# MULTISPECIFIC AND COSTIMULATORY ANTIBODY CONSTRUCTS 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. 63/638,215, filed April 24, 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 content of each of which is incorporated by reference herein for all purposes.

## TECHNICAL FIELD

**[0002]** The present disclosure generally relates to multispecific 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 disease-associated antigen, such as a tumor-associated antigen (TAA) on a tumor cell.

## 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. Bispecific T cell engagers (TCEs) have exhibited clinical successes in the treatment of hematological cancers, while treatment of solid tumors remains a challenge. Treatment of solid tumors with conventional CD3-engaging TCEs can result in limited T cell proliferation and recruitment to the tumor site, and treatment related T cell anergy, thus restricting the ability of bispecific TCEs to inhibit growth of these poorly infiltrated and rapidly growing tumors.

## SUMMARY

**[0004]** In various embodiments, the present disclosure describes multispecific 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 disease-associated antigen, e.g., an antigen on a tumor cell. In certain embodiments of

the present disclosure, described herein are costimulatory, multivalent and multispecific antibody constructs that comprise at least three binding domains, wherein a first binding domain is 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 disease-associated antigen, such as a TAA on a tumor cell. A costimulatory antibody construct of the present disclosure can be multivalent and multispecific and comprises the two effector cell (e.g., T cell) antigen binding domains in a specific configuration, that is the two effector cell antigen binding domains are in a certain proximity and relative orientation to each other, as further described herein.

**[0005]** In some embodiments, the present disclosure describes an antibody construct, comprising: (i) a first immunoglobulin (Ig) heavy chain, H1, comprising, from N- to C-terminus, a first Fab heavy chain coupled to a first Fc polypeptide, (ii) a first Ig light chain, L1, comprising, from N- to C-terminus, a first Fab light chain coupled to a first scFv domain, and (iii) a second Ig heavy chain, H2, comprising a second Fc polypeptide, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain that is capable of binding CD3 (e.g., CD3e or CD3 epsilon) on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (c) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain. In certain embodiments, another (e.g., third) antigen binding domain is coupled to the N- or C-terminus of the second Fc polypeptide, wherein such an additional antigen binding domain is capable of binding a disease-associated antigen, such as a TAA on a tumor cell. The first light chain, L1, of an antibody construct described herein thus comprises the domain structure: $(VL\text{-}CL)_{Fab}$-$(VL\text{-}VH)_{scFv}$ or $(VL\text{-}CL)_{Fab}$-$(VH\text{-}VL)_{scFv}$, optionally including one or more linkers as further described herein. Antibody constructs comprising such light chain L1 can have a format also referred to herein as "Format A."

**[0006]** In various embodiments, the disease-associated antigen is a TAA.

**[0007]** In some embodiments, the present disclosure describes 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; 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.

**[0008]** In some embodiments, the third (disease-associated) antigen binding domain is a second Fab domain. In other embodiments, the third antigen binding domain is a second scFv domain.

**[0009]** In some embodiments, the present disclosure describes an antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and two immunoglobulin light chains, L1 and L2, wherein: (i) 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; (ii) H2 comprises, 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; (iii) L1 comprises, from N- to C-terminus, a first Fab light chain, comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising a third VL sequence coupled to a third VH sequence; and (iv) L2 comprises, from N- to C-terminus, a second Fab light chain comprising a second VL sequence and a second CL sequence, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the second Fab heavy chain of H2 and the second Fab light chain of L2 form a second Fab domain capable of binding a TAA on a tumor cell; (c) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0010]** In some embodiments, the present disclosure describes an antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and one immunoglobulin light chain, L1, wherein: (i) 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; (ii) L1 comprises, from N- to C-terminus, a first Fab light chain, comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising a second VL sequence coupled to a second VH sequence; and (iii) H2 comprises, from N- to C-terminus, a second scFv domain comprising a third VL sequence and a third VH sequence, coupled to a second Fc polypeptide; wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; (c) the second scFv domain is capable of binding a TAA on a tumor cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0011]** Further described herein are pharmaceutical compositions comprising one or more of the antibody constructs of the present disclosure, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

**[0012]** Further described herein is a method of producing an 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 of the polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture.

**[0013]** Further described herein is a method of treating a tumor in a subject in need thereof, the method comprising administering to the subject an antibody construct of the present disclosure. In some embodiments, such tumor can be a solid tumor, such as a carcinoma, a sarcoma, or a lymphoma (e.g., Non-Hodgkin Lymphoma). In other embodiments, the tumor can be a liquid tumor (e.g., a hematologic malignancy) such as a leukemia.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0014]** 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.

**[0015] FIGS. 1A-1D** and **1G-1H** show schematic representations of the format and geometry of trivalent and trispecific antibody constructs that comprise binding domains capable of engaging CD3, CD28, and a TAA, according to certain embodiments of the present disclosure. **FIGS. 1A** and **1B** are representative depictions of antibody constructs having a format referred to herein "Format A." **FIGS. 1E-1F** show schematic representations of bispecific control constructs used as described and used in this disclosure.

**[0016] FIGS. 2A-2B** show binding of certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti-MSLN (scFv) trivalent and trispecific constructs (v34914, v34915, v34916 and v34917, **FIG. 2A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti-MSLN (scFv) trivalent and trispecific constructs (v34913 and v34918, **FIG. 2B**) to CD4+ (left) and CD8+ (right) T cells, as measured by flow cytometry.

**[0017] FIGS. 3A-3B** show binding of certain trivalent and trispecific anti-CD3/anti-CD28/anti-Cldn18.2 antibody constructs to CLDN18.2+ SNU 601 cells as measured by flow cytometry. Both,

the constructs that comprised an anti-CD3 Fab domain and an anti-CD28 scFv domain (v37633, v37634 and v37635) as well as those that comprised an anti-CD28 Fab domain and an anti-CD3 scFv domain (v37638, v37640 and v37642), were compared against an anti-(Cldn18.2xCD3) bispecific construct (v37663), a one-armed anti-Cldn18.2 antibody (v37675), a benchmark control (v35923, also known as AMG910), an anti-Cldn18.2 monoclonal antibody (mAb), and the anti-RSV protein F mAb palivizumab.

**[0018] FIGS. 4A-4B** show binding of certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti- Cldn18.2 (scFv) trivalent and trispecific constructs (v37633-v37637, **FIG. 4A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti- Cldn18.2 (scFv) trivalent and trispecific constructs (v37638-v37642, **FIG. 4B**) to CD4+ (left) and CD8+ (right) T cells, as measured by flow cytometry.

**[0019] FIG. 5** shows binding of selected trivalent and trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs, and the format-matched single arm binding controls, to CD3+CD28+ human T cells measured by flow cytometry.

**[0020] FIG. 6** shows binding curves 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 for certain trivalent and trispecific antibody constructs as well as for bispecific control constructs.

**[0021] FIGS. 7A-7B** show that certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti-MSLN (scFv) trivalent and trispecific constructs (v34914, v34915, v34916 and v34917, **FIG. 7A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti-MSLN (scFv) trivalent and trispecific constructs (v34913 and v34918, **FIG. 7B**) directed T cells from healthy donors to toward MSLN+ H292 target cells, inducing T cell mediated killing of the tumor cells.

**[0022] FIGS. 8A-8D** show *in vitro* T cell-dependent cytotoxicity induced by the trivalent and trispecific antibody constructs v34914 (**FIG. 8A**), v34916 (**FIG. 8B**), v34913 (**FIG. 8C**) and v34918 (**FIG. 8D**) and compared to the bispecific anti-(MSLNxCD3) benchmark control construct v34919 against $MSLN^{high}$ H292 cells (152,986 MSLN/cell) and $MSLN^{low}$ OVTOKO cells (9,752 MSLN/cell).

**[0023] FIGS. 9A-9B** show that the trivalent and trispecific antibody constructs v34913, v34916 and v34918 directed T cells from healthy donors to kill MSLN+ H292 target cells expressing moderate levels of MSLN. This long-term T cell-dependent cellular cytotoxicity (TDCC) study utilized a low E:T ratio of 1:5 and an incubation period of either 3 days (**FIG. 9A**) or 7 days (**FIG.**

**9B**), e.g., long-term co-culture at low E:T ratios to reflect conditions more accurately in certain (e.g., solid) tumor types.

**[0024] FIGS. 10A-10B** show that certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti-Cldn18.2 (scFv) trivalent and trispecific constructs (v37633-v37637, **FIG. 10A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti- Cldn18.2 (scFv) trivalent and trispecific constructs (v37638-v37642, **FIG. 10B**) directed T cells from healthy donors to kill CLDN18.2+ SNU 601 target cells. These trispecific constructs were compared against a bispecific anti-(Cldn18.2xCD3) control (v37663), a bispecific anti-(Cldn18.2xCD28) control (v37665) and a negative control (Het-Fc version of palivizumab, v22277).

**[0025] FIGS. 11A-11D** show *in vitro* T cell-dependent cytotoxicity induced by the trivalent and trispecific antibody constructs v37633 (**FIG. 11A**), v37634 (**FIG. 11B**), v37638 (**FIG. 11C**) and v37642 (**FIG. 11D**) and compared to the bispecific anti-(MSLNxCD3) benchmark control construct v35923 against CLDN18.2$^{high}$ SNU 601 and CLDN18.2$^{low}$ SKOV-3 target cells.

**[0026] FIGS. 12A-12B** show concentration response curves for a long-term (7 days) TDCC study for the tested anti-CLDN18.2 trivalent and trispecific constructs that either comprised anti-CD3 (Fab)/anti-CD28 (scFv)/anti-Cldn18.2 (scFv) (v37633-v36735, **FIG. 12A**) or anti-CD3 (scFv)/anti-CD28 (Fab)/anti- Cldn18.2 (scFv) (v37638, v37640, v37642, **FIG. 12B**) and show that the constructs directed T cells from healthy donors to kill CLDN18.2+ target cells expressing varying levels of CLDN18.2 using an E:T ratio of 1:1. Top left: SNU-601 (276,125 CLDN18.2/cell); top right: KATO-III (63,566 CLDN18.2/cell); bottom: DAN-G (33,164 CLDN18.2/cell).

**[0027] FIG. 13** shows that several tested anti-CLDN18.2 trivalent and trispecific constructs with varying affinity for CD28, v37638, v37683, v37689, v37692 and v37694, directed T cells from healthy donors to kill CLDN18.2+ SNU 601 cells and compared to bispecific (v37663) and negative (v22277) control constructs.

**[0028] FIG. 14A** shows concentration response curves and in vitro cytotoxicities for the 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, 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. **FIG. 14B** 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, 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.

**[0029] FIGS. 14C-14E** 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.

**[0030] FIGS. 15A-15B** show that certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti-MSLN (scFv) trivalent and trispecific constructs (v34914, v34915, v34916 and v34917, **FIG. 15A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti-MSLN (scFv) trivalent and trispecific constructs (v34913 and v34918, **FIG. 15B**) induced Interleukin-2 (IL-2, left) and/or tumor necrosis factor alpha (TNFα, right) production from T cells when co-incubated with MSLN+ H292 cells (E:T of 2:1) for 72 hours (hrs).

**[0031] FIGS. 16A-16B** show that certain anti-CD3 (Fab)/anti-CD28 (scFv)/anti-Cldn18.2 (scFv) trivalent and trispecific constructs (v37633-v37637, **FIG. 16A**), and anti-CD3 (scFv)/anti-CD28 (Fab)/anti- Cldn18.2 (scFv) trivalent and trispecific constructs (v37638-v37642, **FIG. 16B**) induced IL-2 (top) and/or TNFα (bottom) production from T cells co-incubated with CLDN18.2+ SNU 601 cells (E:T of 2:1) for 72 hrs.

**[0032] FIGS. 17A-17B** show that certain anti-CLDN18.2 trivalent and trispecific constructs that either comprised anti-CD3 (Fab)/anti-CD28 (scFv)/anti-Cldn18.2 (scFv) (v37633-v36735, **FIG. 17A**) or anti-CD3 (scFv)/anti-CD28 (Fab)/anti- Cldn18.2 (scFv) (v37638, v37640, v37642, **FIG. 17B**) were capable of inducing the production of several cytokines (i.e., IFNγ (top), IL-2 (middle), TNFα (bottom)) in T cells in the presence of CLDN18.2+ target SNU 601 using a 72 hours incubation period and an E:T ratio of 2:1, and in comparison to certain bispecific and benchmark control constructs.

**[0033] FIGS. 18A-18B** show that several tested anti-CLDN18.2 trivalent and trispecific constructs with varying affinity for CD28, v37638, v37683, v37689, v37692 and v37694, induced IL-2 (**FIG. 18A**) or TNFα (**FIG. 18B**) production from T cells co-incubated with CLDN18.2+

SNU 601 cells (E:T of 2:1) for 72 hrs, and in comparison to bispecific (v37663) and negative (v22277) control constructs.

**[0034] FIGS. 18C-18D** show curves of the percent of cells proliferated with the respective titrated antibody constructs. **FIG. 18C** shows T cell proliferation in the presence of DLL3+ NCI-H82 cells, and **FIG. 18D** shows T cell proliferation in the absence of DLL3+ cells.

**[0035] FIG. 18E** shows upregulation of Bcl-XL expression in activated T-cells using certain trispecific antibody constructs of the present disclosure as well as bispecific and benchmark controls. 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.

**[0036] FIG. 19** shows *in vivo* anti-tumor activities in donor X-engrafted mice treated with either the bispecific control constructs, v35923 or v38417, or the trispecific and trivalent antibody construct, v37634. **FIG. 20** shows the body weights of mice treated over the course of the *in vivo* efficacy study referenced in **FIG. 19**.

**[0037] FIG. 21** shows flow cytometric analysis of T cell populations in blood and tumor following administration of constructs including the trivalent and trispecific antibody construct v37634.

**[0038] FIG. 22** shows percent body weight loss compared to baseline (e.g., body weight measured prior to PBMC engraftment) following treatment with vehicle control, 1 mg/kg of v37634, v37663, or ANC28.1/5D10, as well as 0.25 mg/kg of OKT3 (also known as Orthoclone OKT3 or Muromonab-CD3).

**[0039] FIG. 23A** shows the change in tumor volume and **FIG. 23B** shows the change in body weight following IV administration of 0.05 mg/kg (q1wx4) of either the trivalent and trispecific antibody construct v37634, a clinical benchmark, or a negative control to mice bearing SNU620 gastric tumors.

**[0040] FIG. 24** 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 the clinical benchmark. **FIG. 25** 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 clinical benchmark (Long-rank $p = 0.0047$). **FIG. 26** shows that body weights of mice treated with v38967 over the course of this in vivo study remained stable.

**[0041] FIG. 27** shows tumor regression observed in donor A-engrafted mice treated with the trivalent and trispecific antibody construct v39917 compared to the clinical benchmark (v38589) and irrelevant mAb (v22277). **FIG. 28** and **FIG. 29** show the percent survival and change in body weight, respectively, over the course of this in vivo study.

**[0042] FIG. 30** shows the qualitative food consumption of male cynomolgus monkeys (n=3) that were given a repeat intravenous (i.v.) dose of 3 mg/kg of v40063 once weekly for two weeks. **FIGS. 31A-31C** show hematological changes, more specifically red cell mass parameters (red blood cells (**FIG. 31A**), hematocrit (**FIG. 31B**) and hemoglobin (**FIG. 31C**)), measured in male cynomolgus monkeys (n=3) that were given a repeat i.v. dose of 3 mg/kg of v40063 once weekly for two weeks. **FIGS. 32A-32D** show additional hematological changes observed in the study referred to in **FIG. 30** including lymphocyte count (**FIG. 32A**), reticulocyte count (**FIG. 32B**), neutrophil count (**FIG. 32C**), and monocyte count (**FIG. 32D**). **FIGS. 33A-33D** show changes in serum concentrations of the following cytokines: IL-6 (**FIG. 33A**), IFN-γ (**FIG. 33B**), TNF-α (**FIG. 33C**) and MCP-1 (**FIG. 33D**).

**[0043] FIG. 34** depicts curves showing the percent of cells proliferated with the respective titrated antibody constructs tested.

**[0044] FIG. 35** shows tumor regression observed in donor B-engrafted mice treated with v41086, CD28-KO (v41753), and the clinical benchmark (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. Body weights remained stable for all mice as shown in **FIG. 36**.

**[0045] FIG. 37** 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 clinical benchmark (v38985).

**[0046] FIG. 38** shows serum cytokine (IFNγ, IL-2, TNFα and IL-6; in pg/mL) concentrations measured 72 hours post-treatment of humanized mice with the respective construct.

**[0047] FIG. 39** shows serum concentrations of the measured cytokines IL-6, IL-10, MCP-1 and IL-2 in cynomolgus monkeys that were given two i.v. doses of 10 mg/kg of the respective tested constructs.

**[0048] FIG. 40** shows the impact of certain antibody constructs having a format according to either Format A, B or C, on the viability of T cells in the absence of tumor cells.

**[0049] FIG. 41** shows target-independent cytokine induction by certain antibody constructs having a format according to either Format A, B or C.

**[0050] FIG. 42** shows the percentage bridging of CD3(KO) T cells to CD28(KO) T cells by certain antibody constructs having a format according to either Format A, B or C.

## DETAILED DESCRIPTION

**[0051]** In various embodiments, the present disclosure describes costimulatory antibody constructs that can be multivalent and multispecific and capable of binding two different antigens on a cytotoxic effector cell (e.g., a T cell), and either (i) a TAA on a tumor cell or (ii) an antigen associated with another disease such as an inflammatory or an autoimmune disease. Such antibody construct can comprise at least 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 TAA on a tumor cell or another disease-associated antigen (such as an inflammatory or autoimmune disease-associated antigen).

**[0052]** In certain embodiments, described herein are multivalent and multispecific T cell engaging antibody constructs capable of costimulating an effector cell (e.g., a T cell), e.g., by engaging two antigens on such effector cell. In some embodiments, these effector cell antigens are CD3 (which includes the epsilon chain of CD3, e.g., CD3ε or CD3-epsilon, and CD28. As further described herein, the costimulatory T cell engaging antibody constructs of the present disclosure can comprise a certain format and geometry that enables obligate cis binding of CD3 and CD28 on the same cytotoxic effector cell (as further defined and described herein), rather than binding CD3 on one cell (e.g., a first T cell) and CD28 on another cell (e.g., a second T cell) and cross-linking such effector cells (e.g., T cells).

**[0053]** In various embodiments, described herein are multivalent and multispecific antibody constructs that comprise an immunoglobulin (Ig) light chain, L1, comprising a Fab light chain portion (e.g., a first VL sequence and a CL sequence) and an scFv portion (e.g., a second VL sequence and a VH sequence), also referred to herein as "Format A". In various embodiments, the Fab light chain portion of L1 can form an anti-CD3 Fab domain with a Fab heavy chain portion of a corresponding Ig heavy chain, H1, while the scFv domain of L1 is capable of engaging CD28. The format (e.g., Fab, scFv, etc.), spatial orientation and relative spatial distance between the anti-CD3 and anti-CD28 binding domains was optimized to generate costimulatory antibody constructs with increased target antigen (e.g., TAA)-dependent activity (e.g., T cell activation, cytokine

release, etc.), reduced T cell toxicity, obligate cis binding of CD3 and CD28 on the same T cell (e.g., reducing the risk of T cell mediated fratricide), and conditional engagement of CD28 requiring CD3 binding for induction CD28 signaling.

## I. DEFINITIONS

**[0054]** 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.

**[0055]** 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. It is to be understood that such a variation is always included in any given value provided herein, whether it is specifically referred to or not.

**[0056]** 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."

**[0057]** 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.

**[0058]** 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 or patient is a

mammal. In some embodiments, the subject is a human. In other embodiments, the subject is a rodent or a non-human primate.

**[0059]** An "effective amount" of a costimulatory 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.

**[0060]** 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% or less, 98% or less, or about 97% or less sequence identity.

**[0061]** The term "antibody construct," as used herein, generally refers to an antibody-like molecule comprising at least one immunoglobulin (Ig) domain, such as an Fc domain, a Fab domain, etc., and comprising two or more, three or more, or four or more polypeptide chains that are covalently and/or non-covalently associated with each other. The polypeptide chains of an antibody construct can include one or more Ig heavy chain(s) and one or more Ig light chain(s). A costimulatory antibody construct, according to various embodiments of the present disclosure, comprises at least three or at least four polypeptide chains including two Ig heavy chains (e.g., H1, H2, etc.) and at least one light chain, wherein the light chain is part of a costimulatory portion of the antibody construct and comprises a Fab portion capable of binding a first antigen (e.g., CD3) on a cytotoxic effector cell and an scFv portion capable of binding a second antigen (e.g., CD28)

on a cytotoxic effector cell. **FIGs. 1A** and **1B** illustrate certain embodiments of costimulatory antibody constructs of the present disclosure comprising at least three (e.g., two Ig heavy and one Ig light chain, **FIG. 1A**) or at least four (e.g., two Ig heavy and two Ig light chains, **FIG. 1B**).

**[0062]** 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, e.g., CD3ε) and a second epitope on cluster of differentiation 28 (CD28), or a first epitope on CD3 or 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 epitopes located on two different antigens (e.g., CD3 and CD28, or CD3 and a TAA). In some of these embodiments, a multispecific antibody construct of this disclosure can comprise specificities for at least three different epitopes located on three different antigens (e.g., CD3, CD28, and a TAA). 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), at least trispecific (i.e., comprising three binding domains with specificities for three different antigens or targets, e.g., CD3, CD28, and a TAA), or even tetraspecific and so forth.

**[0063]** 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., a Claudin (Cldn) such as Cldn6 or Cldn18.2, mesothelin (MSLN), or another antigen expressed by a tumor cell). 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 bind to, e.g., a monospecific antibody construct comprises one binding domain with a specificity for one epitope or antigen, a bispecific antibody construct comprises two binding domains with specificities for two different epitopes and/or antigens, and a trispecific antibody construct comprises three binding domains with specificities for three different epitopes and/or antigens, and so forth.

**[0064]** The term "multivalent," 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 bivalent," i.e., it comprises at least two binding domains, e.g., at least a first binding domain and a second binding domain, wherein each of the first and second binding domains is capable of specifically binding an epitope and/or antigen, e.g., CD3, CD28, or a TAA. The at least two binding domains can bind to the same epitope or antigen or to different epitopes or antigens. The term "multivalent" encompasses antibody constructs that are bivalent, trivalent, tetravalent, and so forth.

**[0065]** 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 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, 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 generally is 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 multivalent and multispecific, each of the three binding domains of a construct is capable of binding a different epitope or antigen, and thus the construct engages each of the three epitopes and/or antigens monovalently.

**[0066]** As used herein, the term "format" in the context of an antibody construct described herein generally describes attributes of the antibody construct such as 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), 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 multivalent and multispecific 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.

**[0067]** 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 $K_D$.

**[0068]** 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, such as the relative spatial localization, arrangement and/or connectivity of the various domains of an antibody construct, e.g., the relative arrangement and connectivity of binding domains and Fc domain, as further described herein and as illustrated in **FIGS. 1A-1D** according to certain embodiments of the present disclosure.

**[0069]** As used herein, the term "costimulatory" or "costimulation" in the context of an antibody construct described herein generally refers to the ability of an antibody construct to bind CD3 and CD28 on a cytotoxic effector cell, e.g., a T cell, in vitro and/or in vivo. In various embodiments, the term "costimulatory" in the context of an antibody construct described herein refers to the ability of an antibody construct to bind CD3 and CD28 on the same cytotoxic effector cell, e.g., a

T cell, in vitro and/or in vivo, as compared to binding CD3 and CD28 on the same cytotoxic effector cell on two different cytotoxic effector cells.

**[0070]** 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."

**[0071]** Generally, and unless specified otherwise, an amino acid sequence of a polypeptide described herein is described and defined in the direction from its N- to its C-terminus. As an example, a polypeptide described as comprising an scFv domain that is 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.

**[0072]** 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) sequences.

**[0073]** The term "amino acid modification," as used herein in the context of an amino acid sequence of a polypeptide that has been modified relative to a reference (e.g., wildtype (WT)) sequence, 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) amino acid sequence of the polypeptide.

**[0074]** Descriptions of antibody constructs such as "anti-(TAAxCD28xCD3)" and "anti-TAA/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 TAA, CD28 and CD3, respectively. In various embodiments, such description refers to a costimulatory, multivalent and multispecific antibody construct having at least three binding domains, one capable of binding an epitope on TAA, one an epitope on CD28 and one an epitope on CD3, respectively, wherein the TAA can be DLL3, MSLN, Cldn18.2 or, in various embodiments, any other tumor antigen described herein and known in the art.

**[0075]** 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.

**[0076]** 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. COSTIMULATORY ANTIBODY CONSTRUCTS

**[0077]** As further described herein, in various embodiments, the present disclosure relates to costimulatory, multivalent and multispecific antibody constructs that comprise at least three binding domains, with two of such binding domains directed against CD3 and CD28 expressed by an immune cell, e.g., T cell, and a disease-associated antigen, such as a TAA expressed by a tumor cell, or an antigen associated with an inflammatory or autoimmune disease.

### A. Format and Geometry of Antibody Constructs

**[0078]** In various embodiments, the antibody constructs described in the present disclosure are costimulatory, multivalent and multispecific and comprise at least three antigen binding domains (which can also be referred to herein as "paratopes"), with each of the three binding domains being capable of binding a different antigen.

**[0079]** In certain embodiments, a multivalent and multispecific antibody construct of the present disclosure can consist of or comprise a format of 1+1+1, indicating that each of the at least three binding domains binds a different antigen, and hence such construct binds each antigen in a monovalent manner. Such 1+1+1-format antibody constructs can also be described as trivalent and trispecific.

**[0080]** In other embodiments, a multivalent and multispecific antibody construct of the present disclosure can comprise at least four antigen binding domains and can consist of or comprise a format of 2+1+1, indicating that two of the at least four binding domains each bind a different antigen in a monovalent ("1") manner, and the other two of the at least four binding domains bind a third antigen in a bivalent ("2") manner. Such 2+1+1-format antibody constructs can also be described as tetravalent and trispecific.

**[0081]** In yet other embodiments, a multivalent and multispecific antibody construct of the present disclosure can comprise at least four antigen binding domains and can consist of or comprise a

format of 1+1+1+1, indicating that each of the at least four binding domains binds a different antigen in a monovalent ("1") manner. Such 1+1+1+1-format antibody constructs can be described as tetravalent and tetraspecific.

**[0082]** As further described herein, any one of the 2, 3, 4 or more antigen binding domains of an antibody construct can have a different structure or format. In some embodiments, any one of the 2, 3, 4 or more antigen binding domains of an antibody construct can comprise or consist of a single-domain antibody (e.g., a VH domain), a VHH antibody, an scFv domain, a Fab domain, or any other binding domain structure known in the art, such as a linear polypeptide sequence.

**[0083]** In certain embodiments, a costimulatory antibody construct of the present disclosure comprises one or more scFv domain(s) and/or one or more Fab domain(s) as antigen binding domains.

**[0084]** As further described herein, in various embodiments, a multivalent and multispecific antibody construct of this disclosure can comprise two structural portions: (i) a costimulation (or costimulatory) portion that comprises or consists of a first heavy (H1) and first light (L1) chain pair, e.g., a H1-L1 pair, that comprises at least an anti-CD3 binding domain and an anti-CD28 binding domain, and (ii) a target antigen (e.g., TAA)-binding portion, comprising a target antigen binding domain that can either comprise or consist of a second heavy chain, H2 (e.g., in instances in which the target antigen binding domain is an scFv domain or a single-chain Fab), or a second heavy and light chain pair, e.g., H2-L2 (e.g., in instance in which the target antigen binding domain is a Fab domain). These multivalent and multispecific antibody constructs can have a format also referred to herein as "Format A," as further described elsewhere herein, and which generally refers to an antibody construct in which the costimulatory portion comprises an anti-CD3 Fab domain coupled to the N-terminus of a first Fc polypeptide, and an anti-CD28 scFv domain, and wherein the anti-CD28 scFv domain is coupled to the C-terminus of the light chain of the anti-CD3 Fab domain; the target antigen binding portion may comprise a target antigen binding domain coupled to the N-terminus of a second Fc polypeptide, wherein the format of the target antigen binding domain may vary (e.g., may comprise an scFv domain or a Fab domain, as shown in, e.g., **FIG. 1A** and **1B**).

**[0085]** In various embodiments, described herein are multivalent and multispecific antibody constructs comprising three antigen binding domains, two of which are capable of binding two different antigens on a cytotoxic effector cell (e.g., an immune cell such as a T cell), and one

binding domain is capable of binding a disease-associated antigen (e.g., a TAA) on a target cell (e.g., a tumor cell). In certain embodiments, such costimulatory multivalent and multispecific antibody constructs comprise a (i) costimulation portion comprising a H1-L1 pair that comprises an anti-CD3 Fab domain and an anti-CD28 scFv domain as well as a first Fc polypeptide, and in which the anti-CD28 scFv domain is coupled to the C-terminus of the Fab light chain of the anti-CD3 Fab domain, and (ii) a TAA-binding portion comprising an anti-TAA binding domain and a second Fc polypeptide.

**[0086]** In some embodiments, described herein is a multivalent and multispecific antibody construct, comprising: (i) a first immunoglobulin (Ig) heavy chain, H1, comprising, from N- to C-terminus, a first Fab heavy chain coupled to a first Fc polypeptide, (ii) a first Ig light chain, L1, comprising, from N- to C-terminus, a first Fab light chain coupled to a first scFv domain, and (iii) a second Ig heavy chain, H2, comprising a second Fc polypeptide, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain that is capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (c) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain. In some embodiments, such antibody construct further comprises a binding domain coupled to the N-terminus of the second Fc polypeptide, wherein such a binding domain is capable of binding a TAA on a tumor cell.

**[0087]** In some embodiments, described herein is a multivalent and multispecific antibody construct, comprising: (i) a first immunoglobulin (Ig) heavy chain, H1, comprising, from N- to C-terminus, a first Fab heavy chain coupled to a first Fc polypeptide, (ii) a first Ig light chain, L1, comprising, from N- to C-terminus, a first Fab light chain coupled to a first scFv domain, and (iii) a second Ig heavy chain, H2, comprising a second Fc polypeptide, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain that binds CD3 on a cytotoxic effector cell; (b) the first scFv domain that is coupled to the C-terminus of the first Fab light chain binds CD28 on a cytotoxic effector cell; and (c) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0088]** In some embodiments, such multivalent and multispecific antibody construct further comprises a binding domain coupled to the N-terminus of the second Fc polypeptide, wherein such binding domain binds a TAA on a tumor cell. In some of these embodiments, H2 further comprises a second Fab heavy chain that is coupled to the N-terminus of the second Fc polypeptide and a

second Ig light chain, L2, comprising a second Fab light chain, and wherein the second Fab heavy chain of H2 and the second Fab light chain of L2 form the second Fab domain.

**[0089]** In some embodiments, H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide. Such first linker$^{Fab\text{-}Fc}$ can comprise or consist of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein the derivative has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region. In some embodiments, the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consist of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein each of the derivatives has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0090]** In some embodiments, the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consist of an amino acid sequence having at least about 80%, 85%, 90%, or at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 56 or 54.

**[0091]** In some embodiments, L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain. Such linker$^{Fab\text{-}scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 58, or a sequence having 1, 2, or 3 amino acid modifications relative thereto.

**[0092]** In some embodiments, the first scFv domain that is capable of binding CD28 and comprises an anti-CD28 VH sequence and an anti-CD28 VL sequence can further comprise a first Linker$^{scFv}$ (e.g., Linker$^{scFv1}$). In some embodiments, the first scFv domain has the domain structure, from N- to C-terminus, of VL-Linker$^{scFv1}$-VH. In other embodiments, the first scFv domain has the domain structure, from N- to C-terminus, of VH-Linker$^{scFv1}$-VL.

**[0093]** In some embodiments, described herein are costimulatory, multivalent and multispecific antibody constructs that comprise at least two Fab domains and at least one scFv domain as binding domains that each target a different antigen. Such antibody constructs can also be referred to as heterodimeric Fab or "Het-Fab" antibody constructs because each Fab domain binds a different antigen and can thus comprise VH and VL sequences with different sequences and, optionally, can 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.

**[0094]** Thus, in various embodiments, the binding domain that is coupled to the N-terminus of the second Fc polypeptide of an antibody construct herein can be a second Fab domain. **FIG. 1B** shows

a schematic representation of such an antibody construct according to certain embodiments of this disclosure.

**[0095]** In some embodiments, described herein is a multivalent and multispecific antibody construct (e.g., a $Fab^2$ construct such as a Het-Fab 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 second Fab domain capable of binding a TAA 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 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.

**[0096]** In certain embodiments of a multivalent and multispecific Het-Fab antibody construct, the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab heavy and/or light chain sequence that promotes correct heavy and light chain pairing. 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.

**[0097]** In some embodiments of a multivalent and multispecific Het-Fab antibody construct, the first Fab domain targeting CD3 and the second Fab domain targeting a TAA each comprise a kappa light chain.

**[0098]** In other embodiments of a multivalent and multispecific Het-Fab antibody construct, the first Fab domain and the second Fab domain each comprise a lambda light chain.

**[0099]** In yet other embodiments of a costimulatory Het-Fab antibody construct, one of the first Fab domain and the second Fab domain comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

**[0100]** In certain embodiments of a multivalent and multispecific 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 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 kappa light chain, and the other Fab domain comprising the lambda light chain comprises 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 lambda light chain, wherein the amino acid residues are identified according to the Kabat numbering system.

**[0101]** In certain embodiments of a multivalent and multispecific 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 of the amino acid substitutions 124R and 186K in the CH1 sequence of the heavy chain 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 comprising the lambda light chain comprises one or more of the amino acid substitutions 139W, 143D and 145T in the CH1 sequence of the heavy chain and one or more 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.

**[0102]** In various other embodiments, described herein are multivalent and multispecific antibody constructs that comprise at least one Fab domain and at least two scFv domains as binding domains. Such antibody constructs can also be referred to as costimulatory multivalent and multispecific $scFv^2$ antibody constructs indicating that such constructs comprise at least two scFv binding domains.

**[0103]** In such embodiments, the binding domain that is coupled to the N-terminus of the second Fc polypeptide of an antibody construct herein is a second scFv domain comprising a second VH sequence and a second VL sequence. **FIG. 1A** shows a schematic of such an $scFv^2$ antibody construct according to certain embodiments of this disclosure.

**[0104]** In some embodiments, described herein is a multivalent and multispecific antibody construct (e.g., a $scFv^2$ 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 second scFv domain capable of binding a TAA 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.

**[0105]** In some of these embodiments, H2 can further comprise a first linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide. Such first linker$^{scFv\text{-}Fc}$ can comprise or consist of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region. In some embodiments, the first linker$^{scFv\text{-}Fc}$ can comprise or consist of an amino acid sequence having at least about 805, 85%, 90%, or at least about 95% sequence identity to the sequence set forth in SEQ ID NO: 56 or 57.

**[0106]** In some embodiments, the second scFv domain that is capable of binding a TAA on a tumor cell comprises a second Linker$^{scFv}$ (Linker$^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH-Linker$^{scFv2}$-VL.

**[0107]** In various embodiments, the Linker$^{scFv1}$ and the Linker$^{scFv2}$ of an scFv$^2$ antibody construct herein can each independently comprise or consist of the amino acid sequence of $(G_4S)_x$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 63). In some embodiments, x is 3 or 4.

**[0108]** In certain embodiments, the present disclosure relates to a multivalent and multispecific 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; 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.

**[0109]** Such multivalent and multispecific antibody construct can comprise a first Ig heavy chain (H1) comprising a first Fab heavy chain comprising a first VH sequence and a first CH1 sequence and the first Fc polypeptide, a second Ig heavy chain (H2) comprising the second Fc polypeptide, and a first Ig light chain (L1) comprising a first Fab light chain coupled to the first scFv domain, wherein the first Fab heavy chain and the first Fab light chain form the first Fab domain. 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.

**[0110]** In some embodiments, L1 of such multivalent and multispecific antibody construct 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, wherein the first scFv domain comprises, 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, L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain. Such linker$^{Fab\text{-}scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 58, or a sequence having 1, 2, or 3 amino acid modifications relative thereto.

**[0111]** In some embodiments, the first scFv domain that is capable of binding CD28 further comprises a first Linker$^{scFv}$ (Linker$^{scFv1}$) and has the domain structure, from N- to C-terminus, of VL- Linker$^{scFv1}$-VH.

**[0112]** In various embodiments, such a multivalent and multispecific antibody construct is a Het-Fab construct in which the third binding domain is a second Fab domain.

**[0113]** In such embodiments, the second Ig heavy chain (H2) further comprises a second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, and the antibody construct further comprises a second Ig light chain (L2) comprising a second Fab light chain comprising a second VL sequence and a second CL sequence, and wherein the second Fab heavy chain and the second Fab light chain form the second Fab domain.

**[0114]** 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. The first linker$^{Fab\text{-}Fc}$, the second linker$^{Fab\text{-}Fc}$, or both such linkers of an antibody construct, can be polypeptide linkers independently comprising or consisting of about 5 to about 50 consecutive amino acid residues. In some embodiments, the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consists of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0115]** In certain embodiments of a multivalent and multispecific Het-Fab antibody construct, the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promotes correct heavy and light chain pairing. 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.

**[0116]** As further described herein, the first Fab domain, the second Fab domain, or both Fab domains, can each independently comprise a kappa or a lambda light chain. In some embodiments,

the first Fab domain and the second Fab domain (i) each comprise a kappa light chain, (ii) 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.

**[0117]** In various other embodiments, a multivalent and multispecific antibody construct herein is a $scFv^2$ construct in which the third binding domain is a second scFv domain comprising a second VH sequence and a second VL sequence.

**[0118]** In such embodiments, H2 further comprises a $linker^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide. Such $linker^{scFv\text{-}Fc}$ can comprise or consist of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0119]** In some embodiments, the second scFv domain comprises a second $Linker^{scFv}$ ($Linker^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH-$Linker^{scFv2}$-VL.

**[0120]** In various embodiments, the $Linker^{scFv1}$ and the $Linker^{scFv2}$ each independently comprise or consist of the amino acid sequence of $(G_4S)_x$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 63).

**[0121]** In various embodiments of the present disclosure, a multivalent and multispecific antibody construct described herein can comprise or consist of at least four polypeptide chains. Such four chains can include two Ig heavy chains, H1 and H2, and two Ig light chains, L1 and L2. In various embodiments, such costimulatory multivalent and multispecific antibody constructs can include Het-Fab constructs comprising at least two Fab domains.

**[0122]** Hence, in some embodiments, described herein is a multivalent and multispecific antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and two immunoglobulin light chains, L1 and L2, wherein: (i) 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; (ii) H2 comprises, 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; (iii) L1 comprises, from N- to C-terminus, a first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising a third VL sequence coupled to a third VH sequence; and (iv) L2 comprises, from N- to C-terminus, a second Fab light chain comprising a second VL sequence and a second CL sequence, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the second Fab heavy chain of H2 and the second Fab light chain of L2 form a second Fab domain

capable of binding a TAA on a tumor cell; (c) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0123]** In some embodiments, H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide, and H2 further comprises a second linker$^{Fab\text{-}Fc}$ that couples the second Fab heavy chain to the second Fc polypeptide.

**[0124]** In further embodiments, the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promotes correct heavy and light chain pairing, as further described elsewhere herein. In some embodiments, the first Fab domain and the second Fab domain (i) each comprise a kappa light chain, (ii) 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.

**[0125]** In various embodiments of the present disclosure, a multivalent and multispecific antibody construct can comprise or consist of at least three polypeptide chains. Such three chains can include two Ig heavy chains, H1 and H2, and one Ig light chain, L1. In various embodiments, such multivalent and multispecific antibody constructs can include scFv$^2$ constructs comprising at least two scFv domains.

**[0126]** In certain embodiments, described herein is a multivalent and multispecific antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and one immunoglobulin light chain, L1, wherein: (i) 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; (ii) L1 comprises, from N- to C-terminus, a first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising a second VL sequence coupled to a second VH sequence; and (iii) H2 comprises, from N- to C-terminus, a second scFv domain comprising a third VL sequence and a third VH sequence, coupled to a second Fc polypeptide; wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; (c) the second scFv domain is capable of binding a TAA on a tumor cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0127]** In some of these embodiments, H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide, and H2 further comprises a linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**[0128]** In some embodiments, one or more of the first linker$^{Fab\text{-}Fc}$, the second linker$^{Fab\text{-}Fc}$, and the linker$^{scFv\text{-}Fc}$ of the antibody constructs described herein (e.g., as applicable to Het-Fab or scFv$^2$ antibody constructs) can independently comprise or consists of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0129]** In various embodiments, a multivalent and multispecific antibody construct of the present disclosure can comprise a first light chain, L1, which further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**[0130]** As further described herein, a multivalent and multispecific antibody construct of this disclosure (e.g., a Het-Fab or an scFv$^2$ antibody construct) can comprise a dimeric Fc domain. In various embodiments, such dimeric Fc domain is a heterodimeric Fc domain in which the amino acid sequences of the first Fc polypeptide and the second Fc polypeptide differ in at least one residue, e.g., have about 99%, 98%, 97%, 95% or about 90% sequence identity to one another.

**[0131]** In various embodiments of a costimulatory antibody construct herein that comprises an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, the first Fc polypeptide comprises a first CH2 sequence and a first CH3 sequence and the second Fc polypeptide comprises a second CH2 sequence and a second CH3 sequence.

**[0132]** In some embodiments, one or more of the constant domain sequences (e.g., one or more of CH1, CH2 and CH3 sequences) of an Ig heavy chain of an antibody construct described herein are IgG, IgA or IgE constant domains. In some embodiments, one or more of the constant domain sequences of an Ig heavy chain of an antibody construct described herein are IgG constant domains. Such one or more IgG constant domains can independently be of the IgG1, IgG2, IgG3 or IgG4 subclass. In some embodiments, one or more constant domains of an antibody construct are IgG1 constant domains. In other embodiments, one or more constant domains of an antibody construct are IgG2 constant domains. In yet other embodiments, one or more constant domains of an antibody construct are IgG4 constant domains.

**[0133]** In some of these embodiments, at least one of the first and second IgG CH2 sequences comprises one or more amino acid substitutions relative to a corresponding wildtype IgG CH2 sequence that reduce or ablate binding of the antibody construct to one or more Fcγ receptors.

**[0134]** In further embodiments of herein described antibody constructs, at least one of the first and second IgG CH3 sequences comprises one or more amino acid substitutions relative to a corresponding wildtype IgG CH3 sequence that promotes formation of a heterodimeric Fc domain compared to a formation of a homodimeric Fc domain. As further described herein, several approaches that promote heterodimeric Fc formation are known in the art. In certain 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.

**[0135]** As described further herein, the multivalent and multispecific antibody constructs of the present disclosure can comprise several different immunoglobulin domains (e.g., one or more of an Fc domain, a Fab domain, an scFv domain, etc.) and further exhibit certain physicochemical and biological properties that may be a result of their specific format, geometry and/or structure in combination with certain binding affinities for their biological targets.

**B. Domains of an Antibody Construct**

**[0136]** A costimulatory, multivalent and multispecific antibody construct of the present disclosure can comprise one or more antibody domains. In various embodiments, a multivalent and multispecific antibody construct of the present disclosure comprises a plurality of (i.e., two or more) antibody domains. Such plurality of antibody domains can include Ig-derived domains and comprise (i) one or more 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 heavy chain polypeptide sequence (also referred to as a Fab heavy chain) comprising a heavy chain variable domain ($V_H$) sequence and a heavy constant domain ($C_{H1}$) sequence and a light chain polypeptide sequence (also referred to as a Fab light chain) 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, optionally via a linker$^{scFv}$ as further described herein. The various domains an antibody construct can comprise are further described herein.

**[0137]** In some embodiments, a multivalent and multispecific antibody construct of the present disclosure can comprise additional types of domains, which may or may not be an Ig domain or Ig-derived, such as VHH domains, single-chain Fabs (scFabs), single-domain antibody (sdAb) domains, or other amino acid or non-amino acid-based moieties such as fluorophores, other detectable labels, low molecular weight (MW) organic compounds with, e.g., MW <700 Da, etc.

**[0138]** An Ig structural unit 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.

**[0139]** In various embodiments, a multivalent and multispecific 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 other embodiments, an antibody construct described herein is based on an IgG2 immunoglobulin. In yet other embodiments, an antibody construct described herein is based on an IgG4 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., Fc domain and/or CH1 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.

**[0140]** Generally, in antibodies, the N-terminal domain of each polypeptide chain (e.g., heavy and light 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 multivalent and multispecific 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 multivalent and multispecific 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 at least six variable domain sequences, e.g., three $V_H$ domain sequences and three $V_L$ domain sequences.

**[0141]** 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, or as described for constructs in which an scFv domain (which contains two variable domain sequences coupled to one another in tandem) is coupled N-terminally to a Fab domain via a Fab variable domain sequence, e.g., either the Fab $V_H$ or the Fab $V_L$ sequence, depending on whether the scFv domain is coupled to the Fab heavy or light chain, respectively. In some embodiments, such construct can comprise the heavy chain domain structure, from N- to C-terminus: $[(V_H\text{-}V_L)$ or $(V_L\text{-}V_H)]_{scFv}\text{-}(V_H\text{-}C_{H1})_{Fab}\text{-Fc}$. The one or more variable domains can be coupled to each other in tandem either with or without one or more linkers, as further described herein, e.g., a $linker^{scFv}$, $linker^{scFv\text{-}Fab}$, etc.

**[0142]** 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 a wildtype sequence) 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), 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).

**[0143]** 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***

**[0144]** 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.

**[0145]** 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.

**[0146]** 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.

**[0147]** 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, and light chain CDR1, CDR2 and CDR3) 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, e.g., SEQ ID NOs: 134-139, 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 embodiments, 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, | H51-H57 | H93-102 | L27-L32 | L50-L52 | L89-L97 |

| Definition | Heavy Chain | | | Light Chain | | |
|---|---|---|---|---|---|---|
| | CDR1[2] | CDR2 | CDR3 | CDR1 | CDR2 | CDR3 |
| | H35A or H35B | | | | | |
| 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.

**[0148]** 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.

**[0149]** 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 an antibody construct (e.g., antigen affinity, stability, and/or pharmacokinetics, 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 Fcγ receptors.

**[0150]** In some embodiments, the present disclosure relates to antibody constructs that can have certain valencies, e.g., can be multivalent, such as bivalent, trivalent, tetravalent, and so on. Hence, in various embodiments, an antibody construct herein comprises two, three, or four antigen binding domains, i.e., is at least bivalent, at least trivalent, or at least tetravalent. In certain embodiments of this disclosure, an antibody construct can be at least trispecific and trivalent, and thus such antibody construct can comprise at least three binding domains. Each of these three binding domains has a unique binding specificity for a certain 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).

**[0151]** 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.

**[0152]** In various embodiments, a multivalent and multispecific antibody construct as described herein comprises (i) a first Fab domain capable of binding to a first antigen, (ii) a first scFv domain capable of binding to a second antigen, and (iii) a second Fab domain capable of binding to a third antigen, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

**[0153]** In other embodiments, a multivalent and multispecific antibody construct as described herein comprises (i) a Fab domain capable of binding to a first antigen, (ii) a first scFv domain capable of binding to a second antigen, and (iii) a second scFv domain capable of binding to a third antigen, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide.

**[0154]** In certain embodiments, a multivalent and multispecific antibody construct described herein comprises a fourth binding domain. Such fourth binding domain can bind to an epitope that is either identical or different to the epitopes the other three binding domains bind to.

**[0155]** 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.

**[0156]** 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: $V_L$-$linker^{scFv}$-$V_H$, 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: $V_H$-$linker^{scFv}$-$V_L$.

### *B.2 Binding Domains against Antigens on a Cytotoxic Effector Cell*

**[0157]** As further described herein, 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 a cytotoxic effector cell. Such cytotoxic effector cell can be an immune cell. Such immune cell can comprise a T cell, a macrophage, a dendritic cell, a neutrophil, a B-cell, an NK cell, or a combination thereof.

**[0158]** In various embodiments, an 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. In various embodiments, such first and second antigens are different from each other and are located on the same cytotoxic effector cell, e.g., T cell.

**[0159]** In some embodiments of a multivalent and multispecific T cell-engaging antibody construct herein, the first antigen on the cytotoxic effector cell is CD3, and the second antigen on the cytotoxic effector cell is CD28. In some of these embodiments, both CD3 and CD28 can be engaged by a multivalent and multispecific antibody construct when located on the same cytotoxic effector cell surface. In other embodiments, both antigens CD3 and CD28 can be engaged by a multivalent and multispecific antibody construct when located on the surfaces of different cytotoxic effector cells.

**[0160]** The binding of an antibody construct of the present disclosure 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).

**[0161]** In various embodiments, a multivalent and multispecific antibody construct of the present disclosure that binds CD3 and CD28 and that comprises a light chain, L, comprising an anti-CD3 Fab light chain C-terminally coupled to an anti-CD28 scFv domain, binds CD3 and CD28 in "cis," i.e., on the same effector cell (e.g., T cell).

**[0162]** In some embodiments, the present disclosure relates to multivalent and multispecific antibody constructs comprising at least a first binding domain capable of binding CD3 on a cytotoxic effector cell, a second binding domain capable of binding CD28 on a cytotoxic effector cell, and a third binding domain capable of binding a target antigen, such as a TAA on a tumor cell, wherein the antibody construct binds CD3 and CD28 on the same cytotoxic effector cell. 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 a construct to bind both, antigens on one or more effector cell(s) (e.g., T cell(s)) as well as a TAA on a tumor cell. In some embodiments, the engagement of a multivalent and multispecific antibody construct with two different effector cell antigens on an effector cell and a TAA 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 the TAA. In various embodiments, and as further described herein, a multivalent and multispecific

antibody construct may cause a significantly reduced immune cell (e.g., T cell) activation in the absence of the TAA, e.g., when the immune synapse cannot be fully formed due to an absence of the TAA, and relative to immune cell activation in the presence of the TAA. Such TAA-dependent immune cell activation can provide for a broader therapeutic window compared to conventional T cell engager modalities.

**[0163]** In various embodiments, the 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, the second antigen binding domain capable of binding CD28 on a cytotoxic effector cell can also be a Fab domain or an scFv domain.

**[0164]** In various embodiments of a multivalent and multispecific T cell-engaging antibody construct herein, the first binding domain capable of binding CD3 on a cytotoxic effector cell is a Fab domain, and the second binding domain capable of binding CD28 on a cytotoxic effector cell is an scFv domain, wherein CD3 and CD28 is engaged by the construct on the same effector cell.

**[0165]** In some embodiments, 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.

**[0166]** In some embodiments, 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.

**[0167]** In various embodiments, the cytotoxic effector cell that expresses CD3 and CD28 which the antibody construct engages is a T cell.

***B.2.1 Binding Domains Against CD3***

**[0168]** As described herein, in some embodiments, a multivalent and multispecific antibody construct of the present disclosure comprises a binding domain capable of binding cluster of differentiation 3 (CD3, e.g., CD3ε) on a cytotoxic effector cell (e.g., T cell). Such multivalent and multispecific antibody construct can bind one or more of human, cynomolgus (cyno) and murine CD3.

**[0169]** In various embodiments, an anti-CD3 binding domain (e.g., an scFv domain or a Fab domain) of an antibody construct herein can have an affinity for CD3 (e.g., 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. Thus, in various embodiments, an anti-CD3 binding domain of an antibody construct herein has a $K_D$ value for binding CD3 that is 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, 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 of an antibody construct 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.

**[0170]** In various embodiments, the anti-CD3 binding domain of an antibody construct herein is a Fab domain.

**[0171]** In certain embodiments, the anti-CD3 binding domain of an antibody construct has an affinity for CD3 that is about 2-fold, 3-fold, 5-fold, 7-fold, 10-fold, or about 15-fold higher than the binding affinity of the anti-CD28 domain for CD28. In some of these embodiments, the difference in affinities of the anti-CD3 and anti-CD28 binding domains for their respective targets, and, in some embodiments, in combination with their structure and relative spatial orientation, can provide for conditional binding of CD28 by the construct that is dependent on engaging CD3, i.e., there is no or significantly reduced CD28 binding of the construct in the absence of CD3 engagement.

**[0172]** In some embodiments, an antibody construct herein comprising an anti-CD3 binding domain, an anti-CD28 binding domain, and an anti-TAA binding domain, binds CD3 with a $K_D$ from about 0.1 nM to about 100 nM, from about 1 nM to about 100 nM, or from about 10 nM to about 75 nM.

**[0173]** 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.

**[0174]** 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, and a LCDR3 sequence comprising the sequence QSYSSGFI (SEQ ID NO: 55).

**[0175]** 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.

**[0176]** 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.

**[0177]** In various embodiments of a multivalent and multispecific antibody construct, the anti-CD3 binding domain is a Fab domain as described herein.

**[0178]** 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.

**[0179]** 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.

**[0180]** 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.

**[0181]** 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.

**[0182]** 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.

**[0183]** 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.

**[0184]** 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.

**[0185]** 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.

**[0186]** 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.

**[0187]** 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.

**[0188]** 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.

**[0189]** 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.

**[0190]** 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.

**[0191]** As further described herein, in some embodiments, the binding affinity of a multivalent and multispecific 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.

**[0192]** 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.

**[0193]** 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).

**[0194]** 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.

**[0195]** 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 multivalent and multispecific antibody constructs described herein.

**[0196]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH 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: 82 and a VL 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: 83. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 82 and a VL sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 83. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 82 and a VL sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 83.

**[0197]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence comprising the HCDR1-3 sequence set forth in SEQ ID NOs: 134-136, respectively, and a VL sequence comprising the LCDR1-3 sequence set forth in SEQ ID NOs: 137-139, respectively.

**[0198]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH 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: 84 and a VL 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: 85. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 84 and a VL sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 85. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 84 and a VL sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 85.

**[0199]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence comprising the HCDR1-3 sequence set forth in SEQ ID NOs: 134, 140 and 141, respectively, and a VL sequence comprising the LCDR1-3 sequence set forth in SEQ ID NOs: 142, 138 and 139, respectively.

**[0200]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH 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: 86 and a VL 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: 87. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 86 and a VL sequence having at least about 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 87. In certain embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 86 and a VL sequence having at least about 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 87.

**[0201]** In some embodiments, an antibody construct herein comprises an anti-CD3 Fab domain comprising a VH sequence comprising the HCDR1-3 sequence set forth in SEQ ID NOs: 143, 135 and 141, respectively, and a VL sequence comprising the LCDR1-3 sequence set forth in SEQ ID NOs: 137-139, respectively.

### *B.2.2 Binding Domains Against CD28*

**[0202]** As further described herein, in various embodiments, a multivalent and multispecific antibody construct of the present disclosure (e.g., a Het-Fab or $scFv^2$ antibody construct) comprises at least one binding domain capable of binding cluster of differentiation 28 (CD28). In some embodiments, such construct binds one or more of human, cyno and murine CD28.

**[0203]** In various embodiments, such anti-CD28 binding domain (e.g., an scFv domain of 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 250 nM, from about 15 nM to about 35 nM or from about 5 nM to about 50 nM.

**[0204]** In various embodiments of a multivalent and multispecific antibody construct, the anti-CD28 binding domain comprises or consists of an scFv domain.

**[0205]** In various embodiments, the anti-CD28 binding domain of an antibody construct herein comprises or consists of an scFv domain, and is coupled to the C-terminus of a light chain of an anti-CD3 Fab domain of multivalent and multispecific antibody construct. Such multivalent and multispecific antibody construct can engage CD3 and CD28 in "cis" on the same effector cell, such as a T cell, as further described herein.

**[0206]** In some embodiments, an antibody construct herein comprises an scFv 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).

**[0207]** In some of these embodiments, an antibody construct herein comprises an scFv 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 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.

**[0208]** In other embodiments, a multivalent and multispecific antibody construct of the present disclosure comprises an anti-CD28 binding domain (e.g., an scFv 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, Kabat, or another numbering system known to the skilled artisan.

**[0209]** 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).

**[0210]** Hence, in some embodiments, an anti-CD28 binding domain (e.g., an scFv 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.

**[0211]** In some embodiments, an antibody construct herein binds CD28 with a $K_D$ from about 1 nM to about 100 nM or from about 10 nM to about 75 nM.

**[0212]** 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.

**[0213]** 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, and as determined using the IMGT numbering scheme. In some of these embodiments, the anti-CD28 $V_H$ domain comprises the amino acid sequence set forth in SEQ ID NO: 24.

**[0214]** 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.

**[0215]** 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.

**[0216]** 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.

**[0217]** 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.

**[0218]** 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.

**[0219]** 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.

**[0220]** 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.

**[0221]** 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.

**[0222]** 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.

**[0223]** 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.

**[0224]** 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.

**[0225]** 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, e.g., in the absence of the TAA. In some embodiments, non-specific (e.g., in the absence of the TAA) 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.

**[0226]** 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.

**[0227]** 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.

**[0228]** 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.

**[0229]** 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.

**[0230]** 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.

**[0231]** 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.

**[0232]** 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.

**[0233]** 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.

**[0234]** 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.

**[0235]** 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.

**[0236]** 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.

**[0237]** 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.

**[0238]** 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.

**[0239]** 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.

**[0240]** 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: 16.

**[0241]** In various embodiments of a multivalent and multispecific antibody construct described herein, 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.

**[0242]** 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$^{scFv}$-$V_H$.

**[0243]** 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.

**[0244]** 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.

**[0245]** 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.

**[0246]** 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.

**[0247]** 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).

**[0248]** As further described herein, in various embodiments, an anti-CD28 binding domain of a multivalent and multispecific 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.

**[0249]** 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 multivalent and multispecific antibody constructs described herein.

**[0250]** In certain embodiments, a multivalent and multispecific antibody construct comprises anti-CD28 scFv domain comprising a VH sequence having an amino acid sequence of at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 102, and a VL sequence having an amino acid sequence of at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 103. In some of these embodiments, the anti-CD28 scFv domain comprises a VH sequence having the sequence set forth in SEQ ID NO: 102, and a VL sequence having the sequence set forth in SEQ ID NO: 103.

**[0251]** In some embodiments, an anti-CD28 scFv domain comprises a VH sequence comprising the HCDRs1-3 set forth in SEQ ID NO: 106-108, and a VL sequence comprising the LCDRs1-3 set forth in SEQ ID NO: 109-111.

**[0252]** In certain embodiments, a multivalent and multispecific antibody construct comprises an anti-CD28 scFv domain comprising a VH sequence having an amino acid sequence of at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 104, and a VL sequence having an amino acid sequence of at least about 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence set forth in SEQ ID NO: 105. In some of these embodiments, the anti-CD28 scFv domain comprises a VH sequence having the sequence set forth in SEQ ID NO: 104, and a VL sequence having the sequence set forth in SEQ ID NO: 105.

**[0253]** In some embodiments, an anti-CD28 scFv domain comprises a VH sequence comprising the HCDRs1-3 set forth in SEQ ID NO: 112-114, and a VL sequence comprising the LCDRs1-3 set forth in SEQ ID NO: 115-117.

### C. Binding Domains Against a Disease-Associated Antigen

**[0254]** As described herein, in various embodiments, a multivalent and multispecific antibody construct of the present disclosure comprises at least one binding domain capable of binding a disease-associated antigen.

**[0255]** The disease-associated antigen can be a TAA on tumor cell, or an antigen associated with an inflammatory or autoimmune disease.

**[0256]** In various embodiments, the disease-associated antigen targeted by an antibody construct of the present disclosure is a TAA on tumor cell.

**[0257]** In various embodiments, and as further described herein, an antibody construct herein can be at least trivalent and trispecific and thus 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 the TAA.

**[0258]** In some embodiments, an antibody construct herein binds a TAA monovalently, i.e., via a single binding domain, such as a Fab domain or an scFv domain.

**[0259]** In other embodiments, an antibody construct herein binds to a TAA bivalently, i.e., via two binding domains. In such embodiments, an antibody construct can comprise two anti-TAA binding domains that target the same epitope on the TAA. In other embodiments, an antibody construct can comprise two anti-TAA binding domains that target different epitopes on the TAA, e.g., binds the TAA bi-paratopically.

**[0260]** In various embodiments, and as further described herein, the binding domain of a multivalent and multispecific antibody construct that binds a disease-associated antigen such as a TAA comprises or consists of a Fab domain (e.g., in a Het-Fab antibody construct). In other embodiments, and as further described herein, the binding domain of a multivalent and multispecific antibody construct that binds a disease-associated antigen such as a TAA comprises or consists of as scFv domain (e.g., in a $scFv^2$ antibody construct).

**[0261]** In the context of the present disclosure, the term "tumor-associated antigen" generally relates to proteins that are under normal (e.g., healthy, or non-disease) conditions specifically expressed in a limited number of tissues and/or organs, and/or in specific developmental stages, and are expressed or aberrantly expressed in one or more tumor tissues. In the context of the present disclosure, the TAA can be associated with the cell surface of a tumor cell and may not be or only rarely expressed in normal tissues. A TAA herein can be associated with a certain type of tumor based on the type of cell and/or tissue it originated from.

**[0262]** In some embodiments, a TAA that can be targeted by an antibody construct of this disclosure can be associated with a carcinoma, a sarcoma, a myeloma, a leukemia, a lymphoma, or a mixed type tumor. In certain embodiments, a multivalent and multispecific antibody construct of the present disclosure can bind a TAA of a solid tumor. Solid tumors can include carcinomas, sarcomas, or lymphomas. In other embodiments, a TAA that can be targeted by an antibody construct of the present disclosure can be that of a non-solid tumor (e.g., a liquid tumor), such as a leukemia.

**[0263]** In some embodiments, the TAA can be Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2),

Fibronectin EDB (EDB-FN), fibronectin F.IIIB domain, CGS-2, EpCAM, EGFR, HER-2, HER-3, cMet, GPC3, FOLR1, EpCAM, PSMA, CD38, DLL3, MSLN or Cldn18.2.

**[0264]** In some embodiments, the TAA can be associated with a solid tumor. Examples of such TAAs can include GPC3, MSLN, DLL3, HER2, FAP, EGFRvIII, PSMA, Cldn18.2, Mucin-1 and EpCAM.

**[0265]** In various embodiments, any solid tumor-associated antigen may be targeted by a multivalent and multispecific antibody construct of the present disclosure as long as the antibody construct comprises a binding domain that is capable of engaging the solid tumor-associated antigen with a high enough affinity to allow formation of a TCR-independent immune synapse between an immune cell (e.g., T cell, engaged via the constructs anti-CD3 and anti-CD28 domains) and the tumor cell expressing the antigen, followed by induction of an anti-tumor immune response resulting in tumor cell lysis or other events that are tumor cell-static or -toxic.

**[0266]** In some embodiments, a multivalent and multispecific antibody construct of the present disclosure comprises a binding domain capable of binding Delta-like ligand 3 (DLL3). In various embodiments, an antibody construct herein can be at least 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. 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 multivalent and multispecific antibody construct, the anti-DLL3 binding domain comprises a Fab domain. In other embodiments of a multivalent and multispecific antibody construct, the anti-DLL3 binding domain comprises an scFv domain. The anti-DLL3 binding domains that can be part of a multivalent and multispecific 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 multivalent and multispecific 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. 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 multivalent and multispecific antibody constructs described herein. In various embodiments, a multivalent and

multispecific antibody construct of the present disclosure, e.g., a multivalent and multispecific Het-Fab or $scFv^2$ antibody construct, comprises an anti-DLL3 binding domain (e.g., an scFv domain or a Fab domain) 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 multivalent and multispecific 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 multivalent and multispecific 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.

**[0267]** In some embodiments, an anti-DLL3 binding domain, e.g., an anti-DLL3 VH and/or an anti-DLL3 VL sequence, of a multivalent and multispecific antibody construct of this disclosure can be one that is known in the art, or a variant (e.g., having about  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.

**[0268]** In certain embodiments, a multivalent and multispecific antibody construct of the present disclosure, e.g., a multivalent and multispecific (e.g., a 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: 158, the HCDR2 sequence as set forth in SEQ ID NO: 159 and the HCDR3 sequence as set forth in SEQ ID NO: 160, and a VL sequence comprising the LCDR1 sequence as set forth in SEQ ID NO: 161, the LCDR2 sequence as set forth in SEQ ID NO: 162 and the LCDR3 sequence as set forth in SEQ ID NO: 163.

**[0269]** In some embodiments, a multivalent and multispecific 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: 152, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 153.

**[0270]** In certain embodiments, a multivalent and multispecific 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: 158, the HCDR2 sequence as set forth in SEQ ID NO: 159 and the HCDR3 sequence as set forth in SEQ ID NO: 160, and a VL sequence comprising the LCDR1 sequence as set forth in SEQ ID NO: 164, the LCDR2 sequence as set forth in SEQ ID NO: 165 and the LCDR3 sequence as set forth in SEQ ID NO: 163.

**[0271]** In some embodiments, a multivalent and multispecific 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: 154, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 155.

**[0272]** In some embodiments, a multivalent and multispecific 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: 156, and a VL sequence comprising the amino acid sequence set forth in SEQ ID NO: 157.

**[0273]** As described herein, in some embodiments, the anti-TAA binding domain of an antibody construct herein can be capable of binding mesothelin (MSLN). In various embodiments, the anti-TAA binding domain (e.g., scFv domain or Fab domain) of an antibody construct herein is capable of binding MSLN. In some embodiments, such anti-MSLN binding domain comprises a $V_H$ sequence comprising a HCDR1 having the sequence GYTMN (SEQ ID NO: 118), a HCDR2 having the sequence LITPYSGASSYAQKFQG (SEQ ID NO: 119), and a HCDR3 having the sequence GGYDGRGFDY (SEQ ID NO: 120) and a $V_L$ sequence comprising a LCDR1 having the sequence SASSSVSYMH (SEQ ID NO: 121), a LCDR2 having the sequence DTSKLAS (SEQ ID NO: 122), and a LCDR3 having the sequence QQWSGHPLT (SEQ ID NO: 123). In some embodiments, the anti-MSLN binding domain of an antibody construct herein comprises a VH sequence that comprises or consists 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: 132, and a VL sequence that comprises or consists 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: 133. Other anti-MSLN binding domains that are known in the art can also be used in the context of the multivalent and multispecific antibody constructs described herein.

**[0274]** In yet other embodiments, the anti-TAA binding domain of an antibody construct herein can be capable of binding Claudin18.2 (Cldn18.2). Generally, such anti-TAA binding domain herein can comprise or consist of an scFv domain or a Fab domain. In various embodiments, the anti-TAA binding domain of a multivalent and multispecific antibody construct herein is an scFv domain. In other embodiments, the anti-TAA binding domain of a multivalent and multispecific antibody construct herein is a Fab domain. In various embodiments, the anti-TAA binding domain (e.g., scFv domain or Fab domain) of an antibody construct herein is capable of binding Cldn18.2. In some embodiments, such anti-Cldn18.2 binding domain comprises a HCDR1 having the sequence SNPMI (SEQ ID NO: 124), a HCDR2 having the sequence IIDTDGSTYYADWAKG (SEQ ID NO: 125), and a HCDR3 having the sequence RLHGSSNGYYDDL (SEQ ID NO: 126) and a $V_L$ sequence comprising a LCDR1 having the sequence QASQSIYSYLS (SEQ ID NO: 127), a LCDR2 having the sequence KASTLAS (SEQ ID NO: 128), and a LCDR3 having the sequence QQGYTVTNVDKNT (SEQ ID NO: 129). In some embodiments, an anti-Cldn18.2 binding domain (e.g., scFv domain or Fab domain) of an antibody construct herein comprises a $V_H$ sequence 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: 130, and a $V_L$ sequence 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: 131. In some embodiments, the anti-Cldn18.2 binding domain of an antibody construct herein is an scFv domain and comprises, either from N- to C-terminus or from C- to N-terminus, a $V_H$ sequence comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 130, coupled to a $V_L$ sequence comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 131, via a linker$^{scFv}$ having the amino acid sequence set forth in SEQ ID NO: 59. Other anti-Cldn18.2 binding domains that are known in the art can also be used in the context of the multivalent and multispecific antibody constructs described herein.

**[0275]** Generally, in various embodiments, an antibody construct of this disclosure can bind a TAA with a $K_D$ from about 0.01 nM to about 10 nM, from about 0.05 nM to about 5 nM, or from about 0.1 nM to about 5 nM.

**D. Fc Domains**

**[0276]** As described herein, a multivalent and multispecific 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 about 99% amino acid sequence identity, e.g., each comprising one or more asymmetric amino acid substitutions that can promote preferential pairing for 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).

**[0277]** 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.

**[0278]** As disclosed herein, a multivalent and multispecific 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.

**[0279]** In certain embodiments, an antibody construct herein comprises an Fc domain based on a human IgG Fc domain, such as an IgG1, IgG2, IgG3 or IgG4 domain. In some embodiments, an antibody construct comprises an Fc domain based on a human IgG1 Fc domain. In other embodiments, an antibody construct comprises an Fc domain based on a human 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.

**[0280]** In various embodiments, a multivalent and multispecific 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.

***Modified Fc Domains***

**[0281]** In some embodiments, the present disclosure relates to multivalent and multispecific 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 of the first and the second Fc polypeptide(s) 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.

**[0282]** 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.

**[0283]** 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.

**[0284]** 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.

**[0285]** 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.

**[0286]** 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)** | APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK (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.

**[0287]** 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.

**[0288]** 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**.

**[0289]** 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**.

**[0290]** 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.

**[0291]** 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.

**[0292]** 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 Fcγ receptors 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 Fcγ receptors ("knock-out" variants) can be useful.

**[0293]** Non-limiting examples of amino acid modifications to the CH2 domain that alter binding of the Fc domain by Fcγ receptors 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 Fcγ receptors are described in *Therapeutic Antibody Engineering* (Strohl & Strohl, Woodhead Publishing series in Biomedicine No 11, ISBN 1 907568 37 9, Oct 2012, page 283).

**[0294]** 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 Fcγ receptors (i.e., a "knock-out" or "KO" variant).

**[0295]** 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).

**[0296]** 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 |

**[0297]** 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 Fcγ receptors are described in International Patent Publication No. WO 2014/190441.

**[0298]** 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.

**[0299]** 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.

**E. Linkers**

**[0300]** In various embodiments, a multivalent and multispecific 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.

**[0301]** 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 ($C_{H1}$) of a Fab domain, to an Fc polypeptide. Thus, a linker can be used to couple one domain of an antibody construct to another domain. Examples include (from N- to C-terminus) (i) a linker$^{Fab\text{-}Fc}$ that couples a Fab domain to an Fc domain, (ii) a linker$^{scFv}$ that couples $V_H$ domain to a $V_L$ domain, or vice versa depending on the scFv domain structure, (iii) a linker$^{scFv\text{-}Fc}$ that couples an scFv domain to an Fc domain, and (iv) a linker$^{Fab\text{-}scFv}$ that couples a Fab domain (e.g., a Fab light chain) to an scFv domain. In embodiments in which an antibody construct comprises two scFv domains (e.g., an scFv$^2$ antibody construct), both such scFv domains of the antibody construct can contain a linker$^{scFv}$ with either identical or different amino acid sequence. In embodiments in which both scFv domains contain an identical linker$^{scFv}$, such antibody construct can be described as comprising a linker$^{scFv}$ (instead of specifying that it contains a linker$^{scFv1}$ and a linker$^{scFv2}$ as both linkers contain the same amino acid sequence). In other embodiments in which the two 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$^{scFv2}$, indicating that the two linker sequences differ in at least one amino acid residue.

**[0302]** 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 biological target(s).

**[0303]** Furthermore, 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 the construct may be administered to. In certain embodiments, one or more of the linker(s) used in an antibody construct herein can comprise part or all of (i) a human Ig hinge region, (ii) a stalk region of C-type lectins, (iii) a family of type II membrane proteins, or combinations thereof. In certain embodiments, one or more of the linker(s) used in an antibody construct herein can comprise part or all of a human Ig hinge region, such as an IgG1 hinge region, or is a derivative thereof comprising one or more amino acid modification relative to a wildtype hinge region. Examples of linkers that may comprise part or all of a human Ig hinge region, or a derivative thereof, are linker$^{Fab\text{-}Fc}$ or linker$^{scFv\text{-}Fc}$.

**[0304]** 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.

**[0305]** 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 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: 63) 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.

**[0306]** 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, 5 or more linkers, which can include one or more linker$^{Fab\text{-}Fc}$, one or more linker$^{scFv\text{-}Fc}$, one or more linker$^{Fab\text{-}scFv}$, and/or one or more linker$^{scFv}$.

**[0307]** 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.

**[0308]** 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.

**[0309]** 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.

**[0310]** 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).

**[0311]** In some embodiments, an antibody construct of this disclosure comprises a linker$^{Fab\text{-}Fc}$ 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 or 54. In some embodiments, an antibody construct comprises a linker$^{scFv\text{-}Fc}$ 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$^{Fab\text{-}scFv}$ 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.

**[0312]** In certain embodiments, a multivalent and multispecific (e.g., 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: 54.

**[0313]** 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 or 54, 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.

**[0314]** Any of these antibody constructs can further comprise one or more linker$^{scFv}$ that couple the two variable domains VH and VL of an scFv domain to one another, wherein such linker$^{scFv}$ can comprise or consist of the amino acid sequence set forth in SEQ ID NO: 57, as well as a linker$^{Fab\text{-}scFv}$ that couples the C-terminus of a Fab light chain to the N-terminus of an scFv domain, wherein such linker$^{Fab\text{-}scFv}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 58.

### F. Certain Embodiments of Costimulatory Antibody Constructs

**[0315]** The present disclosure describes multivalent and multispecific antibody constructs that can have different formats and/or geometries, e.g., spatial molecular structures. The multivalent and multispecific antibody constructs described herein have the ability to engage at least three different antigens, two different antigens on one or more cytotoxic effector cell(s) (e.g., T cell(s)) and a

disease-associated antigen, such as TAA on a tumor cell, wherein each of these antigens is engaged at least in a monovalent manner.

**[0316]** Certain embodiments of the present disclosure relate to multivalent and multispecific antibody constructs that are at least trivalent and trispecific Het-Fab antibody constructs. Such Het-Fab antibody constructs can comprise at least three antigen binding domains, two of which are Fab domains. And 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) and thus can be described as hetero-Fabs or in short "Het-Fabs" (e.g., compared to homodimeric Fabs that share heavy and light chains with identical amino acid sequences and target the same antigen, e.g., "Hom-Fabs").

**[0317]** In various embodiments, described herein is a multivalent and multispecific Het-Fab 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 a disease-associated antigen on a target 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 first Fab domain binds CD3 and the first scFv domain binds CD28. In some embodiments, the third binding domain comprises or consists of a second Fab domain or a second scFv domain. In some embodiments, the third binding domain binds a TAA on a tumor cell.

**[0318]** In various embodiments, described herein is a multivalent and multispecific 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) 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.

**[0319]** In some embodiments, a multivalent and multispecific Het-Fab antibody construct of the present disclosure is capable of monovalently engaging CD3, CD28 and a TAA.

**[0320]** In some embodiments, a multivalent and multispecific Het-Fab 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 second Fab domain, wherein the second Fab domain is capable of binding a TAA on the tumor cell; (iii) an scFv domain, wherein the scFv domain is 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 scFv domain is coupled to the C-terminus of the light chain of the first Fab domain.

**[0321]** In some embodiments, a multivalent and multispecific Het-Fab antibody construct of the present disclosure that is capable of binding CD3, CD28 and a TAA comprises or consists of four polypeptide chains, at least two immunoglobulin heavy chains (e.g., H1 and H2) and at least 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 multivalent and multispecific Het-Fab antibody construct.

**[0322]** In some embodiments, a multivalent and multispecific 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 heavy chain heterodimer H1-H2 compared to formation of the 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.

**[0323]** In some embodiments of a multivalent and multispecific 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.

**[0324]** In some embodiments of a multivalent and multispecific 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.

**[0325]** In some embodiments, a multivalent and multispecific 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.

**[0326]** Hence, in various embodiments, a multivalent and multispecific Het-Fab antibody construct of the present disclosure comprises (i) amino acid modification(s) in one or more of its CH3 sequences of the Fc domain to promote correct heavy chain heterodimer pairing (i.e., H1-H2 as compared to H1H1 or H2H2), and in addition to that (ii) amino acid modification(s) in the CH1/CL sequences of its respective Fab domains to promote correct heavy and light chain pairing (i.e., H1-L1 and H2-L2 as compared to H1L1 and H2L2). Together, these amino acid modifications can enable correct chain pairing to yield the antibody construct, which chain configuration can be described as: L1H1:H2L2.

**[0327]** In various embodiments, the present disclosure relates to multivalent and multispecific $scFv^2$ antibody constructs. Such $scFv^2$ antibody constructs can comprise at least three antigen binding domains, at least two of which are scFv domains, thus the descriptor "$scFv^2$".

**[0328]** In some embodiments, a multivalent and multispecific 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 TAA on a tumor cell; (iii) a second 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 Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the second scFv domain is coupled to the C-terminus of the Fab light chain.

**[0329]** In some embodiments, a multivalent and multispecific 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 a TAA on a tumor cell; (iii) the second scFv domain, wherein the second 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 Fab domain is coupled to the N-terminus of the first Fc polypeptide, (b) the first scFv domain is coupled to the N-terminus of the second Fc polypeptide, and (c) the second scFv domain is coupled to the C-terminus of the Fab light chain.

**[0330]** In some embodiments, a multivalent and multispecific scFv$^2$ antibody construct of the present disclosure that is capable of binding CD3, CD28 and a TAA comprises or consists of at least three polypeptide chains, at least two immunoglobulin heavy chains (e.g., H1 and H2) and at least one immunoglobulin light chain (e.g., L). In some embodiments, the three polypeptide chains associate, either covalently and/or non-covalently, to form the multivalent and multispecific scFv$^2$ antibody construct.

**[0331]** In some embodiments, a multivalent and multispecific 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.

**[0332]** In some embodiments of a multivalent and multispecific $scFv^2$ 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.

**[0333]** 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. 1A** (e.g., the third binding domain is a second scFv domain) and **1B** (e.g., the 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.

**[0334]** In various embodiments, a multivalent and multispecific antibody construct (e.g., a Het-Fab or $scFv^2$ 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 $(VL\text{-}CL)_{Fab}\text{-}(VL\text{-}VH)_{scFv}$. In other embodiments, the light chain has the domain structure, from N- to C-terminus, of $(VL\text{-}CL)_{Fab}\text{-}(VH\text{-}VL)_{scFv}$.

**[0335]** In certain embodiments, described herein is a multivalent and multispecific antibody construct (e.g., a Het-Fab or $scFv^2$ 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 multivalent and multispecific 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.

**[0336]** In certain embodiments, described herein is a multivalent and multispecific 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 multivalent and multispecific antibody construct can comprise an amino acid sequence having the amino acid sequence set forth in SEQ ID NO: 13.

**[0337]** In various other embodiments, and as further described herein, a Fab light chain that comprises a Fab portion (e.g., a VL and a CL domain) binding CD3 and an scFv portion binding CD28 can have a different amino acid sequence when compared to SEQ ID NO: 13, e.g., can have less than 98%, 95%, 90%, or less than 85% sequence identity to SEQ ID NO: 13, because different combinations of anti-CD3 and/or anti-CD28 binding domain sequences (i.e., paratopes) may be used in the costimulatory constructs described herein. As further described herein, as long as such anti-CD3 and/or anti-CD28 binding domains fulfill certain criteria such as relative CD3 and CD28 binding affinities, induction of CD3 and CD28 engagement of the construct on an immune cell (e.g., T cell), as determined by, e.g., SPR or a comparable method known in the art, various different anti-CD3 and/or anti-CD28 binding domains known in the art may be used in the antibody constructs described herein.

### G. Certain Properties of Costimulatory Antibody Constructs

**[0338]** The costimulatory multivalent and multispecific 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 the specific combination of antibody format and antigen binding

affinities. As disclosed herein, a costimulatory multivalent and multispecific antibody construct comprises a light chain comprising a Fab portion and an scFv portion, as further described herein, and thus can possess certain properties that can be unique to a construct with that specified format (i.e., Format A).

**[0339]** In some embodiments, binding of a multivalent and multispecific antibody construct to two different antigens on a T cell and to a TAA on a tumor cell, e.g., in a tumor (micro)environment, can be – at least temporarily – simultaneous, thereby establishing a TCR-independent immune synapse, which can direct T cell-mediated cytotoxic activity to a tumor environment that contains tumor cells expressing the TAA. In various embodiments, and as further described herein, a multivalent and multispecific antibody construct may cause a significantly reduced immune cell (e.g., T cell) activation in the absence of the TAA, e.g., when the immune synapse cannot be fully formed due to an absence of the TAA. 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-dependent manner and thus may cause less off-target effects in the subject compared to conventional constructs or antibodies that act in a less TAA-dependent manner.

**[0340]** In various embodiments, and in order to address certain treatment challenges with known and conventional therapeutic T cell engaging antibodies, e.g., durability of response, off-target toxicities and/or sustained T cell activation, the herein disclosed multivalent and multispecific costimulatory T cell engager antibody constructs have been rationally designed to, e.g., optimally engage CD3 and CD28 on a T cell in obligate cis (i.e., on the same T cell), and redirect and enhance cytotoxic T cell responses to TAA-expressing tumor cells while maintaining an improved safety profile when compared to conventional therapeutic modalities.

**[0341]** In some embodiments, the antibody constructs described herein can posses an enhanced anti-tumor activity in tumors that have 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), due to their costimulatory activity by being capable of engaging both CD3 and CD28 on the same immune cell (e.g., T cell).

**[0342]** In various embodiments, binding of a multivalent and multispecific 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.

**[0343]** 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 stimulation. The antibody constructs of the present disclosure have been specifically designed, e.g., through their format and geometry, to provide both CD3 and CD28 costimulation. 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, can be 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 TAA, at the same time allows for the most potent anti-tumor activity, compared to instances in which, e.g., only CD3 and/or CD28 are engaged and bound by the construct.

**[0344]** In some embodiments, costimulatory 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 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.

**[0345]** In some embodiments, a costimulatory antibody construct of the present disclosure does bind CD3 and CD28 on the same cytotoxic effector cells (e.g., T cell) and does not bind CD3 and CD28 on two different effector cells, as measured, e.g., 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. Hence, in various embodiments, a costimulatory antibody construct of the present disclosure co-engages CD3 and CD28 on the surface of the same cytotoxic effector cell (also referred to herein as "cis" binding of CD3 and CD28). In various embodiments, a costimulatory antibody construct binds CD28 with a $K_D$ that is about 2-fold, 4-fold, 6-fold, 8-fold or 10-fold higher compared to that of CD3, when measured using a cytotoxic effector cell that expresses both CD3 and CD28 (and when compared

to a cytotoxic effector cell that expresses CD28 but not CD3), and when measured using SPR or an alternative method known in the art for measuring cell binding of antibody constructs.

**[0346]** In various embodiments, and without being bound by any theory, a costimulatory antibody construct of the present disclosure can bind CD3 on the surface of an effector cell (e.g., T cell) first via its anti-CD3 Fab domain before CD28 on the same effector cell is bound by the anti-CD28 scFv domain. The binding of a costimulatory antibody construct to CD28 hence can be conditional upon CD3 binding, another property specific to costimulatory antibody constructs described herein that comprise an anti-CD3 Fab domain, and wherein the anti-CD3 Fab light chain is C-terminally coupled to an anti-CD28 scFv domain. A multivalent and multispecific 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 (KO) cell line). In some embodiments, a multivalent and multispecific 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.

**[0347]** In some embodiments, a multivalent and multispecific antibody construct of the present disclosure binds a cytotoxic effector cell (e.g., a T cell) that expresses CD3 and CD28 with an affinity (e.g., $K_D$) 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-CD3xTAA and/or anti-CD28xTAA antibody construct.

**[0348]** Furthermore, in various embodiments, the multivalent and multispecific antibody constructs of the present disclosure may provide a target cell (e.g., tumor cell) dependent cytotoxicity profile, e.g., as shown herein when cytokine release is significantly reduced in the presence of isolated T cells only compared to conditions in which the T cells are in co-culture with TAA-expressing tumor cells.

**[0349]** In some embodiments, a multivalent and multispecific 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 TAA) 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-TAA Fab domains as described herein.

**[0350]** In some embodiments, the multivalent and multispecific 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 measured using, e.g., size-exclusion chromatography, or other comparable methods known in the art. In certain embodiments, such constructs can comprise at least one Fab domain capable of binding CD3, and two scFv domains, wherein one such scFv domain is capable of binding CD28, and the other scFv domain is capable of binding a TAA. In other embodiments, such constructs can comprise at least two Fab domains, one of which is capable of binding either CD3 and the other Fab domain is capable of binding a TAA, 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.

**[0351]** In various embodiments, a costimulatory antibody construct comprising a costimulatory portion and a TAA-engaging portion, wherein the costimulatory portion comprises an anti-CD3 Fab domain and an anti-CD28 scFv domain and wherein the anti-CD28 scFv domain is coupled to the C-terminus of the Fab light chain of the anti-CD3 Fab domain, can comprise various different combinations of different anti-CD3, anti-CD28 and anti-TAA binding domain sequences. In some embodiments, the anti-CD3, anti-CD28 and anti-TAA binding domains can have certain ratios of

affinities for their respective targets, and wherein these affinities can be determined either in a one-armed antibody (OAA) context (e.g., a monovalent, monospecific construct comprising an Fc and a single binding domain (e.g., a Fab or an scFv) against a given antigen), or in a multispecific antibody context (e.g., when using a multivalent and multivalent antibody construct as described herein.

**[0352]** In some embodiments, the $K_D$ ratio of the anti-CD3, anti-CD28 and anti-TAA binding domains for their respective targets can be about (1-11)[CD3]:(5-160)[CD28]:(0.01-0.5[TAA], 1[CD3]:(1-10)[CD28]:(0.01-1)[TAA], 1[CD3]:(1-5)[CD28]:(0.01-1)[TAA], 1[CD3]:(1-10)[CD28]:(0.01-0.5)[TAA] or about 1[CD3]:(1-5)[CD28]:(0.01-0.5)[TAA]. Such a ratio of antigen affinities of binding domains, in combination with the structure of the costimulatory portion of an antibody construct as described herein, can provide for, e.g., CD3 dependent engagement of CD28 (e.g., resulting in less CD28-dependent toxicities), TAA-driven cytotoxic activity of the construct.

**III. SEQUENCE IDENTITY OF AMINO ACID AND NUCLEIC ACID SEQUENCES**

**[0353]** 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 multivalent and multispecific 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.

**[0354]** 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 multivalent and multispecific antibody constructs disclosed herein.

**[0355]** 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.

**[0356]** 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.

**[0357]** 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.

**[0358]** 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.

**[0359]** 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).

**[0360]** 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.

**[0361]** 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 |
| 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 |

**[0362]** 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

**[0363]** In certain embodiments, the present disclosure relates to pharmaceutical compositions that can comprise one or more of the multivalent and multispecific 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.

**[0364]** 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.

**[0365]** 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.

**[0366]** 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.

**[0367]** 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.

**[0368]** Hence, also described herein is a pharmaceutical composition comprising any one or more of the multivalent and multispecific antibody construct(s) disclosed herein, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

**V. KITS**

**[0369]** The present disclosure also describes kits comprising one or more of the multivalent and multispecific 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).

**[0370]** 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.

**[0371]** 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.

**[0372]** 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).

**[0373]** A kit herein can further include other materials desirable from a commercial or user standpoint, such as filters, needles, and syringes.

## VI. METHODS

**[0374]** Further described herein are methods of producing and using the multivalent and multispecific antibody constructs of the present disclosure.

### A. Methods of Producing an Antibody Construct

**[0375]** In some embodiments, the present disclosure relates to methods for preparing the multivalent and multispecific 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).

**[0376]** 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).

**[0377]** 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.

**[0378]** 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.

**[0379]** 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).

**[0380]** 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).

**[0381]** In certain embodiments, the host cell used to produce a multivalent and multispecific 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.

**[0382]** 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.

**[0383]** 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.

**[0384]** 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.

**[0385]** 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.

**[0386]** 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.

**[0387]** 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.

**[0388]** 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).

**[0389]** 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.

**[0390]** 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.

**[0391]** 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 $NaBH_4$.

**[0392]** 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.

**[0393]** 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.

**[0394]** In some embodiments, described herein is a method of producing a multivalent and multispecific 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**

**[0395]** In certain embodiments, the present disclosure relates to methods of using multivalent and multispecific 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.

**[0396]** Such method can comprise administering a multivalent and multispecific 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.

**[0397]** In some embodiments, the present disclosure relates to a method of treating a cancer, an autoimmune disease, or an inflammatory condition in a subject in need thereof, the method comprising administering to the subject a multivalent and multispecific antibody construct of the present disclosure, or a pharmaceutical composition comprising such antibody construct, and wherein the antibody construct comprises a binding domain capable of binding an antigen associated with a cancer, an autoimmune disease, or an inflammatory condition.

**[0398]** In various embodiments, the method is directed to treating a cancer in a subject in need thereof. 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.

**[0399]** In certain embodiments, the present disclosure relates to a method of treating a solid tumor in a subject in need thereof, the method comprising administering to the subject a multivalent and multispecific antibody construct of the present disclosure, or a pharmaceutical composition comprising such antibody construct. In some embodiments, solid tumors include carcinomas, sarcomas, and lymphomas. A solid tumor target that can be engaged by an antibody construct of the present disclosure include any known solid tumor target, including MSLN, Cldn18.2, DLL3, HER2, GPC3, amongst others.

**[0400]** In certain embodiments, the present disclosure relates to a method of treating a liquid tumor in a subject in need thereof, the method comprising administering to the subject a multivalent and multispecific antibody construct of the present disclosure, or a pharmaceutical composition comprising such antibody construct. In some embodiments, the liquid tumor can be a leukemia.

**[0401]** 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.

**[0402]** The methods described herein can comprise administering a multivalent and multispecific 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.

**[0403]** A treatment (e.g., of a cancer, an autoimmune and/or inflammatory condition in a subject) can be achieved by administration of a therapeutically effective amount of a multivalent and multispecific 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 multivalent and multispecific antibody construct described herein.

**[0404]** A suitable dosage of a multivalent and multispecific 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.

**[0405]** In some embodiments, a method of treating a disease (e.g., a cancer, an autoimmune and/or inflammatory condition) 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.

**[0406]** 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 a TAA, the method comprising contacting the cell population with an effective amount of a multivalent and multispecific antibody construct of the present disclosure. In some embodiments, such multivalent and multispecific antibody construct binds CD3 and CD28 on one or more cytotoxic effector cell, such as an immune cell, and the TAA 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 at least: (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 the TAA 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 the TAA 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.

**[0407]** In some embodiments, the present disclosure relates to a method of inhibiting the proliferation of tumor cells expressing the TAA 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 multivalent and multispecific antibody construct of the present disclosure. In some embodiments, such multivalent and multispecific antibody construct binds CD3 and CD28 on one or more immune cell(s) and the TAA 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 the TAA 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 the TAA 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.

**[0408]** In some embodiments, the present disclosure relates to a method of killing tumor cells expressing a TAA, such method comprising contacting a cell population comprising the tumor cells and immune cells expressing CD3 and CD28 with an effective amount of a multivalent and multispecific antibody construct of the present disclosure. In various embodiments, such multivalent and multispecific antibody construct binds CD3 and CD28 on one or more immune

cell(s) and the TAA 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 the TAA 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 the TAA 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.

**[0409]** In various embodiments, the TAA is expressed by a solid tumor. The solid tumor can be a carcinoma, a sarcoma, or a lymphoma.

**[0410]** In other embodiments, the TAA is expressed by a liquid tumor such as a leukemia.

**[0411]** 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.

**[0412]** As described herein, in various embodiments, an antibody construct herein can be administered to a subject in need thereof, for example, a subject suffering from a cancer, an autoimmune disease and/or an inflammatory condition, 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.

**[0413]** In various embodiments, an antibody construct described herein, e.g., a multivalent and multispecific 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 a TAA (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.

**[0414]** In some embodiments, the present disclosure relates to a method of inhibiting the growth of a TAA-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 multivalent and multispecific antibody construct of the present disclosure. In various embodiments, such *in vivo* anti-tumor effect is elicited by the multivalent and multispecific antibody construct that binds (e.g., simultaneously) CD3 and CD28 on one or more immune cell(s) and the TAA 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 the TAA 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 the TAA 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.

**[0415]** 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 a TAA 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.

**[0416]** In various embodiments of the methods described herein, the multivalent and multispecific antibody constructs binds CD3 and CD28 on the same immune cell (e.g., T cell). In other embodiments, the multivalent and multispecific 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.

**[0417]** In certain other embodiments, a method herein is directed to treating an autoimmune disease in a subject in need thereof.

**[0418]** In yet other embodiments, a method herein is directed to treating an inflammatory condition in a subject in need thereof.

**[0419]** In various embodiments of the methods described herein, the subject is a rodent, a non-human primate, or a human.

**[0420]** In further embodiments, and in relation to a method described herein, administration of a sufficient amount of a multivalent and multispecific 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.

**[0421]** 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 multivalent and multispecific 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, such as GM-CSF, TNFα, a MIP-1, IFN-γ, IL-2, IL-12, IL-17, IL-21 and/or C-X-C motif ligand 13 (CXCL13) by T effector cells.

**[0422]** As described herein, in various embodiments, a multivalent and multispecific 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 Fcγ receptors (also referred to herein as an Fc "knock-out" or "KO" variant).

### C. Experimental Methods

**[0423]** In some embodiments, the present disclosure relates to experimental methods for analyzing and/or detecting a multivalent and multispecific 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.

**[0424]** Specific binding of a multivalent and multispecific 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.

**[0425]** Additional experimental methods are described in, e.g., **EXAMPLES 1-58** herein.

## VII. CERTAIN EMBODIMENTS OF THE DISCLOSURE

**[0426]** Certain embodiments of the present disclosure relate to embodiments 1-143 below, as well as to any combination of one or more of embodiments 1-143.

**[0427] Embodiment 1.** An antibody construct, comprising: (i) a first immunoglobulin (Ig) heavy chain, H1, comprising, from N- to C-terminus, a first Fab heavy chain coupled to a first Fc polypeptide, (ii) a first Ig light chain, L1, comprising, from N- to C-terminus, a first Fab light chain

coupled to a first scFv domain, and (iii) a second Ig heavy chain, H2, comprising a second Fc polypeptide, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain that is capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (c) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0428] Embodiment 2.** The antibody construct of embodiment 1, wherein the antibody construct further comprises a binding domain coupled to the N-terminus of the second Fc polypeptide, wherein the binding domain is capable of binding a disease-associated antigen, optionally wherein the disease-associated antigen is a TAA on a tumor cell.

**[0429] Embodiment 3.** The antibody construct of embodiment 1 or embodiment 2, wherein H1 further comprises a first $linker^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide.

**[0430] Embodiment 4.** The antibody construct of embodiments 3, wherein the first $linker^{Fab\text{-}Fc}$ comprises or consists of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein the derivative has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0431] Embodiment 5.** The antibody construct of any one of embodiments 1-4, wherein L1 further comprises a $linker^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**[0432] Embodiment 6.** The antibody construct of any one of embodiments 1-5, wherein the first scFv domain comprises a first $Linker^{scFv}$ ($Linker^{scFv1}$) and has the domain structure, from N- to C-terminus, of VL-$Linker^{scFv1}$-VH.

**[0433] Embodiment 7.** The antibody construct of any one of embodiments 2-6, wherein the binding domain is a second Fab domain.

**[0434] Embodiment 8.** The antibody construct of embodiment 7, wherein H2 further comprises a second Fab heavy chain that is coupled to the N-terminus of the second Fc polypeptide and a second Ig light chain, L2, comprising a second Fab light chain, and wherein the second Fab heavy chain of H2 and the second Fab light chain of L2 form the second Fab domain.

**[0435] Embodiment 9.** The antibody construct of embodiment 8, wherein H2 further comprises a second $linker^{Fab\text{-}Fc}$ comprising or consisting of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein the derivative has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0436] Embodiment 10.** The antibody construct of any one of embodiments 7-9, wherein the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promote correct heavy and light chain pairing.

**[0437] Embodiment 11.** The antibody construct of any one of embodiments 7-10, wherein the first Fab domain and the second Fab domain (i) each comprise a kappa light chain, (ii) 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.

**[0438] Embodiment 12.** The antibody construct of any one of embodiments 2-6, wherein the binding domain is a second scFv domain comprising a second VH sequence and a second VL sequence.

**[0439] Embodiment 13.** The antibody construct of embodiment 12, wherein H2 further comprises the second scFv domain coupled to the N-terminus of the second Fc polypeptide.

**[0440] Embodiment 14.** The antibody construct of embodiment 12 or embodiment 13, wherein H2 further comprises a first $linker^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**[0441] Embodiment 15.** The antibody construct of embodiment 14, wherein the first $linker^{scFv\text{-}Fc}$ comprises or consists of an IgG1, IgG2 or IgG4 hinge region, or are a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0442] Embodiment 16.** The antibody construct of any one of embodiments 12-15, wherein the second scFv domain comprises a second $Linker^{scFv}$ ($Linker^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH-$Linker^{scFv2}$-VL.

**[0443] Embodiment 17.** The antibody construct of embodiment 6 or embodiment 16, wherein the $Linker^{scFv1}$ and the $Linker^{scFv2}$ each independently comprise or consist of the amino acid sequence of $(G_4S)_x$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 63).

**[0444] Embodiment 18.** 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; 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.

**[0445] Embodiment 19.** The antibody construct of embodiment 18, wherein the antibody construct comprises a first Ig heavy chain (H1) comprising a first Fab heavy chain comprising a first VH sequence and a first CH1 sequence and the first Fc polypeptide, a second Ig heavy chain (H2) comprising the second Fc polypeptide, and a first Ig light chain (L1) comprising a first Fab light chain and the first scFv domain, wherein the first Fab heavy chain and the first Fab light chain form the first Fab domain.

**[0446] 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 a first CL sequence, coupled to the first scFv domain, wherein the first scFv domain comprises, 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.

**[0447] Embodiment 21.** The antibody construct of embodiment 19 or embodiment 20, wherein L1 further comprises a $linker^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**[0448] Embodiment 22.** The antibody construct of any one of embodiments 18-21, wherein the first scFv domain further comprises a first $Linker^{scFv}$ ($Linker^{scFv1}$) and has the domain structure, from N- to C-terminus, of VL- $Linker^{scFv1}$-VH.

**[0449] Embodiment 23.** The antibody construct of any one of embodiments 19-22, wherein the third binding domain is a second Fab domain.

**[0450] Embodiment 24.** The antibody construct of embodiment 23, wherein the second Ig heavy chain (H2) further comprises a second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, and the antibody construct further comprises a second Ig light chain (L2) comprising a second Fab light chain comprising a second VL sequence and a second CL sequence, and wherein the second Fab heavy chain and the second Fab light chain form the second Fab domain.

**[0451] Embodiment 25.** The antibody construct of embodiment 23 or embodiment 24, 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.

**[0452] Embodiment 26.** The antibody construct of any one of embodiments 20-25, wherein the first linker$^{Fab\text{-}Fc}$, the second linker$^{Fab\text{-}Fc}$, or both, are polypeptide linkers each independently comprising or consisting of about 5 to about 50 consecutive amino acid residues.

**[0453] Embodiment 27.** The antibody construct of embodiment 26, wherein the first linker$^{Fab\text{-}Fc}$ and the second linker$^{Fab\text{-}Fc}$ independently comprise or consists of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90%, 93%, 95%, 97%, or 99% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0454] Embodiment 28.** The antibody construct of any one of embodiments 23-27, wherein the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promotes correct heavy and light chain pairing.

**[0455] Embodiment 29.** The antibody construct of any one of embodiments 23-28, wherein the first Fab domain and the second Fab domain (i) each comprise a kappa light chain, (ii) each comprise a lambda light chain, or (iii) one Fab domain comprises a kappa light chain and the other Fb domain comprises a lambda light chain.

**[0456] Embodiment 30.** The antibody construct of any one of embodiments 19-22, wherein the third binding domain is a second scFv domain comprising a second VH sequence and a second VL sequence.

**[0457] Embodiment 31.** The antibody construct of embodiment 30, wherein H2 further comprises a linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**[0458] Embodiment 32.** The antibody construct of embodiment 31, wherein the linker$^{scFv\text{-}Fc}$ comprises or consists of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0459] Embodiment 33.** The antibody construct of any one of embodiments 30-32, wherein the second scFv domain comprises a second Linker$^{scFv}$ (Linker$^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH- Linker$^{scFv2}$-VL.

**[0460] Embodiment 34.** The antibody construct of embodiment 22 or embodiment 33, wherein the Linker$^{scFv1}$ and the Linker$^{scFv2}$ each independently comprise or consist of the amino acid sequence of $(G_4S)_x$, wherein x is 1, 2, 3, 4 or 5 (SEQ ID NO: 63).

**[0461] Embodiment 35.** An antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and two immunoglobulin light chains, L1 and L2, wherein: (i) 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; (ii) H2 comprises, 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; (iii) L1 comprises, from N- to C-terminus, a first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising a third VL sequence coupled to a third VH sequence; and (iv) L2 comprises, from N- to C-terminus, a second Fab light chain comprising a second VL sequence and a second CL sequence, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the second Fab heavy chain of H2 and the second Fab light chain of L2 form a second Fab domain capable of binding a TAA on a tumor cell; (c) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0462] Embodiment 36.** The antibody construct of embodiment 35, wherein H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide, and H2 further comprises a second linker$^{Fab\text{-}Fc}$ that couples the second Fab heavy chain to the second Fc polypeptide.

**[0463] Embodiment 37.** The antibody construct of embodiment 35 or embodiment 36, wherein the first Fab domain, the second Fab domain, or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promotes correct heavy and light chain pairing.

**[0464] Embodiment 38.** The antibody construct of any one of embodiments 35-37, wherein the first Fab domain and the second Fab domain (i) each comprise a kappa light chain, (ii) each comprise a lambda light chain, or (iii) one Fab domain comprises a kappa light chain and the other Fb domain comprises a lambda light chain.

**[0465] Embodiment 39.** An antibody construct comprising two immunoglobulin heavy chains, H1 and H2, and one immunoglobulin light chain, L1, wherein: (i) 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; (ii) L1 comprises, from N- to C-terminus, a first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to a first scFv domain comprising

a second VL sequence coupled to a second VH sequence; and (iii) H2 comprises, from N- to C-terminus, a second scFv domain comprising a third VL sequence and a third VH sequence, coupled to a second Fc polypeptide; wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; (c) the second scFv domain is capable of binding a TAA on a tumor cell; and (d) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain.

**[0466] Embodiment 40.** The antibody construct of embodiment 39, wherein H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide, and H2 further comprises a linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**[0467] Embodiment 41.** The antibody construct of embodiment 36 or embodiment 40, wherein one or more of the first linker$^{Fab\text{-}Fc}$, the second linker$^{Fab\text{-}Fc}$, and the linker$^{scFv\text{-}Fc}$ independently comprise or consists of an IgG1, IgG2 or IgG4 hinge region, or a derivative thereof, wherein the derivative has at least about 80%, 85%, 90% or 95% sequence identity to a wildtype IgG1, IgG2 or IgG4 hinge region.

**[0468] Embodiment 42.** The antibody construct of any one of embodiments 35-41, wherein L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**[0469] Embodiment 43.** The antibody construct of any one of embodiments 1-42, wherein the first Fc polypeptide comprises a first CH2 sequence and a first CH3 sequence and the second Fc polypeptide comprises a second CH2 sequence and a second CH3 sequence.

**[0470] Embodiment 44.** The antibody construct of any one of embodiments 1-43, wherein the dimeric Fc domain is a heterodimeric Fc domain.

**[0471] Embodiment 45.** The antibody construct of embodiment 43 or embodiment 44, wherein at least one of the first and second CH2 sequences comprises one or more amino acid substitutions relative to a corresponding wildtype CH2 sequence that reduce or ablate binding of the antibody construct to one or more Fcγ receptors.

**[0472] Embodiment 46.** The antibody construct of any one of embodiments 43-45, wherein at least one of the first and second CH3 sequences comprises one or more amino acid substitutions relative to a corresponding wildtype CH3 sequence that promotes formation of a heterodimeric Fc domain compared to a formation of a homodimeric Fc domain.

**[0473] Embodiment 47.** The antibody construct of embodiment 46, 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.

**[0474] Embodiment 48.** The antibody construct of any one of embodiments 1-47, wherein the antibody construct binds CD3 with a $K_D$ from about 0.1 nM to about 100 nM, from about 1 nM to about 100 nM, or from about 10 nM to about 75 nM, optionally, wherein CD3 comprises the CD3 epsilon chain.

**[0475] Embodiment 49.** The antibody construct of any one of embodiments 1-48, wherein the first Fab domain that is capable of binding CD3 comprises HCDR1-3 as set forth in SEQ ID NOs: 49-52 and LCDR1-3 as set forth in SEQ ID NOs: 53-55.

**[0476] Embodiment 50.** The antibody construct of any one of embodiments 1-49, wherein the first Fab domain that is capable of binding CD3 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 1, 2, 82, 84 or 86, and a VL sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 5, 83, 85 or 87.

**[0477] Embodiment 51.** The antibody construct of any one of embodiments 1-50, wherein the antibody construct binds CD28 with a $K_D$ from about 1 nM to about 100 nM or from about 10 nM to about 75 nM.

**[0478] Embodiment 52.** The antibody construct of any one of embodiments 1-51, wherein the first scFv domain that is capable of binding CD28 comprises HCDR1-3 as set forth in SEQ ID NOs: 30, 34 and 40, and LCDR1-3 as set forth in SEQ ID NOs: 43, 47 and 48.

**[0479] Embodiment 53.** The antibody construct of any one of embodiments 1-52, wherein the first scFv domain that is capable of binding CD28 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 15, 18, 20, 21, 22, 23, 24, 25, 27 and 76, and a VL sequence having at least about 90%, 95%, 97%,

or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 16, 17, 19, 26 and 77.

**[0480] Embodiment 54.** The antibody construct of any one of embodiments 1-53, wherein the antibody construct does not bind CD3 and CD28 on two different cytotoxic effector 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.

**[0481] Embodiment 55.** The antibody construct of embodiment 54, wherein the antibody construct co-engages CD3 and CD28 on the surface of the same cytotoxic effector cell.

**[0482] Embodiment 56.** The antibody construct of any one of embodiments 1-55, wherein the antibody construct binds CD28 with a $K_D$ that is about 2-fold, 4-fold, 6-fold, 8-fold or 10-fold higher on a cytotoxic effector cell that expresses both CD3 and CD28, compared to a cytotoxic effector cell that expresses CD28 but not CD3, and when measured using SPR.

**[0483] Embodiment 57.** The antibody construct of any one of embodiments 1-56, wherein the antibody construct binds the TAA with a $K_D$ from about 0.01 nM to about 10 nM, from about 0.05 nM to about 5 nM, or from about 0.1 nM to about 5 nM.

**[0484] Embodiment 58.** The antibody construct of any one of embodiments 1-57, wherein the antibody construct is capable of forming a TCR-independent immune synapse by binding CD3 and CD28 on the surface of the cytotoxic effector cell and the TAA on the tumor cell.

**[0485] Embodiment 59.** The antibody construct of any one of embodiments 1-58, wherein the antibody construct comprises a fourth binding domain.

**[0486] Embodiment 60.** The antibody construct of any one of embodiments 1-59, wherein the cytotoxic effector cell is an immune cell, optionally wherein the immune cell is a T cell.

**[0487] Embodiment 61.** The antibody construct of any one of embodiments 1-60, wherein the TAA is expressed by a solid tumor.

**[0488] Embodiment 62.** The antibody construct of any one of embodiments 1-61, wherein the solid tumor is a carcinoma, a sarcoma, or a lymphoma.

**[0489] Embodiment 63.** A pharmaceutical composition comprising the antibody construct of any one of embodiments 1-62, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

**[0490] Embodiment 64.** 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 1-62.

**[0491] Embodiment 65.** A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 64.

**[0492] Embodiment 66.** A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 64, or the vector or set of vectors of embodiment 65.

**[0493] Embodiment 67.** A method of producing an antibody construct of any one of embodiments 1-62, 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 of the polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture.

**[0494] Embodiment 68.** The method of embodiment 67, further comprising, subsequent to step (b), purifying the antibody construct.

**[0495] Embodiment 69.** 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 1-62, wherein the immune cells express CD3 and CD28 and the tumor cells express a TAA.

**[0496] Embodiment 70.** 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 1-62, wherein the immune cells express CD3 and CD28 and the tumor cells express a TAA.

**[0497] Embodiment 71.** 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 1-62, wherein the immune cells express CD3 and CD28 and the tumor cells express a TAA.

**[0498] Embodiment 72.** The method of any one of embodiments 69-71, wherein the immune cells comprise T cells.

**[0499] Embodiment 73.** The method of any one of embodiments 69-72, wherein the antibody construct binds (i) CD3 and CD28 on the same immune cell and (ii) the TAA on a tumor cell.

**[0500] Embodiment 74.** The method of embodiment 73, wherein the binding of the antibody construct to CD3 and CD28 on the immune cell and the TAA on the tumor cell forms a TCR-independent artificial immune synapse between the immune cell and the tumor cell, thereby eliciting a cytotoxic immune response of the immune cell against the tumor cell.

**[0501] Embodiment 75.** The method of any one of embodiments 69-74, wherein the cell population is within a subject.

**[0502] Embodiment 76.** A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of embodiments 1-62.

**[0503] Embodiment 77.** The method of embodiment 76, wherein a cytotoxic immune response against the tumor is elicited in the subject, thereby treating the tumor in the subject.

**[0504] Embodiment 78.** The method of embodiment 76 or embodiment 77, wherein the tumor is a solid tumor.

**[0505] Embodiment 79.** An antibody construct of any one of embodiments 1-62 for use in the treatment of a tumor, optionally wherein the tumor is a solid tumor or a liquid tumor.

**[0506] Embodiment 80.** Use of an antibody construct of any one of embodiments 1-62 in the manufacture of a medicament for the treatment of a tumor, optionally wherein the tumor is a solid tumor or a liquid tumor.

**[0507] Embodiment 81.** An antibody construct, comprising: (i) a first immunoglobulin (Ig) heavy chain, H1, comprising, from N- to C-terminus, a first Fab heavy chain coupled to a first Fc polypeptide, (ii) a first Ig light chain, L1, comprising, from N- to C-terminus, a first Fab light chain coupled to a first scFv domain, and (iii) a second Ig heavy chain, H2, comprising a second Fc polypeptide, wherein: (a) the first Fab heavy chain of H1 and the first Fab light chain of L1 form a first Fab domain that is capable of binding CD3 on a cytotoxic effector cell; (b) the first scFv domain is capable of binding CD28 on a cytotoxic effector cell; and (c) the first Fc polypeptide and the second Fc polypeptide form a dimeric Fc domain, and wherein the antibody construct: (1) does not bind CD3 and CD28 on two different cytotoxic effector 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; and/or (2) binds CD28 with a $K_D$ that is about 2-fold, 4-fold, 6-fold, 8-fold or 10-fold higher on a cytotoxic effector cell that expresses both CD3 and CD28, compared to a cytotoxic effector cell that expresses CD28 but not CD3, and when measured using SPR.

**[0508] Embodiment 82.** The antibody construct of embodiment 81, further comprising a binding domain capable of binding a TAA on a tumor cell, wherein the binding domain is coupled to the N-terminus of the second Fc polypeptide, and optionally wherein the binding domain is a second scFv domain or a second Fab domain.

**[0509] Embodiment 83.** The antibody construct of embodiment 82, wherein the $K_D$ ratio of the first Fab domain for binding CD3, the first scFv domain for binding CD28, and the anti-TAA binding domain for binding the TAA is about 1:1:0.01 to about 1:5:0.5 for CD3, CD28, and TAA, respectively, wherein such range of $K_D$ ratios can also be expressed as (1-11)[CD3]:(5-160)[CD28]:(0.01-0.5[TAA] or 1[CD3]:(1-5)[CD28]:(0.01-0.5)[TAA].

**[0510] Embodiment 84.** The antibody construct of embodiment 82 or embodiment 83, wherein the antibody construct does not reduce T cell viability by more than 5%, 3%, 1%, or by 0% compared to T cells treated with a negative control construct that does not contain a binding domain against the TAA, and wherein the antibody construct is incubated with the T cells for 48 hours.

**[0511] Embodiment 85.** 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 disease-associated antigen, and (iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that promote formation of the heterodimeric Fc domain; (b) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide; (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain; and (d) the third binding domain is coupled to the N-terminus of the second Fc polypeptide.

**[0512] Embodiment 86.** The antibody construct of embodiment 85, wherein the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that reduce binding to at least one FcγR.

**[0513] Embodiment 87.** 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 disease-associated antigen, and (iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein: (a) the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino

acid modifications that reduce binding to at least one FcγR; (b) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide; (c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain; and (d) the third binding domain is coupled to the N-terminus of the second Fc polypeptide.

**[0514] Embodiment 88.** The antibody construct of embodiment 87, wherein the dimeric Fc domain is a heterodimeric Fc domain and the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that promote formation of the heterodimeric Fc domain.

**[0515] Embodiment 89.** The antibody construct of any one of embodiments 85-88, wherein the third binding domain comprises or consists of a second Fab domain or a second scFv domain.

**[0516] Embodiment 90.** The antibody construct of any one of embodiments 85-89, wherein the disease-associated antigen is a tumor-associated antigen (TAA).

**[0517] Embodiment 91.** The antibody construct of embodiment 90, wherein the TAA is an antigen associated with a solid tumor.

**[0518] Embodiment 92.** The antibody construct of embodiment 90, wherein the TAA is an antigen associated with a liquid tumor.

**[0519] Embodiment 93.** The antibody construct of any one of embodiments 90-92, wherein the $K_D$ ratio of the three binding domains for their respective targets is about (1-11)[CD3]:(5-160)[CD28]:(0.01-0.5[TAA], 1[CD3]:(1-10)[CD28]:(0.01-1)[TAA] or 1[CD3]:(1-5)[CD28]:(0.01-0.5)[TAA].

**[0520] Embodiment 94.** The antibody construct of any one of embodiments 85-93, wherein the first scFv domain has the domain structure, from N- to C-terminus, of: VH-Linker$^{scFv1}$-VL or VL-Linker$^{scFv1}$-VH, and wherein the Linker$^{scFv1}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 63.

**[0521] Embodiment 95.** The antibody construct of any one of embodiments 85-94, wherein the first scFv domain has the domain structure, from N- to C-terminus, of VL-Linker$^{scFv1}$-VH.

**[0522] Embodiment 96.** The antibody construct of any one of embodiments 85-95, wherein the antibody construct comprises at least, from N- to C-terminus, (i) a first immunoglobulin (Ig) heavy chain (H1) comprising a first Fab heavy chain comprising a first VH sequence and a first CH1 sequence, coupled to the first Fc polypeptide, (ii) a second Ig heavy chain (H2) comprising the second Fc polypeptide, and (iii) a first Ig light chain (L1) comprising a first Fab light chain

comprising a first VL sequence and a first CL sequence, coupled to the first scFv domain, wherein the first Fab heavy chain of H1 and the first Fab light chain of L1 form the first Fab domain.

**[0523] Embodiment 97.** The antibody construct of embodiment 96, wherein L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**[0524] Embodiment 98.** The antibody construct of embodiment 97, wherein the linker$^{Fab\text{-}scFv}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 58, or an amino acid sequence having one or more amino acid substitutions thereto.

**[0525] Embodiment 99.** The antibody construct of any one of embodiments 96-98, wherein H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide.

**[0526] Embodiment 100.** The antibody construct of embodiments 99, wherein the first linker$^{Fab\text{-}Fc}$ comprises or consists of an IgG1 hinge region, or are a derivative thereof, wherein the linker$^{Fab\text{-}Fc}$ has at least about 80%, 85%, 90%, 93%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 54 or 56.

**[0527] Embodiment 101.** The antibody construct of any one of embodiments 85-100, wherein the first Fc polypeptide comprises a first CH2 sequence and a first CH3 sequence and the second Fc polypeptide comprises a second CH2 sequence and a second CH3 sequence.

**[0528] Embodiment 102.** The antibody construct of embodiment 101, wherein the first CH2 sequence and/or the second CH2 sequence comprise one or more amino acid modifications that reduce binding to at least one FcγR.

**[0529] Embodiment 103.** The antibody construct of embodiment 102, wherein the first CH2 sequence and/or the second CH2 sequence comprise one or more of the amino acid substitutions L234A, L235A and D265S, and wherein the numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system.

**[0530] Embodiment 104.** The antibody construct of any one of embodiments 101-103, wherein the first CH3 sequence and/or the second CH3 sequence comprise one or more amino acid modifications that promote formation of a heterodimeric Fc domain when compared to the formation of a homodimeric Fc domain.

**[0531] Embodiment 105.** The antibody construct of embodiment 104, 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.

**[0532] Embodiment 106.** The antibody construct of any one of embodiments 85-105, wherein the antibody construct binds CD3 with a $K_D$ from about 0.1 nM to about 100 nM, from about 1 nM to about 100 nM, or from about 10 nM to about 75 nM, optionally, wherein CD3 comprises the CD3 epsilon chain.

**[0533] Embodiment 107.** The antibody construct of any one of embodiments 85-106, wherein the antibody construct binds CD28 with a $K_D$ from about 1 nM to about 100 nM or from about 10 nM to about 75 nM.

**[0534] Embodiment 108.** The antibody construct of any one of embodiments 85-107, wherein the antibody construct co-engages CD3 and CD28 on the surface of 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.

**[0535] Embodiment 109.** The antibody construct of any one of embodiments 85-108, wherein the antibody construct binds CD28 on a cytotoxic effector cell that expresses both CD3 and CD28 with a $K_D$ that is about 2-fold, 4-fold, 6-fold, 8-fold or 10-fold higher when compared to a cytotoxic effector cell that expresses CD28 but not CD3, when measured using SPR.

**[0536] Embodiment 110.** The antibody construct of any one of embodiments 85-109, wherein the cytotoxic effector cell is an immune cell, optionally wherein the immune cell is a T cell.

**[0537] Embodiment 111.** The antibody construct of embodiment 110, wherein the antibody construct does not reduce the viability of a population of T cells by more than 5%, 3%, 1%, or by 0% after incubation for 48 hours when compared to T cells treated with a negative control construct that does not contain a binding domain against the disease-associated antigen.

**[0538] Embodiment 112.** The antibody construct of embodiment 96-111, wherein H2 further comprises a second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, wherein the second Fab heavy chain is coupled to the N-terminus of the second Fc polypeptide.

**[0539] Embodiment 113.** The antibody construct of embodiment 112, wherein H2 further comprises a second linker$^{Fab\text{-}Fc}$ comprising or consisting of an IgG1 hinge region, or are a derivative thereof, wherein the second linker$^{Fab\text{-}Fc}$ has at least about 80%, 85%, 90%, 93%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 54 or 56.

**[0540] Embodiment 114.** The antibody construct of embodiment 112, further comprising a second Ig light chain (L2) comprising a second Fab light chain comprising a second VL sequence and a second CL sequence, wherein the second Fab heavy chain of H2 and the second Fab light chain of L2 form a second Fab domain as the third binding domain.

**[0541] Embodiment 115.** 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 comprising or consisting of a second Fab domain and capable of binding a disease-associated antigen, 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.

**[0542] Embodiment 116.** The antibody construct of any one of embodiments 112-115, wherein one of the Fab domains comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

**[0543] Embodiment 117.** The antibody construct of any one of embodiments 112-116, wherein one or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promote correct heavy and light chain pairing of H1 with L1 as compared to L2, and H2 with L2 as compared to L1.

**[0544] Embodiment 118.** The antibody construct of embodiment 96-111, wherein H2 further comprises a second scFv domain comprising a second VH sequence and a second VL sequence, wherein the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

**[0545] Embodiment 119.** The antibody construct of embodiment 118, wherein H2 further comprises a first linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**[0546] Embodiment 120.** The antibody construct of embodiment 119, wherein the first linker$^{scFv\text{-}Fc}$ comprises or consists of an IgG1 hinge region, or are a derivative thereof, wherein the first

linker$^{scFv\text{-}Fc}$ has at least about 80%, 85%, 90%, 95% or 100% sequence identity to the sequence set forth in SEQ ID NO: 57.

**[0547] Embodiment 121.** The antibody construct of embodiment 119 or embodiment 120, wherein the second scFv domain comprises a second Linker$^{scFv}$ (Linker$^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH-Linker$^{scFv2}$-VL, wherein the Linker$^{scFv2}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 63.

**[0548] Embodiment 122.** The antibody construct of any one of embodiments 85-121, wherein the first Fab domain that is capable of binding CD3 (i) comprises HCDR1-3 as set forth in SEQ ID NOs: 49, 50 and 52, respectively, or 49, 51 and 52, respectively, and LCDR1 and 3 as set forth in SEQ ID NOs: 53 and 55, respectively, with LCDR2 having the sequence of RND, according to the IMGT numbering system; (ii) comprises HCDR1-3 as set forth in SEQ ID NOs: 134, 140 and 141, respectively, and LCDR1-3 set forth in SEQ ID NOs: 142, 138 and 139, respectively; or (iii) comprises HCDR1-3 as set forth in SEQ ID NOs: 143, 135 and 141, respectively, and LCDR1-3 set forth in SEQ ID NOs: 137-139, respectively.

**[0549] Embodiment 123.** The antibody construct of any one of embodiments 85-122, wherein the first Fab domain that is capable of binding CD3 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 1, 2, 82, 84 or 86, and a VL sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 5, 83, 85 or 87.

**[0550] Embodiment 124.** The antibody construct of any one of embodiments 85-123, wherein the first scFv domain that is capable of binding CD28 (i) comprises HCDR1-3 as set forth in SEQ ID NOs: 30, 34 and 40, and LCDR1-3 as set forth in SEQ ID NOs: 43, 47 and 48, (ii) comprises HCDR1-3 as set forth in SEQ ID NOs: 106-108, and LCDR1-3 as set forth in SEQ ID NOs: 109-111, or (iii) comprises HCDR1-3 as set forth in SEQ ID NOs: 112-114, and LCDR1-3 as set forth in SEQ ID NOs: 115-117.

**[0551] Embodiment 125.** The antibody construct of any one of embodiments 85-124, wherein the first scFv domain that is capable of binding CD28 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 15, 18, 20, 21, 22, 23, 24, 25, 27 and 76, and a VL sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 16, 17, 19, 26 and 77.

**[0552] Embodiment 126.** The antibody construct of any one of embodiments 85-125, wherein the antibody construct binds the disease-associated antigen with a $K_D$ from about 0.01 nM to about 10 nM, from about 0.05 nM to about 5 nM, or from about 0.1 nM to about 5 nM.

**[0553] Embodiment 127.** The antibody construct of any one of embodiments 85-126, wherein the antibody construct comprises a fourth binding domain, optionally wherein the fourth binding domain is capable of binding a disease-associated antigen.

**[0554] Embodiment 128.** A pharmaceutical composition comprising the antibody construct of any one of embodiments 85-127, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

**[0555] Embodiment 129.** 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 85-127.

**[0556] Embodiment 130.** A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 129.

**[0557] Embodiment 131.** A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of embodiment 129, or the vector or set of vectors of embodiment 130.

**[0558] Embodiment 132.** A method of producing an antibody construct of any one of embodiments 85-127, 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 of the polypeptide chains that form the antibody construct; and (b) recovering the antibody construct from the host cell culture.

**[0559] Embodiment 133.** A method of eliciting an immune response in a cell population comprising immune cells and target cells, the method comprising contacting the cell population with an effective amount of the antibody construct of any one of embodiments 85-127, wherein the immune cells express CD3 and CD28 and the target cells express a disease-associated antigen.

**[0560] Embodiment 134.** A method of inhibiting the proliferation of target cells, the method comprising contacting a cell population comprising the target cells and immune cells with an effective amount of the antibody construct of any one of embodiments 85-127, wherein the immune cells express CD3 and CD28 and the target cells express a disease-associated antigen.

**[0561] Embodiment 135.** The method of any one of embodiments 133-134, wherein the target cells comprise tumor cells and the disease-associated antigen is a TAA.

**[0562] Embodiment 136.** The method of any one of embodiments 133-135, wherein the cell population is within a subject.

**[0563] Embodiment 137.** A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of embodiments 85-127.

**[0564] Embodiment 138.** The method of embodiment 137, further comprising eliciting a cytotoxic immune response against the tumor in the subject, thereby treating the tumor in the subject.

**[0565] Embodiment 139.** The method of embodiment 137 or embodiment 138, wherein the tumor is a solid tumor or a liquid tumor.

**[0566] Embodiment 140.** An antibody construct of any one of embodiments 85-127 for use in the treatment of a tumor.

**[0567] Embodiment 141.** Use of an antibody construct of any one of embodiments 85-127 in the manufacture of a medicament for the treatment of a tumor.

**[0568] Embodiment 142.** In a method comprising treating a subject having a tumor by administering to the subject an antibody construct targeting a TAA, 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.

**[0569] Embodiment 143.** A method of treating a subject having a tumor by administering to the subject an antibody construct targeting a TAA comprising: (a) means for binding the TAA; 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

**[0570]** The following Examples are provided for illustrative purposes and are not intended to limit the scope of the invention in any way.

**[0571]** 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).

**[0572]** 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 AND PREPARATION OF MULTIVALENT AND MULTISPECIFIC ANTI-(MSLNxCD28xCD3) ANTIBODY CONSTRUCTS

**[0573]** Several multivalent and multispecific anti-(MSLNxCD28xCD3) antibody constructs as well as bispecific anti-(MSLNxCD3) and anti-(MSLNxCD28) control antibody constructs were produced as described below. The multivalent and multispecific antibody constructs and controls were prepared in different formats and geometries, as shown, e.g., in **FIGS. 1A-1F**. Such 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 MSLN-expressing tumor cells.

***Design of multivalent and multispecific antibody constructs targeting MSLN, CD28 and CD3***

**[0574]** Multivalent and multispecific antibody constructs that are monovalent for each antigen were prepared in a format in which the MSLN antigen binding domain was an scFv 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.

**[0575]** Bispecific antibody constructs were prepared in a format in which MSLN antigen-binding domain was an scFv domain and the CD3 or CD28 antigen-binding domain was a Fab domain. These trispecific and bispecific control 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., **TABLE 5**). 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). The antibody constructs in **TABLE 6** are 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.

**[0576]** The multivalent and multispecific antibody constructs against MSLN, CD28 and CD3 and bispecific control constructs were expressed and characterized as described in WO2015109131. Briefly, the genes encoding the antibody constructs' heavy and light chains were constructed via gene synthesis using codons optimized for human/mammalian expression. The anti-MSLN scFv domain sequence was generated from the $V_H$ and $V_L$ sequences of the mouse anti-MSLN antibody (*see*, e.g., Nat Biotechnol. 1999 Jun;17(6):568-72) with additional humanizing mutations. 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 (*see*, e.g., Shiao et al., *Transplantation*. 2007;83(3):304-13).

**[0577]** In the trispecific as well as bispecific antibody constructs of this disclosure containing an anti-CD3 Fab domain or an anti-CD28 Fab domain, the $V_H$ and $V_L$ sequences were coupled to human IgG1 $C_{H1}$ and $C_L$ sequences, respectively. In some trispecific antibody constructs of this disclosure, an anti-CD3 scFv domain was coupled to the N-terminus of the $V_H$ domain of the anti-CD28 Fab domain or to the N- or C-termini of the first or second Fc polypeptide of the heterodimeric Fc domain. In other trispecific antibody constructs of this disclosure, an anti-CD28 scFv domain was coupled to the N-terminus of the $V_H$ or $V_L$ domain of the anti-CD3 Fab domain or to the C-terminus of the $C_L$ domain of the anti-CD3 Fab domain. In various multivalent and multispecific antibody constructs of this disclosure, an anti-MSLN scFv domain was coupled to the N-terminus of the first or second Fc polypeptide of the heterodimeric Fc 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 coupled to human IgG1 $C_{H1}$ and $C_L$ sequences, respectively. In cases of the bispecific control constructs, the scFv domains were coupled to one Fc polypeptide chain of the heterodimeric Fc domain and the $V_H$-$C_{H1}$ domains of the Fab domain were fused to the second Fc polypeptide chain of the heterodimeric Fc domain.

**[0578]** Several heavy chain domain structures and formats that were used to generate certain antibody constructs of the present disclosure, e.g., the multivalent and multispecific as well as bispecific constructs, and/or other constructs for studying the impact of overall construct format and geometry on the properties of an antibody construct, are shown below in, e.g., **TABLE 5**, from N-terminus to C-terminus.

## EXAMPLE 2: DESIGN AND PREPARATION OF MULTIVALENT AND MULTISPECIFIC ANTI-(CLDN18.2xCD28xCD3) ANTIBODY CONSTRUCTS

**[0579]** Several multivalent and multispecific anti-(CLDN18.2(Cldn18.2)xCD28xCD3) antibody constructs and bispecific anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) control antibody constructs were produced as described below. The antibody constructs and controls were prepared in different formats and geometries, as shown, e.g., in **FIGS. 1A-1F**. Such 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 CLDN18.2-expressing tumor cells.

***Design of multivalent and multispecific antibody constructs targeting CLDN18.2, CD28 and CD3***

**[0580]** Multivalent and multispecific antibody constructs were prepared in a format in which the CLDN18.2 antigen-binding arm was an scFv 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.

**[0581]** Bispecific control antibody constructs were prepared in a format in which CLDN18.2 antigen-binding domain was an scFv domain and the CD3 or CD28 antigen-binding domain was a Fab domain.

**[0582]** 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 constructs 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.

**[0583]** The multivalent and multispecific antibody constructs as well as the bispecific control constructs against CLDN18.2, CD28 and/or CD3 were designed, expressed, and characterized as described in WO2015109131. Briefly, the genes encoding the antibody constructs' heavy and light chains 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. The anti-CLDN18.2 scFv domain sequence was generated from the $V_H$ and $V_L$ sequences of the humanized rabbit anti-CLDN18.2 antibody (13B08, *internal Ab*).

**[0584]** In multivalent and multispecific antibody constructs as well as the bispecific control constructs of this disclosure containing an anti-CD3 Fab domain or an anti-CD28 Fab, 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, anti-CD3 scFv domain or an anti-CD28 scFv domain were fused to the N- or C-termini of one of the Fc polypeptides of the heterodimeric Fc domain. In other trispecific antibody constructs of this disclosure, anti-CD3 scFv domain was fused to the N-terminus of the $V_H$ domain of the anti-CD28 Fab domain or to the C-terminus of the $C_L$ domain of the anti-CD28 Fab domain. In other trispecific antibody constructs of this disclosure, 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-CLDN18.2 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-CD3 Fab domain 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. In all embodiments, the scFv domains were fused to one chain of the heterodimeric Fc domain and the $V_H$-$C_{H1}$ domains of the Fabs were fused to the second chain of the heterodimeric Fc domain.

**[0585]** Some heavy chain domain structures and formats that were used to generate certain antibody constructs of the present disclosure, e.g., some multivalent and multispecific 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**, from N-terminus to C-terminus:

**TABLE 5: Heavy Chain Domain Structures Used in Certain Antibody Constructs**

| Heavy Chain Domain Structure: | Certain Antibody Constructs that can comprise the Chain Format: |
|---|---|
| $V_H$-$C_{H1}$-hinge-CH2-CH3 | v34915, v34916, v34919, v34927, v37634, v37639 |
| $V_H$-$V_L$-hinge-CH2-CH3 | v37633, v37634, v37635, v37637, v37638, v37639, v37640, v37642, v37658, v37660, v37662, v37663, v37665, v37667, v37668 |
| $V_L$-$V_H$-hinge-CH2-CH3 | v34913, v34914, v34915, v34916, v34917 v34918, v34919, v34927 |
| $V_H$-$V_L$-$V_H$-$C_{H1}$-hinge-CH2-CH3 | v34913, v34914, v37633, v37637, v37638, v37642, v37658, v37662, v37664, v37666, v37667 |
| $V_H$-$C_{H1}$-hinge-CH2-CH3-$V_L$-$V_H$ | v34917, v34918, v37635, v37640, v37650, v37669 |
| $V_H$-$V_L$-VH-CH1-hinge-CH2-CH3-VL-VH | v37636, v37641 |
| CH2-CH3 | v37636, v37641 |

**[0586] TABLE 6** below provides a description of certain multivalent and multispecific anti-(MSLNxCD28xCD3) and anti-(CLDN18.2xCD28xCD3) construct as well as bispecific control constructs that were prepared herein with varying valencies, formats and geometries. The respective numbers of antigen-targeting domains for each construct are indicated in the "Format" column. As described herein, the "1" indicates monovalent binding against a certain antigen, and a "2" indicates bivalent binding against a certain antigen.

**TABLE 6: Description of Certain Antibody Constructs of the Present Disclosure**

| Construct # | Format | Heavy Chain A | Heavy Chain B | Fc Description |
|---|---|---|---|---|
| 34913 | 1x1x1 | N-terminus C3E6 VH-VL scFv, huTN228 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 34914 | 1x1x1 | N-terminus huTN228 VH-VL scFv, C3E6 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 34915 | 1x1x1 | C3E6 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 34916 | 1x1x1 | C3E6 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 34917 | 1x1x1 | C3E6 Fab, C-terminus huTN228 VL-VH scFv | MSLN VL-VH scFv | Het Fc, FcKO |
| 34918 | 1x1x1 | huTN228 Fab, C-terminus C3E6 VL-VH scFv | MSLN VL-VH scFv | Het Fc, FcKO |
| 34919 | 1x1 | C3E6 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 34927 | 1x1 | huTN228 Fab | MSLN VL-VH scFv | Het Fc, FcKO |
| 37633 | 1x1x1 | N-terminus huTN228 VH-VL scFv, C3E6 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |

| | | | | |
|---|---|---|---|---|
| 37634 | 1x1x1 | C3E6 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37635 | 1x1x1 | C3E6 Fab, C-terminus huTN228 VL-VH scFv | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37636 | 1x1x1 | N-terminus 13B08 VH-VL scFv, C3E6 Fab, C-terminus huTN228 VL-VH scFv | No paratope | Het Fc, FcKO |
| 37637 | 1x1x1 | huTN228 VH-VL scFv | N-terminus 13B08 VH-VL scFv, C3E6 Fab | Het Fc, FcKO |
| 37638 | 1x1x1 | N-terminus C3E6 VH-VL scFv, huTN228 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37639 | 1x1x1 | huTN228 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37640 | 1x1x1 | huTN228 Fab, C-terminus C3E6 VL-VH scFv | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37641 | 1x1x1 | N-terminus 13B08 VH-VL scFv, huTN228 Fab, C-terminus C3E6 VL-VH scFv | No paratope | Het Fc, FcKO |
| 37642 | 1x1x1 | N-terminus 13B08 VH-VL scFv, huTN228 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37658 | 1x1x1 | N-terminus C3E6 VH-VL scFv, Palivizumab Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37660 | 1x1x1 | Palivizumab Fab, C-terminus C3E6 VL-VH scFv | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37662 | 1x1x1 | N-terminus 13B08 VH-VL scFv, Palivizumab Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37663 | 1x1 | C3E6 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37664 | 1x1 | No paratope | N-terminus 13B08 VH-VL scFv, C3E6 Fab | Het Fc, FcKO |
| 37665 | 1x1 | huTN228 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37666 | 1x1 | N-terminus 13B08 VH-VL scFv, huTN228 Fab | No paratope | Het Fc, FcKO |
| 37667 | 2x1 | N-terminus C3E6 VH-VL scFv, C3E6 Fab | 13B08 VH-VL scFv | Het Fc, FcKO |
| 37669 | 2x1 | C3E6 Fab, C-terminus C3E6 VL-VH scFv | 13B08 VH-VL scFv | Het Fc, FcKO |
| 35923 AMG-910 (Benchmark | 1x1 | Anti-CLDN18.2 VH - VL scFv- Anti-CD318.2 VH - VL scFv | n/a | n/a |
| 22277 | 1x1 | Palivizumab Fab | Palivizumab Fab | Het Fc |
| 31926 | 1x1 | N-terminus anti-hemagglutinin VH-VL scFv, C3E6 Fab | Anti-hemagglutinin VH-VL scFv | Het Fc, FcKO |

n/a = not applicable.

**[0587]** By way of example, in **TABLE 6**, the nomenclature "1x1x1" indicates that the trispecific antibody construct is multivalent and multispecific (e.g., engaging each antigen monovalently) and comprised either one MSLN binding domain, one CD28 binding domain and one CD3 binding

domain or one CLDN18.2 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 either one MSLN or CLDN18.2 binding domains and one CD3 or CD28 binding domain, etc. Certain formats (e.g., 1x1x1, 1x1, 2x1, etc.) and geometries (e.g., relative positioning of binding domains) of certain antibody constructs described herein are also illustrated in, e.g., **FIGS. 1A-1L**.

***Production of Multivalent and multispecific Anti-(MSLNxCD3xCD28) and Anti-(CLDN18.2xCD3xCD28) Antibody Constructs***

**[0588]** 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)).

**[0589]** 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), and light chain(s) (LC) that allow for heterodimer formation (e.g., HC-A:HC-B:LC ratios = 1:1:1 or 2:1:1or 1:1.5:1 or 1:2:1.5 or 1:1:1.5 or 2:1:3). 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.

**[0590]** 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™).

**[0591]** 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 A5-NaCl (50 mM sodium acetate, 150 mM sodium chloride, pH 5.0) or H6-NaCl (50 mM histidine, 150 mM sodium chloride, pH 6.0) buffer was used at a flow-rate of 1 mL/min. 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 A5Su (50 mM sodium acetate, 9% sucrose, pH 5.0) 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.

**[0592]** 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.

**[0593]** 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., WO2015109131. All antibody constructs were expressed and purified to >90% heterodimer purity without contaminating homodimers.

**[0594]** **TABLE 7** below describes the post purification yield for the production of certain antibody constructs described herein.

**TABLE 7: Post Purification Yield and Purity for Certain Antibody Constructs**

| Antibody Construct | Yield (mg/L) | Purity (%)* | Buffer |
|---|---|---|---|
| v34913 | 285 | 98.5 | 50 mM NaAc, 9% Sucrose, pH 5.0 |

| | | | |
|---|---|---|---|
| v34914 | 386 | 98.5 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34915 | 260 | 95.1 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34916 | 469 | 97.2 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34917 | 158 | 99.3 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34918 | 304 | 96.9 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34919 | 105 | 97.9 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v34927 | 478 | 98.5 | 50 mM NaAc, 9% Sucrose, pH 5.0 |
| v37633 | 23 | 99.5 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37634 | 29 | 99.8 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37635 | 38 | 99.4 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37636 | 19 | 99.3 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37637 | 33 | 94.2 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37638 | 33 | 99.8 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37639 | 23 | 98.8 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37640 | 20 | 99.8 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37641 | 23 | 99.5 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37642 | 35 | 99.6 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37658 | 39 | 97.4 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37660 | 50 | 99.6 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37662 | 46 | 99.7 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v37663 | 44 | 99.6 | 50 mM Histidine, 9% Sucrose, pH 6.0 |
| v35923 | 7 | 98.4 | 50 mM Histidine, 9% Sucrose, pH 6.0 |

*As determined by HPLC-SEC (size-exclusion chromatography HPLC)

## EXAMPLE 3: DESIGN, STRUCTURE AND PREPARATION OF MULTIVALENT AND MULTISPECIFIC ANTI-(DLL3xCD28xCD3) ANTIBODY CONSTRUCTS

**[0595]** Several multivalent and multispecific 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 and shown and described in **TABLES 9-11** 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 Multivalent and multispecific Antibody Constructs Targeting DLL3, CD28 and CD3***

**[0596]** Several multivalent and multispecific 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.

**[0597]** 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.

**[0598]** 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 9**). 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 constructs in **TABLE 9** 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.

**[0599]** The multivalent and multispecific 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, the genes encoding the antibody constructs' heavy and light chains 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.

**[0600]** In multivalent and multispecific 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.

**[0601]** Certain heavy chain domain structures and formats that were used to generate certain antibody constructs of the present disclosure, e.g., certain multivalent and multispecific 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 8** and **TABLE 9** (domain structures are generally shown from N-terminus to C-terminus, unless otherwise stated). **TABLE 10** shows several production parameters for certain constructs described herein.

**TABLE 8: Various Heavy Chain Domain Structures and Exemplary Multivalent and multispecific Antibody Constructs comprising such Heavy Chains**

| Heavy Chain Domain Structure: | Certain Antibody Constructs that can 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 9: Format and Structure of Certain Multivalent and multispecific Antibody Constructs and Control Constructs**

| Construct # | Format | Heavy Chain A | Heavy Chain B | Fc Description |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| v38578 | 1x1x1 | N-terminus huTN228 VH-VL scFv, C3E6 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38967 | 1x1x1 | C3E6 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v41268 | 1x1x1 | C3E5 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38580 | 1x1x1 | C3E6 Fab, C-terminus huTN228 VL-VH scFv | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38584 | 1x1x1 | huTN228 Fab, C-terminus C3E6 VL-VH scFv | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38582 | 1x1x1 | N-terminus C3E6 VH-VL scFv, huTN228 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38586 | 1x1 | huTN228 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v38588 | 1x1 | 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 | C3E6 Fab | DLL3 Fab | Het Fc, FcKO |
| v39917, v41386 | 1x1x1 | C3E5 Fab | DLL3 Fab | Het Fc, FcKO |
| v39920 | 1x1 | C3E6 Fab | DLL3 Fab | Het Fc, FcKO |
| v39922 | 1x1 | 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 | C3E5 Fab | DLL3 Fab | Het Fc, FcKO |
| v41094 | 1x1x1 | C3E6 Fab | DLL3 Fab | Het Fc, FcKO |
| v41753 | 1x1x1 | C3E5 Fab | DLL3 Fab | Het Fc, FcKO |
| v41754 | 1x1x1 | C3E6 Fab | DLL3 Fab | Het Fc, FcKO |
| v41112 | 1x1x1 | C3E5 Fab | DLL3 Fab | Het Fc, FcKO |
| v41757 | 1x1x1 | C3E5 Fab | DLL3 Fab | Het Fc, FcKO |
| v41759 | 1x1x1 | C3E5 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v41760 | 1x1x1 | CD3 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v41761 | 1x1 | C3E5 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |
| v41762 | 1x1 | CD3 Fab | DLL3 VL-VH scFv | Het Fc, FcKO |

n/a = not applicable.

**TABLE 10: Buffer Systems, Yields and Purity of Various Multivalent and multispecific 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 |

**[0602]** Certain multivalent and multispecific 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 11** below.

**TABLE 11: Anti-CD3 and Anti-CD28 binding domain sequences used in Certain Multivalent and multispecific 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 |

**[0603]** By way of example, in **TABLE 9**, the nomenclature "1x1x1" indicates that the trispecific antibody construct is multivalent and multispecific (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 Multivalent and multispecific Anti-(DLL3xCD3xCD28) Antibody Constructs***

**[0604]** 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)).

**[0605]** 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.

**[0606]** 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™).

**[0607]** 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. 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.

**[0608]** 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.

**[0609]** 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 10** describes the post purification yield for the production of certain antibody constructs described herein.

## EXAMPLE 4: ABILITY OF MULTIVALENT AND MULTISPECIFIC ANTIBODY CONSTRUCTS TO BIND MSLN, CD3 AND CD28 AS ASSESSED BY SURFACE PLASMON RESONANCE (SPR)

**[0610]** This example demonstrates the ability of the anti-MSLN, anti-CD3 and anti-CD28 paratopes when part of a multivalent and multispecific construct to bind to the respective targets as assessed by SPR. The changes in CD3 and CD28 binding properties in the trispecific (e.g., anti-(MSLNxCD3xCD28) and anti-(CLDN18.2xCD3xCD28)) antibody constructs based on their geometry and format is demonstrated as assessed by SPR.

***Binding of Multivalent and multispecific Antibody Constructs to MSLN by SPR***

**[0611]** 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 MSLN his tag protein (R&D systems) was immobilized by amine coupling on CM5 sensor chip and purified bispecific antibody constructs to be tested was flowed on the sensor chip at 30 µL/min

for 120 s, following a dissociation for 600 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 Multivalent and multispecific Antibody Constructs to CD3 by SPR***

**[0612]** 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 30 µL/min for 140-500 s following a dissociation for 600-1200 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 Multivalent and multispecific Antibody Constructs to CD28 by SPR***

**[0613]** 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 injected on the sensor chip at 30 µL/min for 140-500 s following a dissociation for 600-1200 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.

**[0614]** For the multivalent and multispecific construct v37634, an antihuman IgG Fc capture antibody was immobilized on the CM5 sensor ship and the trispecific construct was captured followed by injections of recombinant human/Cynomolgus/Rhesus macaque CD28 Protein, His Tag protein (Acro Biosystems).

***Results***

**[0615] TABLE 15** below shows the binding parameters, including the calculated $K_D$ values, for certain tested multivalent and multispecific antibody constructs binding to MSLN.

**TABLE 15: Binding of Certain Multivalent and multispecific Constructs to MSLN**

| Construct | MSLN | | |
|---|---|---|---|
| | **$k_a$ (1/Ms)** | **$k_d$ (1/s)** | **$K_D$ (M)** |
| v34913 | 3.40E+05 | 2.12E-03 | 6.49E-09 |
| v34914 | 3.74E+05 | 1.97E-03 | 5.39E-09 |
| v34916 | 3.88E+05 | 1.81E-03 | 4.78E-09 |

| | | | |
|---|---|---|---|
| v34917 | 4.11E+05 | 1.99E-03 | 4.96E-09 |
| v34918 | 2.94E+05 | 1.87E-03 | 7.08E-09 |
| v34919 | 4.58E+05 | 1.81E-03 | 4.16E-09 |
| v34927 | 2.70E+05 | 1.87E-03 | 6.86E-09 |

**[0616] TABLE 16** shows the binding parameters, including the calculated $K_D$ values, for certain tested multivalent and multispecific 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 16.**

**TABLE 16: Binding of Certain Multivalent and multispecific 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) |
| v34913 | 2.70E+05 | 1.12E-02 | 4.16E-08 | 4.74E+04 | 2.16E-03 | 4.58E-08 |
| v34914 | 2.37E+05 | 1.91E-02 | 8.11E-08 | 7.02E+04 | 8.49E-03 | 1.22E-07 |
| v34916 | 2.91E+05 | 1.04E-02 | 3.72E-08 | 1.30E+04 | 5.46E-04 | 5.65E-08 |
| v34917 | 4.44E+05 | 1.77E-02 | 4.08E-08 | 8.24E+03 | 2.09E-03 | 3.73E-07 |
| v34918 | 1.18E+05 | 1.35E-02 | 1.18E-07 | 5.27E+04 | 2.59E-03 | 5.00E-08 |
| v34919 | 2.67E+05 | 1.31E-02 | 5.04E-08 | ND | ND | ND |
| v34927 | ND | ND | ND | 5.09E+04 | 2.58E-03 | 5.07E-08 |
| v37633 | 1.30E+05 | 1.76E-02 | 1.35E-07 | 1.13E+05 | 6.20E-03 | 5.51E-08 |
| v37634* | 1.63E+05 | 1.23E-02 | 7.76E-08 | 7.23E+03 | 1.87E-03 | 2.58E-07 |
| v37635 | 1.57E+05 | 1.43E-02 | 9.25E-08 | 4.04E+04 | 1.07E-02 | 2.88E-07 |
| v37636 | 1.34E+05 | 2.10E-02 | 1.60E-07 | 3.04E+04 | 1.32E-02 | 4.34E-07 |
| v37637 | 1.21E+05 | 1.29E-02 | 1.06E-07 | 1.13E+05 | 6.92E-03 | 6.14E-08 |
| v37638 | 1.64E+05 | 1.23E-02 | 7.46E-08 | 9.55E+04 | 2.10E-03 | 2.22E-08 |
| v37639 | 2.92E+04 | 1.44E-02 | 4.92E-07 | 8.41E+04 | 2.01E-03 | 2.39E-08 |
| v37640 | 5.22E+04 | 1.55E-02 | 2.96E-07 | 7.78E+04 | 2.12E-03 | 2.73E-08 |
| v37641 | 6.08E+04 | 1.34E-02 | 2.25E-07 | 6.65E+04 | 2.06E-03 | 3.18E-08 |
| v37642 | 1.89E+05 | 1.17E-02 | 6.20E-08 | 9.82E+04 | 1.98E-03 | 2.02E-08 |
| v37663 | 2.06E+05 | 1.57E-02 | 7.64E-08 | ND | ND | ND |
| v37664 | 1.80E+05 | 2.28E-02 | 1.27E-07 | ND | ND | ND |
| v37665 | ND | ND | ND | 8.94E+04 | 2.24E-03 | 2.51E-08 |
| v37666 | ND | ND | ND | 6.73E+04 | 2.17E-03 | 3.24E-08 |
| v37667 | 1.28E+06 | 7.78E-03 | 5.93E-09 | ND | ND | ND |
| v37669 | 5.89E+05 | 2.55E-03 | 4.38E-09 | ND | ND | ND |
| v35923 | 8.31E+05 | 1.56E-03 | 1.90E-09 | ND | ND | ND |

* Note – CD28 binding of construct v37634 was assessed by a different method; v37634 was captured on anti-human IgG Fc immobilized on CM5 sensor chip and CD28-his tag was flowed.

**TABLE 17: Binding of In-Format Engineered Anti-CD28 Constructs to huCD28**

| Construct | AA subst.* | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) | Fold-change from WT |
|---|---|---|---|---|---|
| v37638 | - | 9.55E+04 | 2.10E-03 | 2.22E-08 | 1.0 |
| v37683 | P1058A ($V_H$) | 9.58E+04 | 4.66E-03 | 4.86E-08 | 2.2 |

| | | | | | |
|---|---|---|---|---|---|
| v37684 | G1064S ($V_H$) | 8.06E+04 | 1.13E-02 | 1.43E-07 | 6.4 |
| v37685 | V1035G ($V_L$) | 6.79E+04 | 2.90E-02 | 6.31E-07 | 28.4 |
| v37686 | D1068E ($V_L$) | 8.93E+04 | 2.52E-03 | 2.81E-08 | 1.3 |
| v37687 | E1080K ($V_H$) | 1.01E+05 | 4.75E-03 | 4.70E-08 | 2.1 |
| v37688 | Y1110S ($V_H$) | 9.73E+04 | 2.53E-03 | 2.58E-08 | 1.2 |
| v37689 | N1111aA ($V_H$) | 8.96E+04 | 2.11E-02 | 2.39E-07 | 10.8 |
| v37690 | Y1112S ($V_H$) | ND | ND | ND | n/a |
| v37691 | L1112aN ($V_H$) | 9.86E+04 | 1.07E-02 | 1.09E-07 | 4.9 |
| v37692 | Y1113S ($V_H$) | 3.22E+04 | 6.13E-02 | 2.11E-06 | 95.1 |
| v37693 | Y1037A ($V_H$) | 1.06E+05 | 9.68E-03 | 9.18E-08 | 4.1 |
| v37694 | Y1031A ($V_L$) | ND | ND | ND | n/a |
| v37695 | N1066A ($V_L$) | 9.51E+04 | 2.66E-03 | 2.77E-08 | 1.3 |
| v37642 | - | 7.04E+04 | 2.86E-03 | 4.23E-08 | 1 |
| v38990 | Y1113S ($V_H$) | 4.61E+04 | 3.30E-02 | 9.51E-06 | 225 |
| v38991 | N1111aA ($V_H$) | 6.29E+04 | 1.45E-02 | 2.57E-07 | 6.1 |
| v38992 | G1064S ($V_H$) | 5.98E+04 | 7.38E-03 | 1.30E-07 | 3.1 |
| v38993 | L1112aN ($V_H$) | 5.90E+04 | 8.17E-03 | 1.44E-07 | 3.4 |

ND = not determined (e.g., no measurable binding signal was observed).
*AA position numbering according to the IMGT numbering system.

## EXAMPLE 5: ABILITY OF ANTI-(MSLNXCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY

**[0617]** To determine the impact of the trispecific construct format on binding to CD3+CD28+ primary human T cells, the binding affinity to human T cells was measured by flow cytometry as described below.

**[0618]** The ability of trispecific anti-MSLN-CD3-CD28 constructs, as well as bispecific and benchmark controls, to bind to CD3+CD28+ primary human T cells was assessed via whole cell FACS binding analysis.

**[0619]** Briefly, primary human T cells (>80% viability) 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 mL FACS buffer and centrifuging for 400xg for 3 minutes. The cell pellet was resuspended in 50 mL FACS buffer with Alexa Fluor 647-labeled goat anti-human IgG (Jackson Labs) and fluorophore-conjugated antibodies specific for CD4 and CD8. Cells were incubated for 1 hour at 4 °C. Following incubation, cells were washed twice by adding 150 mL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer. The apparent binding affinities and $B_{max}$ of

the anti-MSLN-CD3-CD28 (1+1+1) multivalent and multispecific constructs and the bispecific controls to human T cells are shown in **FIGS. 2A-2B**.

***Ability of 1+1+1 Anti-(MSLNxCD3xCD28) Multivalent and multispecific Antibody Constructs to Bind to Native CD3 and CD28 as Assessed by Flow Cytometry***

**[0620]** The anti-CD3 bispecific construct, v34919, exhibited the weakest binding to CD4+ and CD8+ human T cells with a relative binding affinity around 50 nM. The relative binding affinity of the anti-CD28 bispecific constructs, v34927, was higher than that of v34919, measured at around 0.5 nM. The multivalent and multispecific constructs tested displayed a stronger binding to the human T cells, compared to both bispecific control constructs, v34919 and v34927, with two trispecific constructs, v34913 and v34918, exhibiting <1 nM binding.

This observed shift in binding affinity relative to the corresponding bispecific control constructs indicated bivalent binding of the trispecific constructs to T cells via their anti-CD3 and anti-CD28 binding domains.

## EXAMPLE 6: ABILITY OF TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND NATIVE CLDN18.2 AS ASSESSED BY FLOW CYTOMETRY

**[0621]** To determine the impact of construct format on the ability of a construct to bind CLDN18.2, the binding affinity to CLDN18.2 was measured for certain antibody constructs using flow cytometry as described below.

**[0622]** Specifically, the ability of certain trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs as well as bispecific and benchmark controls to bind to CLDN18.2+ SNU 601 cells, was assessed via whole cell FACS binding analysis. To that end, cells (>80% viability) 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 mL FACS buffer and centrifuging for 400xg for 3 minutes. The cell pellet was resuspended in 50 mL 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 mL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer. The apparent binding affinities of the constructs to MSLN are shown in **TABLE 18**.

**TABLE 18: Whole Cell Binding of Engineered Trispecific Constructs to Native CLDN18.2**

| Construct | SNU 601 | |
|---|---|---|
| | Apparent Affinity ($EC_{50}$; nM) | $B_{max}$ (MFI; anti-IgG AF647) |
| v37633 | 40.8 | 8574.75 |
| v37634 | 27.2 | 6835.5 |
| v37635 | 30.1 | 6597.25 |
| v37638 | 47.5 | 9138.25 |
| v37640 | 26.4 | 9767.25 |
| v37642 | 24.1 | 9312.25 |
| v37663 | 31.3 | 9198.5 |
| v37411 | 8.9 | 11690 |
| v37675 | 37.1 | 9825.25 |
| v35923 | 244.6 | 7861.75 |

**[0623]** Binding curves for the tested anti-(CLDN18.2xCD3xCD28) trispecific constructs of varying geometry as well as those of the bispecific controls are shown in **FIGS. 3A-3B**.

**[0624]** All tested multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs, the anti-(CLDN18.2xCD3) bispecific control, as well as the anti-CLDN18.2 scFv-OAA control exhibited similar binding to CLDN18.2+ SNU 601 cells, with apparent affinities in the range of about 25-50 nM. The anti-CLDN18.2 monoclonal antibody (mAb), v37411, exhibited the highest apparent affinity with a $K_D$ of 8.9 nM. However, all of the tested multivalent and multispecific constructs showed an improved binding, with an approximately 5 to 10-fold lower $K_D$ (i.e., higher binding) than the benchmark CLDN18.2xCD3 bispecific control, v35923.

**[0625]** The trispecific constructs, v37633, v37638, v37640, v37642, v37663, and the anti-CLDN18.2 scFv-OAA, v37675, exhibited similar maximal binding, with Bmax values of approximately 9,000. The trispecific constructs, v37634 and v37635, exhibited the lowest maximal binding, with Bmax values around 6,800 to 6,600, respectively. The anti-CLDN18.2 mAb, v37411, showed the highest maximal binding, with a Bmax value of 11,690. No binding was observed for the negative control, v22277.

**[0626]** Taken together, these results demonstrate that the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs were capable of binding native CLDN18.2 expressed on the surface of SNU 601 cells.

**EXAMPLE 7A: ABILITY OF CLDN18.2XCD3XCD28 TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY**

**[0627]** To determine the impact of the trispecific construct format on its ability to bind CD3+CD28+ primary human T cells, the binding affinity to human T cells was measured by flow cytometry as described below.

**[0628]** The ability of trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs, as well as those of some bispecific and benchmark controls, to bind to CD3+CD28+ primary human T cells was assessed via whole cell FACS binding analysis. To that end, primary human T cells (>80% viability) 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 mL FACS buffer and centrifuging for 400xg for 3 minutes. The cell pellet was resuspended in 50 mL FACS buffer with Alexa Fluor 647-labeled goat anti-human IgG (Jackson Labs) and fluorophore-conjugated antibodies specific for CD4 and CD8. Cells were incubated for 1 hour at 4 °C. Following incubation, cells were washed twice by adding 150 mL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer. Data are representative of 3 independent experiments with 3 donors.

**[0629]** The binding curves of the tested constructs to CD3+CD28+ T cells are shown in **FIGS. 4A-4B**.

The anti-(Cldn18.2xCD3) bispecific control construct, v37663, exhibited the weakest binding to CD4+ and CD8+ human T cells with a relative binding affinity around 200 nM. The relative binding affinity of the anti-CD28 bispecific control, v37665, was higher than that of the CD3 bispecific, and measured around 25 nM. The tested trispecific constructs displayed stronger binding to the human T cells, compared to both bispecific controls, with two trispecific constructs, v37638 and v37642, exhibiting sub-1 nM binding. This increase in affinity relative to the bispecific controls indicates bivalent binding to both CD3 and CD28 on T cells of the trispecific constructs. No binding was observed with the negative control, v22277.

**EXAMPLE 7B: ABILITY OF ANTI-(CLDN18.2xCD3xCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY**

**[0630]** To determine the impact of the trispecific construct format and geometry on its ability to bind CD3 and CD28 on primary human T cells, binding by selected constructs – as well as their format-matched single arm binding controls – to primary human CD3+CD28+ T cells was assessed. The multivalent and multispecific constructs, v37634 and v37642, as well as the respective format-matched single arm CD3-binding controls, v37663 and v37662, as well as the respective format-matched single arm CD28-binding controls, v37654 and v37666, were assessed. The construct v35923 was included as a high affinity anti-(CLDN18.2xCD3) bispecific TCE, and v22277 was included as a negative control for T cell binding.

**[0631]** The ability of the tested multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody constructs – as well as that of some single-arm binding controls – to bind to CD3+CD28+ primary human T cells was assessed via whole cell FACS binding analysis. To that end, primary human T cells (>80% viability) 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 1 nM of test article and incubated at 4 °C for one hour. Following incubation, cells were washed twice by adding 150 mL FACS buffer and centrifuging for 400xg for 3 minutes. The cell pellet was resuspended in 50 mL FACS buffer with Alexa Fluor 647-labeled goat anti-human IgG (Jackson Labs) and fluorophore-conjugated antibodies specific for CD4 and CD8. Cells were incubated for 1 hour at 4 °C. Following incubation, cells were washed twice by adding 150 mL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer. Data are representative of 2 independent experiments.

**[0632]** Alexa Fluor 647 GeoMean values (mean +/- SD) of the tested constructs to CD3+CD28+ T cells are shown in **FIG. 5** and **TABLE 19**.

**TABLE 19: On-Cell Binding by Selected Constructs to CD3+CD28+ human T cells**

| Construct | Average GeoMean; AF647 | Standard Deviation |
|---|---|---|
| v35923 | 3818.5 | 270.8 |
| v37634 | 593.5 | 13.4 |
| v37654 | 72 | 2.8 |
| v37642 | 4822.5 | 193.0 |
| v37662 | 2173.5 | 82.7 |
| v37663 | 171.5 | 2.1 |

| | | |
|---|---|---|
| v37666 | 531.5 | 20.5 |
| v22277 | 71.9 | 0.1 |

**[0633]** The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, and it's format-matched CD3-binding control, v37663, exhibited approximately 8.3-fold and 2.4-fold increased binding, respectively, compared to the negative control, v22277. The format-matched, CD28-binding control for v37634, v37654, exhibited no difference in binding relative to the negative control, v22277. The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37642 (which has a different geometry compared to v37634), and it's format-matched CD3- and CD28-binding controls, v37662 and v37666, exhibited approximately 67-fold, 30-fold, and 7.4-fold increased binding, respectively, compared to the negative control, v22277.

**[0634]** Taken together, these data show that binding by individual paratopes within a trispecific format can be impacted by modulating the geometry of trivalent trispecific constructs. As demonstrated, the multivalent and multispecific antibody construct v37634 showed an approximately 8-fold reduced binding to CD28 in the absence of CD3 when compared to the multivalent and multispecific antibody construct v37642 (which has a different geometry compared to v37634).

## EXAMPLE 7C: ABILITY OF ANTI-(CLDN18.2XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE HUMAN OR CYNOMOLGUS MONKEY CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY

**[0635]** Certain multivalent and multispecific antibody constructs were tested for binding to primary cynomolgus monkey or human T cells to assess the cross-reactivity of the anti-(CLDN18.2xCD3xCD28) construct, v37634, and a surrogate anti-(CLDN18.2xCD3xCD28) construct, v39452, which included a higher affinity anti-CD3 paratope against cynomolgus CD3. The constructs v37663 and v39453 were included as anti-(CLDN18.2xCD3) bispecific controls for v37634 and v39452, respectively. v35923 was included as a benchmark anti-(CLDN18.2xCD3) construct and v22277 was included as a negative control.

**[0636]** The ability of the trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs, as well as those of some bispecific and benchmark controls, to bind to CD3+CD28+ primary human or cynomolgus monkey T cells was assessed via whole cell FACS binding analysis. To that end,

primary human or cynomolgus monkey T cells (>80% viability) 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 for 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 fluorophore-conjugated antibodies specific for CD4 and CD8. Cells were incubated for 1 hour at 4 °C. Following incubation, cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 3 minutes. Cells were fixed with 25 µL of FluoroFix Buffer (BioLegend) for 25 mins at 4°C. After fixing, cells were washed twice by adding 150 µL of FACS buffer and centrifuging at 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

**[0637]** The calculated $EC_{50}$ and $B_{max}$ values of the tested constructs for binding to CD3+CD28+ human and cynomolgus monkey T cells are presented in **TABLE 20A**.

**TABLE 20A: Binding of Tested Anti-CLDN18.2 Constructs to Primary Human or Cynomolgus Monkey T Cells**

| Antibody Construct | $EC_{50}$ (nM) | | $B_{max}$ | |
|---|---|---|---|---|
| | Human | Cynomolgus Monkey | Human | Cynomolgus Monkey |
| v22277 | nd* | nd | 60.0 | 202.0 |
| v35923 | 0.8 | 1.5 | 15938 | 5768 |
| v37634 | 9.8 | 28.9 | 12305 | 2520 |
| v37663 | 25.0 | 45 | 13864 | 1111 |
| v39452 | 0.8 | 11.0 | 20858 | 4137 |
| v39453 | 1.1 | 19.4 | 18201 | 3961 |

*nd= not defined

**[0638]** The anti-(CLDN18.2xCD3xCD28) construct, v37634, exhibited an approximate 3-fold reduced apparent affinity for cynomolgus monkey pan T cells compared to human pan T cells with an approximate five-fold reduced Bmax value. v39452 displayed an approximate 10-fold reduced apparent affinity for cynomolgus monkey pan T cells compared to human pan T cells with an approximate five-fold reduced Bmax value.

## EXAMPLE 7D: ABILITY OF CERTAIN ANTI-(CLDN18.2XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND CD3 AND CD28 IN TRANS ON TWO DIFFERENT T CELLS

**[0639]** To determine the impact of antibody construct format on the ability of a construct to bind CD3 and CD28 in trans (i.e., to bind CD3 on one T cell and CD28 on another T cell), cell-cell

bridging of selected antibody constructs, as well as their format-matched single arm binding controls, was assessed. The trispecific constructs, v37634 and v37642, as well as the respective format-matched single arm CD3-binding controls, v37663 and v37662, and the respective format-matched single arm CD28-binding controls, v37654 and v37666, were assessed. The construct v37035 was included as a benchmark CD3xCD28xTAA trispecific antibody, and v39982 was included as a negative control for T cell bridging.

**[0640]** The ability of trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs to bind CD3 and CD28 in trans was assessed via flow cytometry. 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 Fixable viability dye eFluor 660 (eBiosciences). 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.

**[0641]** EC50 values and mean maximum percent double positive events of the tested constructs from the concentration response curves are presented in **TABLE 20B.**

**TABLE 20B: $EC_{50}$ Values and Mean Maximum Percent Double Positive Events of the Tested Constructs**

| Antibody Construct | $EC_{50}$ (pM) | Maximum Percent Double Positive Events (Mean ± SD) |
|---|---|---|
| v37634 | ND | 1.9 ± 0.3 |
| v37642 | 71.1 | 22.9 ± 2.6 |
| v37654 | ND | 1.0 ± 0.6 |
| v37662 | ND | 1.3 ± 0.1 |
| v37666 | ND | 1.5 ± 0.4 |
| v37035 | 865.0 | 9.4 ± 1.6 |
| v39215 | ND | 1.0 ± 0.2 |
| v39982 | ND | 1.4 ± 0.2 |

ND= not determined

**[0642]** The trispecific construct, v37642, and the benchmark trispecific, v37035, exhibited bridging of the Jurkat$^{CD28\text{-}KO}$ and Jurkat$^{CD3\text{-}KO}$cells, with $EC_{50}$ values of 71.1 pM and 865.0 pM, respectively, and maximum percent double positive event values of 22.9 and 9.4, respectively. The anti-CD3- and anti-CD28-single binding arm controls for v37642, v37662 and v37666, respectively, did not exhibit bridging of the Jurkat$^{CD28\text{-}KO}$ and Jurkat$^{CD3\text{-}KO}$cells. The trispecific construct, v37634, that comprises a light chain comprising a Fab light chain portion (anti-CD3) and an scFv portion (anti-CD28) and the anti-CD3- and anti-CD28-single binding arm controls, v39215 and v37654, respectively, did not exhibit bridging of the Jurkat$^{CD28\text{-}KO}$ and Jurkat$^{CD3\text{-}KO}$cells. The negative control, v39982, did not exhibit bridging of the Jurkat$^{CD28\text{-}KO}$ and Jurkat$^{CD3\text{-}KO}$cells.

**[0643]** Taken together, these results demonstrate that the ability to induce trans binding of CD3 and CD28 on two different cells can be modulated by the geometry and/or format of a multivalent and multispecific construct.

## EXAMPLE 8: ABILITY OF MULTIVALENT AND MULTISPECIFIC ANTIBODY CONSTRUCTS TO BIND DLL3, CD3 AND CD28 AS ASSESSED BY SURFACE PLASMON RESONANCE (SPR)

**[0644]** This example demonstrates the ability of the anti-DLL3, anti-CD3 and anti-CD28 paratopes when part of a multivalent and multispecific construct as well as bispecific constructs 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 Multivalent and multispecific Antibody Constructs to DLL3 as assessed by SPR***

**[0645]** 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 multivalent and multispecific 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 Multivalent and multispecific Antibody Constructs to CD3 as assessed by SPR***

**[0646]** 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 Multivalent and multispecific Antibody Constructs to CD28 as assessed by SPR***

**[0647]** 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 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 with reported values as the mean of three independent runs.

***Results***

**[0648]** **TABLE 21** shows the binding parameters, including the calculated $K_D$ values, for certain tested multivalent and multispecific 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 21.**

**TABLE 21: 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 | Not measurable by SPR | Not measurable by SPR | Not measurable by SPR |

| | | | | | | |
|---|---|---|---|---|---|---|
| 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 |

**[0649] TABLE 22** below shows the binding parameters, including the calculated $K_D$ values, for certain tested multivalent and multispecific antibody constructs binding to DLL3.

**TABLE 22: 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 9: ABILITY OF ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND NATIVE DLL3 AS ASSESSED BY FLOW CYTOMETRY

**[0650]** To determine the impact of the format of multivalent and multispecific 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.

**[0651]** Specifically, the ability of certain multivalent and multispecific anti-(DLL3xCD3xCD28) antibody constructs as well as bispecific and benchmark controls 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 mL FACS buffer and centrifuging at 400xg for 3 minutes. The cell pellet was resuspended in 50 mL 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 mL FACS buffer and centrifuging at 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

**[0652]** The apparent binding affinities that were calculated from the binding curves of the tested constructs to DLL3 are shown in **TABLE 23**.

**TABLE 23: Whole Cell Binding of Certain Multivalent and multispecific 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 10A: ABILITY OF ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND TO NATIVE CD3 AND CD28 AS ASSESSED BY FLOW CYTOMETRY

**[0653]** To determine the impact of the format of multivalent and multispecific 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.

**[0654]** The ability of certain multivalent and multispecific anti-(DLL3xCD3xCD28) antibody constructs, as well as that of some bispecific and benchmark control 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. The cells were washed twice by adding 150 mL PBS and centrifuging for 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 mL FACS buffer and centrifuging for 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 150 mL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

**[0655]** Binding curves for some of the tested trispecific constructs and bispecific controls are shown in **FIG. 6.** The apparent binding affinities and $B_{max}$ of the tested constructs are shown in **TABLES 24A** and **24B**.

**TABLE 24A: 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 24B: 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 10B: INVESTIGATION OF THE ABILITY OF TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND CD3 AND CD28 ON THE SAME T CELL**

**[0656]** 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.

**[0657]** 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 Fixable viability dye eFluor 660 (eBiosciences). 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.

**[0658]** $EC_{50}$ values and mean maximum percent double positive events of the tested constructs from the concentration response curves are presented in **TABLE 24C**.

**TABLE 24C: 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 |

ND= not determined

**[0659]** The data demonstrate that the trivalent and trispecific antibody constructs, v39915 and v39917, 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, did show T cell cross-linking and thus appear to bind CD3 and CD28 on different T cells.

## EXAMPLE 11: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON *IN VITRO* CYTOTOXICITY TOWARDS MSLN-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 activity and preferential killing of tumor cells overexpressing MSLN, selected construct were tested in primary human T cell cultures with allogeneic tumor cell lines expressing MSLN.

***Impact of Trispecific Construct Format on In Vitro Cytotoxicity Towards MSLN-expressing Tumor Cell Lines in Co-culture with Primary Human CD3+ T Cells***

**[0661]** Frozen human T cells were thawed at 37 °C and mixed with MSLN+ H292 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 to be tested for 72 hours, after which Hoechst stain (vendor) was added to the cells. After a 30-minute incubation period, tumor cell viability was assessed through live tumor cell counts using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 3 independent experiments with 1 donor.

**[0662]** Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**TABLE 25: Potency and Maximum Cytotoxicity Induced by Anti-MSLN 1+1+1 Multivalent and multispecific Constructs from Primary Human T Cells Co-cultured with H292 Cells**

| Construct | H292 | |
|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) |
| v34913 | 0.70 | 79.1 |
| v34914 | 19.07 | 76.6 |
| v34915 | ND | 57.3 |
| v34916 | 36.60 | 75.6 |
| v34917 | 46.68 | 70.0 |
| v34918 | 3.32 | 72.1 |

| | | |
|---|---|---|
| v34919 | 262.20 | 66.1 |
| v34927 | ND | 7.7 |

**[0663]** Cytotoxicity curves of primary human T cells in c-culture with MSLN+ H292 cells following treatment with the tested anti-MSLN-CD3-CD28 (1+1+1) multivalent and multispecific antibody constructs or bispecific controls are shown in **FIGS. 7A-7B**.

**[0664]** The multivalent and multispecific constructs having formats comprising one CD3 Fab, one CD28 scFv, and one MSLN scFv exhibited a range of potencies against MSLN-overexpressing H292 allogeneic target cells. The most potent of the CD3 Fab-containing constructs, v34914, showed approximately 14-fold higher potency compared to the respective bivalent MSLNxCD3 bispecific control construct, v34919. The constructs v34916 and v34917 exhibited equivalent potencies, which were approximately 5-fold higher compared to v34919. The multivalent and multispecific constructs, v374914, v34916, and v34917 induced greater maximum cytotoxicity than the bivalent MSLNxCD3 bispecific control construct, 34919, with maximum cytotoxicity reaching between about 70-77% compared to the 66% reached by v34919.

**[0665]** The multivalent and multispecific constructs having formats comprising one CD28 Fab, one CD3 scFv, and one MSLN scFv exhibited the highest potencies (i.e., lowest $IC_{50}$ values) against MSLN-overexpressing H292 allogeneic target cells. The most potent of the CD28 Fab-containing constructs, v34913, showed approximately 375-fold higher potency than the corresponding MSLNxCD3 bispecific control construct, v34919. The construct v34918 exhibited an approximately 80-fold greater potency than v34919. Maximum cytotoxicity induced by constructs v34913 and v34918 reached 79.1 and 72.1%, respectively. Negative control, v22277, did not induce any measurable cytotoxicity of H292 cells.

**[0666]** These results demonstrate that the cytotoxicity toward allogeneic target tumor cells can be modulated through modification of format and/or geometry of an antibody construct, with various formats exhibiting a range of potencies against H292 cells. Furthermore, these data show that a several hundred-fold increase in anti-tumor cytotoxicity can be achieved by adding a costimulatory anti-T cell antigen binding domain (e.g., anti-CD28) when compared to corresponding bispecific constructs that only contain one binding domain targeting an immune cell (e.g., T cell) antigen.

**EXAMPLE 12: MSLN-TARGETING OF ANTI-(MSLNXCD3xCD28) TRISPECIFIC ANTIBODY CONSTRUCTS IN *IN VITRO* T-CELL DEPENDENT CYTOTOXICITY ASSAYS WITH TUMOR CELLS EXPRESSING VARYING LEVELS OF MSLN**

**[0667]** To determine whether certain trispecific antibody constructs exhibit tumor antigen dependency with respect to their *in vitro* cytotoxic activity, T-cell dependent cytotoxicity assays were conducted with tumor cells expressing high or low-levels of antigen.

**[0668]** Certain anti-MSLN trispecific constructs as well as benchmark and bispecific control constructs 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 CD3+ T cells were thawed and mixed with H292 (152 986 MSLN/cell) or OVTOKO (9 752 MSLN/cell) target cells at an E:T ratio of 2:1. Plates were incubated for 72 hr at 37 °C and 5% carbon dioxide, after which Hoechst Stain (ThermoFisher) was added to the cells. After a 30-minute incubation period, tumor cell viability was assessed through live tumor cell counts using the Operetta CLS High Content Analysis System (PerkinElmer). Data are representative of 2 independent experiments with 1 donor.

**[0669]** Percent cell cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**[0670]** Concentration response curves for the tested antibody constructs are presented in **FIGS. 8A-8D** and $IC_{50}$ and maximum percent killing from the T cell-dependent cytotoxicity assay comparing the tested trispecific constructs and the bispecific/benchmark control, v34919, are presented in **TABLE 26.**

**TABLE 26: Cytotoxic Activity of Anti-MSLN Multivalent and multispecific Constructs and Bispecific/Benchmarks Towards MSLN High and Low/Negative Tumor Cells Grown in Culture with Primary Human CD3+ T Cells**

| Construct | H292 | | OVTOKO | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) | $IC_{50}$ (pM) | Maximum Killing (%) |
| v34913 | 0.363 | 73.1 | 2228 | 39.2 |
| v34914 | 14.67 | 68.9 | 4260 | 34.6 |
| v34916 | 42.86 | 68.1 | ND | 7.9 |
| v34917 | 36.19 | 67.9 | ND | 13.5 |
| v34918 | 7.214 | 70.0 | 8058 | 38.8 |
| v34919 | 420.7 | 43.4 | ND | 7.9 |

**[0671]** In sum, all multivalent and multispecific constructs tested exhibited measurable cytotoxicity against the MSLN+ cell line, H292, with maximum cytotoxicity reaching between 67-74%, whereas the MSLNxCD3 bispecific control construct, v34919, reached only 43.4%. The most potent (the $IC_{50}$ values were used as a measurement for potency in this study) trispecific construct, v34913, showed approximately 1200-fold higher potency as compared to the corresponding anti-CD3xMSLN bispecific control construct v34919. The constructs v34918 and v34914 exhibited similar potencies, which were approximately 30 to 60-fold higher than that measured for the control v34919. The constructs v34916 and v4917 exhibited similar potencies, which were approximately 10-fold higher than that measured for the control v34919.

**[0672]** Overall cytotoxicity induced by the trispecific constructs, v34913, v34914, v34917, and v34918, was reduced in $MSLN^{low}$ cell lines (compared to the $MSLN^{high}$ H292 cells), with maximum cytotoxicity reaching 39.2%, 34.6%, 13.5% and 38.8%, respectively. The benchmark control anti-(MSLNxCD3) bispecific construct, v34919, induced cytotoxicity toward $MSLN^{low}$ OVTOKO cells reaching a maximum of 7.9%.

**[0673]** These results show that the multivalent and multispecific constructs not only exhibited antigen-dependent cytotoxic activity against tumor cells, with reduced cytotoxicity against $MSLN^{low}$ OVTOKO cells, but also a significantly higher cytotoxic activity (e.g., at least 10-fold higher) than a corresponding control anti-(MSLNxCD3) bispecific construct (v34919).

## EXAMPLE 13: IMPACT OF ANTI-CD28 PARATOPE OF ANTIBODY CONSTRUCTS ON SUSTAINED *IN VITRO* CYTOTOXICITY TOWARDS MSLN-POSITIVE TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ T CELLS

**[0674]** To test the impact of the CD28 paratope on the sustained *in vitro* activity and preferential killing of tumor cells overexpressing MSLN, selected trispecific constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing moderate levels MSLN.

**[0675]** Frozen human T cells were thawed at 37 °C and mixed with H292 (MSLN+) cells such that the ratio of T cells to allogeneic tumor cells was adjusted to an E:T ratio of 1:5, respectively. The mixtures were incubated together with the antibody constructs for 7 days, after which Hoechst stain (vendor) was added to the cells. After a 30-minute incubation period, tumor cell viability was assessed through live tumor cell counts using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 2 independent experiments with 2 donors.

**[0676]** Concentration response curves for the anti-MSLN constructs are presented in **FIGS. 9A-9B** measured at days 3 (**FIG. 9A**) or day 7 (**FIG. 9B**) post-incubation, and $IC_{50}$ and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLE 27**.

**TABLE 27: Cytotoxic Activity of Anti-MSLN Trispecific Constructs and Bispecific as well as Benchmark Constructs Towards MSLN$^+$ H292 Tumor Cells Grown in Culture with Primary Human CD3+ T Cells**

| Construct | Day 3 | | Day 7 | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) | $IC_{50}$ (pM) | Maximum Killing (%) |
| v34913 | 8.921 | 28.0 | 3.535 | 65.3 |
| v34916 | 83.11 | 19.5 | 452.3 | 66.8 |
| v34918 | 162.4 | 20.5 | 77.21 | 63.7 |
| v34919 | 2775 | 23.0 | ND | 0 |

**[0677]** After 7 days of co-culture, the multivalent and multispecific constructs v34913, v34916 and v34918, exhibited $IC_{50}$ values of 3.535 pM, 452.3 pM, and 77.2 pM, respectively, toward H292 cells, whereas no cytotoxic effect was determined for the bispecific control construct v34919. All trispecific constructs reached between about 63-67% maximum cytotoxicity of H292 target cells, whereas the control v34919 as well as the negative control v22277 did not induce any measurable cytotoxicity of H292 cells.

Taken together, these results demonstrate that the multivalent and multispecific anti-(MSLNxCD3xCD28) constructs can be superior in inducing sustained *in vitro* tumor cell-directed cytotoxicity toward target cells expressing moderate levels of MSLN when compared to corresponding bivalent anti-(MSLNxCD3) bispecific constructs. Furthermore, these data show that the multivalent and multispecific antibody constructs of the present disclosure are capable of inducing strong anti-tumor T cell responses over long incubation periods in long-term co-cultures (e.g., may be more reflective of conditions in solid tumors), and, particularly noteworthy, at low effector (e.g., T cell) to tumor cell ratio. This ability appears particularly striking when compared to the corresponding anti-(MSLNxCD3) bispecific construct, v34919, which, especially in the 7-day long-term co-culture, showed no significant anti-tumor activity.

**EXAMPLE 14: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON *IN VITRO* CYTOTOXICITY TOWARDS CLDN18.2-EXPRESSING TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ T CELLS**

**[0678]** To test the impact of antibody construct format on its anti-tumor cell activity and preferential killing of such tumor cells overexpressing CLDN18.2, selected constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing CLDN18.2.

**[0679]** Frozen human T cells were thawed at 37 °C and mixed with CLDN18.2+ SNU 601-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 live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 3 independent experiments with 2 donors.

**[0680]** Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**TABLE 28: Potency and Maximum Cytotoxicity Induced by Anti-CLDN18.2 Trispecific Antibody Constructs from Primary Human T Cells Co-cultured with SNU 601 Target Cells**

| Construct | SNU 601 | |
|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) |
| v37633 | 0.098 | 86.1 |
| v37634 | 0.032 | 88.0 |
| v37635 | 0.099 | 83.4 |
| v37636 | 6.082 | 81.5 |
| v37637 | 0.328 | 85.0 |
| v37638 | 0.006 | 84.8 |
| v37639 | 3.350 | 83.4 |
| v37640 | 0.126 | 87.8 |
| v37641 | 0.178 | 85.3 |
| v37642 | 0.001 | 87.0 |
| v37663 | 0.461 | 84.3 |
| v37665 | ND | 8.2 |

**[0681]** Concentration response cytotoxicity curves of primary human T cells in co-culture with CLDN18.2+ SNU 601 cells following treatment with tested anti-(CLDN18.2xCD3xCD28) trispecific constructs and bispecific controls are shown in **FIGS. 10A-10B**.

**[0682]** The multivalent and multispecific constructs that comprised one anti-CD3 Fab domain, one anti-CD28 scFv domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies against CLDN18.2-overexpressing SNU 601 allogeneic target cells. The most potent of the construct, v37634, showed approximately 15-fold higher potency compared to the respective bivalent anti-(CLDN18.2xCD3) bispecific control, v37663. The constructs v37633 and v37635 exhibited similar potencies, which were approximately 5-fold higher than v37663. The construct v37637 exhibited a similar potency when compared to v37663, with $IC_{50}$ values of 0.328 pM and 0.461 pM, respectively. The multivalent and multispecific constructs v37634, v37633, v37635, and v37637 all induced similar maximum cytotoxicities when compared to the bivalent anti-(CLDN18.2xCD3) bispecific control construct, with maximum cytotoxicities reaching between 83-88%. The construct v37636 exhibited a reduced cytotoxicity against SNU 601 cells compared to v37663, with an approximate 10-fold reduction in the $IC_{50}$ value and a maximum cytotoxicity of 81.5%.

**[0683]** The multivalent and multispecific constructs that comprised one anti-CD28 Fab domain, one anti-CD3 scFv domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies against CLDN18.2-overexpressing SNU 601 allogeneic target cells. The most potent of the anti-CD28 Fab-containing constructs, v37642, and the second most potent construct, v37638, showed approximately a 450-fold and 80-fold higher potency, respectively, when compared to the bivalent anti-(CLDN18.2xCD3) bispecific control construct, v37663. The constructs v37640 and v37641 exhibited equivalent potencies, which were approximately 4 to 2.5-fold higher, respectively, than that of the control construct v37663. The multivalent and multispecific constructs v37638, v37640, v37641, and v37642 all induced similar maximum cytotoxicities when compared to the bivalent anti-(CLDN18.2xCD3) bispecific control, with maximum cytotoxicity reaching between 84-88%. The construct v37639 exhibited reduced cytotoxicity against SNU 601 cells compared to v37663, with an approximate 10-fold reduction in the $IC_{50}$ value and a maximum cytotoxicity of 83.4%. Negative control, v22277, did not induce cytotoxicity of SNU 601 cells.

**[0684]** These results demonstrate that the cytotoxicity of the multivalent and multispecific constructs toward allogeneic target tumor cells can be modulated through modification of the format and/or geometry of an antibody construct, with various formats exhibiting a range of potencies against SNU 601 cells, and wherein the highest performing constructs demonstrated

superior anti-tumor cytotoxicities when compared to bispecific controls that only contain a single binding domain against an immune cells antigen.

**EXAMPLE 15: CLDN18.2-TARGETING OF ANTI-(CLDN18.2XCD3XCD28) TRISPECIFIC CONSTRUCTS IN *IN VITRO* T-CELL DEPENDENT CYTOTOXICITY ASSAYS WITH TUMOR CELLS EXPRESSING VARYING LEVELS OF CLDN18.2**

**[0685]** To determine if certain trispecific antibody constructs exhibit tumor antigen dependency with respect to their *in vitro* cytotoxic activity, T-cell dependent cytotoxicity assays were conducted with tumor cells expressing different, e.g., high or low-levels, of antigen.

**[0686]** The selected anti-CLDN18.2 trispecific constructs as well as benchmark or bispecific control constructs 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 CD3+ T cells were thawed and mixed SNU 601 (276,125 CLDN18.2/cell) or SKOV-3 (12,951 CLDN18.2/cell) target cells at an E:T ratio of 2:1. Plates were incubated for 72 hrs at 37 °C and 5% carbon dioxide, after which Hoechst Stain was added to the cells. After a 30-minute incubation period, tumor cell viability was assessed through live tumor cell counts using the Operetta CLS High Content Analysis System (PerkinElmer). Data are representative of 3 independent experiments with 2 donors.

**[0687]** Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**[0688]** Concentration response curves for the anti-CLDN18.2 constructs are presented in **FIGS. 11A-11D** and $IC_{50}$ and maximum percent killing from the T cell-dependent cytotoxicity assay comparing the trispecific antibody constructs to the benchmark control, v35923, are presented in **TABLE 29.**

**TABLE 29: Cytotoxic Activity of Anti-CLDN18.2 Trispecific Antibody Constructs Towards CLDN18.2 high and low/negative Tumor Cells Grown in Culture CD3+ T Cells**

| Construct | SNU 601 | | SKOV-3 | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) | $IC_{50}$ (pM) | Maximum Killing (%) |
| v37633 | 1.238 | 87.6 | ND | 27.67 |
| v37634 | 1.791 | 89.3 | ND | 0 |
| v37635 | 4.634 | 88.0 | ND | 21.3 |
| v37638 | 0.498 | 91.8 | ND | 36.1 |
| v37640 | 4.618 | 90.0 | ND | 34.4 |

| | | | | |
|---|---|---|---|---|
| v37642 | 0.183 | 89.9 | 126.8 | 34.6 |
| v35923 | 5.652 | 88.7 | ND | 0 |

**[0689]** All multivalent and multispecific constructs tested were highly potent against the CLDN18.2+ cell line, SNU 601, with maximum cytotoxicity reaching between about 86-92%. The most potent construct, v37642, and the second most potent construct, v37638, showed an approximately 40-fold and 15-fold higher potency, respectively, when compared to the bivalent anti-(CLDN18.2xCD3) benchmark bispecific construct, v35923. The constructs v37633 and v37634 exhibited similar potencies, which were approximately 5-fold higher than v35923. The constructs v37640 and v37635 exhibited a similar potency to v35923, with $IC_5$ values of 4.618 pM and 4.634 pM, respectively.

**[0690]** The measured cytotoxicities induced by all tested trispecific constructs, except for v37634, were reduced in the CLDN18.2$^{low}$ SKOV-3 cell line, with maximum cytotoxicity reaching between 21% to 36%. The construct v37642 exhibited an approximate 700-fold reduction in cytotoxic potency against the CLDN18.2$^{low}$ SKOV-3 cells when compared to its potency against CLDN18.2$^{high}$ SNU 601 cells. The benchmark anti-(CLDN18.2xCD3) bispecific control, v35923, however, induced no cytotoxicity towards CLDN18.2$^{low}$ SKOV-3 cells.

**[0691]** These results demonstrate that the multivalent and multispecific constructs exhibit antigen-dependent cytotoxic activity, as shown first in the CLDN18.2$^{high}$ cell line, SNU 601, followed by testing in CLDN18.2$^{low}$ SKOV-3 cells and measuring a reduced cytotoxic activity.

## EXAMPLE 16: IMPACT OF THE PRESENCE OF THE ANTI-CD28 PARATOPE IN THE TRISPECIFIC CONSTRUCTS ON SUSTAINED *IN VITRO* CYTOTOXICITY TOWARDS CLDN18.2-POSITIVE TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ T CELLS

**[0692]** To test the impact of the CD28 paratope used in the multivalent and multispecific constructs herein on their sustained *in vitro* activity and preferential killing of tumor cells overexpressing CLDN18.2, selected constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing moderate levels CLDN18.2.

**[0693]** Frozen human T cells were thawed at 37 °C and mixed with CLDN18.2+ SNU 601-RFP, KATO III-RFP, or DAN G-RFP cells such that the ratio of T cells to allogeneic tumor cells was adjusted to an E:T ratio of 1:5. The mixtures were incubated together with the antibody constructs

for 7 days, after which tumor cell viability was assessed through live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 2 independent experiments with 2 donors.

**[0694]** Concentration response curves for the anti-CLDN18.2 constructs are presented in **FIGS. 12A-12B** (left: SNU-601; middle: KATO-III; right: DAN-G) and $IC_{50}$ values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLE 30.**

**TABLE 30: Cytotoxic Activity of Anti-CLDN18.2 Trispecific Constructs Towards CLDN18.2$^{med}$ Tumor Cells Grown in Culture with Primary Human CD3+ T Cells**

| Construct # | SNU 601 | | KATO III | | DAN G | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) | $IC_{50}$ (pM) | Maximum Killing (%) | $IC_{50}$ (pM) | Maximum Killing (%) |
| v37633 | 10.1 | 100 | 221.9 | 89 | 546.1 | 69 |
| v37634 | 9.6 | 100 | 483.1 | 89 | 4316 | 69 |
| v37635 | 9.7 | 100 | 161.9 | 92 | 1844 | 76 |
| v37638 | 1.8 | 100 | 54.5 | 89 | 18.98 | 83 |
| v37640 | 7.3 | 100 | 106.4 | 90 | 141.7 | 91 |
| v37642 | 1.0 | 100 | 7.3 | 90 | 27.25 | 80 |
| v37663 | 96.2 | 99 | ND | 58 | 5170 | 41 |
| v35923 | 28.6 | 100 | 388.1 | 78 | ND | 2 |

**[0695]** The measured cytotoxic activities of the different constructs tested largely correlated with the level of CLDN18.2 expressed by the cell lines, with the highest potency observed toward SNU 601 cells (~276,125 CLDN18.2/cell), and the lowest toward DAN G cells (~33,164 CLDN18.2/cell). The most potent constructs, v37638 and v37642, exhibited approximately 10 to 100-fold higher potency toward SNU 601 cells when compared to the anti-(CLDN18.2xCD3) bispecific control constructs, v35923 and v37663, respectively. The constructs v37633, v37634, v37635, and v37640 exhibited similar potency toward SNU 601 cells, with an approximately 3 to 10-fold greater potency compared to those of the constructs v37663 and v35923, respectively. All tested multivalent and multispecific constructs induced 100% maximum cytotoxicity against SNU 601 target cells.

**[0696]** The most potent constructs, v37638 and v37642, exhibited an approximately 7 to 50-fold higher potency, respectively, toward KATO III cells when compared to the anti-(CLDN18.2xCD3) bispecific control, v35923. The constructs v37633, v37634, v37635, and v37640 exhibited an approximately 4-fold potency toward KATO III cells when compared of the potency of v35923. All trispecific constructs tested reached approximately 90% maximum cytotoxicity of KATO III

target cells, whereas maximum cytotoxicity against KATO III induced by the bispecific controls, v37663 and v35923, reached 58% and 78%, respectively. Negative control, v22277, did not induce cytotoxicity of any cell line.

**[0697]** The most potent constructs, v37642 and v37638, exhibited an approximately 200 to 300-fold higher potency, respectively, toward DAN G cells than the anti-(CLDN18.2xCD3) bispecific control, v37663. The constructs v37640 and v37633 exhibited approximately 35 to 100-fold higher potency, respectively, toward DAN G cells when compared to v37663. The construct v37635 exhibited an approximately 10-fold higher potency toward DAN G cells when compared to v37663. The construct v37634 exhibited similar potency toward DAN G compared to v37663. All tested trispecific constructs reached between 70-90% maximum cytotoxicity against DAN G target cells, whereas maximum cytotoxicity against DAN G induced by the bispecific controls v37663 and v35923 reached 2% and 40%, respectively.

**[0698]** Taken together, these results suggest that the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs can be superior to the corresponding bivalent anti-(CLDN18.2xCD3) bispecific control constructs that only contain one immune cell antigen targeting domain at inducing a sustained *in vitro* cytotoxicity toward target cells expressing various levels of CLDN18.2.

## EXAMPLE 17A: IMPACT OF ANTI-CD28 PARATOPE AFFINITY ON VITRO ACTIVITY OF TRISPECIFIC CONSTRUCTS IN T-CELL DEPENDENT CYTOTOXICITY ASSAY WITH TUMOR CELLS EXPRESSING VARYING LEVELS OF CLDN18.2

**[0699]** To assess the effect of reducing the affinity of the trispecific antibody construct toward CD28, selected constructs, including v37638, were tested in T cell-dependent cytotoxicity assays with tumor cell lines expressing varying levels of CLDN18.2. Four additional constructs with the same format as v37638 but with reduced affinity toward CD28, v37683, v37689, v37692, v37694 were included, as was a CLDN18.2xCD3 bispecific control, v37663.

**[0700]** Test articles (e.g., antibody constructs, controls, etc.) 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). Human panT cells were thawed and mixed with SNU 601-RFP (high CLDN18.2 density) cells at an E:T ratios of 2:1. Plates were incubated for 168 hrs at 37 °C and 5% carbon dioxide, after which tumor cell viability was assessed through live tumor

cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 3 independent experiments with 3 donors.

**[0701]** Concentration response curves for some of the anti-CLDN18.2 trispecific constructs with reduced affinity for CD28 are presented in **FIG. 13** and $IC_{50}$ values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLE 31.**

**TABLE 31: Cytotoxic Activity of Trispecific Constructs with Reduced Affinity for CD28 Towards CDLN18.2+ Tumor Cells Grown in Culture with Primary Human T Cells**

| Construct # | SNU 601 | |
|---|---|---|
| | $IC_{50}$ (pM) | Maximum Killing (%) |
| v37638 | 0.17 | 88.2 |
| v37683 | 0.25 | 88.6 |
| v37684 | 0.34 | 89.3 |
| v37685 | 0.81 | 88.7 |
| v37686 | 0.09 | 88.5 |
| v37687 | 0.10 | 88.5 |
| v37688 | 0.18 | 87.8 |
| v37689 | 0.12 | 93.4 |
| v37690 | 1.21 | 92.3 |
| v37691 | 0.08 | 91.0 |
| v37692 | 0.63 | 91.5 |
| v37693 | 0.13 | 95.3 |
| v37694 | 1.44 | 93.1 |
| v37695 | 0.04 | 89.1 |
| v37663 | 1.542 | 89.5 |

**[0702]** All tested multivalent and multispecific constructs that have mutations in the anti-CD28 Fab domain (mutations relative to the hTN228 wildtype $V_H$ and/or $V_L$ sequences) induced similar maximum cytotoxicity when compared to both (i) the trispecific constructs that comprise the wildtype TN228 paratope, e.g., construct v37638, and (ii) the bivalent and bispecific control construct, v37663, with maximum cytotoxicity reaching between 88-95%. Trispecific constructs that showed a 10-fold reduction or less in affinity for CD28 as measured by SPR maintained similar cytotoxic potency (i.e., $IC_{50}$ values) toward CLDN18.2+ SNU 601 cells as that of the trispecific construct with the wild type TN228 Fab domain, v37638. Trispecific constructs, v37685, and v37692, with an approximately 30 and 90-fold reduction in affinity, respectively, for CD28 by SPR exhibited a 3 to 5-fold reduction in cytotoxic potency toward CLDN18.2+ SNU 601 cells when compared to the trispecific construct with the wild type TN228 Fab domain, v37638. The trispecific constructs, v37690, and v37694, which did not exhibit measurable binding to CD28 by

SPR still exhibited similar cytotoxic potency toward CLDN18.2+ SNU 601 cells when compared the bispecific construct, v37663. Negative control, v22277, did not induce cytotoxicity of SNU 601 cells.

**[0703]** Taken together, these results demonstrate that modifications to the anti-CD28 Fab domain that reduce the affinity of the construct for CD28 within 10-fold of wild type TN228, have minimal impact on the ability of the trispecific constructs to induce cytotoxicity toward CLDN18.2+ target cells. Reduction in affinity to CD28 greater than 10-fold compared to wild type TN228, still maintained quantifiable binding to CD28 by SPR, reduces cytotoxic potency toward CLDN+ target cells, but still maintained cytotoxic superiority to that of the corresponding bispecific control construct.

## EXAMPLE 17B: IMPACT OF ANTI-CD28 PARATOPE AFFINITY ON IN VITRO ACTIVITY OF MULTIVALENT AND MULTISPECIFIC ANTIBODY CONSTRUCTS IN T CELL DEPENDENT CYTOTOXICITY ASSAY WITH CLDN18.2+ SNU-601 TUMOR CELLS

**[0704]** To assess the impact of reducing the affinity of certain anti-(CLDN18.2xCD3xCD28) trispecific antibody constructs toward CD28, selected constructs with the same format as v37642 but with reduced binding affinity towards CD28 were tested in T cell-dependent cytotoxicity assays with CLDN18.2+ tumor cell line SNU-601. Additionally, anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific control constructs, v37663 and v37665, respectively, were included. A trispecific construct, v37662, containing a paratope against an irrelevant antigen, RSV protein F (RSV-F), in place of the anti-CD28 paratope in the same format as v37642, was included as a format control. A monoclonal anti-RSV protein F antibody, v22277, was included as a negative control.

**[0705]** Test articles (e.g., antibody constructs, controls, etc.) 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). Human panT cells were thawed and mixed with SNU 601-RFP (high CLDN18.2 density) cells at E:T ratios of 2:1 and 1:5 and plates were incubated for 72 hrs and 168 hrs, respectively, at 37 °C and 5% carbon dioxide, after which tumor cell viability was assessed through live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Data are representative of 3 independent experiments with 2 donors.

**[0706]** Calculated $IC_{50}$ values and maximum mean cytotoxicity from the T cell-dependent cytotoxicity assay are presented in **TABLE 32A**.

**[0707]** Percent cytotoxicity was calculated using the following formula:

$$\%\ cytotoxicity = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**TABLE 32A: Cytotoxic Activity of Trispecific Constructs with Reduced Affinity for CD28 Towards CDLN18.2+ Tumor Cells Grown in Culture with Primary Human T Cells**

| Construct # | 2:1 E:T | | 1:5 E:T | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | Maximum Mean Cytotoxicity (%) | $IC_{50}$ (pM) | Maximum Mean Cytotoxicity (%) |
| v37642 | 0.166 | 98.74 | 2.295 | 96.94 |
| v37662 | 17.92 | 96.92 | ND | 4.01 |
| v38991 | 0.8377 | 98.24 | 12.71 | 95.57 |
| v38990 | 3.579 | 97.79 | 145.3 | 95.4 |
| v38994 | 0.1584 | 98.03 | 2.42 | 94.09 |
| v37663 | 13.23 | 97.69 | 206.7 | 92.95 |
| v37665 | ND | -9.28 | ND | -4.68 |
| v22277 | ND | 8.47 | ND | 0.72 |

**[0708]** All tested multivalent and multispecific constructs that have mutations in the anti-CD28 Fab domain (mutations relative to the hTN228 wildtype $V_H$ and/or $V_L$ sequences) induced similar maximum cytotoxicity when compared to both (i) the trispecific constructs that comprise the wildtype TN228 paratope, e.g., construct v37642, and (ii) the bivalent and bispecific control construct, v37663, with maximum cytotoxicity reaching between 93-99 % for both of the E:T ratios tested. The multivalent and multispecific construct v37642 exhibited the highest potency in a CLDN18.2$^+$ SNU-601 co-culture at both 2:1 and 1:5 E:T ratios, with $IC_{50}$ values of 0.166 pM and 2.295 pM, respectively. At 2:1 E:T ratio, the multivalent and multispecific constructs with mutations in the anti-CD28 paratope as well as the CD28 KO anti-(CD3xRSV-FxCLDN18.2) control construct exhibited similar maximum cytotoxicity relative to the trispecific construct with the wild-type anti-CD28, v37642. At 1:5 E:T ratio, while the multivalent and multispecific constructs bearing mutations in the anti-CD28 paratope, v38990, v38991 and v38994, showed similar maximum cytotoxicity, the CD28 KO control, v37662, displayed a 24-fold reduction in cytotoxicity. The multivalent and multispecific construct, v38990, having a 225-fold reduction in binding affinity compared to anti-CD28 WT, as determined by SPR, showed a 22-fold and 60-fold reduced cytotoxic potency for the 2:1 and 1:5 E:T ratios, respectively, as compared to v37642

(containing the WT (unmodified) anti-CD28 domain). The construct v38991 which exhibits a 6.1-fold reduced binding affinity to CD28 as shown by SPR, showed a 5-fold reduction in cytotoxic potency towards CLDN18.2+ SNU-601 cells, relative to the wild-type multivalent and multispecific construct, v37642, for both E:T ratios. The multivalent and multispecific construct, v38994, displayed comparable potency to the wild-type multivalent and multispecific construct v37642 at both E:T ratios. The negative control, v22277, did not induce cytotoxicity of SNU 601 cells.

**[0709]** Taken together, these results demonstrate that mutations in the anti-CD28 paratope that reduce the binding affinity of the multivalent and multispecific antibody constructs can also impact the cytotoxic potency of these constructs in a co-culture of CLDN18.2+ SNU-601 target cells and human CD3+ T cells. This reduction in cytotoxic potency became more distinct in the lower E:T condition experiments of 1:5 ratio for, e.g., v38990 (225-fold binding affinity reduction determined by SPR) but was maintained for v38991 (6-fold binding affinity reduction determined by SPR). All of the trispecific constructs tested that contained mutations in the CD28 paratope exhibited higher cytotoxic potency than the corresponding bispecific controls (anti-(CD3xCLDN18.2) and anti-(CD28xCLDN18.2)).

**EXAMPLE 17C: T CELL FUNCTION AND VIABILITY FOLLOWING REPETITIVE STIMULATION WITH TRISPECIFIC ANTI-(CLDN18.2xCD3xCD28) CONSTRUCTS**

**[0710]** To investigate the ability of trispecific antibody constructs described herein to induce repetitive anti-tumor cytotoxicity, certain constructs were assessed in a repeat challenge assay with primary human pan T cells in culture with SNU 601 target cells.

**[0711]** For the initial stimulation of human pan T cells, frozen T cells were thawed at 37 °C and mixed with SNU 601-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 SNU-601 cell co-cultures and incubated for 48 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 SNU 601-RFP target cells at an E:T ratio of 5:1. Test articles were diluted in assay media, added to the cell mixture, and cultures were incubated for 48-72 hours. Following each round of stimulation, tumor cell cytotoxicity was assessed through live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin

Elmer). T cell viability and T cell fold-expansion was also determined. Data are representative of two independent experiments with two donors.

**[0712]** Percent cytotoxicity was determined using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ tumor\ cell-only\ wells}\right) \times 100$$

T cell fold expansion was determined using the following formula:

$$\text{Fold-expansion} = \left(\frac{number\ of\ T\ cells\ after\ stimulation}{number\ of\ T\ cells\ seeded}\right)$$

**[0713]** Percent cytotoxicity of target cells (mean ± SEM) in co-culture with human pan T cells following each round of stimulation with tested constructs is shown in **TABLE 32B**. Percent T cell viability following each round of T cell stimulation. Percent viability of T cells in co-culture with SNU 601 target cells following each round of stimulation with tested constructs is shown in **TABLE 32C**. Fold-expansion of T cells in co-culture with SNU 601 target cells following each round of stimulation with tested constructs is shown in **TABLE 32D**.

**TABLE 32B: Cytotoxicity of Target Cells Induced by T cells Repetitively Stimulated with Tested Constructs**

| Stimulation Round | Percent Cytotoxicity (%; mean ± SD) | | | |
|---|---|---|---|---|
| | Construct # | | | |
| | v37634 | v37663 | v35923 | v40297 |
| 1 | 89.9 ± 0.7 | 90.0 ± 0.8 | 89.0 ± 1.3 | 87.7 ± 0.9 |
| 2 | 91.9 ± 0.9 | 91.7 ± 0.8 | 91.9 ± 0.2 | 90.3 ± 1.0 |
| 3 | 96.4 ± 0.4 | 95.5 ± 0.5 | 95.3 ± 1.0 | 95.2 ± 0.3 |
| 4 | 91.4 ± 0.3 | 64.5 ± 1.6 | 71.4 ± 1.0 | 62.7 ± 1.9 |
| 5 | 89.0 ± 0.6 | 14.6 ± 2.8 | 26.6 ± 1.9 | 34.3 ± 7.7 |
| 6 | 94.7 ± 0.3 | nd | nd | nd |
| 7 | 7.9 ± 3.3 | nd | nd | nd |

**TABLE 32C: Viability of T Cells Repetitively Stimulated With Tested Constructs**

| Stimulation Round | Percent Viability (%) | | | |
|---|---|---|---|---|
| | Construct # | | | |
| | v37634 | v37663 | v35923 | v40297 |
| 1 | 99 | 99 | 99 | 99 |
| 2 | 63 | 66 | 68 | 60 |
| 3 | 86 | 88 | 86 | 85 |
| 4 | 91 | 72 | 75 | 75 |
| 5 | 85 | 47 | 42 | 38 |
| 6 | 75 | nd | nd | nd |

| | | | | |
|---|---|---|---|---|
| 7 | 74 | nd | nd | nd |

**TABLE 32D: Fold-Expansion of T cells Repetitively Stimulated with Tested Constructs**

| Stimulation Round | Fold-Expansion | | | |
|---|---|---|---|---|
| | Construct # | | | |
| | v37634 | v37663 | v35923 | v40297 |
| 1 | 0.5 | 0.5 | 0.5 | 0.5 |
| 2 | 5.9 | 3.2 | 3.2 | 3.0 |
| 3 | 8.6 | 1.9 | 2.1 | 2.0 |
| 4 | 1.4 | 0.3 | 0.3 | 0.3 |
| 5 | 1.4 | nd | nd | nd |
| 6 | 0.4 | nd | nd | nd |

**[0714]** The trivalent and trispecific construct, v37634, resulted in greater than 85% cytotoxicity of target cells for up to 6 rounds of repetitive stimulation. In comparison, the bispecific control and benchmark constructs, v37663, v35923, and v40297, resulted in greater than 85% cytotoxicity of target cells for up to 3 rounds of stimulation. The trispecific construct, v37634, resulted in greater than 70% T cell viability over 7 rounds of repetitive stimulation. Bispecific control and benchmark constructs, v37663, v35923, and v40297, resulted in greater than 70% T cell viability over 4 rounds of stimulation. The trispecific construct, v37634, resulted in an 8.6-fold expansion of T cells following stimulation 3, and an AUC value of 43.64. Bispecific control and benchmark constructs, v37663, v35923, and v40297, resulted in an approximate 2-fold expansion of T cells following the third round of stimulation, and AUC values of 13.6, 14.1, and 13.1, respectively.

**[0715]** Taken together, these data demonstrate that the trispecific constructs engaging CLDN18.2, CD3 and CD28 can modify T cell function and T cell viability over repetitive cycles of T cell stimulation.

## EXAMPLE 17D: IMPACT OF TRISPECIFIC ANTI-(CLDN18.2XCD3XCD28) ANTIBODY CONSTRUCTS ON VIABILITY OF PRIMARY CD3+ T CELLS

**[0716]** To determine the impact of the format of trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs on the viability of human CD3+ T cells, selected constructs were assessed in monocultures of primary human CD3+ T cells. Selected trispecific constructs containing a paratope against an irrelevant antigen, anti-RSV protein F (RSV-F), in place of the CD3, CD28, or CLDN18.2 paratopes were included as format controls for each binding arm of the trispecific constructs, v37634 and v37642. A benchmark trispecific antibody that includes a CD3-CD28

CODV-Fab, v37035 was included. Bispecific anti-CLDN18.2-CD3 constructs, v37663 and v35923, as well as bispecific anti-CLDN18.2-CD28 construct, v37665, were included as controls. Puromycin (Life Technologies) was included as a positive control for T cell death.

**[0717]** Constructs were diluted to 45 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 (ThermoFisher Scientific). Frozen human CD3+ T cells were thawed at 37 °C and combined with CellTox™ Green dye (Promega). CellTox™ Green measured changes in membrane integrity as a result of cell death – as cells lost viability, cells fluoresced green. CD3+ 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™ (PerkinElmer) and data were analyzed using the Operetta CLS High Content Analysis System (PerkinElmer). Data are representative of one experiment with two donors. Background-adjusted Mean Fluorescence Intensity (MFI) was determined using the following formula:

$$\text{Background-Adjusted MFI} = MFI_{Treated\ well} - Mean\ MFI_{T\ cell-only\ wells}$$

**[0718]** Background-adjusted MFI following treatment of primary CD3+ T cells with trispecific anti-CLDN18.2-CD3-CD28 constructs is summarized in **TABLE 32E.**

**TABLE 32E: MFI of CellTox Green by CD3+ T cells Incubated with Certain Trispecific anti-(CLDN18.2xCD3xCD28) Antibody Constructs**

| Construct # | MFI, CellTox™ Green | |
|---|---|---|
| | Mean | Standard Deviation |
| v37035 | 511.1 | 6.393 |
| v37634 | 98.47 | 5.587 |
| v37642 | 335.1 | 71.01 |
| v39215 | 59.93 | 9.452 |
| v39982 | 100.3 | 84.91 |
| Puromycin | 1421.1 | 103.0 |

**[0719]** Treatment with the positive control resulted in the greatest reduction of T cell viability, with a background-adjusted MFI of 1421.1. Treatment with the negative control, v39982, resulted in a background-adjusted MFI of 100.3. Treatment with the trispecific anti-(CLDN18.2x CD3xCD28) construct, v37634, or with the format-matched single arm anti-CD3 controls, v39215 or v37662, resulted in no T cell death relative to the negative control, with background-adjusted MFI values within one-fold of that induced by v39982. Treatment with v37035 and v37642 resulted in reduced T cell viability relative to v39982, with background-adjust MFI values of 511.1

and 335.1, respectively, which were approximately 5-and 3-fold higher, respectively, than that induced by v39982.

**[0720]** The results show that the format of a trispecific anti-(CLDN18.2xCD3xCD28) antibody construct can impact the viability of monocultures of human primary CD3+ T cells.

**EXAMPLE 18A: 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**

**[0721]** To test the ability of certain multivalent and multispecific anti-(DLL3xCD3xCD28) antibody constructs to induce sustained *in vitro* cytotoxic activity towards DLL3+ tumor cells relative to bispecific and benchmark controls, selected constructs were tested in primary human T cell cultures with tumor cell lines expressing DLL3.

**[0722]** 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).

**[0723]** Concentration response curves for the anti-DLL3 constructs are presented in **FIG. 14A** and $IC_{50}$ values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLE 33.**

**TABLE 33: Cytotoxic Activity of Anti-DLL3 Trispecific, Bispecific and Benchmark 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 | ND | -4.8 | ND | 1.9 | ND | 6.9 |

**[0724]** 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).

**[0725]** Concentration response curves for the anti-DLL3 constructs are presented in **FIG. 14B** and $IC_{50}$ values and maximum percent killing from the T cell-dependent cytotoxicity assay are presented in **TABLES 34** and **35A.**

**TABLE 34: $IC_{50}$ 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 35A: $IC_{50}$ 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 18B: INDUCTION OF T CELL CYTOXICITY FOLLOWING REPETITIVE STIMULATION WITH TRISPECIFIC ANTI-(DLL3xCD3xCD28) CONSTRUCT

**[0726]** 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.

**[0727]** 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. 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.

**[0728]** Percent cytotoxicity was determined using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{MFI\ of\ treatment\ wells}{MFI\ of\ no\ treatment\ wells}\right) \times 100$$

**[0729]** Percent cytotoxicity of target cells (mean ± SEM) in co-culture with human pan T cells following each round of stimulation with select constructs is shown in **Table 35B.**

**TABLE 35B: 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 |

**[0730]** The data demonstrate that the trivalent and trispecific Het-Fab antibody constructs, v39915 and particularly v39917, 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) as well as the clinical benchmark v38589 elicited significantly lower anti-tumor cytotoxicity starting at stimulation round 3 onwards.

## EXAMPLE 18C: IMPACT OF TRISPECIFIC ANTI-(DLL3XCD3XCD28) ANTIBODY CONSTRUCTS ON THE VIABILITY OF PRIMARY CD3+ T CELLS

**[0731]** 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.

**[0732]** 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 (ThermoFisher Scientific). 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™ (PerkinElmer) and data were analyzed using the Operetta CLS High Content Analysis System (PerkinElmer). ΔMFI of CellTox™ Green was calculated by subtracting the mean MFI of no treatment wells.

**[0733]** ΔMFI of CellTox™ Green of Pa1n T cells following treatment is summarized in **TABLES 35C** and **35D.**

**TABLE 35C: 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 35D: 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 |

**[0734]** The data demonstrate that the trivalent and trispecific antibody constructs, v39915 and particularly v39917, 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 18D: 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**

**[0735]** To test the impact of antibody construct format on its 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.

**[0736]** 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).

**[0737]** Percent cytotoxicity was calculated using the following formula:

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

**[0738]** 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. 14C-14E**, respectively. Calculated $IC_{50}$ and maximum cytotoxicity (%) are shown in **TABLE 35D** (primary human T cells) and **TABLE 35E** (primary human Pan-T cells) below:

**TABLE 35D: 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 35E: 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 19: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH MSLN-POSITIVE TUMOR CELL LINES

**[0739]** This example demonstrates that the tested trispecific antibody constructs of the present disclosure activated T cells by simultaneously binding to tumor-associated antigen on cancer cells and both CD3e and CD28 on T cells, leading to cross-linking of CD3e and CD28. The resulting signalling cascade triggered T cell activation, leading to cytokine secretion. To assess the impact of the formats of an antibody construct on its ability to activate T cells, TNFα and IL-2 production was measured for various constructs with varying formats/geometries as described below.

**[0740]** Test articles (e.g., antibody constructs) 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). Human CD3+ T cells were thawed and mixed with H292 (high MSLN density) cells at an E:T ratio of 2:1 then added to the plates. Plates were incubated for 72 hrs 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.

**[0741]** 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 1 independent experiments with 1 donor.

**[0742]** Potency and maximum cytokine production of TNFα and IL-2 by human CD3+ T cells in the presence of anti-MSLN-CD3-CD28 trispecific constructs are summarized in **TABLE 36**.

**TABLE 36: Potency and Maximum Cytokine Production Induced by Anti-MSLN Trispecific Constructs from Primary Human T Cells Co-cultured with H292 Target Cells**

| Antibody Construct | IL-2 | | TNFα | |
|---|---|---|---|---|
| | $EC_{50}$ (pM) | Maximum Cytokine (pg/mL) | $EC_{50}$ (pM) | Maximum Cytokine (pg/mL) |
| v34913 | 4.242 | 1810.0 | 17.22 | 128.2 |
| v34914 | 1014 | 3830.9 | ND | 196.4 |
| v34915 | 154.3 | 350.6 | 184.8 | 38.1 |
| v34916 | 508.9 | 2470.4 | 1143 | 109.1 |
| v34917 | 134.7 | 622.5 | 72.48 | 30.6 |
| v34918 | 134.2 | 3080.6 | 287.4 | 180.03 |
| v34919 | 226.6 | 15.7 | ND | 11.8 |
| v34927 | ND | 2.8 | ND | 2.1 |

**[0743]** Human CD3+ T cell TNFα and IL-2 release after incubation in the presence of anti-(MSLNxCD3xCD28) trispecific constructs with different formats are summarized in **FIGS. 15A-15B**.

**[0744]** Of the trispecific constructs that comprised one anti-CD3 Fab domain, one anti-CD28 scFv domain, and one anti-MSLN scFv domain, the constructs v34915 and v34917 showed similar $EC_{50}$ values, with approximately 1.5-fold higher potency for IL-2 production compared to the bivalent

anti-(MSLNxCD3) bispecific control, v34919. Maximum levels of IL-2 production induced by v34915 and v34917 reached 350.6 pg/mL and 622.5 pg/mL, respectively, which was approximately 20 to 40-fold higher than that induced by v34919. The constructs v34914 and v34916 exhibited reduced potency of IL-2 production compared to v34919, with an approximate 2-5-fold increase in their $EC_{50}$ values, respectively. Maximum cytokine production induced by v34914 and v34916 reached 3830.9 pg/mL and 2470.4 pg/mL, which was approximately 150 to 250-fold higher than that induced by v34919. The construct v34913 exhibited the highest potency in IL-2 production of the trispecific construct that comprised one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-MSLN scFv domain, which was 53-fold greater than that measured for the control v34919. The construct v34918 displayed an $EC_{50}$ of 134.2 pM, which was approximately 2-fold greater than that of v34919. The constructs v34913 and v34918 induced maximum cytokine levels of 1810.0 pg/mL and 3080.6 pg/mL, respectively, which was approximately 115 to 196-fold greater than that induced by v34919. The negative control, v22277, and the bivalent anti-(MSLNxCD28) bispecific control, v34927, did not induce any measurable IL-2 production.

**[0745]** Of the trispecific constructs that comprised one anti-CD3 Fab domain, one anti-CD28 scFv domain, and one anti-MSLN scFv domain, the construct v34917 exhibited the highest potency for TNFα production, followed by the constructs v34915 and v34916, with $EC_{50}$ values of 72.48 pM, 184.8 pM, and 1143 pM, respectively. TNFα production induced by construct v34914 did not reach plateau, and therefore the $EC_{50}$ value was not determined. Maximum TNFα induced by the constructs v34914 and v34916 reached 196.4 pg/mL and 109.1 pg/mL, respectively. Maximum TNFα induced by the constructs v34915 and v34917 reached 38.1 pg/mL and 30.6 pg/mL, respectively. Of the trispecific constructs that comprised one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-MSLN scFv domain, the construct v34913 exhibited the highest potency for TNFα production, followed by v34918, with $EC_{50}$ values of 17.22 pM, 287.4 pM, respectively. Maximum TNFα induced by the constructs v34913 and v34918 reached 128.2 pg/mL and 180.03 pg/mL, respectively. The negative control, v22277, and the bivalent anti-(MSLNxCD28) and anti-(MSLNxCD3) bispecific controls, v34927 and v34919, did not induce any measurable TNFα production.

**[0746]** These results show that induction of T cell mediated cytokine production can be significantly enhanced using antibody constructs comprising two T cell antigen binding domains

(e.g., against CD and CD28) compared to conventional constructs targeting only one T cell antigen. Furthermore, cytokine production can be modulated through modification of the format and/or geometry of an antibody coonstruct, with various construct formats/geometries exhibiting a range in potency and maximum cytokine production of IL-2 and TNFα.

**EXAMPLE 20: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH CLDN18.2-POSITIVE TUMOR CELLS**

**[0747]** The trispecific antibody constructs described herein can activate T cells, e.g., by simultaneously binding a tumor-associated antigen (e.g., Cldn18.2) on cancer cells 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 trispecific construct formats and/or geometries on T cell activation, TNFα and IL-2 production was measured as described below.

**[0748]** 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). Human CD3+ T cells were thawed and mixed with SNU 601 (high CLDN18.2 density) cells at an E:T ratio of 2:1 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.

**[0749]** 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.

**[0750]** Potency and maximum cytokine production of TNFα and IL-2 by human CD3+ T cells in the presence of the tested anti-(CLDN18.2xCD3xCD28) constructs are summarized in **TABLE 37**.

**TABLE 37: Potency and Maximum Cytokine Production Induced by Anti-CLDN18.2 Trispecific Constructs from Primary Human T Cells Co-cultured with SNU 601 Cells**

| Construct | IL-2 | | TNFα | |
|---|---|---|---|---|
| | $EC_{50}$ (pM) | Maximum cytokine conc. (pg/mL) | $EC_{50}$ (pM) | Maximum cytokine conc. (pg/mL) |
| v37633 | ND | 9069.5 | 146.8 | 810.1 |
| v37634 | 67.3 | 2557.3 | 10.9 | 382.2 |
| v37635 | 102.5 | 3968.5 | 25.4 | 503.4 |
| v37636 | ND | 8172.0 | ND | 775.3 |
| v37637 | 500.8 | 4677.0 | 304.6 | 571.4 |
| v37638 | 1.2 | 3321.3 | 84.9 | 385.3 |
| v37639 | ND | 8813.0 | 640.5 | 426.8 |
| v37640 | 48.1 | 5743.4 | 35.9 | 574.9 |
| v37641 | 55.0 | 4982.6 | 41.9 | 577.3 |
| v37642 | 0.8 | 3633.0 | 1.0 | 422.1 |
| v37663 | 284.4 | 499.2 | 192.3 | 170.3 |
| v37665 | ND | 8.6 | ND | 0.5 |

**[0751]** Human CD3+ T cell IL-2 (left) and TNFα (right) release after incubation in the presence of anti-(CLDN18.2xCD3xCD28) trispecific constructs with different formats are summarized in **FIGS. 16A-16B**.

**[0752]** The multivalent and multispecific constructs that comprised one anti-CD3 Fab domain, one anti-CD28 scFv domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies (e.g., $EC_{50}$ values) for T cell-mediated IL-2 production. The most potent and second most potent of these constructs, v37634 and v37635, respectively, showed approximately 3 to 4-fold higher potency for IL-2 production when compared to the bivalent anti-(CLDN18.2xCD3) bispecific control construct, v37663. Maximum levels of IL-2 production induced by the constructs v37634 and v37635 reached 2557.3 pg/mL and 3968.5 pg/mL, respectively, which was approximately 5 to 7-fold higher than IL-2 production induced by v37663. The construct v37637 exhibited a potency similar to v37663, with a less than 2-fold reduction in potency but resulted in an approximately 10-fold greater maximum level of IL-2 production. The potencies for the constructs v37633 and v37636 were not determined, as the curve did not reach plateau, but maximum IL-2

production reached 9096.5 pg/mL and 8172.0 pg/mL, respectively, which was approximately 15 to 20-fold greater than that induced by the control v37663.

**[0753]** The multivalent and multispecific constructs that contained one anti-CD3 Fab domain, one anti-CD28 scFv domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies for T cell mediated TNFα production. The most potent and second most potent constructs, v37634 and v37635, showed approximately an 8 to 15-fold higher potency for TNFα production compared to the bivalent anti-(CLDN18.2xCD3) bispecific control construct, v37663. Maximum levels of TNFα production induced by the constructs v37634 and v37635 reached 382.2 pg/mL and 503.4 pg/mL, respectively, which was approximately 2 to 4-fold higher than the TNFα production induced by the control v37663. The constructs v37633 and v37637 exhibited a similar potency to v37663 but induced an approximately 3 to 5-fold higher production of TNFα when compared to v37663. The potency for v37636 was not determined, as the concentration response curve did not reach plateau, but maximum TNFα production reached 775.3 pg/mL, which was approximately 5-fold higher than that induced by v37663.

**[0754]** The multivalent and multispecific constructs that contained one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies for T cell-mediated IL-2 production. The most potent and second most potent constructs, v37642 and v37638, showed an approximately 250 to 350-fold higher potency for IL-2 production compared to the bivalent anti-(CLDN18.2xCD3) bispecific control, v37663. Maximum levels of IL-2 production induced by the constructs v37642 and v37638 reached 3633.0 pg/mL and 3321.3 pg/mL, respectively, which was approximately 6-fold higher than the IL-2 production induced by the control v37663. The constructs v37640 and v37641 exhibited similar potencies, with an approximately 5-fold increase in potency when compared to v37663, and maximum IL-2 production reached 5743.4 pg/mL and 4982.6 pg/mL, respectively, which was approximately 10-fold greater than that induced by v37663. The multivalent and multispecific constructs that comprised one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-CLDN18.2 scFv domain exhibited a range of potencies in T cell-mediated TNFα production. The most potent of these constructs, v37642, showed an approximately 200-fold higher potency for TNFα production compared to the bivalent anti-(CLDN18.2xCD3) bispecific control, v37663. Maximum levels of TNFα production induced by the construct v37642 reached 422.1 pg/mL, which was approximately 2-fold higher than TNFα production induced by the control v37663. The constructs

v37640 and v37641 exhibited similar potencies, with an approximate 5-fold increase in potency compared to v37663, and maximum TNFα production reached 574.9 pg/mL and 577.3 pg/mL, respectively, which was approximately 3-fold greater than that induced by the control v37663. The construct v37638 exhibited an approximate 2-fold increase in potency compared to v37663, with maximum TNFα production reaching 385.3 pg/mL, approximately 2-fold greater than that induced by the control v37663. The construct v37639 showed an approximate 3-fold reduction in potency compared to v37663, with maximum TNFα production reaching 426.8 pg/mL, approximately 2.5-fold greater than that induced by the control v37663. Negative control, v22277, did not induce any measurable production of IL-2 or TNFα.

**[0755]** These results demonstrate that induction of cytokine production by immune cells (e.g., T cells) can be modulated through modification of the format and/or geometry of an antibody construct, with various formats exhibiting a range in potency and maximum cytokine production of IL-2 and TNFα.

## EXAMPLE 21: IMPACT OF ANTI-CD28 PARATOPE USED IN TRISPECIFIC CONSTRUCTS ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH CLDN18.2-POSITIVE TUMOR CELLS

**[0756]** To assess the impact of the presence of both anti-CD3 as well as the anti-CD28 paratopes in the multivalent and multispecific antibody constructs described herein as well as that of their formats on their ability to activate T cells, IFNγ, TNFα, and IL-2 production was measured as described below. These experiments compared the trispecific constructs of this disclosure to the bispecific control constructs, v37663 and v35923. In addition, bispecific control constructs in which the anti-CD28 Fab domain was replaced with an anti-RSV Fab domain were also included.

**[0757]** Test articles (e.g., antibody constructs, controls, etc.) 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). Human CD3+ T cells were thawed and mixed with SNU 601 (high CLDN18.2 density) cells at an E:T ratio of 2:1 then added to the plates. Plates were incubated for 72 hrs 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.

**[0758]** IFNγ, 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 IFNγ, 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 IFNγ, TNFα and IL-2 concentrations in pg/mL. Data are representative of 1 independent experiments with 1 donors.

**[0759]** Potency and maximum cytokine production of IFNγ, TNFα and IL-2 by human CD3+ T cells in the presence of anti-(CLDN18.2xCD3xCD28) trispecific constructs and anti-CLDN18.2 bispecific controls are summarized in **TABLE 38**.

**TABLE 38: Maximal Cytokine Production Induced by Anti-CLDN18.2 Trispecific Constructs from Primary Human T Cells C o-cultured with SNU 601 Target Cells**

| Construct # | Maximum IFNγ (pg/mL) | Maximum IL-2 (pg/mL) | Maximum TNFα (pg/mL) |
|---|---|---|---|
| v37633 | 4970.5 | 6918.9 | 653.6 |
| v37634 | 2962.7 | 1607.1 | 275.2 |
| v37635 | 4307.2 | 4129.8 | 513.5 |
| v37638 | 4070.5 | 2239.1 | 446.7 |
| v37640 | 4192.4 | 7497.7 | 780.7 |
| v37642 | 3160.2 | 3070.0 | 426.3 |
| v37658 | 716.4 | 34.0 | 40.2 |
| v37660 | 789.1 | 46.4 | 55.4 |
| v37662 | 249.9 | 13.7 | 17.6 |
| v37663 | 1958.8 | 199.9 | 120.6 |
| v37665 | 0.4 | 0.5 | 0.6 |
| v35923 | 2839.6 | 492.6 | 204.2 |

**[0760]** Human CD3+ T cell IFNγ, TNFα and IL-2 release after incubation in the presence of anti-(CLDN18.2xCD3xCD28) trispecific antibody constructs with different formats and anti-

(CLDN18.2xCD3) bispecific controls are summarized in **FIGS. 17A-17B** (top left: IFNγ, top right: IL-2, bottom: TNFα).

**[0761]** IFNγ production induced by all tested trispecific constructs was similar to that induced by the benchmark bivalent anti-(CLDN18.2xCD3) bispecific construct, v35923, and was approximately 2-fold higher than that induced by v37663. IFNγ production induced by the trispecific constructs that comprised one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-CLDN18.2 scFv domain, v37638, v37640, and v37642, was approximately 5- to 10-fold greater than that induced by the trivalent, format-matched bispecific constructs, v37658, v37660, v37662, in which the anti-CD28 Fab domain was replaced with an anti-RSV Fab domain. Similar trends were observed for TNFα production of the tested multivalent and multispecific constructs.

**[0762]** The IL-2 production that was induced by the tested multivalent and multispecific antibody constructs was significantly higher than that produced by the corresponding bivalent anti-(CLDN18.2xCD3) constructs or the trivalent format-matched anti-(CLDN18.2xCD3xRSV) constructs. The tested trispecific constructs induced an approximately 10- to 40-fold increase in IL-2 production compared to the bivalent anti-(CLDN18.2xCD3) bispecific construct, v37663, and approximately 5- to 15-fold increase compared to the benchmark bivalent anti-(CLDN18.2xCD3) bispecific construct, v35923. IL-2 production induced by the trispecific construct that comprised one anti-CD3 scFv domain, one anti-CD28 Fab domain, and one anti-CLDN18.2 scFv domain, v37638, v37640, and v37642, was approximately 5- to 10-fold greater than that induced by the trivalent, format-matched bispecific constructs, v37658, v37660, v37662, in which the anti-CD28 Fab domain was replaced with an anti-RSV Fab domain.

These results demonstrate that induction of cytokine production can be significantly enhanced for T cell engaging therapeutics by using the multivalent and multispecific constructs of the present disclosure that can bind two antigens, e.g., CD3 and CD28, on T cells.

## EXAMPLE 22: IMPACT OF ANTI-CD28 PARATOPE AFFINITY ON CONSTRUCTS' ABILITY TO INDUCE PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH CLDN18.2+ TARGET CELLS

**[0763]** To assess the effect of reducing the affinity of the trispecific antibody construct to CD28 on its ability to induce cytokine production, selected constructs, including v37638, were assessed for IL-2 and TNFα production by primary human CD3+ T cells in co-culture with CLDN18.2+ SNU 601 target cells. Four additional molecules with the same format as v37638 but with reduced

affinity to CD28, v37683, v37689, v37692, v37694 were included, as was an anti-(CLDN18.2xCD3) bispecific control, v37663.

**[0764]** Test articles (e.g., antibody constructs, controls, etc.) 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). Human CD3+ T cells were thawed and mixed with SNU 601 (high CLDN18.2 density) cells at an E:T ratio of 2:1 then added to the plates. Plates were incubated for 72 hrs 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.

**[0765]** 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 antibody constructs 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.

**[0766]** Potency and maximum cytokine production of TNFα and IL-2 by human CD3+ T cells in the presence of Cldn-expressing SNU601 cells and anti-(CLDN18.2xCD3xCD28) trispecific constructs or anti-CLDN18.2 bispecific controls are summarized in **TABLE 39**, as are maximum cytokine production values for experiments in which the trispecific constructs were incubated with isolated T cells only (i.e., no TAA-expressing tumor cells present). Concentration response curves for some of the tested constructs in co-cultures of T cells and SNU601 cells are illustrated in **FIGS. 18A-18B**.

**TABLE 39: Potency and Maximal Cytokine Production Induced by Anti-CLDN18.2 Trispecific Constructs with Reduced Affinity to CD28 from Primary Human T Cells Co-cultured with SNU 601 Target Cells**

| Construct # | With SNU 601 Cells | | T cells Alone | |
|---|---|---|---|---|
| | IL-2 | TNFα | IL-2 | TNFα |
| | Maximum cytokine (pg/mL) | Maximum cytokine (pg/mL) | Maximum cytokine (pg/mL) | Maximum cytokine (pg/mL) |
| v37638 | 3706.7 | 669.5 | 1753.9 | 115.4 |
| v37683 | 4028.1 | 513.0 | 82.9 | 25.0 |
| v37684 | 3431.1 | 515.9 | 292.6 | 56.2 |
| v37685 | 3397.3 | 408.6 | 190.0 | 25.0 |
| v37686 | 4182.7 | 612.7 | 388.1 | 41.0 |
| v37687 | 4139.2 | 411.6 | 170.5 | 21.7 |
| v37688 | 3779.5 | 513.7 | 279.3 | 39.6 |
| v37689 | 3212.5 | 543.0 | 676.7 | 58.9 |
| v37690 | 2703.8 | 518.3 | 243.2 | 23.0 |
| v37691 | 3358.0 | 473.2 | 184.7 | 33.56 |
| v37692 | 2891.1 | 476.3 | 119.6 | 20.2 |
| v37693 | 3600.3 | 452.8 | 104.0 | 19.7 |
| v37694 | 226.4 | 166.0 | 29.9 | 12.9 |
| v37695 | 3473.7 | 403.5 | 28.9 | 13.0 |
| v37663 | 584.1 | 42.7 | 1.9 | 3.8 |

**[0767]** Maximum IL-2 and TNFα productions from T cells in co-culture with CLDN18.2+ SNU 601 cells induced by all constructs tested with affinity-reducing mutations in their anti-CD28 Fab domain, with the exception of construct v37694, were similar to that induced by the construct with the wild type anti-CD28 Fab, v37638. Maximum IL-2 and TNFα production induced by these trispecific constructs was on average 6-fold and 12-fold higher, respectively, than that induced by the corresponding anti-(CLDN18.2xCD3) bispecific, v37663. The construct v37694 did not induce greater maximum IL-2 production by T cells in co-culture with SNU-601 than that induced by control v37663. Furthermore, the maximum production of TNFα induced by v37694 was approximately 4-fold greater than that induced by v37663.

**[0768]** Target-independent (i.e., isolated T cells only in culture, no co-culture with TAA-expressing tumor cells) maximum IL-2 and TNFα productions from stimulated T cells induced by all tested constructs with affinity-reducing mutations in their anti-CD28 Fab domain was reduced compared to that induced by the construct with the wild type anti-CD28 Fab, v37638.

**[0769]** The construct v37695, which exhibited similar binding to CD28 as v37638, as measured by SPR, showed surprisingly an approximate 60-fold and 10-fold reduction in maximum non-

specific IL-2 and TNFα production, respectively, compared to that induced by v37638, while exhibiting an approximate 15-fold and 3-fold increase in IL-2 and TNFα production, respectively, compared to that induced of v37663. The construct v37694, which did not show binding to CD28 by SPR, induced similar levels of non-specific IL-2 and TNFα production as v37695. Negative control, v22277, did not induce IL-2 or TNFα production in T cells. These data suggest that certain mutations in the anti-CD28 paratope region unexpectedly led to a reduction in non-specific activity while still providing for potent cytokine production.

**[0770]** Taken together, these results show that induction of nonspecific cytokine production can be modulated (e.g., reduced) by introducing specific amino acid substitutions in the anti-CD28 Fab domain, without significantly impacting the trispecific constructs' ability to induce cytokine production by T cells in co-culture with CLDN18.2+ SNU 601 cells. Furthermore, these results show that the multivalent and multispecific antibody constructs of the present disclosure can provide a strict tumor cell dependent cytotoxicity profile, with no significant activation of isolated T cells (i.e., no presence of tumor cells).

## EXAMPLE 23: IMPACT OF CD3 & CD28 CO-ENGAGEMENT WITH A TRISPECIFIC ANTIBODY COMPARED TO COMBINATION THERAPY USING ANTI-(CD3xCLDN18.2) & ANTI-(CD28x CLDN18.2) BISPECIFIC ANTIBODIES

**[0771]** To interrogate the difference in cytotoxicity and induction of proinflammatory cytokine release induced by the trispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, in comparison to a combination of the bispecific anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific antibody constructs, v37663 and v37665 (i.e., v37663 + v37665 vs. v37634 alone), respectively, selected constructs were tested in primary human T cell cultures with allogeneic tumor cell lines expressing CLDN18.2. Trispecific control constructs containing a paratope against the irrelevant antigen, RSV protein F (RSV-F), in place of the CD3, CD28, or CLDN18.2 paratopes, v37654, v39215, and v37644, respectively, were included as format controls for each binding arm of the tested trispecific antibody construct, v37634. A monoclonal anti-RSV protein F construct, v22277, was included as a negative control.

**[0772]** Frozen human CD3+ T cells were thawed at 37 °C and mixed with CLDN18.2-expressing SNU 601-and KATO III-RFP cells such that the ratio of T cells to allogeneic tumor cells was adjusted to effector-to-target (E:T) ratios of 2:1 and 1:5. The mixtures were incubated together with the constructs for 72 and 168 hours, respectively, after which tumor cell viability was assessed

through live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020).

**[0773]** Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**[0774]** Supernatant was collected from the 2:1 E:T experiment at the 72-hour timepoint and analyzed for IFN$\gamma$, TNF$\alpha$, IL-2, and IL-6 by MSD (Mesoscale Discovery) using 4-plex U-plex MSD kits. The night before cytokine quantification, MSD plates were blocked and coated in capture constructs 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 IFN$\gamma$, TNF$\alpha$, IL-2, and IL-6 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 constructs 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 IFN$\gamma$, TNF$\alpha$, IL-6, and IL-2 concentrations in pg/mL.

**TABLE 40: Potency and Maximum Mean Cytotoxicity (MMC) Induced by Anti-CLDN18.2 Multivalent and multispecific Antibody Constructs, Bispecific Constructs alone, or a Combination of Anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) Bispecific Constructs, from Primary Human T cells co-cultured with SNU 601-RFP Target Cells**

| Construct # | 2:1 E:T | | 1:5 E:T | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | MMC (%) | $IC_{50}$ (pM) | MMC (%) |
| v37634 | 1.01 | 98.3 | 14.94 | 99.7 |
| v39215 | 3.95 | 97.9 | 116.9 | 97.2 |
| v37644 | ND | 19.1 | ND | 14.3 |
| v37654 | ND | 11.4 | ND | 6.8 |
| v37663 | 2.47 | 98.6 | 33.17 | 98.6 |
| v37663+v37665 | 13.89 | 99.2 | 19.44 | 99.8 |
| v37665 | ND | 18.2 | ND | 4.2 |
| v22277 | ND | 20.4 | ND | 5.4 |

**TABLE 41: Potency and Maximum Mean Cytotoxicity (MMC) Induced by Anti-CLDN18.2 Multivalent and multispecific Antibody Constructs or a Combination of Anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) Bispecific Constructs from Primary Human T cells co-cultured with KATO III-RFP Target Cells**

| Construct # | 2:1 E:T | | 1:5 E:T | |
|---|---|---|---|---|
| | $IC_{50}$ (pM) | MMC (%) | $IC_{50}$ (pM) | MMC (%) |
| v37634 | 4.112 | 89.9 | 41.17 | 89.7 |
| v39215 | 50.65 | 84.7 | 1330 | 67.4 |
| v37644 | ND | 14.5 | ND | 9.5 |
| v37654 | ND | 26.7 | ND | 8.9 |
| v37663 | 21.38 | 81.1 | 722.3 | 59.5 |
| v37663+v37665 | 19.97 | 87.0 | 36.94 | 88.5 |
| v37665 | ND | 9.1 | ND | -0.4 |
| v22277 | ND | 16.0 | ND | 23.3 |

**[0775]** Cytotoxic potency and maximum cytotoxicity values from T cells in co-culture with CLDN18.2-expressing SNU 601- and KATO III-RFP cells following treatment with a trivalent anti-(CLDN18.2xCD3xCD28) trispecific construct, combination therapy of anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific constructs, and bispecific and negative control constructs are shown in **TABLE 40**, and from T cells co-cultured with KATO III-RFP cells are shown in **TABLE 41**. Potency and maximum cytokine concentrations (as well as standard deviation, "SD") of IFN$\gamma$, IL-2, TNF$\alpha$, and IL-6 production by primary human T cells in co-culture with CLDN18.2-expressing SNU 601 cells following treatment with trivalent anti-(CLDN18.2xCD3xCD28) trispecific construct, combination therapy of anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific constructs, and bispecific and negative control constructs are shown in **TABLE 42**. Data from T cells in co-culture with SNU 601-RFP cells are representative of two independent donors from three independent experiments.

**[0776]** The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) trispecific construct, v37634, exhibited the highest cytotoxic potency in high E:T T cell co-cultures with SNU 601- and KATO III-RFP cells, with $IC_{50}$ values of 1.01 and 4.112 pM, respectively, and reaching a maximum cytotoxicity of 98.3 and 89.9%, respectively. The bispecific anti-(CLDN18.2xCD3) and trispecific anti-(CLDN18.2xCD3xRSV-F) constructs, v37663 and v39215, respectively, induced approximately 2.5- to 4-fold reduced potency in high E:T T cell co-cultures with SNU 601-RFP cells compared to v37634, with $IC_{50}$ values of 2.47 and 3.95 pM, respectively, and reaching a maximum cytotoxicity of 98.6% and 97.9%, respectively. In high E:T KATO III-RFP co-cultures,

v37663 and v39215 induced approximately 5- to 10-fold reduced potency compared to v37634, with $IC_{50}$ values of 21.38 and 50.65 pM, respectively and reaching 81.1% and 84.7% maximum cytotoxicity. Combination of anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific constructs, v37663+v37665, induced reduced cytotoxic potency in high E:T co-cultures with SNU 601- or KATO III-RFP cell lines compared to v37634, with $IC_{50}$ values of 13.89 and 19.97 pM, respectively, and reaching maximum cytotoxicity of 99.2% and 87.0%, respectively.

**[0777]** The construct v37634 alone and the combination v37663+v37665 induced similar cytotoxic potency in low E:T T cell co-cultures with SNU 601- and KATO III-RFP cells, with $IC_{50}$ values of 14.94 and 19.44 pM, respectively, in SNU 601-RFP co-cultures, and 41.17 and 36.94 pM, respectively, in KATO III-RFP co-cultures. Maximum cytotoxicity induced by v37634 and v37663+v37665 in SNU 601-RFP co-cultures reached 99.7% and 99.8%, respectively, and in KATO III-RFP co-cultures, reached 89.7% and 88.5%, respectively. The bispecific anti-(CLDN18.2xCD3) and trispecific format control anti-(CLDN18.2xCD3xRSV-F) constructs, v37663 and v39215, induced reduced potency in low E:T T cell co-cultures with SNU 601-RFP cells compared to v37634 and v37663+v37665, with $IC_{50}$ values of 33.17 and 116.9 pM, respectively, and reaching a maximum cytotoxicity of 98.6% and 97.2%, respectively. In low E:T KATO III-RFP co-cultures, v37663 and v39215 induced reduced potency compared to v37634, with $IC_{50}$ values of 722.3 and 1330 pM, respectively and reaching 59.5% and 67.4% maximum cytotoxicity.

**[0778]** Control constructs anti-(CLDN18.2xRSV-FxCD28), v37654, and anti-(RSV-FxCD3xCD28), v37644, and bispecific anti-(CLDN18.2xCD28), v37665, as well as the negative control construct, v22277, did not induce cytotoxicity against SNU 601-RFP or KATO III-RFP cell lines at either high or low E:T co-cultures.

**TABLE 42: Maximum IFN$\gamma$, IL-2, TNF$\alpha$, and IL-6 Production Induced by Anti-CLDN18.2 Multivalent and multispecific Antibody Constructs, Bispecific Constructs alone, or a Combination of Anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) Bispecific Constructs, from Primary Human T cells co-cultured with SNU 601-RFP Target Cells**

| Construct # | Maximum Mean Cytokine Concentration (pg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IFN$\gamma$ | SD | IL-2 | SD | TNF$\alpha$ | SD | IL-6 | SD |
| v37634 | 33195.9 | 3441.1 | 3642.3 | 411.1 | 367.8 | 92.3 | 16.1 | 4.6 |
| v39215 | 14430.4 | 2136.3 | 113.5 | 20.2 | 87.1 | 13.0 | 10.6 | 0.8 |
| v37644 | 58.6 | 18.7 | 12.2 | 5.8 | 2.6 | 1.0 | 1.6 | 0.7 |
| v37654 | 5.3 | 1.3 | 1.1 | 0.4 | 1.2 | 0.7 | 3.9 | 5.6 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| v37663 | 13624.5 | 1932.7 | 108.0 | 8.9 | 108.0 | 14.4 | 9.3 | 3.2 |
| v37663+ v37665 | 65422.1 | 10791.5 | 9116.4 | 1095.8 | 1037.3 | 253.7 | 19.7 | 7.5 |
| v37665 | 4.1 | 0.2 | 3.2 | 2.3 | 1.5 | 0.3 | 2.4 | 3.3 |
| v22277 | 8.6 | 7.4 | 1.8 | 1.1 | 1.2 | 0.7 | 2.0 | 0.8 |

**[0779]** The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, induced IFN$\gamma$ production that reached a maximum of 33195.9 pg/mL. IFN$\gamma$ production induced by v39215 and v37663 was approximately 2-fold reduced compared to v37634, reaching a maximum of 14430.4 and 13624.5 pg/mL, respectively. The combination of bispecific anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific constructs, v37663+v37665, induced the highest level of IFN$\gamma$ production, reaching a maximum of 65422.1 pg/mL, approximately 2-fold higher than that induced by v37634. v37634 induced IL-2 production that reached a maximum of 3642.3 pg/mL. IL-2 production induced by v39215 and v37663 was approximately 30-fold reduced compared to v37634, reaching a maximum of 113.5 and 108.0 pg/mL, respectively. The combination, v37663+v37665, induced the highest level of IL-2 production, reaching a maximum of 9116.4 pg/mL, approximately 2.5-fold higher than that induced by v37634. v37634 induced TNF$\alpha$ production that reached a maximum of 367.8 pg/mL. TNF$\alpha$ production induced by v39215 and v37663 was approximately 3.5- to 4-fold reduced compared to v37634, reaching a maximum of 87.1 and 108.0 pg/mL, respectively. The combination, v37663+v37665, induced the highest level of TNF$\alpha$ production, reaching a maximum of 1037.3 pg/mL, approximately 3-fold higher than that induced by v37634. v37634 and the combination, v37663+v37665, induced similar levels of IL-6 production that reached a maximum of 16.1 and 19.7 pg/mL, respectively. IL-6 production induced by v39215 and v37663 was approximately 1.5- to 2-fold reduced compared to v37634 and v37663+v37665, reaching a maximum of 10.6 and 9.3 pg/mL, respectively.

**[0780]** Control anti-(CLDN18.2xRSV-FxCD28) construct, v37654, and anti-(RSV-FxCD3xCD28) construct, v37644, the bispecific anti-(CLDN18.2xCD28) construct, v37665, as well as the negative control construct, v22277, did not induce a measurable production of IFN$\gamma$, IL-2, TNF$\alpha$, or IL-6 following co-culture of human Pan T cells with SNU 601-RFP cell lines.

**[0781]** These results show that the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, can induce non-inferior or similar cytotoxicity of target tumor cells but with a reduced induction of cytokine production by T cells when compared to a combination treatment of anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific constructs.

**EXAMPLE 24: IMPACT OF ANTI-CD28 PARATOPE AFFINITY ON CONSTRUCTS' ABILITY TO INDUCE PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN CD3+ T CELLS IN CO-CULTURE WITH CLDN18.2+ TARGET CELLS**

**[0782]** To assess the effect of reducing the affinity of the trispecific antibody construct to CD28 on its ability to induce cytokine production, selected constructs, including v37642, were assessed for IL-2 and IFNγ production by primary human CD3+ T cells in co-culture with CLDN18.2+ SNU 601 target cells. Four additional molecules with the same format and geometry as v37642, but with reduced affinity to CD28, v38990, v38991, v38992, and v38993, were included, as was a trivalent anti-(CLDN18.2xCD3xRSV-F) control construct, v37662, a CLDN18.2xCD3 bispecific control construct, v35923, and a negative control construct, v22277.

**[0783]** Test articles (e.g., antibody constructs, controls, etc.) were titrated in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) and added to appropriate wells of the 384-well black flat bottom assay plates (ThermoFisher, Watham, MA). Human CD3+ T cells were thawed and resuspended as monocultures of CD3+ T cells or mixed with SNU 601 (high CLDN18.2 density) cells at an E:T ratio of 2:1 then added to the plates. Plates were incubated for 72 hrs 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.

**[0784]** IFNγ and IL-2 were quantified using MSD (Mesoscale Discovery, Piscataway, NJ). The night before cytokine quantification, MSD plates were blocked and coated in capture constructs 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 and IL-2 standard (calibrator 1) and IFNγ standard (R&D Biosystems) 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 antibody constructs 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 IFNγ and IL-2 concentrations in pg/mL.

**[0785]** Production of IFNγ and IL-2 by human CD3+ T cells in the presence of 45 nM of anti-(CLDN18.2xCD3xCD28) trispecific constructs and anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific controls are summarized in **TABLE 43**.

**TABLE 43: Mean Cytokine Production Induced by Anti-CLDN18.2 Multivalent and multispecific Antibody Constructs with Reduced Affinity to CD28 from Primary Human T Cells Co-cultured with SNU 601 Target Cells**

| Construct # | T cells Alone | | | |
|---|---|---|---|---|
| | IL-2 | | IFNγ | |
| | Mean Cytokine (pg/mL) | Standard Deviation | Mean Cytokine (pg/mL) | Standard Deviation |
| v37642 | 2933.9 | 1010.9 | 857.4 | 493.0 |
| v37662 | 0.1 | 0.1 | 1.7 | 0.6 |
| v38990 | 1.8 | 2.2 | 0.9 | 0.1 |
| v38991 | 26.5 | 19.2 | 15.4 | 5.6 |
| v38992 | 1690.2 | 63.7 | 559.3 | 114.0 |
| v38993 | 1524.2 | 163.6 | 474.6 | 145.3 |
| v35923 | 1.3 | 0.2 | 1.0 | 0.5 |
| v22277 | 0.5 | 0.1 | 0.3 | 0.3 |

**TABLE 44: Potency of Cytokine Production Induced by Anti-CLDN18.2 Multivalent and multispecific Antibody Constructs with Reduced Affinity to CD28 from Primary Human T Cells Co-cultured with SNU 601 Target Cells**

| Construct # | T cells + SNU 601 | | | |
|---|---|---|---|---|
| | IL-2 | | IFNγ | |
| | $EC_{50}$ (pM) | Maximum Cytokine (pg/mL) | $EC_{50}$ (pM) | Maximum Cytokine (pg/mL) |
| v37642 | 2.666 | 5065 | 3.669 | 3041 |
| v37662 | ND | 49.04 | 12.37 | 391.7 |
| v38990 | 1559 | 5244 | 603.7 | 3291 |
| v38991 | 22.24 | 9615 | 90.92 | 6146 |
| v38992 | 9.157 | 5556 | 23.02 | 3992 |
| v38993 | 28.69 | 8414 | 79.32 | 4449 |
| v35923 | 76.33 | 5643 | 26 | 1999 |
| v22277 | ND | 52.79 | ND | 51.57 |

**[0786]** Maximum IL-2 and IFNγ production from monocultures of T cells induced by the anti-(CLDN18.2xCD3xCD28) construct with the wild type anti-CD28 Fab, v37642, was 2933.9 and 857.4 pg/mL, respectively. IL-2 and IFNγ production induced by v38992 and 38993 – with 3.1-fold and 3.4-fold reduction in CD28 affinity by SPR, respectively – induced approximately 1.5-

fold to 2-fold reduced production if IL-2 and IFNγ compared to v37642, with maximum IL-2 production of 1690.2 and 1524.2 pg/mL, respectively, and maximum IFNγ production of 559.3 and 474.6 pg/mL, respectively. The trispecific construct, v38991 – with 6.1-fold reduction in CD28 affinity by SPR – exhibited an approximate 100-fold reduction in IL-2 production, with maximum of 26.5 pg/mL induced, and an approximately 55-fold reduction in IFNγ production, with a maximum of 15.4 pg/mL induced. The trispecific construct, v38990, with a 225-fold reduction in CD28 by SPR, did not induce IL-2 or IFNγ production, relative to the negative control construct, v22277.

**[0787]** Maximum IL-2 and IFNγ produced by T cells in co-culture with SNU 601 cells induced by the trispecific constructs with affinity attenuated CD28, v38990, 38991, 38992, and 38993, remained within two-fold of that induced by the trispecific construct with a wild type anti-CD28 domain, v37642. Trispecific construct, v37642, exhibited the highest potency of IL-2 and IFNγ production by T cells co-cultured with SNU 601 target cells, with $EC_{50}$ value of 2.66 pM and 3.689 pM, respectively. The constructs v38992, 38993, and v38991 – with 3.1-, and 3.4-, and 6.1-fold reduction in CD28 affinity by SPR, respectively – exhibited between 3-fold to 10-fold fold reduction in potency of IL-2 production compared to v37642, with $EC_{50}$ values of 9.175, 28.69, and 22.24 pM, respectively, and a 6-fold to 25-fold reduction in potency of IFNγ production, with $EC_{50}$ values of 23.02, 79.32, and 90.92 pM, respectively. The construct v38990 – with 225-fold reduction in CD28 affinity by SPR – exhibited approximately a 586-fold reduction in potency of IL-2 production compared to v37642, with an $EC_{50}$ value of 1559 pM, and 165-fold reduction in potency of IFNγ production, with an $EC_{50}$ value of 603.7 pM.

**[0788]** Taken together, these results show that induction of nonspecific cytokine production can be modulated (e.g., reduced) by introducing specific amino acid substitutions in the anti-CD28 Fab domain, without significantly impacting the trispecific constructs' ability to induce cytokine production by T cells in co-culture with CLDN18.2+ SNU 601 cells. Furthermore, these results show that the multivalent and multispecific antibody constructs of the present disclosure can provide a strict tumor cell dependent cytotoxicity profile, with no significant activation of isolated T cells (i.e., no presence of tumor cells).

**EXAMPLE 25: IMPACT OF FORMAT AND GEOMETRY OF IMMOBILIZED MULTIVALENT AND MULTISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO PREDICTIVE ASSAY FOR CYTOKINE RELEASE SYNDROME**

**[0789]** To interrogate the impact of the format and geometry of certain multivalent and multispecific antibody constructs on the induction of proinflammatory cytokine release in in vitro predictive assays of cytokine release syndrome, selected multivalent and multispecific anti-(CLDN18.2xCD3xCD28) 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 (catalogue: MAB9929, R&D Systems) and anti-CD28, v36577, monoclonal antibodies, 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-TNF$\alpha$ 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 (v29982) backbones were included as negative isotype control constructs.

**[0790]** 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 IFN$\gamma$, IL-2, and IL-6 by MSD (Mesoscale Discovery).

**[0791]** 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 IFN$\gamma$, IL-2, and IL-6 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 IFN$\gamma$, IL-6, and IL-2 concentrations in pg/mL. Data are pooled from 2-5 independent experiments with 2-4 individual donors.

**[0792]** Production of IL-2, IFN$\gamma$ and IL-6 by human PBMCs in the presence of the tested anti-(CLDN18.2xCD3xCD28) multivalent and multispecific antibody constructs and controls are summarized in **TABLE 45**.

**TABLE 45: Mean Cytokine Production (and Standard Error of the Mean = SEM) Induced by Immobilized Anti-(CLDN18.2xCD3xCD28) Multivalent and multispecific Antibody Constructs from Monocultures of Primary PBMCs**

| Construct/Catalogue # | PBMCs | | | | | |
|---|---|---|---|---|---|---|
| | IL-2 | | IFN$\gamma$ | | IL-6 | |
| | Mean Cytokine (pg/mL) | SEM | Mean Cytokine (pg/mL) | SEM | Mean Cytokine (pg/mL) | SEM |
| v37634 | 86.6 | 14.9 | 3.39E+4 | 1.03E+4 | 245.0 | 58.4 |
| v39215 | 43.8 | 12.0 | 1.55E+4 | 3.92E+3 | 124.3 | 25.4 |
| v37654 | 7.1 | 1.2 | 380.4 | 147.4 | 14.6 | 4.8 |
| v37638 | 3.88E+4 | 2.83E+3 | 7.83E+5 | 3.45E+5 | 1302.1 | 819.5 |
| v39231 | 4.9 | 4.9 | 0.0 | 0.0 | 6.2 | 0.9 |
| v37658 | 75.5 | 9.8 | 1.95E+4 | 5.51E+3 | 212.7 | 66.3 |
| v37692 | 1.67E+4 | 4.01E+3 | 2.80E+5 | 4.71E+4 | 1107.0 | 246.8 |
| v39232 | 38.1 | 35.6 | 232.7 | 232.7 | 0.9 | 0.9 |
| v37642 | 5.25E+4 | 1.84E+4 | 5.51E+5 | 7.71E+4 | 941.5 | 454.0 |
| v37662 | 45.6 | 21.5 | 1.60E+4 | 8.39E+2 | 184.4 | 1.0 |
| v37665 | 21.2 | 14.3 | 625.7 | 625.0 | 156.9 | 139.8 |
| v37663 | 51.0 | 17.8 | 6.71E+4 | 4.28E+4 | 256.1 | 40.3 |
| v35923 | 41.6 | 12.8 | 1.59E+4 | 8.52E+3 | 121.8 | 25.0 |
| v36992 | 20.8 | 16.8 | 120.1 | 63.8 | 64.5 | 26.9 |
| v39982 | 14.7 | 6.3 | 175.9 | 142.9 | 231.0 | 217.2 |
| v36577 | 7.44E+3 | 1.49E+3 | 4.28E+4 | 7.80E+3 | 349.1 | 51.0 |
| A2019 | 10.6 | 3.5 | 0.5 | 0.5 | 207.9 | 170.0 |
| MAB9929 | 32.8 | 13.2 | 1.01E+4 | 7.79E+3 | 879.9 | 796.7 |
| PHA | 423.4 | 174.9 | 1.73E+5 | 4.69E+4 | 2.44E+4 | 8.73E+3 |

**[0793]** The trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs, v37638, v37692, and v37642, induced higher levels of IL-2, IFN$\gamma$, and IL-6 than the positive control anti-CD28 construct, v36577. The trispecific anti-(CLDN18.2xCD3xCD28) construct, v37634, induced markedly lower IL-2 production compared to v36577, and an approximate 2- to 3-fold reduction

in IL-6 production, relative to that induced by v36577 and MAB9929. IL-6 production by v37634 was similar to that induced by the negative CRS control, A2019.

Taken together, these data show that induction of cytokine in in vitro predictive assays of cytokine release syndrome can be modified by modulating the geometry and/or format of trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs.

**EXAMPLE 26A: IMPACT OF THE FORMAT AND GEOMETRY OF TRISPECIFIC ANTI-(CLDN18.2xCD3xCD28) CONSTRUCTS ON TARGET-INDEPENDENT CYTOKINE PRODUCTION BY PRIMARY HUMAN IMMUNE CELLS**

**[0794]** To assess the impact of the format and geometry of certain multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs on target-independent activation of primary human immune cells, selected constructs were assessed for their ability to stimulate cytokine production following incubation with human PBMCs and CD3+ T cells.

**[0795]** Test articles (e.g., antibody constructs, controls, etc.) were diluted to 45 nM, titrated 1:9 in RPMI1640 (Gibco) + 10% FBS (ThermoFisher), and added to appropriate wells of the 384-well black flat bottom assay plates (ThermoFisher, Watham, MA). Human PBMCs or CD3+ T cells were thawed and resuspended as monocultures of cells and added to the plates. Plates were incubated for 72 hrs 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.

**[0796]** IFNγ, 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 and IL-2 standard (calibrator 1) and IFNγ standard (R&D Biosystems) 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 antibody constructs 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 IFNγ, TNFα, and IL-2 concentrations in pg/mL.

**[0797]** Maximum cytokine production of IFNγ, TNFα and IL-2 by human CD3+ T cells in the presence of anti-(CLDN18.2xCD3xCD28) trispecific constructs and anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific control constructs are summarized in **TABLE 46**. Maximum cytokine production of IFNγ, TNFα and IL-2 by human PBMCs in the presence of anti-(CLDN18.2xCD3xCD28) trispecific constructs and anti-(CLDN18.2xCD3) and anti-(CLDN18.2xCD28) bispecific control constructs are summarized in **TABLE 47A**.

**TABLE 46: Mean Maximum Cytokine Production Induced by Anti-CLDN18.2 Trispecific Constructs from Monocultures of Primary Human CD3+ T Cells**

| Construct # | CD3+ T Cells | | | | | |
|---|---|---|---|---|---|---|
| | IL-2 | | IFNγ | | TNFα | |
| | Mean Cytokine (pg/mL) | Standard Deviation | Mean Cytokine (pg/mL) | Standard Deviation | Mean Cytokine (pg/mL) | Standard Deviation |
| v37633 | 2141.1 | 1514.3 | 430.0 | 316.7 | 149.5 | 100.4 |
| v37634 | 4.2 | 4.0 | 0.5 | 0.5 | 2.4 | 1.0 |
| v37635 | 1559.4 | 404.4 | 1079.0 | 392.6 | 340.3 | 98.4 |
| v37638 | 936.0 | 422.4 | 448.0 | 248.5 | 198.7 | 77.6 |
| v37640 | 2392.5 | 1214.7 | 808.8 | 500.6 | 280.9 | 122.4 |
| v37642 | 1458.2 | 935.9 | 1040.7 | 698.8 | 291.0 | 186.9 |
| v37663 | 0.8 | 0.2 | 9.9 | 9.0 | 0.7 | 0.4 |
| v37665 | 0.0 | 0.0 | 0.6 | 0.2 | 0.2 | 0.1 |
| v35923 | 0.2 | 0.3 | 0.2 | 0.6 | 0.2 | 0.2 |
| v22277 | 0.3 | 0.3 | 0.2 | 0.5 | 0.3 | 0.2 |

**TABLE 47A: Mean Maximum Cytokine Production Induced by Anti-CLDN18.2 Trispecific Constructs from Monocultures of Primary Human PBMCs**

| Construct # | PBMCs | | | | | |
|---|---|---|---|---|---|---|
| | IL-2 | | IFNγ | | TNFα | |
| | Mean Cytokine (pg/mL) | Standard Deviation | Mean Cytokine (pg/mL) | Standard Deviation | Mean Cytokine (pg/mL) | Standard Deviation |
| v37634 | 7.6 | 9.3 | 17.2 | 12.9 | 2.1 | 1.4 |
| v37638 | 116.6 | 96.0 | 823.0 | 713.6 | 23.4 | 20.8 |
| v37642 | 3545.3 | 369.3 | 20049.8 | 5800.2 | 866.8 | 30.9 |
| v37663 | 5.8 | 8.0 | 17.9 | 28.9 | 2.0 | 3.2 |
| v37665 | 2.5 | 0.9 | 3.5 | 2.5 | 0.2 | 0.2 |
| v35923 | 1.1 | 0.6 | 3.0 | 2.8 | 0.3 | 0.4 |
| v22277 | 1.8 | 0.9 | 2.0 | 1.7 | 0.4 | 0.4 |

**[0798]** The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs, v37633, v37635, v37638, v37640, and v37642, induced nonspecific cytokine production by monocultures of T cells, ranging from approximately 1000-2500 pg/mL of IL-2, 400-1100 pg/mL of IFN$\gamma$, and 150-350 pg/mL of TNF$\alpha$. Surprisingly, the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) construct, v37634, which has a different format than the other WT trispecific constructs v37638 and v37642, induced less than 10 pg/mL of IL-2, IFN$\gamma$, or TNF$\alpha$; similar values for measured for the bispecific anti-(CLDN18.2xCD3) control constructs, v37663 and 35923, the bispecific anti-(CLDN18.2xCD28) control construct, v37665, and the negative control, v22277.

**[0799]** The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) constructs, v37638 and v37642, induced nonspecific cytokine production by monocultures of PBMCs, ranging from approximately 100-3500 pg/mL of IL-2, 800-20000 pg/mL of IFN$\gamma$, and 23-900 pg/mL of TNF$\alpha$. The multivalent and multispecific anti-(CLDN18.2xCD3xCD28) construct, v37634, which has a different format than the other WT trispecific constructs v37638 and v37642, induced less than 20 pg/mL of IL-2, IFN$\gamma$, or TNF$\alpha$; similar values for measured for the bispecific anti-(CLDN18.2xCD3) control constructs, v37663 and 35923, the bispecific anti-(CLDN18.2xCD28) control construct, v37665, and the negative control, v22277.

**[0800]** Taken together, these results show that induction of nonspecific cytokine production can be modulated (e.g., reduced) through modification of format and/or geometry of an antibody construct.

## EXAMPLE 26B: IMPACT OF IMMOBILIZED TRISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO PREDICTIVE ASSAY FOR CYTOKINE RELEASE SYNDROME

**[0801]** Cytokine release syndrome (CRS) is a systemic inflammatory response that can be triggered by various T cell-engaging immunotherapeutic agents and represents one of the most frequent serious adverse effects of these therapies. To interrogate the impact of the format of an antibody construct on its ability to induce proinflammatory cytokine release in in vitro predictive assays of cytokine release syndrome, selected anti-(CLDN18.2xCD3xCD28) and control constructs were immobilized onto the surface of 96 well microtiter plates and tested in monocultures of primary human PBMCs. A benchmark trispecific antibody that includes a

CD3xCD28 CODV-Fab, v37035, was included for comparison (see, e.g., Steinmetz et al., MAbs. 2016 Jul; 8(5): 867–878.). Monoclonal anti-CD3 (catalogue: MAB9929, R&D Systems) and anti-CD28, v36577, antibodies, which have been shown to induce cytokine release syndrome in patients as well as two mitogens, PHA (catalogue: 00497793, Invitrogen) and SEB (catalogue: S4881, Sigma Aldrich), were included as positive controls. An anti-TNF$\alpha$ monoclonal antibody (catalogue: A2019, Universal Biologicals) was included as a negative control for cytokine release syndrome. An anti-RSV-F monoclonal antibody with IgG1 LALADS (v39982) backbones was included as a negative isotype control construct.

**[0802]** 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).

**[0803]** 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 IL-2 concentrations in pg/ml. Data are pooled from one independent experiment with 3 individual donors.

**[0804]** Production of IL-2 by human PBMCs in the presence of anti-(CLDN18.2xCD3xCD28) trispecific constructs and controls are summarized in **TABLE 47B**.

**TABLE 47B: Mean Cytokine Production Induced by Immobilized Trispecific Anti-(CLDN18.2xCD3xCD28) Antibody Constructs from Monocultures of Primary PBMCs**

| Construct/ Catalogue # | PBMCs | |
|---|---|---|
| | IL-2 | |
| | Mean Cytokine (pg/mL) | SEM |
| v36577 | 3247.2 | 61.0 |
| v37035 | 1942.8 | 130.4 |
| v37634 | 383.5 | 9.5 |
| v37663 | 138.4 | 9.7 |
| v39982 | 0.0 | 0.0 |
| v40062 | 265.6 | 11.7 |
| A2019 | 0.0 | 0.0 |
| SEB | 2072.5 | 64.6 |
| PHA | 696.6 | 37.8 |

**[0805]** The trispecific anti-(CLDN18.2xCD3xCD28) construct, v40062, induced approximately 10-fold lower IL-2 production by PBMCs than that induced by the bivalent anti-CD28 monoclonal antibody, v36577, and the benchmark anti-(CD3xCD28) trispecific construct, v37035.

**[0806]** Taken together, these data show that cytokine induction in in vitro predictive assays of cytokine release syndrome can be modified by modulating the format of a trispecific anti-(CLDN18.2xCD3xCD28) antibody construct, and that certain constructs of the present disclosure can possess more favorable cytokine release profiles when compared to conventional and/or current benchmark constructs.

## EXAMPLE 27A: IMPACT OF IMMOBILIZED ANTI-(DLL3XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO PREDICTIVE ASSAY FOR CYTOKINE RELEASE SYNDROME

**[0807]** 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: 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-TNF$\alpha$ 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 (v29982) backbones were included as negative isotype control constructs.

**[0808]** 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).

**[0809]** 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.

**[0810]** Production of IL-2 by human PBMCs in the presence of the tested trispecific antibody constructs and controls are summarized in **TABLE 48**.

**TABLE 48: 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 | |
| v29982 | 24.3 | 12.1 | |
| v36992 | 16.7 | 8.3 | |

**EXAMPLE 27B: IMPACT OF TRISPECIFIC CONSTRUCT STRUCTURE ON PROLIFERATION OF HUMAN CD3+ T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES**

**[0811]** To test the impact of antibody construct format on its 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 clinical benchmarks as described below.

**[0812]** 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 mL 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 mL 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 RT for 60 minutes. Following staining of cells, plates were washed three times with FACS Buffer. Following the final wash, cells were resuspended in 150 mL 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).

**[0813]** Curves showing the percent of cells proliferated with the respective titrated antibody constructs tested are shown in **FIGS. 18C-18D (FIG. 18C:** T cells in the presence of DLL3+ NCI-H82 cells; **FIG. 18D:** T cells alone).

**EXAMPLE 27C: 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**

**[0814]** 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.

**[0815]** 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 °C 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 °C and washed with FACS buffer. Then, cells were fixed and permeabilized for intracellular staining with 1x Fix/Perm concentrate (Invitrogen eBiosciences) diluted in fix/perm diluent (Invitrogen eBiosciences). Cells were incubated for 30 minutes away from light at room temperature (RT). After staining, cells were washed twice with 1X Perm buffer and stained with anti-Bcl-xL or isotype intracellular stains (CellSignaling) diluted in 1X perm buffer (Invitrogen eBiosciences) and incubated for 30 minutes at room temperature away from light. Finally, cells were washed twice with 1X perm buffer (Invitrogen eBiosciences), 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).

**[0816]** Upregulation of Bcl-XL expression in activated T-cells using certain trispecific antibody constructs of the present disclosure as well as bispecific and benchmark controls are shown in **FIG. 18E**.

**EXAMPLE 28: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING MULTIVALENT AND MULTISPECIFIC CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(CLDN18.2XCD3) BISEPCIFIC CONTROL CONSTRUCTS**

**[0817]** This example assessed the ability of certain multivalent and multispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SNU620 gastric cancer model and compares their performance against corresponding bispecific control constructs.

*SNU620 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity*

**[0818]** NCG female mice (Jiangsu GemPharmatech Co, Ltd.) are inoculated subcutaneously in the right front flank region with SNU620 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. The date of tumor inoculation is denoted as day 0.

**[0819]** Animals with a single tumor of a volume of 100-120 $mm^3$ 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 ($5x10^6$ cells) from donor A or donor B.

**[0820]** 24 hours after randomization, animals are treated intravenously as per the study design shown in **TABLE 49**. Test article administration and animal numbers for each study group are also shown in **TABLE 49**.

**[0821]** 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 will be 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 reached a maximal tumor volume of 2 000 $mm^3$ or when tumor exceeds 10% of normal body weight or when greater that 20% body weight loss, or after a maximum of 40 days post tumor injection.

**TABLE 49: *In Vivo* Study Groups and Dosing Regiment**

| Study Group | Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|---|
| 1A (6 mice-donor A) | v37634 | 0.2 mg/kg | Q7Dx4 |
| 1B (6 mice-donor B) | | | |
| 2A (6 mice-donor A) | v37634 | 1 mg/kg | Q7Dx4 |
| 2B (6 mice-donor B) | | | |
| 3A (6 mice-donor A) | v37634 | 5 mg/kg | Q7Dx4 |
| 3B (6 mice-donor B) | | | |
| 4A (6 mice-donor A) | v37663 | 0.2 mg/kg | Q7Dx4 |
| 4B (6 mice-donor B) | | | |
| 5A (6 mice-donor A) | v37663 | 1 mg/kg | Q7Dx4 |
| 5B (6 mice-donor B) | | | |
| 6A (6 mice-donor A) | v37663 | 5 mg/kg | Q7Dx4 |
| 6B (6 mice-donor B) | | | |
| 7A (6 mice-donor A) | v37638 | 0.2 mg/kg | Q7Dx4 |
| 7B (6 mice-donor B) | | | |
| 8A (6 mice-donor A) | v37638 | 1 mg/kg | Q7Dx4 |
| 8B (6 mice-donor B) | | | |
| 9A (6 mice-donor A) | v37638 | 5 mg/kg | Q7Dx4 |
| 9B (6 mice-donor B) | | | |
| 10A (6 mice-donor A) | v37692 | 0.2 mg/kg | Q7Dx4 |
| 10B (6 mice-donor B) | | | |
| 11A (6 mice-donor A) | v37692 | 1 mg/kg | Q7Dx4 |
| 11B (6 mice-donor B) | | | |
| 12A (6 mice-donor A) | v37692 | 5 mg/kg | Q7Dx4 |
| 12B (6 mice-donor B) | | | |
| 13A (6 mice-donor A) | v37658 | 0.2 mg/kg | Q7Dx4 |
| 13B (6 mice-donor B) | | | |
| 14A (6 mice-donor A) | v37658 | 1 mg/kg | Q7Dx4 |
| 14B (6 mice-donor B) | | | |
| 15A (6 mice-donor A) | v37658 | 5 mg/kg | Q7Dx4 |
| 15B (6 mice-donor B) | | | |
| 16A (6 mice-donor A) | v35923 | 0.2 mg/kg | Q7Dx4 |
| 16B (6 mice-donor B) | | | |
| 17A (6 mice-donor A) | v35923 | 1 mg/kg | Q7Dx4 |
| 17B (6 mice-donor B) | | | |
| 18A (6 mice-donor A) | v22277 | 5 mg/kg | Q7Dx4 |
| 18B (6 mice-donor B) | | | |
| 19A (6 mice-No PBMC) | v38874 | 0 mg/kg | Q7Dx4 |
| 19B (6 mice-No PBMC) | | | |

**EXAMPLE 29: *IN VIVO* TUMOR GROWTH INHIBITION USING MULTIVALENT AND MULTISPECIFIC CONSTRUCTS OF THE PRESENT DISCLOSURE COMPARED TO CORRESPONDING ANTI-(CLDN18.2xCD3) BISPECIFIC CONTROL CONSTRUCTS**

**[0822]** The ability of certain multivalent and multispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SNU620 gastric cancer model was assessed and their performance was compared against that of corresponding bispecific control constructs.

***SNU620 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity***

**[0823]** NCG female mice (Jiangsu GemPharmatech Co, Ltd.) were inoculated subcutaneously in the right front flank region with SNU620 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. The date of tumor inoculation was denoted as day 0.

**[0824]** Animals with a single tumor of a volume of 100-120 $mm^3$ 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 ($5x10^6$ cells) from donor A or donor B. 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 50** below. The test article administration and the animal numbers in each study group are also shown in **TABLE 50.**

**TABLE 50: *In Vivo* Study Groups and Dosing Regiment**

| Study Group | Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|---|
| 1A (6 mice-donor A) | v37634 | 0.2 mg/kg | Q7Dx4 |
| 1B (6 mice-donor B) | | | |
| 2A (6 mice-donor A) | v37634 | 1 mg/kg | Q7Dx4 |
| 2B (6 mice-donor B) | | | |
| 3A (6 mice-donor A) | v37634 | 5 mg/kg | Q7Dx4 |
| 3B (6 mice-donor B) | | | |
| 4A (6 mice-donor A) | v37663 | 0.2 mg/kg | Q7Dx4 |
| 4B (6 mice-donor B) | | | |
| 5A (6 mice-donor A) | v37663 | 1 mg/kg | Q7Dx4 |
| 5B (6 mice-donor B) | | | |
| 6A (6 mice-donor A) | v37663 | 5 mg/kg | Q7Dx4 |
| 6B (6 mice-donor B) | | | |
| 7A (6 mice-donor A) | v37638 | 0.2 mg/kg | Q7Dx4 |
| 7B (6 mice-donor B) | | | |
| 8A (6 mice-donor A) | v37638 | 1 mg/kg | Q7Dx4 |
| 8B (6 mice-donor B) | | | |
| 9A (6 mice-donor A) | v37638 | 5 mg/kg | Q7Dx4 |

| | | | |
|---|---|---|---|
| 9B (6 mice-donor B) | | | |
| 10A (6 mice-donor A)<br>10B (6 mice-donor B) | v37692 | 0.2 mg/kg | Q7Dx4 |
| 11A (6 mice-donor A)<br>11B (6 mice-donor B) | v37692 | 1 mg/kg | Q7Dx4 |
| 12A (6 mice-donor A)<br>12B (6 mice-donor B) | v37692 | 5 mg/kg | Q7Dx4 |
| 13A (6 mice-donor A)<br>13B (6 mice-donor B) | v37658 | 0.2 mg/kg | Q7Dx4 |
| 14A (6 mice-donor A)<br>14B (6 mice-donor B) | v37658 | 1 mg/kg | Q7Dx4 |
| 15A (6 mice-donor A)<br>15B (6 mice-donor B) | v37658 | 5 mg/kg | Q7Dx4 |
| 16A (6 mice-donor A)<br>16B (6 mice-donor B) | v35923 | 0.2 mg/kg | Q7Dx4 |
| 17A (6 mice-donor A)<br>17B (6 mice-donor B) | v35923 | 1 mg/kg | Q7Dx4 |
| 18A (6 mice-donor A)<br>18B (6 mice-donor B) | v22277 | 5 mg/kg | Q7Dx4 |
| 19A (6 mice-No PBMC)<br>19B (6 mice-No PBMC) | v38874 | 0 mg/kg | Q7Dx4 |

**[0825]** 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 2 000 $mm^3$ or when tumor exceeded 10% of normal body weight or when greater than 20% body weight loss, or after a maximum of 40 days post tumor injection. Tumor volume data were analyzed using a mixed-effect model.

**EXAMPLE 30: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING MULTIVALENT AND MULTISPECIFIC CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(CLDN18.2xCD3) BISPECIFIC CONTROL CONSTRUCTS**

**[0826]** The ability of certain multivalent and multispecific antibody constructs of the present disclosure to inhibit tumor growth and/or reduce tumor volume in the SNU620 gastric cancer

model was assessed and their performance was compared against corresponding bispecific control constructs.

***SNU620 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity***

**[0827]** NCG female mice (Jiangsu GemPharmatech Co, Ltd.) were inoculated subcutaneously in the right front flank region with SNU620 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. The date of tumor inoculation was denoted as day 0.

**[0828]** Animals with a single tumor of a volume of 100-120 $mm^3$ 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 ($5x10^6$ cells) from donor X.

**[0829]** 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 51**. The test article administration and the animal numbers in each study group are also shown in **TABLE 51.**

**[0830]** 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 2 000 $mm^3$ or when tumor exceeded 10% of normal body weight or when greater than 20% body weight loss, or after a maximum of 40 days post tumor injection. Tumor volume data were analyzed using a mixed-effect model.

**TABLE 51: *In Vivo* Study Groups and Dosing Regiment**

| Study Group | Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|---|
| 1A (6 mice-donor X) | v37634 | 0.001 mg/kg | Q7Dx4 |
| 2A (6 mice-donor X) | v37634 | 0.01 mg/kg | Q7Dx4 |
| 3A (6 mice-donor X) | v37634 | 0.1 mg/kg | Q7Dx4 |
| 4A (6 mice-donor X) | v38417 | 0.001 mg/kg | Q7Dx4 |
| 5A (6 mice-donor X) | v38417 | 0.01 mg/kg | Q7Dx4 |
| 6A (6 mice-donor X) | v38417 | 0.1 mg/kg | Q7Dx4 |
| 7A (6 mice-donor X) | v35923 | 0.001 mg/kg | Q7Dx4 |
| 8A (6 mice-donor X) | v35923 | 0.01 mg/kg | Q7Dx4 |

| | | | |
|---|---|---|---|
| 9A (6 mice-donor X) | v35923 | 0.1 mg/kg | Q7Dx4 |
| 10A (6 mice-donor X) | v22277 | 0.11 mg/kg | Q7Dx4 |

**[0831]** As shown in **FIG. 19** and **TABLE 52**, tumor regression was observed in all donor X-engrafted mice treated with 0.1 mg/kg of either the bispecific constructs, v35923 or v38417, or the trispecific construct, v37634. Tumor growth rate inhibition was significantly higher in mice receiving 0.01 mg/kg of v37634 compared to mice receiving equivalent doses of either v35923 or v38417. Tumor outgrowth was observed in animals treated with the negative control (v22277). Body weights remained stable for all mice (**FIG. 20**).

**TABLE 52: Tumor Growth Inhibition Constants and Rate Inhibition for Donor X**

| Test Article | Dose | Growth Constant (1/day; 95% CI) | Growth Rate Inhibition |
|---|---|---|---|
| v37634 | 0.1 mg/kg | -0.2271 (-0.2668, -0.1875) | 319.63% |
| | 0.01 mg/kg | -0.1429 (-0.1800, -0.1057) | 238.2% |
| | 0.001 mg/kg | 0.0211 (-0.0069, 0.0490) | 79.59% |
| v38417 | 0.1 mg/kg | -0.2717 (-0.3119, -0.2315) | 362.77% |
| | 0.01 mg/kg | 0.0276 (-0.0035, 0.0586) | 73.31% |
| | 0.001 mg/kg | 0.0435 (-0.0193, 0.0677) | 57.93% |
| v35923 | 0.1 mg/kg | -0.2837 (-0.3234, -0.2440) | 374.37% |
| | 0.01 mg/kg | 0.0229 (-0.0017, 0.0476) | 77.85% |
| | 0.001 mg/kg | 0.0877 (0.0748, 0.1006) | 15.18% |
| v22277 | 0.11 mg/kg | 0.1034 (0.0910, 0.1157) | 0% |

## EXAMPLE 31: IN VIVO TUMOR PHARMACODYNAMIC STUDY

**[0832]** The ability of certain multivalent and multispecific antibody constructs of the present disclosure to induce immune cell changes in the SNU620 gastric cancer model was assessed and their performance was compared against corresponding bispecific control constructs.

***SNU620 Tumor Induction and PBMC Engraftment in NCG Mice for Antitumor Activity***

**[0833]** NCG female mice (Jiangsu GemPharmatech Co, Ltd.) were inoculated subcutaneously in the right front flank region with SNU620 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. Animals with a single tumor of a volume of 200-300 $mm^3$ 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 ($5x10^6$ cells) from donor X. 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 53**. Test article administration

and the animal numbers in each study group are also shown in **TABLE 53**. The date of treatment was denoted as day 0.

**[0834]** 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. Whole blood was collected via terminal cardiac puncture at 3-, 6- and 8-days post first dose. Tumors were collected and processed for flow cytometric analysis, fixed for FFPE analysis or snap frozen.

**TABLE 53: *In Vivo* Study Groups and Dosing Regiment**

| Study Group | Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|---|
| 1 (15 mice-donor X) | v37634 | 0.01 mg/kg | Day 0, 7 |
| 2 (15 mice-donor X) | v37634 | 0.05 mg/kg | Day 0, 7 |
| 3 (15 mice-donor X) | v37663 | 0.01 mg/kg | Day 0, 7 |
| 4 (15 mice-donor X) | v37663 | 0.05 mg/kg | Day 0, 7 |
| 5 (15 mice-donor X) | v35923 | 0.01 mg/kg | Day 0, 7 |
| 6 (15 mice-donor X) | v35923 | 0.05 mg/kg | Day 0, 7 |
| 7 (15 mice-donor X) | v22777 | 0.01 mg/kg | Day 0, 7 |
| 8 (15 mice-donor X) | v22777 | 0.05 mg/kg | Day 0, 7 |

**[0835]** At 3-, 6- and 8-days post first dose, the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, lead to increased counts of CD45+CD3+ cells in the tumor compared to the v22277 negative control, e.g., as shown in **FIG. 21**. At 3- and 6-days post first dose, CD45+CD3+ counts for the construct v37634 in the tumor were higher than those of the bispecific control, v37663, and the benchmark v35923. The antibody construct, v37634, did not lead to increased CD45+CD3+ numbers in the blood, compared to the negative control v22277. One day post second dose, CD4+ and CD8+ T cells in the tumor had increased CD69 MFI compared to the v35923 benchmark and v22277 negative control.

## EXAMPLE 32: IN VIVO CYTOKINE RELEASE SYNDROME STUDY USING MULTIVALENT AND MULTISPECIFIC ANTIBODY CONSTRUCTS AS WELL AS CORRESPONDING ANTI-CLDN18.2XCD3 BISPECIFIC CONTROL CONSTRUCTS

**[0836]** The ability of certain multivalent and multispecific antibody constructs of the present disclosure to induce cytokine release and body weight loss in PBMC humanized NSG-MHCI/II

double knockout mouse model was assessed, and their performance was compared against corresponding bispecific control constructs.

***Irradiation and PBMC Engraftment of NSG-MHC I/II DKO mice***

**[0837]** NSG-MHC I/II DKO female mice (JAX Stock#025216) were irradiated with 100cGy of total body irradiation and injected IV via tail vein with ($15x10^6$) PBMCs from donor A or B. The date of PBMC injection was denoted as day 0. On day 5, animals were weighed, randomized and distributed into experimental groups according to the study protocol. On day 6, animals were treated intravenously via tail vein as per the study design shown in **TABLE 54**. The test article administration and the animal numbers in each study group are also shown in **TABLE 54.**

**TABLE 54: *In Vivo* Study Groups and Dosing Regiment**

| Study Group | Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|---|
| 1 (6 mice per donor) | PBS | n/a | Day 6 |
| 2 (6 mice per donor) | v37634 | 0.01 mg/kg | Day 6 |
| 3 (6 mice per donor) | v37634 | 0.1 mg/kg | Day 6 |
| 4 (6 mice per donor) | v37634 | 1 mg/kg | Day 6 |
| 5 (6 mice per donor) | v36577 | 0.01 mg/kg | Day 6 |
| 6 (6 mice per donor) | v36577 | 0.1 mg/kg | Day 6 |
| 7 (6 mice per donor) | v36577 | 1 mg/kg | Day 6 |
| 8 (6 mice per donor) | v37663 | 0.1 mg/kg | Day 6 |
| 9 (6 mice per donor) | v37663 | 1 mg/kg | Day 6 |
| 10 (6 mice per donor) | OKT3 | 0.25 mg/kg | Day 6 |
| 11 (6 mice per donor) | Anti-CD28 ANC | 1 mg/kg | Day 6 |

**[0838]** 2 hours and 6 hours post dosing and then daily, each mouse was observed and given a health score. Body weights were measured twice per week after PBMC injection and daily after dosing. 2 hours and 6 hours post dosing blood was collected via retro-orbital route and processed to serum. Serum samples were analyzed for human cytokines using BD™ CBA Human Th1/Th2 Cytokine Kit II (Catalog No. 551809). On study day 10, animals were euthanized via $CO_2$ asphyxiation. Spleen, the large lobe of the liver and stomach with a small part of the duodenum were collected and placed in 10% neutral buffered formalin. Tissue samples were transferred to ethanol and then paraffin embedded.

**[0839]** Percent body weight loss compared to baseline (e.g., body weight measured prior to PBMC engraftment) following treatment with vehicle control, 1 mg/kg of v37634, v37663, or ANC28.1/5D10, as well as 0.25 mg/kg of OKT3 is shown in **FIG. 22**. Cytokine induction

following treatment with antibody constructs is shown in **TABLE 55**. Data are representative of two independent experiments with two independent donors.

**TABLE 55: Peripheral Cytokine Concentrations produced by NSG MCH Class I/II DKO Mice engrafted with human PBMCs 6 hours post-treatment with Selected Constructs**

| | | Vehicle | v37634 | | | v37663 | | OKT3 | ANC28.1/5D10 |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 mg/kg | 0.1 mg/kg | 0.01 mg/kg | 1 mg/kg | 0.1 mg/kg | 0.25 mg/kg | 1 mg/kg |
| **IFNg** | **Mean** | 184.0 | 120.6 | 134.8 | 153.9 | 88.9 | 167.6 | 4567.0 | 960.3 |
| | **SEM** | 23.4 | 16.7 | 8.5 | 42.6 | 23.2 | 22.3 | 623.0 | 139.1 |
| **TNFa** | **Mean** | 3.1 | 1.0 | 1.7 | 3.2 | 0.7 | 2.6 | 735.3 | 25.7 |
| | **SEM** | 1.0 | 0.6 | 0.8 | 1.5 | 0.4 | 1.4 | 81.2 | 3.1 |
| **IL-2** | **Mean** | 5.2 | 3.5 | 3.2 | 5.0 | 3.2 | 4.6 | 491.4 | 54.8 |
| | **SEM** | 1.0 | 0.5 | 0.7 | 0.8 | 0.5 | 0.4 | 55.5 | 5.8 |
| **IL-10** | **Mean** | 29.7 | 24.2 | 19.3 | 26.7 | 19.1 | 25.1 | 597.9 | 133.9 |
| | **SEM** | 6.7 | 2.4 | 5.8 | 6.1 | 4.2 | 2.1 | 94.8 | 24.6 |
| **IL-6** | **Mean** | 9.5 | 6.8 | 4.9 | 9.2 | 5.7 | 8.2 | 89.4 | 20.2 |
| | **SEM** | 2.1 | 1.1 | 1.6 | 1.9 | 1.7 | 1.2 | 12.7 | 1.9 |
| **IL-4** | **Mean** | 0.3 | 0.6 | 0.0 | 0.1 | 0.0 | 0.7 | 52.6 | 17.8 |
| | **SEM** | 0.2 | 0.3 | 0.0 | 0.1 | 0.0 | 0.3 | 8.9 | 2.5 |

At 6 hours post-treatment, all doses of the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, and bispecific anti-(CLDN18.2xCD3) construct, v37663, induced similar levels of IFNg, TNFa, IL-2, IL-10, IL-6, and IL-4 production compared to that induced by treatment with the vehicle control. Treatment with 0.25 mg/kg of OKT3 resulted in about 25-fold increased IFNg, 240-fold increased TNFa, 95-fold increased IL-2, 20-fold increased IL-10, 9.4-fold increased IL-6, and about 178-fold increased IL-4 production relative to that induced by treatment with vehicle control. Treatment with 1 mg/kg of ANC28.1/5D10 resulted in about 5-fold increased IFNg, 8.4-fold increased TNFa, 10.5-fold increased IL-2, 4.5-fold increased IL-10, 2-fold increased IL-6, and 60.5-fold increased IL-4 production relative to that induced by treatment with vehicle control. Similar trends in cytokine production were observed after 2 hours post-treatment. No body weight loss was observed following treatment with 1 mg/kg of either the multivalent and multispecific anti-(CLDN18.2xCD3xCD28) antibody construct, v37634, or the bispecific anti-(CLDN18.2xCD3) bispecific construct, v37663, relative to mice treated with vehicle control. Treatment with 0.25 mg/kg OKT3 or 1 mg/kg ANC28.1/5D10 resulted in greater than 10% body weight loss relative to mice treated with vehicle control. Similar trends in body weight loss were observed with 0.1 and 0.01 mg/kg doses of the multivalent and multispecific anti-(CLDN18.2xCD3xCD28)

construct, v37634, and the 0.1 mg/kg dose of the bispecific anti-(CLDN18.2xCD3) bispecific construct, v37663.

**EXAMPLE 33: IN VIVO STUDY**

**[0840]** This in vivo study examined the performance of a trivalent and trispecific antibody construct, v37634, compared to a clinical benchmark molecule (AMG910) in a murine gastric cancer model.

**[0841]** NCG female mice (Jiangsu GemPharmatech Co, Ltd.) were inoculated subcutaneously in the right front flank region with SNU620 tumor cells ($5x10^6$ cells) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development. The date of tumor inoculation was denoted as day 0. Animals with a single tumor of a volume of 100-120 $mm^3$ 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 ($5x10^6$ cells). 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 56**. The test article administration and the animal numbers in each study group are also shown in **TABLE 56**.

**[0842]** 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 will be 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 2 000 $mm^3$ or when tumor exceeded 10% of normal body weight or when greater than 20% body weight loss, or after a maximum of 40 days post tumor injection.

**[0843]** A linear mixed-effects model was fit to log-transformed tumor volumes over time to compare differences in tumor growth rate between treatment groups. Tumor growth rate differences between treatment groups were assessed using an F-test and the resulting p-values were adjusted for multiple tests according to the Tukey procedure. Adjusted p values $<0.05$ were considered statistically significant. Percent tumor growth rate inhibition (% TGRI) for a given treatment group relative to the control was computed using the following expression:

$$\%\,\mathrm{TGRI} = \left(1 - \frac{k_{treatment}}{k_{control}}\right) \times\ 100\%$$

where $k_{treatment}$ is the growth rate constant of a given treatment group, and $k_{control}$ is the growth rate constant of the control group. A TGRI value of zero indicates no change from control, while a value >100% indicates tumor regression. Negative RI values denote a growth rate faster than the control.

**TABLE 56: *In Vivo* Study Groups and Dosing Regiment**

| Test Article | Dose (IV) | Treatment Schedule |
|---|---|---|
| v37634 | 0.05 mg/kg | Q7Dx4 |
| v37663 | 0.05 mg/kg | Q7Dx4 |
| v35923 | 0.05 mg/kg | Q7Dx4 |
| v22277 | 0.05 mg/kg | Q7Dx4 |

**TABLE 57: Tumor Growth Rate Inhibition (%) and Statistical Summary**

| Treatment Group | % TGRI | p-value (vs. v22277) |
|---|---|---|
| v22277 | 0 | n/a |
| v37634 | 131.1 | <0.01 |
| v37663 | 68.9 | 0.06 |
| v35923 | 12.2 | 0.99 |

**[0844]** Tumor growth rate inhibition values are documented in **Table 57**. Tumor volumes are presented in **FIG. 23A**. Body weight changes following treatment are shown in **FIG. 23B**. **FIGS. 23A-23B** indicate that the trivalent and trispecific construct, v37634, achieved superior tumor growth control compared to the clinical benchmark molecule, with 5/6 mice that showed complete responses compared to 0/6 for the tested benchmark. Moreover, the trivalent and trispecific construct, v37634, was well tolerated based on % change in body weight over the course of this study. Treatment with v37634 resulted in complete tumor regression in 5 out of the 6 mice treated and resulted in tumor growth rate inhibition of 131.1%. Treatment with v37663 resulted in complete tumor regression in 3 out of the 6 mice treated and resulted in tumor growth rate inhibition of 68.9%. Treatment with v35923 resulted in complete tumor regression in 0 out of the 6 mice treated and resulted in tumor growth rate inhibition of 12.2%. Body weight loss did not exceed 10% in any of the treatment group.

## EXAMPLE 34: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING A MULTIVALENT AND MULTISPECIFIC CONSTRUCT TO A CLINICAL BENCHMARK CONSTRUCT

**[0845]** The ability of a certain multivalent and multispecific 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 benchmark construct.

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

**[0846]** 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.

**[0847]** 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.

**[0848]** When the average tumor volume reached 100-120 $mm^3$, animals were treated intravenously as per the study design shown in **TABLE 58**. The test article administration and the animal numbers in each study group are also shown in **TABLE 58**.

**[0849]** 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.

**TABLE 58: *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 |

As shown in **FIG. 24**, tumor growth delay was observed in donor A-engrafted mice treated with 2.5 mg/kg of the multivalent and multispecific 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 clinical benchmark (Long-rank p = 0.0047) (**FIG. 25**). Body weights remained stable for all mice (**FIG. 26**).

**EXAMPLE 35: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS**

**[0850]** 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*

**[0851]** 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.

**[0852]** 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.

**[0853]** 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 59**. The test article administration and the animal numbers in each study group are also shown in **TABLE 59**.

**[0854]** 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.

**[0855]** As shown in **FIG. 27**, tumor regression was observed in donor A-engrafted mice treated with v39917 compared to the clinical benchmark (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 clinical benchmark (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 clinical benchmark (Log-rank p= 0.0106) or irrelevant mAb (Log-rank p= 0.0153 ) (**FIG. 28**). Body weights remained stable for all mice (**FIG. 29**).

**TABLE 59: *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 36: NON-HUMAN PRIMATE TOXICOLOGY STUDY

### *Ability of Anti-(cldn18.2xcd3xcd28) Trispecific Antibody Constructs to Bind to Native Human or Cynomolgus Monkey CD3 and CD28 as Assessed by Flow Cytometry*

**[0856]** First, certain trivalent and trispecific antibody constructs were tested for binding to primary cynomolgus monkey or human T cells to assess the cross-reactivity of the anti-(CLDN18.2xCD3xCD28) construct, v37634, and a surrogate anti-(CLDN18.2xCD3xCD28) construct, v39452, which included a higher affinity anti-CD3 paratope against cynomolgus CD3.

The constructs v37663 and v39453 were included as anti-(CLDN18.2xCD3) bispecific controls for v37634 and v39452, respectively. v35923 was included as a benchmark anti-(CLDN18.2xCD3) construct and v22277 was included as a negative control.

**[0857]** The ability of the trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs, as well as those of some bispecific and benchmark controls, to bind to CD3+CD28+ primary human or cynomolgus monkey T cells was assessed via whole cell FACS binding analysis. To that end, primary human or cynomolgus monkey T cells (>80% viability) 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 for 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 fluorophore-conjugated antibodies specific for CD4 and CD8. Cells were incubated for 1 hour at 4 °C. Following incubation, cells were washed twice by adding 150 µL FACS buffer and centrifuging at 400xg for 3 minutes. Cells were fixed with 25 µL of FluoroFix Buffer (BioLegend) for 25 mins at 4°C. After fixing, cells were washed twice by adding 150 µL of FACS buffer and centrifuging at 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD LSRFortessa Cell Analyzer.

**[0858]** The calculated $EC_{50}$ and $B_{max}$ values of the tested constructs for binding to CD3+CD28+ human and cynomolgus monkey T cells are presented in **TABLE 60**.

**TABLE 60: Binding of Tested Anti-CLDN18.2 Constructs to Primary Human or Cynomolgus Monkey T Cells**

| Antibody Construct | $EC_{50}$ (nM) | | $B_{max}$ | |
|---|---|---|---|---|
| | Human | Cynomolgus Monkey | Human | Cynomolgus Monkey |
| v22277 | nd* | nd | 60.0 | 202.0 |
| v35923 | 0.8 | 1.5 | 15938 | 5768 |
| v37634 | 9.8 | 28.9 | 12305 | 2520 |
| v37663 | 25.0 | 45 | 13864 | 1111 |
| v39452 | 0.8 | 11.0 | 20858 | 4137 |
| v39453 | 1.1 | 19.4 | 18201 | 3961 |

*nd= not defined

**[0859]** The anti-(CLDN18.2xCD3xCD28) construct, v37634, exhibited an approximate 3-fold reduced apparent affinity for cynomolgus monkey pan T cells compared to human pan T cells with an approximate five-fold reduced Bmax value. v39452 displayed an approximate 10-fold reduced

apparent affinity for cynomolgus monkey pan T cells compared to human pan T cells with an approximate five-fold reduced Bmax value.

***Induction of In Vitro Cynomolgus Monkey T Cell-Mediated Cytotoxic Activity of Trispecific Constructs***

**[0860]** Certain trivalent and trispecific antibody constructs were tested in cynomolgus monkey or human T cell-dependent cytotoxicity assays with CLDN18.2-expressing SNU 620-RFP tumor cells to determine the functional cross-reactivity of the anti-(CLDN18.2xCD3xCD28) construct, v40062, and a surrogate anti-(CLDN18.2xCD3xCD28) construct, v40063, which included a higher affinity anti-CD3 paratope against cynomolgus monkey CD3. As further described herein, the construct v40062 and v40063 are essentially identical to v37634 and v39452, respectively, except that the two heavy chains each contained an additional C-terminal lysine residue and was used in this study solely for production purposes and the C-terminal lysine residues undergo cleavage after expression. v37663 and v39453 were included as anti-(CLDN18.2xCD3) bispecific controls for v40062 and v40063, respectively. v35923 was included as a benchmark anti-(CLDN18.2xCD3) construct and v39982 was included as a negative control. Test articles (e.g., antibody constructs, controls, etc.) 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). Human or cynomolgus monkey pan T cells were thawed and mixed with SNU 620-RFP cells at an E:T ratio of 10:1. Plates were incubated for 72 hrs at 37 °C and 5% carbon dioxide, after which tumor cell viability was assessed through live tumor cell counts (RFP+) using the Operetta CLS High Content Analysis System (Perkin Elmer; HH16000020). Percent cytotoxicity was calculated based on the following formula:

$$\% \text{ cytotoxicity} = \left(1 - \frac{number\ of\ tumor\ cells\ left\ in\ sample}{total\ number\ of\ tumor\ cells\ in\ untreated\ condition}\right) \times 100$$

**[0861]** Determined mean $IC_{50}$ values from pooled data of 4 independent experiments with 2 independent cynomolgus monkey or human Pan T cells in the T cell-dependent cytotoxicity assay comparing the functional cross-reactivity of the trispecific constructs are presented in **TABLE 61.**

**TABLE 61: Cytotoxic Activity of Tested Anti-Cldn18.2 Trispecific Constructs Towards Cldn18.2-expressing Tumor Cells Grown in Culture with Primary Human or Cynomolgus Monkey CD3+ T cells**

| Antibody Construct | Cytotoxic Potency ($IC_{50}$; pM) |
|---|---|

| | Human | | Cynomolgus | |
|---|---|---|---|---|
| | Mean | SEM* | Mean | SEM |
| v39982 | nd# | nd | nd | nd |
| v35923 | 18.0 | 0.5 | 174.9 | 48.7 |
| v37663 | 39.4 | 1.6 | nd | nd |
| v39453 | 4.6 | 0.3 | 20887.3 | 5539.1 |
| v40062 | 25.7 | 1.4 | 30255.7 | 5212.5 |
| v40063 | 5.3 | 0.4 | 3107.5 | 687.4 |

*SEM = standard error of the mean; #nd = not defined.

**[0862]** The construct v40063 exhibited an approximately 10-fold increased cytotoxic potency against CLDN18.2-expressing tumor cells in co-culture with primary cynomolgus monkey T cells compared to that induced by v40062. v40063 displayed an approximately 120-fold reduced cytotoxic potency against CLDN18.2-expressing tumor cells in co-culture with primary cynomolgus monkey T cells compared to that induced by v40062 against CLDN18.2-expressing tumor cells in co-culture with primary human T cells.

***Non-Human Primate Toxicology Study***

**[0863]** This example describes a toxicology study that was conducted in cynomolgus monkeys using the multivalent and multispecific antibody construct, v40063, which exhibited an approximately 10-fold increased cytotoxic potency in an in vitro TDCC assay with cynomolgus monkey T cells compared to v40062.

**[0864]** Male cynomolgus monkeys (n=3) were given a repeat i.v. dose of 3 mg/kg of v40063 once weekly for two weeks. Mean body weight decreased by 6.5% during the 1-week interval following the first dose (Days 1-8) and 0.7% during the week after the second dose (Days 8-15). These changes corresponded to decreased qualitative food consumption during the first week which improved during the second week **FIG. 30**. Hematological changes included decreased red cell mass parameters (red blood cells, hematocrit and hemoglobin); decreased lymphocytes; increased neutrophils; increased monocytes; and increased reticulocytes, as shown in **FIGS. 31A-31C** and **FIGS. 32A-32D**. There were transient increases in serum concentrations of the cytokines IL-6, TNF-α, IFN-γ, and MCP-1, most notably after the first dose, as shown in **FIGS. 33A-33D**. Histopathological changes were secondary to decreased food consumption and body weight loss and included vacuolation in epithelial crypt cells of the duodenum, jejunum, and ileum; vacuolation in liver sinusoids; decreased cellularity in thymic cortex; and adipose atrophy in multiple tissues.

**[0865]** Altogether, these data indicate that v40063 administered at 3 mg/kg once weekly for two weeks is well-tolerated in cynomolgus monkeys.

## EXAMPLE 37: ENGINEERING OF ANTI-CD28 PARATOPE FOR GENERATING CONSTRUCTS WITH VARYING BINDING AFFINITY FOR CD28

**[0866]** This example demonstrates the engineering of the anti-CD28 paratope TN228 to modulate binding affinity for CD28 engagement.

**[0867]** 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.

**[0868]** 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. It was contemplated that CDR and adjacent framework regions that differ from the murine germline sequences may have been mutated due to affinity selection pressure. It was hypothesized that reversion of such sites in the humanized construct back to the murine germline parental sequence may modulate antigen binding affinity. Such positions, once identified, may also be swapped to other amino acid residues that may either increase or decrease affinity to differing extents, for example, by hypothetically strengthening or weakening electrostatic interactions or changing steric complementarity with the antigen. The extent of D-gene expansion within the CDR H3 loop may also suggest sites for affinity modulating mutations. Mutation sites may also be selected within CDRs, and adjacent framework regions based on 3D structure modeling to identify residues that are likely to be solvent exposed, not participating in intra-domain interactions and positioned to hypothetically contribute to antigen binding interactions. Regions thus identified may be mutated to an alternative amino acid residue to modulate antigen binding affinity. While the primary goal of the affinity modulation effort was to generate a panel of anti-CD28 paratopes with weaker affinity to varying degrees, such engineering may also uncover mutations that strengthen antigen binding affinity.

**[0869] TABLE 12** 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.

**TABLE 12: 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 38: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS

**[0870]** 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*

**[0871]** 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.

**[0872]** 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.

**[0873]** 24 hours after randomization, animals are treated intravenously as per the study design shown in **TABLE 13**. The test article administration and the animal numbers in each study group are also shown in **TABLE 13**.

**[0874]** 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 13: *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 39: *IN VIVO* TUMOR GROWTH INHIBITION STUDY USING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS AS WELL AS CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS

**[0875]** 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*

**[0876]** 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.

**[0877]** 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).

**[0878]** On the day of randomization, animals are treated intravenously as per the study design shown in **TABLE 14**. The test article administration and the animal numbers in each study group are also shown in **TABLE 14**.

**[0879]** 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 14: *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 40: INVESTIGATION OF THE ABILITY OF TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND CD3 AND CD28 ON THE SAME T CELL

**[0880]** 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.

**[0881]** 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.

**[0882]** $EC_{50}$ values and mean maximum percent double positive events of the tested constructs from the concentration response curves are presented in **TABLES 62A** and **62B**.

**TABLE 62A: Summary Table of $EC_{50}$ and Maximum Percent Double Positive Events of Cell Cross-linking Induced by Certain Trispecific and Bispecific Constructs**

| Antibody Construct | EC50 (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 |

ND= not determined

**TABLE 62B: Summary Table of $EC_{50}$ and Maximum Percent Double Positive Events of Cell Cross-linking Induced by Certain Trispecific and Bispecific Constructs**

| Antibody Construct | EC50 (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 |

ND= not determined

**[0883]** 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 41: THERMAL STABILITY OF ANTI-(DLL3xCD3xCD28) TARGETING TRIVALENT AND TRISPECIFIC ANTIBODY CONSTRUCTS

**[0884]** 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).

**[0885]** 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.

**[0886] TABLE 63** 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 63**: **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 |

**[0887]** The results shown in **TABLE 63** demonstrate that the tested antibody constructs exhibited thermostability profiles comparable to those of conventional IgG1 antibodies.

**[0888]** The trivalent and trispecific antibody constructs were further tested for (i) its freeze-thaw stability, in which it 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.

**[0889]** The trivalent and trispecific antibody constructs were tested in an accelerated stress test for its stability, in which it showed minimal change in purity post 14 days incubation at 4°C and 40 °C, using a construct concentration of 1 mg/mL. 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 64** below.

**TABLE 64: 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 42: 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**

**[0890]** 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.

**[0891]** 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).

**[0892]** Percent cytotoxicity was calculated using the following formula:

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

**[0893]** Calculated $IC_{50}$ and maximum cytotoxicity (%) are shown in **TABLE 65.** Data are representative of 3 independent experiments with 3 donors.

**TABLE 65: 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 43: IMPACT OF TRISPECIFIC CONSTRUCT FORMAT ON INDUCTION OF PRO-INFLAMMATORY CYTOKINE RELEASE BY HUMAN PAN-T CELLS IN CO-CULTURE WITH DLL3-POSITIVE AND DLL3-NEGATIVE TUMOR CELL LINES**

**[0894]** 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.

**[0895]** 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.

**[0896]** 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 ℃ 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 ℃. 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.

**[0897]** 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 66A** below.

**TABLE 66A: 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 |

**[0898]** 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 66B** below.

**TABLE 66B: 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 |

**[0899]** 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 66C** below.

**TABLE 66C: 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 |

**[0900]** 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 66D-66E**.

**TABLE 66D: 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 66E: 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 |

**[0901]** 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 66F-66G**.

**TABLE 66F: 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 66G: 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 44: ABILITY OF ANTI-(DLL3xCD3xCD28) TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND NATIVE DLL3 AS ASSESSED BY FLOW CYTOMETRY**

**[0902]** 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.

**[0903]** Specifically, the ability of certain trispecific anti-(DLL3xCD3xCD28) antibody constructs as well as bispecific and benchmark controls 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.

**[0904]** The apparent binding affinities that were calculated from the binding curves of the tested constructs to DLL3 are shown in **TABLE 67**.

**TABLE 67: 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 45: IMPACT OF FORMAT OF TRISPECIFIC ANTIBODY CONSTRUCTS ON PROLIFERATION OF HUMAN CD3+ T CELLS IN CO-CULTURE WITH DLL3-POSITIVE TUMOR CELL LINES

**[0905]** To test the impact of antibody construct format on its 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 clinical benchmarks as described below.

**[0906]** 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).

**[0907]** Curves showing the percent of cells proliferated with the respective titrated antibody constructs tested are shown in **FIG. 34**.

## EXAMPLE 46: IMPACT OF IMMOBILIZED TRISPECIFIC ANTIBODY CONSTRUCTS ON INDUCTION OF CYTOKINE IN AN IN VITRO PREDICTIVE ASSAY FOR CYTOKINE RELEASE SYNDROME

**[0908]** 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-TNF$\alpha$ 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.

**[0909]** 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).

**[0910]** 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.

**[0911]** 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 68A** and **68B**.

**TABLE 68A: 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 68B: 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 47: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING TRIVALENT AND TRISPECIFIC ANTI-DLL3 CONSTRUCTS TO CORRESPONDING ANTI-(DLL3XCD3) BISPECIFIC CONTROL CONSTRUCTS AND A CLINICAL BENCHMARK CONSTRUCT**

**[0912]** 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*

**[0913]** 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.

**[0914]** 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.

**[0915]** 24 hours after randomization, animals were treated intravenously as per the study design shown in **TABLE 69**. The test article administration and the animal numbers in each study group are also shown in **TABLE 69**.

**[0916]** 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.

**[0917]** As shown in **FIG. 35**, tumor regression was observed in donor B-engrafted mice treated with v41086, CD28-KO (v41753), and the clinical benchmark (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. 36**).

**TABLE 69: *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 48: *IN VIVO* TUMOR GROWTH INHIBITION STUDY COMPARING A TRIVALENT AND TRISPECIFIC CONSTRUCT TO A CLINICAL BENCHMARK CONSTRUCT

**[0918]** 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 benchmark construct.

*NCI-H82 co-inoculation with human T cells in NCG Mice for Antitumor Activity*

**[0919]** 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 70**.

**[0920]** The test article administration and the animal numbers in each study group are also shown in **TABLE 70.**

**[0921]** 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.

**[0922]** As shown in **FIG. 37**, 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 clinical benchmark (v38985) and untreated control.

**TABLE 70: *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 | BIWx6 |
| 4 (6 mice-donor C) | v38589 | 2.85 nmol/kg | BIWx6 |
| 5 (6 mice-donor D) | - | - | - |
| 6 (6 mice-donor D) | v41086 | 2.85 nmol/kg | BIWx6 |
| 7 (6 mice-donor D) | v38589 | 2.85 nmol/kg | BIWx6 |
| 8 (6 mice-donor E) | - | - | - |
| 9 (6 mice-donor E) | v41086 | 2.85 nmol/kg | BIWx6 |
| 10 (6 mice-donor E) | v38589 | 2.85 nmol/kg | BIWx6 |

## EXAMPLE 49: STUDY ASSESSING *IN VIVO* SYSTEMIC CYTOKINE INDUCTION USING A HUMANIZED MOUSE MODEL

**[0923]** 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.

**[0924]** Mice were dosed with the respective construct at 2 mg/kg, except for OKT3 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 (containing the TGN1412 paratope) and v38589 (clinical benchmark control).

**[0925]** **FIG. 38** 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 clinical benchmark tested in this study.

## EXAMPLE 50: NON-HUMAN PRIMATE TOXICOLOGY STUDY

**[0926]** 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).

**[0927]** 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. 39**. The peak 10 mg/kg surrogate values were measured in a male with an increased cytokine release profile.

**[0928]** Overall, these data indicate that the tested trivalent and trispecific antibody constructs were well-tolerated in cynomolgus monkeys.

## EXAMPLE 51: DESIGN AND PREPARATION OF CERTAIN TRIVALENT AND TRISPECIFIC ANTIBODY CONSTRUCTS WITH VARIOUS CD3 AND CD28 PARATOPES

**[0929]** Several trivalent and trispecific anti-(MSLNxCD28xCD3) antibody constructs as well as bispecific anti-(MSLNxCD3) and anti-(MSLNxCD28) control antibody constructs were produced to demonstrate the advantage of an antibody format in which an anti-CD28 scFv domain is coupled

to the C-terminus of a light chain of an anti-CD3 Fab domain (e.g., as shown in **FIGS. 1A-B**, also referred to herein as "Format A") over alternate formats (e.g., formats B and C). To that end, trispecific antibody constructs and respective controls were prepared using 3 different formats, as shown, in **FIGS. 1A, 1B** (representative depictions of Format A)**, 1G** (representative depiction of Format B) and **1H** (representative depiction of Format C), and which are further described in **TABLE 71** below. Three different anti-CD28 and three different anti-CD3 paratopes with varying properties (e.g., target affinity, binding epitope, etc.) were selected to demonstrate that Format A retains its superior properties when different combinations of anti-CD3, anti-CD28 and/or anti-TAA paratope sequences are used. As further described and demonstrated herein, use of different combinations of anti-CD3, anti-CD28 and/or anti-TAA paratope sequences in Format A did not alter the general properties of the resulting trivalent and trispecific antibody constructs, e.g., similar properties were observed including the constructs' capability of specifically targeting and killing TAA-expressing tumor cells, their ability to activate T cells in an antigen-dependent manner, cytokine induction in the presence/absence of tumor cells, and their ability to bind CD3 and CD28 on the same T cell thereby avoiding T-cell cross-linking.

***Design of trivalent and trispecific antibody constructs targeting MSLN, CD28 and CD3***

**[0930]** Trivalent and Trispecific antibody constructs that are monovalent for each antigen were prepared in a format in which the MSLN antigen binding domain was an scFv 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, depending on the overall format and geometry of a given construct.

**[0931]** The anti-MSLN scFv domain sequence was generated as described in **EXAMPLE 1**.

**[0932]** The first set of anti-CD3 paratope sequences used was generated from the anti-CD3 monoclonal antibody C3E6 (*see*, e.g., U.S. Pat. Publ. No. 2019/0359712), which binds a discontinuous epitope on CD3ε. The second set of anti-CD3 paratope sequences was generated from the humanized anti-CD3 monoclonal antibody as described in, e.g., U.S. Pat. Publ. No. 2016/0297881, which targets a non-overlapping linear epitope including residues 87 to 91 (FSELE) of the CD3ε antigen. The third set of anti-CD3 paratope sequences was generated from the anti-CD3 monoclonal antibody described in, e.g., U.S. Pat. Publ. No. 2018/0273622, which binds an undetermined epitope on the CD3 antigen, but was expected to exhibit different properties (e.g., CD3 affinity) compared to the other two anti-CD3 paratopes.

**[0933]** The first set of anti-CD28 paratope sequences was generated from the conventional partial agonist anti-CD28 monoclonal antibody huTN228 as described elsewhere herein. The second set of anti-CD28 paratope sequences was generated from the superagonist anti-CD28 monoclonal antibody TGN1412 (*see*, e.g., U.S. Pat. Publ. No. US2012/0082683), which targets the C'-D loop of the immunoglobulin-like domain of CD28 (*see*, e.g., Luhder et al, J. Exp. Med. 2003, 197(8), 955-966). The third set of anti-CD28 paratope sequences was generated from the antagonist anti-CD28 monoclonal antibody CD28.3 (*see*, e.g., U.S. Pat. Publ. No. 2013/0078236), which targets a discontinuous epitope on the CDEF loops (Poirier et al., American Journal of Transplantation, 2012, 12;1682-1690).

**[0934]** The trispecific and bispecific antibody constructs tested herein 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., **TABLE 5**). 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). The antibody construct in **TABLE 71** 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.

**[0935]** Several heavy chain domain structures and formats that were used to generate certain antibody constructs of the present disclosure, e.g., the trivalent and trispecific as well as bispecific constructs, and/or other constructs for studying the impact of overall construct format and geometry on the properties of an antibody construct, are shown below in **TABLE 71**. Constructs used to demonstrate the transferability of the biological properties recapitulated in format A geometry when including different anti-CD3, anti-CD28 and/or anti-TAA are described in **TABLE 72**.

**TABLE 71: Format and Structure of Certain Trivalent and Trispecific Antibody Constructs and Control Constructs (*Domain structure shown from N-terminus to C-terminus*)**

| Format | Heavy Chain A | Heavy Chain B | Light Chain A |
|---|---|---|---|
| A | CD3 VH-CH1-hinge-FcKO | TAA scFv-hinge-FcKO | CD3 VL-CL-linker-CD28 scFv |

| | | | |
|---|---|---|---|
| **B** | CD3 scFv-linker-CD28 VH-CH1-hinge-FcKO | TAA scFv-hinge-FcKO | CD28 VL-CL |
| **C** | TAA scFv-linker-CD28 VH-CH1-hinge-FcKO | CD3 scFv-hinge-FcKO | CD28 VL-CL |
| **Bisp. Ctrl.** | CD3 VH-CH1-hinge-FcKO | TAA scFv-hinge-FcKO | CD3 VL-CL |

**TABLE 72A: Format and Structure of Certain Trivalent and Trispecific Antibody Constructs and Control Constructs Used to Demonstrate Superior Properties of Format A compared to Formats B and C using Various Paratope Combinations**

| **Format** (representative figure): | **Antibody Constructs having the specified Format:** |
|---|---|
| **Format A** (*see*, e.g., FIG. 1A) | 37634, 42671, 42672, 42673, 42674, 42675, 42676, 42677, 42678, 34916, 42652, 42655, 34910, 42651, 42654, 34898, 42650, 42653 |
| **Format B** (*see*, e.g., FIG. 1G) | 37638, 42679, 42680, 42681, 42682, 42683, 42684, 42685, 42686, 34913, 42658, 42661, 34907, 42657, 42660, 34895, 42656, 42659 |
| **Format C** (*see*, e.g., FIG. 1H) | 37642, 42687, 42688, 42689, 42690, 42691, 42692, 42693, 42694, 42664, 42667, 42670, 42663, 42666, 42669, 42662, 42665, 42668 |
| Bispecific controls | 42706, 42705, 42704, 42701, 42702, 42703, 37663, 39495, 38335, 34919, 36682, 36681 |

**TABLE 72B: Heavy and Light Chain Clone Numbers of Certain Trivalent and Trispecific Antibody Constructs (referred to as "variant ID") of this Disclosure (Corresponding Amino Acid Sequences are Provided in the Sequence Listing)**

| Variant ID | H1 | H2 | L1 |
|---|---|---|---|
| 42671 | CL_#28373 | CL_#25100 | CL_#31598 |
| 42672 | CL_#28373 | CL_#12989 | CL_#32590 |
| 42673 | CL_#28373 | CL_#25094 | CL_#26140 |
| 42674 | CL_#28373 | CL_#25100 | CL_#32587 |
| 42675 | CL_#28373 | CL_#12989 | CL_#32589 |
| 42676 | CL_#28373 | CL_#25094 | CL_#26128 |
| 42677 | CL_#28373 | CL_#25100 | CL_#32586 |
| 42678 | CL_#28373 | CL_#12989 | CL_#32588 |
| 37638 | CL_#26149 | CL_#28373 | CL_#26120 |
| 42679 | CL_#28373 | CL_#32593 | CL_#26120 |
| 42680 | CL_#28373 | CL_#32596 | CL_#26120 |
| 42681 | CL_#28373 | CL_#26137 | CL_#26111 |
| 42682 | CL_#28373 | CL_#32592 | CL_#26111 |
| 42683 | CL_#28373 | CL_#32595 | CL_#26111 |
| 42684 | CL_#28373 | CL_#26125 | CL_#26099 |
| 42685 | CL_#28373 | CL_#32591 | CL_#26099 |
| 42686 | CL_#28373 | CL_#32594 | CL_#26099 |

| | | | |
|---|---|---|---|
| 37642 | CL_#28382 | CL_#28383 | CL_#26120 |
| 42687 | CL_#32601 | CL_#28382 | CL_#26120 |
| 42688 | CL_#32602 | CL_#28382 | CL_#26120 |
| 42689 | CL_#28383 | CL_#32599 | CL_#26111 |
| 42690 | CL_#32601 | CL_#32599 | CL_#26111 |
| 42691 | CL_#32602 | CL_#32599 | CL_#26111 |
| 42692 | CL_#28383 | CL_#32600 | CL_#26099 |
| 42693 | CL_#32601 | CL_#32600 | CL_#26099 |
| 42694 | CL_#32602 | CL_#32600 | CL_#26099 |
| 34916 | CL_#25094 | CL_#26161 | CL_#26152 |
| 42652 | CL_#26161 | CL_#25100 | CL_#31598 |
| 42655 | CL_#26161 | CL_#12989 | CL_#32590 |
| 34910 | CL_#25094 | CL_#26161 | CL_#26140 |
| 42651 | CL_#26161 | CL_#25100 | CL_#32587 |
| 42654 | CL_#26161 | CL_#12989 | CL_#32589 |
| 34898 | CL_#25094 | CL_#26161 | CL_#26128 |
| 42650 | CL_#26161 | CL_#25100 | CL_#32586 |
| 42653 | CL_#26161 | CL_#12989 | CL_#32588 |
| 34913 | CL_#26149 | CL_#26161 | CL_#26120 |
| 42658 | CL_#26161 | CL_#32593 | CL_#26120 |
| 42661 | CL_#26161 | CL_#32596 | CL_#26120 |
| 34907 | CL_#26137 | CL_#26161 | CL_#26111 |
| 42657 | CL_#26161 | CL_#32592 | CL_#26111 |
| 42660 | CL_#26161 | CL_#32595 | CL_#26111 |
| 34895 | CL_#26125 | CL_#26161 | CL_#26099 |
| 42656 | CL_#26161 | CL_#32591 | CL_#26099 |
| 42659 | CL_#26161 | CL_#32594 | CL_#26099 |
| 42664 | CL_#28383 | CL_#27562 | CL_#26120 |
| 42667 | CL_#32601 | CL_#27562 | CL_#26120 |
| 42670 | CL_#32602 | CL_#27562 | CL_#26120 |
| 42663 | CL_#28383 | CL_#32598 | CL_#26111 |
| 42666 | CL_#32601 | CL_#32598 | CL_#26111 |
| 42669 | CL_#32602 | CL_#32598 | CL_#26111 |
| 42662 | CL_#28383 | CL_#32597 | CL_#26099 |
| 42665 | CL_#32601 | CL_#32597 | CL_#26099 |
| 42668 | CL_#32602 | CL_#32597 | CL_#26099 |

***Design of trivalent and trispecific antibody constructs targeting CLDN18.2, CD28 and CD3***

**[0936]** Similar constructs targeting CLDN18.2 were designed, as explained above in the MSLN example, except that all anti-MSLN paratopes were substituted with a CLDN18.2 targeting paratope. The anti-CLDN18.2 paratope sequences are further described (*see*, e.g., **EXAMPLE 2**).

***Production of Antibody Constructs***

**[0937]** 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)) or ExpiCHO-S™ cells (Thermofisher). The CHO cells were transfected in exponential growth phase with polyethylenimine (PEI, Polysciences) or ExpiFectamine™ CHO at an optimal DNA ratios of heavy chain A (HC-A), heavy chain B (HC-B), and light chain(s) (LC) that allow for heterodimer formation. Transfected cells were harvested after 5-6 days. The clarified culture medium was purified with MabSelect SuRe (GE Healthcare) protein-A. The eluted fractions containing multispecific antibodies were neutralized and analyzed by non-reducing and reducing High Throughput Protein Express assay using Caliper LabChip GXII (Perkin Elmer, Waltham, MA). The antibody constructs were further purified by CH1 select affinity chromatography and/or gel filtration chromatography. Fractions corresponding to the purified antibody constructs were collected, buffer exchanged into H6Su (50 mM histidine, 9% sucrose, pH 6.0) and concentrated to 0.5-2 mg/mL. The apparent purity of the final antibody construct was evaluated by UPLC-SEC and non-reducing CE-SDS. All antibody constructs were expressed and purified to >90% heterodimer purity.

## EXAMPLE 52: ABILITY OF TRIVALENT AND TRISPECIFIC ANTIBODY CONSTRUCTS TO BIND CD3 AND CD28 AS ASSESSED BY SURFACE PLASMON RESONANCE (SPR)

**[0938]** This example demonstrates the ability of some of the above-described anti-CD3 and anti-CD28 paratopes when part of a trivalent and trispecific construct to bind to the respective targets as assessed by SPR. SPR experiments were carried out as described in **EXAMPLES 4** and **8**.

**[0939] TABLE 73** shows the binding parameters, including the calculated $K_D$ values, for anti-CLDN18.2 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 antibody constructs can be demonstrated by the binding parameters reported below in **TABLE 73.**

**TABLE 73: CD3 and CD28 Binding Data of Certain Anti-CLDN18.2 Antibody Constructs**

| 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) |
| v37634 | 5.36E+04 | 1.14E-02 | 2.10E-07 | 4.47E+03 | 3.16E-03 | 1.77E-06 |
| v37638 | 5.48E+04 | 1.24E-02 | 2.32E-07 | 2.81E+04 | 1.09E-03 | 4.51E-08 |
| v37642 | 7.11E+04 | 9.82E-03 | 1.43E-07 | 2.68E+04 | 9.78E-04 | 4.71E-08 |

| | | | | | | |
|---|---|---|---|---|---|---|
| v42671 | 5.86E+04 | 2.58E-03 | 4.41E-08 | 1.17E+04 | 3.83E-03 | 3.25E-07 |
| v42673 | 5.17E+04 | 1.29E-02 | 2.52E-07 | 7.57E+03 | 1.94E-03 | 4.17E-07 |
| v42674 | 5.73E+04 | 2.75E-03 | 4.92E-08 | 3.30E+03 | 1.34E-03 | 4.06E-07 |
| v42676 | 4.81E+04 | 1.48E-02 | 3.09E-07 | 4.81E+03 | 9.81E-04 | 2.14E-07 |
| v42677 | 6.23E+04 | 2.64E-03 | 4.24E-08 | 1.07E+04 | 1.23E-03 | 1.22E-07 |
| v42679 | 4.94E+04 | 3.42E-03 | 7.56E-08 | 3.66E+04 | 9.09E-04 | 3.16E-08 |
| v42681 | 5.61E+04 | 1.23E-02 | 2.25E-07 | 2.24E+04 | 2.74E-04 | 1.33E-08 |
| v42682 | 5.14E+04 | 4.65E-03 | 1.17E-07 | 2.61E+04 | 1.92E-04 | 7.67E-09 |
| v42684 | 4.49E+04 | 9.17E-03 | 2.05E-07 | 1.57E+04 | 1.04E-03 | 6.78E-08 |
| v42685 | 5.52E+04 | 4.22E-03 | 1.10E-07 | 2.28E+04 | 1.31E-03 | 5.66E-08 |
| v42687 | 4.77E+04 | 3.64E-03 | 7.93E-08 | 3.63E+04 | 8.61E-04 | 3.07E-08 |
| v42689 | 5.39E+04 | 1.22E-02 | 2.41E-07 | 1.93E+04 | 3.65E-04 | 1.88E-08 |
| v42690 | 4.92E+04 | 4.15E-03 | 9.68E-08 | 2.38E+04 | 1.69E-04 | 8.39E-09 |
| v42693 | 5.66E+04 | 3.43E-03 | 6.90E-08 | 2.84E+04 | 1.07E-03 | 3.79E-08 |

**[0940] TABLE 74** shows the binding parameters, including the calculated $K_D$ values, for anti-MSLN 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 antibody constructs can be demonstrated by the binding parameters reported in **TABLE 74.**

**TABLE 74: CD3 and CD28 Binding Data of Tested Anti-MSLN Antibody Constructs**

| 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) |
| v34895 | 6.41E+04 | 9.41E-03 | 1.46E-07 | 2.84E+04 | 1.51E-03 | 6.46E-08 |
| v34907 | 5.31E+04 | 9.85E-03 | 1.87E-07 | 2.07E+04 | 1.39E-04 | 9.51E-09 |
| v34910 | 6.50E+04 | 1.34E-02 | 2.06E-07 | 1.12E+04 | 3.51E-03 | 3.64E-07 |
| v34913 | 5.00E+04 | 1.25E-02 | 2.49E-07 | 3.88E+04 | 1.04E-03 | 4.67E-08 |
| v34916 | 5.32E+04 | 1.41E-02 | 2.70E-07 | 3.31E+03 | 7.49E-03 | 4.48E-06 |
| v42650 | 7.99E+04 | 2.77E-03 | 3.72E-08 | 7.94E+03 | 8.51E-03 | 4.35E-07 |
| v42652 | 8.87E+04 | 2.79E-03 | 3.19E-08 | 2.01E+04 | 7.56E-03 | 3.90E-07 |
| v42656 | 3.00E+04 | 4.46E-03 | 1.60E-07 | 3.60E+04 | 1.67E-03 | 5.19E-08 |
| v42657 | 1.75E+04 | 3.64E-03 | 2.11E-07 | 2.63E+04 | 1.49E-04 | 7.60E-09 |
| v42658 | 1.47E+04 | 3.56E-03 | 2.52E-07 | 4.53E+04 | 1.05E-03 | 3.59E-08 |
| v42662 | 5.64E+04 | 1.04E-02 | 1.85E-07 | 3.37E+04 | 1.51E-03 | 4.94E-08 |
| v42663 | 6.10E+04 | 1.16E-02 | 1.90E-07 | 2.22E+04 | 2.23E-04 | 1.43E-08 |
| v42664 | 3.03E+04 | 1.07E-02 | 3.09E-05 | 3.73E+04 | 1.04E-03 | 3.54E-08 |
| v42665 | 1.87E+04 | 3.77E-03 | 2.05E-07 | 3.98E+04 | 1.99E-03 | 5.97E-08 |
| v42666 | 7.05E+03 | 2.46E-03 | 2.34E-06 | 2.79E+04 | 1.36E-04 | 6.95E-09 |
| v42667 | 6.14E+04 | 1.04E-03 | 1.71E-08 | 4.53E+04 | 1.07E-03 | 3.84E-08 |

***Binding of Certain Antibody Constructs to MSLN as Measured by SPR***

**[0941]** All SPR binding experiments were carried out using a Sierra SPR-32 Pro (Bruker) instrument at 25 °C with 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 at pH 7.4. Anti-human IgG antibody was immobilized by amine coupling to a High Capacity Amine

Sensor (HCA) chip to approximately 10000 RU. Trispecifc antibody constructs to be tested were captured on the sensor at 10 uL/min for 90 s to reach a surface density of roughly 300 RU. Recombinant human mesothelin his tag protein (Acro Biosystems) was flowed on the captured sensor chip at 50 µL/min for 240 s, following a dissociation for 800 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 repeated runs.

**[0942] TABLE 75** below shows the average binding parameters, including the calculated $K_D$ values, for certain tested trivalent and trispecific antibody constructs binding to mesothelin:

| Construct | Human mesothelin | | |
|---|---|---|---|
| | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
| v34913 | 1.32E+05 | 2.37E-03 | 1.79E-08 |
| v34916 | 1.52E+05 | 2.12E-03 | 1.40E-08 |
| v42664 | 1.48E+05 | 2.45E-03 | 1.66E-08 |

## EXAMPLE 53: IMPACT OF FORMAT AND PARATOPE COMBINATIONS OF TRISPECIFIC ANTIBODY CONSTRUCTS ON VIABILITY OF PRIMARY CD3+ T CELLS

**[0943]** To determine to what extent format and paratope combinations of tested constructs impact the viability of human CD3+ T cells, selected constructs were assessed in monocultures of primary human CD3+ T cells. More specifically, tested anti-(TAAxCD3xCD28) constructs encompassed those listed in **TABLE 72** above, and included MSLN-targeting as well as Cldn18.2-targeting constructs, and wherein each construct comprised 1 of 3 different anti-CD3 paratopes and 1 of 3 different anti-CD28 paratopes.

**[0944]** Briefly, constructs were diluted to 45 nM in RPMI1640 (Gibco) + 10% FBS (Gibco) + 1% Pen/Strep (Gibco) and plated in respective wells on a 384-well flat, optical-bottom assay plate (ThermoFisher Scientific). Frozen human CD3+ T cells were thawed at 37 °C and combined with CellTox™ Green dye (Promega). CD3+ T cells mixed 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, representing cell death, was detected using the Operetta® CLS™ (PerkinElmer) and data were analyzed using the Operetta CLS High Content Analysis System (PerkinElmer). Data are presented as pooled means of technical triplicates from two independent T cell donors. Background-adjusted Median Fluorescence Intensity (MFI) was determined using the following formula:

$$\text{Background-Adjusted MFI} = MFI_{Treated\ well} - Mean\ MFI_{T\ cell-onl\ \ wells}$$

**[0945]** Pooled mean background-adjusted MFI following treatment of primary CD3+ T cells with trispecific anti-TAAxCD3xCD28 constructs are summarized in **TABLE 76.** Statistical difference of T cell viability was determined using one way ANOVA followed by a Tukey's test using the pooled means of the independent donors.

**TABLE 76: Pooled Mean Background-adjusted MFI of CellTox™ Green by CD3+ T Cells Incubated in the Presence of Trispecific anti-(TAAxCD3xCD28) Constructs with different CD3, CD28, and TAA paratopes in different geometries.**

| Format | Mean | SEM | p-value (vs. Format A) |
|---|---|---|---|
| A | 26.9 | 5.2 | n/a |
| B | 129.8 | 12.9 | **** |
| C | 82.7 | 13.0 | ** |
| CD3xTAA Bispecific Control | 14.9 | 8.2 | ns |
| Negative Control (Palivizumab) | 4.3 | 6.8 | ns |
| Positive Control (Puromycin) | 483.4 | 47.9 | **** |

MFI= median fluorescence intensity; SEM = standard error of the mean; n/a = not applicable; ** = $p < 0.005$; **** = $p < 0.0001$; ns = not significant

**[0946]** This data (see also, **FIG. 40**) show that trivalent and trispecific anti-(CLDN18.2xCD3xCD28) antibody constructs of each format class tested (i.e., Formats A, B and C) had format-dependent effects on human primary CD3+ T cell viability, irrespective of the anti-TAA, -CD3, and -CD28 paratopes included in the respective construct. Specifically, these data demonstrate that constructs having format A exhibit significantly lower T cell cytotoxic effects when compared to constructs having format B or C.

## EXAMPLE 54: BINDING OF CD3 AND CD28 ON JURKAT CELLS BY SELECTED TRISPECIFIC ANTIBODY CONSTRUCTS WITH DIFFERENT PARATOPES AND GEOMETRIES

**[0947]** This example investigates via flow cytometry the impact of the format (e.g., having format A, B or C) of an antibody construct (tested were those listed in **TABLE 72A**) on its ability to bind CD3 and CD28 in either "cis" (on the same T cell) or in "trans" (on two different T cell).

**[0948]** 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 PBS (Gibco) and incubated at 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 Fixable viability dye eFluor 660 (eBiosciences). After viability staining, 1x$10^5$ 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).

**[0949]** Cells were then mixed with 5 nM of test construct and incubated at 4 °C for one hour. Following incubation, cells were acquired on the BD FACSymphony™ A1 Cell Analyzer and analyzed for percent of CellTrace™ Violet and CellTracker™ Green double positive events. Mean maximum percent double positive events of the tested constructs from the concentration response curves are presented in **TABLE 77.**

**[0950]** These data show (see also, **FIG. 42**) that antibody constructs having a Format A configuration of anti-CD3 and anti-CD28 binding domains exhibit significantly less T cell-T cell cross-linking compared to antibody constructs having a Format B or Format C configuration of anti-CD3 and anti-CD28 binding domains.

**TABLE 77: Maximum Percent Double-Positive Events of Cell-Cell Cross-linking Induced by Tested Antibody Constructs**

| **Representative Format** | **Maximum Percent Double Positive Events (Mean ± SEM)** | **p-value** (vs Format A) |
|---|---|---|
| Format A | 2.05 ± 0.26 | n/a |
| Format B | 7.34 ± 0.87 **** | ** |
| Format C | 10.34 ± 1.36 **** | **** |
| TAAxCD3 Bispecific Controls | 1.12 ± 0.35 ns | ns |
| Negative Control (Palivizumab) | 1.43 ± 0.13 ns | ns |

SEM = standard error of the mean; n/a = not applicable; ** = P <0.005; **** = p <0.0001; ns = not significant

## EXAMPLE 55: ABILITY OF TRISPECIFIC ANTIBODY CONSTRUCTS WITH DIFFERENT CD3 AND CD28 PARATOPES AND FORMATS TO BIND TO NATIVE CD28 ON JURKAT CELLS AS ASSESSED BY FLOW CYTOMETRY

**[0951]** The impact of antibody construct format on its ability to bind natively expressed CD28 on Jurkat$^{CD3\text{-}KO}$ cells was measured by flow cytometry as described below.

**[0952]** To that end, TCR knockout Jurkat cells (Jurkat$^{CD3\text{-}KO}$, BPS Bioscience) were washed twice by adding 150 µL PBS (Gibco) and centrifuged 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. The cells were resuspended to 1.25x10$^6$ 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 150 µL FACS buffer and centrifuging for 400xg for 3 minutes. Cells were resuspended in FACS buffer and acquired on the BD FACSymphony™ A1 Cell Analyzer.

**[0953]** The apparent binding affinities and $B_{max}$ of the tested constructs are shown in **TABLE 78**.

**TABLE 78: Whole Cell Binding of Trispecific Constructs to TCR knockout (Jurkat CD3-KO) Jurkat cells**

| | Jurkat CD3-KO | |
|---|---|---|
| **Antibody Construct (Format)** | **$EC_{50}$ (nM)** | **Bmax** |
| v37634 (A) | ND | 180 |
| v42673 (A) | ND | 421 |
| v42676 (A) | ND | 926 |
| v37638 (B) | 8.83 | 9401 |
| v42681 (B) | 3.14 | 18686 |
| v42684 (B) | 31.13 | 15550 |
| v37642 (C) | 8.79 | 8542 |
| v42689 (C) | 4.87 | 16291 |
| v42692 (C) | 26.59 | 13433 |
| v22277 (neg. ctrl.) | ND | 157 |

ND= Not Defined

## EXAMPLE 56: IMPACT OF ANTI-(MSLNXCD3XCD28) TRISPECIFIC CONSTRUCTS WITH DIFFERENT CD3 AND CD28 PARATOPES AND FORMATS ON *IN VITRO* CYTOTOXICITY TOWARDS MSLN-EXPRESSING TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ CD28+ T CELLS

**[0954]** To test the impact of antibody construct format on its anti-tumor cell activity and preferential killing of such tumor cells expressing MSLN, certain constructs were tested in primary human T cell co-cultured with allogeneic tumor cell lines expressing MSLN.

**[0955]** To that end, frozen human T cells were thawed at 37 °C and mixed with MSLN+ NCI-H292-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 respective antibody constructs titrated in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) 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).

**[0956]** Percent cytotoxicity was calculated using the following formula:

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

**[0957]** Calculated $IC_{50}$ values and maximum cytotoxicity (%) are shown in **TABLE 79.** Data are representative of 2 donors.

**TABLE 79: Potency and Maximum Cytotoxicity of Certain Trispecific Anti-MSLN Antibody Constructs Incubated with Primary Human T Cells in Co-culture with NCI-H292 Target Cells for 72 hours**

| | NCI-H292 | |
|---|---|---|
| **Antibody Construct** (see also, Table 62) | **$IC_{50}$ (pM)** | **Maximum Cytotoxicity (%)** |
| v34919 | 1025.00 | 62.84 |
| v36682 | 47.22 | 73.94 |
| v36681 | 9.42 | 75.15 |
| v34898 | 2748.00 | 80.31 |
| v34910 | 39.29 | 84.01 |
| v34916 | 183.20 | 85.55 |
| v42650 | 3151.00 | 83.86 |
| v42651 | 41.49 | 87.46 |
| v42652 | 84.64 | 88.17 |
| v42653 | 652.00 | 65.28 |
| v42654 | 10.17 | 86.83 |
| v42655 | 30.80 | 83.60 |
| v34895 | 3.61 | 85.20 |
| v34907 | 9.36 | 79.00 |
| v34913 | 6.12 | 79.81 |
| v42656 | 85.73 | 82.52 |
| v42657 | 211.70 | 79.87 |
| v42658 | 248.70 | 77.23 |
| v42659 | 3071.00 | 72.78 |
| v42660 | 110.00 | 78.58 |
| v42661 | 819.70 | 29.18 |
| v42662 | 2.19 | 87.96 |

| | | |
|---|---|---|
| v42663 | 2.31 | 83.28 |
| v42664 | 2.55 | 83.71 |
| v42665 | 207.10 | 86.50 |
| v42666 | 217.30 | 83.48 |
| v42667 | 340.10 | 78.45 |
| v42669 | 101.60 | 83.25 |
| v42670 | 5115.00 | 70.76 |
| v42702 | 10395.00 | 75.34 |

**EXAMPLE 57: IMPACT OF ANTI-(CLDN18.2XCD3XCD28) TRISPECIFIC ANTIBODY CONSTRUCTS WITH DIFFERENT CD3 AND CD28 PARATOPES AND FORMATS ON *IN VITRO* CYTOTOXICITY TOWARDS CLDN18.2-EXPRESSING TUMOR CELL LINES IN CO-CULTURE WITH PRIMARY HUMAN CD3+ CD28+ T CELLS**

**[0958]** To test the impact of antibody construct format on its anti-tumor cell activity and preferential killing of such tumor cells expressing CLDN18.2, selected constructs were tested in primary human T cell co-cultured with allogeneic tumor cell lines expressing CLDN18.2.

**[0959]** To that end, frozen human T cells were thawed at 37 °C and mixed with CLDN18.2+ SNU-601-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 titrated in RPMI1640 (Gibco) + 10% FBS (ThermoFisher) 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).

**[0960]** Percent cytotoxicity was calculated using the following formula:

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

**[0961]** Calculated $IC_{50}$ and maximum cytotoxicity (%) are shown in **TABLE 80.** Data are representative of 2 donors.

**TABLE 80: Potency and Maximum Cytotoxicity of anti-CLDN18.2 Trispecific Antibody Constructs Incubated with Primary Human T Cells in Co-culture with SNU-601 Target Cells for 72 hours.**

| | SNU-601 | |
|---|---|---|
| **Antibody Construct** | **$IC_{50}$ (pM)** | **Maximum Cytotoxicity (%)** |
| v37663 | 10.03 | 93.10 |
| v39495 | 4.53 | 93.61 |

| | | |
|---|---|---|
| v38335 | 0.66 | 93.28 |
| v42676 | 3.35 | 94.11 |
| v42673 | 2.10 | 94.56 |
| v37634 | 7.95 | 94.30 |
| v42677 | 4.90 | 94.60 |
| v42674 | 6.86 | 94.65 |
| v42671 | 5.49 | 94.54 |
| v42678 | 1.84 | 94.35 |
| v42675 | 1.56 | 94.96 |
| v42672 | 1.67 | 94.65 |
| v42684 | 1.53 | 93.67 |
| v42681 | 1.75 | 94.45 |
| v37638 | 1.70 | 94.47 |
| v42685 | 4.56 | 92.98 |
| v42682 | 6.71 | 94.49 |
| v42679 | 4.33 | 94.20 |
| v42686 | 9.23 | 93.08 |
| v42683 | 1.34 | 93.22 |
| v42680 | 2.04 | 94.13 |
| v42692 | 0.68 | 94.29 |
| v42689 | 0.67 | 94.41 |
| v37642 | 0.41 | 95.11 |
| v42693 | 2.59 | 93.35 |
| v42690 | 2.35 | 93.81 |
| v42687 | 1.54 | 93.84 |
| v42694 | 1.20 | 93.47 |
| v42691 | 0.37 | 94.23 |
| v42688 | 0.52 | 93.67 |

**EXAMPLE 58: TARGET INDEPENDENT CYTOKINE PRODUCTION BY PRIMARY HUMAN PBMCS IN THE PRESENCE OF TRISPECIFIC ANTI-(TAAxCD3xCD28) ANTIBODY CONSTRUCTS WITH VARYING CD3. CD28, AND TAA PARATOPES**

**[0962]** To determine the impact of format and different combinations of anti-CD3, anti-CD28, and/or anti-TAA paratopes of certain trispecific antibody constructs on induction of target-independent cytokine production, tested constructs (see, **TABLE 72A**) were incubated with primary human PBMCs and THP-1 cells, a TAA-negative cell line.

**[0963]** Test articles (e.g., antibody constructs, controls, etc.) were titrated in RPMI 1640 (Gibco) + 10% FBS (ThermoFisher) and added to appropriate wells of the 384-well black flat bottom assay plates (ThermoFisher, Watham, MA). Human PBMCs were thawed and resuspended as monocultures of PBMCs cells then added to the plates. Plates were incubated for 72 hrs 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.

**[0964]** IL-2 was quantified using MSD (Mesoscale Discovery, Piscataway, NJ). The night before cytokine quantification, MSD plates were blocked and coated in capture constructs 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 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 antibody constructs 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.

**[0965]** Pooled mean cytokine concentrations following treatment of primary PBMCs with the tested constructs are summarized in **TABLE 81** below (see also, **FIG. 41**). Statistical differences of mean IL-2 concentrations were determined using one way ANOVA followed by a Tukey's test using the pooled means of the constructs with two independent donors.

**TABLE 81: Pooled Mean IL-2 production by PBMCs incubated with Certain Antibody Constructs or Controls**

| Antibody Constructs with Format | Pooled Mean IL-2 Concentration (pg/mL) | SEM | P value (vs. Format A) |
|---|---|---|---|
| A | 33.5 | 10.9 | n/a |
| B | 157.2 | 51.1 | ns |
| C | 1691.4 | 463.1 | **** |
| Bispecific anti TAAxCD3 controls | 45.9 | 30.6 | ns |
| Negative Control (v39982) | 19.2 | 6.1 | ns |

SEM = standard error of the mean; n/a = not applicable; **** = $p < 0.0001$; ns = not significant

**[0966]** 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.

**[0967]** 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

**[0968]** 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 |
| | $V_H$ | CD3 | 82 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMN WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKD RFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHKN FGNSYVTWFAYWGQGTLVTVSS |
| | $V_L$ | CD3 | 83 | QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYA NWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLL GGKAALTITGAQAEDEADYYCALWYSNLWVFGG GTKLTVL |
| | $V_H$ | CD3 | 84 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG NFGNSYVSWFAYWGQGTLVTVSS |
| | $V_L$ | CD3 | 85 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG TKLTVL |
| | $V_H$ | CD3 | 86 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYGMN WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKD RFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGN FGNSYVSWFAYWGQGTLVTVSS |
| | $V_L$ | CD3 | 87 | QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYA NWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLL GGKAALTITGAQAEDEADYYCALWYSNLWVFGG GTKLTVL |
| ***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<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | Linker$^{scFv\text{-}Fc}$-CH2-CH3 | HC B | 12 | EPKSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL<br>MISRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVH<br>NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY<br>KCKVSNKALPAPIEKTISKAKGQPREPQVYVLPPSR<br>DELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENN<br>YLTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS<br>CSVMHEALHNHYTQKSLSLSPG |
| 26152 | Anti-CD3 Fab-Linker$^{Fab\text{-}scFv}$-anti-CD28 scFv | LC | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| | Fab heavy chain | CD3 | 14 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKV |
| | VH | Cldn18.2 | 130 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSS |
| | VL | Cldn18.2 | 131 | DGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIK |
| | VH | MSLN | 132 | QVQLVQSGAEVKKPGASVKISCKASGYSFTGYTM<br>NWVRQAPGQGLEWIGLITPYSGASSYAQKFQGRA |

| | | | | |
|---|---|---|---|---|
| | | | | TLTVDKSTSTAYMELSSLRSEDTAVYYCARGGYD GRGFDYWGQGTNVTVSS |
| | VL | MSLN | 133 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI K |
| | VH | DLL3 | 152 | QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS |
| | VL | DLL3 | 153 | DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK |
| | VH | DLL3 | 154 | QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV HWVRQAPGQCLEWMVIIDPGGGTTSYAQKFQGRV TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS |
| | VL | DLL3 | 155 | DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK |
| | VH | DLL3 | 156 | QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFQGRV TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV TGNYFYYGMDVWGQGTTVTVSS |
| | VL | DLL3 | 157 | DIVMTQSPDSLAVSLGERATINCKSSQGISNYLAW YQQKPGQPPKLLIYAVSTRESGVPDRFSGSGSGTDF TLTISSLQAEDVAVYYCLQHDSYPYTFGQGTKLEIK |
| ***v37634 – CD3xCD28xCldn18.2 Trispecific Antibody Construct*** | | | | |
| **25094** | H | CD3 | 11 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **28373** | H | CLDN 18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW |

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| | | | | YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26152** | L | CD3/ CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| ***v40062 – Cldn18.2xCD28xCD3 Trispecific Antibody Construct*** | | | | |
| **30477** | H | CD3 | 89 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| **30478** | H | Cldn18.2 | 90 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV |

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| | | | | DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| | L | CD3/CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| ***v39452 – Cldn18.2xCD28xCD3 Trispecific Antibody Construct*** | | | | |
| **25096** | H | CD3 | 91 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **28373** | H | CLDN 18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE |

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| | | | | SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26152** | L | CD3/CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| ***v40063 – Cldn18.2xCD28xCD3 Trispecific Antibody Construct*** | | | | |
| **25096** | H | CD3 | 91 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| **28373** | H | CLDN 18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| **26152** | L | CD3/CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS |

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| | | | | KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| ***v34916 – CD3xCD28xMSLN Trispecific Antibody Construct*** | | | | |
| **25094** | H | CD3 | 11 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26161** | H | MSLN | 92 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY<br>TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFPP<br>KPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVD<br>GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL<br>NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY<br>VLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESN<br>GQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRWQ<br>QGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26152** | L | CD3/ CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT |

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| | | | | ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| ***v34916 – CD3xCD28xCldn18.2 Trispecific Antibody Construct*** | | | | |
| **25100** | H | CD3 | 93 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPL<br>APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL<br>TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| **28373** | H | Cldn18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **29931** | L | CD3/CD28 | 94 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG<br>TKLTVLSSGQPKAAPSVTLFPPSSEELQANKATLVC<br>LISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN<br>NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK<br>TVAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTIT<br>CRASESVEYYVTSLMQWYQQKPGKAPKLLIYAAS<br>NVDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQ |

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| | | | | QSRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSG<br>GGGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSY<br>GVHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSR<br>LTISEDTSKNQVSLKLSSVTAADTAVYYCARDRAY<br>GNYLYAMDYWGQGTLVTVSS |
| ***v39499 – CD3xCD28xCldn18.2 Trispecific Antibody Construct*** | | | | |
| **29934** | H | CD3 | 95 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYGMN<br>WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKD<br>RFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGN<br>FGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPLAP<br>SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS<br>GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN<br>VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG<br>GPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPE<br>VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV<br>LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK<br>GQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPS<br>DIAVEWESNGQPENNYKTTPPVLDSDGSFALVSKL<br>TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS<br>PG |
| **28373** | H | Cldn18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **29935** | L | CD3/CD28 | 96 | QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYA<br>NWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLL<br>GGKAALTITGAQAEDEADYYCALWYSNLWVFGG<br>GTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVC<br>LISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN<br>NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK<br>TVAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTIT<br>CRASESVEYYVTSLMQWYQQKPGKAPKLLIYAAS<br>NVDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQ<br>QSRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSG<br>GGGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSY<br>GVHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSR |

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| | | | | LTISEDTSKNQVSLKLSSVTAADTAVYYCARDRAY<br>GNYLYAMDYWGQGTLVTVSS |
| ***Costimulatory Heavy-Light Chain Pair: anti-CD3 Fab domain and LC-coupled anti-CD28 scFv domain*** | | | | |
| **30609** | H | CD3 | 97 | EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVGRIRSKYNNYATYYADSVKD<br>RFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHKN<br>FGNSYVTWFAYWGQGTLVTVSSASTKGPSVFPLAP<br>SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS<br>GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN<br>VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG<br>GPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPE<br>VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV<br>LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK<br>GQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPS<br>DIAVEWESNGQPENNYKTTPPVLDSDGSFALVSKL<br>TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS<br>PG |
| **29935** | L | CD3/CD28 | 98 | QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYA<br>NWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLL<br>GGKAALTITGAQAEDEADYYCALWYSNLWVFGG<br>GTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVC<br>LISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN<br>NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK<br>TVAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTIT<br>CRASESVEYYVTSLMQWYQQKPGKAPKLLIYAAS<br>NVDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQ<br>QSRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSG<br>GGGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSY<br>GVHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSR<br>LTISEDTSKNQVSLKLSSVTAADTAVYYCARDRAY<br>GNYLYAMDYWGQGTLVTVSS |
| *v39496 – CD3xCD28xCldn18.2 Trispecific Antibody Construct* | | | | |
| **25100** | H | CD3 | 93 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPL<br>APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL<br>TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK |

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| | | | | LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| **28373** | H | Cldn18.2 | 88 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **29931** | L | CD3/CD28 | 99 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG<br>TKLTVLSSGQPKAAPSVTLFPPSSEELQANKATLVC<br>LISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN<br>NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK<br>TVAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTIT<br>CRASESVEYYVTSLMQWYQQKPGKAPKLLIYAAS<br>NVDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQ<br>QSRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSG<br>GGGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSY<br>GVHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSR<br>LTISEDTSKNQVSLKLSSVTAADTAVYYCARDRAY<br>GNYLYAMDYWGQGTLVTVSS |
| *v34910 – CD3xCD28(CD28.3)xMSLN Trispecific Antibody Construct* | | | | |
| **25094** | H | CD3 | 11 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26161** | H | MSLN | 92 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY |

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| | | | | TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFPP<br>KPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVD<br>GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL<br>NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY<br>VLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESN<br>GQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRWQ<br>QGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26140** | L | CD3/CD28 | 100 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCKTNE<br>NIYSNLAWYQQKDGKSPQLLIYAATHLVEGVPSRF<br>SGSGSGTQYSLTISSLQPEDFGNYYCQHFWGTPATF<br>GGGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLQ<br>QSGAELKKPGASVKVSCKASGYTFTEYIIHWIKLRS<br>GQGLEWIGWFYPGSNDIQYNAQFKGKATLTADKS<br>SSTVYMELTGLTPEDSAVYFCARRDDFSGYDALPY<br>WGQGTLVTVSA |
| *v34898 – CD3xCD28 (TGN1412)xMSLN Trispecific Antibody Construct* | | | | |
| **25094** | H | CD3 | 11 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26161** | H | MSLN | 92 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY<br>TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM |

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| | | | | ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFPP<br>KPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVD<br>GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL<br>NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY<br>VLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESN<br>GQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRWQ<br>QGNVFSCSVMHEALHNHYTQKSLSLSPG |
| **26128** | L | CD3/CD28 | 101 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCHASQ<br>NIYVWLNWYQQKPGKAPKLLIYKASNLHTGVPSR<br>FSGSGSGTDFTLTISSLQPEDFATYYCQQGQTYPYT<br>FGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQVQL<br>VQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVR<br>QAPGQGLEWIGCIYPGNVNTNYNEKFKDRATLTV<br>DTSISTAYMELSRLRSDDTAVYFCTRSHYGLDWNF<br>DVWGQGTTVTVSS |
| **v41086** (Het-Fab) | H1 (31129) | CD3 | 166 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | H2 (31130) | DLL3 | 167 | QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV<br>HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV<br>TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV<br>TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA<br>PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT<br>SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC<br>NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA<br>GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP<br>EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP |

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| | | | | SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | L1 (31131) | CD3/CD28 | 168 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| | L2 (31132) | DLL3 | 169 | DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW<br>FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF<br>TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK<br>RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>EC |
| **v41759** (scFv$^2$) | H1 (31417) | CD3 | 91 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | H2 (31703) | DLL3 | 170 | DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW<br>FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF<br>TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK<br>GGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKK<br>PGSSVKVSCKASGYTFVHYYVHWVRQAPGQCLE<br>WMVIIDPGGGTTSYAQKFQGRVTMTRDTSTNTVY<br>MELSSLRSEDTAVYYCARGERVTGNYFYYGMDV<br>WGQGTTVTVSSEPKSSDKTHTCPPCPAPEAAGGPS<br>VFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKF |

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| | | | | NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV<br>LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP<br>REPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAV<br>EWESNGQPENNYLTWPPVLDSDGSFFLYSKLTVDK<br>SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | L (31416) | CD3/CD28 | 13 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| **v41760** (scFv$^2$) | H1 (31418) | CD3 | 93 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPL<br>APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL<br>TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | H2 (31703) | DLL3 | 170 | DIQMTQSPSTLSASVGDRVTITCVANQGISNYLVW<br>FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF<br>TLTISSLQPDDFATYYCLQHDSYPYTFGCGTKLEIK<br>GGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKK<br>PGSSVKVSCKASGYTFVHYYVHWVRQAPGQCLE<br>WMVIIDPGGGTTSYAQKFQGRVTMTRDTSTNTVY<br>MELSSLRSEDTAVYYCARGERVTGNYFYYGMDV<br>WGQGTTVTVSSEPKSSDKTHTCPPCPAPEAAGGPS<br>VFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKF<br>NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV<br>LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP<br>REPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAV |

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| | | | | EWESNGQPENNYLTWPPVLDSDGSFFLYSKLTVDK<br>SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | L (31700) | CD3/CD28 | 171 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG<br>TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI<br>SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN<br>KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKT<br>VAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTITC<br>RASESVEYYVTSLMQWYQQKPGKAPKLLIYAASN<br>VDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQ<br>SRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSGG<br>GGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSYG<br>VHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSRL<br>TISEDTSKNQVSLKLSSVTAADTAVYYCARDRAYG<br>NYLYAMDYWGQGTLVTVSS |
| **v41112** (Het-Fab) | H1 (31129) | CD3 | 166 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITRSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | H2 (31141) | DLL3 | 172 | QVQLVQSGAEVKKPGSSVKVSCKASGYTFVHYYV<br>HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFQGRV<br>TMTRDTSTNTVYMELSSLRSEDTAVYYCARGERV<br>TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA<br>PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT<br>SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC<br>NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA<br>GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP<br>EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP<br>SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | L1 (31131) | CD3/CD28 | 168 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT |

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| | | | | VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| | L2 (31144) | DLL3 | 173 | DIVMTQSPDSLAVSLGERATINCKSSQGISNYLAW<br>YQQKPGQPPKLLIYAVSTRESGVPDRFSGSGSGTDF<br>TLTISSLQAEDVAVYYCLQHDSYPYTFGQGTKLEIK<br>RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>E |
| **v41094** (Het-Fab) | H1 (31145) | CD3 | 174 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLRPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE<br>SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | H2 (31130) | DLL3 | 167 | QVQLVQSGAEVKKPGASVKVSCKASGYTFVHYYV<br>HWVRQAPGQGLEWMVIIDPGGGTTSYAQKFLGRV<br>TMTRDTSTNTVYMELKSLRSEDTAVYYCARGERV<br>TGNYFYYGMDVWGQGTTVTVSSASTKGPSVFPLA<br>PSSKSTSGGTAALGCEVTDYFPEPVTVSWNSGALT<br>SGVHTFPAVLESSGLYSLSSVVTVPSSSLGTQTYIC<br>NVNHKPSNTKVDKKVEPDSCDKTHTCPPCPAPEAA<br>GGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHEDP<br>EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVLPPSRDELTKNQVSLLCLVKGFYP<br>SDIAVEWESNGQPENNYLTWPPVLDSDGSFFLYSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |

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| | L1 (31131) | CD3/CD28 | 168 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSDEELQANTATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>ESELSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV<br>PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| | L2 (31132) | DLL3 | 169 | DIQMTQSPSAMSASVGDRVTITCVANQGISNYLVW<br>FQQKPGKAPKRLIYSVNSLYSGVPSRFSGSGSGTEF<br>TLTISSLQPEDFATYYCLQHDSYPYTFGQGTKLEIK<br>RTVAAPSVFIFPPKDERLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>RLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>EC |
| | CL_#12989 | | 175 | QVQLVESGGGVVQPGRSLRLSCKASGYTFTRSTM<br>HWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRF<br>TISADKSKSTAFLQMDSLRPEDTGVYFCARPQVHY<br>DYNGFPYWGQGTPVTVSSASTKGPSVFPLAPSSKS<br>TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH<br>TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH<br>KPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS<br>VFLFPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKF<br>NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV<br>LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP<br>REPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIA<br>VEWESNGQPENNYKTTPPVLDSDGSFALVSKLTVD<br>KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | CL_#25094 | | 176 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG<br>GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA<br>VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN<br>TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF<br>PPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWY<br>VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE |

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| | | | | SNGQPENNYKTTPPVLDSDGSFALVSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | CL_#25100 | | 177 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPL<br>APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL<br>TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | CL_#26099 | | 178 | DIQMTQSPSSLSASVGDRVTITCHASQNIYVWLNW<br>YQQKPGKAPKLLIYKASNLHTGVPSRFSGSGSGTD<br>FTLTISSLQPEDFATYYCQQGQTYPYTFGGGTKVEI<br>KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR<br>EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS<br>STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR<br>GEC |
| | CL_#26111 | | 179 | DIQMTQSPSSLSASVGDRVTITCKTNENIYSNLAWY<br>QQKDGKSPQLLIYAATHLVEGVPSRFSGSGSGTQY<br>SLTISSLQPEDFGNYYCQHFWGTPATFGGGTKLEIK<br>RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>EC |
| | CL_#26120 | | 180 | DIQMTQSPSSLSASVGDRVTITCRASESVEYYVTSL<br>MQWYQQKPGKAPKLLIYAASNVDSGVPSRFSGSG<br>SGTDFTLTISSLQPEDIATYYCQQSRKVPFTFGGGT<br>KVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNN<br>FYPREAKVQWKVDNALQSGNSQESVTEQDSKDST<br>YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK<br>SFNRGEC |
| | CL_#26125 | | 181 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSGGGGSGGGGSGGGGSGGG<br>GSNFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYV<br>NWYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRS<br>SKSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGGGGSGQVQLVQSGAEVKKPGASVKVSCKASG<br>YTFTSYYIHWVRQAPGQGLEWIGCIYPGNVNTNYN |

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| | | | | EKFKDRATLTVDTSISTAYMELSRLRSDDTAVYFC<br>TRSHYGLDWNFDVWGQGTTVTVSSASTKGPSVFP<br>LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA<br>LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK<br>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | CL_#26128 | | 182 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCHASQ<br>NIYVWLNWYQQKPGKAPKLLIYKASNLHTGVPSR<br>FSGSGSGTDFTLTISSLQPEDFATYYCQQGQTYPYT<br>FGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQVQL<br>VQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVR<br>QAPGQGLEWIGCIYPGNVNTNYNEKFKDRATLTV<br>DTSISTAYMELSRLRSDDTAVYFCTRSHYGLDWNF<br>DVWGQGTTVTVSS |
| | CL_#26137 | | 183 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSGGGGSGGGGSGGGGSGGG<br>GSNFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYV<br>NWYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRS<br>SKSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGGGGSGQVQLQQSGAELKKPGASVKVSCKASG<br>YTFTEYIIHWIKLRSGQGLEWIGWFYPGSNDIQYNA<br>QFKGKATLTADKSSSTVYMELTGLTPEDSAVYFCA<br>RRDDFSGYDALPYWGQGTLVTVSAASTKGPSVFPL<br>APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL<br>TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI<br>CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEA<br>AGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSHED<br>PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA<br>KGQPREPQVYVYPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTPPVLDSDGSFALVSK |

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| | | | | LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL<br>SPG |
| | CL_#26140 | | 184 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCKTNE<br>NIYSNLAWYQQKDGKSPQLLIYAATHLVEGVPSRF<br>SGSGSGTQYSLTISSLQPEDFGNYYCQHFWGTPATF<br>GGGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLQ<br>QSGAELKKPGASVKVSCKASGYTFTEYIIHWIKLRS<br>GQGLEWIGWFYPGSNDIQYNAQFKGKATLTADKS<br>SSTVYMELTGLTPEDSAVYFCARRDDFSGYDALPY<br>WGQGTLVTVSA |
| | CL_#26149 | | 185 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSGGGGSGGGGSGGGGSGGG<br>GSNFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYV<br>NWYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRS<br>SKSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGGGGSGQVQLQESGPGLVKPSETLSLTCAVSGF<br>SLTSYGVHWIRQPPGKGLEWLGVIWPGGGTNFNS<br>ALMSRLTISEDTSKNQVSLKLSSVTAADTAVYYCA<br>RDRAYGNYLYAMDYWGQGTLVTVSSASTKGPSV<br>FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS<br>GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ<br>TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA<br>PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVS<br>HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR<br>VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI<br>SKAKGQPREPQVYVYPPSRDELTKNQVSLTCLVKG<br>FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFAL<br>VSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK<br>SLSLSPG |
| | CL_#26152 | | 186 | NFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYVN<br>WYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRSS<br>KSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFY<br>PGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAA<br>SSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT<br>ECSGGGGSGDIQMTQSPSSLSASVGDRVTITCRASE<br>SVEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSG<br>VPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKV |

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| | | | | PFTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQ<br>VQLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWI<br>RQPPGKGLEWLGVIWPGGGTNFNSALMSRLTISED<br>TSKNQVSLKLSSVTAADTAVYYCARDRAYGNYLY<br>AMDYWGQGTLVTVSS |
| | CL_#26161 | | 187 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY<br>TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFPP<br>KPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVD<br>GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL<br>NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY<br>VLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESN<br>GQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRWQ<br>QGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | CL_#27562 | | 188 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY<br>TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSGGGGSGQVQLQESGPGLVKPSETLSLTCAVS<br>GFSLTSYGVHWIRQPPGKGLEWLGVIWPGGGTNF<br>NSALMSRLTISEDTSKNQVSLKLSSVTAADTAVYY<br>CARDRAYGNYLYAMDYWGQGTLVTVSSASTKGP<br>SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW<br>NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG<br>TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC<br>PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVS<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST<br>YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE<br>KTISKAKGQPREPQVYVYPPSRDELTKNQVSLTCL<br>VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS<br>FALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#28373 | | 189 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF |

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| | | | | TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| | CL_#28382 | | 190 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKGGGGSGQVQLQESGPGLVKPSETLSLTCAV<br>SGFSLTSYGVHWIRQPPGKGLEWLGVIWPGGGTNF<br>NSALMSRLTISEDTSKNQVSLKLSSVTAADTAVYY<br>CARDRAYGNYLYAMDYWGQGTLVTVSSASTKGP<br>SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW<br>NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG<br>TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC<br>PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVS<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST<br>YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE<br>KTISKAKGQPREPQVYVYPPSRDELTKNQVSLTCL<br>VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS<br>FALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#28383 | | 191 | EVQLVESGGGLVQPGGSLRLSCAASGVTFNYYGM<br>SWIRQAPGKGLEWVASITSSGGRIYYPDSVKGRFTI<br>SRENTQKTLYLQMNSLRAEDTAVYYCTLDGRDG<br>WVAYWGQGTLVTVSSGGGGSGGGGSGGGGSGGG<br>GSNFMLTQPHSVSESPGKTVTISCKRNTGNIGSNYV<br>NWYQQHEGSSPTTIIYRNDKRPDGVSDRFSGSIDRS<br>SKSASLTISNLKTEDEADYFCQSYSSGFIFGGGTKLT<br>VLAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFPPKP<br>KDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVDG<br>VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN<br>GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYV<br>LPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNG<br>QPENNYLTWPPVLDSDGSFFLYSKLTVDKSRWQQ<br>GNVFSCSVMHEALHNHYTQKSLSLSPG |
| | CL_#31598 | | 192 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG |

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| | | | | TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI<br>SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN<br>KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKT<br>VAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTITC<br>RASESVEYYVTSLMQWYQQKPGKAPKLLIYAASN<br>VDSGVPSRFSGSGSGTDFTLTISSLQPEDIATYYCQQ<br>SRKVPFTFGGGTKVEIKGGGGSGGGGSGGGGSGG<br>GGSQVQLQESGPGLVKPSETLSLTCAVSGFSLTSYG<br>VHWIRQPPGKGLEWLGVIWPGGGTNFNSALMSRL<br>TISEDTSKNQVSLKLSSVTAADTAVYYCARDRAYG<br>NYLYAMDYWGQGTLVTVSS |
| | CL_#32586 | | 193 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG<br>TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI<br>SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN<br>KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKT<br>VAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTITC<br>HASQNIYVWLNWYQQKPGKAPKLLIYKASNLHTG<br>VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGQT<br>YPYTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGS<br>QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIH<br>WVRQAPGQGLEWIGCIYPGNVNTNYNEKFKDRAT<br>LTVDTSISTAYMELSRLRSDDTAVYFCTRSHYGLD<br>WNFDVWGQGTTVTVSS |
| | CL_#32587 | | 194 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYA<br>NWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLG<br>GKAALTLSGAQPEDEAEYYCALWYSNLWVFGGG<br>TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI<br>SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN<br>KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKT<br>VAPTECSGGGGSGDIQMTQSPSSLSASVGDRVTITC<br>KTNENIYSNLAWYQQKDGKSPQLLIYAATHLVEG<br>VPSRFSGSGSGTQYSLTISSLQPEDFGNYYCQHFWG<br>TPATFGGGTKLEIKGGGGSGGGGSGGGGSGGGGS<br>QVQLQQSGAELKKPGASVKVSCKASGYTFTEYIIH<br>WIKLRSGQGLEWIGWFYPGSNDIQYNAQFKGKAT<br>LTADKSSSTVYMELTGLTPEDSAVYFCARRDDFSG<br>YDALPYWGQGTLVTVSA |
| | CL_#32588 | | 195 | DIQMTQSPSSLSASVGDRVTMTCSASSSVSYMNWY<br>QQKPGKAPKRWIYDSSKLASGVPARFSGSGSGTDY<br>TLTISSLQPEDFATYYCQQWSRNPPTFGGGTKLQIT<br>RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>ECGGGGSGDIQMTQSPSSLSASVGDRVTITCHASQ |

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| | | | | NIYVWLNWYQQKPGKAPKLLIYKASNLHTGVPSR<br>FSGSGSGTDFTLTISSLQPEDFATYYCQQGQTYPYT<br>FGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQVQL<br>VQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVR<br>QAPGQGLEWIGCIYPGNVNTNYNEKFKDRATLTV<br>DTSISTAYMELSRLRSDDTAVYFCTRSHYGLDWNF<br>DVWGQGTTVTVSS |
| | CL_#32589 | | 196 | DIQMTQSPSSLSASVGDRVTMTCSASSSVSYMNWY<br>QQKPGKAPKRWIYDSSKLASGVPARFSGSGSGTDY<br>TLTISSLQPEDFATYYCQQWSRNPPTFGGGTKLQIT<br>RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>ECGGGGSGDIQMTQSPSSLSASVGDRVTITCKTNE<br>NIYSNLAWYQQKDGKSPQLLIYAATHLVEGVPSRF<br>SGSGSGTQYSLTISSLQPEDFGNYYCQHFWGTPATF<br>GGGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLQ<br>QSGAELKKPGASVKVSCKASGYTFTEYIIHWIKLRS<br>GQGLEWIGWFYPGSNDIQYNAQFKGKATLTADKS<br>SSTVYMELTGLTPEDSAVYFCARRDDFSGYDALPY<br>WGQGTLVTVSA |
| | CL_#32590 | | 197 | DIQMTQSPSSLSASVGDRVTMTCSASSSVSYMNWY<br>QQKPGKAPKRWIYDSSKLASGVPARFSGSGSGTDY<br>TLTISSLQPEDFATYYCQQWSRNPPTFGGGTKLQIT<br>RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE<br>AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS<br>TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG<br>ECGGGGSGDIQMTQSPSSLSASVGDRVTITCRASES<br>VEYYVTSLMQWYQQKPGKAPKLLIYAASNVDSGV<br>PSRFSGSGSGTDFTLTISSLQPEDIATYYCQQSRKVP<br>FTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQV<br>QLQESGPGLVKPSETLSLTCAVSGFSLTSYGVHWIR<br>QPPGKGLEWLGVIWPGGGTNFNSALMSRLTISEDT<br>SKNQVSLKLSSVTAADTAVYYCARDRAYGNYLYA<br>MDYWGQGTLVTVSS |
| | CL_#32591 | | 198 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSGGGGSGGGGS<br>GGGGSGGGGSQAVVTQEPSLTVSPGGTVTLTCGSS<br>TGAVTTSNYANWVQEKPGQAFRGLIGGTNKRAPG<br>TPARFSGSLLGGKAALTLSGAQPEDEAEYYCALW<br>YSNLWVFGGGTKLTVLGGGGSGQVQLVQSGAEV<br>KKPGASVKVSCKASGYTFTSYYIHWVRQAPGQGL<br>EWIGCIYPGNVNTNYNEKFKDRATLTVDTSISTAY<br>MELSRLRSDDTAVYFCTRSHYGLDWNFDVWGQG |

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| | | | | TTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV<br>KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS<br>LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE<br>PKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM<br>ISRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVHN<br>AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK<br>CKVSNKALPAPIEKTISKAKGQPREPQVYVYPPSRD<br>ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY<br>KTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCS<br>VMHEALHNHYTQKSLSLSPG |
| | CL_#32592 | | 199 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSGGGGSGGGGS<br>GGGGSGGGGSQAVVTQEPSLTVSPGGTVTLTCGSS<br>TGAVTTSNYANWVQEKPGQAFRGLIGGTNKRAPG<br>TPARFSGSLLGGKAALTLSGAQPEDEAEYYCALW<br>YSNLWVFGGGTKLTVLGGGGSGQVQLQQSGAELK<br>KPGASVKVSCKASGYTFTEYIIHWIKLRSGQGLEWI<br>GWFYPGSNDIQYNAQFKGKATLTADKSSSTVYME<br>LTGLTPEDSAVYFCARRDDFSGYDALPYWGQGTL<br>VTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVK<br>DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL<br>SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP<br>KSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI<br>SRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVHNA<br>KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC<br>KVSNKALPAPIEKTISKAKGQPREPQVYVYPPSRDE<br>LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK<br>TTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSV<br>MHEALHNHYTQKSLSLSPG |
| | CL_#32593 | | 200 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSGGGGSGGGGS<br>GGGGSGGGGSQAVVTQEPSLTVSPGGTVTLTCGSS<br>TGAVTTSNYANWVQEKPGQAFRGLIGGTNKRAPG<br>TPARFSGSLLGGKAALTLSGAQPEDEAEYYCALW<br>YSNLWVFGGGTKLTVLGGGGSGQVQLQESGPGLV<br>KPSETLSLTCAVSGFSLTSYGVHWIRQPPGKGLEW<br>LGVIWPGGGTNFNSALMSRLTISEDTSKNQVSLKLS<br>SVTAADTAVYYCARDRAYGNYLYAMDYWGQGT<br>LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK<br>DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL<br>SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP<br>KSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI |

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| | | | | SRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVHNA<br>KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC<br>KVSNKALPAPIEKTISKAKGQPREPQVYVYPPSRDE<br>LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK<br>TTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSV<br>MHEALHNHYTQKSLSLSPG |
| | CL_#32594 | | 201 | QVQLVESGGGVVQPGRSLRLSCKASGYTFTRSTM<br>HWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRF<br>TISADKSKSTAFLQMDSLRPEDTGVYFCARPQVHY<br>DYNGFPYWGQGTPVTVSSGGGGSGGGGSGGGGSG<br>GGGSDIQMTQSPSSLSASVGDRVTMTCSASSSVSY<br>MNWYQQKPGKAPKRWIYDSSKLASGVPARFSGSG<br>SGTDYTLTISSLQPEDFATYYCQQWSRNPPTFGGGT<br>KLQITGGGGSGQVQLVQSGAEVKKPGASVKVSCK<br>ASGYTFTSYYIHWVRQAPGQGLEWIGCIYPGNVNT<br>NYNEKFKDRATLTVDTSISTAYMELSRLRSDDTAV<br>YFCTRSHYGLDWNFDVWGQGTTVTVSSASTKGPS<br>VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN<br>SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT<br>QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP<br>APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSV<br>SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY<br>RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK<br>TISKAKGQPREPQVYVYPPSRDELTKNQVSLTCLV<br>KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF<br>ALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#32595 | | 202 | QVQLVESGGGVVQPGRSLRLSCKASGYTFTRSTM<br>HWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRF<br>TISADKSKSTAFLQMDSLRPEDTGVYFCARPQVHY<br>DYNGFPYWGQGTPVTVSSGGGGSGGGGSGGGGSG<br>GGGSDIQMTQSPSSLSASVGDRVTMTCSASSSVSY<br>MNWYQQKPGKAPKRWIYDSSKLASGVPARFSGSG<br>SGTDYTLTISSLQPEDFATYYCQQWSRNPPTFGGGT<br>KLQITGGGGSGQVQLQQSGAELKKPGASVKVSCK<br>ASGYTFTEYIIHWIKLRSGQGLEWIGWFYPGSNDIQ<br>YNAQFKGKATLTADKSSSTVYMELTGLTPEDSAV<br>YFCARRDDFSGYDALPYWGQGTLVTVSAASTKGP<br>SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW<br>NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG<br>TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC<br>PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVS<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST<br>YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE<br>KTISKAKGQPREPQVYVYPPSRDELTKNQVSLTCL<br>VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS |

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| | | | | FALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#32596 | | 203 | QVQLVESGGGVVQPGRSLRLSCKASGYTFTRSTM<br>HWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRF<br>TISADKSKSTAFLQMDSLRPEDTGVYFCARPQVHY<br>DYNGFPYWGQGTPVTVSSGGGGSGGGGSGGGGSG<br>GGGSDIQMTQSPSSLSASVGDRVTMTCSASSSVSY<br>MNWYQQKPGKAPKRWIYDSSKLASGVPARFSGSG<br>SGTDYTLTISSLQPEDFATYYCQQWSRNPPTFGGGT<br>KLQITGGGGSGQVQLQESGPGLVKPSETLSLTCAV<br>SGFSLTSYGVHWIRQPPGKGLEWLGVIWPGGGTNF<br>NSALMSRLTISEDTSKNQVSLKLSSVTAADTAVYY<br>CARDRAYGNYLYAMDYWGQGTLVTVSSASTKGP<br>SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW<br>NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG<br>TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC<br>PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVS<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST<br>YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE<br>KTISKAKGQPREPQVYVYPPSRDELTKNQVSLTCL<br>VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS<br>FALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#32597 | | 204 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY<br>TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSGGGGSGQVQLVQSGAEVKKPGASVKVSCKA<br>SGYTFTSYYIHWVRQAPGQGLEWIGCIYPGNVNTN<br>YNEKFKDRATLTVDTSISTAYMELSRLRSDDTAVY<br>FCTRSHYGLDWNFDVWGQGTTVTVSSASTKGPSV<br>FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS<br>GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ<br>TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA<br>PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVS<br>HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR<br>VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI<br>SKAKGQPREPQVYVYPPSRDELTKNQVSLTCLVKG<br>FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFAL<br>VSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK<br>SLSLSPG |
| | CL_#32598 | | 205 | EIVLTQSPATLSLSPGERVTMSCSASSSVSYMHWY<br>QQKPGQAPRLWIYDTSKLASGVPARFSGSGSGTDY |

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| | | | | TLTISSVEPEDNAVYYCQQWSGHPLTFGQGTKVEI<br>KGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVK<br>KPGASVKISCKASGYSFTGYTMNWVRQAPGQGLE<br>WIGLITPYSGASSYAQKFQGRATLTVDKSTSTAYM<br>ELSSLRSEDTAVYYCARGGYDGRGFDYWGQGTNV<br>TVSSGGGGSGQVQLQQSGAELKKPGASVKVSCKA<br>SGYTFTEYIIHWIKLRSGQGLEWIGWFYPGSNDIQY<br>NAQFKGKATLTADKSSSTVYMELTGLTPEDSAVYF<br>CARRDDFSGYDALPYWGQGTLVTVSAASTKGPSV<br>FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS<br>GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ<br>TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA<br>PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVS<br>HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR<br>VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI<br>SKAKGQPREPQVYVYPPSRDELTKNQVSLTCLVKG<br>FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFAL<br>VSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK<br>SLSLSPG |
| | CL_#32599 | | 206 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF<br>TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKGGGGSGQVQLQQSGAELKKPGASVKVSCK<br>ASGYTFTEYIIHWIKLRSGQGLEWIGWFYPGSNDIQ<br>YNAQFKGKATLTADKSSSTVYMELTGLTPEDSAV<br>YFCARRDDFSGYDALPYWGQGTLVTVSAASTKGP<br>SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW<br>NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG<br>TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC<br>PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVS<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST<br>YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE<br>KTISKAKGQPREPQVYVYPPSRDELTKNQVSLTCL<br>VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS<br>FALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#32600 | | 207 | QVQLVESGGGLVQPGGSLRLSCSVSGIDLSSNPMI<br>WVRQAPGKGLQYIGIIDTDGSTYYADWAKGRFTIS<br>KDSTTVYLQINSPRAEDTAVYYCARRLHGSSNGYY<br>DDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG<br>SDGQMTQSPSSVSASVGDRVTITCQASQSIYSYLSW<br>YQQKPGQRPKLLIYKASTLASGVPSRFSGSGSGTDF |

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| | | | | TLTISSVQPEDFATYYCQQGYTVTNVDKNTFGGGT<br>KVEIKGGGGSGQVQLVQSGAEVKKPGASVKVSCK<br>ASGYTFTSYYIHWVRQAPGQGLEWIGCIYPGNVNT<br>NYNEKFKDRATLTVDTSISTAYMELSRLRSDDTAV<br>YFCTRSHYGLDWNFDVWGQGTTVTVSSASTKGPS<br>VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN<br>SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT<br>QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP<br>APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSV<br>SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY<br>RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK<br>TISKAKGQPREPQVYVYPPSRDELTKNQVSLTCLV<br>KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF<br>ALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYT<br>QKSLSLSPG |
| | CL_#32601 | | 208 | EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMN<br>WVRQAPGKGLEWVSRIRSKYNNYATYYADSVKG<br>RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHG<br>NFGNSYVSWFAYWGQGTLVTVSSGGGGSGGGGS<br>GGGGSGGGGSQAVVTQEPSLTVSPGGTVTLTCGSS<br>TGAVTTSNYANWVQEKPGQAFRGLIGGTNKRAPG<br>TPARFSGSLLGGKAALTLSGAQPEDEAEYYCALW<br>YSNLWVFGGGTKLTVLAAEPKSSDKTHTCPPCPAP<br>EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVSVSH<br>EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV<br>VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS<br>KAKGQPREPQVYVLPPSRDELTKNQVSLLCLVKGF<br>YPSDIAVEWESNGQPENNYLTWPPVLDSDGSFFLY<br>SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL<br>SLSPG |
| | CL_#32602 | | 209 | QVQLVESGGGVVQPGRSLRLSCKASGYTFTRSTM<br>HWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRF<br>TISADKSKSTAFLQMDSLRPEDTGVYFCARPQVHY<br>DYNGFPYWGQGTPVTVSSGGGGSGGGGSGGGGSG<br>GGGSDIQMTQSPSSLSASVGDRVTMTCSASSSVSY<br>MNWYQQKPGKAPKRWIYDSSKLASGVPARFSGSG<br>SGTDYTLTISSLQPEDFATYYCQQWSRNPPTFGGGT<br>KLQITAAEPKSSDKTHTCPPCPAPEAAGGPSVFLFP<br>PKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYV<br>DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD<br>WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ<br>VYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWE<br>SNGQPENNYLTWPPVLDSDGSFFLYSKLTVDKSRW<br>QQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| ***Certain Anti-CD28 Binding Domain Sequences (Mutation Positions According to the IMGT Numbering Scheme)*** | | | | |

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| Hu_TN228_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 |
| CD28.3 | VH | CD28 | 102 | QVQLQQSGAELKKPGASVKVSCKASGYTFTEYIIH WIKLRSGQGLEWIGWFYPGSNDIQYNAQFKGKAT LTADKSSSTVYMELTGLTPEDSAVYFCARRDDFSG YDALPYWGQGTLVTVSA |
| CD28.3 | VL | CD28 | 103 | DIQMTQSPSSLSASVGDRVTITCKTNENIYSNLAWY QQKDGKSPQLLIYAATHLVEGVPSRFSGSGSGTQY SLTISSLQPEDFGNYYCQHFWGTPATFGGGTKLEIK |
| TGN1412 | VH | CD28 | 104 | QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIH WVRQAPGQGLEWIGCIYPGNVNTNYNEKFKDRAT LTVDTSISTAYMELSRLRSDDTAVYFCTRSHYGLD WNFDVWGQGTTVTVSS |
| TGN1412 | VL | CD28 | 105 | DIQMTQSPSSLSASVGDRVTITCHASQNIYVWLNW YQQKPGKAPKLLIYKASNLHTGVPSRFSGSGSGTD FTLTISSLQPEDFATYYCQQGQTYPYTFGGGTKVEI K |
| ***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 | AASNVES |
| N1066A | LCDR2 | CD28 | 46 | AASAVDS |
| Cons. | LCDR2 | CD28 | 47 | AASX$_9$VX$_{10}$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 |
| CD28.3 | HCDR1 | CD28 | 106 | EYIIH |
| CD28.3 | HCDR2 | CD28 | 107 | WFYPGSNDIQYNAQFKG |
| CD28.3 | HCDR3 | CD28 | 108 | RDDFSGYDALPY |
| CD28.3 | LCDR1 | CD28 | 109 | KTNENIYSNLA |
| CD28.3 | LCDR2 | CD28 | 110 | AATHLVE |
| CD28.3 | LCDR3 | CD28 | 111 | QHFWGTPAT |
| TGN1412 | HCDR1 | CD28 | 112 | SYYIH |
| TGN1412 | HCDR2 | CD28 | 113 | CIYPGNVNTNYNEKFKD |
| TGN1412 | HCDR3 | CD28 | 114 | SHYGLDWNFDV |
| TGN1412 | LCDR1 | CD28 | 115 | HASQNIYVWLN |
| TGN1412 | LCDR2 | CD28 | 116 | KASNLHT |
| TGN1412 | LCDR3 | CD28 | 117 | QQGQTYPYT |
| | HCDR1 | MSLN | 118 | GYTMN |
| | HCDR2 | MSLN | 119 | LITPYSGASSYAQKFQG |
| | HCDR3 | MSLN | 120 | GGYDGRGFDY |
| | LCDR1 | MSLN | 121 | SASSSVSYMH |
| | LCDR2 | MSLN | 122 | DTSKLAS |
| | LCDR3 | MSLN | 123 | QQWSGHPLT |
| | HCDR1 | Cldn18.2 | 124 | SNPMI |
| | HCDR2 | Cldn18.2 | 125 | IIDTDGSTYYADWAKG |
| | HCDR3 | Cldn18.2 | 126 | RLHGSSNGYYDDL |
| | LCDR1 | Cldn18.2 | 127 | QASQSIYSYLS |
| | LCDR2 | Cldn18.2 | 128 | KASTLAS |
| | LCDR3 | Cldn18.2 | 129 | QQGYTVTNVDKNT |
| | HCDR1 | CD3 | 134 | TYAMN |
| | HCDR2 | CD3 | 135 | RIRSKYNNYATYYADSVKD |
| | HCDR3 | CD3 | 136 | HKNFGNSYVTWFAY |
| | LCDR1 | CD3 | 137 | RSSTGAVTTSNYAN |
| | LCDR2 | CD3 | 138 | GTNKRAP |
| | LCDR3 | CD3 | 139 | ALWYSNLWV |
| | HCDR1 | CD3 | 134 | TYAMN |
| | HCDR2 | CD3 | 140 | RIRSKYNNYATYYADSVKG |
| | HCDR3 | CD3 | 141 | HGNFGNSYVSWFAY |
| | LCDR1 | CD3 | 142 | GSSTGAVTTSNYAN |

| | | | | |
|---|---|---|---|---|
| | LCDR2 | CD3 | 138 | GTNKRAP |
| | LCDR3 | CD3 | 139 | ALWYSNLWV |
| | HCDR1 | CD3 | 143 | TYGMN |
| | HCDR2 | CD3 | 135 | RIRSKYNNYATYYADSVKD |
| | HCDR3 | CD3 | 141 | HGNFGNSYVSWFAY |
| | LCDR1 | CD3 | 137 | RSSTGAVTTSNYAN |
| | LCDR2 | CD3 | 138 | GTNKRAP |
| | LCDR3 | CD3 | 139 | ALWYSNLWV |
| Kabat | HCDR1 | DLL3 | 158 | HYYVH |
| Kabat | HCDR2 | DLL3 | 159 | IIDPGGGTTSYAQKFQG |
| Kabat | HCDR3 | DLL3 | 160 | GERVTGNYFYYGMDV |
| Kabat | LCDR1 | DLL3 | 161 | VANQGISNYLV |
| Kabat | LCDR2 | DLL3 | 162 | SVNSLYS |
| Kabat | LCDR3 | DLL3 | 163 | LQHDSYPYT |
| Kabat | HCDR1 | DLL3 | 158 | HYYVH |
| Kabat | HCDR2 | DLL3 | 159 | IIDPGGGTTSYAQKFQG |
| Kabat | HCDR3 | DLL3 | 160 | GERVTGNYFYYGMDV |
| Kabat | LCDR1 | DLL3 | 164 | KSSQGISNYLA |
| Kabat | LCDR2 | DLL3 | 165 | AVSTRES |
| Kabat | LCDR3 | DLL3 | 163 | LQHDSYPYT |
| | | | | |
| ***Linkers*** | | | | |
| | $Linker^{Fab\text{-}Fc}$ | Hinge | 56 | EPKSCDKTHTCPPCP |
| | $Linker^{Fab\text{-}Fc}$ | Hinge | 54 | 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 a disease-associated antigen, and

(iv) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide,

wherein:

(a) the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that promote formation of the heterodimeric Fc domain;

(b) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide;

(c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain; and

(d) the third binding domain is coupled to the N-terminus of the second Fc polypeptide.

**2.** The antibody construct of claim 1, wherein the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that reduce binding to at least one FcγR.

**3.** 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 disease-associated antigen, and

(iv) a dimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide,

wherein:

(a) the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that reduce binding to at least one FcγR;

(b) the first Fab domain is coupled to the N-terminus of the first Fc polypeptide;

(c) the first scFv domain is coupled to the C-terminus of the light chain of the first Fab domain; and

(d) the third binding domain is coupled to the N-terminus of the second Fc polypeptide.

**4.** The antibody construct of claim 3, wherein the dimeric Fc domain is a heterodimeric Fc domain and the first Fc polypeptide and/or the second Fc polypeptide comprise one or more amino acid modifications that promote formation of the heterodimeric Fc domain.

**5.** The antibody construct of any one of claims 1-4, wherein the third binding domain comprises or consists of a second Fab domain or a second scFv domain.

**6.** The antibody construct of any one of claims 1-5, wherein the disease-associated antigen is a tumor-associated antigen (TAA).

**7.** The antibody construct of claim 6, wherein the TAA is an antigen associated with a solid tumor.

**8.** The antibody construct of claim 6, wherein the TAA is an antigen associated with a liquid tumor.

**9.** The antibody construct of any one of claims 6-8, wherein the $K_D$ ratio of the three binding domains for their respective targets is about (1-11)[CD3]:(5-160)[CD28]:(0.01-0.5[TAA], 1[CD3]:(1-10)[CD28]:(0.01-1)[TAA] or 1[CD3]:(1-5)[CD28]:(0.01-0.5)[TAA].

**10.** The antibody construct of any one of claims 1-9, wherein the first scFv domain has the domain structure, from N- to C-terminus, of: VH-Linker$^{scFv1}$-VL or VL-Linker$^{scFv1}$-VH, and wherein the Linker$^{scFv1}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 63.

**11.** The antibody construct of any one of claims 1-10, wherein the first scFv domain has the domain structure, from N- to C-terminus, of VL-Linker$^{scFv1}$-VH.

**12.** The antibody construct of any one of claims 1-11, wherein the antibody construct comprises at least, from N- to C-terminus, (i) a first immunoglobulin (Ig) heavy chain (H1) comprising a first Fab heavy chain comprising a first VH sequence and a first CH1 sequence, coupled to the first Fc polypeptide, (ii) a second Ig heavy chain (H2)

comprising the second Fc polypeptide, and (iii) a first Ig light chain (L1) comprising a first Fab light chain comprising a first VL sequence and a first CL sequence, coupled to the first scFv domain, wherein the first Fab heavy chain of H1 and the first Fab light chain of L1 form the first Fab domain.

**13.** The antibody construct of claim 12, wherein L1 further comprises a linker$^{Fab\text{-}scFv}$ that couples the first Fab light chain to the first scFv domain.

**14.** The antibody construct of claim 13, wherein the linker$^{Fab\text{-}scFv}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 58, or an amino acid sequence having one or more amino acid substitutions thereto.

**15.** The antibody construct of any one of claims 12-14, wherein H1 further comprises a first linker$^{Fab\text{-}Fc}$ that couples the first Fab heavy chain to the first Fc polypeptide.

**16.** The antibody construct of claims 15, wherein the first linker$^{Fab\text{-}Fc}$ comprises or consists of an IgG1 hinge region, or are a derivative thereof, wherein the linker$^{Fab\text{-}Fc}$ has at least about 80%, 85%, 90%, 93%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 54 or 56.

**17.** The antibody construct of any one of claims 1-16, wherein the first Fc polypeptide comprises a first CH2 sequence and a first CH3 sequence and the second Fc polypeptide comprises a second CH2 sequence and a second CH3 sequence.

**18.** The antibody construct of claim 17, wherein the first CH2 sequence and/or the second CH2 sequence comprise one or more amino acid modifications that reduce binding to at least one FcγR.

**19.** The antibody construct of claim 18, wherein the first CH2 sequence and/or the second CH2 sequence comprise one or more of the amino acid substitutions L234A, L235A and D265S, and wherein the numbering of amino acid residues in the Fc polypeptides is according to the EU numbering system.

**20.** The antibody construct of any one of claims 17-19, wherein the first CH3 sequence and/or the second CH3 sequence comprise one or more amino acid modifications that promote formation of a heterodimeric Fc domain when compared to the formation of a homodimeric Fc domain.

**21.** The antibody construct of claim 20, 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.

**22.** The antibody construct of any one of claims 1-21, wherein the antibody construct binds CD3 with a $K_D$ from about 0.1 nM to about 100 nM, from about 1 nM to about 100 nM, or from about 10 nM to about 75 nM.

**23.** The antibody construct of any one of claims 1-22, wherein the antibody construct binds CD28 with a $K_D$ from about 1 nM to about 100 nM or from about 10 nM to about 75 nM.

**24.** The antibody construct of any one of claims 1-23, wherein the antibody construct co-engages CD3 and CD28 on the surface of 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.

**25.** The antibody construct of any one of claims 1-24, wherein the antibody construct binds CD28 on a cytotoxic effector cell that expresses both CD3 and CD28 with a $K_D$ that is about 2-fold, 4-fold, 6-fold, 8-fold or 10-fold higher when compared to a cytotoxic effector cell that expresses CD28 but not CD3, when measured using SPR.

**26.** The antibody construct of any one of claims 1-25, wherein the cytotoxic effector cell is an immune cell, optionally wherein the immune cell is a T cell.

**27.** The antibody construct of claim 26, wherein the antibody construct does not reduce the viability of a population of T cells by more than 5%, 3%, 1%, or by 0% after incubation for 48 hours when compared to T cells treated with a negative control construct that does not contain a binding domain against the disease-associated antigen.

**28.** The antibody construct of claim 12-27, wherein H2 further comprises a second Fab heavy chain comprising a second VH sequence and a second CH1 sequence, wherein the second Fab heavy chain is coupled to the N-terminus of the second Fc polypeptide.

**29.** The antibody construct of claim 28, wherein H2 further comprises a second $linker^{Fab\text{-}Fc}$ comprising an IgG1 hinge region, or are a derivative thereof, wherein the second

linker$^{Fab\text{-}Fc}$ has at least about 80%, 85%, 90%, 93%, 95%, 97%, 99% or 100% sequence identity to the sequence set forth in SEQ ID NO: 54 or 56.

**30.** The antibody construct of claim 28, further comprising a second Ig light chain (L2) comprising a second Fab light chain comprising a second VL sequence and a second CL sequence, wherein the second Fab heavy chain of H2 and the second Fab light chain of L2 form a second Fab domain as the third binding domain.

**31.** 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 comprising a second Fab domain and capable of binding a disease-associated antigen, 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.

**32.** The antibody construct of any one of claims 28-31, wherein one of the Fab domains comprises a kappa light chain and the other Fab domain comprises a lambda light chain.

**33.** The antibody construct of any one of claims 28-32, wherein one or both Fab domains comprise one or more amino acid substitutions relative to a reference Fab sequence that promote correct heavy and light chain pairing of H1 with L1 as compared to L2, and H2 with L2 as compared to L1.

**34.** The antibody construct of claim 12-27, wherein H2 further comprises a second scFv domain comprising a second VH sequence and a second VL sequence, wherein the second scFv domain is coupled to the N-terminus of the second Fc polypeptide.

**35.** The antibody construct of claim 34, wherein H2 further comprises a first linker$^{scFv\text{-}Fc}$ that couples the second scFv domain to the second Fc polypeptide.

**36.** The antibody construct of claim 35, wherein the first linker$^{scFv\text{-}Fc}$ comprises or consists of an IgG1 hinge region, or are a derivative thereof, wherein the first linker$^{scFv\text{-}Fc}$ has at least about 80%, 85%, 90%, 95% or 100% sequence identity to the sequence set forth in SEQ ID NO: 57.

**37.** The antibody construct of claim 35 or claim 36, wherein the second scFv domain comprises a second Linker$^{scFv}$ (Linker$^{scFv2}$) and has the domain structure, from N- to C-terminus, of VH-Linker$^{scFv2}$-VL, wherein the Linker$^{scFv2}$ comprises or consists of the amino acid sequence set forth in SEQ ID NO: 63.

**38.** The antibody construct of any one of claims 1-37, wherein the first Fab domain that is capable of binding CD3 (i) comprises HCDR1-3 as set forth in SEQ ID NOs: 49, 50 and 52, respectively, or 49, 51 and 52, respectively, and LCDR1 and 3 as set forth in SEQ ID NOs: 53 and 55, respectively, with LCDR2 having the sequence of RND, according to the IMGT numbering system; (ii) comprises HCDR1-3 as set forth in SEQ ID NOs: 134, 140 and 141, respectively, and LCDR1-3 set forth in SEQ ID NOs: 142, 138 and 139, respectively; or (iii) comprises HCDR1-3 as set forth in SEQ ID NOs: 143, 135 and 141, respectively, and LCDR1-3 set forth in SEQ ID NOs: 137-139, respectively.

**39.** The antibody construct of any one of claims 1-38, wherein the first Fab domain that is capable of binding CD3 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 1, 2, 82, 84 or 86, and a VL sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 5, 83, 85 or 87.

**40.** The antibody construct of any one of claims 1-39, wherein the first scFv domain that is capable of binding CD28 (i) comprises HCDR1-3 as set forth in SEQ ID NOs: 30, 34 and 40, and LCDR1-3 as set forth in SEQ ID NOs: 43, 47 and 48, (ii) comprises HCDR1-3 as set forth in SEQ ID NOs: 106-108, and LCDR1-3 as set forth in SEQ ID NOs: 109-111, or (iii) comprises HCDR1-3 as set forth in SEQ ID NOs: 112-114, and LCDR1-3 as set forth in SEQ ID NOs: 115-117.

**41.** The antibody construct of any one of claims 1-40, wherein the first scFv domain that is capable of binding CD28 comprises a VH sequence having at least about 90%, 95%, 97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 15, 18, 20, 21, 22, 23, 24, 25, 27 and 76, and a VL sequence having at least about 90%, 95%,

97%, or 99% sequence identity to any one of the sequences set forth in SEQ ID NOs: 16, 17, 19, 26 and 77.

**42.** The antibody construct of any one of claims 1-41, wherein the antibody construct binds the disease-associated antigen with a $K_D$ from about 0.01 nM to about 10 nM, from about 0.05 nM to about 5 nM, or from about 0.1 nM to about 5 nM.

**43.** The antibody construct of any one of claims 1-42, wherein the antibody construct comprises a fourth binding domain.

**44.** A pharmaceutical composition comprising the antibody construct of any one of claims 1-43, and a pharmaceutically acceptable carrier, excipient, diluent, or combination thereof.

**45.** 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 1-43.

**46.** A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of claim 45.

**47.** A cell comprising the nucleic acid molecule or the set of nucleic acid molecules of claim 45, or the vector or set of vectors of claim 46.

**48.** A method of producing an antibody construct of any one of claims 1-43, 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 of the polypeptide chains that form the antibody construct; and

(b) recovering the antibody construct from the host cell culture.

**49.** A method of eliciting an immune response in a cell population comprising immune cells and target cells, the method comprising contacting the cell population with an effective amount of the antibody construct of any one of claims 1-43, wherein the immune cells express CD3 and CD28 and the target cells express a disease-associated antigen.

**50.** A method of inhibiting the proliferation of target cells, the method comprising contacting a cell population comprising the target cells and immune cells with an effective amount of the antibody construct of any one of claims 1-43, wherein the immune cells express CD3 and CD28 and the target cells express a disease-associated antigen.

**51.** The method of any one of claims 49-50, wherein the target cells comprise tumor cells and the disease-associated antigen is a TAA.

**52.** The method of any one of claims 49-51, wherein the cell population is within a subject.

**53.** A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject an antibody construct of any one of claims 1-43.

**54.** The method of claim 53, further comprising eliciting a cytotoxic immune response against the tumor in the subject, thereby treating the tumor in the subject.

**55.** The method of claim 53 or claim 54, wherein the tumor is a solid tumor or a liquid tumor.

**56.** An antibody construct of any one of claims 1-43 for use in the treatment of a tumor.

**57.** Use of an antibody construct of any one of claims 1-43 in the manufacture of a medicament for the treatment of a tumor.