the

anti-CD3 Fab domain or to the C-terminus of the C_L domain of the anti-CD3 Fab domain. In certain trispecific and bispecific antibody constructs of this disclosure, an anti-DLL3 scFv domain was fused either to one of the heterodimeric Fc domains or to the N-terminus of the V_H domain of the anti-CD28 Fab domain. In bispecific control antibody constructs containing an anti-CD3 Fab domain or an anti-CD28 Fab domain, the anti-CD3 or anti-CD28 V_H and V_L domain sequences were fused to human IgG1 C_{H1} and C_L sequences, respectively. For various antibody constructs described herein, one scFv domain was fused to one chain of the heterodimeric Fc domain and the V_H-C_{H1} domains of a Fab domain was fused to the second chain of the heterodimeric Fc domain.

[0567] Certain heavy chain domain structures and formats that were used to generate certain antibody constructs of the present disclosure, e.g., certain trivalent and trispecific DLL3-targeting constructs, bispecific control constructs, as well as other constructs for studying the impact of overall construct format and geometry, are shown below in **TABLE 5** and **TABLE 6** (domain structures are generally shown from N-terminus to C-terminus, unless otherwise stated). **TABLE 7** shows several production parameters for certain constructs described herein.

TABLE 5: Various Heavy Chain Domain Structures and Exemplary Trivalent and Trispecific Antibody Constructs comprising such Heavy Chains

Heavy Chain Domain Structure:	**Certain Antibody Constructs that comprise the Chain Format:**
V_H-C_{H1}-hinge-CH2-CH3	v41268, v38967, v38580, v38584, v38586, v38588, v22277, v39915, v39917, v39920, v39922, v41086, v41094, v41753, v41754, v41112, v41757
V_L-V_H-hinge-CH2-CH3	v41268, v38578, v38967, v38580, v38584, v38582, v38961, v38962, v38963, v38964, v38965, v38966, v38586, v38588, v41759, v41760, v41761, v41762
V_H-V_L-V_H-C_{H1}-hinge-CH2-CH3	v38578, v38582, v38961, v38962, v38963, v38964, v38965, v38966
V_H-C_{H1}-hinge-CH2-CH3-V_L-V_H	v38580, v38584
V_H-V_L-V_H-V_L-hinge-CH2-CH3-hinge-CH2-CH3	v38589
VHH-VHH-V_H-V_L	v40309
V_H-V_L-VHH-VHH	v40310
V_H-V_H-C_{H1}-hinge-CH2-CH3	v37035

TABLE 6: Format and Structure of Certain Trivalent and Trispecific Antibody Constructs and Control Constructs

Construct #	**Format** (representative Figure (if applicable))	**Heavy Chain A**	**Heavy Chain B**	**Fc Description**

v38578	1x1x1 (Fig. 1A)	N-terminus huTN228 VH-VL scFv, C3E6 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v38967	1x1x1 (Fig. 1B)	C3E6 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v41268	1x1x1 (Fig. 1B)	C3E5 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v38580	1x1x1 (Fig. 1C)	C3E6 Fab, C-terminus huTN228 VL-VH scFv	DLL3 VL-VH scFv	Het Fc, FcKO
v38584	1x1x1 (Fig. 1C)	huTN228 Fab, C-terminus C3E6 VL-VH scFv	DLL3 VL-VH scFv	Het Fc, FcKO
v38582	1x1x1 (Fig. 1A)	N-terminus C3E6 VH-VL scFv, huTN228 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v38586	1x1 (Fig. 1F)	huTN228 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v38588	1x1 (Fig. 1F)	C3E6 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v38589 (AMG757)	1x1	Anti-DLL3 VH - VL scFv-Anti-CD3 VH - VL scFv	n/a	n/a
v22277	1x1	Palivizumab Fab	Palivizumab Fab	Het Fc
v39915	1x1x1 (Fig. 1D)	C3E6 Fab	DLL3 Fab	Het Fc, FcKO
v39917, v41386	1x1x1 (Fig. 1D)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v39920	1x1 (Fig. 1E)	C3E6 Fab	DLL3 Fab	Het Fc, FcKO
v39922	1x1 (Fig. 1E)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v40309	1x1	Anti-DLL3 VHH – Anti-HSA VHH – Anti-CD3 V_L-V_H scFv	n/a	n/a
v40310	1x1	Anti-CD3 V_H-V_L scFv – Anti-HSA VHH – Anti-DLL3 - VHH	n/a	n/a
v37035	1x1x1	Anti-MSLN Fab	Anti-CD3 VH – Anti-CD28 VH	n/a
v41086	1x1x1 (Fig. 1D)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v41094	1x1x1 (Fig. 1D)	C3E6 Fab	DLL3 Fab	Het Fc, FcKO
v41753	1x1x1 (Fig. 1D)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v41754	1x1x1 (Fig. 1D)	C3E6 Fab	DLL3 Fab	Het Fc, FcKO
v41112	1x1x1 (Fig. 1D)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v41757	1x1x1 (Fig. 1D)	C3E5 Fab	DLL3 Fab	Het Fc, FcKO
v41759	1x1x1 (Fig. 1B)	C3E5 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v41760	1x1x1 (Fig. 1B)	SP34 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v41761	1x1 (Fig. 1F)	C3E5 Fab	DLL3 VL-VH scFv	Het Fc, FcKO
v41762	1x1 (Fig. 1F)	SP34 Fab	DLL3 VL-VH scFv	Het Fc, FcKO

n/a = not applicable.

TABLE 7: Buffer Systems, Yields and Purity of Various Trivalent and Trispecific Antibody Constructs

Antibody Construct	Yield (mg/L)	Purity (%)*	Buffer
v38578	5.6	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38967	9.8	99.6	50 mM Histidine, 9% Sucrose, pH 6.0
v38580	12	99.4	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38584	31	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38582	1.8	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38961	13.9	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38962	0.5	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38963	24	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38964	7.9	98.3	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38965	22	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38966	2.6	95.5	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38586	8.3	97.4	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38588	25.5	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v38589	28.3	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v22277	99.4	99.7	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v39915	5.2	99.9	50 mM Histidine, 9% Sucrose, pH 6.0
v39917	5.1	99.9	50 mM Histidine, 9% Sucrose, pH 6.0
v39920	2.6	98.7	50 mM Histidine, 9% Sucrose, pH 6.0
v39922	2.3	98.8	50 mM Histidine, 9% Sucrose, pH 6.0
v40309	8.3	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v40310	16.3	100	1 mM Potassium Phosphate monobasic, 155 mM Sodium Chloride, 3 mM Sodium Phosphate dibasic, pH 7.4
v37035	16	97.1	50 mM Histidine, 9% Sucrose, pH 6.0
v41086	59.7	99.1	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41094	65.5	99.7	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41753	450.9	98.5	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41754	418.7	99.0	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41112	368.7	99.9	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41757	658.2	99.2	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80

v41759	72.4	99.5	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41760	71.2	99.7	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41761	91.3	100	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80
v41762	64.5	99.9	50 mM Histidine, 9% Sucrose, pH 6.0, 0.02% Polysorbate 80

[0568] Certain trivalent and trispecific antibody constructs of the present disclosure comprise one or more of the following anti-CD3 and anti-CD28 binding domain sequences as shown in **TABLE 8** below.

TABLE 8: Anti-CD3 and Anti-CD28 binding domain sequences used in Certain Trivalent and Trispecific Antibody Constructs

Name	**Sequence VH**	**SEQ ID NO**
Anti-CD3 C3E5	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGMSWIRQAPGK GLEWVASITRSGGRIYYPDSVKGRFTISRENTQKTLYLQMNSLR AEDTAVYYCTLDGRDGWVAYWGQGTLVTVSS	2
Anti-CD3 C3E6	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGMSWIRQAPGK GLEWVASITSSGGRIYYPDSVKGRFTISRENTQKTLYLQMNSLR AEDTAVYYCTLDGRDGWVAYWGQGTLVTVSS	1
Anti-CD28 TN228	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWIRQPPGKG LEWLGVIWPGGGTNFNSALMSRLTISEDTSKNQVSLKLSSVTAA DTAVYYCARDRAYGNYLYAMDYWGQGTLVTVSS	15
Name	**Sequence VL**	**SEQ ID NO**
Anti-CD3 C3E5/E6	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVNWYQQHEGSS PTTIIYRNDKRPDGVSDRFSGSIDRSSKSASLTISNLKTEDEADYF CQSYSSGFIFGGGTKLTVL	5
Anti-CD28 TN228	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSLMQWYQQKP GKAPKLLIYAASNVDSGVPSRFSGSGSGTDFTLTISSLQPEDIATY YCQQSRKVPFTFGGGTKVEIK	16

[0569] By way of example, in **TABLE 6**, the nomenclature "1x1x1" indicates that the trispecific antibody construct is trivalent and trispecific (e.g., engaging each antigen monovalently) and comprised of one DLL3 binding domain, one CD28 binding domain and one CD3 binding domain. Accordingly, "1x1" indicates that the bispecific control antibody construct is bivalent and comprised of one DLL3 binding domains and one CD3 or CD28 binding domain, etc. Certain formats (e.g., 1x1x1, 1x1) and geometries (e.g., relative positioning of binding domains) of certain antibody constructs described herein are also illustrated in, e.g., **FIGS. 1A-1F**.

Production of Trivalent and Trispecific Anti-(DLL3xCD3xCD28) Antibody Constructs

[0570] Generally, the final gene products were sub-cloned into the mammalian expression vector pTT5 (NRC-BRI, Canada) and expressed in CHO cells (Durocher, Y., Perret, S. & Kamen, A. High-level and high-throughput recombinant protein production by transient transfection of

suspension-growing CHO cells. See, e.g., *Nucleic acids research* 30, E9 (2002)). The constructs v41086, v41094, v41753, v41754, v41112, v41757, v41759, v41760, v41761, and v41762 were transiently transfected into CHO-K1 cells using the WuXian™ Transient Platform (WuXi Biologics).

[0571] The CHO cells were transfected in exponential growth phase (1.5 to 2 million cells/mL) with aqueous 1 mg/mL 25 kDa polyethylenimine (PEI, Polysciences) using an PEI:DNA ratio of 2.5:1 (*see*, e.g., Raymond C. et al. A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. Methods. 55(1):44-51 (2011)). In order to determine the optimal concentration range for forming heterodimers, the DNA was transfected in optimal DNA ratios of the heavy chain A (HC-A), heavy chain B (HC-B), light chain A (LC-A), and light chain B (LC-B) that allow for heterodimer formation (e.g., HC-A:HC-B:LC-A ratios = 1:1:1 or 2:1:2 or 1:2:1 or 1:3:1 and HC-A:HC-B:LC-A:LC-B ratios = 15:15:35:35 or 8:22:35:35 or 22:8:35:35). Transfected cells were harvested after 5-6 days with the culture medium collected after centrifugation at 4000 rpm and clarified using a 0.45 µm filter.

[0572] The clarified culture medium was loaded onto a MabSelect SuRe (GE Healthcare) protein-A column and washed with 10 column volumes of PBS buffer at pH 7.2 - 7.4. The antibody construct was eluted with 10 column volumes of 0.1 M citrate buffer at pH 3.6 with the pooled fractions containing the antibody construct neutralized with 1 M TRIS at pH 9 or 11. Antibody construct was then quantified based on A280 nm (NanoDrop™).

[0573] The antibody constructs were further purified by gel filtration chromatography using a Superdex 200 HiLoad 16/600 200pg column (GE Healthcare) via an AKTA Express FPLC or AKTA Pure at a flowrate of 1 mL/min. PBS buffer at pH 7.4 or H6-NaCl (50 mM histidine, 150 mM sodium chloride, pH 6.0) buffer was used at a flow-rate of 1 mL/min. Additional purifications using POROS XS (Thermo) and/or KappaSelect (Cytiva) were used depending on purity. Fractions of eluted antibody construct were collected based on absorbance at A280 nm and the fractions were assessed by non-reducing and reducing High Throughput Protein Express assay using Caliper LabChip GXII (Perkin Elmer, Waltham, MA). Procedures were carried out according to HT Protein Express LabChip User Guide version2 LabChip GXII User Manual, with the following modifications. Antibody construct samples, at either 2 µl or 5 µl (concentration range 5-2000 ng/µl), were added to separate wells in 96 well plates (BioRad, Hercules, CA) along with 7 µl of HT Protein Express Sample Buffer (Perkin Elmer # 760328). Antibody construct samples were

then denatured at 90 °C for 5 mins. The LabChip instrument was operated using the HT Protein Express Chip (Perkin Elmer, Waltham, MA) and the Ab-200 assay setting. Fractions corresponding to the purified antibody constructs were collected, buffer exchanged into PBS pH7.4 or H6Su (50 mM histidine, 9% sucrose, pH 6.0) using a Zeba Spin desalting column (Thermo Scientific), concentrated to ~1 mg/mL, and stored at -80 °C.

[0574] Endotoxin levels were determined by the LAL (limulus amebocyte lysate) assay using the Endosafe Portable Test System (PTS, Charles River, Wilmington, MA). Antibody constructs were qualified based on A280 nm absorbance (nanodrop) post protein-A and SEC purification. UPLC-SEC was performed using a Waters Acquity BEH200 SEC column (2.5 mL, 4.6x150 mm, stainless steel, 1.7 µm particles) (Waters LTD, Mississauga, ON) set to 30 °C or 25 °C and mounted on a Waters Acquity UPLC H-Class Bio system with a PDA detector. Run times consisted of 7 min and a total volume per injection of 2.8 µL or 5 µL with a running buffer of DPBS or DPBS with 0.02% Tween 20 pH 7.4 - or 200 mM Potassium Phosphate with 200 mM Potassium Chloride pH 7.0 at 0.4 mL/min. Elution was monitored by UV absorbance in the range 210-500 nm, and chromatograms were extracted at 280 nm. Peak integration was performed using Empower 3 software.

[0575] The apparent purity and yield of the final antibody construct was estimated by UPLC-SEC and LC/MS as described in detail in, e.g., International Patent Publication No. WO 2015/109131. All antibody constructs were expressed and purified to >90% heterodimer purity without contaminating homodimers. **TABLE 7** describes the post purification yield for the production of certain antibody constructs described herein.

EXAMPLE 2: ABILITY OF TRIVALENT AND TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND DLL3, CD3 AND CD28 AS ASSESSED BY SURFACE PLASMON RESONANCE (SPR)

[0576] This example demonstrates the ability of the anti-DLL3, anti-CD3 and anti-CD28 paratopes when part of a trivalent and trispecific construct and bispecific controls to bind to the respective targets as assessed by SPR. The changes in CD3 and CD28 binding properties in the trispecific (e.g., anti-(DLL3xCD3xCD28)) antibody constructs based on their geometry and format is demonstrated as assessed by SPR.

Binding of Trivalent and Trispecific Antibody Constructs to DLL3 as assessed by SPR

[0577] All SPR binding experiments were carried out using a BiacoreT200 instrument at 25 °C with 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 at pH 7.4. Recombinant human and cyno DLL3 his tag protein (Acro Biosystems) was immobilized by amine coupling on CM5 sensor chip and purified trivalent and trispecific as well as bispecific antibody constructs to be tested was flowed on the sensor chip at 50 µL/min for 180 s, following a dissociation for 1200 seconds (s). Resultant k_{on} (also referred to as "k_a"), k_{off} (also referred to as "k_d") and K_D values were determined from fitting the sensorgrams using the 1:1 binding model with reported values as the mean of three independent runs.

Binding of Trivalent and Trispecific Antibody Constructs to CD3 as assessed by SPR

[0578] All SPR binding experiments were carried out using a BiacoreT200 instrument at 25 °C with 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 at pH 7.4. Recombinant human CD3 epsilon/delta heterodimer, his tag protein (Acro Biosystems) was covalently immobilized on CM5 sensor chips by amine coupling. Purified antibody constructs to be tested were indirectly captured on the sensor chip by binding to the recombinant CD3 protein when injected at 50 µL/min for 100 s following a dissociation for 100-200 s. Resultant k_{on}, k_{off} and K_D values were determined from fitting the sensorgrams using the 1:1 binding model with reported values as the mean of three independent runs.

Binding of Trivalent and Trispecific Antibody Constructs to CD28 as assessed by SPR

[0579] All SPR binding experiments were carried out using a BiacoreT200 instrument at 25 °C with 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 at pH 7.4. Recombinant human/cynomolgus/rhesus macaque CD28 mouse IgG2a Fc fusion protein (Acro Biosystems) was covalently immobilized on CM5 sensor chips by amine coupling. Purified antibody constructs to be tested were flowed on the sensor chip coated with recombinant CD3 protein at 50 µL/min for 120 s following a dissociation for 200-300 s. Resultant k_{on}, k_{off} and K_D values were determined from fitting the sensorgrams using the 1:1 binding model.

[0580] All SPR binding experiments were carried out using a BiacoreT200 instrument at 25 °C with 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 at pH 7.4. Recombinant human/cynomolgus/rhesus macaque CD28 protein (Acro Biosystems) was covalently immobilized on CM5 sensor chips by amine coupling. Purified antibody constructs to be tested were injected on the sensor chip at 50 µL/min for 100 s following a dissociation for 100-200 s.

Resultant k_{on}, k_{off} and K_D values were determined from fitting the sensorgrams using the 1:1 binding model

Results

[0581] TABLE 9 shows the binding parameters, including the calculated K_D values, for certain tested trivalent and trispecific antibody constructs binding to CD3 and CD28. The impact of format and geometry of the binding properties of the anti-CD3 and anti-CD28 paratopes in all the trispecific and bispecific antibody constructs can be demonstrated by the binding parameters reported in **TABLE 9.**

TABLE 9: Binding of Certain Antibody Constructs to CD3 and CD28

Construct	CD3			CD28		
	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)
v38967	2.66E+05	6.68E-03	3.61E-08	6.20E+03	5.12E-03	8.61E-07
v38580	4.14E+05	3.12E-03	7.49E-09	4.44E+04	5.32E-03	1.23E-07
v38584	3.70E+05	1.08E-02	3.14E-08	2.14E+05	1.84E-03	8.62E-09
v38582	4.06E+05	9.04E-03	2.23E-08	2.82E+05	2.59E-03	9.27E-09
v38961	4.31E+05	9.66E-03	2.25E-08	2.66E+05	2.69E-03	1.02E-08
v38963	4.44E+05	8.17E-03	1.84E-08	2.03E+05	1.24E-02	6.53E-08
v38964	4.54E+05	7.19E-03	1.58E-08	9.60E+04	1.51E-02	1.60E-07
v38965	3.90E+05	5.06E-03	1.26E-08	5.50E+04	2.74E-02	5.34E-07
v38966	3.33E+05	1.01E-02	3.23E-08	ND	ND	ND
v38586	ND	ND	ND	2.47E+05	2.72E-03	1.12E-08
v38588	5.78E+05	1.39E-02	2.41E-08	ND	ND	ND
v38589	9.30E+05	2.13E-03	2.30E-09	ND	ND	ND
v39915	3.74E+05	1.55E-02	4.49E-08	8.28E+03	8.59E-03	1.05E-06
v39917	5.36E+05	4.33E-03	8.60E-09	1.57E+04	9.59E-03	6.14E-07
v39920	6.76E+05	1.73E-02	2.55E-08	ND	ND	ND
v39922	1.05E+06	3.81E-03	3.63E-09	ND	ND	ND
v41086	8.00E+05	2.56E-03	3.19E-09	3.21E+04	3.07E-03	9.57E-08
v41094	8.55E+05	2.45E-02	2.87E-08	6.61E+03	6.93E-03	1.05E-06
v41112	5.57E+05	3.46E-03	6.21E-09	3.09E+04	5.71E-03	1.85E-07
v41759	5.69E+05	3.06E-03	5.38E-09	1.64E+04	8.11E-03	4.94E-07
v41760	3.94E+05	2.38E-03	6.04E-09	2.04E+04	8.57E-03	4.26E-07

[0582] TABLE 10 below shows the binding parameters, including the calculated K_D values, for certain tested trivalent and trispecific antibody constructs binding to DLL3.

TABLE 10: Binding of Certain Antibody Constructs to DLL3

Construct	Human DLL3			Cyno DLL3		
	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)
v38967	2.13E+05	5.69E-05	2.67E-10	2.08E+05	5.79E-05	2.78E-10
v39915, v39917	3.01E+05	5.84E-05	2.15E-10	2.69E+05	6.09E-05	2.42E-10

v41759	5.12E+05	1.27E-04	2.49E-10	4.29E+05	1.03E-04	2.39E-10
v41760	4.62E+05	1.29E-04	2.78E-10	4.04E+05	1.05E-04	2.59E-10
v41086	2.66E+05	2.17E-04	8.15E-10	2.35E+05	2.15E-04	9.1E-10
v41904	3.17E+06	2.14E-03	6.74E-10	1.0E+07	8.9E-03	8.92E-10
v41112	2.43E+08	1.56E-04	6.39E-10	2.07E+05	1.7E-04	8.17E-10

EXAMPLE 3: ENGINEERING OF ANTI-CD28 PARATOPE FOR GENERATING CONSTRUCTS WITH VARYING BINDING AFFINITY FOR CD28

[0583] This example demonstrates the engineering of the anti-CD28 paratope TN228 to modulate binding affinity for CD28 engagement.

[0584] A panel of constructs encompassing a wide range of affinities against CD28 was constructed by mutating residues in the V_H and V_L domains of the anti-CD28 antibody, specifically focusing on residues within the CDR domains by IMGT definition. A panel was created as "in-format", i.e., in a trivalent and trispecific anti-DLL3/anti-CD3/anti-CD28 antibody construct format in the same format as that of construct v38582 (see, e.g., **FIG. 1A** for format).

[0585] Positions were selected for mutation by comparing the huTN228 V_H and V_L sequences with the parental murine sequences and the closest murine germlines to form hypotheses about which sites may have been subject to affinity maturation-driven mutations.

[0586] TABLE 11 below shows the varying binding affinities (K_D) for anti-CD28 engineered constructs in the trivalent and trispecific format similar to v38582, with comparison to huTN228 parental (unmutated) construct in the same format, indicating that most constructs comprising an affinity-engineered anti-CD28 paratope showed reduced binding affinity to CD28 compared to constructs comprising the unmodified anti-CD28 paratope.

TABLE 11: Binding Data Against CD28 for Affinity Engineered anti-CD28 Binding Domains Tested in a Trivalent and Trispecific Format

Construct	AA subst.	k_a (1/Ms)	k_d (1/s)	K_D (M)	Fold-change from WT
v38582 (parental huTN228)	none	2.82E+05	2.59E-03	9.27E-09	
v38961	N1066A (V_L)	2.66E+05	2.69E-03	1.02E-08	1
v38963	L1112AN (V_H)	2.03E+05	1.24E-02	6.53E-08	6.4
v38964	V1035G (V_L)	9.60E+04	1.51E-02	1.60E-07	15.7
v38965	Y1113S (V_H)	5.50E+04	2.74E-02	5.34E-07	52.5

EXAMPLE 4: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON *IN VITRO* CYTOTOXICITY TOWARDS DLL3-EXPRESSING TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ CD28+ T CELLS

[0587] To test the impact of antibody construct format on anti-tumor cell activity and preferential killing of such tumor cells expressing DLL3, selected antibody constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing DLL3.

[0588] Frozen human T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP cells such that the ratio of T cells to allogeneic tumor cells was adjusted to an E:T ratio of 5:1. The mixtures were incubated together with the respective antibody construct for 72 hours, after which tumor cell viability was assessed through whole well Mean Fluorescence Intensity using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Background subtraction was performed using the sliding parabola filter in the Harmony High-Content Imaging and Analysis Software (Perkin Elmer).

[0589] Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{MFI\ of\ treatment\ well}{MFI\ of\ untreated\ well}\right) \times 100$$

[0590] Dose response curves of primary human T cells in co-culture with DLL3+ NCI-H82-RFP cells as well as primary human Pan-T cells in co-culture with DLL3+ NCI-H82-RFP cells following treatment with anti-(DLL3xCD3xCD28) trivalent and trispecific antibody constructs and bispecific controls are shown in **FIGS. 2A-2C**, respectively. Calculated IC_{50} and maximum cytotoxicity (%) are shown in **TABLE 12** (primary human T cells) and **TABLE 13** (primary human Pan-T cells) below.

[0591] The trivalent and trispecific antibody constructs v39915 and v39917 showed maximum killing of at least 87%.

TABLE 12: Potency and Maximum Cytotoxicity of Anti-DLL3 Trivalent and Trispecific Antibody Constructs from Primary Human T Cells Co-cultured with NCI-H82 Target Cells for 72 Hours

Construct	NCI-H82	
	IC_{50} (pM)	Maximum Killing (%)
v38578	27.59	89.15
v38967	23.31	93.49
v38580	26.02	91.13
v38582	3.4	95.49

v38961	2.975	94.80
v38962	2.444	94.80
v38963	3.646	97.01
v38964	2.743	95.52
v38965	14.49	94.01
v38966	24.46	90.05
v38584	8.097	88.72
v38586	ND	27.04
v38588	17.08	85.78
v38589	25.25	89.16
v22277	ND	11.00

TABLE 13: Potency and Maximum Cytotoxicity Values of Anti-DLL3 Trivalent and Trispecific Antibody Constructs from Primary Human Pan-T Cells Co-cultured with NCI-H82 Target Cells for 72 Hours

Construct	NCI-H82	
	IC_{50} (pM)	Maximum Killing (%)
v38967	12.15	87.5
v39915	16.64	87.3
v39917	1.795	92.4
v39920	15.27	76.5
v39922	1.842	86.7
v38589	20.41	86.8
v22277	ND	9.75

EXAMPLE 5: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY PRIMARY HUMAN PAN-T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES AND DLL3-NEGATIVE TUMOR CELL LINES

[0592] The trivalent and trispecific antibody constructs described herein can activate T cells, e.g., by binding (in some instances simultaneously binding) DLL3 on a cancer cell and both CD3e and CD28 on one or more T cell(s), which can lead to cross-linking of CD3e and CD28. The resulting signalling cascade can trigger T cell activation, leading to cytokine secretion. To assess the impact of the format of trispecific antibody constructs on target-dependent and target-independent T cell activation, TNFα and IL-2 production was measured as described below.

[0593] Test articles (e.g., antibody constructs and controls) were diluted in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) and added to appropriate wells of the 384-well black flat bottom assay plates (ThermoFisher, Watham, MA). Primary human T cells were thawed and either plated alone or mixed with NCI-H82-RFP cells at an E:T ratio of 5:1 then added to the plates. Plates were

incubated for 72 hr at 37 °C with 5% carbon dioxide. Post incubation, 30 µL/well of supernatant was transferred to non-binding 384-well plates (Greiner-Bio-One, Kremsmünster, Austria) and stored at -20°C.

[0594] TNFα and IL-2 were quantified using MSD (Mesoscale Discovery, Piscataway, NJ). The night before cytokine quantification, MSD plates were blocked and coated in capture antibodies according to the manufacturers' instructions. The following day, plates were washed in PBS-T and 5 µl of assay diluent was added to each plate. The supplied TNFα and IL-2 standard (calibrator 1) was titrated as per manufacturer's instructions. Supernatants were thawed at room temperature and 5 µL of samples or standards were transferred to MSD plates. Detection antibodies were prepared at appropriate dilutions and 10 µL was added to each sample and standard well in MSD plates. The plates were sealed with aluminum foil and incubated away from light at room temperature for two hours. Plates were washed 3x in PBS-T and 40 µL MSD Gold read buffer T was added to each well. Plates were read on the MESO SECTOR 6000 and cytokine concentration was determined using MSD software. Data from a standard curve and samples were used to perform a nonlinear curve-fit with x-interpolation to obtain TNFα and IL-2 concentrations in pg/mL. Data are representative of 3 independent experiments with 2 donors.

[0595] Potency and maximum cytokine production of TNFα and IL-2 by primary human T cells when co-cultured with DLL3+ NCI-H82-RFP cells are shown in **TABLES 14-15**.

[0596] The results demonstrate potent cytokine induction in the presence of target cells, and low (or non detectable) nonspecific cytokine induction in the presence of DLL3-negative cells, for constructs comprising a costimulatory portion comprising an anti-CD3 Fab light C-terminally coupled to an anti-CD28 scFv domain (e.g., v39915, v39917, etc.).

TABLE 14: Potency and Maximum Cytokine Production of Tested Constructs

	With NCI-H82 Cells			
	IL-2		TNFα	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v38578	652.9	627.3	352.2	207.8
v38967	230.1	266.7	90.2	132.4
v38580	607.1	353.5	246.1	142.9
v38582	8.621	827.5	6.2	340.4
v38584	132.6	1808.5	63.8	658.6
v38961	ND	1044.9	5.9	293.5
v38962	8.7	859.3	9.4	352.0
v38963	14.9	673.1	18.5	327.2
v38964	10.6	736.6	8.5	233.9
v38965	29.9	398.0	30.2	179.6

v38966	215.4	53.2	99.8	72.4
v38586	ND	17.6	ND	8.2
v38588	1652	32.4	373.1	44.3
v38589	154	55.4	69.1	107.7
v22277	ND	0.3	ND	1.3

TABLE 15: Potency and Maximum Cytokine Production of Tested Constructs

	With NCI-H82 Cells			
	IL-2		TNFα	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v38967	362.8	1251.3	214.2	227.0
v39915	208.6	1184.7	216	184.7
v39917	71.3	2234.7	66.1	273.2
v39922	1903.0	59.6	95.4	5.9
v39920	68.0	13.5	5704	29.6
v38588	228.5	43.3	544.7	36.3
v38589	195.1	113.0	100.4	43.8
v22277	ND	2.2	ND	0.4

[0597] Potency and maximum cytokine production of TNFα and IL-2 by primary human T cells when co-cultured with DLL3 negative NCI-H460-RFP for 72 hours are shown in **TABLES 16-17**.

TABLE 16: Potency and Maximum Cytokine Production of Tested Constructs

	With NCI-H460 Cells			
	IL-2		TNFα	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v38578	ND	86.8	ND	20.8
v38967	ND	1.3	ND	0.6
v38580	ND	17.5	ND	3.0
v38582	517.7	132.4	774.8	120.6
v38584	371.1	5520.1	197.5	811.7
v38961	893.5	182.3	763	132.3
v38962	2539	244.4	1687	159.0
v38963	720.7	226.1	579.8	129.9
v38964	3130	62.6	1753	36.7
v38965	310.1	5.8	369.1	5.2
v38966	ND	4.0	ND	1.2
v38586	ND	1.4	ND	0.4
v38588	ND	1.9	ND	0.8
v38589	ND	4.9	ND	2.1
v22277	ND	0.8	ND	0.5

TABLE 17: Potency and Maximum Cytokine Production of Tested Constructs

	With NCI-H460 Cells

	IL-2		TNFα	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v38967	ND	21.6	ND	0.8
v39915	ND	89.4	ND	1.7
v39917	ND	57.1	ND	0.7
v38965	ND	6.4	ND	0.3
v39922	ND	4.6	ND	0.2
v39920	ND	7.1	ND	0.7
v38588	ND	13.2	ND	1.8
v38589	ND	5.4	ND	0.9
v22277	ND	21.6	ND	0.8

EXAMPLE 6: ABILITY OF ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND NATIVE DLL3 AS ASSESSED BY FLOW CYTOMETRY

[0598] To determine the impact of the format of trivalent and trispecific antibody constructs on their ability to bind DLL3 on DLL3-positive cells, the binding affinity to DLL3 was measured for certain constructs using flow cytometry as described below.

[0599] Specifically, the ability of certain trivalent and trispecific anti-(DLL3xCD3xCD28) antibody constructs as well as bispecific and reference constructs to bind to DLL3+ tumor cell lines NCI-H82, SHP-77 and COR-L279 cells, was assessed via whole cell FACS binding analysis. To that end, tumor cells were resuspended to $1x10^6$ cells/mL in FACS buffer (PBS + 2% FBS; Gibco) and seeded in a 96 well v-bottom plate (Sarstedt). Cells were mixed with antibody dilutions and incubated at 4 °C for one hour. Following incubation, cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 3 minutes. The cell pellet was resuspended in 50 µL FACS buffer with Alexa Fluor 647-labeled goat anti-human IgG (Jackson Labs) and incubated for 1 hour at 4 °C. Cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

[0600] The apparent binding affinities that were calculated from the binding curves of the tested constructs to DLL3 are shown in **TABLE 18**.

TABLE 18: Whole Cell Binding of Certain Trivalent and Trispecific Antibody Constructs to Native DLL3.

Construct	NCI-H82		SHP-77		COR-L279	
	Apparent Affinity (EC_{50}; nM)	B_{max} (MFI; anti-IgG AF647)	Apparent Affinity (EC_{50}; nM)	B_{max} (MFI; anti-IgG AF647)	Apparent Affinity (EC_{50}; nM)	B_{max} (MFI; anti-IgG AF647)
v38967	49.1	5423.5	159.6	1890.5	116.2	1353
v38588	32.3	5667	159.9	2627	97.3	1719
v39915	19.4	7650	61.1	4520	30.1	2422
v39920	13.4	6318	51.9	3936.5	28.7	2328
v39917	9.5	6348	34.1	3641.5	21.1	2080.5
v39922	12.6	6636	43.8	3720.5	28.0	2233
v22277	446.7	1096	279.8	671.5	777.6	570

EXAMPLE 7: ABILITY OF ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY

[0601] To determine the impact of the format of trivalent and trispecific antibody constructs on their ability to bind natively expressed CD3 and CD28, the binding affinity to CD3+CD28+ wild-type Jurkats (WT), CD3 knockout and CD28 knockout Jurkat cells was measured by flow cytometry as described below.

[0602] The ability of certain trivalent and trispecific anti-(DLL3xCD3xCD28) antibody constructs, as well as that of some bispecific and reference constructs, to bind to CD3 and CD28 on Jurkat cells was assessed via whole cell FACS binding analysis. To that end, a TCR knockout Jurkat cell line (CD3 KO) was purchased (BPS Bioscience) and a CRISPR CD28 knockout Jurkat cell line (CD28 KO) was engineered. Jurkat cells were collected and pelleted by centrifuging at 500xg for 5 min following by resuspension in 5 mL of Zombie Violet Live/Dead Stain (BioLegend) diluted in PBS and incubated for 20 minutes at 4 °C. Following incubation, cells were washed once by adding 5 mL FACS buffer and centrifuging at 400xg for 3 minutes. The cells were resuspended to $1.2x10^6$ cells/mL in FACS buffer (PBS + 2% FBS; Gibco) and seeded in a 96 well v-bottom plate (Sarstedt). Titrated antibody constructs pre-labeled overnight with Fab anti-Hu IgG Alexa Fluor 647 (Jackson) were added to the cells and incubated at 4 °C for one hour. Following incubation, cells were washed twice by adding 200 µL FACS buffer and centrifuging for 500xg for 5 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

[0603] Binding curves for the tested trispecific constructs and bispecific controls are shown in **FIGS. 3A** and **3B.** The apparent binding affinities and B_{max} of the tested constructs are shown in **TABLES 19A** and **19B**.

TABLE 19A: Whole Cell Binding of Tested Constructs to wild-type, TCR knockout (CD3 KO) and CD28 KO Jurkat cells.

	Jurkat WT		Jurkat CD28 KO		Jurkat CD3 KO	
	EC_{50} (nM)	Bmax	EC_{50} (nM)	Bmax	EC_{50} (nM)	Bmax
v38967	30.9	12274	60.4	5300	ND	151
v39915	34.7	11898.5	49.7	4664.5	ND	229.5
v39917	4.3	14016.5	10.8	5323	ND	142
v39920	42.8	8320.5	42.3	5195	ND	116.5
v39922	12.1	11448	11.3	6525.5	ND	142
v38589	20.6	1876	21.8	1207.5	ND	80.35
v38586	3.2	16898	ND	112	3.3	5840.5
v38588	59.1	9660	58.2	5125	ND	202.5
v22277	ND	65.45	ND	50.3	ND	94.3

TABLE 19B: Whole Cell Binding of Tested Constructs to wild-type, TCR knockout (CD3 KO) and CD28 KO Jurkat cells.

	Jurkat WT		Jurkat CD28 KO		Jurkat CD28 KO	
	EC_{50} (nM)	Bmax	EC_{50} (nM)	Bmax	EC_{50} (nM)	Bmax
v41086	0.8	25401	4.6	19054	ND	1199
v41112	0.6	26559	2.4	14543	ND	1221
v41759	0.5	28933	1.7	20423	ND	1149
v41760	2.4	30445	3.7	22788	ND	1299
v41753	1.8	16405	1.2	14428	ND	561
v41757	2.3	14464	1.9	14006	ND	486
v41761	1.3	20200	1.1	18735	ND	750
v41762	3.9	22837	2.9	20467	ND	543
v38589	6.5	18378	5.5	18308	ND	604
v22277	ND	840	ND	831	ND	543

EXAMPLE 8: ABILITY OF ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO INDUCE SUSTAINED *IN VITRO* CYTOTOXICITY TOWARDS DLL3-POSITIVE TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN PAN T-CELLS

[0604] To test the ability of certain trivalent and trispecific anti-(DLL3xCD3xCD28) antibody constructs to induce sustained *in vitro* cytotoxic activity towards DLL3+ tumor cells relative to

bispecific and reference constructs, selected constructs were tested in primary human T cell cultures with tumor cell lines expressing DLL3.

[0605] Frozen human T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP, SHP-77-RFP or COR-L279-RFP cells such that the ratio of T cells to tumor cells was adjusted to an effector to target cell (E:T) ratio of 1:5, 1:2 and 1:2 for the H82, SHP-77 and COR-L279 cell lines, respectively. The mixtures were incubated together with the titrated antibody constructs for 7 days, after which tumor cell viability was assessed through whole well Mean Fluorescence Intensity using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020).

[0606] Concentration response curves for the anti-DLL3 constructs are presented in **FIG. 4A** and IC_{50} values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLE 20A.** The results demonstrate that the trivalent and trispecific costimulatory construct v41086 induced cytotoxic effects toward DLL3-positive target cells in the presence of human T cells.

TABLE 20A: Cytotoxic Activity of Anti-DLL3 Trispecific, Bispecific and Reference Constructs Towards DLL3+ Tumor Cell Lines Grown in Culture with Primary Human T Cells

Construct	NCI-H82		SHP-77		COR-L279	
	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)
v38967	124.2	74.03	2449	93.18	517.1	68.46
v39915	229.7	75.48	1051	94.73	ND	53.68
v39917	78.18	94.88	143.6	95.90	73.59	82.08
v39920	ND	15.76	ND	50.18	ND	18.37
v39922	ND	30.11	79.26	95.13	ND	63.55
v38589	128	45.04	125.4	96.17	ND	43.91
v22277	ND	12.18	ND	14.51	ND	10.35
	NCI-H82		SHP-77		NCI-H524	
v41086	46.0	68.7	397.7	82.8	181.2	71.8
v41759	37.0	90.2	1719.0	73.5	69.2	76.3
v41760	53.9	59.4	19884.0	58.6	179.1	77.8
v41112	222.3	84.7	2062.0	76.8	583.6	76.4
v41094	285.8	73.4	2176.0	85.5	783.4	58.1
v41753	ND	34.2	999.8	80.0	ND	63.4
v41761	42.0	71.0	598.4	60.5	55.5	80.4
v41762	88.2	45.9	1939.0	62.2	ND	58.7
v41757	ND	31.8	16255.0	78.7	1570.0	73.7
v41754	ND	2.1	ND	20.8	ND	10.2
v38589	ND	19.8	666.2	67.6	ND	31.4

v22277 (Pali)	ND	-4.8	ND	1.9	ND	6.9

[0607] In a subsequent follow-on experiment, frozen human T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP, SHP-77-RFP, COR-L279-RFP, NCI-H69-RFP, NCI-H524-RFP and NCI-H526-RFP cells such that the ratio of T cells to tumor cells was adjusted to an effector to target cell (E:T) ratio of 1:5 for NCI-H82 and 1:2 for the remaining cell lines. The mixtures were incubated together with the antibody constructs titrated in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) + 15 mg/mL human serum albumin (MilliporeSigma) for 7 days, after which tumor cell viability was assessed through whole well Mean Fluorescence Intensity using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020).

[0608] Concentration response curves for the anti-DLL3 constructs are presented in **FIG. 4B** and IC_{50} values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLES 20B-C.**

TABLE 20B: IC_{50} Values and Maximum Percent Killing of Tested Constructs

Construct	NCI-H82		SHP-77		COR-L279	
	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)
v38967	66.4	95.28	ND	68.49	523.6	78.58
v39915	135	95.21	1639	91.83	619.5	83.53
v39917	30.94	95.01	110.9	97.58	82.06	94.83
v39920	152.2	23.99	ND	44.20	ND	42.27
v39922	63.31	71.24	79.64	95.72	83.99	82.20
v38588	556.7	68.20	ND	42.63	ND	62.14
v38589	257.6	77.89	127.6	96.03	786.6	81.95
v40309	147.4	87.39	ND	33.23	8816	76.59
v40310	64.73	90.14	ND	81.71	74864	81.12
v22277	ND	10.76	ND	11.79	ND	4.42

TABLE 20C: IC_{50} Values and Maximum Percent Killing of Tested Constructs

Construct	NCI-H69		NCI-H524		NCI-H526	
	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)	IC_{50} (pM)	Maximum Killing (%)
v38967	389.6	90.48	67.07	97.57	ND	90.96
v39915	266.7	85.74	71.23	96.60	2234	84.90
v39917	26.08	95.07	21.4	98.22	487.3	95.16
v39920	277.2	56.21	7470	90.01	ND	47.81
v39922	22.53	91.63	12.72	96.79	294.1	95.83
v38588	629.8	67.86	217.1	95.22	1812	50.56
v38589	136.8	86.96	109.2	95.19	3541	48.28
v40309	1100	81.98	742.1	92.65	ND	8.89

v40310	191	88.59	253.3	93.46	ND	19.57
v22277	ND	8.65	ND	8.03	ND	11.40

EXAMPLE 9: IMPACT OF TRISPECIFIC CONSTRUCT STRUCTURE ON PROLIFERATION OF HUMAN CD3+ T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES

[0609] To test the impact of antibody construct format on the ability to induce T cell proliferation in the presence and absence of DLL3+ tumor cell lines, T cell proliferation was measured with certain trispecific and bispecific constructs and reference constructs as described below.

[0610] Test articles (e.g., antibody constructs, controls, etc.) were diluted in RPMI1640 (Gibco) + 10% FBS (ThermoFisher Scientific) + 1% Pen/Strep (Gibco) and titrated 1:5 in appropriate wells of 96-well flat bottom assay plates (ThermoFisher Scientific). Human pan T cells were thawed and incubated with 10 µM CellTrace Violet staining solution (ThermoFisher Scientific) for 20 minutes at 37 °C. Following incubation with CellTrace Violet staining solution, T cells were washed and either mixed with NCI-H82 cells at an E:T ratio of 2:1 or plated alone. Plates were incubated at 37 °C, with 5% carbon dioxide, for 5 days, after which cells were transferred to the wells of a V-bottom 96 well plate and washed twice by adding 150 µL 1X PBS (Gibco) and centrifuging at 400xg for 3 minutes. Cells were resuspended in Zombie Violet Live/Dead Stain (BioLegend) diluted in PBS and incubated for 20 minutes at 4 °C. Following incubation, cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 3 minutes. Cells were then stained with FITC-conjugated anti-human CD4, PE-conjugated CD8, and incubated at 4 °C for 60 minutes. Following staining of cells, plates were washed three times with FACS Buffer. Following the final wash, cells were resuspended in 80 µL FCS Buffer and acquired on the LSRFortessa Cell Analyzer (BD Biosciences). Data analysis and T cell proliferation modeling were completed using FlowJo software (BD Biosciences).

[0611] Curves showing the percent of cells proliferated with the respective titrated antibody constructs tested and in either the presence or absence of target cells are shown in **FIG. 5A** and **FIG. 5B**. The data demonstrate that the trivalent and trispecific costimulatory construct v39917 induced proliferation of T cells in the presence of target cells (**FIG. 5A**) but did not induce proliferation in the absence of target cells (**FIG. 5B**).

EXAMPLE 10: IMPACT OF IMMOBILIZED ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO ASSAY FOR CYTOKINE RELEASE SYNDROME

[0612] To interrogate the impact of the format and geometry of certain trispecific antibody constructs on the induction of proinflammatory cytokine release in in vitro assays of cytokine release syndrome, selected trispecific anti-(DLL3xCD3xCD28) antibody constructs and control constructs were immobilized onto the surface of 96 well microtiter plates and tested in monocultures of primary human PBMCs. Monoclonal anti-CD3 antibody (catalogue: MAB9929, R&D Systems) and anti-CD28 antibody, v36577, which have been shown to induce cytokine release syndrome in patients as well as a mitogen, PHA (Invitrogen), were included as positive controls. An anti-TNFα monoclonal antibody (catalogue: A2019, Universal Biologicals) was included as a negative control for cytokine release syndrome. Anti-RSV-F monoclonal antibodies with IgG4 (v36992) and IgG1 LALADS (v39982) backbones were included as negative isotype control constructs.

[0613] Tested antibody constructs were immobilized onto the surface of 96 well U bottom polypropylene plates by incubating 1 µg of antibody in 60 µL of PBS for 2 hours at room temperature. Plates were washed twice with 200 µL of PBS to remove any unbound antibody. Frozen human PBMCs were thawed at 37 °C, washed in fresh cell culture media (RPMI + 10% heat-inactivated human AB serum) and 200 µL of 200 000 cells were added to the wells of the plate containing the immobilized antibody constructs. PBMCs were incubated with immobilized antibody constructs for 48 hours, after which supernatant was harvested and analyzed for IL-2 by MSD (Mesoscale Discovery).

[0614] The night before cytokine quantification, MSD plates were blocked and coated in capture antibodies according to the manufacturer's instructions. The following day, plates were washed in PBS-T and 5 µl of assay diluent was added to each plate. The supplied IL-2 standard (calibrator 1) was titrated as per manufacturer's instructions. Supernatants were thawed at room temperature and 5 µL of samples or standards were transferred to MSD plates. Detection antibodies were prepared at appropriate dilutions and 10 µL was added to each sample and standard well in MSD plates. The plates were sealed with aluminum foil and incubated away from light at room temperature for two hours. Plates were washed 3x in PBS-T and 40 µL MSD Gold read buffer T was added to each well. Plates were read on the MESO SECTOR 6000 and cytokine concentration

was determined using MSD software. Data from a standard curve and samples were used to perform a nonlinear curve-fit with x-interpolation to obtain IL-2 concentrations in pg/mL. Data are pooled from 4 individual donors.

[0615] Production of IL-2 by human PBMCs in the presence of the tested trispecific antibody constructs and controls are summarized in **TABLE 21**. The data demonstrate that the trivalent and trispecific costimulatory construct v39917, for example, induced significantly less IL-2 production compared to the positive control and trivalent and trispecific constructs with other formats and geometries, e.g., v38582, v38965.

TABLE 21: Mean IL-2 Production (and Standard Error of the Mean = SEM) Induced by Immobilized Anti-(DLL3xCD3xCD28) Trispecific Antibody Constructs from Monocultures of Primary PBMCs

Construct/Catalogue #	PBMCs	
	IL-2	
	Mean Cytokine (pg/mL)	SEM
v38967	85.2	42.6
v38582	2.59E+04	1.30E+04
v38965	1.02E+04	5.09E+03
v38964	1.91E+04	9.57E+03
v39915	47.3	23.6
v39920	26.5	13.3
v39917	305.8	152.9
v39922	240.2	120.1
v38588	22.1	11.1
v38589	25.3	12.6
v36577	6.02E+03	3.01E+03
MAB9929-100	33.8	16.9
A2019	32.1	16.1
PHA	318.5	159.3
v39982	24.3	12.1
v36992	16.7	8.3

EXAMPLE 11: IMPACT OF TRISPECIFIC ANTIBODY CONSTRUCT FORMAT ON UPREGULATION OF BCL-XL BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES

[0616] Activation of T cells through cross-linking of CD3e and CD28 using a trispecific and trivalent anti-(DLL3xCD3xCD28) antibody construct of the present disclosure can lead to

upregulation of anti-apoptotic, pro-survival signals in T cells. Hence, this example aimed at assessing the target-dependent upregulation of Bcl-xL expression in activated T-cells using certain constructs of the present disclosure and compare those to corresponding bispecific control constructs, as further described below.

[0617] To that end, tested antibody constructs were diluted to 20 nM in RPMI1640 (Gibco) + 10% FBS (ThermoFisher Scientific) + 1% Pen/Strep (Gibco) and plated in respective wells on a 96-well flat-bottom assay plate (ThermoFisher Scientific). Human CD3+ Pan T cells were thawed and mixed with NCI-H82 (DLL3-positive) tumor cell line at 1:1 E:T ratio and seeded into wells with 5 nM antibody constructs at 37 ℃ with 5% CO_2 overnight. Cells were transferred to 96-well V-bottom plates (Sarstedt) and washed twice with cold 1X PBS (Gibco). Cells were then stained with 1:1000 Zombie Violet Dye (BioLegend) in PBS and washed 2 times with FACS buffer. Next, cells were stained with cocktail of anti-CD45, anti-CD4, and anti-CD8 or single stain (BioLegend) controls for 60 minutes at 4 ℃ and washed with FACS buffer. Then, cells were fixed and permeabilized for intracellular staining with eBioscience Fixation/Permeabilization Concentrate (Invitrogen) diluted in eBioscience Fixation/Permeabilization Diluent (Invitrogen eBioscience). Cells were incubated for 30 minutes away from light at room temperature (RT). After staining, cells were washed twice with 1X eBioscience Permeabilization Buffer (Invitrogen) and stained with anti-Bcl-XL or isotype intracellular stains (CellSignaling) diluted in 1X Permeabilization buffer and incubated for 30 minutes at room temperature away from light. Finally, cells were washed twice with 1X eBioscience Permeabilization buffer, resuspended in FACS buffer, and events were captured on the BD LSRFortessa X-20 (BD Biosciences). Data were analyzed using FlowJo software (BD Biosciences) to generate values representing median fluorescence intensity (MFI).

[0618] Upregulation of Bcl-XL expression in activated T-cells using certain trispecific antibody constructs of the present disclosure as well as bispecific and reference constructs are shown in **FIGS. 6A** and **6B** (**** = $p < 0.0001$). The data show that the trivalent and trispecific antibody construct, v39917, which comprised a light chain comprising a C-terminally attached anti-CD28 scFv domain, induced Bcl-XL upregulation similar to that of the combination treatment including the two bispecific anti-(CD28xDLL3) and anti-(CD3xDLL3) control constructs.

EXAMPLE 12: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS

[0619] This example assessed the ability of certain trivalent and trispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SHP-77 small cell lung carcinoma model and compared their performance against corresponding bispecific control constructs.

SHP-77 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity

[0620] CR NCG female mice (Charles River Laboratories International, Inc.) were inoculated subcutaneously in the right flank region with SHP-77 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

[0621] Animals with a single tumor of a volume of 70-100 mm^3 were randomized and distributed in experimental groups according to the study protocol. Randomization was performed based on "Matched distribution" method (StudyDirectorTM software, version 3.1.399.19). Immediately after randomization, animals were inoculated intravenously with PBMCs ($1x10^7$ cells) from donor A. The date of PBMC inoculation is denoted as day 0.

[0622] 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 22**. The test article administration and the animal numbers in each study group are also shown in **TABLE 22**.

[0623] After randomization, tumor volumes were measured twice per week in two dimensions using a caliper. The animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals in detail. Animals were euthanized and necropsied when tumor volume reaches a maximal tumor volume of 2000 mm^3 or when tumor exceeded 10% of normal body weight, or when greater that 20% body weight loss, or after a maximum of 60 days post PBMC injection. Tumor volume data was analyzed using a mixed-effect model. Cumulative survival was analyzed using the Kaplan-Meier method.

[0624] As shown in **FIG. 10**, tumor regression was observed in donor A-engrafted mice treated with v39917 compared to the reference construct (v38589) and irrelevant mAb (v22277).

Treatment with 6.6 nmol/kg of v39917 resulted in full tumor regression in 3 out of 7 mice, and partial tumor regression in 1 mouse. No tumor regression was observed in mice treated with the reference construct (v38589) or the irrelevant mAb (v22277) at any of the doses tested. Percent survival was significantly higher in mice receiving 6.6 nmol/kg of v39917 compared to mice receiving equivalent doses of reference construct (Log-rank p= 0.0106) or irrelevant mAb (Log-rank p= 0.0153) (**FIG. 11**). Body weights remained stable for all mice (**FIG. 12**).

TABLE 22: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (7 mice-donor A)	v38967	6.6 nmol/kg	Q7Dx4
2 (7 mice-donor A)	v38967	33 nmol/kg	Q7Dx4
3 (7 mice-donor A)	v39917	1.32 nmol/kg	Q7Dx4
4 (7 mice-donor A)	v39917	6.6 nmol/kg	Q7Dx4
5 (7 mice-donor A)	v39917	33 nmol/kg	Q7Dx4
6 (7 mice-donor A)	v39915	1.32 nmol/kg	Q7Dx4
7 (7 mice-donor A)	v39915	6.6 nmol/kg	Q7Dx4
8 (7 mice-donor A)	v39915	33 nmol/kg	Q7Dx4
9 (7 mice-donor A)	v38588	6.6 nmol/kg	Q7Dx4
10 (7 mice-donor A)	v38588	33 nmol/kg	Q7Dx4
11 (7 mice-donor A)	v38589	1.32 nmol/kg	Q7Dx4
12 (7 mice-donor A)	v38589	6.6 nmol/kg	Q7Dx4
13 (7 mice-donor A)	v38589	33 nmol/kg	Q7Dx4
14 (7 mice-donor A)	v22277	33 nmol/kg	Q7Dx4

EXAMPLE 13: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS

[0625] This example assesses the ability of certain trivalent and trispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the NCI-H82 small cell lung carcinoma model and compares their performance against corresponding bispecific control constructs.

NCI-H82 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity

[0626] CR NCG female mice (Charles River Laboratories International, Inc.) are inoculated subcutaneously in the right flank region with NCI-H82 tumor cells ($2.5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

[0627] Animals with a single tumor of a volume of 60-100 mm^3 are randomized and distributed in experimental groups according to the study protocol. Randomization is performed based on "Matched distribution" method (StudyDirectorTM software, version 3.1.399.19). Immediately

after randomization, animals are inoculated intravenously with PBMCs ($1x10^7$ cells) from donor A. The date of PBMC inoculation is denoted as day 1.

[0628] 24 hours after randomization, animals are treated intravenously as per the study design shown in **TABLE 23**. The test article administration and the animal numbers in each study group are also shown in **TABLE 23**.

[0629] After randomization, tumor volumes are measured twice per week in two dimensions using a caliper. The animals are checked daily for morbidity and mortality. During routine monitoring, the animals are checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights are measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs are recorded for individual animals in detail. Animals are euthanized and necropsied when tumor volume reaches a maximal tumor volume of 2000 mm^3 or when tumor exceeds 10% of normal body weight, or when greater that 20% body weight loss, or after a maximum of 45 days post PBMC injection.

TABLE 23: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (9 mice-donor A)	v38967	33 nmol/kg	Q7Dx4
2 (9 mice-donor A)	v38967	53 nmol/kg	Q7Dx4
3 (9 mice-donor A)	v39917	6.6 nmol/kg	Q7Dx4
4 (9 mice-donor A)	v39917	33 nmol/kg	Q7Dx4
5 (9 mice-donor A)	v39917	53 nmol/kg	Q7Dx4
6 (9 mice-donor A)	v39915	6.6 nmol/kg	Q7Dx4
7 (9 mice-donor A)	v39915	33 nmol/kg	Q7Dx4
8 (9 mice-donor A)	v39915	53 nmol/kg	Q7Dx4
9 (9 mice-donor A)	v38588	33 nmol/kg	Q7Dx4
10 (9 mice-donor A)	v38588	53 nmol/kg	Q7Dx4
11 (9 mice-donor A)	v38589	6.6 nmol/kg	Q7Dx4
12 (9 mice-donor A)	v38589	33 nmol/kg	Q7Dx4
13 (9 mice-donor A)	v38589	53 nmol/kg	Q7Dx4
14 (7 mice-donor A)	v22277	53 nmol/kg	Q7Dx4

EXAMPLE 14: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS

[0630] This example assesses the ability of certain trivalent and trispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the NCI-H69 small

cell lung carcinoma model and compares their performance against corresponding bispecific control constructs.

NCI-H69 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity

[0631] CR NCG female mice (Charles River Laboratories International, Inc.) are inoculated subcutaneously in the right flank region with NCI-H69 tumor cells ($13.5x10^6$ cells) in 0.2 ml of RPMI 1640 without phenol red for tumor development.

[0632] Animals with a single tumor of a volume of 100-150 mm^3 are inoculated intravenously with PBMCs ($1x10^7$ cells) from donor A. Two (2) days after PBMC inoculation mice are randomized and distributed in experimental groups according to the study protocol. Homogeneity between groups is tested by analysis of variance (ANOVA).

[0633] On the day of randomization, animals are treated intravenously as per the study design shown in **TABLE 24**. The test article administration and the animal numbers in each study group are also shown in **TABLE 24**.

[0634] After tumor inoculation, tumor volumes are measured twice per week in two dimensions using a caliper. The animals are checked daily for morbidity and mortality. During routine monitoring, the animals are checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights are measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs are recorded for individual animals in detail. Animals are euthanized and necropsied when tumor volume reaches a maximal tumor volume of 2000 mm^3 or when tumor exceeds 10% of normal body weight, or when greater that 20% body weight loss, or after a maximum of 8 weeks post PBMC injection.

TABLE 24: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (7 mice-donor A)	v38967	6.6 nmol/kg	Q7Dx4
2 (7 mice-donor A)	v38967	33 nmol/kg	Q7Dx4
3 (7 mice-donor A)	v39917	1.32 nmol/kg	Q7Dx4
4 (7 mice-donor A)	v39917	6.6 nmol/kg	Q7Dx4
5 (7 mice-donor A)	v39917	33 nmol/kg	Q7Dx4
6 (7 mice-donor A)	v39915	1.32 nmol/kg	Q7Dx4
7 (7 mice-donor A)	v39915	6.6 nmol/kg	Q7Dx4
8 (7 mice-donor A)	v39915	33 nmol/kg	Q7Dx4
9 (7 mice-donor A)	v38588	6.6 nmol/kg	Q7Dx4
10 (7 mice-donor A)	v38588	33 nmol/kg	Q7Dx4
11 (7 mice-donor A)	v38589	1.32 nmol/kg	Q7Dx4
12 (7 mice-donor A)	v38589	6.6 nmol/kg	Q7Dx4

13 (7 mice-donor A)	v38589	33 nmol/kg	Q7Dx4
14 (7 mice-donor A)	v22277	33 nmol/kg	Q7Dx4

EXAMPLE 15: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING A TRIVALENT AND TRISPECIFIC CONSTRUCT TO A CLINICAL REFERENCE CONSTRUCT

[0635] The ability of a certain trivalent and trispecific antibody construct of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SHP-77 small cell lung carcinoma model was assessed and its performance was compared against a clinical reference construct.

SHP-77 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity

[0636] CR NCG female mice (Charles River Laboratories International, Inc.) were inoculated subcutaneously in the right flank region with SHP-77 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

[0637] Animals with a single tumor of a volume of 70-100 mm^3 were randomized and distributed in experimental groups according to the study protocol. Randomization was performed based on "Matched distribution" method (StudyDirectorTM software, version 3.1.399.19). Immediately after randomization, animals were inoculated intravenously with PBMCs ($1x10^7$ cells) from donor A. The date of PBMC inoculation was denoted as day 0.

[0638] When the average tumor volume reached 100-120 mm^3, animals were treated intravenously as per the study design shown in **TABLE 25**. The test article administration and the animal numbers in each study group are also shown in **TABLE 25**.

[0639] After randomization, tumor volumes were measured twice per week in two dimensions using a caliper. The animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals in detail. Animals were euthanized and necropsied when tumor volume reached a maximal tumor volume of 2000 mm^3 or when tumor exceeded 10% of normal body weight or when greater than 20% body weight loss, or after a maximum of 60 days post PBMC injection. Tumor volume data were analyzed using a mixed-effect model. Cumulative survival was analyzed. Cumulative survival was analyzed using the Kaplan-Meier method.

TABLE 25: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (7 mice-donor A)	v38967	2.5 mg/kg	Q7Dx4
2 (7 mice-donor A)	v38589	2.5 mg/kg	Q7Dx4

[0640] As shown in **FIG. 7**, tumor growth delay was observed in donor A-engrafted mice treated with 2.5 mg/kg of the trivalent and trispecific antibody construct v38967 when compared to the clinical benchmark. Percent survival was significantly higher in mice receiving 2.5 mg/kg of v38967 compared to mice receiving equivalent doses of reference construct (Long-rank p = 0.0047) (**FIG. 8**). Body weights remained stable for all mice (**FIG. 9**).

EXAMPLE 16: INVESTIGATION OF THE ABILITY OF TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND CD3 AND CD28 ON THE SAME T CELL

[0641] To determine the impact of the format of trispecific antibody constructs on their ability to bind CD3 and CD28 in either cis (i.e., on the same cell) or in trans (i.e., on two different cells), cell-cell bridging of Jurkat cells was assessed via flow cytometry.

[0642] Briefly, Jurkat T cells, with either the expression of CD28 knocked out ($Jurkat^{CD28\text{-}KO}$) or CD3 knocked out ($Jurkat^{CD3\text{-}KO}$), such that they only expressed CD3 or CD28, respectively, were resuspended in 5 µM of CellTrace™ Violet (Invitrogen) or CellTracker™ Green CMFDA (Invitrogen), respectively, in serum-free media (RPMI 1640; Gibco) and incubated for 37 °C for 20 minutes. Following incubation, RPMI 1640 + 10% FBS (Gibco) was added to cells, centrifuged at 500 xg for 5 minutes and washed twice with PBS (Gibco). CellTrace™ Violet-stained $Jurkat^{CD28\text{-}KO}$ and CellTracker™ Green-Stained $Jurkat^{CD3\text{-}KO}$ cells were mixed 1:1 and stained with eBioscience Fixable Viability Dye eFluor 660 (Invitrogen). After viability staining, $1x10^5$ cells of the $Jurkat^{CD28\text{-}KO}$ and $Jurkat^{CD3\text{-}KO}$ mixture were added to the wells of a 96-well V bottom plate (Sarstedt). Cells were mixed with titrating amount of test article and incubated at 4 °C for one hour. Following incubation, cells were acquired on the BD LSRFortessa Cell Analyzer and analyzed for percent of CellTrace™ Violet and CellTracker™ Green double positive events that indicate T cell-T cell bridging.

[0643] EC_{50} values and mean maximum percent double positive events of the tested constructs from the concentration response curves are presented in **TABLES 26A** and **26B**.

TABLE 26A: Summary Table of EC_{50} and Maximum Percent Double Positive Events of Cell Cross-linking Induced by Certain Trispecific and Bispecific Constructs

Antibody Construct	EC_{50} (pM)	Maximum Percent Double Positive Events (Mean ± SD)
v38967	ND	1.08 ± 0.03
v38588	ND	0.91 ± 0.57
v39915	ND	0.96 ± 0.40
v39920	ND	0.66 ± 0.04
v39917	ND	0.67 ± 0.40
v39922	ND	0.69 ± 0.21
v38582	157.5	16.7 ± 2.55
v38584	240.4	26.6 ± 0.57
v22277	ND	0.75 ± 0.17

TABLE 26B: Summary Table of EC_{50} and Maximum Percent Double Positive Events of Cell Cross-linking Induced by Certain Additional Trispecific and Bispecific Constructs

Antibody Construct	EC_{50} (pM)	Maximum Percent Double Positive Events (Mean ± SD)
v41086	ND	0.88 ± 0.20
v41753	ND	0.64 ± 0.09
v38584	213.9	20.45 ± 0.35
v22277	ND	0.73 ± 0.23

[0644] The data demonstrate that the trivalent and trispecific antibody constructs, v39915 and v39917, and v41086 which comprise a light chain comprising an anti-CD3 Fab portion (e.g., a VL and CL domain) coupled via its C-terminus to an anti-CD28 scFv domain, do not cross-link T cells, i.e., did not bind two T cells in trans (e.g., did not show any double positive events), but instead seem to engage CD3 and CD28 in cis, on the same T cell. Other trivalent and trispecific constructs, e.g., v38582 and v38584, that possessed a different format and geometry compared to v39915 and v39917, and v41086, did show T cell cross-linking and thus appear to bind CD3 and CD28 on different T cells.

EXAMPLE 17: IMPACT OF TRISPECIFIC ANTI-(DLL3XCD3XCD28) ANTIBODY CONSTRUCTS ON THE VIABILITY OF PRIMARY CD3+ T CELLS

[0645] To determine the impact of the format of trispecific anti-(DLL3xCD3xCD28) antibody constructs on the viability of human CD3+ T cells, selected constructs were assessed in monocultures of primary human Pan T cells. Puromycin (Life Technologies) was included as a positive control for T cell death.

[0646] Antibody constructs were diluted to 50 nM in RPMI1640 (Gibco) + 10% FBS (ThermoFisher Scientific) + 1% Pen/Strep (Gibco) and plated in respective wells on a 384-well flat, optical-bottom assay plate (Revvity). Frozen human Pan T cells were thawed at 37 °C and combined with CellTox™ Green dye (Promega). CellTox™ Green binds to the DNA of cells with impaired membrane integrity and fluoresces green providing a fluorescent read-out for cell death. Pan T cells with CellTox™ Green dye were added to diluted constructs and incubated at 37 °C with 5% CO_2 for 48 hours. After 48 hours, fluorescence was detected using the Operetta® CLS™ (Revvity) and data were analyzed using the Harmony High-Content Imaging and Analysis System (Revvity). ΔMFI of CellTox™ Green was calculated by subtracting the mean MFI of no treatment wells.

[0647] ΔMFI of CellTox™ Green of Pan T cells following treatment is summarized in **TABLES 27A** and **27B.**

TABLE 27A: ΔMFI CellTox Green of Pan T cells Incubated with Certain Bispecific and Trispecific Antibody Constructs

Construct	MFI, CellTox™ Green	
	Mean	Standard Deviation
v38967	58.1	9.6
v38588	42.6	36.3
v39915	119.2	33.3
v39920	119.6	26.7
v39917	41.3	13.4
v39922	50.1	27.7
v38589	15.5	15.1
v37035	298.0	110.1
v38582	307.2	8.1
v38584	180.2	40.4
v22277	82.1	26.4
Puromycin	1245.1	120.4

TABLE 27B: ΔMFI CellTox Green of Pan T cells Incubated with Certain Bispecific and Trispecific Antibody Constructs

Construct	MFI, CellTox™ Green	
	Mean	Standard Deviation
v41086	0.2	6.5
v41753	2.9	1.1
v38584	114.8	10.6
v37035	115.2	7.4
v22277	9.3	7.8
Puromycin	555.6	5.1

[0648] The data demonstrate that the trivalent and trispecific antibody constructs, v39915, v39917, and v41086, which comprise a light chain comprising an anti-CD3 Fab portion (e.g., a VL and CL domain) coupled via its C-terminus to an anti-CD28 scFv domain, reduced T cell viability to a significantly lower degree when compared to trispecific constructs with different formats and/or geometries, e.g., the trispecific constructs v38582 and v38584.

EXAMPLE 18: INDUCTION OF T CELL CYTOXICITY FOLLOWING REPETITIVE STIMULATION WITH TRISPECIFIC ANTI-(DLL3xCD3xCD28) CONSTRUCT

[0649] To investigate the ability of certain trivalent and trispecific antibody constructs to induce repetitive anti-tumor cytotoxicity, select constructs were assessed in a repeat challenge assay with primary human pan T cells in culture with NCI-H82 target cells.

[0650] For the initial stimulation, frozen human pan T cells were thawed at 37 °C and mixed with NCI-H82-RFP target cells in assay media (RPMI 1640 + 10% FBS; Gibco) at an E:T ratio of 5:1. Test articles were diluted in assay media and added to human pan T and NCI-H82 cell co-cultures and incubated for 72 hours. For the second round of stimulation and all subsequent stimulations, cells were collected from culture, centrifuged at 500 xg for 5 minutes, replenished with fresh assay media, and diluted with fresh NCI-H82-RFP target cells at an E:T ratio of 5:1 (**TABLE 28A**) or 4:1 (**TABLE 28B**). Test articles were diluted in assay media, added to the cell mixture, and cultures were incubated for 72-96 hours. Following each round of stimulation, tumor cell cytotoxicity was assessed through whole well mean fluorescent intensity (MFI) of the red fluorescent NCI-H82-RFP cells, using the Operetta CLS High Content Analysis System (Perkin Elmer). Data are representative of two independent experiments with two donors.

[0651] Percent cytotoxicity was determined using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{MFI\ of\ treatment\ wells}{MFI\ of\ no\ treatment\ wells}\right) \times 100$$

[0652] Percent cytotoxicity of target cells (mean ± SD) in co-culture with human pan T cells following each round of stimulation with select constructs is shown in **TABLES 28A** and **28B.**

TABLE 28A: Cytotoxicity of Target Cells Induced by T Cells Repetitively Stimulated with Tested Constructs

Stimulation Round	Percent Cytotoxicity (%; mean ± SD)					
	Construct #					
	v38967	v38588	v39917	v39922	v39915	v38589
1	94.6 ± 0.4	88.4 ± 1.9	94.8 ± 0.8	92.3 ± 0.6	93.0 ± 0.9	91.2 ± 1.6
2	95.4 ± 0.3	94.4 ± 0.1	95.0 ± 0.2	94.6 ± 0.1	95.5 ± 0.2	94.8 ± 0.2
3	96.8 ± 0.4	53.2 ± 1.9	97.0 ± 0.8	7.9 ± 0.6	97.0 ± 0.9	15.3 ± 1.6
4	86.8 ± 0.4	ND	86.1 ± 0.8	ND	86.2 ± 0.9	ND
5	79.2 ± 0.4	ND	64.1 ± 1.9	ND	40.6 ± 0.8	ND

TABLE 28B: Cytotoxicity of Target Cells Induced by T Cells Repetitively Stimulated with Tested Constructs

Stimulation Round	Percent Cytotoxicity (%; mean ± SD)		
	Construct #		
	v41086	v41753	v38589
1	98.2 ± 0.1	97.7 ± 0.2	97.7 ± 0.1
2	97.9 ± 0.1	97.1 ± 0.2	97.1 ± 0.2
3	97.6 ± 0.3	4.6 ± 1.4	10.1 ± 0.7
4	94.1 ± 0.1	ND	ND
5	91.3 ± 0.2	ND	ND

[0653] The data demonstrates that the trivalent and trispecific Het-Fab antibody constructs, v39915, v39917 and v41086, as well as the trivalent and trispecific $scFv^2$ antibody construct, v38967, all of which comprising a light chain comprising an anti-CD3 Fab light chain (e.g., a VL and CL domain) coupled via its C-terminus to an anti-CD28 scFv domain, elicited anti-tumor T cell cytotoxicity across all rounds of stimulation, whereas the anti-(DLL3xCD3) bispecific control constructs v38588 (scFv-Fab) and v39922 (Fab-Fab), the anti-(DLL3xCD3xHA) trispecific control with an irrelevant anti-HA (hemagglutinin) ScFv domain in place of the anti-CD28 ScFv domain, as well as the reference construct v38589 elicited significantly lower anti-tumor cytotoxicity starting at stimulation round 3 onwards.

EXAMPLE 19: THERMAL STABILITY OF ANTI-(DLL3xCD3xCD28) TARGETING TRIVALENT AND TRISPECIFIC ANTIBODY CONSTRUCTS

[0654] The thermal stability of engineered full-sized trispecific anti-DLL3/anti-CD3/anti-CD28 antibody constructs, and bispecific anti-DLL3/anti-CD3 constructs was assessed by differential scanning calorimetry (DSC).

[0655] All DSC experiments were carried out using a VP-Capillary DSC (Malvern Instruments Ltd) instrument. The constructs were diluted to 0.4 mg/mL with 0.4 mL loaded into the 96 well plates and measured with a scan rate of 1 °C/min from 20 to 100 °C. Data was analyzed using the Origin 7 software with the VP-Capillary DSC option with the H6Su (50 mM histidine, 9% sucrose, pH 6.0, 0.02% PS80) buffer background subtracted.

[0656] TABLE 29 below shows the maximum melting temperatures (Tm) for each of the peaks in the thermograms of the trispecific and bispecific antibody constructs tested. The term "$scFv^2$" indicates that the construct comprised two scFv domains as binding domains, which are not coupled to one another in tandem, e.g., as shown in **FIG. 1B** or **1C**, etc.

TABLE 29: **Thermostability Data of Tested Antibody Constructs**

Antibody Construct	Targeting Domains	Tm1 (°C)	Tm2 (°C)	Tm3 (°C)
v41086	scFv, Fab, Fab	68.3	73.9	84.4
v41094	scFv, Fab, Fab	68.6	74.3	84.7
v41753	scFv, Fab, Fab	58.5	72.4	84.3
v41754	scFv, Fab, Fab	58.3	72.4	84.3
v41112	scFv, Fab, Fab	68.3	78.1	78.5
v41757	scFv, Fab, Fab	58.7	74.9	78.2
v41759	$scFv^2$, Fab	63.5	81.6	
v41760	$scFv^2$, Fab	63.8	67.7	83.8
v41761	scFv, Fab	62.3	81.6	
v41762	scFv, Fab	64.8	68.0	83.3

[0657] The results shown in **TABLE 29** demonstrate that the tested antibody constructs exhibited thermostability profiles comparable to those of conventional IgG1 antibodies.

[0658] The trivalent and trispecific antibody constructs were further tested (i) for freeze-thaw stability, and showed no significant change in purity post 3 cycles freezing to -80 °C followed by thawing to 4 °C, and (ii) in an accelerated stress test in which the construct showed only minimal change in purity post 14 days incubation at 40 °C, using a construct concentration of 1 mg/mL.

[0659] Further, the trivalent and trispecific antibody constructs were tested for its stability upon treatment with low pH (pH 3.5) and high pH (pH 10.0), in which the constructs showed no significant change in purity post 3 hours and 4 days, respectively. The purity of the trispecific antibody construct v37634 post each of the stress condition is compared in the **TABLE 30** below.

TABLE 30: Monomer Purity (%) of Trivalent and Trispecific Construct Post Stress Treatment Compared to No Treatment Condition

	Monomer Purity (%) by HPLC-SEC Analysis				
	v41086	**v41094**	**v41112**	**v41759**	**v41760**
No Treatment	98.6	100	100	98.8	99
3 cycles freeze-thaw	98.4	99.5	99.2	99	99.1
14 days incubation at 4 °C	98.3	99.4	99.7	98.8	99
14 days incubation at 40 °C	98.3	99.5	99.3	97.1	97.6
2 hours at pH 3.3	94.1	94.2	95.3	69.4	68.6
4 days at pH 10	95.9	96	95.1	93.2	92.3

EXAMPLE 20: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON *IN VITRO* CYTOTOXICITY TOWARDS DLL3-EXPRESSING TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ CD28+ T CELLS

[0660] To test the impact of antibody construct format on its anti-tumor cell activity and preferential killing of such tumor cells expressing DLL3, selected constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing DLL3.

[0661] Frozen human T cells were thawed at 37 °C and mixed with DLL3+ NCI-H82-RFP cells such that the ratio of T cells to allogeneic tumor cells was adjusted to an E:T ratio of 2:1. The mixtures were incubated together with the antibody constructs for 72 hours, after which tumor cell viability was assessed through whole well Mean Fluorescence Intensity using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Background subtraction was performed using the sliding parabola filter in the Harmony High-Content Imaging and Analysis Software (Perkin Elmer).

[0662] Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{MFI\ of\ treatment\ well}{MFI\ of\ untreated\ well}\right) \times 100$$

[0663] Calculated IC_{50} and maximum cytotoxicity (%) are shown in **Table 31.** Data are representative of 3 independent experiments with 3 donors. The data demonstrate that the trivalent

and trispecific costimulatory antibody construct, v41086, exhibited maximum cytotoxicity of at least 74%.

TABLE 31: Potency and Maximum Cytotoxicity of anti-DLL3 Trispecific Antibody Constructs from Primary Human T Cells Co-cultured with NCI-H82 Target Cells for 72 hours

Construct	NCI-H82	
	IC_{50} (pM)	Maximum Cytotoxicity (%)
v41086	9.175	74.89
v41094	40.06	73.39
v41112	38.52	77.63
v41759	5.649	75.40
v41760	11.16	71.55
v38589	22.09	59.48
v22277	ND	12.76

EXAMPLE 21: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY PRIMARY HUMAN PAN-T CELLS IN CO-CULTURE WITH DLL3-POSITIVE AND DLL3-NEGATIVE TUMOR CELL LINES

[0664] The trispecific antibody constructs described herein can activate T cells, e.g., by simultaneously binding a tumor-associated antigen (e.g., DLL3) on a cancer cell and both CD3e and CD28 on one or more T cell(s), which can lead to cross-linking of CD3e and CD28. The resulting signalling cascade can trigger T cell activation, leading to cytokine secretion. To assess the impact of the format of various trispecific constructs on target-dependent and target-independent T cell activation, IL-2, TNFα and IFNγ production was measured as described below.

[0665] Test articles (e.g., antibody constructs and controls) were diluted in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) and added to appropriate wells of the 384-well black flat bottom assay plates (ThermoFisher). Primary human T cells were thawed and plated alone or mixed with NCI-H82-RFP or NCI-H292-RFP cells at an E:T ratio of 2:1 then added to the plates 384-well optical bottom plates (Revvity). Plates were incubated for 72 hr at 37 °C with 5% carbon dioxide. Post incubation, 40 µL/well of supernatant was transferred to non-binding 384-well plates (Greiner-Bio-One) and stored at -20°C.

[0666] IL-2, TNFα and IFNγ were quantified using 3-plex MSD (Mesoscale Discovery, Piscataway, NJ). Multiplex capture antibody solution was prepared according to the

manufacturers' instructions and 10 µL was added to each well of the 3-plex MSD plates. Plates were sealed and incubated at 4 ºC overnight. The following day, plates were washed with 3x with PBS-T and 10 µl of assay diluent was added to each plate. The supplied IL-2, TNFα and IFNγ standard (calibrator 1) was titrated as per manufacturer's instructions. Supernatants were thawed at room temperature and 10 µL of samples or standards were transferred to MSD plates. The plates were sealed and incubated overnight at 4 ºC. The following day, plates were washed 3X with PBS-T. Detection antibody cocktail was prepared according to manufacturer's instructions and 10 µL was added to each sample and standard well in MSD plates. The plates were sealed and incubated away from light at room temperature for two hours with shaking. Plates were washed 3x in PBS-T and 40 µL MSD Gold read buffer B was added to each well. Plates were read on the MESO SECTOR 6000, and cytokine concentration was determined using MSD Workbench software. Data from the standard curve and samples were used to perform a nonlinear curve-fit with x-interpolation to obtain IL-2, TNFα and IFNγ concentrations in pg/mL. Data are representative of 3 independent experiments with 3 donors.

[0667] Potency and maximum cytokine production of IL-2, TNFα and IFNγ by primary human T cells when co-cultured with DLL3 positive NCI-H82-RFP for 72 hours in the presence of anti-DLL3 antibody constructs are shown in **TABLE 32** below.

[0668] The data in the tables below demonstrate that the trivalent and trispecific costimulatory antibody construct, v41086, exhibited potent cytokine induction in the presence of target cells, but induced no significant amounts of cytokines in the presence of DLL3-negative cells.

TABLE 32: Potency and Maximum Cytokine Production Elicited by Tested Constructs in the Presence of DLL3-Positive Cells

	With NCI-H82 Cells					
	IL-2		TNFα		IFNγ	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v41086	42.9	1679.3	39.8	340.8	40.7	14639.0
v41094	289.2	1792.0	173.3	337.7	183.0	14269.9
v41112	207.7	1498.2	127.4	290.7	155.0	13228.5
v41759	42.2	1981.7	25.7	352.4	36.6	16793.3
v41760	94.6	776.2	60.9	230.6	85.4	13098.7
v41753	127.6	148.4	130.5	121.1	261.4	8084.8
v41754	40.1	19.2	3117.0	15.8	31741.0	1020.8
v41757	452.9	114.3	295.3	93.4	607.5	7328.1
v41761	23.8	869.8	18.3	283.6	25.8	15260.8
v41762	63.9	663.3	49.6	232.4	52.8	12709.6

v38589	1663.0	353.9	267.5	152.1	268.1	7566.0
v22277	ND	4.0	ND	1.7	ND	9.7

[0669] Potency and maximum cytokine production of TNFα and IL-2 by primary human T cells when co-cultured with DLL3 negative NCI-H292-RFP for 72 hours in the presence of anti-DLL3 antibody constructs are shown in **TABLE 33** below.

TABLE 33: Potency and Maximum Cytokine Production Elicited by Tested Constructs in the Presence of DLL3-Negative Cells

	With NCI-H292 Cells					
	IL-2		TNFα		IFNγ	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v41086	ND	6.5	ND	7.9	ND	99.6
v41094	ND	5.9	ND	5.0	ND	23.9
v41112	ND	6.2	ND	6.3	ND	24.0
v41759	ND	5.4	ND	4.8	ND	39.8
v41760	ND	4.1	ND	5.3	ND	29.7
v41753	ND	3.9	ND	4.0	ND	14.7
v41754	ND	4.6	ND	4.0	ND	11.6
v41757	ND	4.4	ND	4.1	ND	18.7
v41761	ND	5.1	ND	5.5	ND	28.7
v41762	ND	6.6	ND	6.1	ND	30.7
v38589	ND	4.8	ND	5.5	ND	21.0
v22277	ND	5.0	ND	6.7	ND	27.1

[0670] Potency and maximum cytokine production of IL-2, TNFα and IFNγ by primary human T cells when cultured alone for 72 hours in the presence of anti-DLL3 antibody constructs are shown in **TABLE 34** below.

TABLE 34: Potency and Maximum Cytokine Production Elicited by Tested Constructs in the Presence of T cells alone (i.e., Absence of Tumor Cells)

	T Cells Alone					
	IL-2		TNFα		IFNγ	
	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)	EC_{50} (pM)	Maximum cytokine (pg/mL)
v41086	ND	5.5	ND	2.1	ND	29.6
v41094	ND	4.9	ND	2.1	ND	5.5
v41112	ND	3.6	ND	2.3	ND	6.2
v41759	ND	5.3	ND	2.3	ND	12.8
v41760	ND	3.2	ND	2.0	ND	23.0
v41753	ND	3.3	ND	2.1	ND	6.9
v41754	ND	4.5	ND	1.7	ND	7.3
v41757	ND	3.4	ND	2.1	ND	9.9

v41761	ND	4.3	ND	2.5	ND	10.2
v41762	ND	4.3	ND	3.1	ND	15.4
v38589	ND	7.9	ND	3.2	ND	12.9
v22277	ND	6.4	ND	2.4	ND	9.0

EXAMPLE 22: ABILITY OF ANTI-(DLL3xCD3xCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND NATIVE DLL3 AS ASSESSED BY FLOW CYTOMETRY

[0671] To determine the impact of the format of trivalent and trispecific antibody constructs on their ability to specifically bind DLL3 on DLL3-positive cells with minimal binding to DLL3-negative cells, the binding affinity to DLL3 was measured for certain constructs using flow cytometry as described below.

[0672] Specifically, the ability of certain trispecific anti-(DLL3xCD3xCD28) antibody constructs as well as bispecific controls and reference constructs to bind to DLL3-positive tumor cell line NCI-H82 with minimal binding to DLL3-negative tumor cell line NCI-H292, was assessed via whole cell FACS binding analysis. To that end, tumor cells were resuspended in Zombie Violet Live/Dead Stain (BioLegend) diluted in PBS and incubated for 20 minutes at 4 °C. Following the incubation, cells were washed once with FACS Buffer and seeded in a 96 well v-bottom plate (Sarstedt). Cells were mixed with antibody dilutions and incubated at 4 °C for one hour. Following incubation, cells were washed twice by adding 200 µL FACS buffer per well and centrifuging at 500xg for 5 minutes. The cell pellet was resuspended in 50 µL FACS buffer with Alexa Fluor 647-labeled goat anti-human IgG (Jackson Labs) and incubated for 1 hour at 4 °C. Cells were washed twice by adding 200 µL FACS buffer and centrifuging at 500xg for 5 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

[0673] The apparent binding affinities that were calculated from the binding curves of the tested constructs to DLL3 are shown in **TABLE 35**.

TABLE 35: Whole Cell Binding of Certain Trivalent and Trispecific Antibody Constructs to Native DLL3.

Construct	NCI-H82		NCI-H292	
	Apparent Affinity (EC_{50}; nM)	B_{max} (MFI; anti-IgG AF647)	Apparent Affinity (EC_{50}; nM)	B_{max} (MFI; anti-IgG AF647)
v41386	2.37	10316	ND	822
v41086	2.68	8822	ND	367
v41094	4.86	8472	ND	242
v41112	11.42	7895	ND	240
v41759	5.01	8946	ND	480

v41760	5.97	8348	ND	326
v41753	3.02	9169	ND	312
v41754	4.10	8280	ND	282
v41757	11.87	7768	ND	266
v41761	3.64	8864	ND	517
v41762	4.18	7951	ND	325
v22277	ND	774	ND	872

EXAMPLE 23: IMPACT OF FORMAT OF TRISPECIFIC ANTIBODY CONSTRUCTS ON PROLIFERATION OF HUMAN CD3+ T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES

[0674] To test the impact of format on the ability of an antibody construct to induce T cell proliferation in the presence and absence of DLL3+ tumor cell lines, T cell proliferation was measured with certain trispecific and bispecific constructs and clinical benchmarks as described below.

[0675] Test articles (e.g., antibody constructs, controls, etc.) were diluted in RPMI1640 (Gibco) + 10% FBS (ThermoFisher Scientific) + 1% Pen/Strep (Gibco) and titrated 1:5 in appropriate wells of 96-well flat bottom assay plates (ThermoFisher Scientific). Human pan T cells were thawed and incubated with 10 µM CellTrace Violet staining solution (ThermoFisher Scientific) for 20 minutes at 37 °C. Following incubation with CellTrace Violet staining solution, T cells were washed and either mixed with NCI-H82 cells at an E:T ratio of 2:1 or plated alone. Plates were incubated at 37 °C, with 5% carbon dioxide, for 5 days, after which cells were transferred to the wells of a V-bottom 96 well plate and washed twice by adding 150 µL 1X PBS (Gibco) and centrifuging at 400xg for 5 minutes. Cells were resuspended in Fixable Viability Dye eFluor 660 (Invitrogen) diluted in PBS and incubated for 20 minutes at 4 °C. Following incubation, cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 5 minutes. Cells were then stained with APC-Cy7-conjugated anti-human CD45 and incubated at 4 °C for 60 minutes. Following staining of cells, plates were washed three times with FACS Buffer. Following the final wash, cells were resuspended in 80 µL FCS Buffer and acquired on the LSRFortessa Cell Analyzer (BD Biosciences). Data analysis and T cell proliferation modeling were completed using FlowJo software (BD Biosciences).

[0676] Curves showing the percent of cells proliferated with the respective titrated antibody constructs tested are shown in **FIG. 13A**. Histograms of proliferation at 200 pM are displayed in **FIG. 13B**.

EXAMPLE 24: IMPACT OF IMMOBILIZED TRISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO ASSAY FOR CYTOKINE RELEASE SYNDROME

[0677] To interrogate the impact of the format and geometry of certain trispecific antibody constructs on the induction of proinflammatory cytokine release in *in vitro* predictive assays of cytokine release syndrome, selected trispecific anti-(DLL3xCD3xCD28) antibody constructs and control constructs were immobilized onto the surface of 96 well microtiter plates and tested in monocultures of primary human PBMCs. Monoclonal anti-CD3 antibody (catalogue: MA5-41754, Invitrogen) and anti-CD28 antibody, v36577, which have been shown to induce cytokine release syndrome in patients as well as a mitogen, PHA (Invitrogen), were included as positive controls. An anti-TNFα monoclonal antibody (catalogue: A2019, Universal Biologicals) was included as a negative control for cytokine release syndrome. Anti-RSV-F monoclonal antibody with an IgG1 LALADS (v39982) backbone was included as a negative isotype control construct.

[0678] Tested antibody constructs were immobilized onto the surface of 96 well U bottom polypropylene plates by incubating 1 µg of antibody construct in 60 µL of PBS for 2 hours at room temperature. Plates were washed twice with 200 µL of PBS to remove any unbound antibody construct. Frozen human PBMCs were thawed at 37 °C, washed in fresh cell culture media (RPMI + 10% heat-inactivated human AB serum) and 200 µL containing 200,000 cells were added to the wells of the plate containing the immobilized antibody constructs. PBMCs were incubated with immobilized antibody constructs for 48 hours, after which supernatant was harvested and analyzed for IL-2 & IL-6 by MSD (Mesoscale Discovery).

[0679] The night before cytokine quantification, MSD plates were blocked and coated in capture antibodies according to the manufacturer's instructions. The following day, plates were washed in PBS-T and 5 µl of assay diluent was added to each plate. The supplied IL-2 standard (calibrator 1) was titrated as per manufacturer's instructions. Supernatants were thawed at room temperature and 5 µL of samples or standards were transferred to MSD plates. Detection antibodies were prepared at appropriate dilutions and 10 µL was added to each sample and standard well in MSD plates. The plates were sealed with aluminum foil and incubated away from light at room temperature for two hours. Plates were washed 3x in PBS-T and 40 µL MSD Gold read buffer T was added to each well. Plates were read on the MESO SECTOR 6000 and cytokine concentration was determined using MSD software. Data from a standard curve and samples were used to

perform a nonlinear curve-fit with x-interpolation to obtain IL-2 concentrations in pg/mL. Data are pooled from 11 individual donors.

[0680] Production of IL-2 and IL-6 by human PBMCs in the presence of the tested trispecific antibody constructs and controls are summarized in **TABLES 36A** and **36B**. The data demonstrate that the trivalent and trispecific costimulatory antibody construct, v41086, induced significantly lower amounts of IL-2 and IL-6 in the presence of human PBMCs only (no DLL3-expressing target cells present) when compared to the positive control.

TABLE 36A: Mean IL-2 Production (and Standard Error of the Mean = SEM) Induced by Immobilized Anti-(DLL3xCD3xCD28) Trispecific Antibody Constructs from Monocultures of Primary PBMCs

Construct/ Catalogue #	PBMCs	
	IL-2	
	Mean Cytokine (pg/mL)	SEM
v39982	14.2	3.3
v41753	297.5	46.9
v41086	370.0	57.0
v41759	347.8	54.7
v38589	31.3	3.9
v36577	3201.0	687.7
A2019	9.1	2.1
MA-41754	23.3	4.7
PHA	538.3	110.1
No Treatment	8.6	2.1

TABLE 36B: Mean IL-6 Production (and Standard Error of the Mean = SEM) Induced by Immobilized Anti-(DLL3xCD3xCD28) Trispecific Antibody Constructs from Monocultures of Primary PBMCs

Construct/ Catalogue #	PBMCs	
	IL-6	
	Mean Cytokine (pg/mL)	SEM
v39982	435.4	196.2
v41753	482.4	110.0
v41086	1426.0	446.9
v41759	2993.0	984.8
v38589	5398.0	1375.0
v36577	2765.0	840.2
A2019	7537.0	1649.0
MA-41754	3605.0	1101.0
PHA	8703.0	1210.0

No Treatment	2290.0	1004.0

EXAMPLE 25: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS TO CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS AND A CLINICAL REFERENCE CONSTRUCT

[0681] This example assessed the ability of certain trivalent and trispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SHP-77 small cell lung carcinoma model and compared their performance against corresponding bispecific control constructs and a clinical benchmark.

SHP-77 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity

[0682] CR NCG female mice (Charles River Laboratories International, Inc.) were inoculated subcutaneously in the right flank region with SHP-77 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

[0683] Animals with a single tumor of a volume of 70-100 mm^3 were randomized and distributed in experimental groups according to the study protocol. Randomization was performed based on "Matched distribution" method (StudyDirectorTM software, version 3.1.399.19). Immediately after randomization, animals were inoculated intravenously with PBMCs ($1x10^7$ cells) from donor B. The date of PBMC inoculation is denoted as day 0.

[0684] 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 37**. The test article administration and the animal numbers in each study group are also shown in **TABLE 37**.

[0685] After randomization, tumor volumes were measured twice per week in two dimensions using a caliper. The animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals in detail. Animals were euthanized and necropsied when tumor volume reaches a maximal tumor volume of 2000 mm^3 or when tumor exceeded 10% of normal body weight, or when greater that 20% body weight loss, or after a maximum of 60 days post PBMC injection. Tumor volume data was analyzed using a mixed-effect model.

[0686] As shown in **FIG. 14** (arrows indicate time points of test article administration), tumor regression was observed in donor B-engrafted mice treated with v41086, CD28-KO (v41753), and the reference construct (v38589), compared to the irrelevant mAb (v40552). Treatment with 6.6 nmol/kg and 3.3 nmol/kg of v41086 resulted in full tumor regression in 7 out of 7 mice. Similar results were observed after treatment with v38589. Treatment with v41753 at 6.6 nmol/kg resulted in full tumor regression in 7 out of 7 mice. At 3.3 nmol/kg, treatment with v41753 lead to partial tumor regression in 5 out of 7 mice, and tumor growth delay in 2 out of 7 mice. No tumor regression was observed in mice treated with the irrelevant mAb (v40552). Body weights remained stable in all groups until day 21 post-PBMC injection, when body weight loss was observed in some mice across different treatment groups indicating graft versus host disease onset (**FIG. 15**).

TABLE 37: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (7 mice-donor B)	v41086	6.6 nmol/kg	Q7Dx6
2 (7 mice-donor B)	v41086	3.3 nmol/kg	Q7Dx6
3 (7 mice-donor B)	v41086	1.1 nmol/kg	Q7Dx6
4 (7 mice-donor B)	v41086	0.37 nmol/kg	Q7Dx6
5 (7 mice-donor B)	v41753	33 nmol/kg	Q7Dx6
6 (7 mice-donor B)	v41753	1.32 nmol/kg	Q7Dx6
7 (7 mice-donor B)	v41753	6.6 nmol/kg	Q7Dx6
8 (7 mice-donor B)	v41753	33 nmol/kg	Q7Dx6
9 (7 mice-donor B)	v38589	6.6 nmol/kg	Q7Dx6
10 (7 mice-donor B)	v38589	33 nmol/kg	Q7Dx6
11 (7 mice-donor B)	v38589	1.32 nmol/kg	Q7Dx6
12 (7 mice-donor B)	v38589	6.6 nmol/kg	Q7Dx6
13 (7 mice-donor B)	v40552	33 nmol/kg	Q7Dx6

EXAMPLE 26: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING A TRIVALENT AND TRISPECIFIC CONSTRUCT TO A CLINICAL REFERENCE CONSTRUCT

[0687] This example assessed the ability of a certain trivalent and trispecific antibody construct of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the NCI-H82 co-inoculated small cell lung carcinoma model and compared its performance against a clinical reference construct.

NCI-H82 co-inoculation with human T cells in NCG Mice for Antitumor Activity

[0688] CR NCG female mice (Charles River Laboratories International, Inc.) were inoculated subcutaneously in the right flank region with a mix of NCI-H82 tumor cells ($5x10^6$ cells) and human CD3+ T cells ($1x10^6$ cells) from 3 different donors in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. The date of tumor/T cell implantation was denoted as day 0. 24h after tumor/T cell implantation, animals were randomized and distributed in experimental groups according to the study protocol. Randomization was performed based on "Block randomization" method (Benchling In Vivo software). Immediately after randomization, animals were treated intraperitoneally as per study design shown in **TABLE 38**.

[0689] The test article administration and the animal numbers in each study group are also shown in **TABLE 38** (BIW = twice/week).

[0690] One week post-randomization, tumor volumes were measured twice per week in two dimensions using a caliper. The animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals in detail. Animals were euthanized and necropsied when tumor volume reached a maximal tumor volume of 2000 mm^3 or when tumor exceeded 10% of normal body weight or when greater than 20% body weight loss.

[0691] As shown in **FIG. 16**, tumor growth inhibition was observed in all donor-engrafted mice treated with 2.85 nmol/kg of the trivalent and trispecific antibody construct v41086 when compared to treatment with the reference construct (v38985) and untreated control.

TABLE 38: *In Vivo* Study Groups and Dosing Regiment

Study Group	Test Article	Dose (IV)	Treatment Schedule
1 (6 mice – No donor)	-	-	-
2 (6 mice-donor C)	-	-	-
3 (6 mice-donor C)	v41086	2.85 nmol/kg	BIW
4 (6 mice-donor C)	v38589	2.85 nmol/kg	BIW
5 (6 mice-donor D)	-	-	-
6 (6 mice-donor D)	v41086	2.85 nmol/kg	BIW
7 (6 mice-donor D)	v38589	2.85 nmol/kg	BIW
8 (6 mice-donor E)	-	-	-
9 (6 mice-donor E)	v41086	2.85 nmol/kg	BIW
10 (6 mice-donor E)	v38589	2.85 nmol/kg	BIW

EXAMPLE 27: STUDY ASSESSING *IN VIVO* SYSTEMIC CYTOKINE INDUCTION USING A HUMANIZED MOUSE MODEL

[0692] This study assessed systemic cytokine induction *in vivo* of the trivalent and trispecific antibody construct v41086 and certain control constructs using a humanized mouse model.

[0693] Mice were dosed with the respective construct at 2 mg/kg, except for OKT3 (also known as Orthoclone OKT3 or Muromonab-CD3) which was dosed at 0.25 mg/kg. Serum cytokine concentrations (IFNγ, IL-2, TNFα and IL-6; calculated in pg/mL) were measured 72 hours post-treatment with the respective construct. Tested constructs included the trivalent and trispecific antibody construct v41086, v41753 (a bispecific anti-(CD3xDLL3) control antibody), v36577 (a control antibody containing the TGN1412 paratope) and v38589 (clinical reference construct; Pali FcKO = v40522).

[0694] **FIG. 17** shows the results of this study, indicating that the tested trivalent and trispecific antibody construct v41086 induced less systemic cytokines compared to, e.g., the CD28-binder v36577, and amounts comparable to the reference construct tested in this study.

EXAMPLE 28: NON-HUMAN PRIMATE TOXICOLOGY STUDY

[0695] This example describes a toxicology study that was conducted in cynomolgus monkeys using the trivalent and trispecific Het-Fab antibody constructs v41386 (test construct) and v41387 (surrogate, identical to v41386 except for the anti-CD3 binding domain which has a higher binding affinity to cynomolgus CD3 compared to that of v41386).

[0696] Cynomolgus monkeys were given two i.v. doses of 10 mg/kg of the respective tested constructs. Transient, minor increases in serum concentrations of the measured cytokines IL-6, IL-10, MCP-1 and IL-2 were observed as shown in **FIG. 18**. The peak 10 mg/kg surrogate values were measured in a male with an increased cytokine release profile.

[0697] Overall, these data indicate that the tested trivalent and trispecific antibody constructs were well-tolerated in cynomolgus monkeys.

[0698] The description, for purposes of explanation, has been described with reference to specific implementations. However, the illustrative discussions above are not intended to be exhaustive or to limit the implementations to the precise forms disclosed. Many modifications and variations are possible in view of the embodiments described herein. The implementations were chosen and described in order to best explain the principles and their practical applications, to thereby enable

others skilled in the art to best utilize the implementations and various implementations with various modifications as are suited to the particular use contemplated.

[0699] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference in their entireties to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

SEQUENCE TABLE

[0700] Selected embodiments and amino acid sequences of antibody constructs and portions or fragments thereof (e.g., chain portions, scFv, Fab or Fc domains, linker, etc.) that are disclosed and described herein are shown in the sequence table below. Unless otherwise defined herein, antibody construct polypeptide chains labeled with "H" refer to an Ig, or Ig-derived, heavy chain, and polypeptide chains labeled with "L" refer to an Ig, or Ig-derived, light chain.

Variant #	Description	Type/ Target	SEQ ID NO	Sequence
Certain Anti-CD3 Binding Domain Sequences				
	V_H	CD3	1	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSS
	V_H	CD3	2	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSS
	C_{H1}		3	ASTKGPSVFPLRPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTV PSSSLGTQTYICNVNHKPSNTKVDKKV
	C_{H1}		4	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV PSSSLGTQTYICNVNHKPSNTKVDKKV
	V_L	CD3	5	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VL
	C_L		6	GQPKAAPSVTLFPPSDEELQANTATLVCLISDFYPG AVTVAWKADSSPVKAGVETTTPSKQSNNKYAAES ELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC S

	C_L		7	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPG AVTVAWKADSSPVKAGVETTTPSKQSNNKYAASS YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC S
	VH		82	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG NFGNSYVSWFAYWGQGTLVTVSS
	VL		83	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG TKLTVL
Other Antibody Construct Domain and Chain Sequences				
	CH2		8	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSV SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAK
	CH3		9	GQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFALVSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PG
	CH3		10	GQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPS DIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PG
30875/ 25094	Anti-CD3 Fab-Linker$^{Fab\text{-}Fc}$-CH2-CH3	HC A	11	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG
	Linker$^{scFv\text{-}Fc}$-CH2-CH3	HC B	12	EPKSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL MISRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYVLPPSR DELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENN YLTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPG

26152	Anti-CD3 Fab-Linker$^{Fab\text{-}scFv}$-anti-CD28 scFv	LC	13	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY AMDYWGQGTLVTVSS
	Fab heavy chain	CD3	14	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKV
	VH	DLL3	99	QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS
	VL	DLL3	100	DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK
	VH	DLL3	101	QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV HWVRQAPGQCLEWMVIIDPGGGTTSYAQKFQGRV TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS
	VL	DLL3	102	DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK
	VH	DLL3	103	QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFQGRV TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS
	VL	DLL3	104	DIVMTQSPDSLAVSLGERATINCKSSQGISNYLAW YQQKPGQPPKLLIYAVSTRESGVPDRFSGSGSGTDF TLTISSLQAEDVAVYYCLQHDSYPYTFGQGTKLEIK
Certain Anti-CD28 Binding Domain Sequences (Mutation Positions According to the IMGT Numbering Scheme)				
Hu_TN2 28_WT	V_H	CD28	15	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS

				EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H		15	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L (D1068E)		17	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVESGVPSRFSGSGS GTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGTK VEIK
Mod. Hu_TN228	V_H (Y1110S)		18	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRASGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H		15	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L (N1066A)		19	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASAVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (E1080K)		20	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS KDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (P1058A)		21	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWAGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK

Mod. Hu_TN228	V_H (Y1037A)		22	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSAGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (L1112aN)		23	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY NYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (G1064S)		24	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGSTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (N1111aA)		25	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGAY LYAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H		15	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LYAMDYWGQGTLVTVSS
	V_L (V1035G)		26	DIQMTQSPSSLSASVGDRVTITCRASESVEYYGTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (Y1113S)		27	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNY LSAMDYWGQGTLVTVSS
	V_L		16	DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG

				SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Mod. Hu_TN228	V_H (Y1112S)	CD28	76	QVQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVH WIRQPPGKGLEWLGVIWPGGGTNFNSALMSRLTIS EDTSKNQVSLKLSSVTAADTAVYYCARDRAYGNS LYAMDYWGQGTLVTVSS
Mod. Hu_TN228	V_L (Y1031A)	CD28	77	DIQMTQSPSSLSASVGDRVTITCRASESVEAYVTSL MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT KVEIK
Amino Acid Sequences of Certain Anti-(DLL3xCD3xCD28) Trispecific Antibody Constructs *(a construct can also be referred to by a specific variant ("v") number (#) and its heavy and light chain sequences given as H1, H2, L, L1, L2, etc. merely for reference)*				
v41086 (Het-Fab)	H1 (31129)	CD3	105	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG
	H2 (31130)	DLL3	106	QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG
	L1 (31131)	CD3/CD28	107	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT

				ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY AMDYWGQGTLVTVSS
	L2 (31132)	DLL3	108	DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG EC
v41759 (scFv^2)	H1 (31417)	CD3	109	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG
	H2 (31703)	DLL3	110	DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK GGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKK PGSSVKVSCKASGYTFVHYYVHWVRQAPGQCLE WMVIIDPGGGTTSYAQKFQGRVTMTRDTSTNTVY MELSSLRSEDTAVYYCARGERVTGNYFYYGMDV WGQGTTVTVSSEPKSSDKTHTCPPCPAPEAAGGPS VFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP REPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAV EWESNGQPENNYLTWPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
	L (31416)	CD3/CD28	111	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY

				PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY AMDYWGQGTLVTVSS
v41760 (scFv^2)	H1 (31418)	CD3	112	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG NFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG
	H2 (31703)	DLL3	113	DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK GGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKK PGSSVKVSCKASGYTFVHYYVHWVRQAPGQCLE WMVIIDPGGGTTSYAQKFQGRVTMTRDTSTNTVY MELSSLRSEDTAVYYCARGERVTGNYFYYGMDV WGQGTTVTVSSEPKSSDKTHTCPPCPAPEAAGGPS VFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP REPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAV EWESNGQPENNYLTWPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
	L (31700)	CD3/CD28	114	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKT VAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTITC RASESVEYYVTSLMQWYQQKPGKAPKLLIYAASN

				VDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQ SRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSGG GGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSYG VHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSRL TISEDTSKNQVSLKLSSVTAADTAVYYCARDRAYG NYLYAMDYWGQGTLVTVSS
v41112 (Het-Fab)	H1 (31129)	CD3	105	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG
	H2 (31141)	DLL3	115	QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFQGRV TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG
	L1 (31131)	CD3/CD28	107	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED

				TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY AMDYWGQGTLVTVSS
	L2 (31144)	DLL3	116	DIVMTQSPDSLAVSLGERATINCKSSQGISNYLAW YQQKPGQPPKLLIYAVSTRESGVPDRFSGSGSGTDF TLTISSLQAEDVAVYYCLQHDSYPYTFGQGTKLEIK RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG E
v41094 (Het-Fab)	H1 (31145)	CD3	117	EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG
	H2 (31130)	DLL3	106	QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG
	L1 (31131)	CD3/CD28	107	NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ

				VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY AMDYWGQGTLVTVSS
	L2 (31132)	DLL3	108	DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG EC
CDRs (cons. = consensus sequence)				
WT	HCDR1	CD28	28	SYGVH
	HCDR1	CD28	29	S**A**GVH
Cons.	HCDR1	CD28	30	SX_1GVH, X_1 = Y, A (“,” is equivalent to “or”)
WT	HCDR2	CD28	31	VIWPGGGTNFNSALMS
	HCDR2	CD28	32	VIW**A**GGGTNFNSALMS
	HCDR2	CD28	33	VIWPGG**S**TNFNSALMS
Cons.	HCDR2	CD28	34	$VIWX_2GGX_3TNFNSALMS$, X_2 = P, A; X_3 = G, S
WT	HCDR3	CD28	35	DRAYGNYLYAMDY
	HCDR3	CD28	36	DRA**S**GNYLYAMDY
	HCDR3	CD28	37	DRAYG**A**YLYAMDY
	HCDR3	CD28	38	DRAYGNY**N**YAMDY
	HCDR3	CD28	39	DRAYGNYL**S**AMDY
Cons.	HCDR3	CD28	40	$DRAX_4GX_5YX_6X_7AMDY$, X_4 = S, Y; X_5 = N, A; X_6 = L, N; X_7 = S, Y
WT	LCDR1	CD28	41	RASESVEYYVTSLMQ
V1035G	LCDR1	CD28	42	RASESVEYY**G**TSLMQ
Cons.	LCDR1	CD28	43	$RASESVEYYX_8TSLMQ$, X_8 = G, V
WT	LCDR2	CD28	44	AASNVDS
D1068E	LCDR2	CD28	45	AASNV**E**S
N1066A	LCDR2	CD28	46	AAS**A**VDS
Cons.	LCDR2	CD28	47	$AASX_9VX_{10}S$, X_9 = N, A; X_{10} = E, D
WT	LCDR3	CD28	48	QQSRKVPFT
IMGT	HCDR1	CD3	49	GVTFNYYG
IMGT	HCDR2	CD3	50	ITSSGGRI
IMGT			51	ITRSGGRI
IMGT	HCDR3	CD3	52	TLDGRDGWVAY
IMGT	LCDR1	CD3	53	TGNIGSNY
IMGT	LCDR2	CD3	n/a	RND
IMGT	LCDR3	CD3	55	QSYSSGFI
Kabat	HCDR1	CD3	84	TYAMN
Kabat	HCDR2	CD3	85	RIRSKYNNYATYYADSVKG
Kabat	HCDR3	CD3	86	HGNFGNSYVSWFAY
Kabat	LCDR1	CD3	87	GSSTGAVTTSNYAN

Kabat	LCDR2	CD3	88	GTNKRAP
Kabat	LCDR3	CD3	89	ALWYSNLWV
Kabat	HCDR1	DLL3	90	HYYVH
Kabat	HCDR2	DLL3	91	IIDPGGGTTSYAQKFLG
Kabat	HCDR3	DLL3	92	GERVTGNYFYYGMDV
Kabat	LCDR1	DLL3	93	VANQGISNYLV
Kabat	LCDR2	DLL3	94	SVNSLYS
Kabat	LCDR3	DLL3	95	LQHDSYPYT
Kabat	HCDR1	DLL3	90	HYYVH
Kabat	HCDR2	DLL3	96	IIDPGGGTTSYAQKFQG
Kabat	HCDR3	DLL3	92	GERVTGNYFYYGMDV
Kabat	LCDR1	DLL3	93	VANQGISNYLV
Kabat	LCDR2	DLL3	94	SVNSLYS
Kabat	LCDR3	DLL3	95	LQHDSYPYT
Kabat	HCDR1	DLL3	90	HYYVH
Kabat	HCDR2	DLL3	96	IIDPGGGTTSYAQKFQG
Kabat	HCDR3	DLL3	92	GERVTGNYFYYGMDV
Kabat	LCDR1	DLL3	97	KSSQGISNYLA
Kabat	LCDR2	DLL3	98	AVSTRES
Kabat	LCDR3	DLL3	95	LQHDSYPYT
Linkers				
	$Linker^{Fab\text{-}Fc}$	Hinge	56	EPKSCDKTHTCPPCP
	$Linker^{Fab\text{-}Fc}$	Hinge	120	EPDSCDKTHTCPPCP
	$Linker^{scFv\text{-}Fc}$	Hinge	57	EPKSSDKTHTCPPCP
	$Linker^{Fab\text{-}scFv}$		58	GGGGSG
	$Linker^{scFv}$		59	GGGGSGGGGSGGGGSGGGGS
	Linker		60	$(EAAAK)_n$, n = 1, 2, 3, 4 or 5
	Linker		61	EAAAK
	Linker		62	EAAAKEAAAK
	Linker		n/a	PPP
	Linker		64	PPPP
	Linker		65	GPPPG
	Linker		66	GGPPPGG
	Linker		67	GPPPPG
	Linker		68	GGPPPPGG
	Linker		69	$(G_nS)_m$, n, m can independently be 1, 2, 3, 4 or 5
	Linker		70	$(G_3S)_n(G_4S)_1$, n = 1, 2, 3, 4 or 5
	Linker		71	$(G_3S)_1(G_4S)_n$, n = 1, 2, 3, 4 or 5
	Linker		72	$(G_3S)_n(G_4S)_n$, n = 1, 2, 3, 4 or 5
	Linker		73	$(G_mS)_n$-GG, m, n are independently integers from 1 to 20
	Linker		74	$(SG_n)_m$, m, n are independently integers from 1 to 20
	Linker		75	$(SEG_n)_m$, m, n are independently integers from 1 to 20

CLAIMS

WHAT IS CLAIMED IS:

1. An antibody construct, comprising:

(i) a first Fab domain capable of binding CD3 on a cytotoxic effector cell;

(ii) a first scFv domain capable of binding CD28 on a cytotoxic effector cell;

(iii) a third binding domain capable of binding DLL3 on a tumor cell; and

(iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide,

wherein:

(a) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide,

(b) the third binding domain is coupled to the N-terminus of the second Fc polypeptide, and

(c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

2. The antibody construct of claim 1, wherein the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITSSGGRI (SEQ ID NO: 50) or ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.

3. The antibody construct of claim 1 or claim 2, wherein the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system.

4. The antibody construct of any one of claims 1-3, wherein the first Fab domain comprises a V_H domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 2, and a V_L

domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5.

5. The antibody construct of any one of claims 1-4, wherein the first Fab domain comprises a V_H domain comprising the amino acid sequence set forth in SEQ ID NO: 2, and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 5.

6. The antibody construct of any one of claims 1-5, wherein the first scFv domain comprises a V_H domain comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system.

7. The antibody construct of any one of claims 1-6, wherein the first scFv domain comprises a V_H sequence comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a V_L domain comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16.

8. The antibody construct of any one of claims 1-7, wherein the first scFv domain comprises a V_H sequence comprising the amino acid sequence set forth in SEQ ID NO: 15, and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 16.

9. The antibody construct of any one of claims 1-8, wherein the third binding domain is a second Fab domain or a second scFv domain.

10. The antibody construct of any one of claims 1-9, wherein the third binding domain comprises a V_H domain comprising a HCDR1 having the sequence HYYVH (SEQ ID NO: 90), a HCDR2 having the sequence IIDPGGGTTSYAQKFLG (SEQ ID NO: 91) and a HCDR3 having the sequence GERVTGNYFYYGMDV (SEQ ID NO: 92), and a VL domain comprising a LCDR1 having the sequence VANQGISNYLV (SEQ ID NO: 93), a LCDR2 having the sequence SVNSLYS (SEQ ID NO: 94) and a LCDR3 having the sequence LQHDSYPYT (SEQ ID NO: 95), according to the Kabat numbering system.

11. The antibody construct of any one of claims 1-10, wherein the third binding domain comprises a V_H sequence comprising the amino acid sequence set forth in SEQ ID NO: 99, and a V_L domain comprising the amino acid sequence the sequence set forth in SEQ ID NO: 100.

12. The antibody construct of claim 1, wherein (i) the first Fab domain comprises a V_H domain comprising a HCDR1 having the sequence GVTFNYYG (SEQ ID NO: 49), a HCDR2 having the sequence ITRSGGRI (SEQ ID NO: 51), and a HCDR3 having the sequence TLDGRDGWVAY (SEQ ID NO: 52), and a V_L domain comprising a LCDR1 having the sequence TGNIGSNY (SEQ ID NO: 53), a LCDR2 having the sequence RND, and a LCDR3 having the sequence QSYSSGFI (SEQ ID NO: 55), according to the IMGT numbering system, (ii) the first scFv domain comprises a V_H domain comprising a HCDR1 having the sequence SYGVH (SEQ ID NO: 28), a HCDR2 having the sequence VIWPGGGTNFNSALMS (SEQ ID NO: 31), and a HCDR3 having the sequence DRAYGNYLYAMDY (SEQ ID NO: 35), and a V_L domain comprising a LCDR1 having the sequence RASESVEYYVTSLMQ (SEQ ID NO: 41), a LCDR2 having the sequence AASNVDS (SEQ ID NO: 44), and a LCDR3 having the sequence QQSRKVPFT (SEQ ID NO: 48), according to the Kabat numbering system, and (iii) the third binding domain comprises a V_H domain comprising a HCDR1 having the sequence HYYVH (SEQ ID NO: 90), a HCDR2 having the sequence IIDPGGGTTSYAQKFLG (SEQ ID NO: 91) and a HCDR3 having the sequence GERVTGNYFYYGMDV (SEQ ID NO: 92), and a VL domain comprising a LCDR1 having the sequence VANQGISNYLV (SEQ ID NO: 93), a LCDR2 having the sequence SVNSLYS (SEQ ID NO: 94) and a LCDR3 having the sequence LQHDSYPYT (SEQ ID NO: 95), according to the Kabat numbering system.

13. The antibody construct of claim 1, wherein (i) the first Fab domain comprises the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 2, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 5, (ii) the first scFv domain comprises the HCDR1-3 sequences of the VH sequence set forth in SEQ ID NO: 15, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 16, and (iii) the third binding domain comprises the HCDR1-3 sequences of the VH sequence set forth in

SEQ ID NO: 99, and the LCDR1-3 sequences of the VL sequence set forth in SEQ ID NO: 100.

14. The antibody construct of claim 1, wherein (i) the first Fab domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 2, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 5, (ii) the first scFv domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 15, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 16, and (iii) the third binding domain comprises a VH sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 99, and a VL sequence comprising an amino acid sequence having at least about 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 100.

15. The antibody construct of any one of claims 1-14, wherein the third binding domain is a second Fab domain.

16. The antibody construct of claim 15, wherein (i) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via a first linker$^{Fab\text{-}Fc}$ and (ii) the second Fab domain is coupled to the N-terminus of the second Fc polypeptide via a second linker$^{Fab\text{-}Fc}$.

17. The antibody construct of claim 16, wherein the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consist of the amino acid sequence set forth in SEQ ID NOs: 56 or 120.

18. The antibody construct of any one of claims 15-17, wherein one Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

19. The antibody construct of claim 18, wherein the Fab domain comprising the kappa light chain comprises one or more of the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence and one or more of the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the Fab domain comprising the lambda light chain comprises one or more of the amino acid substitutions 125R and 188K in the CH1 sequence and one or more of the amino acid substitutions 122D, 129T, 176E

and 178E in the CL sequence of the lambda light chain, and wherein the amino acid residues are identified according to the Kabat numbering system.

20. The antibody construct of claim 19, wherein the Fab domain comprising the kappa light chain comprises the amino acid substitutions 143E, 145T, 179E and 228D in the CH1 sequence and the amino acid substitutions 121K, 124R and 178R in the CL sequence of the kappa light chain, and the Fab domain comprising the lambda light chain comprises the amino acid substitutions 125R and 188K in the CH1 sequence the amino acid substitutions 122D, 129T, 176E and 178E in the CL sequence of the lambda light chain.

21. The antibody construct of any one of claims 1-14, wherein the third binding domain is a second scFv domain.

22. The antibody construct of claim 21, wherein (i) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide via a first linker$^{Fab\text{-}Fc}$ and (ii) the second scFv domain is coupled to the N-terminus of the second Fc polypeptide via a first linker$^{scFv\text{-}Fc}$.

23. The antibody construct of claim 22, wherein the first linker$^{Fab\text{-}Fc}$ and the first linker$^{scFv\text{-}Fc}$ independently comprise or consist of the amino acid sequence set forth in SEQ ID NOs: 56, 120 or 57.

24. The antibody construct of any one of claims 1-23, wherein the first Fc polypeptide and the second Fc polypeptide each comprise a CH2 domain that independently comprise an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 8.

25. The antibody construct of claim 24, wherein the first Fc polypeptide and the second Fc polypeptide each comprise the amino acid substitutions L234A_L235A_D265S that reduce binding of the Fc domain to one or more FcγRs, and wherein the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

26. The antibody construct of any one of claims 1-25, wherein the first Fc polypeptide and the second Fc polypeptide each comprise an IgG1 CH3 domain, and wherein each CH3 domain comprises one or more amino acid substitutions compared to a corresponding wild-type IgG1 CH3 domain sequence, wherein the one or more amino acid substitutions promote preferential pairing of the first and second Fc polypeptides to form a heterodimeric Fc domain.

27. The antibody construct of claim 26, wherein the first or the second Fc polypeptide comprises the amino acid substitutions T350V_L351Y_F405A_Y407V, and the other Fc polypeptide comprises the amino acid substitutions T350V_T366L_K392L_T394W, and the numbering of amino acid residues in the first and second Fc polypeptides is according to the EU numbering system.

28. The antibody construct of any one of claims 1-27, wherein the C-terminus of the light chain of the first Fab domain is coupled to the N-terminus of the first scFv domain via a $linker^{Fab\text{-}scFv}$ comprising the amino acid sequence set forth in SEQ ID NO: 58.

29. The antibody construct of any one of claims 1-28, wherein the first scFv domain has the domain structure, from N- to C-terminus, of: VL-$Linker^{scFv}$-VH, and wherein the $Linker^{scFv}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 59.

30. The antibody construct of any one of claims 1-20, comprising: (i) a first heavy chain (H1) comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 105; (ii) a second heavy chain (H2) comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 106; (iii) a first light chain (L1) comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 107; and (iv) a second light chain (L2) comprising an amino acid sequence having at least about 90%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 108.

31. The antibody construct of claim 30, comprising: (i) a first heavy chain (H1) comprising the amino acid sequence set forth in SEQ ID NO: 105; (ii) a second heavy chain (H2) comprising the amino acid sequence set forth in SEQ ID NO: 106; (iii) a first light chain (L1) comprising the amino acid sequence set forth in SEQ ID NO: 107; and (iv) a second light chain (L2) comprising the amino acid sequence set forth in SEQ ID NO: 108.

32. The antibody construct of any one of claims 1-31, wherein the cytotoxic effector cell is a T cell, and wherein the antibody construct binds CD3 and CD28 on the same T cell as compared to binding CD3 and CD28 on different T cells, as measured in a cell-cell bridging assay with recorded double positive events of less than about 5, less than about 3, or less than about 2.

33. A pharmaceutical composition comprising the antibody construct of any one of claims 1-32, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

34. A nucleic acid molecule or a set of nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct of any one of claims 1-32.

35. A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of claim 34.

36. A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of claim 34, or the vector or set of vectors of claim 35.

37. A method of producing an antibody construct of any one of claims 1-32, the method comprising:

(a) obtaining a host cell culture comprising at least one host cell comprising one or more nucleic acid molecules encoding one or more, two or more, three or more, or four or more polypeptide chains that form the antibody construct; and

(b) recovering the antibody construct from the host cell culture.

38. A method of eliciting an anti-tumor immune response in a cell population comprising T cells and tumor cells, the method comprising contacting the cell population with an effective amount of the antibody construct of any one of claims 1-32, wherein the T cells express CD3 and CD28 and the tumor cells express DLL3.

39. A method of treating a cancer in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of claims 1-32.

40. An antibody construct of any one of claims 1-32 for use in the treatment of cancer.

41. Use of an antibody construct of any one of claims 1-32 in the manufacture of a medicament for the treatment of cancer.