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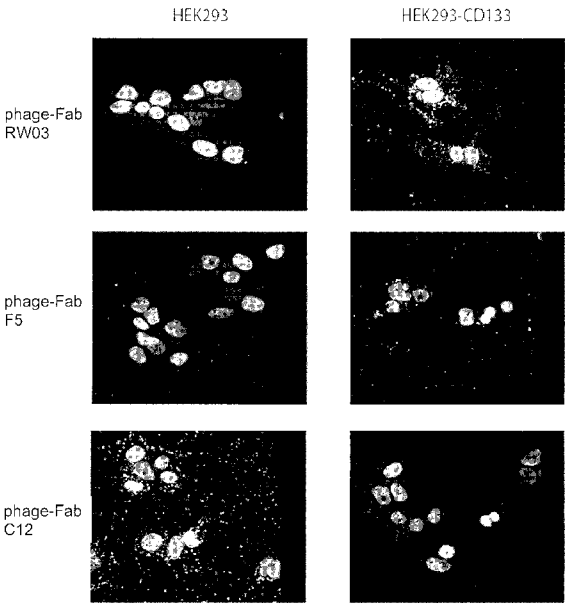
(54) Title: CD133-BINDING AGENTS AND USES THEREOF

FIGURE 2



(57) Abstract: This disclosure is directed to novel CD133-binding agents. The disclosure is also directed to uses of novel CD133-binding agents for detecting CD133-expressing cells and/or quantitating levels of cellular CD133 expression, for targeting CD133-expressing cells, for decreasing levels of CD133 in CD133-expressing cells and for treating or preventing cancer.

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[Continued on next page]

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TITLE: CD133-BINDING AGENTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 62/410,162 filed October 19, 2016, and U.S. Provisional Application No. 62/472,209 filed March 16, 2017, the contents of both of which are incorporated herein by reference in their entirety.

FIELD

[0002] This disclosure relates generally to CD133-binding agents, and to methods and uses of these binding agents.

BACKGROUND

[0003] CD133 has been identified as a marker in melanoma, brain tumors and various carcinomas, including breast, colon, gastric, prostate, liver, pancreatic, lung cancer and head and neck squamous cell carcinoma (Boman et al., 2008; Ferrandina et al., 2009). CD133 expression is often associated with poor survival, drug resistance and metastasis. A correlation between CD133 overexpression, histopathological factors and poor patient outcome has been reported in hepatocellular carcinoma (Zhong et al., 2015). CD133 is a membrane-bound pentaspan glycoprotein, the exact physiologic role of which remains unclear. It is thought to be involved in primitive cell differentiation and epidermal-mesenchymal interaction (Bauer et al., 2008; Ulasov et al., 2011; Evangelista et al., 2006), and to be associated with the WNT signaling pathway and associated cell proliferation (Rappa et al., 2008; Mak et al., 2012a; Takenobu et al., 2011). Downregulation of CD133 in a metastatic melanoma cell line has been shown to result in reduced metastatic capacity of xenografts (Rappa et al., 2008).

[0004] Glioblastoma (GBM) is a uniformly fatal primary brain tumor, characterized by a diverse cellular phenotype and genetic heterogeneity. Despite the use of aggressive cellular multi-modal treatment including surgical resection, radiotherapy and chemotherapy, the outcome of patients with GBM has failed to

improve significantly. Numerous studies have implicated CD133+ brain tumor initiating cells (BTICs) as drivers of chemo- and radio-resistance in GBM. It has also recently been demonstrated that a CD133-driven gene signature is predictive of poor overall survival (Venugopal et al, 2015) and targeting CD133+ treatment refractory cells may be an effective strategy to block GBM recurrence. Medulloblastoma cells that are CD133+ have also been associated with increased multipotency and enriched brain cancer stem cell activity (Singh et al, 2004).

[0005] Anti-CD133 antibody-based drugs have been proposed for treatment of cancer (reviewed in Schmohl and Vallera, 2016). Significant but temporary regression of recurrent glioblastoma after anti-IL13alpha chimeric antigen receptor T-cell therapy has been reported in a human patient (Brown et al., 2016). A need remains for novel agents that bind CD133 with high affinity and specificity.

SUMMARY

[0006] The present inventors have described novel antibody variable regions RW01 and RW03 capable of specifically binding both cell surface-expressed as well as denatured human CD133, and demonstrated specific CD133-binding by binding agents (e.g. antibodies, Fabs, scFvs, Fab-based bispecific antibodies/bispecific T cell engagers (BiTEs) and/or scFab-based bispecific antibodies/BiTEs) comprising these CD133-binding variable regions. It was shown that antibodies with the presently disclosed variable regions specifically bind cell surface-expressed/native human CD133 with a dissociation constant (K_D) in the subnanomolar/nanomolar range. It was shown that such CD133-binding antibodies can be used to specifically detect CD133 expressed on the surface of cells, such as cancer cells (e.g. pancreatic cancer cells, colorectal cancer cells); specifically bind and detect denatured CD133, e.g. in cell lysates; specifically bind, detect and subcellularly localize cellular CD133 via immunofluorescence analysis; and significantly reduce levels of CD133 protein in CD133-positive (CD133+) cancer cells. It was further shown that Fab comprising antibody variable region RW01 and Fab comprising antibody variable region

RW03 do not compete with IgG RW03 and IgG RW01, respectively, for binding to CD133.

[0007] The present inventors have also shown that CD133 specific CAR-T cells specifically induce CD133-positive glioblastoma (GBM) cell death and induce GBM tumor regression *in vivo*. The inventors have also shown that CD133-specific BiTEs recruit T cells to CD133+ human GBM cells and cause cell death. In addition, tumors formed in mouse brain intracranially treated with CD133-specific BiTEs were less aggressive and invasive.

[0008] Accordingly, the present disclosure provides a CD133-binding agent which specifically binds cell surface-expressed/native CD133 and which specifically binds denatured CD133.

[0009] In one embodiment, the CD133-binding agent specifically binds a CD133 epitope bound by an antibody comprising: (a) a light chain having the amino acid sequence of SEQ ID NO: 2, and a heavy chain having the amino acid sequence of SEQ ID NO: 3; and/or (b) a light chain having the amino acid sequence of SEQ ID NO: 4 and a heavy chain having the amino acid sequence of SEQ ID NO: 5.

[0010] In another embodiment, the CD133-binding agent specifically binds a CD133 epitope of cell surface-expressed CD133 which is bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 2 and a heavy chain having the amino acid sequence of SEQ ID NO: 3, and/or which is bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 4 and a heavy chain having the amino acid sequence of SEQ ID NO: 5.

[0011] In a further embodiment, the CD133-binding agent specifically binds a CD133 epitope of denatured CD133 which is bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 2 and a heavy chain having the amino acid sequence of SEQ ID NO: 3, and/or which is bound by an antibody comprising a light chain having the amino acid sequence

of SEQ ID NO: 4 and a heavy chain having the amino acid sequence of SEQ ID NO: 5.

[0012] In one embodiment, the CD133-binding agent comprises an antibody light chain variable domain and an antibody heavy chain variable domain which form an antigen binding site that specifically binds human CD133.

[0013] In another embodiment, the antibody light chain variable domain comprises a light chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8; and the antibody heavy chain variable domain comprises a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 9, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 10, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 11, wherein the light chain variable domain and the heavy chain variable domain form the antigen binding site that binds human CD133. Optionally, the heavy chain variable domain further comprises a Met residue at position 39, a Ser residue at position 55 and a Tyr residue at position 66.

[0014] In a further embodiment, the antibody light chain variable domain comprises a light chain complementarity-determining region (CDR)1 consisting of the amino acid sequence of SEQ ID NO: 6, a light chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 7, and a light chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 8; and the antibody heavy chain variable domain comprises a heavy chain CDR1 consisting of the amino acid sequence of SEQ ID NO: 9, a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 10, and a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 11, wherein the light chain variable domain and the heavy chain variable domain form the antigen binding site that binds human CD133. Optionally, the heavy chain variable domain further comprises a Met residue at position 39, a Ser residue at position 55 and a Tyr residue at position 66.

[0015] In another embodiment, the antibody light chain comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 2.

[0016] In another embodiment, the antibody light chain consists of the amino acid sequence of SEQ ID NO: 2.

[0017] In another embodiment, the heavy chain comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3.

[0018] In another embodiment, the antibody heavy chain consists of the amino acid sequence of SEQ ID NO: 3.

[0019] In another embodiment, the light chain comprises (i) the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 2, and (ii) the heavy chain comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3.

[0020] In another embodiment, the light chain consists of the amino acid sequence of SEQ ID NO: 2, and the heavy chain consists of the amino acid sequence of SEQ ID NO: 3.

[0021] In another embodiment, the antibody light chain variable domain comprises a light chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 12, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 13, and a light chain CDR3 comprising the amino acid sequence of SEQ ID NO: 14; and the antibody heavy chain variable domain comprises a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 15, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17, wherein the light chain variable domain and the heavy chain variable domain form the antigen binding site that binds human CD133.

Optionally, the heavy chain variable domain further comprises a Ile residue at position 39, a Tyr residue at position 55 and a Tyr residue at position 66.

[0022] In one embodiment, the antibody light chain variable domain comprises a light chain complementarity-determining region (CDR)1 consisting of the amino acid sequence of SEQ ID NO: 12, a light chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 13, and a light chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 14; and the antibody heavy chain variable domain comprises a heavy chain CDR1 consisting of the amino acid sequence of SEQ ID NO: 15, a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 17, wherein the light chain variable domain and the heavy chain variable domain form the antigen binding site that binds human CD133. Optionally, the heavy chain variable domain further comprises a Ile residue at position 39, a Tyr residue at position 55 and a Tyr residue at position 66.

[0023] In another embodiment, the antibody light chain comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4.

[0024] In another embodiment, the antibody light chain consists of the amino acid sequence of SEQ ID NO: 4.

[0025] In another embodiment, the antibody heavy chain comprises the amino acid sequence of SEQ ID NO: 5 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 5.

[0026] In another embodiment, the antibody heavy chain consists of the amino acid sequence of SEQ ID NO: 5.

[0027] In another embodiment, the light chain comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4, and the heavy chain comprises the amino acid sequence of SEQ ID NO: 5 or an amino acid

sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 5.

[0028] In another embodiment, the light chain consists of the amino acid sequence of SEQ ID NO: 4, and the heavy chain consists of the amino acid sequence of SEQ ID NO: 5.

[0029] In another embodiment, the CD133-binding agent is selected from the group consisting of an antibody, an antibody fragment, a single-chain Fv (scFv), a bispecific antibody, a phage-Fab wherein the Fab binds CD133 and a phage-scFv wherein the scFv binds CD133.

[0030] In another embodiment, the CD133-binding agent comprises an antibody that binds human CD133.

[0031] In another embodiment, the CD133-binding agent comprises an antibody fragment that binds human CD133.

[0032] In another embodiment, the CD133-binding agent comprises a single-chain Fv (scFv) that binds human CD133.

[0033] In another embodiment, the CD133-binding agent comprises a bispecific antibody that binds human CD133.

[0034] In another embodiment, the CD133-binding agent comprises a phage-Fab, wherein the Fab binds human CD133.

[0035] In another embodiment, the CD133-binding agent comprises a phage-scFv that binds human CD133, wherein the scFv binds human CD133.

[0036] In still another embodiment, the antibody fragment is a fragment antigen-binding (Fab).

[0037] In another embodiment, the CD133-binding agent is (a) a bispecific antibody comprising a CD133-binding single-chain Fab and a non-CD133-binding scFv, (b) a bispecific antibody comprising a CD133-binding Fab and a non-CD133-binding scFv, or (c) a CD133-binding and CD3-binding bispecific antibody.

[0038] In yet another embodiment, the CD133-binding agent is (a) a bispecific antibody comprising a CD133-binding single-chain Fab and a CD3-binding scFv or (b) a bispecific antibody comprising a CD133-binding Fab and a CD3-binding scFv.

[0039] In another embodiment, the CD133-binding agent is a chimeric antigen receptor (CAR) comprising (i) a CD133-binding antibody variable region and (ii) a CAR signaling domain comprising one or more immune cell receptor signaling domains.

[0040] In one embodiment, the CD133-binding agent comprises human antibody constant regions.

[0041] In another embodiment, the CD133-binding agent is an IgG molecule.

[0042] In a further embodiment, the IgG molecule is an IgG1 molecule.

[0043] In another embodiment, the CD133-binding agent is labelled with a detection agent.

[0044] The disclosure also provides an immunoconjugate comprising (1) the binding agent described above attached to (2) an effector agent. Optionally, the effector agent is an anti-neoplastic agent or a toxin.

[0045] The disclosure also provides a pharmaceutical composition comprising the CD133-binding agent or the immunoconjugate described above and a carrier.

[0046] The disclosure also provides a use of the CD133-binding agent, immunoconjugate or the pharmaceutical composition described above for targeting CD133-expressing cells.

[0047] The disclosure also provides a use of the CD133-binding agent, immunoconjugate or the pharmaceutical composition described above for binding CD133-expressing cells.

[0048] The disclosure further provides a use of the CD133-binding agent, immunoconjugate or the pharmaceutical composition described above for detecting CD133-expressing cells and/or quantitating levels of cellular CD133 expression.

[0049] The disclosure additionally provides a use of the CD133-binding agent or the pharmaceutical composition described herein for reducing levels of CD133 protein in CD133-expressing cells.

[0050] In one embodiment, the use of the CD133-binding agent is for detecting and/or quantitating levels of cell-surface expressed CD133 in cells. In another embodiment, the use of the CD133-binding agent is for detecting and/or quantitating total levels of CD133 in cells.

[0051] Optionally, detecting CD133-expressing cells and/or quantitating levels of cellular CD133 expression is done by Western blotting, enzyme linked immunosorbent assay (ELISA), immunofluorescence, immunohistochemistry or flow cytometry.

[0052] In another embodiment, the cells are cancer cells, optionally CD133-expressing cancer cells or cancer cells detectably expressing CD133.

[0053] In yet another embodiment, the cancer cells are melanoma cancer cells, pancreatic cancer cells, brain cancer cells or colorectal cancer cells. In another embodiment, the cancer cells are glioblastoma cells. In another embodiment, the cancer cells are medulloblastoma cells.

[0054] The disclosure additionally provides use of a CD133-binding agent, immunoconjugate or the pharmaceutical composition described herein for treating or preventing a cancer.

[0055] In one embodiment, the cancer is a CD133-expressing cancer or a cancer detectably expressing CD133. In another embodiment, the cancer is metastatic melanoma, brain, prostate, pancreatic or colon cancer.

[0056] In another embodiment, the brain cancer is a glioblastoma, optionally a CD133-expressing glioblastoma or a glioblastoma detectably

expressing CD133. In another embodiment, the brain cancer is a medulloblastoma, optionally a CD133-expressing medulloblastoma or a medulloblastoma detectably expressing CD133.

[0057] The disclosure also provides a use of a CD133-binding agent comprising (a) a CD133-binding single-chain Fab and a non-CD133-binding scFv, (b) a bispecific antibody comprising a CD133-binding Fab and a non-CD133-binding scFv, or (c) a CD133-binding and CD3-binding bispecific antibody, for treating glioblastoma, optionally CD133-expressing glioblastoma or glioblastoma detectably expressing CD133.

[0058] The disclosure also provides a use of a CD133-binding agent comprising (a) a CD133-binding single-chain Fab and a CD3-binding scFv or (b) a bispecific antibody comprising a CD133-binding Fab and a CD3-binding scFv for treating glioblastoma, optionally CD133-expressing glioblastoma or glioblastoma detectably expressing CD133.

[0059] The disclosure also provides a use of a CD133-binding agent comprising (a) a CD133-binding single-chain Fab and a non-CD133-binding scFv, (b) a bispecific antibody comprising a CD133-binding Fab and a non-CD133-binding scFv, or (c) a CD133-binding and CD3-binding bispecific antibody, for treating medulloblastoma, optionally CD133-expressing medulloblastoma or medulloblastoma detectably expressing CD133.

[0060] The disclosure also provides a use of a CD133-binding agent comprising (a) a CD133-binding single-chain Fab and a CD3-binding scFv or (b) a bispecific antibody comprising a CD133-binding Fab and a CD3-binding scFv for treating medulloblastoma, optionally CD133-expressing medulloblastoma or medulloblastoma detectably expressing CD133.

[0061] In one embodiment, the bispecific antibody comprises an amino acid sequence comprising:

(a) SEQ ID NO: 22 and SEQ ID NO: 23,

(b) SEQ ID NO: 24 and SEQ ID NO: 25,

(c) SEQ ID NO: 26,

(d) SEQ ID NO: 27, or functional variants thereof.

[0062] The disclosure also provides a use of a T-cell expressing a chimeric antigen receptor (CAR) described herein for treating glioblastoma, optionally CD133-expressing glioblastoma or glioblastoma detectably expressing CD133.

[0063] The disclosure further provides a use of a T-cell expressing a chimeric antigen receptor (CAR) described herein for treating medulloblastoma, optionally CD133-expressing medulloblastoma or medulloblastoma detectably expressing CD133.

[0064] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating embodiments of the disclosure are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

DRAWINGS

[0065] Embodiments are described below in relation to the drawings in which:

[0066] FIGURE 1A is a table depicting the results of a cell-based ELISA for CD133-binding of phage-Fab clones and phage-scFv clones selected from Library F or Library G, respectively, for binding to cell-surface CD133 using the Cellectseq method. Following cell selections, round four output phage for each library was plated for single colonies. These colonies were grown up in an overnight culture and tested for binding to cells by cell-based ELISA. The plates were read and OD450 nm was detected and recorded. The assay measured binding of clones to CD133-overexpressing HEK293-CD133 cells (under

"CD133" column headers) vs parental HEK293 cells (under "HEK293" column headers).

[0067] FIGURE 1B is a histogram depicting a cell-based ELISA for CD133-binding of the three clones (phage-Fab RW03, phage-Fab C12 and phage-Fab F5) which were selected from Library F for binding to cell-surface CD133 using the Cellectseq method, and which were found, after DNA sequencing, to represent the three unique antibody variable regions present in 77 clones found to preferentially bind to HEK293-CD133 cells vs HEK293 cells by at least 1.5-fold.

[0068] FIGURE 2 is a series of fluorescence photomicrographs depicting that phage-Fab clone RW03 specifically binds to CD133 overexpressing cells. The three clones with unique sequences obtained from the Library F cell-based ELISA were used as probes in an immunofluorescence assay. Phage-Fab clones C12 and F5 are shown to bind to HEK293 cells non-specifically whereas the RW03 clone binds to HEK293-CD133 cells specifically with little background binding to HEK293 cells.

[0069] FIGURE 3A and FIGURE 3B are a histogram and a set of fluorescence photomicrographs, respectively, depicting that purified Fab RW03 specifically binds to HEK293-CD133 as opposed to HEK293 cells. Expressed and purified Fab RW03 was tested for binding by cell-based ELISA (Figure 3A) and immunofluorescence (IF) assay (Figure 3B).

[0070] FIGURE 4A and FIGURE 4B are line graphs depicting binding curves for binding of IgG RW01 and IgG RW03, respectively, to HEK293-CD133 cells, for estimation of binding affinity (EC50). Cells were incubated with stepwise dilutions of either IgG RW01 or IgG RW03 to determine a half-maximal binding curve for the antibodies. Using the SigmaPlot graphing software the EC50 for IgG RW01 was calculated as 2.5 nM (Figure 4A) and the EC50 for IgG RW03 was calculated as 0.5 nM (Figure 4B).

[0071] FIGURE 5 is a set of fluorescence histograms depicting that IgG RW01 and IgG RW03 can be used to specifically bind and detect cell surface

CD133 in pancreatic cancer cells and colorectal cancer cells, as shown via flow cytometry analysis.

[0072] FIGURE 6 is a set of fluorescence photomicrographs depicting that IgG RW01 and IgG RW03 can be used to specifically bind, detect and subcellularly localize cellular CD133, as shown via immunofluorescence analysis. The antibodies were tested for binding to HEK293-CD133 and HEK293 cells.

[0073] FIGURE 7 is a set of photographs of a Western blot analysis depicting that IgG RW01 and IgG RW03 can be used to detect denatured CD133/cellular CD133 in colorectal cancer cells, as shown via Western blot analysis. Whole cell lysates of HEK293, HEK293-CD133 and Caco-2 cells were probed with IgG RW01 and IgG RW03 and binding was detected with an anti-human HRP-conjugated secondary antibody. Beta-actin was used as a loading control.

[0074] FIGURE 8 shows that Fab comprising antibody variable region RW01 and Fab comprising antibody variable region RW03 do not compete with IgG RW03 and IgG RW01, respectively, for binding to CD133. RW01 and RW03 were tested for binding to CD133 in a competitive flow cytometry experiment. In (a) cells were incubated with stepwise dilutions of IgG RW01 (circles) or IgG RW01 in the presence of Fab RW03 (squares). Similarly, in (b) cells were incubated stepwise with dilutions of IgG RW03 (circles) or IgG RW03 in the presence of Fab RW01 (squares).

[0075] FIGURE 9 is a set of photographs of a Western blot analysis depicting that treatment with IgG RW01 or IgG RW03 significantly reduces total cellular levels of CD133 protein in Caco-2 colorectal cancer cells. Caco-2 cells were incubated with the indicated antibody for 24-hours at 37°C, whole cell lysates were prepared and probed with AC133 anti-CD133 antibody. Anti-human IgG (H+L) antibody was used as negative antibody control, and GAPDH was used as a loading control.

[0076] FIGURE 10A is a set of schematic diagrams depicting the configurations of BiTE #1, BiTE #2, BiTE #3 and BiTE #4.

[0077] FIGURE 10B is a photograph of a Western blot analysis depicting that BiTE #1, BiTE #2, BiTE #3 and BiTE #4 could each be expressed and purified from HEK293 cells by transient transfection protocol.

[0078] FIGURE 11A and FIGURE 11B are sets of fluorescence histograms depicting that BiTE #1 (Figure 10A), BiTE #2 (Figure 10A), BiTE #3 (Figure 10B) and BiTE #4 (Figure 10B) each binds to HEK293-CD133 cells significantly more than to the parental HEK293 cells, even at concentrations as low as 0.073-0.11 microgram/ml, as determined via flow cytometry. The BiTE concentrations employed are indicated for each histogram. In each histogram the rightmost peak represents binding to HEK293-CD133 cells and the leftmost peak represents binding to the parental HEK293 cells.

[0079] FIGURE 12 is a histogram of ELISA results depicting that BiTE #1, BiTE #2, BiTE #3 and BiTE #4 each binds to CD3 in the form of CD3 epsilon/gamma and CD3 epsilon/delta. Anti-CD3 antibodies UCHT1 and OKT3 were used as positive antibody controls and BSA was used as no antibody control.

[0080] FIGURE 13 depicts constructs for the generation of CD133-specific chimeric antigen receptors (CARs).

[0081] FIGURE 14 shows the characterization of CAR-T cells.

[0082] FIGURE 15 shows the validation of CD133-specific CAR-T cells.

[0083] FIGURE 16 shows that CD133-specific CAR-T cells are activated in presence of CD133+ human GBM cells.

[0084] FIGURE 17 shows that activated CD133-specific CAR-T cells have enhanced proliferation ability and specifically induce CD133-positive GBM cell death and CD133-positive medulloblastoma cell death. CAR-T cells were co-cultured with $CD133^{high}$ and $CD133^{low}$ GBM cells as well as $CD133^{high}$ medulloblastoma cells. Flow cytometry was based on live-dead staining with IR-dye.

[0085] FIGURE 18A-C shows that CD133-specific T cells induce GBM tumor regression *in vivo.* Treatment was delivered intracranially at a dose of 1 million X 2 doses (2 week).

[0086] FIGURE 19A and B shows the development of CD133xCD3 BiTEs.

[0087] FIGURE 20A and B shows that CD133xCD3 BiTEs bind to CD133+ GBM tumor cells and CD3+ T lymphocytes.

[0088] FIGURE 21 shows that CD133-specific BiTEs activate T cells.

[0089] FIGURE 22A-C shows that CD133-specific BiTEs recruit T cells to CD133+ human GBM cells and cause cell death.

[0090] FIGURE 23A-D shows a CD133xCD3 BiTE mediated antitumor response.

DESCRIPTION OF VARIOUS EMBODIMENTS

[0091] Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. For example, the term "a cell" includes a single cell as well as a plurality or population of cells. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligonucleotide or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art (see, e.g. Green and Sambrook, 2012).

[0092] Terms of degree such as "about", "substantially", and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a deviation of at least ±5% of the modified term if this deviation would not negate the meaning of the word it modifies.

Compositions of Matter:

[0093] The present inventors have provided novel synthetic antibody variable regions which are capable of specifically binding surface expressed/native human CD133 and also denatured human CD133, and which specifically bind human CD133 with a dissociation constant (K_D) in the subnanomolar/nanomolar range (see Examples 3 and 6, below).

[0094] The inventors have particularly provided CD133-binding phage-scFv clone RW01 wherein the scFv comprises CD133-binding antibody variable region RW01, and CD133-binding phage-Fab clone RW03 wherein the Fab comprises CD133-binding antibody variable region RW03. These clones were selected from phage display libraries for their capacity to specifically bind CD133-expressing cells, as initially confirmed via enzyme-linked immunosorbent assay (ELISA; see Examples 1 and 2, below). The inventors have further particularly provided that CD133-binding antibody variable region RW01 comprises an antibody light chain variable domain corresponding to the Asp1 to Lys106 segment of SEQ ID NO: 2, wherein the amino acid sequences of the light chain CDR1, light chain CDR2 and light chain CDR3 thereof correspond to SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8, respectively (see Example 2, below). The inventors have additionally provided that antibody variable region RW01 comprises an antibody heavy chain variable domain corresponding to the Glu1 to Thr120 segment of SEQ ID NO: 3, wherein the amino acid sequences of the heavy chain CDR1, heavy chain CDR2 and heavy chain CDR3 thereof correspond to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively, and wherein the (framework region) residues at positions 39, 55 and 66 are Met, Ser and Tyr residues (see Example 2, below).

[0095] The inventors have yet further provided that CD133-binding antibody variable region RW03 comprises an antibody light chain variable domain corresponding to the Asp1 to Lys109 segment of SEQ ID NO: 4, wherein the amino acid sequences of the light chain CDR1, light chain CDR2 and light chain CDR3 thereof correspond to SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID

NO: 14, respectively (see Example 2, below). The inventors have still further provided that antibody variable region RW03 comprises an antibody heavy chain variable domain corresponding to the Glu1 to Ser118 segment of SEQ ID NO: 5; wherein the amino acid sequences of the heavy chain CDR1, heavy chain CDR2 and heavy chain CDR3 thereof correspond to SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17, respectively; and wherein the (framework region) residues at positions 39, 55 and 66 are Ile, Tyr and Tyr residues, respectively; (see Example 2, below).

[0096] The inventors have further particularly provided that IgG1 antibodies "IgG RW01" and "IgG RW03", which comprise CD133-binding antibody variable region RW01 and CD133-binding antibody variable region RW03, respectively, can be used to: (i) specifically bind and detect cell surface CD133 in pancreatic cancer cell lines and colorectal cancer cell lines, as shown via flow cytometry analysis (see Example 4, below); (ii) specifically bind, detect and subcellularly localize cellular CD133 in CD133-expressing cells, as shown via immunofluorescence analysis (see Example 5, below); (iii) detect denatured CD133 in whole cell lysate of colorectal cancer cells, as shown via Western blot analysis (see Example 6, below); and (iv) significantly reduce total cellular CD133 protein levels in a colorectal cancer cell line (see Example 8, below). The inventors disclose that antibody IgG RW01 comprises a light chain having the amino acid sequence of SEQ ID NO: 2 and a heavy chain having the amino acid sequence of SEQ ID NO: 3, and antibody IgG RW03 comprises a light chain having the amino acid sequence of SEQ ID NO: 4 and a heavy chain having the amino acid sequence of SEQ ID NO: 5 (Example 2). The inventors have still further disclosed that Fab comprising antibody variable region RW01 and Fab comprising antibody variable region RW03 do not compete with IgG RW03 and IgG RW01, respectively, for binding to CD133 (Example 7, below).

[0097] The present inventors have also described a chimeric antigen receptor (CAR) T-cell-based strategy that specifically targets CD133+ GBM cells. CD133-specific CAR-expressing T cells were activated in presence of $CD133^{high}$ GBM cells, and showed increased surface expression of activation markers

CD69 and CD25. Both, CD4+ and CD8+ CD133-specific CAR-T cells showed upregulation in surface expression levels of activation markers. The inventors further demonstrated CAR-T cell-induced cytotoxicity against treatment-resistant and evasive CD133+ GBM BTICs (Example 10, below).

[0098] The inventors have further particularly disclosed that multiple configurations of a bispecific antibody/bispecific T cell engager (BiTE) comprising a scFv which binds the T cell coreceptor CD3, and further comprising a Fab or single-chain Fab (scFab) incorporating CD133-binding antibody variable region RW03, can specifically bind both CD133-positive cells and CD3 (see Example 9, below). The inventors also showed that a recombinant CD133xCD3 bispecific T-cell engager (BiTE) redirects human polyclonal T cells to CD133+ GBM cells, inducing a potent anti-tumor response (see Example 11, below).

[0099] The CD133 molecule is a transmembrane protein having an extracellular N-terminal region, five transmembrane domains with alternating short and long intracellular and extracellular domains, respectively, and an intracellular C-terminal region. As used herein, CD133 may be from any species or source and includes isoforms, analogs, variants or functional derivatives of such a CD133 protein. In one embodiment, CD133 is human CD133. The human CD133 gene or protein may have any of the known published sequences for CD133 which can be obtained from public sources such as GenBank. An example of such a protein sequence includes, but is not limited to the sequence set out as SEQ ID NO: 1. Human CD133 is alternately referred to in the art as Prominin-1.

CD133-Binding Agents

[00100] Accordingly, the disclosure provides a CD133-binding agent which specifically binds cell surface-expressed/native CD133 and which specifically binds denatured CD133.

[00101] As used herein, a CD133-binding agent which "specifically binds cell surface-expressed/native CD133" is an agent which binds CD133-expressing cells. Cells expressing CD133 can be identified as such, e.g. via flow cytometric

analysis, e.g. as described in Example 4. Alternately, a CD133-binding agent which "specifically binds cell surface-expressed/native CD133" is an agent which binds CD133-expressing cells expressing CD133 at undetectable levels, e.g. at levels below the limit of detection of an assay such flow cytometric analysis. As used herein, a CD133-binding agent which "specifically binds denatured CD133" is an agent which binds CD133 in a sample of denatured whole cell protein of CD133-expressing cells as opposed to the other polypeptides in the sample (as determined, e.g. via Western blot analysis, e.g. as described in Example 6).The terms "immunoreacts with CD133", or "is directed against CD133", or is characterized as "anti-CD133" are also used herein for the same purpose.

[00102] In one embodiment, the CD133-binding agent specifically binds a CD133 epitope bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 2 and a heavy chain having the amino acid sequence of SEQ ID NO: 3 (i.e. antibody IgG RW01), and/or specifically binds a CD133 epitope bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 4 and a heavy chain having the amino acid sequence of SEQ ID NO: 5 (i.e. antibody IgG RW03). In an embodiment, the CD133 epitope is a human CD133 epitope.

[00103] As used herein, the term "epitope" refers to the specific site or specific combination of sites/amino acids on an antigen that are bound by antibody IgG RW01 and/or antibody IgG RW03, for example, unmodified or modified (e.g. post-translationally modified, e.g. glycosylated) amino acid residues of human CD133, the minimal polypeptide segment of human CD133 encompassing these amino acid residues, or any combination of polypeptide segments of human CD133 encompassing these amino acid residues. Epitopic determinants usually consist of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics.

[00104] As used herein, unless otherwise specified, an antibody or a bivalent antibody fragment (e.g. $F(ab')_2$) referred to as comprising "a" specific

light chain or “a” specific heavy chain in the singular refers to an antibody or a bivalent antibody fragment in which both light chains or both heavy chains are identical, respectively.

[00105] Embodiments of the CD133-binding agent include any type of CD133-binding molecule, macromolecule, substance, compound, material, composition, or complex, without limitation.

[00106] In one embodiment, the CD133-binding agent is a polypeptide. In other embodiments, the CD133-binding agent is a non-polypeptidic agent, such as a CD133-binding nucleic acid or a CD133-binding organic compound. The CD133-binding agent may be monomeric or multimeric. The CD133-binding agent may be polymeric or non-polymeric. Alternately, the CD133-binding agent may be an engineered polypeptide (e.g. a naturally occurring polypeptide engineered to have a modified amino acid sequence; or a chimeric polypeptide engineered to comprise two or more naturally occurring amino acid sequences; or an engineered polypeptide selected from a library of engineered polypeptides having randomized amino acid sequences), or a chemically modified polypeptide.

[00107] In one embodiment, the CD133-binding agent comprises a CD133-binding antibody variable region.

[00108] As used herein, a CD133-binding antibody variable region is a combination of an antibody heavy chain variable domain and an antibody light chain variable domain, where the antibody heavy chain variable domain and the antibody light chain variable domain form an antigen-binding site that specifically binds CD133.

[00109] The CD133-binding agent is optionally an antibody, an antigen-binding fragment of an antibody, or an agent comprising a CD133-binding antibody variable region.

[00110] As used herein, and unless otherwise specified, the term “antibody” refers to an immunoglobulin (Ig) molecule. The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs

of polypeptide chains, each pair having one light ("L") (about 25 kDa) and one heavy ("H") chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition, and described in more detail below. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. The term "antigen-binding site" or "binding portion" refers to the part of the binding protein that participates in antigen binding. In an antibody, the antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy and light chains. Three highly divergent stretches within the V regions of the heavy and light chains, referred to as "hypervariable regions", are interposed between more conserved flanking stretches known as "framework regions", or "FRs". Thus, the term "FR" refers to amino acid sequences which are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three-dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs". All CDRs and framework regions (FRs) disclosed herein, amino acid sequences of CDRs and FRs disclosed herein, and CDR-encoding or FR-encoding nucleic acid sequences disclosed herein, are intended to be defined in accordance with IMGT numbering (Lefranc et al., 2003). Another system alternately employed in the art for such definitions is that of Kabat numbering (Kabat et al., 1991).

[00111] The CD133-binding agent may be an antibody, such as a human antibody, containing engineered variable regions (e.g. containing variable regions selected from a phage display library displaying engineered antibody variable regions, e.g. a phage-Fab library or a phage-scFv library, e.g. as described in Example 1), or a chimeric antibody comprising human constant regions and an antibody variable region of a non-human mammal. The CD133-binding agent

may be a humanized antibody, e.g. an antibody comprising human constant regions, human variable region framework regions, and CD133-binding CDRs generated in a non-human mammal. The non-human mammal may be a rodent, such as a mouse, rat, rabbit, guinea pig or hamster. Alternately, the non-human mammal may be an ungulate, such as a camelid or a bovid. The CD133-binding agent may be an antibody comprising heavy chain constant regions belonging to any type of class, or subclass. The CD133-binding agent may comprise any type of light chain.

[00112] In one embodiment, the CD133-binding agent is a human antibody, such as an IgG1 antibody, wherein the heavy chain constant regions are gamma1 heavy chain constant regions. In other embodiments, the CD133-binding agent is a human antibody, such as an IgA1, IgA2, IgD, IgG2, IgG3, IgG4, IgE or IgM antibody, wherein the heavy chain constant regions are alpha1, alpha2, delta, gamma2, gamma3, gamma4, epsilon or mu heavy chain constant regions, respectively.

[00113] In yet a further embodiment, the CD133-binding agent is an antibody wherein the light chains comprise human kappa light chain constant domains, or wherein the light chains are human kappa light chains. Alternately, the CD133-binding agent is an antibody wherein the light chains comprise human lambda light chain constant domains, or wherein the light chains are human lambda light chains.

[00114] In still a further embodiment the CD133-binding agent is an antibody comprising human gamma1 heavy chain constant regions and human kappa light chains.

[00115] Embodiments of CD133-binding agents of the present disclosure further include, but are not limited to, fragment antigen-binding (Fab), single-chain Fv (scFv), single-chain Fab (scFab), Fab', Fv, chemically linked $F(ab')_2$, dsFv, dsFv', $sc(Fv)_2$, ds-scFv, (dsFv)2, scFv-Fc, scFv-based chimeric antigen receptors (CARs), Fab-based CARs, scFab-based CARs, single-chain immunoglobulin (e.g. scIgG), single-domain antibody (sdAb, nanobody), scFv-Fc,

minibody (scFv-CH3), diabody, tribody, tetrabody, multimeric antibody (e.g. scFv dimer, bivalent diabody), multispecific antibody (e.g. bispecific antibody, trispecific antibody, di-scFv, tri-scFv, bispecific Fab_2, trispecific Fab_2, trispecific triabody, trispecific Fab_3), multimeric/multispecific antibody (e.g. scFv dimer, bispecific diabody, dsFv-dsFv'), heavy-chain antibody, Fab_3, divalent VHH, pentavalent VHH (pentabody), $(scFv\text{-}SA)_4$ and, $[sc(Fv)2]_2$.

[00116] In another embodiment, the CD133-binding agent is a phage displaying a polypeptide comprising a CD133-binding antibody variable region, such as a phage-Fab or phage-scFv (see Examples 1 and 2, below).

[00117] Embodiments of CD133-binding agents of the present disclosure still further include CD133-binding nucleic acid aptamers (e.g. RNA aptamers or DNA aptamers; see, e.g. Lipi et al., 2016), peptide aptamers (see, e.g. Parashar, 2016), and chemically synthesized agents (e.g. synthetic antibody mimics; see, e.g. McEnaney et al., 2014).

[00118] In another embodiment, the CD133-binding agent is a peptide analog. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or 'peptidomimetics" (see, e.g. Fauchere, 1986); Veber and Freidinger, 1985; and Evans et al., 1987). Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to biologically useful peptides may be used to produce an equivalent biological effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: –CH2NH–, –CH2S–, –CH2-CH2–, –CH=CH– (cis and trans), –COCH2–, CH(OH)CH2– and –CH2SO–, by methods well known in the art. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g. D-lysine in place of L-lysine) may be used to

generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (see, e.g. Rizo and Gierasch, 1992), for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

[00119] In an embodiment, the CD133-binding agent comprises antibody variable region RW01, which comprises (a) an antibody light chain variable domain (SEQ ID NO: 28) corresponding to the Asp1 to Lys106 segment of SEQ ID NO: 2, wherein the amino acid sequences of the light chain CDR1, light chain CDR2 and light chain CD3 thereof correspond to SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8, respectively (see Example 2, below) and (b) an antibody heavy chain variable domain (SEQ ID NO: 29) corresponding to the Glu1 to Thr120 segment of SEQ ID NO: 3, wherein the amino acid sequences of the heavy chain CDR1, heavy chain CDR2 and heavy chain CDR3 thereof correspond to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively, and wherein the (framework region) residues at positions 39, 55 and 66 are Met, Ser and Tyr residues (see Example 2, below).

[00120] In a further embodiment, the CD133-binding agent comprises antibody variable region RW03, which comprises (a) an antibody light chain variable domain (SEQ ID NO: 30) corresponding to the Asp1 to Lys109 segment of SEQ ID NO: 4, wherein the amino acid sequences of the light chain CDR1, light chain CDR2 and light chain CD3 thereof correspond to SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14, respectively (see Example 2, below) and (b) an antibody heavy chain variable domain (SEQ ID NO: 31) corresponding to the Glu1 to Ser118 segment of SEQ ID NO: 5; wherein the amino acid sequences of the heavy chain CDR1, heavy chain CDR2 and heavy chain CD3 thereof correspond to SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17, respectively; and wherein the (framework region) residues at positions 39, 55 and 66 are Ile, Tyr and Tyr residues, respectively (see Example 2, below).

[00121] Also particularly disclosed herein is a CD133-binding agent IgG RW01 comprising a light chain amino acid sequence as shown in SEQ ID NO: 2 and a heavy chain amino acid sequence as shown in SEQ ID NO: 3 (see Example 2, below).

[00122] Yet further particularly disclosed herein is a CD133-binding agent IgG RW03 comprising a light chain amino acid sequence as shown in SEQ ID NO: 4 and a heavy chain amino acid sequence as shown in SEQ ID NO: 5 (see Example 2, below).

[00123] Accordingly, the disclosure also provides a CD133-binding agent comprising:

(i) an antibody light chain variable domain comprising a light chain CDR1 comprising the amino acid sequence of SEQ ID NO: 6, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and/or a light chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8; and/or

(ii) an antibody heavy chain variable domain comprising a Met residue at position 39 and a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 9; a Ser residue at position 55, a Tyr residue at position 66 and a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 10; and/or a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 11, wherein the antibody light chain variable domain and the antibody heavy chain variable domain form an antigen binding site that binds human CD133.

[00124] In one embodiment, the antibody light chain variable domain comprises a light chain CDR1, a light chain CDR2, and a light chain CDR3 comprising the amino acid sequences of SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively; and the antibody heavy chain variable domain comprises a heavy chain CDR1, a heavy chain CDR2, and a heavy chain CDR3 comprising the amino acid sequences of SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11, respectively; and the antibody heavy chain variable domain comprises Met, Ser and Tyr residues at positions 39, 55 and 66, respectively.

[00125] In another embodiment, the antibody light chain variable domain comprises a light chain CDR1, a light chain CDR2, and a light chain CDR3 consisting of the amino acid sequences of SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively; and the antibody heavy chain variable domain comprises an amino acid sequence (SEQ ID NO: 32) composed of a heavy chain CDR1 consisting of the amino acid sequence of SEQ ID NO: 9, and a Met residue flanking the heavy chain CDR1 at position 39; an amino acid sequence (SEQ ID NO: 33) composed of a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 10, a Ser residue flanking the heavy chain CDR2 at position 55, and a Tyr residue flanking the heavy chain CDR2 at position 66; and a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 11.

[00126] In a further embodiment, the light chain comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 2; and/or the heavy chain comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3.

[00127] In still a further embodiment, the light chain consists of the amino acid sequence of SEQ ID NO: 2, and/or the heavy chain consists of the amino acid sequence of SEQ ID NO: 3.

[00128] Accordingly, the disclosure also provides a CD133-binding agent comprising:

(i) an antibody light chain variable domain comprising a light chain CDR1 comprising the amino acid sequence of SEQ ID NO: 12, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 13, and/or a light chain CDR3 comprising the amino acid sequence of SEQ ID NO: 14; and/or

(ii) an antibody heavy chain variable domain comprising a Ile residue at position 39 and a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 15; Tyr residues at positions 55 and 66 and a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16; and/or a heavy chain

CDR3 comprising the amino acid sequence of SEQ ID NO: 17, wherein the antibody light chain variable domain and the antibody heavy chain variable domain form an antigen binding site that binds human CD133.

[00129] In one embodiment, the antibody light chain variable domain comprises a light chain CDR1, a light chain CDR2, and a light chain CDR3 comprising the amino acid sequences of SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, respectively; and the antibody heavy chain variable domain comprises a heavy chain CDR1, a heavy chain CDR2, and a heavy chain CDR3 comprising the amino acid sequences of SEQ ID NO: 15, SEQ ID NO: 16 and SEQ ID NO: 17, respectively; and the antibody heavy chain variable domain comprises Ile, Tyr and Tyr residues at positions 39, 55 and 66, respectively.

[00130] In another embodiment, the antibody light chain variable domain comprises a light chain CDR1, a light chain CDR2, and a light chain CDR3 consisting of the amino acid sequences of SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, respectively; and the antibody heavy chain variable domain comprises an amino acid sequence (SEQ ID NO: 34) composed of a heavy chain CDR1 consisting of the amino acid sequence of SEQ ID NO: 15 and a Ile residue flanking the heavy chain CDR1 at position 39; an amino acid sequence (SEQ ID NO: 35) composed of a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 16, a Tyr residue flanking the heavy chain CDR2 at position 55, and a Tyr residue flanking the heavy chain CDR2 at position 66; and a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 17.

[00131] In a further embodiment, the antibody light chain comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4; and/or the antibody heavy chain comprises the amino acid sequence of SEQ ID NO: 5 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 5.

[00132] In a still further embodiment, the light chain consists of the amino acid sequence of SEQ ID NO: 4, and the heavy chain consists of the amino acid sequence of SEQ ID NO: 5.

[00133] Any of the CD133-binding agents of the present disclosure may be obtained and suitably prepared for use using well-known techniques.

[00134] Polypeptidic CD133-binding agents of the disclosure can be synthesized by recombinant techniques which are well known and routinely practiced in the art. A polypeptidic CD133-binding agent of the disclosure may be produced in recombinant sources, such as recombinant cell lines or transgenic animals. Techniques can be adapted for the production of single-chain antibodies, such as a scFv, specific to CD133 (see, e.g. U.S. Patent No. 4,946,778).

[00135] Alternatively, a polypeptidic CD133-binding agent of the disclosure, such as a CD133-binding antibody of the disclosure may be obtained by immunizing an animal with CD133, or with a polypeptide comprising a suitable CD133 epitope, so as to generate the antibody in the animal's serum.

[00136] A CD133-binding IgG antibody of the disclosure can be purified from a biological sample, such as serum, via techniques such as affinity chromatography using protein A or protein G (see, e.g. Wilkinson, 2000). Additionally or alternatively, CD133, or a polypeptide comprising an epitope thereof, which is specifically bound by the CD133-binding agent may be immobilized on a column to purify the CD133-binding agent from a sample by immunoaffinity chromatography.

[00137] A CD133-binding antibody fragment of the disclosure may be obtained from an antibody using conventional techniques. For example, F(ab')2 fragments can be generated by treating an antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

[00138] Methods of producing polypeptidic CD133-binding agents of the disclosure are described in further detail below.

[00139] As set forth above, in an embodiment, the CD133-binding agent may be a bispecific antibody.

[00140] As used herein, bispecific antibodies are binding agents comprising two different antibody variable regions which confer binding specificities for at least two different antigens or two different epitopes of the same antigen.

[00141] The presently disclosed bispecific antibodies specifically bind CD133 and another antigen or specifically bind different epitopes of CD133. Optionally, the bispecific antibody binds CD133 and a cell-surface protein, receptor or receptor subunit.

[00142] In one embodiment, the bispecific antibody comprises a CD133-binding single-chain Fab and a non-CD133-binding scFv. Alternately, the bispecific antibody comprises a CD133-binding Fab and a non-CD133-binding scFv.

[00143] In another embodiment, the CD133-binding agent is a bispecific antibody that targets, binds and/or engages immune cells such as T cells, macrophages or NK cells. According to this embodiment, the CD133-binding agent is a bispecific antibody where one of the binding specificities is for CD133 and the other binding specificity is for an antigen expressed on the surface of T cells, macrophages or NK cells. For example, the bispecific antibody may bind CD133 and an immune cell receptor, such a receptor of a T cell, which when bound activates or inhibits activity of the immune cell.

[00144] Various techniques for making and isolating bispecific antibodies directly from recombinant cell culture have been described. For example, bispecific antibodies have been produced using leucine zippers (see, e.g. Kostelny et al., 1992), using “diabody” technology (see, e.g. Hollinger et al., 1993), and using single-chain Fv (scFv) dimers (see , e.g. Gruber et al., 1994).

[00145] A bispecific antibody that engages T cells may be referred to as a bispecific T-cell engager (BiTE). In one embodiment of the present disclosure, the bispecific antibody/BiTE specifically binds both CD133 and the T cell co-receptor CD3 (also referred to herein as CD133-binding/CD3-binding bispecific antibody). Accordingly, provided herein is a bispecific antibody/BiTE which comprises a CD133-binding antibody variable region of the disclosure and a CD3-binding antibody variable region. Such bispecific antibodies/BiTEs allow targeting of a T cell to a cell, such as a cancer cell, expressing surface CD133. Various configurations of the bispecific antibodies/BiTEs are contemplated herein. For example, in one embodiment, the bispecific antibody/BiTE comprises an anti-CD133 Fab and an anti-CD3 scFv. Optionally, either the light chain or the heavy chain of the anti-CD133 Fab is linked to the heavy-chain of the anti-CD3 scFv. In another embodiment, the bispecific antibody/BiTE comprises an anti-CD133 single chain Fab (scFab) and an anti-CD3 ScFv. Optionally, either the light chain or the heavy chain of the anti-CD133 Fab or anti-CD133 scFab is linked to the heavy-chain of the anti-CD3 scFv. In one embodiment, the anti-CD3 scFv binds CD3 epsilon/gamma. In one embodiment, the BiTE/bispecific antibody binds CD3 epsilon/delta. See, for example Figure 10A. Examples of configurations and amino acid sequences of various embodiments of the BiTE are provided in Table 6. Accordingly, the present disclosure also provides a BiTE comprising one or more amino acid sequences selected from (a) SEQ ID NO: 22 and SEQ ID NO: 23, (b) SEQ ID NO: 24 and SEQ ID NO: 25, (c) SEQ ID NO: 26, and (d) SEQ ID NO: 27, or functional variants thereof. In one embodiment, the BiTE comprises an amino acid sequence having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% sequence identity to any one of SEQ ID Nos: 22-27.

[00146] In a further embodiment, the bispecific antibody binds CD133 and the NK cell surface receptor CD16.

[00147] In another embodiment of the present disclosure, the CD133-binding agent is a bispecific antibody comprising antibody variable region RW01 and antibody variable region RW03.

[00148] As described above, the CD133-binding agent may have any number of valencies and/or specificities. For example, a trispecific and/or trivalent CD133-binding agent can be prepared (see, e.g. Tutt et al., 1991).

[00149] As further described above, embodiments of the CD133-binding agents also include CD133-binding chimeric antigen receptors (CARs).

[00150] Accordingly, provided herein is a chimeric antigen receptor comprising (i) a CD133-binding agent of the disclosure and (ii) a CAR signaling domain comprising one or more immune cell receptor signaling domains. Chimeric antigen receptors are engineered receptors wherein a polypeptide comprising a CD133-binding variable region of the present disclosure, for example a CD133-binding scFv, is fused, for example via a hinge domain and transmembrane domain, to a CAR signaling domain comprising one or more intracellular signaling domains of one or more immune cell receptors. The CAR can be a monomeric polypeptide (e.g. anti-CD133 scFv-based) or a multimeric polypeptide (e.g. anti-CD133 Fab-based). Expression of such a CAR in an immune effector cell allows targeting of the immune cell to a cell expressing surface CD133, where binding of the CAR to the cell surface expressed CD133 activates effector functions of the immune effector cell.

[00151] In one embodiment, the CAR signaling domain comprises a signaling domain of the T cell co-receptor CD3 (e.g. CD3zeta or CD3gamma). In another embodiment, the CAR comprises a signaling domain of the T cell co-receptor CD3 fused to a signaling domain of one or more T cell costimulatory molecules (e.g. CD28, 4-1BB, CD137, OX40, ICOS and/or CD27). In yet another embodiment, the CAR signaling domain comprises CD3zeta, and portions of CD8 and CD28. In another embodiment, the CAR signaling domain comprises a human CD8 leader sequence and a CD8a transmembrane domain. In one embodiment the CAR comprises a CD28 signaling domain, and a terminal CD3zeta signaling domain. In a further embodiment, the CAR signaling domain comprises a CD3zeta signaling domain, a 4-1BB signaling domain, and a CD28 signaling domain. Different configurations of a scFv comprised in the CAR are

contemplated including VL-linker-VH and VH-linker-VL. The construction of suitable CARs and their use for targeting antigen expressing cells, commonly referred to in the art as “CAR T cell therapy”, is well known in the art (see, e.g. Maus and June, 2016; Abate-Daga and Davila, 2016; Resetca et al., 2016; and Wang and Rivière, 2016).

[00152] In an additional embodiment, the CD133-binding agent is a phage-Fab or phage-scFv, where the Fab or scFv specifically binds CD133. The disclosure also provides a T cell expressing a CAR as described herein.

[00153] It can be desirable to modify a binding agent disclosed herein with respect to effector function, so as to enhance its effectiveness in binding/targeting CD133-expressing cells and/or reducing levels of CD133 in CD133-expressing cells. For example, where the binding agent comprises an antibody Fc region, such as an antibody, cysteine residue(s) can be introduced into the COOH terminal of the Fc region, thereby allowing interchain disulfide bond formation between antibody monomers in this region. The homodimeric antibody thus generated can have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC) (see, e.g. Caron et al., 1992; and Shopes, 1992). Alternatively, an antibody can be engineered that has dual Fc regions and can thereby have enhanced complement lysis and ADCC capabilities (see, e.g. Stevenson et al., 1989). Functional variants of the CD133-binding agents described herein are also encompassed by the present disclosure. The term "functional variant" as used herein includes modifications or chemical equivalents of the amino acid and nucleic acid sequences disclosed herein that perform substantially the same function as the polypeptides or nucleic acid molecules disclosed herein in substantially the same way. For example, functional variants of polypeptides disclosed herein include, without limitation, conservative amino acid substitutions.

[00154] A "conservative amino acid substitution" as used herein, is one in which one amino acid residue is replaced with another amino acid residue are

substitutions that change an amino acid to a different amino acid with similar biochemical properties (e.g. charge, hydrophobicity and size). Variants of polypeptides also include additions and deletions to the polypeptide sequences disclosed herein. In addition, variant nucleotide sequences include analogs and derivatives thereof. A variant of the binding agents disclosed herein include agents that bind to the same antigen or epitope as the binding agents.

[00155] In one embodiment, the present disclosure includes functional variants to the amino acid sequences disclosed herein. In particular, the disclosure provides functional variants of the amino acid sequences of the light chain and heavy chain of IgG RW01 (SEQ ID NO: 2 and SEQ ID NO: 3, respectively), functional variants of the amino acid sequences of the CDRs of antibody variable region RW01 (SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11), and functional variants of the amino acid sequences corresponding to SEQ ID NO: 32 and SEQ ID NO: 33 of the heavy chain of antibody variable region RW01. The disclosure further particularly provides functional variants of the amino acid sequences of the light chain and heavy chain of IgG RW03 (SEQ ID NO: 4 and SEQ ID NO: 5, respectively), functional variants of the amino acid sequences of the CDRs of antibody variable region RW03 (SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 and SEQ ID NO: 17), and functional variants of the amino acid sequences corresponding to SEQ ID NO: 34 and SEQ ID NO: 35 of the heavy chain of antibody variable region RW03.

[00156] In another embodiment, the present disclosure includes functional variants to the nucleic acid sequences that encode the amino acid sequences disclosed herein. Particularly provided are functional variants of the nucleotide sequences encoding the light chain and heavy chain of IgG RW01 (SEQ ID NO: 18 and SEQ ID NO: 19, respectively), functional variants of the nucleotide sequences encoding the light chain and heavy chain variable domains of antibody variable region RW01 (SEQ ID NO: 52 and SEQ ID NO: 53, respectively), functional variants of the nucleotide sequences encoding the amino acid sequences of the CDRs of antibody variable region RW01 (SEQ ID NO: 36,

SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40 and SEQ ID NO: 41), and functional variants of the nucleotide sequences encoding the amino acid sequences corresponding to SEQ ID NO: 32 and SEQ ID NO: 33 of the heavy chain of antibody variable region RW01 (SEQ ID NO: 42 and SEQ ID NO: 43, respectively).

[00157] The disclosure further particularly provides functional variants of the nucleotide sequences encoding the light chain and heavy chain of IgG RW03 (SEQ ID NO: 20 and SEQ ID NO: 21, respectively), functional variants of the nucleotide sequences encoding the light chain and heavy chain variable domains of antibody variable region RW03 (SEQ ID NO: 54 and SEQ ID NO: 55, respectively), functional variants of the nucleotide sequences encoding the amino acid sequences of the CDRs of antibody variable region RW03 (SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48 and SEQ ID NO: 49), and functional variants of the nucleotide sequences encoding the amino acid sequences corresponding to SEQ ID NO: 34 and SEQ ID NO: 35 of the heavy chain of antibody variable region RW03 (SEQ ID NO: 50 and SEQ ID NO: 51, respectively) .

[00158] In addition, the functional variants include nucleotide sequences that hybridize to the nucleic acids encoding the amino acid sequences of the present disclosure, or the complement thereof, under at least moderately stringent hybridization conditions. Such functional variants include nucleotide sequences that hybridize to SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54 or SEQ ID NO: 55, or the complement thereof, under at least moderately stringent hybridization conditions.

[00159] By “at least moderately stringent hybridization conditions” it is meant that conditions are selected which promote selective hybridization

between two complementary nucleic acid molecules in solution. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrids, is determined by the Tm, which in sodium containing buffers is a function of the sodium ion concentration and temperature (Tm = 81.5°C – 16.6 (Log10 [Na+]) + 0.41(%(G+C) – 600/l), or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In order to identify molecules that are similar, but not identical, to a known nucleic acid molecule a 1% mismatch may be assumed to result in about a 1°C decrease in Tm, for example if nucleic acid molecules are sought that have a >95% identity, the final wash temperature will be reduced by about 5°C. Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions. In some embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5x sodium chloride/sodium citrate (SSC)/5x Denhardt's solution/1.0% SDS at Tm - 5°C based on the above equation, followed by a wash of 0.2x SSC/0.1% SDS at 60°C. Moderately stringent hybridization conditions include a washing step in 3x SSC at 42°C. It is understood, however, that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 2002, and in: Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001.

[00160] In one embodiment, the variant amino acid sequences of the amino acid sequences disclosed herein comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% sequence identity to SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO:

15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 or SEQ ID NO: 35.

[00161] In another embodiment, the variant amino acid sequences of the amino acid sequences disclosed herein comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% sequence identity to the framework regions of SEQ ID NOS: 2, 3, 4 or 5. In another embodiment, the variant nucleotide sequences encoding the amino acid sequences disclosed herein comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% sequence identity to SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54 or SEQ ID NO: 55.

[00162] In another embodiment, the variant nucleotide sequences encoding amino acid sequences comprising heavy and light chain variable domains disclosed herein comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% sequence identity to the nucleotide sequences encoding such amino acid sequences, including SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54 and SEQ ID NO: 55.

[00163] The term "sequence identity" as used herein refers to the percentage of sequence identity between two amino acid sequences or two nucleic acid sequences. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g. gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at

corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical overlapping positions/total number of positions.times.100%). In one embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. One non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g. for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present disclosure. BLAST protein searches can be performed with the XBLAST program parameters set, e.g. to score-50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g. of XBLAST and NBLAST) can be used (see, e.g. the NCBI website). Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent identity between two sequences can be determined using techniques similar to those described above, with or without

allowing gaps. In calculating percent identity, typically only exact matches are counted.

Nucleic Acids and Vectors

[00164] Also provided are nucleic acids encoding the antibody variable regions described herein and nucleic acids encoding polypeptides comprising these antibody variable regions. As used herein, the term “nucleic acids” includes isolated nucleic acids.

[00165] In particular the present disclosure provides nucleic acids encoding the CDR regions of antibody variable region RW01 as set out in SEQ ID NOs: 36-41, and functional variants thereof; and nucleic acids encoding the amino acid sequences of the heavy chain variable domain of RW01 as set out in SEQ ID NOs: 42 and 43, and functional variants thereof. Also provided are nucleic acids encoding the CDR regions of RW03 as set out in SEQ ID NOs: 44-49, and functional variants thereof; and nucleic acids encoding the amino acid sequences of the heavy chain variable domain of RW03 as set out in SEQ ID NOs: 50 and 51, and functional variants thereof.

[00166] Further provided is a nucleic acid (SEQ ID NO: 52) encoding the light chain variable domain of antibody variable region RW01, and functional variants thereof. In one embodiment, the nucleic acid encodes (a) the amino acid sequence of SEQ ID NO: 28 or (b) an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 28. Also provided is a nucleic acid (SEQ ID NO: 53) encoding the heavy chain of IgG RW01. In one embodiment, the nucleic acid encodes (a) the amino acid sequence of SEQ ID NO: 29 or (b) an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 29.

[00167] Further provided is a nucleic acid (SEQ ID NO: 54) encoding the light chain variable domain of antibody variable region RW03, and functional variants thereof. In one embodiment , the nucleic acid encodes (a) the amino acid sequence of SEQ ID NO: 30 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 30. Also provided is a

nucleic acid (SEQ ID NO: 55) encoding the heavy chain variable domain of antibody variable region RW03. In one embodiment, the nucleic acid encodes (a) the amino acid sequence of SEQ ID NO: 31 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 31.

[00168] The disclosure also provides nucleic acids encoding the light chain and heavy chain of IgG RW01 and IgG RW03 as set out in SEQ ID Nos: 18, 19, 20 and 21, and functional variants thereof.

[00169] The disclosure also provides nucleic acids encoding the variable domains of the light chain and heavy chain of antibody variable region RW01 and antibody variable region RW03 as set out in SEQ ID Nos: 52, 53, 54 and 55, and functional variants thereof.

[00170] Polypeptidic binding agents disclosed herein can be expressed by a vector containing a nucleic acid encoding the polypeptide of interest using methods which are well known and routinely practiced in the art. Accordingly, the present disclosure also provides a vector expressing any of the nucleic acids described herein.

[00171] The polypeptidic binding agents can be prepared by constructing a nucleic acid encoding a polypeptidic binding agent, inserting the construct into an expression vector, and then expressing it in appropriate host cells. Vectors useful for expressing the polypeptidic binding agents disclosed herein are well known in the art. In one embodiment, the vector includes suitable translation initiation and termination signals in operable reading phase with a functional promoter and can comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and, if desirable, to provide amplification within the host. In addition to vectors, the nucleic acids of the present disclosure can be delivered to a cell or a subject via any other method known in the art including, but not limited to, liposomes, naked DNA, adjuvant-assisted DNA, gene gun, catheters, etc.

Monoclonal polypeptides / Monoclonal Antibodies

[00172] As described above, the CD133-binding agent can be a polypeptide comprising a CD133-binding antibody variable region, such as an antibody specifically comprising antibody variable region RW01 or antibody variable region RW03. Accordingly, the disclosure further provides a monoclonal polypeptidic CD133-binding agent of the disclosure, such as a monoclonal CD133-binding antibody of the disclosure.

[00173] As used herein, a “monoclonal” polypeptidic CD133-binding agent of the disclosure refers to a population of identical polypeptidic CD133-binding agent molecules. For example, in the case of a monoclonal polypeptidic CD133-binding agent of the disclosure comprising a CD133-binding antibody variable region, such as a monoclonal CD133-binding antibody of the disclosure, the CDRs are identical in all the molecules of the population. Various procedures known within the art may be used for the production of monoclonal polypeptides, such as monoclonal antibodies of the disclosure (see, for example, Greenfield, 2013). Monoclonal antibodies are commonly alternatively referred to using the abbreviations “mAb” or “MAb".

[00174] Monoclonal antibodies can be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies and antigen-binding fragments thereof can be readily isolated and sequenced using conventional procedures (e.g. by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

[00175] Monoclonal antibodies may also be generated, e.g. by immunizing an animal with CD133, such as, for example, murine, rat or human CD133 or an

immunogenic fragment, derivative or variant thereof. Alternatively, the animal is immunized with cells transfected with a vector containing a nucleic acid molecule encoding CD133 that is expressed and associated with the surface of the transfected cells. Alternatively, the antibodies are obtained by screening a library that contains antibody or antigen binding domain sequences for binding to CD133. This library is prepared, e.g. in bacteriophage as protein or peptide fusions to a bacteriophage coat protein that is expressed on the surface of assembled phage particles and the encoding DNA sequences contained within the phage particles (i.e., "phage displayed library"). Hybridomas resulting from myeloma/B cell fusions are then screened for reactivity to CD133.

[00176] Monoclonal antibodies may be prepared, for example, using hybridoma methods (see, for example, Kohler and Milstein, 1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes can be immunized in vitro.

Affinity

[00177] Non-covalent interactions occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_D) of the interaction, wherein a smaller K_D represents a greater affinity. Immunological binding properties of specific polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation (see, e.g. Malmqvist, 1993). The ratio of K_{off}/K_{on} enables the cancellation of all parameters

not related to affinity, and is equal to the dissociation constant K_D (see, e.g. Davies et al., 1990).

[00178] A bivalent CD133-binding agent disclosed herein, such as a CD133-binding agent comprising two CD133-binding antibody variable regions (e.g. an antibody or $F(ab')_2$), is considered to specifically bind CD133 when the dissociation constant (K_D) of the binding is ≤ 1 micromolar. A monovalent CD133-binding agent disclosed herein (i.e. which has single CD133-binding site, such as a single CD133-binding antibody variable region, e.g. a scFv or a Fab) is said to specifically bind CD133 when the dissociation constant (K_D) of the binding of the CD133-binding agent in bivalent form is ≤ 1 micromolar. Methods for joining monovalent binding agents of the disclosure for generating suitable bivalent forms thereof are well known in the art (e.g. where the monovalent agent comprises a single antibody variable region, production of bivalent antibodies/$F(ab')_2$ comprising two copies of the antibody variable region; or e.g. using suitable linkers, such as polypeptide linkers, nucleic acid linkers or chemically synthesized linkers).

[00179] In various embodiments, the CD133-binding agent binds CD133 with a dissociation constant (K_D) of ≤ 1 micromolar, ≤ 900 nM, ≤ 800 nM, ≤ 700 nM, ≤ 600 nM, ≤ 500 nM, ≤ 400 nM, ≤ 300 nM, ≤ 200 nM, ≤ 100 nM, ≤ 90 nM, ≤ 80 nM, ≤ 70 nM, ≤ 60 nM, ≤ 50 nM, ≤ 40 nM, ≤ 30 nM, ≤ 20 nM, ≤ 10 nM, ≤ 9 nM, ≤ 8 nM, ≤ 7 nM, ≤ 6 nM, ≤ 5 nM, ≤ 4 nM, ≤ 2 nM, ≤ 1 nM, ≤ 0.9 nM, ≤ 0.8 nM, ≤ 0.7 nM, ≤ 0.6 nM, ≤ 0.5 nM, ≤ 0.4 nM to 0.3 nM, ≤ 0.2 nM, or ≤ 100 pM to about 1 pM.

[00180] In further various embodiments, the CD133-binding agent binds CD133 with a dissociation constant (K_D) of ≤ 1 micromolar to 100 nM, ≤ 100 nM to 10 nM, ≤ 10 nM to 1 nM, ≤ 1 nM to 0.1 nM, or ≤ 0.1 nM to 10 pM.

[00181] In additional various embodiments, the CD133-binding agent binds CD133 with a dissociation constant (K_D) of ≤ 3 nM to 2 nM, ≤ 2.6 nM to 2.4 nM, ≤ 2.5 nM, about 2.5 nM, ≤ 2 nM to 1 nM, ≤ 0.6 nM to 0.4 nM, ≤ 0.5 nM, or about 0.5 nM.

[00182] As disclosed herein, the dissociation constant K_D for the binding of a bivalent CD133-binding agent, such as a CD133-binding antibody of the disclosure or a monovalent CD133-binding agent in bivalent form, is considered to approximately correspond to the half-maximal concentration ("EC50") of the CD133-binding agent required to saturate binding to a population of CD133-overexpressing cells, such as HEK293-CD133 cells, as determined via flow cytometry (see Example 3, below). This method is useful for determining the affinity of a binding agent for a cell surface molecule which cannot be suitably purified, as is often the case with transmembrane proteins, such as CD133. Alternate methods of determining a dissociation constant (K_D) for binding of a CD133-binding agent to cell surface-expressed CD133 include radioligand binding assays, and similar assays known to those skilled in the art. Alternately, where CD133 or a portion thereof bound by a CD133-binding agent of the disclosure is available in purified form, the dissociation constant (K_D) can be measured by assays such as surface plasmon resonance (SPR; Biacore) assay and other suitable assays known in the art.

[00183] As described above, the disclosure provides a CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03.

[00184] Any one of various methods known in the art can be used to identify a CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03. A person skilled in the art will appreciate that binding assays such as a competition binding assay can be used for this purpose. Those skilled in the art will recognize that it is possible to determine, without undue experimentation, if a binding agent specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03 by ascertaining whether the binding agent prevents antibody IgG RW01 and/or antibody IgG RW03 from binding to human CD133. If the binding agent being tested competes with antibody IgG RW01 and/or antibody IgG RW03, as shown by a decrease in binding to human CD133 by antibody IgG RW01 and/or antibody IgG RW03,

then the binding agent binds to the same epitope as antibody IgG RW01 and/or antibody IgG RW03. Methods for the testing the specificity of binding agents include, but are not limited to, enzyme linked immunosorbent assay (ELISA) and other immunologically mediated techniques known within the art.

[00185] In one embodiment, the CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03 is a CD133-binding agent which competes with antibody IgG RW01 and/or antibody IgG RW03 for binding to the surface of CD133-expressing cells. In one embodiment, the CD133 epitope is a human CD133 epitope. For example, a CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03 will partially or fully inhibit binding of antibody IgG RW01 and/or antibody IgG RW03 to CD133-expressing cells pre-incubated with a saturating concentration of such a binding agent, as determined via flow cytometry analogously to the experiments disclosed in Example 7. Alternately, a CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03 will partially or fully inhibit binding of antibody IgG RW01 and/or antibody IgG RW03 to denatured CD133, e.g. denatured CD133 electroblotted onto a membrane (e.g. nitrocellulose or PVDF) following electrophoretic separation under reducing conditions of denatured protein of whole cell lysate (see, e.g. Example 6), where the membrane has been pre-incubated with a saturating concentration of such binding agent.

[00186] In another embodiment, the CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a CD133 epitope bound by antibody IgG RW03 is a monovalent CD133-binding agent (e.g. a Fab or a scFv) which competes with Fab comprising antibody variable region RW01 and/or Fab comprising antibody variable region RW03 for binding to the surface of CD133-expressing cells. In one embodiment, the CD133 epitope is a human CD133 epitope. For example, a monovalent CD133-binding agent which specifically binds a CD133 epitope bound by antibody IgG RW01 and/or a

CD133 epitope bound by antibody IgG RW03 will partially or fully inhibit binding of Fab comprising antibody variable region RW01 and/or Fab comprising antibody variable region RW03 to CD133-expressing cells pre-incubated with a saturating concentration of such a binding agent, as determined via flow cytometry analogously to the experiments disclosed in Example 7. Alternately, the monovalent agent will partially or fully inhibit binding of Fab comprising antibody variable region RW01 and/or Fab comprising antibody variable region RW03 to denatured CD133, e.g. denatured CD133 electroblotted onto a membrane (e.g. nitrocellulose or PVDF) following electrophoretic separation under reducing conditions of denatured protein of whole cell lysate (see, e.g. Example 6), where the membrane has been pre-incubated with a saturating concentration of such binding agent following electroblotting. In one embodiment, the CD133 is human CD133.

Detection Agents

[00187] The binding agents described herein are optionally labeled with a detection agent. As used herein, the term "detection agent" refers to any agent that allows the presence of the binding agent to be detected and/or quantified. Examples of detection agents include, but are not limited to, peptide tags, enzymes (for example, HRP or alkaline phosphatase), proteins (for example phycoerythrin or biotin/streptavidin), magnetic particles, chromophores, fluorescent molecules, chemiluminescent molecules, radioactive labels and dyes. The binding agent may be labeled directly or indirectly with the detection agent.

Humanized Antibodies

[00188] The nucleotide sequence encoding a non-human, e.g. murine, CD133-binding agent disclosed herein can be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous non-human, e.g. murine, sequences (see, e.g. U.S. Patent No. 4,816,567; and Morrison, 1994) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can

be substituted for the constant domains of an antibody disclosed herein, or can be substituted for the variable domains of one antigen-combining site of an antibody disclosed herein to create a chimeric bivalent antibody.

[00189] The non-human binding agents comprising Fc regions, e.g. non-human antibodies, described herein may be humanized in order to make them better tolerated for use in humans. For example, amino acid residues in the framework regions may be humanized by replacing them with amino acid residues and the human framework regions as long as the replacement does not impair the ability of the binding agents to bind to CD133 (see, e.g. Vincke et al., 2008).

[00190] It will be appreciated that murine antibodies or antibodies from other species can be humanized or primatized using techniques well known in the art (see, e.g. Winter and Harris, 1993; and Wright et al., 1992). An antibody may be engineered by recombinant DNA techniques to substitute the CH1, CH2, CH3, hinge domains, and/or the framework domain with the corresponding human sequence (see, e.g. WO 92102190 and U.S. Patent Nos 5,530,101; 5,585,089; 5,693,761; 5,693,792; 5,714,350; and 5,777,085). Also, the use of Ig cDNA for construction of chimeric immunoglobulin genes is known in the art (see, e.g. Liu et al., 1987a; and Liu et al., 1987b). mRNA is isolated from a hybridoma or other cell producing the antibody and used to produce cDNA. The cDNA of interest may be amplified by the polymerase chain reaction using specific primers (U.S. Pat. Nos. 4,683,195 and 4,683,202). Alternatively, a library is made and screened to isolate the sequence of interest. The DNA sequence encoding the variable region of the antibody is then fused to human constant region sequences. Human C region genes are readily available from known clones. The choice of isotype will be guided by the desired effecter functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity. Either of the human light chain constant regions, kappa or lambda, may be used. The chimeric, humanized antibody may then be expressed by conventional methods.

[00191] As described above, the CD133-binding agent may be a human antibody. Fully human antibodies are antibody molecules in which the entire sequence of both the light chain and the heavy chain, including the CDRs, arise from human genes. Such antibodies are termed "human antibodies" or "fully human antibodies" herein. Human monoclonal antibodies can be prepared by using the trioma technique; the human B-cell hybridoma technique (see, e.g. Kozbor, et al., 1983), and the EBV hybridoma technique to produce human monoclonal antibodies (see, e.g. Cole et al., 1985). Human monoclonal antibodies may be utilized and may be produced by using human hybridomas (see, e.g. Cote, et al., 1983) or by transforming human B-cells with Epstein Barr Virus in vitro (see, e.g. Cole et al., 1985).

[00192] A CD133-binding polypeptide comprising a CD133-binding antibody variable region, such as a CD133-binding scFv or CD133-binding Fab may be developed, for example, using phage-display methods using antibodies containing only human sequences. Such approaches are well-known in the art (see, e.g. WO92/01047 and U.S. Pat. No. 6,521,404, which are hereby incorporated by reference). In this approach, a combinatorial library of phage carrying random pairs of light and heavy chains are screened using natural or recombinant source of CD133 or fragments thereof. In another approach, an antibody or fragment can be produced by a process wherein at least one step of the process includes immunizing a transgenic, non-human animal with a CD133 protein. In this approach, some of the endogenous heavy and/or kappa light chain loci of this xenogeneic/non-human animal have been disabled and are incapable of the rearrangement required to generate genes encoding immunoglobulins in response to an antigen. In addition, at least one human heavy chain locus and at least one human light chain locus have been stably transfected into the animal. Thus, in response to an administered antigen, the human loci rearrange to provide genes encoding human variable regions immunospecific for the antigen. Upon immunization, therefore, the animal produces B-cells that secrete fully human immunoglobulins.

[00193] A variety of techniques are well-known in the art for producing xenogeneic/non-human animals (see, e.g. U.S. Pat. Nos. 6,075,181 and 6,150,584, which are hereby incorporated by reference; Green et al., 1994, which is hereby incorporated by reference in its entirety; U.S. Patent Nos. 6,162,963, 6,150,584, 6,114,598, 6,075,181, and 5,939,598; Japanese Patent Nos. 3 068 180 B2, 3 068 506 B2, and 3 068 507 B2; European Patent No., EP 0 463 151 B1; and International Patent Application Nos. WO 94/02602, WO 96/34096, WO 98/24893, and WO 00/76310).

[00194] Alternatively, a "minilocus" approach may be used, in which an exogenous Ig locus is mimicked through the inclusion of pieces (individual genes) from the Ig locus (see, e.g. U.S. Patent Nos. 5,545,806, 5,545,807, 5,591,669, 5,612,205, 5,625,825, 5,625,126, 5,633,425, 5,643,763, 5,661,016, 5,721,367, 5,770,429, 5,789,215, 5,789,650, 5,814,318, 5,877,397, 5,874,299, 6,023,010, and 6,255,458; European Patent No. 0 546 073 B1; and International Patent Application Nos. WO 92/03918, WO 92/22645, WO 92/22647, WO 92/22670, WO 93/12227, WO 94/00569, WO 94/25585, WO 96/14436, WO 97/13852, and WO 98/24884). Thus, one or more VH genes, one or more DH genes, one or more JH genes, a mu constant region, and a second constant region (preferably a gamma constant region) are formed into a construct for insertion into an animal.

[00195] Generation of human antibodies from mice in which, through microcell fusion, large pieces of chromosomes, or entire chromosomes, have been introduced, has also been demonstrated (see, e.g. European Patent Application Nos. 773 288 and 843 961).

Immunoconjugates

[00196] The present disclosure also includes an immunoconjugate comprising (1) a CD133-binding agent, optionally an antibody or an antibody antigen binding fragment, that has been attached to (2) an effector agent. As used herein, the term "immunoconjugate" encompasses CD133-binding agents of the disclosure which do not comprise an antibody variable region, and further

encompasses CD133-binding agents disclosed herein which comprise an antibody variable region.

[00197] In one embodiment, the effector agent is a label, which can generate a detectable signal, directly or indirect. Examples of labels include radioactive isotopes (i.e., a radioconjugate).

[00198] In another embodiment, the effector agent is a therapeutic agent. Therapeutic agents include, but are not limited to, cancer therapeutic agents/antineoplastic agents. In yet another embodiment, the therapeutic agent is a toxin.

[00199] The term “cancer therapeutic agent” or “antineoplastic agent” is used herein to refer to agents that have the functional property of decreasing levels of CD133 in cancer cells, such as pancreatic cancer cells, colorectal cancer cells, breast cancer cells, colon cancer cells, gastric cancer cells, prostate cancer cells, liver cancer cells, lung cancer cells, melanoma cells, brain cancer cells (optionally glioblastoma or medulloblastoma cells) and head and neck squamous cell carcinoma cells.

[00200] The toxin may be an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or a fragment thereof. Toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), Momordica charantia inhibitor, curcin, crotin, Saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[00201] Radioconjugated CD133-binding agents of the disclosure, such as antibodies of the disclosure, may be employed to bind radionuclides to CD133-expressing cells, for example to visualize the cells or as a cytotoxic treatment of the cells. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include 212Bi, 131I, 131In, 90Y, and 186Re.

[00202] Those of ordinary skill in the art will recognize that a large variety of possible moieties can be coupled to the polypeptidic CD133-binding agents of the disclosure, such as those comprising an antibody variable region (e.g. antibodies or antibody fragments comprising a CD133-binding antibody variable region) (see, for example, Cruse and Lewis, 1989, the entire contents of which are incorporated herein by reference). Coupling may be accomplished by any chemical reaction that will bind a moiety and a CD133-binding agent of the disclosure, so long as these retain their respective activities/characteristics for the intended use thereof. This linkage can include many chemical mechanisms, for instance covalent binding, affinity binding, intercalation, coordinate binding and complexation.

[00203] For example, conjugates of a polypeptidic CD133-binding agent of the disclosure, such as an antibody and an effector agent can be made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987).

[00204] Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see, e.g. W094/11026).

Pharmaceutical compositions

[00205] The disclosure also provides pharmaceutical compositions comprising a CD133-binding agent or immunoconjugate or radioconjugate described herein as an active ingredient and a pharmaceutically acceptable carrier.

[00206] As used herein, the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Optional examples of such carriers or diluents include, but are not limited to, water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin.

[00207] A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g. intravenous, intradermal, subcutaneous, oral (e.g. inhalation), transdermal (i.e., topical), transmucosal, and rectal administration.

[00208] In one embodiment, the active ingredient is prepared with a carrier that will protect it against rapid elimination from the body, such as a sustained/controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

[00209] In one embodiment, oral or parenteral compositions are formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms are dictated by and directly dependent on the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and the limitations inherent in the art of preparing such an active ingredient for the treatment of individuals.

[00210] The formulation can also contain more than one active ingredient as necessary for the particular indication being treated, optionally those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the pharmaceutical composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

Methods and Uses:

[00211] The disclosure also provides uses and methods relating to the CD133-binding agents described herein.

Detecting CD133-expressing cells

[00212] The CD133-binding agents, immunoconjugates and pharmaceutical compositions of the present disclosure are useful for detecting cells that express CD133. Accordingly, the disclosure provides a use of the CD133-binding agents described herein for targeting, binding and/or detecting CD133-expressing cells. Optionally, the cells are cancer cells, including, but not limited to, pancreatic cancer cells, colorectal cancer cells, breast cancer cells, colon cancer cells, gastric cancer cells, prostate cancer cells, liver cancer cells, lung cancer cells, melanoma cells, brain cancer cells and head and neck squamous cell carcinoma cells.

[00213] In one embodiment, the CD133-binding agents, immunoconjugates, and pharmaceutical compositions described herein are useful for targeting, binding and/or detecting cell surface expression of CD133-expressing cells.

[00214] In another embodiment, the CD133-binding agents, immunoconjugates and pharmaceutical compositions described herein are useful for targeting, binding, detecting and/or localizing intracellular CD133.

[00215] In another embodiment, the CD133-binding agents, immunoconjugates and pharmaceutical compositions described herein are useful for targeting, binding and/or detecting CD133 in cell lysates.

[00216] In yet another embodiment, the CD133-binding agents, immunoconjugates and pharmaceutical compositions described herein are useful for detecting and/or quantitating levels of expression of CD133 in a sample, optionally in a CD133 expressing cell. In one embodiment, the CD133-binding agents, immunoconjugates and pharmaceutical compositions are used to detect and/or quantitate cellular CD133 levels. In another embodiment, the CD133-binding agents, immunoconjugates and pharmaceutical compositions are useful for detecting and/or quantitating cell surface CD133 levels.

[00217] The CD133-binding agents of the disclosure may be used for detecting/quantitating both native/cell-surface expressed as well as denatured CD133. Overexpression of CD133 often correlates with a cancerous phenotype. For example, Western blotting detection of CD133 protein levels in denatured whole cell lysates using CD133-binding agents of the disclosure can be used to characterize/confirm the ability of a treatment to reduce the metastatic capacity of CD133-expressing cancer cells, since reduced total cellular CD133 protein levels has been shown to correlate with reduced metastatic capacity of the cells (see, e.g. Rappa et al. 2008).

[00218] In general, the use of binding agents for detection of analytes, such as intracellular, total cellular or surface-expressed CD133 protein, is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as radioactive, fluorescent, biological and enzymatic tags. Examples of methods include, but are not limited to, Western blotting, enzyme linked immunosorbent assay (ELISA), immunofluorescence, immunohistochemistry and flow cytometry.

[00219] The CD133-binding agents, immunoconjugates and pharmaceutical compositions of the present disclosure are also useful for reducing and/or eliminating the level or amount of CD133 protein in a cell. Optionally, the cell is a CD133-positive cancer cell, including, but not limited to, a pancreatic cancer cell, colorectal cancer cell, breast cancer cell, colon cancer cell, gastric cancer cell,

prostate cancer cell, liver cancer cell, pancreatic cancer cell, lung cancer cell, melanoma cell, brain cancer cell (optionally a glioblastoma or medulloblastoma cell) and head and neck squamous cell carcinoma cell. Reduction of total cellular CD133 protein levels in cancer cells can be used to reduce the metastatic capacity thereof (see, e.g. Rappa et al. 2008). The CD133 protein of which the levels are reduced is optionally cell surface-expressed and/or intracellular CD133. The CD133 protein in a CD133-expressing cell is optionally reduced by at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98 or 99%; or by 100%.

Targeting CD133-expressing cells to immune cells

[00220] Further, the CD133-binding agents, immunoconjugates and pharmaceutical compositions of the present disclosure are useful for engaging, targeting and/or binding cells of the immune system.

[00221] For example, in one embodiment described above, the CD133-binding agent is a bispecific antibody where one of the binding specificities is for CD133 and the other binding specificity is for an antigen expressed on an immune cell such as a T cell, macrophage or NK cell. As described above, one example of a bispecific antibody that targets T cells is a bispecific T-cell engager (BiTE).

[00222] In another embodiment described above, the CD133-binding agent is a CD133-binding chimeric antigen receptor (CAR) which includes a CD133-binding agent of the disclosure, such as a CD133-binding scFv as its antigen-binding/targeting domain.

[00223] The construction of suitable CARs and their use for targeting antigen expressing cells, commonly referred to in the art as “CAR T cell therapy”, is well known in the art (see, e.g. Maus and June, 2016; Abate-Daga and Davila, 2016; Resetca et al., 2016; and Wang and Rivière, 2016). Accordingly, the bispecific antibodies and chimeric antigen receptors described herein are useful for targeting immune effector cells to CD133-expressing cells (CD133+ cells).

[00224] Also provided are methods for targeting CD133+ cells comprising exposing the CD133+ cells to an immune effector cell expressing a CAR of the disclosure, or to a combination of a bispecific antibody of the disclosure and an immune effector cell specifically bound by the bispecific antibody.

[00225] Targeting immune effector cells to CD133+ cells through these methods may be useful for eliminating, and/or shifting the phenotype of, CD133+ cancer cells from a cancerous phenotype towards a less cancerous or non-cancerous phenotype. In addition, targeting immune effector cells to CD133+ cells may be useful for treating diseases where CD133 is expressed or overexpressed such as cancer.

Diagnostic Methods

[00226] The CD133-binding agents disclosed herein are useful in the detection/quantitation of CD133 in patient samples or in control samples of healthy individuals and accordingly may be useful diagnostics. For example, the binding agents of the disclosure can be used to detect/quantitate total cellular expression of CD133 and/or cell-surface expressed CD133. As used herein, the term “diagnostics” encompasses screening, stratification, monitoring and the like.

[00227] In one embodiment, the CD133-binding agents are used to detect CD133 expressing cells, optionally cancer cells such as pancreatic cancer cells, colorectal cancer cells, breast cancer cells, colon cancer cells, gastric cancer cells, prostate cancer cells, liver cancer cells, lung cancer cells, melanoma cells, brain cancer cells and head and neck squamous cell carcinoma cells.

[00228] In another embodiment, the CD133-binding agents are used for detecting/quantitating cell surface expression of CD133. In another embodiment, the CD133-binding agents are used for detecting/quantitating intracellular expression of CD133. In another embodiment, the CD133-binding agents described herein can be used to detect/quantitate expression of CD133 in a sample.

[00229] For example, CD133-binding agents of the disclosure, such as the antibodies and antibody fragments of the disclosure, may be used for practicing any one of various assays, e.g. immunofluorescence, flow cytometry or ELISAs, to detect/quantitate CD133 levels in a sample.

[00230] In one embodiment, the sample is a patient sample, such as a cancer sample from a cancer patient. Alternately, the sample may be a control sample from a healthy individual. Embodiments of the sample include but are not limited to, a sample of cultured cells, cultured cell supernatant, cell lysate, serum, blood plasma, biological fluid or biological tissue. In other embodiments, the sample is obtained from a cancer. In certain embodiments, the sample is a biopsy sample.

Treatment of cancer

[00231] CD133 has been shown to play an important role in various cancers. For example, CD133 has been identified as a marker for cancer stem cells (CSCs) in brain tumors (Singh et al., 2003) and it has been demonstrated that as few as 100 CD133+ cells from brain tumor fractions are sufficient to generate a tumor in NOD/SCID mice (Singh et al., 2004). Additionally, CD133+ glioma cells have been shown to have increased resistance to radiation in a DNA checkpoint dependent manner as compared with CD133-negative (CD133-) cells (Bao et al., 2006). Pancreatic CSCs have been isolated using anti-CD133 antibodies and it has been demonstrated that these cells are tumorigenic and highly resistant to standard chemotherapy (Hermann et al., 2007). Similarly, it has also been shown that increased CD133 expression in pancreatic cancer cells correlates with a more aggressive nature including increased migration and invasion and heightened tumor aggressiveness (Moriyama et al., 2010). Stem-like cells from prostate cancer tissues have been identified (Collins, 2005) and it has been demonstrated that CD133+ cells isolated from prostate cancer tissues and in an immortalized prostate cancer cell line exhibit stem cell features (Miki et al., 2007; Wei et al., 2007), however other studies have failed to confirm this (Missol-Kolka et al., 2010) and have not reported the stem-cell characteristics

that others attribute to pancreatic cancer lines like the DU145 line (Pfeiffer & Schalken, 2010). Another cancer type for which CD133 is used as a CSC marker is colorectal carcinoma. Separate groups have identified CD133 as a marker of CSCs in colon cancer (O'Brien et al., 2006; Ricci-Vitiani et al., 2006). It has been demonstrated that CD133+ cells readily recapitulate tumors in SCID mice (Ricci-Vitiani et al., 2006), and that there is an enrichment of colon cancer initiating cells purified CD133+ cells compared to unfractionated tumor cells (O'Brien et al., 2006). It has been demonstrated that both CD133+ and CD133-negative cells from colon metastases are able to form colon-spheres and recapitulate tumors in NOD/SCID mice (Shmelkov, 2004). Additionally, several groups have concluded that CD133 is associated with worse clinical prognosis, disease progression and metastasis and that CD133 protein can be used as an independent prognostic marker for colorectal cancer patients (Horst et al., 2009). However, other groups have failed to find a relationship between CD133 and disease progression or survival in colon cancer patients but have instead found a relationship between the expression of the protein and tumor stage (Lugli et al., 2010).

[00232] It has been demonstrated that downregulation of CD133 in the metastatic melanoma cell line FEMX-I resulted in slower cell growth, decreased cell motility, decreased ability to form spheres in stem-cell growth conditions and a reduced metastatic capacity of tumor xenografts (Rappa et al., 2008). It has been shown that the anti-CD133 antibody AC133 conjugated to monomethyl auristatin F inhibits the growth of hepatocellular and gastric cancer cells in vitro (Smith et al., 2008), it was further demonstrated that secondary antibody conjugated to saporin in the presence of AC133 is toxic to FEMX-I cells but not control human fibroblasts (Rappa et al., 2008). Additionally, AC133 directly conjugated to saporin has been shown to be more effective against FEMX-I cells than FEMX-I cells with CD133 expression knocked down (Rappa et al., 2008). It was observed that in cells in which CD133 is knocked down, genes that became upregulated coded for wnt inhibitors (Mak et al., 2012b).

[00233] In addition, numerous studies have implicated CD133+ brain tumor initiating cells (BTICs) as drivers of chemo- and radio-resistance in glioblastoma

(GBM). It has also recently been demonstrated that a CD133-driven gene signature is predictive of poor overall survival (Venugopal et al, 2015) and targeting CD133+ treatment refractory cells may be an effective strategy to block GBM recurrence.

[00234] Accordingly, these results provide support for targeting CD133 as an effective therapeutic strategy and as an effective diagnostics strategy. In addition, the present inventors have described a CAR T-cell-based strategy whereby CD133+ GBM cells are specifically targeted and killed. The present inventors have also shown that a BiTE antibody that redirects human polyclonal T cells to CD133+ GBM cells induces a potent anti-tumor response.

[00235] Accordingly, the CD133-binding agents and pharmaceutical compositions of the present disclosure are useful for treating or preventing a cancer, for example a metastatic melanoma, brain, prostate, pancreatic and/or colon/colorectal cancer. In one embodiment, the cancer is a glioblastoma. In another embodiment, the cancer is a medulloblastoma.

[00236] In another embodiment, the cancer is a CD133-positive cancer (also referred to as a CD133-expressing cancer). In one embodiment, a CD133-positive cancer is defined as a cancer with greater than 80%, 85%, 90%, 95% or 99% CD133-positive cells (i.e., CD133-expressing cells). The percentage of cells expressing CD133 may be determined, for example, in a tumor cell culture. Accordingly, in particular embodiments, the cancer is a CD133-positive glioblastoma or a CD133-positive medulloblastoma. In another embodiment, the cancer is a glioblastoma detectably expressing CD133 or a medulloblastoma detectably expressing CD133.

[00237] In one embodiment, the CD133-binding agents and pharmaceutical compositions described herein are used in a method for treating or preventing cancer, the method comprising administering an effective amount of a CD133-binding agent or pharmaceutical composition disclosed herein to an animal or cell in need thereof, optionally wherein the cancer is metastatic melanoma, brain,

prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00238] In another embodiment, an effective amount of a CD133-binding agent or pharmaceutical composition disclosed herein is used for treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In another embodiment, a CD133-binding agent or pharmaceutical composition disclosed herein is used in the preparation of a medicament for treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00239] In yet another embodiment, an effective amount of a CD133-binding agent or pharmaceutical composition disclosed herein is used for in treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00240] As described above, the present disclosure provides immunoconjugates comprising (1) a CD133-binding agent and (2) an effector agent, where the effector agent is optionally a toxin or an anti-neoplastic agent.

[00241] Accordingly, the present disclosure provides a method of using an immunoconjugate disclosed herein for treating or preventing a cancer, the method comprising administering an effective amount of an immunoconjugate disclosed herein to an animal or cell in need thereof, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00242] In one embodiment, an effective amount of an immunoconjugate disclosed herein is used for treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In another embodiment, an immunoconjugate disclosed herein is used in the preparation of a medicament for treating or preventing a cancer, optionally

wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00243] The present disclosure also provides CARs that target CD133 expressing cells, and T-cells expressing the CARs. Accordingly, in another embodiment, the present disclosure provides a method of using a T cell expressing a CAR disclosed herein for treating or preventing a cancer, the method comprising administering an effective amount of a T cell expressing a CAR disclosed herein to an animal or cell in need thereof, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00244] In one embodiment, an effective amount of a T cell expressing a CAR disclosed herein is used for treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In another embodiment, a T cell expressing a CAR disclosed herein disclosed herein is used in the preparation of a medicament for treating or preventing a cancer, optionally wherein the cancer is metastatic melanoma, brain, prostate, pancreatic and/or colon cancer. In one embodiment, the cancer is a glioblastoma or a medulloblastoma.

[00245] As used herein, the terms “subject” and “animal” include all members of the animal kingdom, in one embodiment the subject is a mammal. In a further embodiment the subject is a human being. In one embodiment, the subject is a patient having a disease, such as a cancer, associated with CD133-expressing cells.

[00246] The term "a cell" includes a single cell as well as a plurality or population of cells.

[00247] An effective amount of a CD133-binding agent, immunoconjugate or pharmaceutical composition of the disclosure relates generally to the amount needed to achieve a therapeutic objective. As noted above, this may be a binding

interaction between the CD133-binding agent and CD133 that, in certain cases, interferes with the functioning of CD133.

[00248] The amount required to be administered will furthermore depend on the binding affinity of the CD133-binding agent for CD133, and will also depend on the rate at which an administered CD133-binding agent is depleted from the free volume of the subject to which it is administered. Common ranges for therapeutically effective dosing of a CD133-binding agent, immunoconjugate or pharmaceutical composition of the disclosure may be, by way of non-limiting example, from about 0.1 mg kg body weight to about 50 mg/kg body weight. Common dosing frequencies may range, for example, from twice daily to once a week.

[00249] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular cancer. Alleviation of one or more symptoms of the cancer indicates that the antibody confers a clinical benefit.

[00250] As used herein, "treating a cancer" includes, but is not limited to, reversing, alleviating or inhibiting the progression of the cancer or symptoms or conditions associated with the cancer. "Preventing a cancer" includes preventing incidence or recurrence of the cancer. "Treating a cancer" also includes extending survival in a subject. Survival is optionally extended by at least 1, 2, 3, 6 or 12 months, or at least 2, 3, 4, 5 or 10 years over the survival that would be expected without treatment with a CD133-binding agent, immunoconjugate or pharmaceutical composition as described herein. "Treating a cancer" also includes reducing tumor mass and/or reducing tumor burden (for example, brain tumor mass and/or brain tumor burden). Optionally, tumor mass and/or tumor burden is reduced by at least 5, 10, 25, 50, 75 or 100% following treatment with a CD133-binding agent, immunoconjugate or pharmaceutical composition as described herein. In other embodiments, "treating a cancer" includes reducing the aggressiveness, grade and/or invasiveness of a tumor. The tumor is

optionally a newly formed tumor or a tumor already present at the time of treatment.

[00251] In one embodiment, the active ingredient may be used in combination with at least one additional therapeutic agent. Accordingly, the application provides a method of preventing or treating a cancer using the CD133-binding agents, immunoconjugates or pharmaceutical compositions disclosed herein in combination with at least one additional therapeutic agent. An additional therapeutic agent may be administered prior to, overlapping with, concurrently, and/or after administration of the active ingredients. When administered concurrently, the CD133-binding agents, immunoconjugates or pharmaceutical compositions and an additional therapeutic agent may be administered in a single formulation or in separate formulations, and if administered separately, then optionally, by different modes of administration. The combination of one or more CD133-binding agents, immunoconjugates or pharmaceutical compositions and one or more other therapeutic agents may synergistically act to combat the cancer.

[00252] Embodiments of the additional therapeutic agent include additional CD133-binding agents, additional CD133-binding immunoconjugates, additional CD133-binding pharmaceutical compositions, cytokines, growth factor inhibitors, immunosuppressants, anti-inflammatory agents, metabolic inhibitors, enzyme inhibitors, anti-neoplastic agents, cytotoxic agents and/or cytostatic agents. Such combination therapies may advantageously utilize lower dosages of an administered active ingredient, thus avoiding possible toxicities or complications associated with monotherapy.

Screening Assays

[00253] The disclosure also provides methods (also referred to herein as "screening assays") for identifying modulators, i.e., test agents (e.g. peptides, peptidomimetics, small molecules or other drugs) that modulate or otherwise interfere with the binding of a protein disclosed herein with the CD133.

[00254] The test agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection (see, e.g. Lam, 1997).

[00255] The above disclosure generally describes the present application. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the disclosure. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

[00256] The following non-limiting examples are illustrative of the present disclosure:

Examples

EXAMPLE 1: Cell Selections and Sequencing

[00257] In order to attempt to discover novel antibodies capable of specifically binding CD133, two phage-display libraries, Library F and Library G, were screened for cell surface CD133 binders using the "Cellectseq" method (the Cellectseq method, Library F and Library G have been previously described, e.g. in US Patent Application No. 13/629,520). Briefly, Library F is a Fab library with diversity in light chain complementarity-determining region (CDR) 3 and in all

three heavy chain CDRs, and Library G is scFv library with diversity in all six CDRs. The cells used for the selections were HEK293 cells engineered to overexpress CD133 (GenBank Accession O43490) for the positive selection and the parental HEK293 cells were used for the negative selection. After four rounds of positive and negative selection, serial dilutions were made of the round four output phage (10E-1 to 10E-3) for each library and used to infect XL1-blue cells. The infected cells were plated for isolating single colonies to be used in a clonal cell-based ELISA for isolating CD133-specific binders. Clones that bound to the HEK293-CD133 cells and generated an ELISA signal that was at least 1.5 fold higher than background binding to HEK293 cells were classified as CD133-specific binders. Sequencing was performed by amplifying the antibody variable (VL and VH) domains from clone DNA with M13-tagged sequencing primers, and the amplified sequences were sequenced via Illumina sequencing. The full-length amino acid sequence of human CD133 expressed in HEK293-CD133 cells is indicated in Table 1. The amino acid sequence coordinates of the three extracellular domains of CD133 are as follows: Gly20–Gly108, Ala179–Tyr433 and Gly508–Asn792. The amino acid sequence coordinates of the segment spanning all three extracellular domains is Gly20–Asn792. The signal sequence corresponding to Met1–Ser19 of the full-length sequence is absent from the mature, cell-surface expressed CD133 protein.

Table 1. Amino acid sequence of human CD133 (GenBank Accession O43490).

*MALVLGSLLLLGLCGNSFS*GGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVHIFLYVVQPRD FPEDTLRKFLQKAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLFIILMPLVGYFFCMCRCCNKCG GEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVANHQVRTRIKRSRKLADSNFKDLRTLLNETPE QIKYILAQYNTTKDKAFTDLNSINSVLGGGILDRLRPNIIPVLDEIKSMATAIKETKEALENMNSTLKSL HQQSTQLSSSLTSVKTSLRSSLNDPLCLVHPSSETCNSIRLSLSQLNSNPELRQLPPVDAELDNVNNVLR TDLDGLVQQGYQSLNDIPDRVQRQTTTVVAGIKRVLNSIGSDIDNVTQRLPIQDILSAFSVYVNNTESYI HRNLPTLEEYDSYWWLGGLVICSLLTLIVIFYYLGLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLS FLFCWILMIIVVLTFVFGANVEKLICEPYTSKELFRVLDTPYLLNEDWEYYLSGKLFNKSKMKLTFEQVY SDCKKNRGTYGTLHLQNSFNISEHLNINEHTGSISSELESLKVNLNIFLLGAAGRKNLQDFAACGIDRMN YDSYLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVLPIEQSLSTLYQSVK ILQRTGNGLLERVTRILASLDFAQNFITNNTSSVIIEETKKYGRTIIGYFEHYLQWIEFSISEKVASCKP VATALDTAVDVFLCSYIIDPLNLFWFGIGKATVFLLPALIFAVKLAKYYRRMDSEDVYDDVETIPMKNME NGNNGYHKDHVYGIHNPVMTSPSQH (SEQ ID NO: 1)
The 3 extracellular domains of CD133 are underlined. Italics indicate signal sequence absent from mature, surface-expressed polypeptide.

EXAMPLE 2: Discovery and characterization of novel antibody variable regions RW01 and RW03 capable of specifically binding human CD133

Cell Selections and Sequencing Data:

[00258] Cell-based selections for CD133-binding phage-Fab or phage-scFv were performed using the Cellectseq method as described in Example 1. Out of 94 phage-Fab clones selected from the Library F output and subjected to ELISA for determining their CD133-binding specificity, 77 clones were found to bind to HEK293-CD133 cells at levels at least 1.5-fold higher than to control HEK293 cells (Figure 1A). In contrast, none of the clones selected from the Library G output exhibited this CD133-binding specificity. The 94 clones selected from Library F were sequenced by amplifying DNA encoding the Fab variable regions (VL and VH regions) using M13-tagged sequencing primers. The Library F and Library G round three and four positive and negative outputs were sequenced via Illumina sequencing to identify binders enriched in the positive selection output pools. The sequencing results indicated that 91 of the 94 clones were composed of clones each having one of 3 unique antibody variable regions, as represented by clones “phage-Fab RW03”, “phage-Fab C12” and “phage-Fab F5”. Specifically, out of the 94 clones, 89 clones shared the same variable region

sequences, 2 clones had unique variable region sequences, and no sequence was obtained for 3 clones.

[00259] Figure 1B shows representative cell-based ELISA results for binding to HEK293-CD133 and HEK293 cells by phage-Fab RW03 comprising "antibody variable region RW03", phage-Fab C12 and phage-Fab F5. Only phage-Fab RW03 showed preferential binding to HEK293-CD133 cells vs HEK293 cells by a factor of at least 1.5. The Fab-encoding DNA sequences of phage-Fab RW03, phage-Fab C12 and phage-Fab F5 were each cloned into an IPTG-inducible vector for protein expression and the expressed Fabs ("Fab RW03", "Fab C12" and "Fab F5", respectively) were purified for testing via immunofluorescence (IF) assay, the results of which are shown in Figure 2. The IF assay showed that Fab RW03 demonstrated highly specific binding to the HEK293-CD133 cells, with very little background binding to HEK293 cells, whereas Fab C12 and Fab F5 were found to bind non-specifically to both HEK293-CD133 cells and HEK293 cells. The specific binding of Fab RW03 to HEK293-CD133 cells was also confirmed by cell-based ELISA (Figure 3A). Furthermore, Fab RW03 also was found to bind to Caco-2 cells, which is a colorectal cancer cell line known to express CD133 (Figure 3B). These results were consistent with the Illumina sequencing results in which the variable region DNA sequences of phage-Fab clone C12 and phage-Fab clone F5 appeared in both the positive as well as the negative selection output pools whereas those of phage-Fab RW03 appear only in the positive selection pool (data not shown). Additionally, the phage-Fab RW03 DNA sequence was the most abundant sequence in both the round 3 and 4 output pools, which is also consistent with the results of the cell-based ELISA in which the DNA sequences of 94% of the phage-Fab binders were those of phage-Fab RW03.

[00260] In addition to determining the DNA sequences encoding CD133-binding phage-Fabs from Library F, the Illumina sequencing data from Library G selection output pools was also analyzed to identify enriched binders (data not shown). DNA encoding the variable regions of 12 selected phage-scFv clones was rescued via PCR amplification and used to generate expression vectors for

expression of 12 IgGs having variable regions corresponding to those of the 12 selected phage-scFv clones. Of these 12 IgGs, "IgG RW01" containing "antibody variable region RW01" derived from phage-scFv clone RW01 was validated for specifically binding to CD133+ cells by flow cytometry analysis. Similarly, "IgG RW03" having "antibody variable region RW03" corresponding to that of Fab RW03 was produced and IgG RW01 and IgG RW03 were further tested in parallel.

[00261] The amino acid sequences of light chain (hK) and heavy chain of IgG RW01 and IgG RW03, and of the heavy and light chain variable domains of antibody variable regions RW01 and RW03 are shown in Table 2, and the amino acid sequences of complementarity-determining regions (CDRs) and heavy chain variable domain residues at positions 39, 55 and 66 of antibody variable region RW01 comprised in IgG RW01 and of antibody variable region RW03 comprised in IgG RW03 are indicated in Table 3. The nucleotide sequences encoding the CDRs and heavy chain variable domain residues at positions 39, 55 and 66 of antibody variable region RW01 comprised in IgG RW01 and of antibody variable region RW03 comprised in IgG RW03 are indicated in Table 4. The amino acid sequence of the light chain variable domain of IgG RW01 corresponds to that of the Asp1 to Lys106 segment of SEQ ID NO: 2; the amino acid sequence of the heavy chain variable domain of IgG RW01 corresponds to that of the Glu1 to Thr120 segment of SEQ ID NO: 3; the amino acid sequence of the light chain variable domain of IgG RW03 corresponds to that of the Asp1 to Lys109 segment of SEQ ID NO: 4; and the amino acid sequence of the heavy chain variable domain of IgG RW03 corresponds to that of the Glu1 to Ser118 segment of SEQ ID NO: 5.

Table 2. Amino acid sequences of light chain (hK) and heavy chain of IgG RW01 and IgG RW03, and of heavy and light chain variable domains of antibody variable regions RW01 and RW03.

Light chain variable domain of antibody variable region RW01: DIQMTQSPSSLSASVGDRVTITCRASQGSSYVAWYQQKPGKAPKLLIYSASYLYSGVPSRFSGSRSGTDFT LTISSLQPEDFATYYCQQGVWSLITFGQGTKVEIK (SEQ ID NO: 28)
Heavy chain variable domain of antibody variable region RW01: EVQLVESGGGLVQPGGSLRLSCAASGFNIYYYGSMHWVRQAPGKGLEWVASISPYYGSTYYADSVKGRF TISADTSKNTAYLQMNSLRAEDTAVYYCARHASSGYGHYAVYGIDYWGQGTLVTVSS (SEQ ID NO: 29)
IgG RW01 - Light chain (hK) amino acid sequence: DIQMTQSPSSLSASVGDRVTITCRASQGSSYVAWYQQKPGKAPKLLIYSASYLYSGVPSRFSGSRSGTDFT LTISSLQPEDFATYYCQQGVWSLITFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO: 2)
IgG RW01 - Heavy chain (hG1) amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGFNIYYYGSMHWVRQAPGKGLEWVASISPYYGSTYYADSVKGRF TISADTSKNTAYLQMNSLRAEDTAVYYCARHASSGYGHYAVYGIDYWGQGTLVTVSS*ASTKGPSVFPLAPS SKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO: 3)
Light chain variable domain of antibody variable region RW03: DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDF TLTISSLQPEDFATYYCQQYSHAGHLFTFGQGTKVEIK (SEQ ID NO: 30)
Heavy chain variable domain of antibody variable region RW03: EVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIHWVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDYWGQGTLVTVSS (SEQ ID NO: 31)
IgG RW03 - Light chain (hK) amino acid sequence: DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDF TLTISSLQPEDFATYYCQQYSHAGHLFTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR GEC* (SEQ ID NO: 4)
IgG RW03 - Heavy chain (hG1) amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIHWVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLP PSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO: 5)

Underline identifies CDR sequence amino acid residues.
Large font identifies FR amino acid residues at positions 39, 55 or 66 randomized in the selection library used to identify antibody variable regions.
Italics identifies immunoglobulin constant region amino acid residues or nucleotide sequences encoding immunoglobulin constant region amino acid residues.

Table 3. Amino acid sequences of CDRs and of FR residues at heavy chain variable domain positions 39, 55 and 66 of antibody variable region RW01 and antibody variable region RW03.

Antibody	Antibody segment	Amino acid sequence
IgG RW01	CDR-L1	QGSSY (SEQ ID NO: 6)
	CDR-L2	SAS (SEQ ID NO: 7)
	CDR-L3	QQGVWSLIT (SEQ ID NO: 8)
	CDR-H1	GFNIYYYGS (SEQ ID NO: 9)
	CDR-H2	ISPYYGST (SEQ ID NO: 10)
	CDR-H3	ARHASSGYGHYAVYGIDY (SEQ ID NO: 11)
	VH domain position 39 (adjacent to carboxy terminal residue of CDR-H1; FR2 residue)	Met
	VH domain position 55 (adjacent to amino terminal residue of CDR-H2; FR2 residue)	Ser
	VH domain position 66 (adjacent to carboxy terminal residue of CDR-H2; FR3 residue)	Tyr
	Segment spanning CDR-H1 (underlined) and VH domain position 39	GSMIYYYGS (SEQ ID NO: 32)
	Segment spanning VH domain position 55, CDR-H2 (underlined) and VH domain position 66	SISPYYGSTY (SEQ ID NO: 33)
IgG RW03	CDR-L1	QSVSSA (SEQ ID NO: 12)
	CDR-L2	SAS (SEQ ID NO: 13)
	CDR-L3	QQYSHAGHLFT (SEQ ID NO: 14)
	CDR-H1	GFNLSSSS (SEQ ID NO: 15)
	CDR-H2	IYPYYSYT (SEQ ID NO: 16)
	CDR-H3	ARFGSVAGFDY (SEQ ID NO: 17)
	VH domain position 39 (adjacent to carboxy terminal residue of CDR-H1; FR2 residue)	Ile
	VH domain position 55 (adjacent to amino terminal residue of CDR-H2; FR2 residue)	Tyr
	VH domain position 66 (adjacent to carboxy terminal residue of CDR-H2; FR3 residue)	Tyr
	Segment spanning CDR-H1 (underlined) and VH domain position 39	GFNLSSSSI (SEQ ID NO: 34)

	Segment spanning VH domain position 55, CDR-H2 (underlined) and VH domain position 66	YIYPYYSYTY (SEQ ID NO: 35)

Table 4. Nucleotide sequences encoding complementarity-determining regions (CDRs) and FR residues at heavy chain variable domain positions 39, 55 and 66 of antibody variable region RW01 and antibody variable region RW03.

Antibody	Antibody segment	Nucleotide sequence
IgG RW01	CDR-L1	CAGGGTTCTTCTTAC (SEQ ID NO: 36)
	CDR-L2	TCTGCATCC (SEQ ID NO: 37)
	CDR-L3	CAGCAAGGTGTTTGGTCTCTGATCACG (SEQ ID NO: 38)
	CDR-H1	GGCTTCAACATCTACTACTACGGTTCT (SEQ ID NO: 39)
	CDR-H2	ATTTCTCCTTACTACGGCTCTACT (SEQ ID NO: 40)
	CDR-H3	GCTCGCCATGCTTCTTCTGGTTACGGTCATTACGCTGTTTACGGTATTGACTAC (SEQ ID NO: 41)
	VH domain position 39 (adjacent to carboxy terminal residue of CDR-H1; FR2 residue)	ATG
	VH domain position 55 (adjacent to amino terminal residue of CDR-H2; FR2 residue)	TCT
	VH domain position 66 (adjacent to carboxy terminal residue of CDR-H2; FR3 residue)	TAC
	Segment spanning CDR-H1 (underlined)	GGCTTCAACATCTACTACTACGGTTCTATG (SEQ ID NO: 42)

	and VH domain position 39	
	Segment spanning VH domain position 55, CDR-H2 (underlined) and VH domain position 66	TCTATTTCTCCTTACTACGGCTCTACTTAC (SEQ ID NO: 43)
IgG RW03	CDR-L1	CAGTCCGTGTCCAGCGCT (SEQ ID NO: 44)
	CDR-L2	TCGGCATCC (SEQ ID NO: 45)
	CDR-L3	CAGCAATACTCTCATGCTGGTCATCTGTTCACG (SEQ ID NO: 46)
	CDR-H1	GGCTTCAACCTCTCTTCTTCTTCT (SEQ ID NO: 47)
	CDR-H2	ATTTATCCTTATTATAGCTATACT (SEQ ID NO: 48)
	CDR-H3	GCTCGCTTCGGTTCTGTTGCTGGTTTTGACTAC (SEQ ID NO: 49)
	VH domain position 39 (adjacent to carboxy terminal residue of CDR-H1; FR2 residue)	ATC
	VH domain position 55 (adjacent to amino terminal residue of CDR-H2; FR2 residue)	TAT
	VH domain position 66 (adjacent to carboxy terminal residue of CDR-H2; FR3 residue)	TAT
	Segment spanning CDR-H1 (underlined) and VH domain position 39	GGCTTCAACCTCTCTTCTTCTTCTATC (SEQ ID NO: 50)

	Segment spanning VH domain position 55, CDR-H2 (underlined) and VH domain position 66	TATATTTATCCTTATTATAGCTATACTTAT (SEQ ID NO: 51)

[00262] The nucleotide sequences of DNA encoding light chain (hK) and heavy chain of IgG RW01 and IgG RW03, and the heavy chain and light chain variable domains of antibody variable regions RW01 and RW03 are shown in Table 5.

Table 5. Nucleotide sequences of DNA encoding light chain (hK) and heavy chain of IgG RW01 and IgG RW03, and heavy chain and light chain variable domains of antibody variable regions RW01 and RW03.

Light chain variable domain of antibody variable region RW01: 5′-GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTG CCGTGCCAGTCAGGGTTCTTCTTACGTAGCCTGGTATCAACAGAAACCAGGAAAAGCTCCGAAGCTTCTGA TTTACTCTGCATCCTACCTCTACTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGTAGCCGTTCCGGGACGG ATTTCACTCTGACCATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAAGGTGTTTGG TCTCTGATCACGTTCGGACAGGGTACCAAGGTGGAGATCAAA-3′ (SEQ ID NO: 52)
Heavy chain variable domain of antibody variable region RW01: 5′-GAGGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGC AGCTTCTGGCTTCAACCTCTCTTCTTCTTCTATCCACTGGGTGCGTCAGGCCCCGGGTAAGGGCCTGGA ATGGGTTGCATATATTTATCCTTATTATAGCTATACTTATATGCCGATAGCGTCAAGGGCCGTTTCA CTATAAGCGCAGACACATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCC GTCTATTATTGTGCTCGCTTCGGTTCTGTTGCTGGTTTTGACTACTGGGGTCAAGGAACCCTGGTCACCGT CTCCTCG-3′ (SEQ ID NO: 53)
IgG RW01 - Light chain (hK) nucleotide sequence: 5′-GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTG CCGTGCCAGTCAGGGTTCTTCTTACGTAGCCTGGTATCAACAGAAACCAGGAAAAGCTCCGAAGCTTCTGA TTTACTCTGCATCCTACCTCTACTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGTAGCCGTTCCGGGACGG ATTTCACTCTGACCATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAAGGTGTTTGG TCTCTGATCACGTTCGGACAGGGTACCAAGGTGGAGATCAAA*CGTACGGTGGCTGCACCATCTGTCTTCAT* *CTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATC* *CCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACA* *GAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAA* *ACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGG*

GAGAGTGT-3′ (SEQ ID NO: 18)

IgG RW01 - Heavy chain (hG1) nucleotide sequence:

5′-GAGGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGC
AGCTTCTGGCTTCAACATCTACTACTACGGTTCTATGCACTGGGTGCGTCAGGCCCCGGGTAAGGGCCT
GGAATGGGTTGCATCTATTTCTCCTTACTACGGCTCTACTTACTATGCCGATAGCGTCAAGGGCCGTT
TCACTATAAGCGCAGACACATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACT
GCCGTCTATTATTGTGCTCGCCATGCTTCTTCTGGTTACGGTCATTACGCTGTTTACGGTATTGACTACTG
GGGTCAAGGAACCCTGGTCACCGTCTCCTCG*GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCT*
CCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTG
ACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGG
ACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACG
TGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACA
TGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGA
CACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGG
TCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTAC
AACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAA
GTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCC
GAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGC
CTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTA
CAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGA
GCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAG
AAGAGCCTCTCCCTGTCTCCGGGTAAA-3′ (SEQ ID NO: 19)

Light chain variable domain of antibody variable region RW03:

5′-GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTG
CCGTGCCAGTCAGTCCGTGTCCAGCGCTGTAGCCTGGTATCAACAGAAACCAGGAAAAGCTCCGAAGCTTC
TGATTTACTCGGCATCCAGCCTCTACTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGTAGCCGTTCCGGGA
CGGATTTCACTCTGACCATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAATACTCT
CATGCTGGTCATCTGTTCACGTTCGGACAGGGTACCAAGGTGGAGATCAAA-3′ (SEQ ID NO: 54)

Heavy chain variable domain of antibody variable region RW03:

5′-GAGGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGC
AGCTTCTGGCTTCAACCTCTCTTCTTCTTCTATCCACTGGGTGCGTCAGGCCCCGGGTAAGGGCCTGGA
ATGGGTTGCATATATTTATCCTTATTATAGCTATACTTATTATGCCGATAGCGTCAAGGGCCGTTTCA
CTATAAGCGCAGACACATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCC
GTCTATTATTGTGCTCGCTTCGGTTCTGTTGCTGGTTTTGACTACTGGGGTCAAGGAACCCTGGTCACCGT
CTCCTCG-3′ (SEQ ID NO: 55)

IgG RW03 - Light chain (hK) nucleotide sequence:

5′-GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTG
CCGTGCCAGTCAGTCCGTGTCCAGCGCTGTAGCCTGGTATCAACAGAAACCAGGAAAAGCTCCGAAGCTTC
TGATTTACTCGGCATCCAGCCTCTACTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGTAGCCGTTCCGGGA
CGGATTTCACTCTGACCATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAATACTCT
CATGCTGGTCATCTGTTCACGTTCGGACAGGGTACCAAGGTGGAGATCAAA*CGTACGGTGGCTGCACCATC*
TGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATA
ACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGA
CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCT
TCAACAGGGGAGAGTGT-3′ (SEQ ID NO: 20)

IgG RW03 - Heavy chain (hG1) nucleotide sequence:

5′-GAGGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGC

AGCTTCTGGCTTCAACCTCTCTTCTTCTTCTATCCACTGGGTGCGTCAGGCCCCGGGTAAGGGCCTGGA ATGGGTTGCATATATTTATCCTTATTATAGCTATACTTATTATGCCGATAGCGTCAAGGGCCGTTTCA CTATAAGCGCAGACACATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCC GTCTATTATTGTGCTCGCTTCGGTTCTGTTGCTGGTTTTGACTACTGGGGTCAAGGAACCCTGGTCACCGT CTCCTCG*GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCA* *CAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCC* *CTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGT* *GACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCA* *AGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA* *CTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCC* *TGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG* *GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGC* *GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT* *CCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGC* *CCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGC* *GACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGA* *CTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCT* *TCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGT* *AAA*-3′ (SEQ ID NO: 21)
Underline identifies nucleotide sequences encoding CDR amino acid residues. Large-font identifies nucleotide sequences encoding FR amino acid residues at positions 39, 55 and 66 randomized in the selection library used to identify antibody variable regions. Italics identify nucleotide sequences encoding immunoglobulin constant region amino acid residues.

EXAMPLE 3: IgG RW01 and IgG RW03 bind CD133 expressed at the cell surface with approximately single-digit nanomolar and subnanomolar affinity, respectively

[00263] The IgG RW01 and IgG RW03 antibodies were tested via flow cytometry for their capacity to bind HEK293-CD133 cells, after which half maximal binding concentrations of each antibody to the cells were estimated. Specifically, HEK293-CD133 cells were incubated with serial dilutions of each antibody, binding was detected with an anti-human Fab'2 secondary antibody and the data was fitted to a line of best fit using the Sigma Plot graphing program. Figure 4 shows the EC50 curve for IgG RW01, which had a calculated EC50 of 2.5nM and the curve for IgG RW03, which had a calculated EC50 of 0.5 nM.

EXAMPLE 4: IgG RW01 and IgG RW03 can be used to specifically bind and detect cell surface CD133 in pancreatic cancer cells and colorectal cancer cells, as shown via flow cytometry analysis

[00264] The IgG RW01 and IgG RW03 antibodies were assessed via flow cytometry for binding to the following cancer cell lines: Caco-2, a colon cancer cell line known to express CD133; the pancreatic cancer cell lines HPAC, PL45, RWP-1 and SU8686; and the ovarian carcinoma cell line Ovcar-8. HEK293 and HEK293-CD133 cells were used as negative and positive controls, respectively. As shown in Figure 5, both antibodies, at 5 µg/ml, bind CD133 at the surface of HEK293-CD133 control cells, at the surface of Caco-2 colorectal cancer cells and at the surface of HPAC, PL45 and RWP-1 pancreatic cancer cells. Bimodal staining peaks are observed in HPAC and PL45 cells and broader peaks such as those observed for the engineered cell line HEK293-CD133 are most likely a result of a heterogeneously expressing population of cells, contrasted with the narrow peak observed with RWP-1 cells indicating a more homogeneously expressing cell population.

EXAMPLE 5: IgG RW01 and IgG RW03 can be used to specifically bind, detect and subcellularly localize cellular CD133, as shown via immunofluorescence analysis

[00265] The IgG RW01 and IgG RW03 antibodies were tested for binding to cell-expressed CD133 in an immunofluorescence assay (Figure 6). The assay shows that IgG RW01 and IgG RW03 bind to HEK293-CD133 cells (indicating cellular sublocalization of CD133), but not to HEK293 cells.

EXAMPLE 6: IgG RW01 and IgG RW03 can each be used to detect denatured CD133 in whole cell lysate of colorectal cancer cells, as shown via Western blot analysis

[00266] Whole cell lysates of Caco-2 colorectal cancer cells were prepared and the capacity of IgG RW01 and IgG RW03 to detect denatured CD133 in the lysate was analyzed via Western blotting assay. HEK293-CD133 cells and HEK293 cells were used as positive and negative controls, respectively. Figure 7

shows that IgG RW01 and IgG RW03 each detect denatured CD133 in lysate of Caco-2 cells and HEK293-CD133 cells, which are both CD133-positive, but not in that of HEK293 cells.

EXAMPLE 7: Epitopes - Fab RW01 and Fab RW03 do not compete with IgG RW03 and IgG RW01, respectively, for binding to CD133

[00267] Experiments were performed to determine whether antibody variable region RW01 and antibody variable region RW03 compete with each other for binding to cell-expressed CD133. Cells were initially incubated with a 25 nM concentration of either Fab RW01 or Fab RW03, and serial dilutions of IgG RW03 or IgG RW01, respectively, were added, and the IgG binding was detected using a secondary antibody against human Fc. The results for each antibody can be seen in Figure 8 (a) and (b) and indicate that IgG RW01 can bind the cells in the presence of saturating RW03 Fab and IgG RW03 can bind in the presence of RW01 Fab.

EXAMPLE 8: Treatment with IgG RW01 or IgG RW03 can be used to significantly reduce total cellular CD133 protein levels in Caco-2 colorectal cancer cells

[00268] To investigate the effect of IgG RW01 and IgG RW03 on CD133 protein levels in cancer cells, Caco-2 colorectal cancer cells were incubated with either IgG RW01 or IgG RW03 for 24 hours at 37°C, and whole cell lysates were analyzed by Western blot using AC133 anti-CD133 antibody (Miltenyi Biotec) as probe and anti-human IgG (H+L) antibody (Jackson ImmunoResearch) as negative antibody control. As Figure 9 shows, treatment with RW01 IgG or RW03 IgG significantly reduced CD133 protein levels, as compared to the untreated and anti-human IgG control treatments. To assess the effect of the observed reduction in CD133 protein levels on Wnt signaling in the cells, β-catenin levels were also analyzed, however there were no observed differences on β-catenin protein stability between control antibody-treated and IgG RW01- or IgG RW03-treated samples.

EXAMPLE 9: Single-chain Fab RW03 can be used for targeting bispecific T cell engagers (BiTEs) to CD133-positive cells

[00269] Vectors were constructed for expression of four novel anti-CD133 x anti-CD3 bi-specific T-cell engagers (BiTEs; Figure 10A). Each of the four BiTEs incorporates an anti-CD3 scFv, and further incorporates either anti-CD133 Fab RW03 (Fab-based BiTEs) or an anti-CD133 single-chain Fab (scFab) incorporating the variable regions of Fab RW03 ("scFab RW03"; scFab-based BiTEs). In each of the 4 BiTEs, the Fab or scFab is linked to the VH domain of the scFv. The 4 BiTEs include two Fab-based variants, in which the scFv is linked either to the light chain ("BiTE #1") or heavy chain-derived portion ("BiTE #2") of the Fab. The other two BiTEs are scFab-based variants, in which the scFv is similarly linked either to the heavy chain-derived segment ("BiTE #3") or light chain segment ("BiTE #4") of the scFab.

[00270] The amino acid sequences of BiTE #1, BiTE #2, BiTE #3 and BiTE #4 are shown in Table 6.

Table 6. Configurations and amino acid sequences of BiTE #1, BiTE #2, BiTE #3 and BiTE #4 polypeptides.

BiTE #1

Configuration:
• VL-*CL* (RW03)-**(G4S)**-VH-**(G4S)3**-VL (CD3) • VH-*CH* (RW03)

Amino acid sequence of VL-*CL* (RW03)-**(G4S)**-VH-**(G4S)3**-VL:

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFT
LTISSLQPEDFATYYCQQYSHAGHLFTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR*
EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
GGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDK
ATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS**GGGGSGGGGSGGGGS**DIQLT
QSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSM
EAEDAATYYCQQWSSNPLTFGAGTKLELK(SEQ ID NO: 22)

Amino acid sequence of VH-*CH* (RW03):

EVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIHWVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFTISA
DTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALG*
CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHT(SEQ ID NO: 23)

BiTE #2

Configuration:
• VH-*CH* (RW03)-**(G4S)**-VH-**(G4S)3**-VL (CD3)
• VL-*CL* (RW03)

Amino acid sequence of VH-*CH* (RW03)-(G4S)-VH-(G4S)3-VL (CD3):

EVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIHWVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFTISA
DTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDYWGQGTLVTV*SSASTKGPSVFPLAPSSKSTSGGTAALG*
CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
*EPKSCDKTHT***GGGGS**DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGY
TNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS**GGGGSGGGGS**
GGGGSDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSG
TSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELK (SEQ ID NO: 24)

Amino acid sequence of VL-*CL* (RW03):

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFT
LTISSLQPEDFATYYCQQYSHAGHLFTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR*
EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO: 25)

BiTE #3

Configuration:
• VL-*CL*-**linker**-VH-*CH* (RW03)-**(G4S)**-VH-**(G4S)3**-VL (CD3)

Amino acid sequence:

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFT
LTISSLQPEDFATYYCQQYSHAGHLFTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR*
EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
GGSSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSGEVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIH
WVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDY
WGQGTLVTV*SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG*
*LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT***GGGGS**DIKLQQSGAELARPGASVKM
SCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSA
VYYCARYYDDHYCLDYWGQGTTLTVSS**GGGGSGGGGSGGGGS**DIQLTQSPAIMSASPGEKVTMTCRASSSVS
YMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTK
LELK (SEQ ID NO: 26)

BiTE #4

Configuration:
• VH-*CH*-**linker**-VL-*CL* (RW03)-**(G4S)**-VH-**(G4S)3**-VL (CD3)

Amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFNLSSSSIHWVRQAPGKGLEWVAYIYPYYSYTYYADSVKGRFTISA
DTSKNTAYLQMNSLRAEDTAVYYCARFGSVAGFDYWGQGTLVTV*SSASTKGPSVFPLAPSSKSTSGGTAALG*
CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
*EPKSCDKTHT***GGSSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG**DIQMTQSPSSLSASVGDRVTITCRA
SQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQYSHAGHL
FTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD*
*SKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC***GGGGS**DIKLQQSGAELARPGASVKM
SCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSA

VYYCARYYDDHYCLDYWGQGTTLTVSS**GGGGSGGGGSGGGGS**DIQLTQSPAIMSASPGEKVTMTCRASSSVS YMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTK LELK (SEQ ID NO: 27)
Underline identifies CDR sequence. Italics represent immunoglobulin constant region amino acids. Linker amino acids indicated in bold

[00271] HEK293 cells transiently transfected with the BiTE-expression vectors were capable of readily expressing BiTE #1, BiTE #2, BiTE #3 and BiTE #4 expressed and purified from, as shown via Western blot analysis of reduced and non-reduced whole cell lysates of transfectants (Figure 10B).

BiTE #1, BiTE #2, BiTE #3 and BiTE #4 have the capacity to bind to cell surface CD133 and CD3

[00272] The capacity of the purified BiTEs to specifically bind cell surface CD133 was determined using flow cytometry analysis of staining of HEK293-CD133 cells vs HEK293 cells with the BiTEs. As shown in Figure 11A and Figure 11B, BiTE #1, BiTE #2, BiTE #3 and BiTE #4 each binds to HEK293-CD133 cells significantly more than to HEK293 cells, even at concentrations as low as 0.073-0.11 microgram/ml. The purified BiTEs were further tested in an ELISA to determine their capacity to bind to CD3 in the form of CD3 epsilon/gamma and CD3 epsilon/delta. As shown in Figure 12, the purified BiTEs bind to CD3 as well as the positive control antibodies (UCHT1, OKT3). These BiTEs can bind to both CD133 and CD3.

EXAMPLE 10: Human CD133-specific Chimeric Antigen Receptor (CAR) modified T cells target patient-derived glioblastoma brain tumors

[00273] A single chain variable fragment (scFv) was derived from RW03 (described above) and a second-generation CAR was generated. Anti-CD133 scFv with a myc tag was cloned in frame with a human CD8 leader sequence, CD8a transmembrane domain, CD28, and hCD3ζ (human CD3zeta) signaling tail in the lentiviral construct pCCL-ΔNGFR vector in two different orientations: Light chain-linker-Heavy chain (CD133 CAR-LH) and Heavy chain-linker-Light chain (CD133 CAR-HL).

[00274] Following lentiviral preparation, T cells isolated from PBMCs were transduced with CD133 CAR-LH and CD133 CAR-VH constructs. After successful T cell engineering, the expression of ΔNGFR and myc tag was analyzed using flow cytometry to confirm the efficiency of transduction and surface expression of anti-CD133 respectively. While expression of ΔNGFR was observed in all CAR T cells (including controls), expression of the c-myc tag in both variations of CARs, CD133 CAR-HL and CD133 CAR-LH was found. Furthermore, Presto Blue-based killing assays were used to test the ability of CD133 CARs to selectively bind and kill CD133+ GBM brain tumor initiating cell lines (BTICs). CD133-specific CAR-T cells were cytotoxic to CD133+ GBMs. Co-culturing CD133 CAR-T cells with GBMs triggered T cell activation and proliferation, validating this adoptive T-cell therapeutic strategy.

[00275] Human T cells expressing CD133-specific CARs were engineered by cloning scFv comprising the heavy and light chain variable domains of antibody variable region RW03 (disclosed above), a short marker epitope of c-myc, the hinge region from murine CD8 and the transmembrane and cytoplasmic portions of murine CD28 and CD3ζ (FIGURE 13). The human CD133-CAR was cloned into the lentiviral transfer vector pCCL-ΔNGFR and packaged as lentiviruses. The c-myc tag in the extracellular domain was used to validate CAR expression. ΔNGFR was used as a cell-surface marker for tracking and sorting of transduced cells.

[00276] Transduced T cells consisted of CD4-positive and CD8-positive cells with both subsets expressing CD133-specific CARs (FIGURE 14). After successful transduction, the expression of ΔNGFR (CD271) was observed in all CAR T cells (including controls), however increased expression of the c-myc tag was found in the CD133 CAR-HL and CD133 CAR-LH cells only (FIGURE 15).

[00277] CD133-specific CAR-T cells were incubated with GBM BT459, stained and analyzed for surface expression of T cell activation markers CD69 and CD25 after 18 hours (FIGURE 16). Both, CD4+ (T-helper) and CD8+ (T-cytotoxic) cells showed upregulation in surface expression levels of activation markers. The

elevated expression was detected only in the presence of CD133-specific CAR and not CAR-T control.

[00278] CD133-specific CAR T-cells showed enhanced proliferation capability after being co-cultured with $CD133^{high}$ GBM cells (FIGURE 17, top row). CD133-specific CAR T cells specifically recognized and kill tumor cells in $CD133^{high}$ GBMs and medulloblastoma (Control), while having no effect on $CD133^{low}$ GBM cells (FIGURE 17, bottom row). $CD133^{high}$ and $CD133^{low}$ GBMs were defined based on the percentage of CD133-positive cells present in the tumor cell culture. GBM cultures with >90% CD133+ cells were defined as $CD133^{high}$ GBMs and cultures with <5% CD133+ cells were defined as $CD133^{low}$ GBMs.

[00279] Tumor-engrafted mice were injected i.e. with CAR-CON (control) T cells (FIGURE 18A) and with CAR-CD133 T cells at an effector to target ratio (E:T) = 2:1 (FIGURE 18B). Tumors formed in mouse brain intracranially treated with CAR-CD133 T cells were significantly less aggressive and invasive compared to control (as assessed by COX IV staining for human cells) (n=4). Mouse xenografts generated after CAR-CD133 T cell treatment had significant less tumor mass (FIGURE 18C).

[00280] Treatment of GBM tumor-bearing mice with CD133-specific CAR-T cells yielded extended survival in mice and significant reductions in brain tumor burden.

EXAMPLE 11. Preclinical validation of a novel CD133/CD3 bispecific T-cell engager (BiTE) antibody to target patient-derived glioblastoma cells

[00281] The BiTE format has been evaluated against a variety of tumor-associated antigens, including CD19, CD20, EpCAM, EGFR, MUC-1, CEA, CD133, EphA2 and HER2/neu (reviewed in Baeuerle et al. 2009). Clinical trials investigating BiTEs include Blinatumomab for leukemia patients (NCT00274742), AMG110/MT110 for lung/colorectal/breast/ovarian cancer patients (NCT00635596), AMG211/MEDI565 for gastrointestinal adenocarcinoma patients (NCT01284231) and AMG212/BAY2010112 for prostate cancer patients

(NCT0173475). BiTEs exhibiting specificity for two GBM tumor cell surface antigens CD133 (Prasad et al, 2015) and EGFRvIII (Choi et al, 2013) have also been shown to be induce anti-tumorigenic activity in xenograft tumor models. Importantly, preclinical evaluation of EGFRvIII-specific BiTEs delivered intravenously showed tumor reduction/shrinkage, extending survival in mice with well-established EGFRvIII-expressing GBM.

[00282] As described in Example 9, CD133-specific BiTEs or RW03xCD3 were constructed that consist of two arms; one arm recognizes the tumor antigen (CD133) while the second is specific to CD3 antigen. The BiTEs were constructed in four different conformations and dual binding specificity was confirmed using flow cytometry. Using $CD133^{high}$ and $CD133^{low}$ primary GBM lines, the binding of BiTEs to CD133+ cells was validated. Further analysis showed binding of BiTEs to human T cells known to express CD3 within a population of healthy donor peripheral blood mononuclear cells. BiTEs redirecting T cells to kill GBMs were observed, with greater efficiency observed in $CD133^{high}$ GBMs, validating BiTE target specificity.

[00283] Specifically, using CellectSeq, a novel methodology that combines use of phage-displayed synthetic antibody libraries and high-throughput DNA sequencing technology, CD133-specific monoclonal antibody 'RW03' was developed (FIGURE 19A). Following validation of CD133 RW03 antibody, CD133-specific BiTEs or RW03xCD3 was constructed that had two arms; one arm recognizes the tumor antigen (CD133) while the second is specific to CD3 antigen. The BiTEs were constructed in four different conformations (FIGURE 19B).

[00284] Flow cytometry was used to confirm dual specificity of CD133xCD3 BiTEs against cells expressing the appropriate antigen. CD133xCD3 BiTEs bind to GBM tumor expressing CD133 in similar capacity when compared with binding to commercially available CD133 (Miltenyi) monoclonal antibody (FIGURE 20A). Analyses revealed binding of CD133xCD3 BiTEs to human T cells known to

express CD3 within a population of healthy donor peripheral blood mononuclear cells (PBMCs) (FIGURE 20B).

[00285] T cells incubated with BiTEs and $CD133^{high}$ GBM BT459 were stained and analyzed for surface expression of activation markers CD69 and CD25 (FIGURE 21). Both, CD4+ (T-helper) and CD8+ (T-cytotoxic) cells showed upregulation in surface expression levels of the activation markers. The elevated expression was detected only in the presence of CD133 BiTE in the co-culture.

[00286] GBM cells form a monolayer when plated alone (FIGURE 22A(a) or plated with T cells (FIGURE 22A(b)); however, addition of BiTEs (FIGURE 22A, c and d) recruits T cells to GBM cells forming spherical clusters. Addition of BiTEs to GBM cells co-cultured with T cells leads to significant cell death of GBM cells as determined through alamar-blue cytotoxicity assay (FIGURE 22B). Quantification of tumor cell lysis by flow cytometry-based Live-Dead staining (with IR dye) (FIGURE 22C). Tumor cells (CFSE-labeled) and T cells (E:T ratio, 1:2) in presence and absence of CD133 BiTEs after 24 hours show BiTE-mediated GBM cell death.

[00287] NSG (NOD scid gamma) mice were implanted i.c. with $CD133^{high}$ GBM cells and upon successful engraftment, were treated with unstimulated PBMCs with or without BiTEs (Total 4 doses over 2 weeks) (FIGURE 23). Tumors formed in mouse brain intracranially treated with CD133 BiTEs were significantly less aggressive and invasive compared to control (as assessed by COX IV staining for human cells) (n=4) (FIGURE 23, A and B)). Mouse xenografts generated after BiTE treatment had less tumor burden (FIGURE 23C) and maintained a significant survival advantage over control mice (n=7) (FIGURE 23D).

METHODS FOR EXAMPLES 10 and 11

[00288] **Flow cytometric characterization:** Patient-derived GBM lines were dissociated and single cells resuspended in PBS+2mM EDTA. Cell suspensions were stained with anti-CD133, anti-CD69, anti-CD25, anti-CD8, anti-CD4 or matched isotype controls (Miltenyi/BD Biosciences). Samples were

run on a MoFlo XDP Cell Sorter (Beckman Coulter). Dead cells were excluded using the viability dye 7AAD. (1:10; Beckman Coulter). Compensation was performed using mouse IgG CompBeads (BD). Surface marker expression was defined as positive or negative based on the analysis regions established using the isotype control.

[00289] **Cell Proliferation Assay:** Single cells were plated in a 96-well plate at a density of 1,000 cells/200 µL per well in quadruplicate and incubated for four days. 20 µL of Presto Blue (Invitrogen) was added to each well approximately 2h prior to the readout time point. Fluorescence was measured using a FLUOstar Omega Fluorescence 556 Microplate reader (BMG LABTECH) at excitation and emission wavelengths of 535 nm and 600 nm, respectively. Readings were analyzed using Omega analysis software.

[00290] **Quantitative cytotoxicity assays**: Prestoblue killing and LDH cytotoxicity assays with different effector/target (E:T) ratios was performed to determine the efficiency of BiTEs to redirect T cells and kill CD133-expressing GBM BTICs.

[00291] ***In vivo*** **GBM intracranial injections and H&E staining of xenograft tumors***:* All experimental procedures involving animals were reviewed and approved by McMaster University Animal Research Ethics Board (AREB). NOD-SCID mice were used for all experiments. Mice were anaesthesized using gas anaesthesia (Isoflurane: 5% induction, 2.5% maintenance) identified using ear notches following minimally invasive surgery, and monitored for recovery. Intracranial injections were performed as previously described (1). Briefly, 10µL of cell suspension was injected into the right frontal lobe of 8-10 week old mice. Mice were monitored weekly for signs of illness, and upon reaching endpoint, brains and lungs (for IT and ICa injections) were harvested, sectioned, and paraffin-embedded for hematoxylin and eosin (H&E). Images were scanned using an Aperio Slide Scanner and analyzed by ImageScope v11.1.2.760 software (Aperio).

[00292] While the present application has been described with reference to examples, it is to be understood that the scope of the claims should not be limited by the embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

[00293] All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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Winter and Harris, 1993. Immunol Today 14:43

Wright et al., 1992. Crit. Reviews in Immunol. 12:125-168

Zhong et al., 2015. Tumor Biol. 36:7623

CLAIMS:

1. A CD133-binding agent which specifically binds (a) a CD133 epitope bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 2, and a heavy chain having the amino acid sequence of SEQ ID NO: 3; and/or (b) a CD133 epitope bound by an antibody comprising a light chain having the amino acid sequence of SEQ ID NO: 4, and a heavy chain having the amino acid sequence of SEQ ID NO: 5.

2. A CD133-binding agent which specifically binds cell surface-expressed CD133 and which specifically binds denatured CD133.

3. The CD133-binding agent of claim 1 or 2, comprising an antibody light chain variable domain and an antibody heavy chain variable domain which form an antigen binding site that specifically binds human CD133.

4. The CD133-binding agent of claim 3, wherein:

(a) (i) the antibody light chain variable domain comprises a light chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8; and (ii) the antibody heavy chain variable domain comprises a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 9, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 10, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 11, a Met residue at position 39, a Ser residue at position 55 and a Tyr residue at position 66; or

(b) (i) the antibody light chain variable domain comprising a light chain complementarity-determining region (CDR)1 comprises the amino acid sequence of SEQ ID NO: 12, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO: 13, and a light chain CDR3 comprising the amino acid sequence of

SEQ ID NO: 14; and (ii) the antibody heavy chain variable domain comprises a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 15, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17, a Ile residue at position 39, a Tyr residue at position 55 and a Tyr residue at position 66.

5. The CD133-binding agent of claim 3 or 4, wherein the light chain comprises (a) the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 2 or (b) the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4.

6. The CD133-binding agent of claim 3 or 4, wherein the heavy chain comprises (a) the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3 or (b) the amino acid sequence of SEQ ID NO: 5 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 5.

7. The CD133-binding agent of claim 4 wherein: (a) the light chain comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 2, and the heavy chain comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3; or (b) the light chain comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4, and the heavy chain comprises the amino acid sequence of SEQ ID NO: 5 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 5.

8. The CD133-binding agent of any one of claims 1-7, wherein the binding agent is selected from the group consisting of an antibody, an antibody fragment, a single-chain Fv (scFv), a bispecific antibody, a phage-Fab and a phage-scFv.

9. The CD133-binding agent of any one of claims 1-8, wherein the binding agent is a fragment antigen-binding (Fab).

10. The CD133-binding agent of any one of claims 1-8, wherein the binding agent is (a) a bispecific antibody comprising a CD133-binding single-chain Fab and a non-CD133-binding scFv, (b) a bispecific antibody comprising a CD133-binding Fab and a non-CD133-binding scFv, or (c) a CD133-binding and CD3-binding bispecific antibody.

11. The CD133-binding agent of any one of claims 1-8 and 10, wherein the binding agent is (a) a bispecific antibody comprising a CD133-binding single-chain Fab and a CD3-binding scFv or (b) a bispecific antibody comprising a CD133-binding Fab and CD3-binding scFv.

12. The CD133-binding agent of any one of claims 1-8, wherein the binding agent is a chimeric antigen receptor (CAR) comprising (i) a CD133-binding antibody variable region and (ii) a CAR signaling domain comprising one or more immune cell receptor signaling domains.

13. The CD133-binding agent of any one of claims 8-12, wherein the CD133-binding agent comprises human antibody constant regions.

14. The CD133-binding agent of any one of claims 1-8, wherein the CD133-binding agent is an IgG molecule.

15. The CD133-binding agent of any one of claims 1-14, wherein the binding agent is labelled with a detection agent.

16. An immunoconjugate comprising (1) the binding agent of any one of claims 1 to 14 attached to (2) an effector agent.

17. The immunoconjugate of claim 16, wherein the effector agent is an anti-neoplastic agent.

18. The immunoconjugate of claim 16, wherein the effector agent is a toxin.

19. A pharmaceutical composition comprising the CD133-binding agent of any one of claims 1-15 or the immunoconjugate of any one of claims 16-18 and a carrier.

20. A use of the CD133-binding agent of any one of claims 1-15, the immunoconjugate of any one of claims 16-18 or the pharmaceutical composition of claim 19 for targeting CD133-expressing cells.

21. A use of the CD133-binding agent of any one of claims 1-15, the immunoconjugate of any one of claims 16-18 or the pharmaceutical composition of claim 19 for binding CD133-expressing cells.

22. A use of the CD133-binding agent of any one of claims 1-15, the immunoconjugate of any one of claims 16-18 or the pharmaceutical composition of claim 19 for detecting CD133-expressing cells and/or for quantitating levels of cellular CD133 expression.

23. A use of the CD133-binding agent of any one of claims 1-15 or the pharmaceutical composition of claim 19 for reducing levels of CD133 protein in CD133-expressing cells.

24. The use of any one of claims 20-23, wherein the CD133-expressing cells are cancer cells.

25. A use of the CD133-binding agent of any one of claims 1-15, the immunoconjugate of any one of claims 16-18 or the pharmaceutical composition of claim 19 for treating or preventing a cancer.

26. The use of claim 25, wherein the cancer is a metastatic melanoma, a brain cancer, a prostate cancer, a pancreatic cancer or a colon cancer.

27. The use of claim 26, wherein the brain cancer is a glioblastoma.

28. The use of claim 27, wherein the glioblastoma is a glioblastoma detectably expressing CD133.

29. A use of the CD133-binding agent of claim 10 or 11 for treating glioblastoma, optionally glioblastoma detectably expressing CD133.

30. The use of claim 26, wherein the brain cancer is a medulloblastoma.

31. The use of claim 30, wherein the medulloblastoma is a medulloblastoma detectably expressing CD133.

32. A use of the CD133-binding agent of claim 10 or 11 for treating medulloblastoma, optionally medullolastoma detectably expressing CD133.

33. The use of claim 29 or 32, wherein the bispecific antibody comprises an amino acid sequence comprising:

(a) SEQ ID NO: 22 and SEQ ID NO: 23,

(b) SEQ ID NO: 24 and SEQ ID NO: 25,

(c) SEQ ID NO: 26,

(d) SEQ ID NO: 27,

or functional variants thereof.

34. A use of a T-cell expressing the chimeric antigen receptor (CAR) of claim 12 for treating glioblastoma, optionally glioblastoma detectably expressing CD133.

35. A use of a T-cell expressing the chimeric antigen receptor (CAR) of claim 12 for treating medulloblastoma, optionally medulloblastoma detectably expressing CD133.

FIGURE 1A

Library F

CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293
1.797	0.93	1.665	0.956	0.956	0.822	1.639	0.934	1.576	0.948	1.644	0.848
1.704	0.947	1.685	0.875	0.875	0.905	1.64	0.771	1.668	0.845	1.681	0.834
1.739	0.83	1.579	0.808	0.808	0.811	1.611	0.875	1.609	0.714	1.609	0.652
1.788	0.933	1.65	0.838	0.838	0.841	1.572	0.734	1.641	0.744	1.642	0.663
1.778	0.824	1.663	0.638	0.638	0.751	1.498	0.718	1.426	0.715	1.54	0.706
1.759	0.787	1.471	0.64	0.64	1.303	1.605	0.688	0.948	0.674	1.463	0.567
1.744	0.802	1.808	0.831	0.831	0.853	1.529	0.706	1.701	0.77	1.62	0.71
1.621	0.765	1.153	0.465	0.465	0.666	1.692	0.793	1.602	0.729	1.56	0.66

CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293
1.642	0.746	1.62	0.903	1.474	0.778	1.584	0.873	1.52	1.011	1.603	0.685
1.551	0.76	1.628	0.787	1.568	0.777	1.588	0.845	1.52	0.856	1.615	0.87
1.513	0.676	1.561	0.734	1.573	0.821	1.62	0.859	1.565	0.927	1.223	1.049
1.545	0.729	1.51	0.732	1.564	0.735	1.666	0.777	1.502	0.735	1.673	0.725
1.495	0.686	1.592	0.639	1.545	0.718	1.551	0.665	1.621	0.73	1.531	0.674
1.596	0.704	1.501	0.682	1.568	0.72	1.563	0.713	1.608	0.812	1.568	0.678
1.634	0.743	1.538	0.814	1.601	0.68	1.527	0.762	1.705	0.757	1.697	0.716
1.592	0.699	1.674	0.656	1.464	0.658	1.505	0.639	1.701	0.667	0.191	0.161

Library G

CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293
0.6	0.411	0.44	0.355	0.365	0.335	0.369	0.325	0.334	0.259	0.456	0.354
0.405	0.424	0.537	0.518	0.477	0.377	0.405	0.334	0.301	0.248	0.313	0.257
0.36	0.339	0.422	0.331	0.291	0.257	0.317	0.245	0.376	0.268	0.384	0.26
0.304	0.338	0.253	0.236	0.411	0.3	0.235	0.223	0.337	0.258	0.351	0.302
0.236	0.265	0.301	0.319	0.353	0.292	0.331	0.289	0.331	0.287	0.294	0.242
0.226	0.259	0.207	0.236	0.311	0.267	0.443	0.388	0.214	0.2	0.301	0.269
0.248	0.261	0.334	0.304	0.459	0.309	0.311	0.245	0.235	0.223	0.274	0.224
0.248	0.183	0.239	0.203	0.369	0.241	0.232	0.176	0.243	0.163	0.244	0.175

CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293	CD133	HEK293
0.378	0.359	0.307	0.265	0.661	0.639	0.341	0.312	0.424	0.477	0.311	0.295
0.321	0.237	0.42	0.338	0.443	0.303	0.435	0.364	0.319	0.328	0.456	0.416
0.288	0.207	0.293	0.234	0.364	0.275	0.562	0.42	0.359	0.273	0.355	0.374
0.268	0.244	0.32	0.354	0.525	0.33	0.335	0.262	0.489	0.345	0.279	0.227
0.342	0.332	0.305	0.277	0.259	0.235	0.288	0.24	0.241	0.223	0.252	0.215
0.258	0.201	0.314	0.303	0.322	0.277	0.262	0.222	0.356	0.313	0.313	0.228
0.369	0.267	0.457	0.51	0.276	0.2	0.55	0.442	0.273	0.211	0.347	0.272
0.254	0.161	0.291	0.196	0.22	0.186	0.512	0.345	0.326	0.239	0.186	0.168

FIGURE 1B

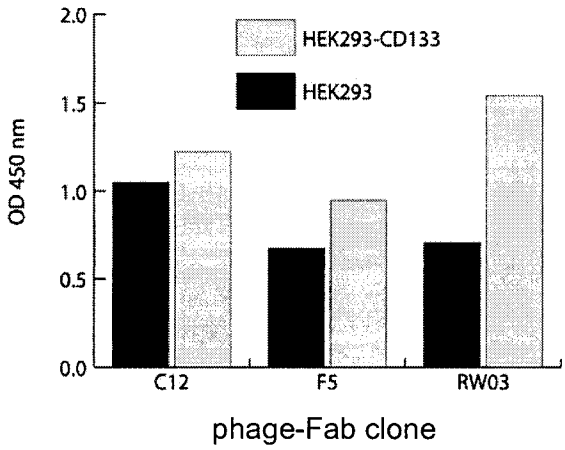

2.0
1.5
1.0
0.5
0.0
OD 450 nm
HEK293-CD133
HEK293
C12
F5
RW03
phage-Fab clone

FIGURE 2

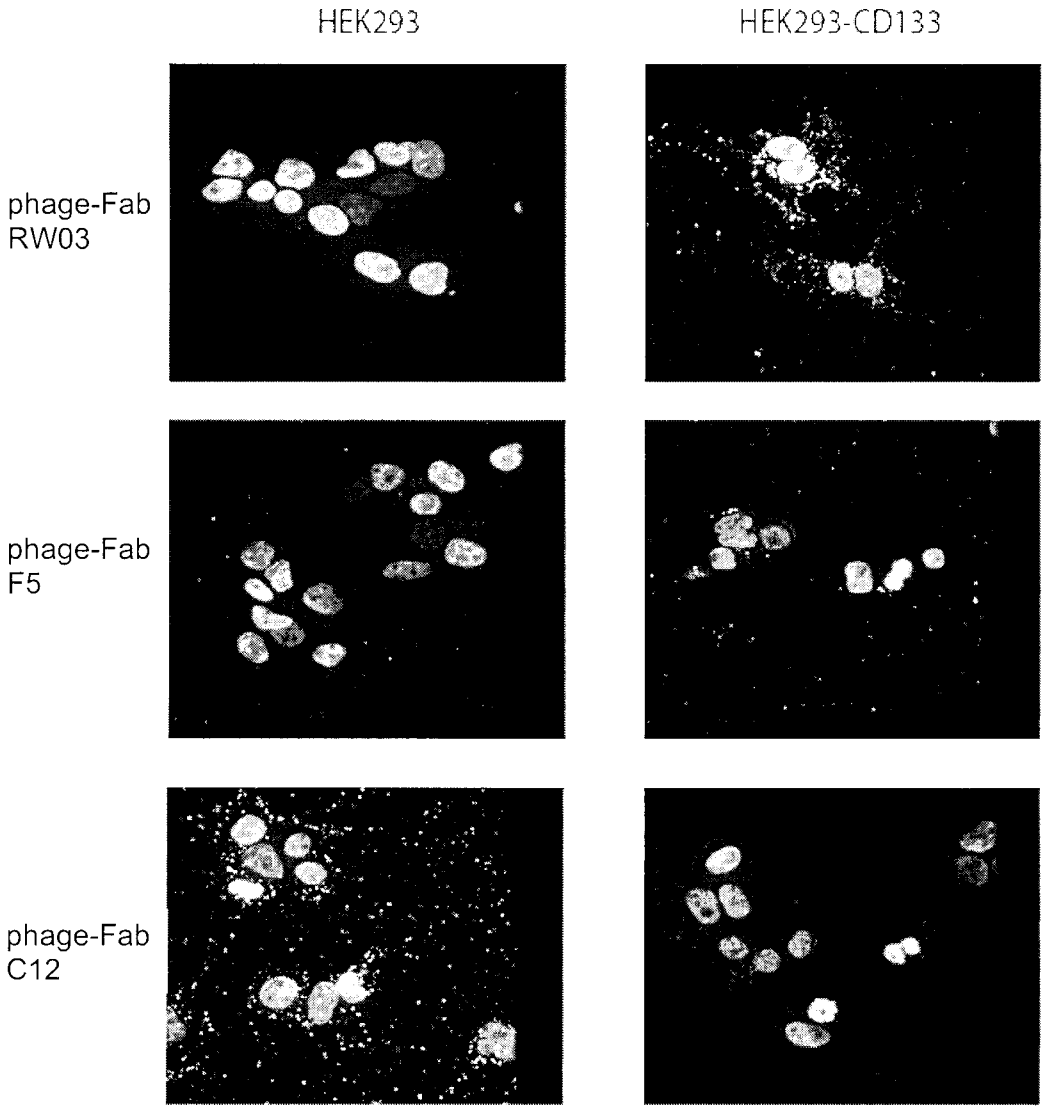

HEK293
HEK293-CD133
phage-Fab RW03
phage-Fab F5
phage-Fab C12

FIGURE 3A

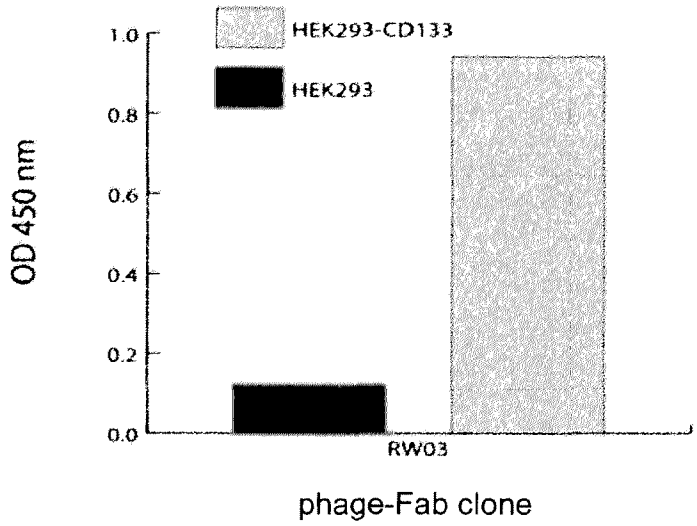


FIGURE 3B

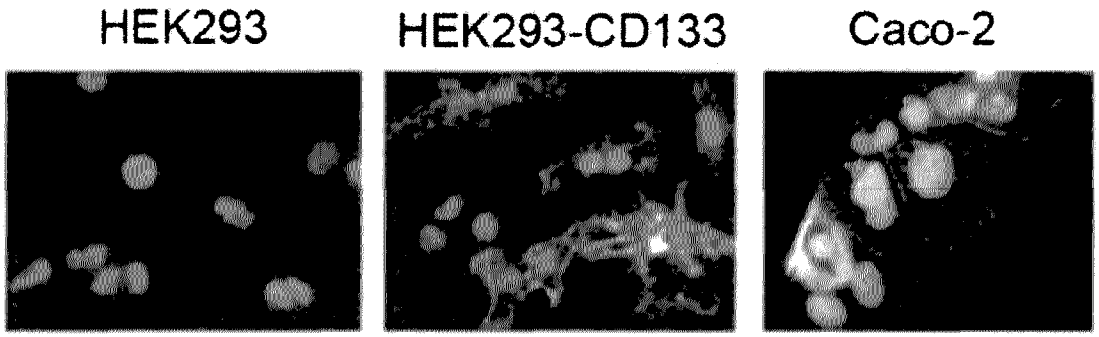


FIGURE 4A

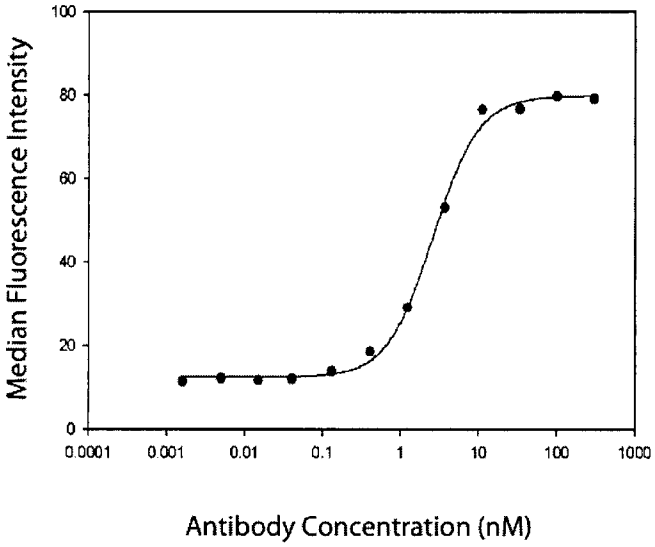


FIGURE 4B

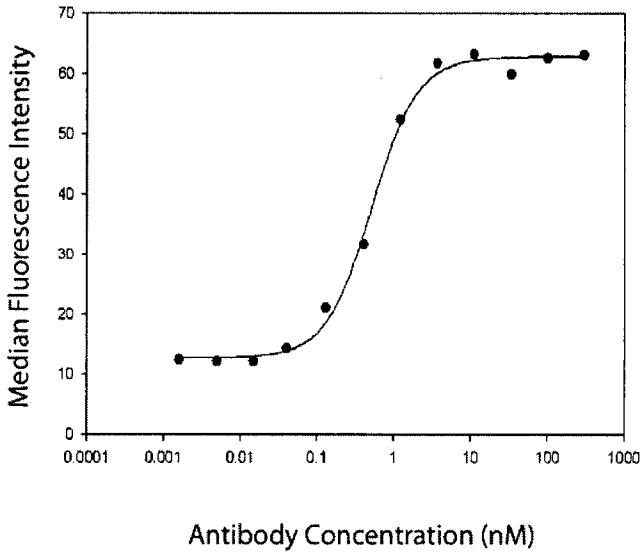


FIGURE 5

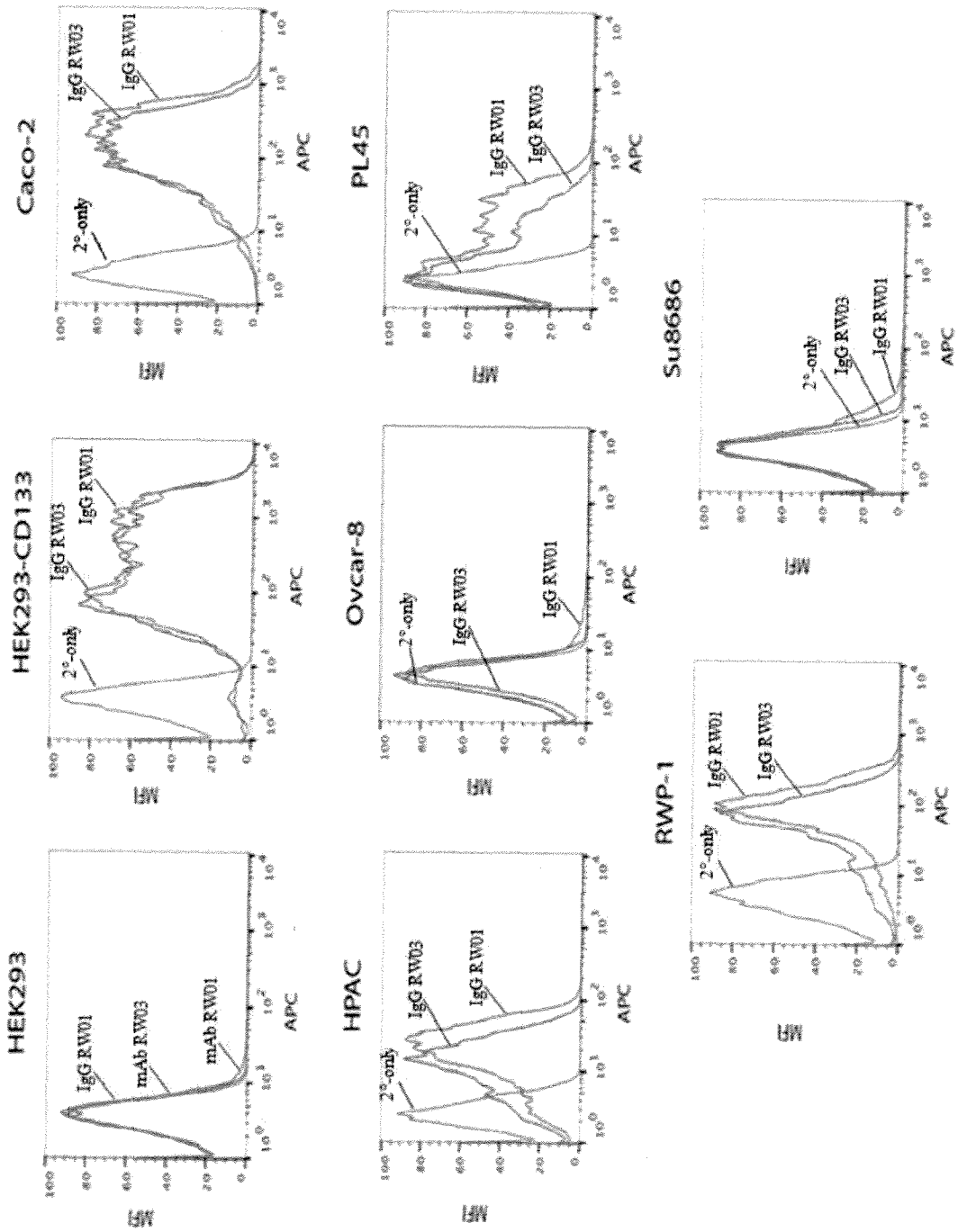

HEK293
IgG RW01
mAb RW03
mAb RW01
HEK293-CD133
2°-only
IgG RW03
IgG RW01
Caco-2
2°-only
IgG RW03
IgG RW01
Ovcar-8
2°-only
IgG RW03
IgG RW01
PL45
2°-only
IgG RW01
IgG RW03
HPAC
2°-only
IgG RW03
IgG RW01
Su8686
2°-only
IgG RW03
IgG RW01
RWP-1
2°-only
IgG RW01
IgG RW03
APC
MFI

FIGURE 6

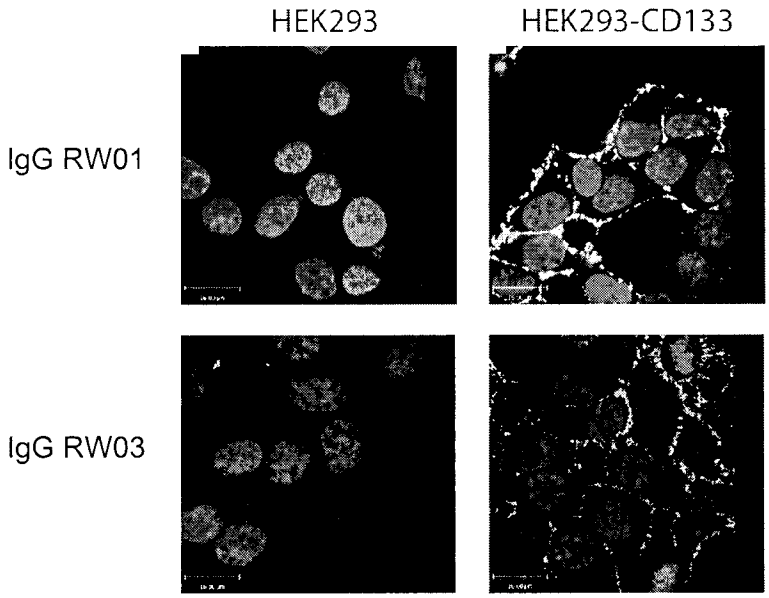


FIGURE 7

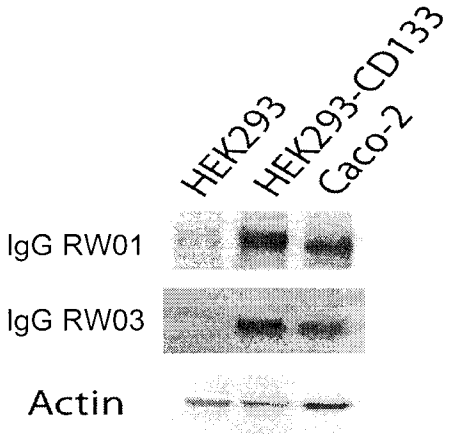
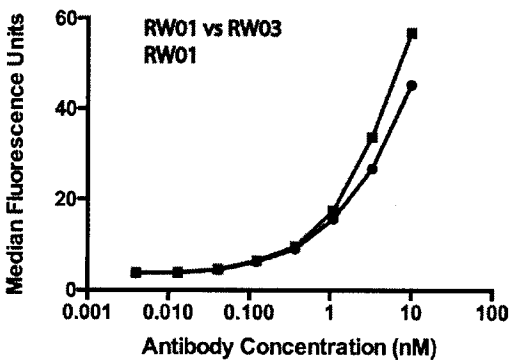


FIGURE 8

a.


RW01 vs RW03
RW01
Median Fluorescence Units
60
40
20
0
0.001
0.010
0.100
1
10
100
Antibody Concentration (nM)

b.

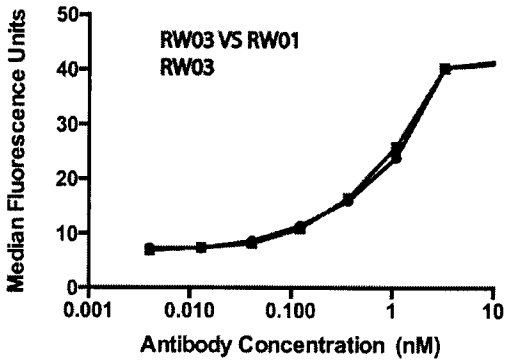

RW03 VS RW01
RW03
Median Fluorescence Units
50
40
30
20
10
0
0.001
0.010
0.100
1
10
Antibody Concentration (nM)

FIGURE 9

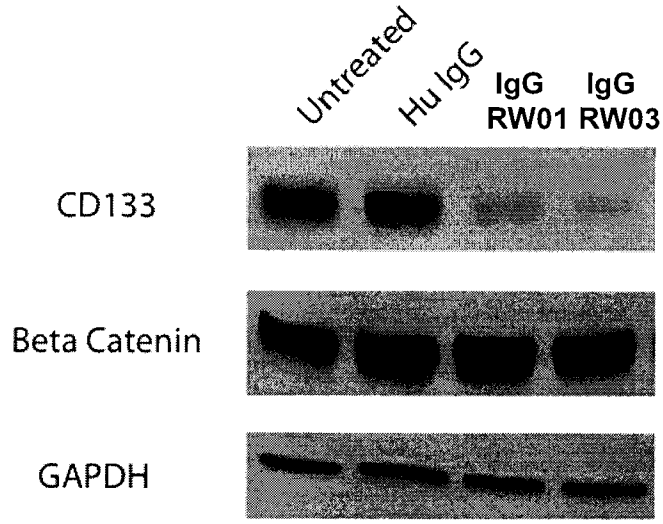


FIGURE 10A

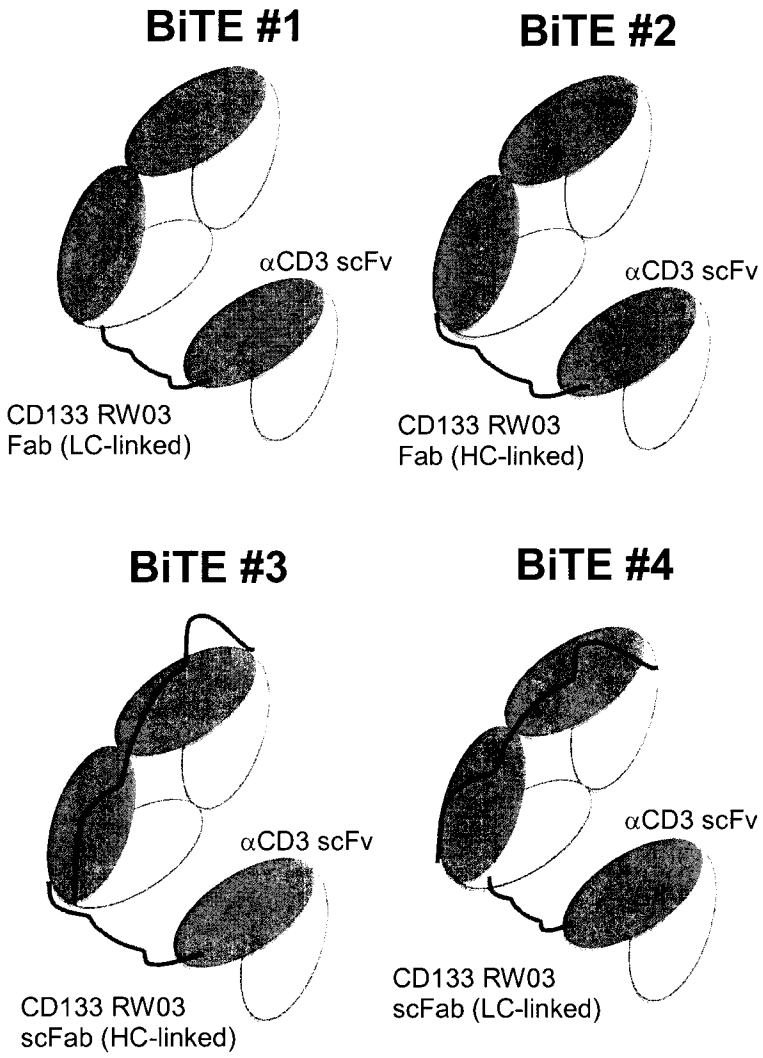

BiTE #1
αCD3 scFv
CD133 RW03
Fab (LC-linked)
BiTE #2
αCD3 scFv
CD133 RW03
Fab (HC-linked)
BiTE #3
αCD3 scFv
CD133 RW03
scFab (HC-linked)
BiTE #4
αCD3 scFv
CD133 RW03
scFab (LC-linked)

FIGURE 10B

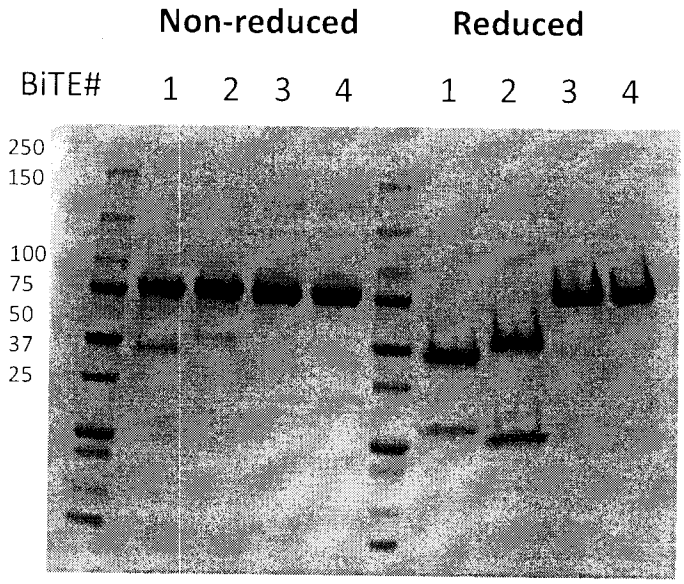
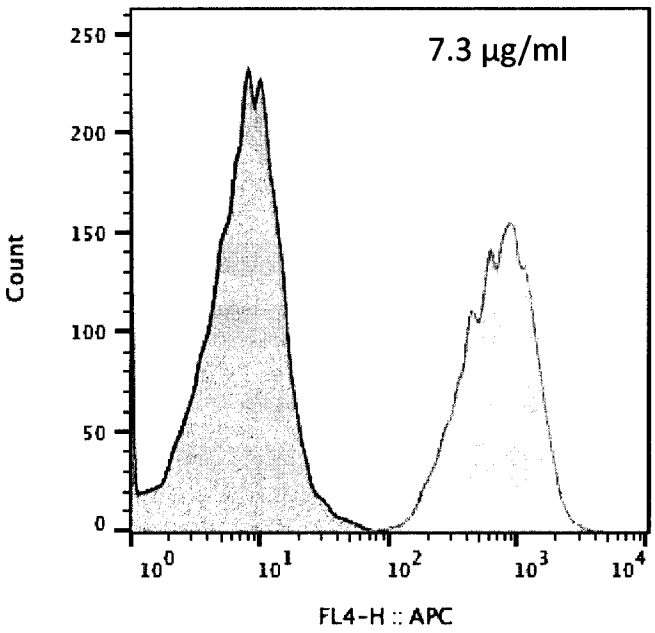


FIGURE 11A

BiTE #1



BiTE #2

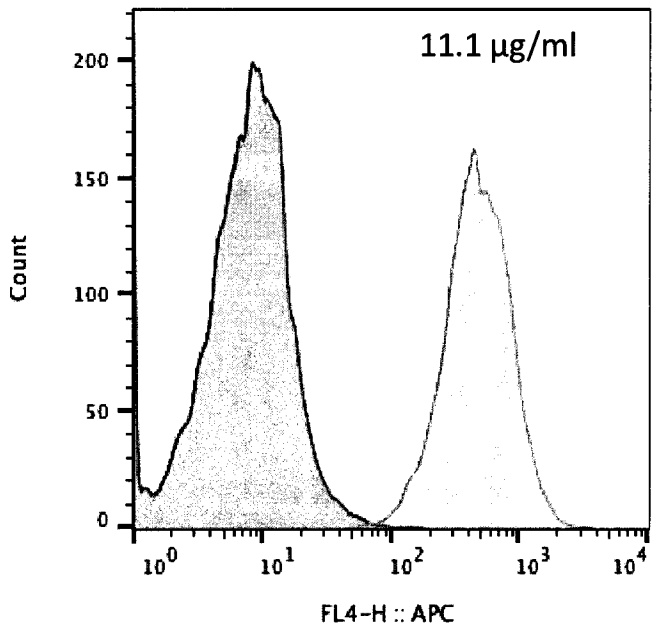
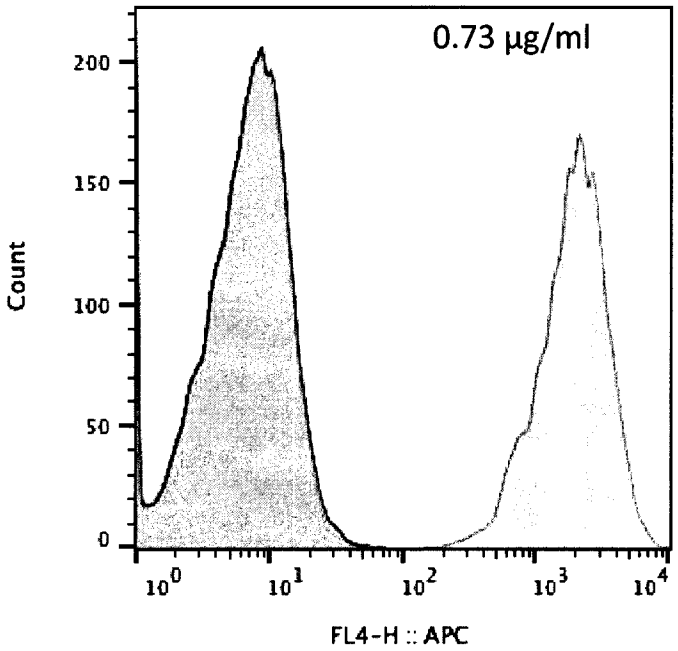


FIGURE 11A con't

BiTE #1



BiTE #2

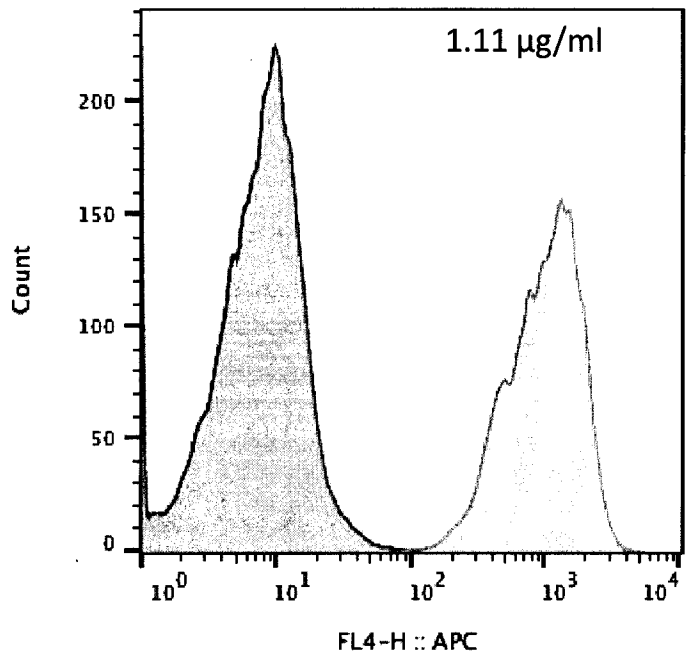
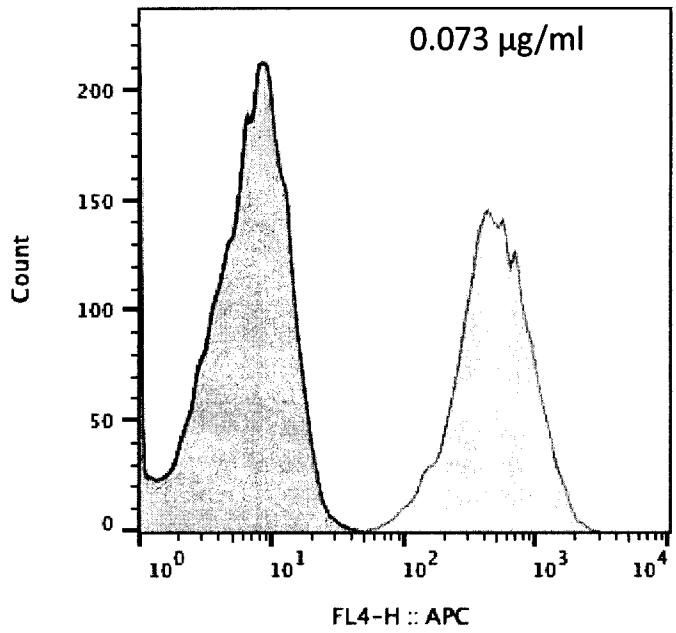


FIGURE 11A con't

BiTE #1



BiTE #2

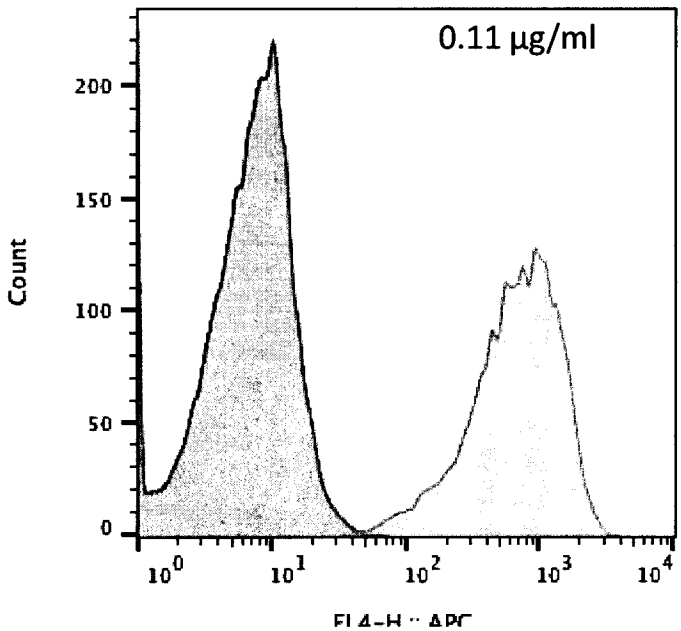
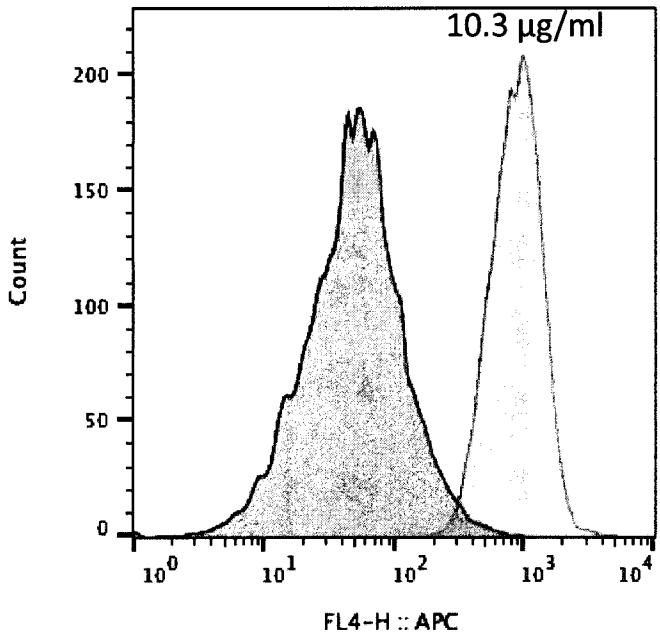


FIGURE 11B

BiTE #3



BiTE #4

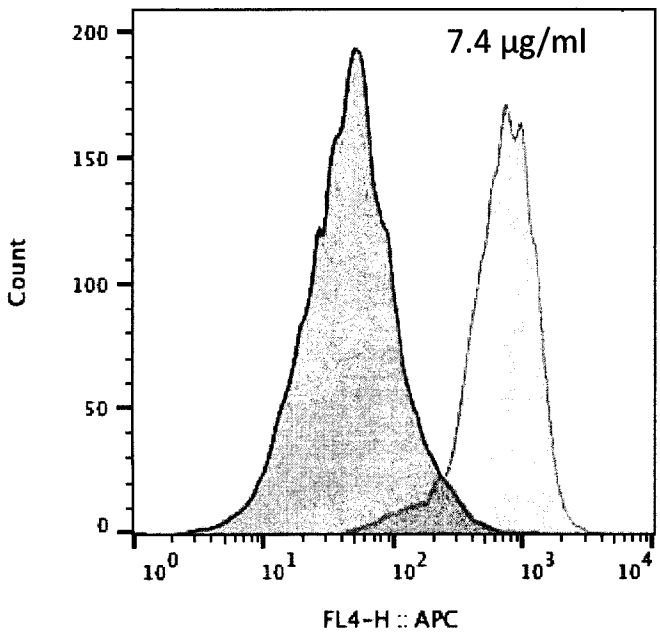
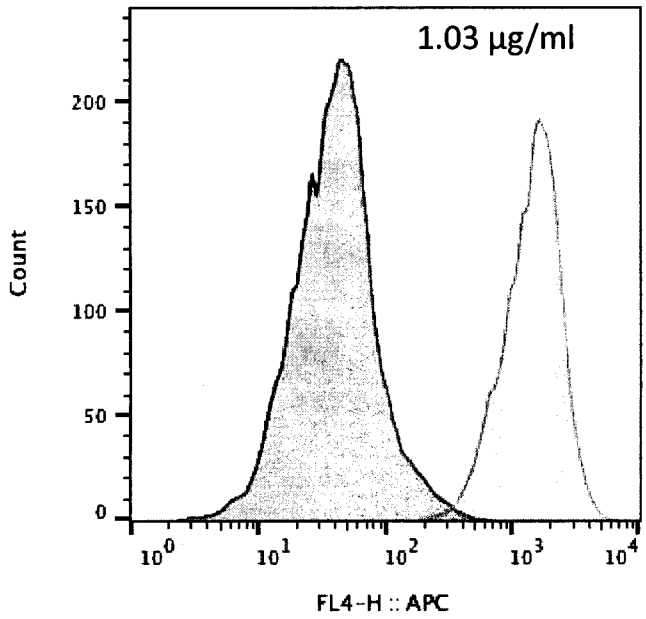


FIGURE 11B con't

BiTE #3



BiTE #4

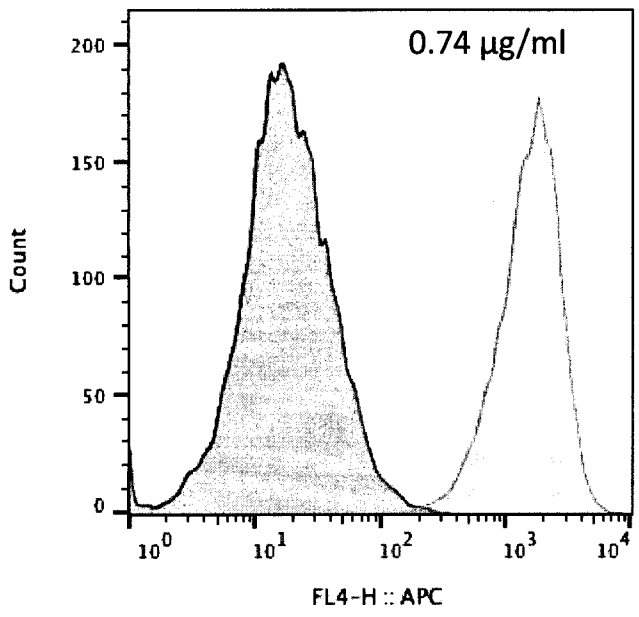
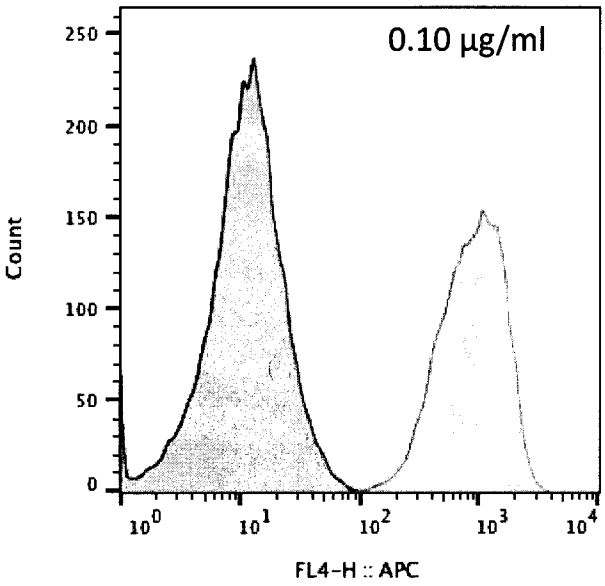


FIGURE 11B con't

BiTE #3



BiTE #4

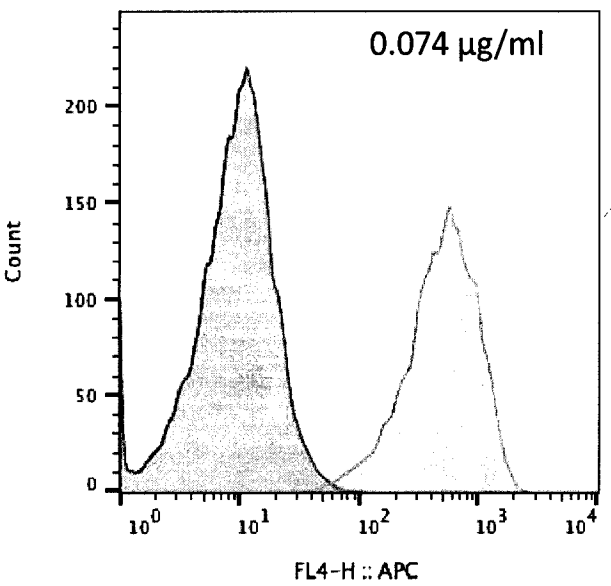
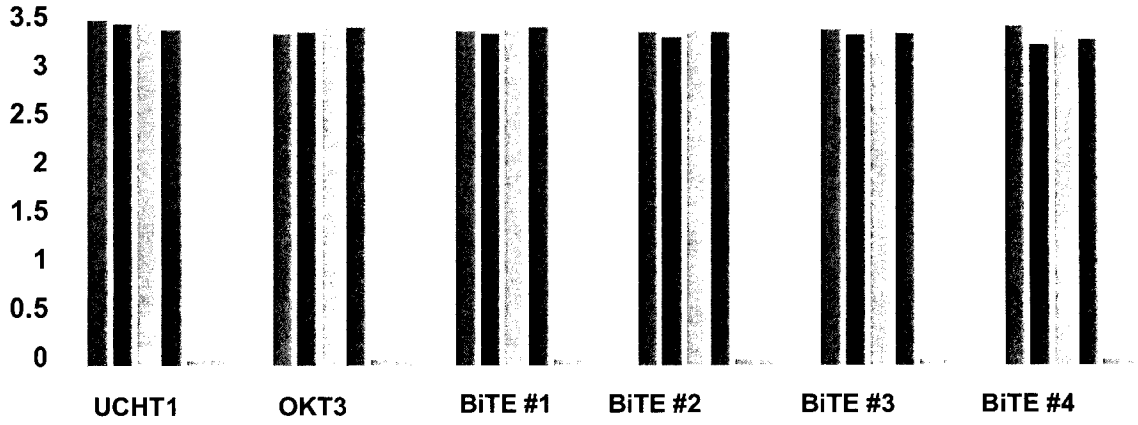


FIGURE 12



Legend:
(bars as numbered from left to right)
Bars 1 and 2: CD3 Epsilon/Gamma
Bars 3 and 4: CD3 Epsilon/Delta
Bars 5 and 6: bovine serum albumin (BSA)

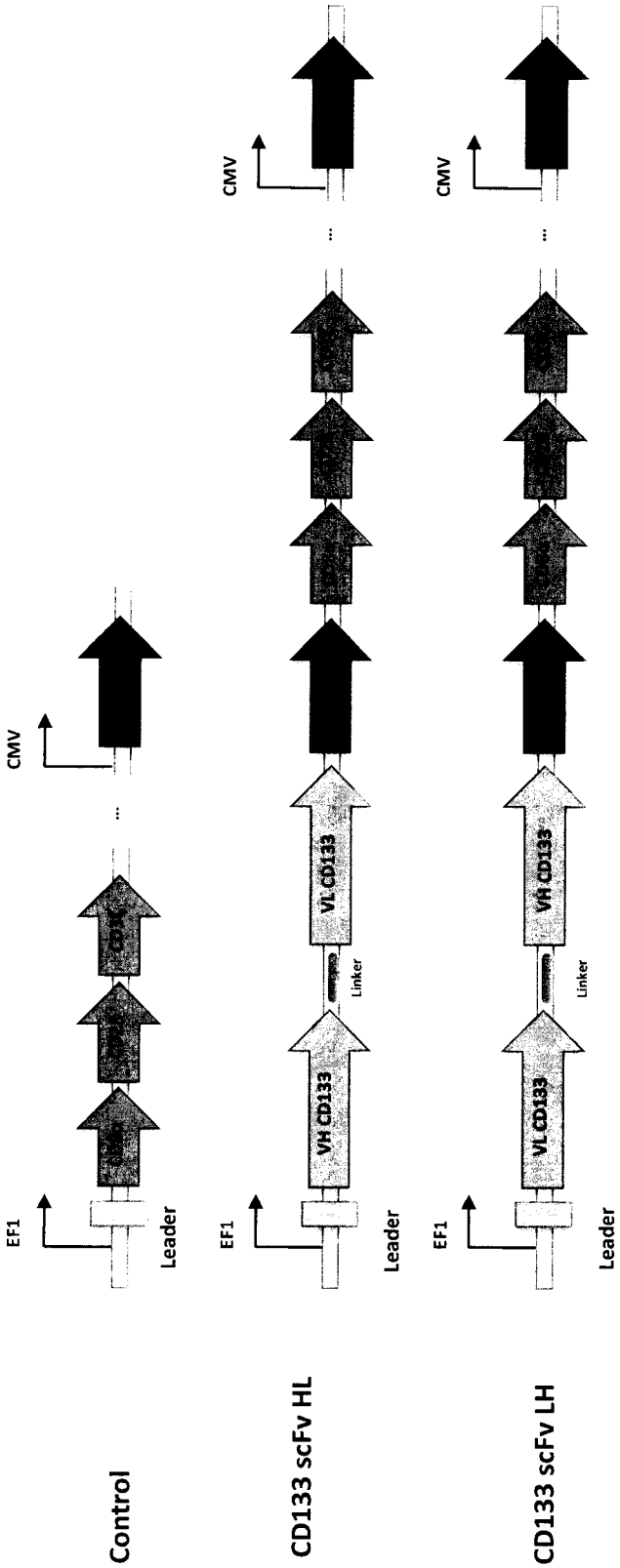
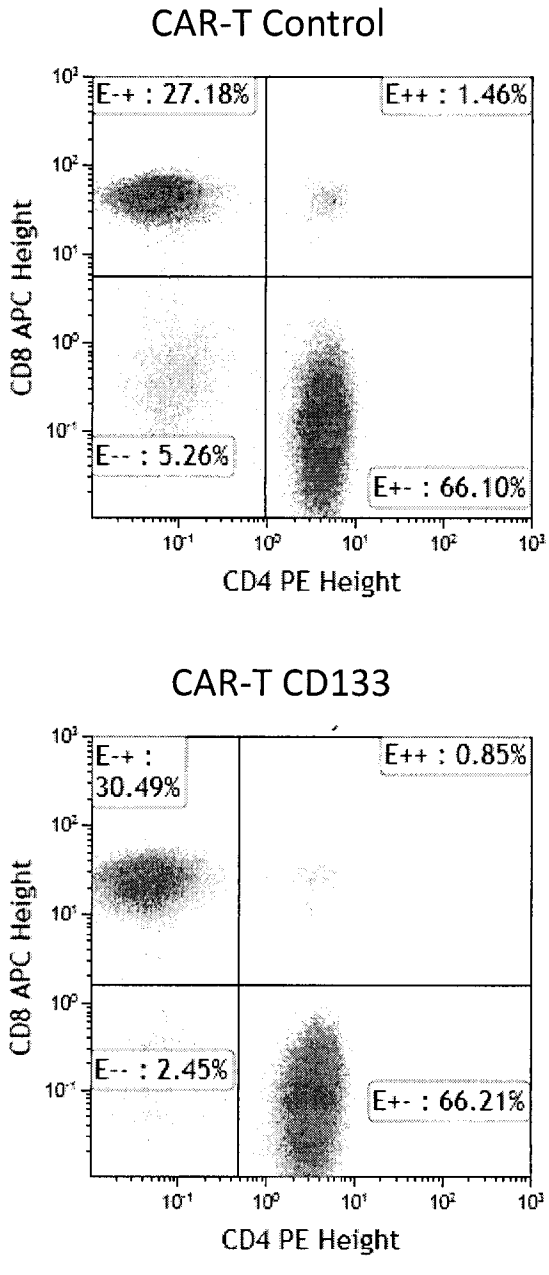

CMV
EF1
Leader
Control
CMV
EF1
Leader
VH CD133
Linker
VL CD133
CD133 scFv HL
CMV
EF1
Leader
VL CD133
Linker
VH CD133
CD133 scFv LH

FIGURE 13

FIGURE 14


CAR-T Control
E-+ : 27.18%
E++ : 1.46%
E-- : 5.26%
E+- : 66.10%
CD8 APC Height
CD4 PE Height
CAR-T CD133
E-+ : 30.49%
E++ : 0.85%
E-- : 2.45%
E+- : 66.21%
CD8 APC Height
CD4 PE Height

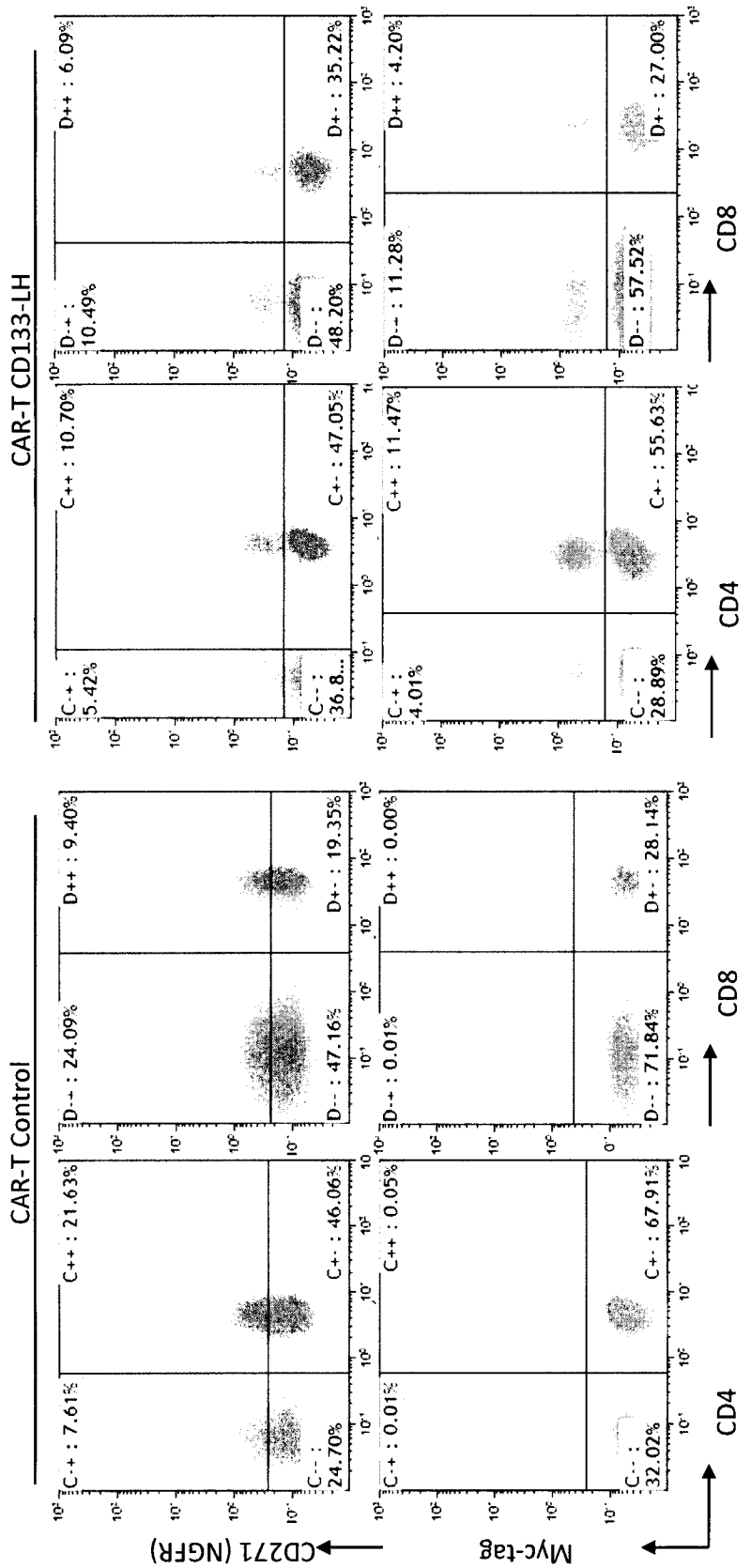

CAR-T Control
CAR-T CD133-LH
CD271 (NGFR)
Myc-tag
CD4
CD8
C-+ : 7.61%
C++ : 21.63%
C-- : 24.70%
C+- : 46.06%
D-+ : 24.09%
D++ : 9.40%
D-- : 47.16%
D+- : 19.35%
C-+ : 0.01%
C++ : 0.05%
C-- : 32.02%
C+- : 67.91%
D-+ : 0.01%
D++ : 0.00%
D-- : 71.84%
D+- : 28.14%
C-+ : 5.42%
C++ : 10.70%
C-- : 36.8...
C+- : 47.05%
D-+ : 10.49%
D++ : 6.09%
D-- : 48.20%
D+- : 35.22%
C-+ : 4.01%
C++ : 11.47%
C-- : 28.89%
C+- : 55.63%
D-+ : 11.28%
D++ : 4.20%
D-- : 57.52%
D+- : 27.00%

FIGURE 15

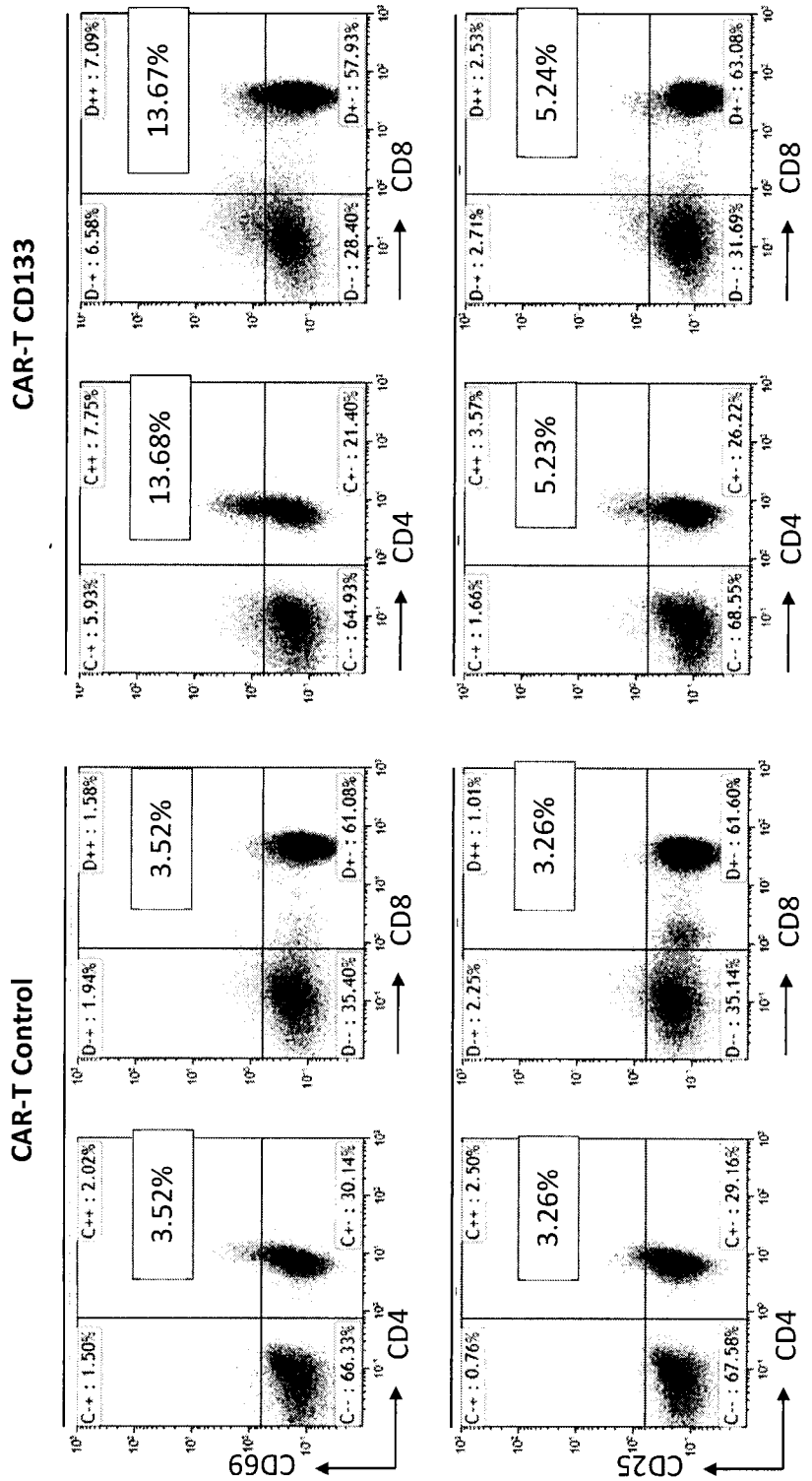
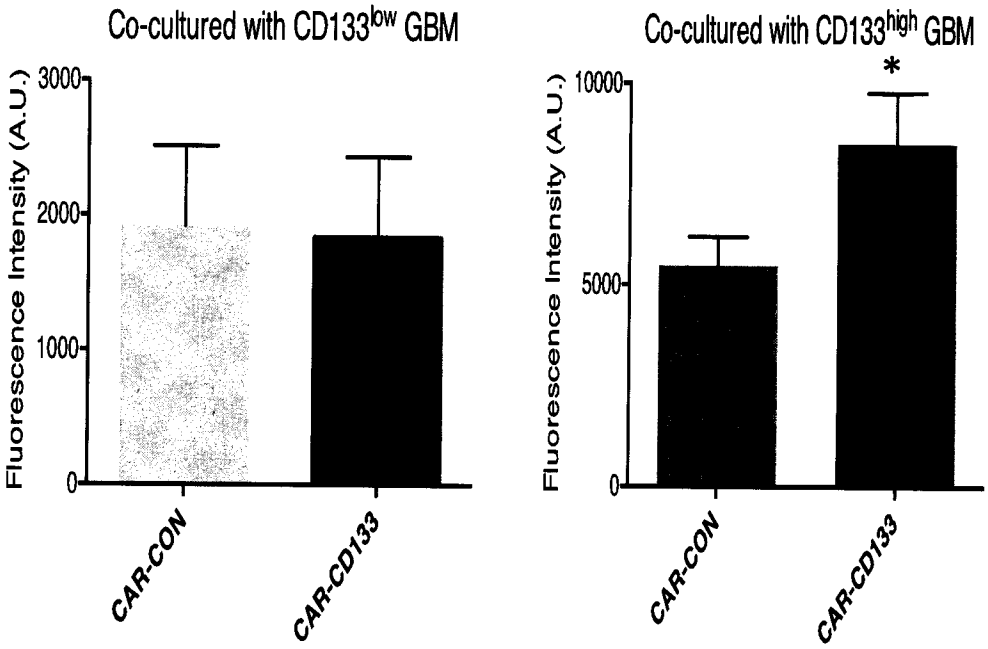

CAR-T Control
CAR-T CD133
CD69
CD25
CD4
CD8
C-+ : 1.50%
C++ : 2.02%
C-- : 66.33%
C+- : 30.14%
3.52%
D-+ : 1.94%
D++ : 1.58%
D-- : 35.40%
D+- : 61.08%
3.52%
C-+ : 0.76%
C++ : 2.50%
C-- : 67.58%
C+- : 29.16%
3.26%
D-+ : 2.25%
D++ : 1.01%
D-- : 35.14%
D+- : 61.60%
3.26%
C-+ : 5.93%
C++ : 7.75%
C-- : 64.93%
C+- : 21.40%
13.68%
D-+ : 6.58%
D++ : 7.09%
D-- : 28.40%
D+- : 57.93%
13.67%
C-+ : 1.66%
C++ : 3.57%
C-- : 68.55%
C+- : 26.22%
5.23%
D-+ : 2.71%
D++ : 2.53%
D-- : 31.69%
D+- : 63.08%
5.24%

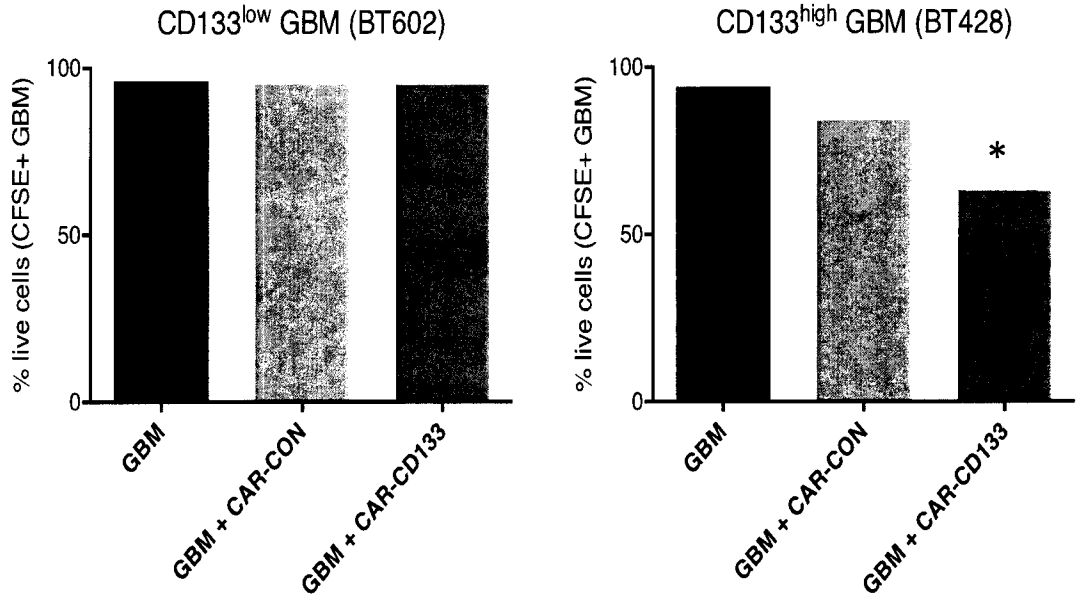
FIGURE 16

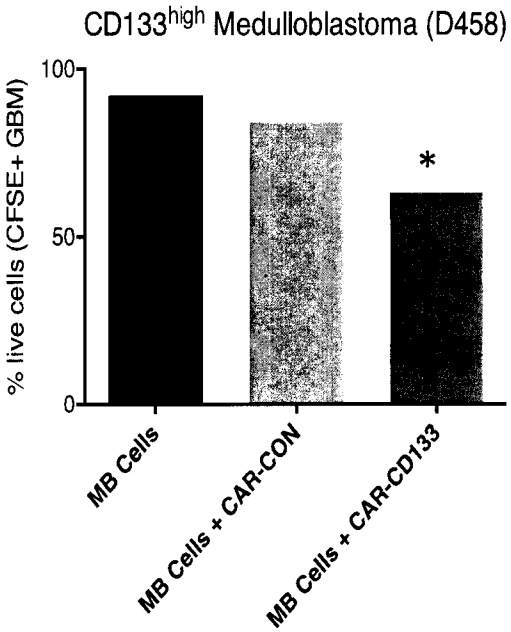
FIGURE 17



E:T = 1:1
Time = 18 hours

FIGURE 17 con't

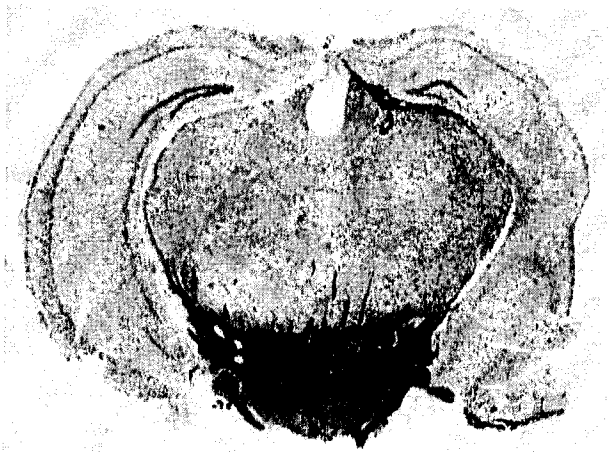




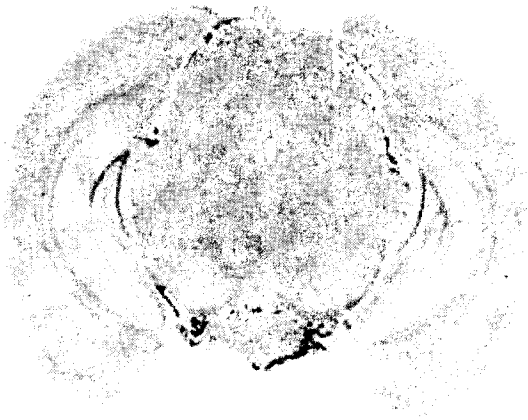
E:T = 1:1
Time = 18 hours

FIGURE 18

A



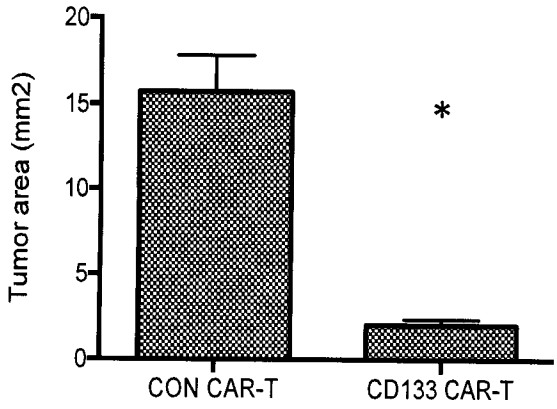
B



CD133-specific CAR-T

Treatment Delivery: Intracranial
Dose: 1million x 2 doses (2 week)

C



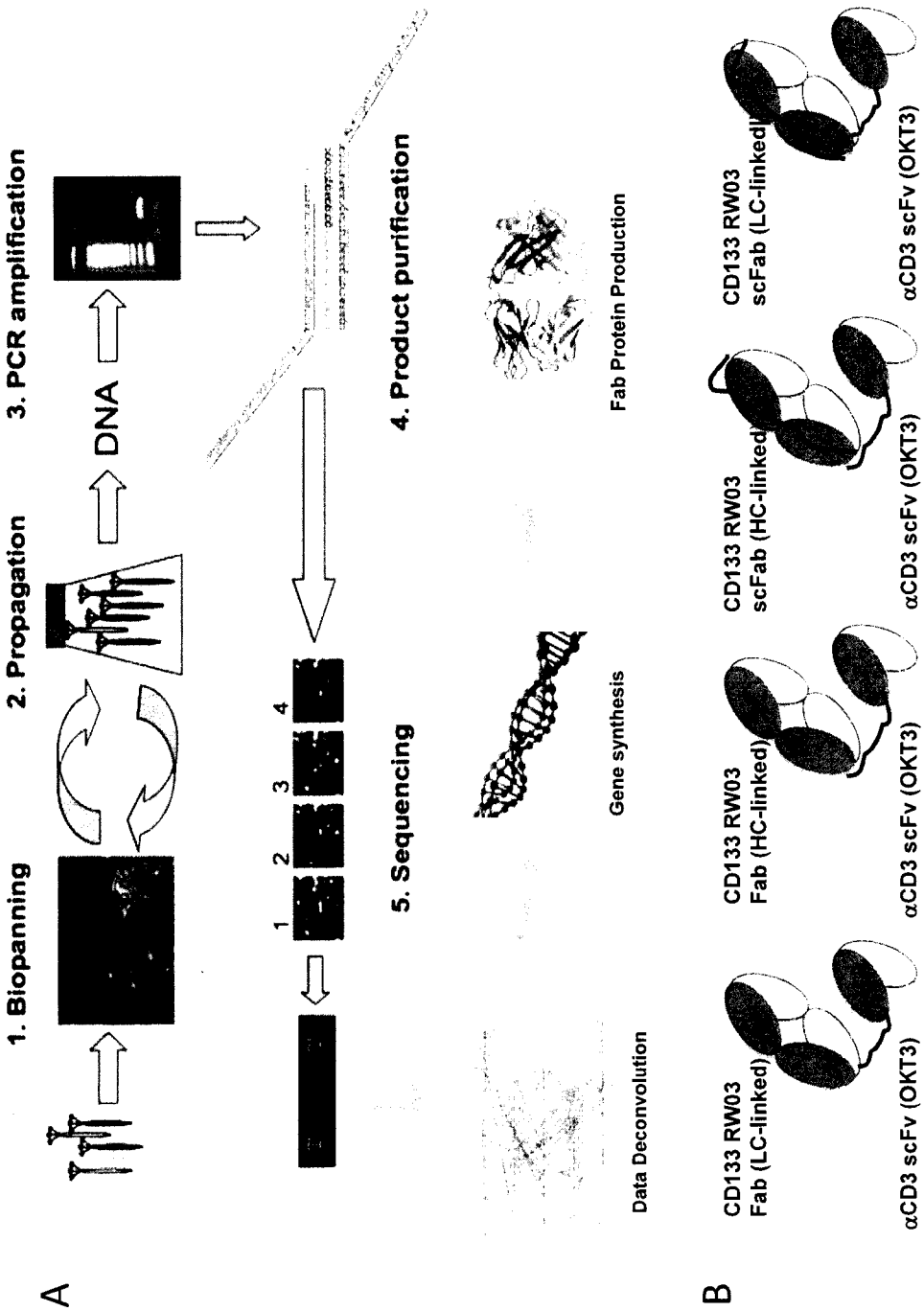

A
1. Biopanning
2. Propagation
3. PCR amplification
DNA
4. Product purification
5. Sequencing
1
2
3
4
Data Deconvolution
Gene synthesis
Fab Protein Production
B
CD133 RW03
Fab (LC-linked)
αCD3 scFv (OKT3)
CD133 RW03
Fab (HC-linked)
αCD3 scFv (OKT3)
CD133 RW03
scFab (HC-linked)
αCD3 scFv (OKT3)
CD133 RW03
scFab (LC-linked)
αCD3 scFv (OKT3)

FIGURE 19

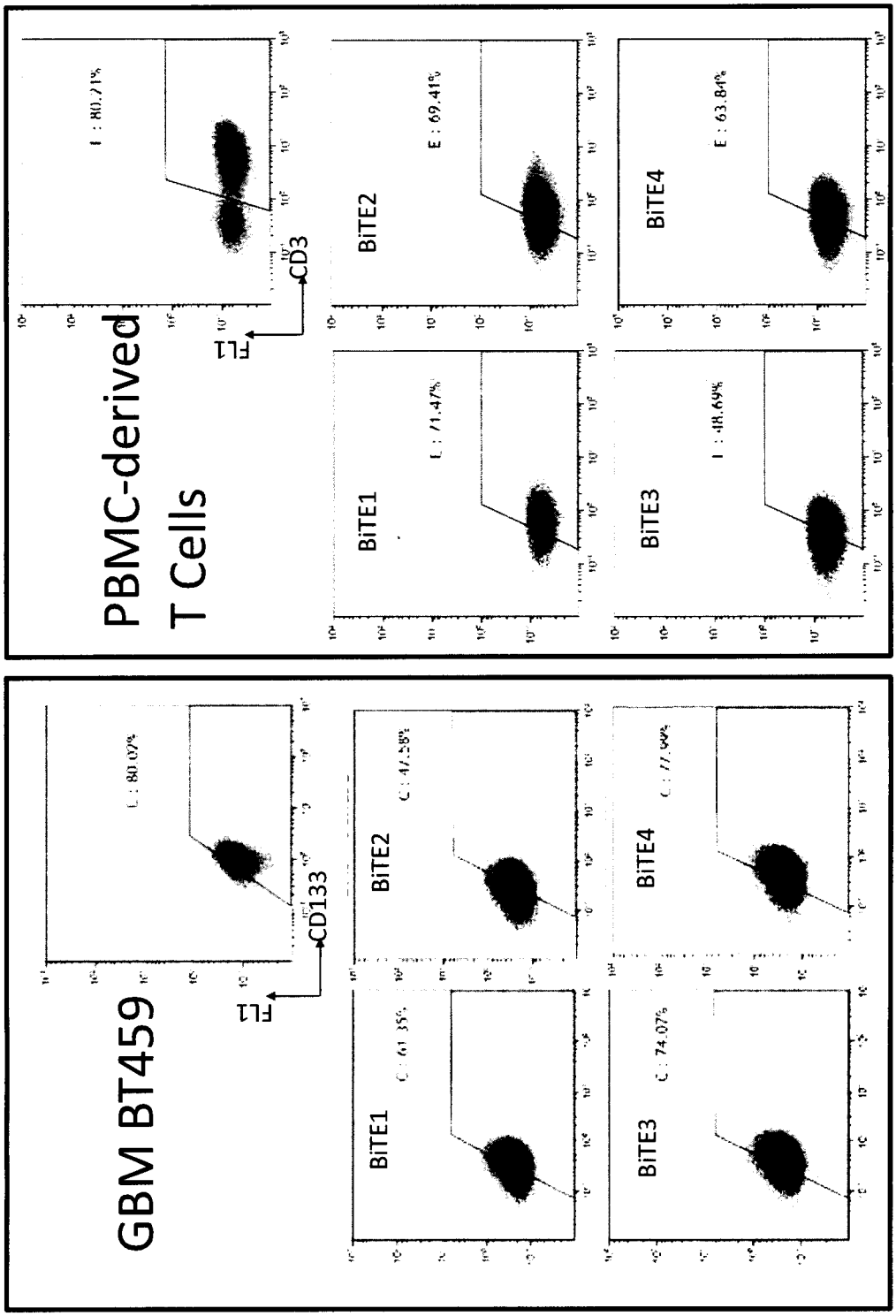

PBMC-derived
T Cells
BiTE1
BiTE2
BiTE3
BiTE4
CD3
FL1
GBM BT459
BiTE1
BiTE2
BiTE3
BiTE4
CD133
FL1

FIGURE 20

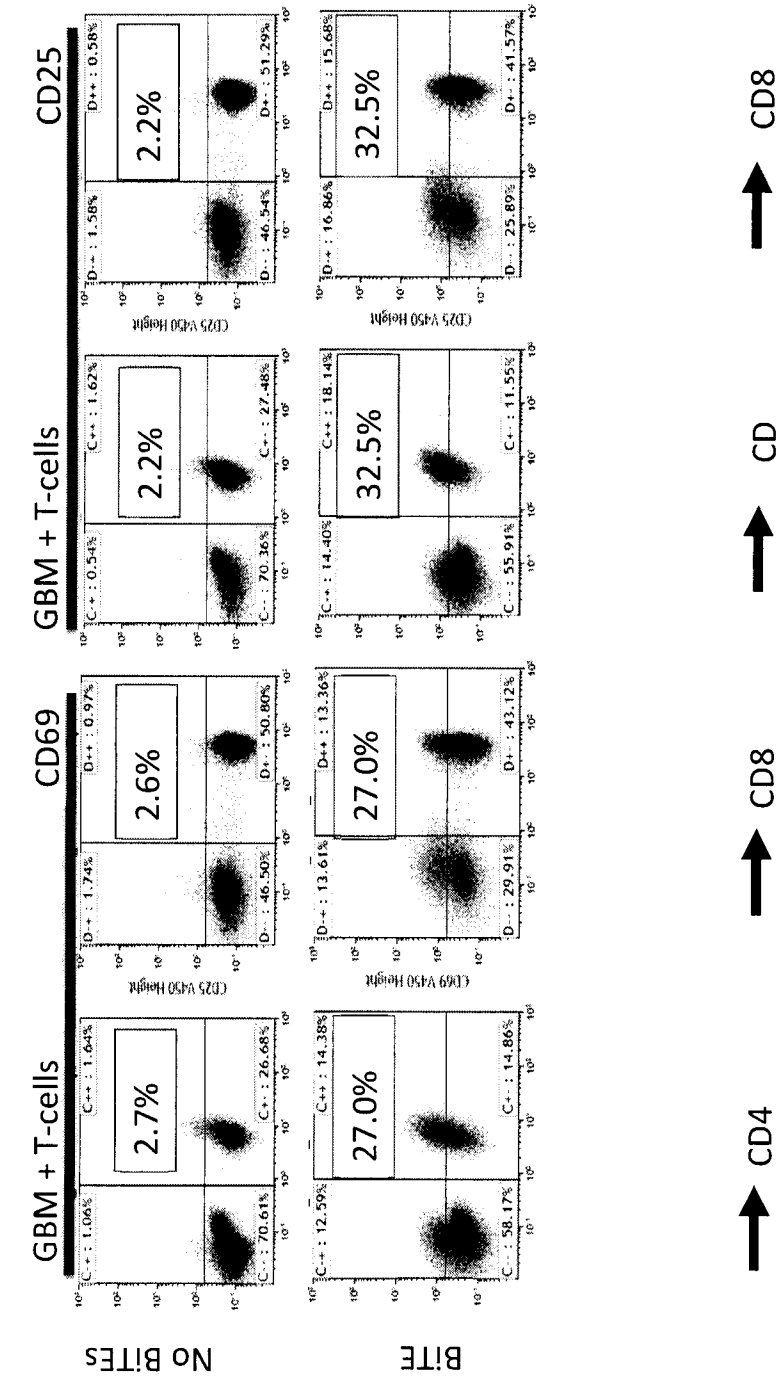

No BiTEs
BiTE
GBM + T-cells
CD69
CD25
2.7%
2.6%
2.2%
27.0%
32.5%
CD4
CD8
CD
CD8

FIGURE 21

FIGURE 22

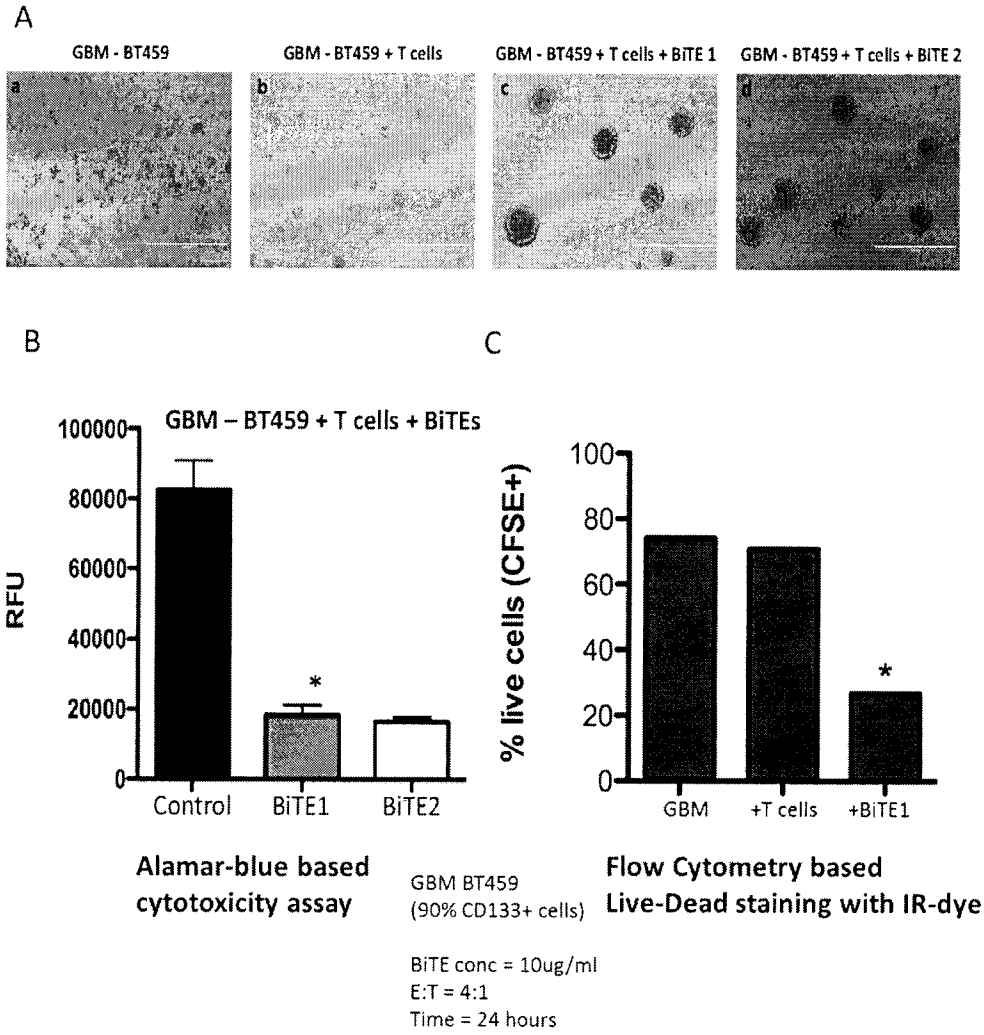
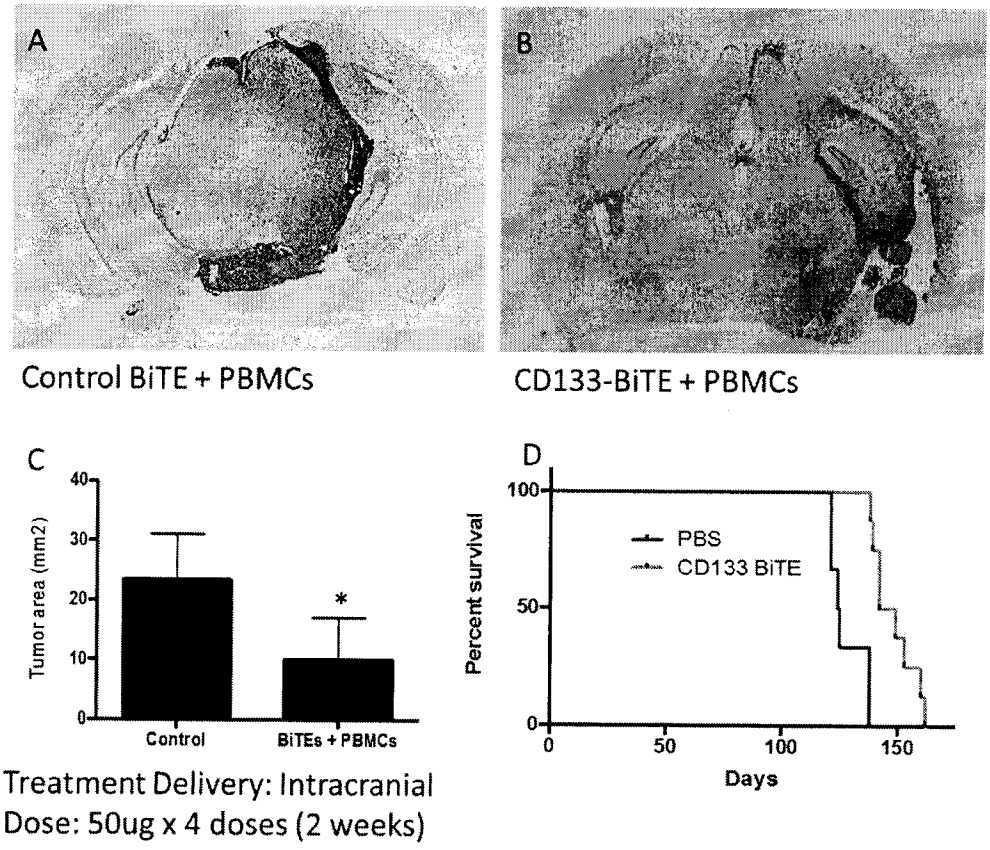

A
GBM - BT459
GBM - BT459 + T cells
GBM - BT459 + T cells + BiTE 1
GBM - BT459 + T cells + BiTE 2
a
b
c
d
B
GBM – BT459 + T cells + BiTEs
RFU
100000
80000
60000
40000
20000
0
*
Control
BiTE1
BiTE2
C
% live cells (CFSE+)
100
80
60
40
20
0
*
GBM
+T cells
+BiTE1
Alamar-blue based cytotoxicity assay
GBM BT459
(90% CD133+ cells)
BiTE conc = 10ug/ml
E:T = 4:1
Time = 24 hours
Flow Cytometry based Live-Dead staining with IR-dye

FIGURE 23


A
B
Control BiTE + PBMCs
CD133-BiTE + PBMCs
C
Tumor area (mm2)
40
30
20
10
0
*
Control
BiTEs + PBMCs
D
Percent survival
100
50
0
PBS
CD133 BiTE
0
50
100
150
Days
Treatment Delivery: Intracranial
Dose: 50ug x 4 doses (2 weeks)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2017/051245

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 16/28*** (2006.01), ***A61K 35/17*** (2015.01), ***A61K 39/395*** (2006.01), ***A61K 47/68*** (2017.01), ***A61P 35/00*** (2006.01), ***C07K 14/705*** (2006.01), ***C07K 16/30*** (2006.01), ***C07K 16/46*** (2006.01), ***C07K 19/00*** (2006.01), ***C12N 5/10*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C07K, A61K, A61P, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Databases: Google scholar, Google patents, Questel orbit

Keywords: CD133, antibody, RW01, RW03, bispecific, CAR-T, BiTE, medulloblastoma, immunoconjugate, cell-surface

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WILLIAMS, R. (11-12-2013) Generation of Anti-CD133 Human Synthetic Antibodies as Tools for Exploring CD133 Function. [online] [Retrieved on: 06 -12-2017]. Retrieved from: https://tspace.library.utoronto.ca/handle/1807/43333. *whole document*	1-9 and 13-28 10-12 and 29-35
X Y	VORA, P. et al. (09-2016) The efficacy of CD133 BiTEs and CAR-T cells in preclinical model of recurrent glioblastoma. CANCER IMMUNOL RES 4 Suppl. Abstract No. B079 ISSN: 2326-6074. *whole document*	1-29, 33, and 34 30-32 and 35
X Y	VORA, P. et al. (04-2016) Preclinical validation of a novel CD133/CD3 bispecific T-cell engager (BiTE) antibody to target patient-derived glioblastoma cells. CANCER RES 76 Suppl. Abstract No. 1481, ISSN: 1538-7445. *whole document*	1-11, 13-29, 33, and 34 12, 30-32, 34, and 35
Y	SINGH, S. K. et al. (18-11-2004) Identification of human brain tumour initiating cells. NATURE 432: 396-401, ISSN: 1476-4687. *abstract*	30-32 and 35

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 8 December 2017 (08-12-2017)	Date of mailing of the international search report 15 December 2017 (15-12-2017)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer Kerry Ferguson (819) 639-7804

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOSTAD, M. et al. (07-03-2015) Light-controlled endosomal escape of the novel CD133-targeting immunotoxin AC133-saporin by photochemical internalization – A minimally invasive cancer stem cell-targeting strategy. JOURNAL OF CONTROLLED RELEASE 206: 37-48 ISSN: 0168-3659 *whole document*	2, 3, 8, 14, and 16-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2017/051245

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Sheet 1

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Sheet 1

Continuation of Box III:

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-35 are directed to a CD133-binding agent which specifically binds cell surface expressed CD133 and denatured CD133 comprising a light chain amino acid sequence as set forth in SEQ ID NO: 2 and a heavy chain amino acid sequence as set forth in SEQ ID NO: 3, associated immunoconjugates, pharmaceutical compositions, the use of the CD133-binding agent for targeting and binding CD133-expressing cells, detecting CD133-expressing cells and/or quantitating levels of cellular CD133 expression, reducing levels of CD133 protein, treating or preventing a cancer, including glioblastoma and medulloblastoma, and the use of a T-cell expressing the CD133-binding agent for treating glioblastoma or medulloblastoma; and

Group B - Claims 1-35 are directed to a CD133-binding agent which specifically binds cell surface expressed CD133 and denatured CD133 comprising a light chain amino acid sequence as set forth in SEQ ID NO: 4 and a heavy chain amino acid sequence as set forth in SEQ ID NO: 5, associated immunoconjugates, pharmaceutical compositions, the use of the CD133-binding agent for targeting and binding CD133-expressing cells, detecting CD133-expressing cells and/or quantitating levels of cellular CD133 expression, reducing levels of CD133 protein, treating or preventing a cancer, including glioblastoma and medulloblastoma, and the use of a T-cell expressing the CD133-binding agent for treating glioblastoma or medulloblastoma.

Although all claims encompass a CD133-binding agent, this is not new (see D1-D3) and cannot unify the claims in a single inventive concept.

The claims must be limited to one inventive concept as set out in PCT Rule 13.