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(54) Title: COMPOSITIONS AND METHODS FOR CD20 IMMUNOTHERAPY

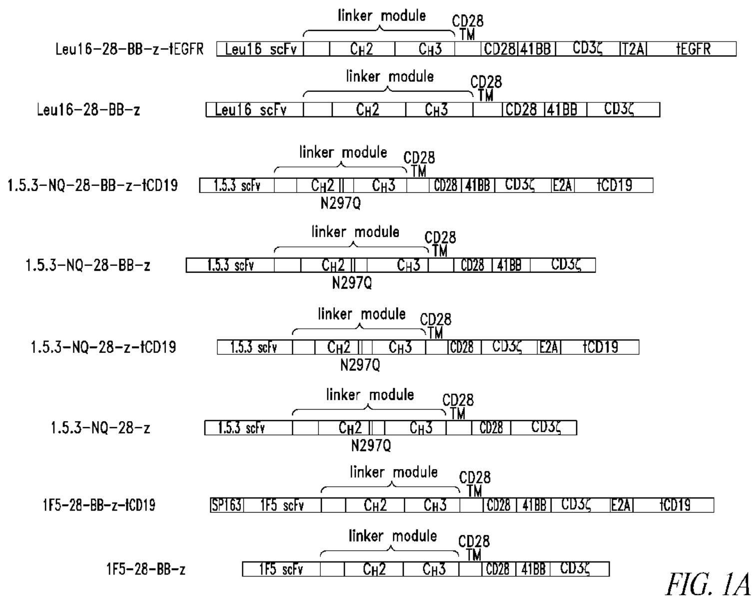

FIG. 1A

(57) **Abstract**: The present disclosure provides compositions and uses thereof for treating a disease or disorder associated with CD20 expression. Treatments of this disclosure include use of a host cell expressing a fusion protein, such as an anti-CD20 CAR, optionally in combination with a CD20-specific binding molecule, a chemotherapeutic, an inhibitor of an immunosuppression component, or combinations thereof.

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COMPOSITIONS AND METHODS FOR CD20 IMMUNOTHERAPY

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under CA154874 awarded by the National Institutes of Health. The government has certain rights in the invention.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 360056_441WO_SEQUENCE_LISTING.txt. The text file is 210 KB, was created on March 16, 2017, and is being submitted electronically via EFS-Web.

BACKGROUND

Adoptive transfer of genetically modified T cells has emerged as a potent therapy for various malignancies. The most widely employed strategy has been infusion of patient-derived T cells expressing chimeric antigen receptors (CARs) targeting tumor-associated antigens. This approach has numerous theoretical advantages, including the ability to target T cells to any cell surface antigen, circumvent loss of major histocompatibility complex as a tumor escape mechanism, and employ a single vector construct to treat any patient, regardless of human leukocyte antigen haplotype. For example, CAR clinical trials for B-cell non-Hodgkin's lymphoma (NHL) have, to date, targeted CD19, CD20, or CD22 antigens that are expressed on malignant lymphoid cells as well as on normal B cells (Brentjens *et al.*, *Sci Transl Med* 2013;5(177):177ra38; Haso *et al.*,*Blood* 2013;121(7):1165-74; James *et al.*, *J Immunol* 2008;180(10):7028-38; Kalos *et al.*, *Sci Transl Med* 2011;3(95):95ra73; Kochenderfer *et al.*, *J Clin Oncol* 2015;33(6):540-9; Lee *et al.*, *Lancet* 2015;385(9967):517-28; Porter *et al.*, *Sci Transl Med* 2015;7(303):303ra139; Savoldo *et al.*, *J Clin Invest* 2011;121(5):1822-6; Till *et al.*, *Blood* 2008;112(6):2261-71; Till *et al.*, *Blood* 2012;119(17):3940-50; Coiffier *et al.*, *N Engl J Med* 2002;346(4):235-42). Most investigators studying therapies for lymphoid malignancies have chosen to target CD19 since this molecule is expressed from earlier stages of B-cell differentiation than CD20 or CD22. CAR T cells targeting CD19 can therefore be used to treat a slightly wider range of B-cell malignancies, including acute lymphoblastic leukemia, which arises at the pro- or pre-B cell stage of differentiation.

CD20 remains an appealing antigen, however, due to its extensive clinical record as a successful immunotherapy target, as demonstrated in trials using rituximab, a monoclonal antibody targeting CD20 (Coiffier *et al.*, *N Engl J Med* 2002;346(4):235-42; Lenz *et al.*, *J Clin Oncol* 2005;23(9):1984-92; Marcus R, *et al.*, *J Clin Oncol* 2008;26(28):4579-86; Pfreundschuh *et al.*, *Lancet Oncol* 2011;12(11):1013-22). In contrast to CD19, which is readily internalized upon antibody binding (Pulczynski *et al.*, *Blood* 1993;81(6):1549-57), CD20 undergoes endocytosis much more slowly after antibody binding (Press *et al.*, *Blood* 1994;83(5):1390-7; Pulczynski *et al.*, *Leuk Res* 1994;18(7):541-52). This stability could theoretically impact the quality of the immunological synapse and subsequent CAR triggering and T cell activation. Loss of CD19 expression on tumor cells has been described as an escape mechanism in patients treated with CD19-targeted T cells (Grupp *et al.*, *N Engl J Med* 2013;368(16):1509-18). Although CD20 loss has also been described following anti-CD20 antibody therapy, CD20-specific CAR T cells provide an alternative target that would allow sequential therapy, or could be used in concert with CD19 CAR T cells to target multiple antigens simultaneously, reducing the risk of immune escape by antigen loss.

One potential limitation of CD20 as a target antigen for CARs is that patients with relapsed or refractory lymphoma who are likely to be candidates for CAR T cell therapy trials will often have been treated recently with rituximab-containing regimens. Since antibody can persist in the serum for months, residual rituximab could theoretically block the binding of CARs to CD20 and prevent or weaken T-cell activation, potentially rendering therapy ineffective. In previous CD20 CAR T cell trials (Till *et al.*, *Blood* 112:2261-71, 2008; Till *et al.*, *Blood* 119:3940-50, 2012), eligibility criteria excluded patients recently treated with rituximab. However, this approach significantly impacts accrual and would ultimately limit the availability of this therapy for patients most in need of novel treatment options.

Currently, there remains a need in the immunotherapy field for compositions and methods for additional or alternative immunotherapies directed against various diseases, including cancer (*e.g.*, leukemia, lymphoma). Presently disclosed embodiments address this need and provide other related advantages.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show schematic diagrams of CD20-specific CAR constructs containing scFvs from different anti-CD20 antibodies (Leu16, 1F5, and 1.5.3). (A) Shows CD20-specific CAR constructs and their respective mature CAR proteins. (B) Shows additional mature CD20-specific CAR proteins.

Figures 2A-2F show rituximab and ofatumumab block antigen binding of antibody used to generate a CAR scFv. Ramos cells ($CD20^{+}$) were incubated with the indicated rituximab (A-C) or ofatumumab (D-F) concentrations for 30 minutes, followed by incubation with PE-labeled anti-CD20 antibody (clone Leu16) or isotype control at either 4°C (A and. D) or 37°C (B and E) for 30 minutes. Cells were washed and analyzed by flow cytometry to determine available CD20 binding sites as measured by PE fluorescence intensity. The graphs depicted in Fig. 2C and Fig. 2F summarize the geometric mean fluorescence intensity (MFI) at either 4°C or 37°C as a function of rituximab or ofatumumab concentration, respectively. The data are representative of three independent experiments.

Figures 3A and 3B show the effect of rituximab on CAR T cell function *in vitro*. The indicated B-cell NHL cell lines were irradiated and incubated for 30 minutes at room temperature with varying rituximab concentrations (at 2x the concentrations during incubation to yield the indicated final concentrations after addition of T cells). CFSE-stained T cells expressing the Leu16-28-BB-z-tEGFR CD20-specific CAR were added to the target cells at a 1:1 volume and ratio. (A) Proliferation of the T cells was analyzed 4 days later by flow cytometry for CFSE dilution. The percent divided $CD3^{+}$ T cells relative to unstimulated T cells are shown on the left axis (filled bars). Cell size of $CD3^{+}$ T cells as determined by geometric mean of forward scatter (subtracting size of cells in media only) is shown on the right axis (open bars). (B) Cytokine secretion of these T cells was measured by Luminex assay using supernatants from 24 hours after restimulation. Interleukin (IL)-2 concentrations are shown on the left y-axis and

Interferon (IFN)-γ and tumor necrosis factor (TNF)-α on the right y-axis. The data shown are representative of 3 independent experiments.

Figure 4 shows the effect of rituximab on CAR T cell-mediated cytotoxicity. The indicated ^{51}Cr-labeled target cells were pre-incubated for 30 minutes with rituximab (at 2x the concentrations during incubation to yield the indicated final concentrations after addition of T cells), and then CD8^{+} T cells expressing the Leu16-28-z CAR were added at the E:T ratios shown in a standard 5-hour ^{51}chromium-release assay. Mock-transduced T cells, and samples with rituximab and target cells only ("0:1") were used as negative controls. The average value of duplicate wells is shown, with error bars representing standard deviation. The data are representative of results from 4 independent experiments.

Figures 5A-5C show that sensitivity to rituximab blockade is dependent on CD20 antigen density on target cells. K562 cells transduced with CD80 and CD20 ("K80-20") were cloned by limiting dilution, selected for high, medium, or low levels of CD20 expression (Fig. 10), and used as target cells in assays for (Fig. 5A) proliferation and cell size (geometric mean forward scatter of gated CD3^{+} cells minus the size of cells in media only) using CFSE-labeled Leu16-28-z CAR-transduced T cells as described in Fig. 3; (Fig. 5B) cytokine secretion of the Leu16-28-z CAR-transduced T cells at 24 hours from (Fig. 5A) above, measured by Luminex assay; and (Fig. 5C) cytotoxicity using Leu16-28-z CAR-transduced CD8^{+} T cells by ^{51}Cr-release assay as described in Fig. 4. Data are representative of three independent experiments. Absolute values for cytokine secretion are shown in Fig. 11.

Figures 6A and 6B show proliferation and cytokine secretion by T cells expressing an anti-CD20 CAR. Healthy donor T cells were sorted and stimulated using anti-CD3/28 beads, followed by transduction with lentiviral vector encoding the 1F5-28-BB-z CAR construct. (A) At day 9 after stimulation, CAR T cells were labeled with CFSE, restimulated with either K562-CD80-CD20 (“K80-20”) or K562-CD80 (“K80”) target cells that had been irradiated, and CFSE dilution was measured by flow cytometry 4 days later to measure T cell proliferation. The percent divided CD3^{+} T cells relative to unstimulated T cells are shown. Mock-transduced T cells were used as a negative control. (B) Cytokine secretion by the T cells was determined by harvesting supernatant samples from the above cultures at 24 hours after restimulation and analyzing the indicated cytokine concentration by Luminex assay.

Figures 7A-7E show the *in vivo* effect of rituximab on CD20 CAR T cell function. Nod-SCID-$\gamma^{-/-}$ (NSG) mice were injected intravenously (i.v.) with 5 x 10^5 rituximab-refractory Raji-ffLuc lymphoma cells, followed by one of the following treatments: no treatment, rituximab only (25 μg or 200 μg) intraperitoneally (i.p.) 5 days later, 10^7 1.5.3-NQ-28-BBz CAR T cells only 6 days after tumor, or rituximab (25 μg or 200 μg) i.p. at 5 days followed by 10^7 CAR T cells at 6 days after tumor. Mice were imaged twice weekly for bioluminescence. (A) Schema of mouse experiment. (B) Average tumor burden per group over time as measured by total body bioluminescence. The geometric mean luminescence values with 95% confidence intervals are shown, and to prevent misleading fluctuations in tumor volume graphs, the last bioluminescence level of each mouse was carried forward after it was killed until no mice in that group remained. Individual bioluminescence traces are shown in Fig. 13. (C) Kaplan-Meier plot showing overall survival of each treatment group. (D) Serum rituximab levels on the day of T cell infusion (day 6) and 1 week post T cell infusion (day 13). The horizontal bars denote the median values. (E) Serum rituximab levels from lymphoma patients who underwent rituximab-containing salvage chemotherapy within the 4 preceding months. The gray horizontal bar line indicates the median, and black horizontal bar lines indicate the interquartile range (25-75%).

Figures 8A-8C show the effect of ofatumumab on CD20 CAR T cell function *in vitro*. Irradiated Rec-1 or Raji-ffLuc cells or non-irradiated ^{51}Cr-labeled Rec-1 cells were pre-incubated for 30 minutes with 2x the indicated concentrations of ofatumumab, followed by experiments to determine function of T cells expressing the 1.5.3-NQ-28-BB-z CAR, using the methodologies described in the legend of Figs. 3 and 4. (A) The percent divided $CD3^+$ T cells relative to unstimulated T cells are shown on the left axis (filled bars). Cell size of $CD3^+$ T cells as determined by geometric mean of forward scatter (subtracting size of cells in media only) is shown on the right axis (open bars). (B) Cytokine secretion of these T cells was measured by Luminex assay using supernatants from 24 hours after restimulation. IL-2 concentrations are shown on the left y-axis and IFN-γ on the right y-axis. (C) Cytotoxicity of 1.5.3-NQ-28-BB-z CAR T cells was determined using a standard 4-hour ^{51}Cr-release assay with Rec-1 target cells. The average value of duplicate wells is shown, with error bars representing standard deviation.

Figure 9 shows CD20 expression of K80-20^{low}, K80-20^{med}, K80-20^{high} as determined by flow cytometry. Open histograms represent cells stained with FITC-conjugated 1F5 antibody (anti-CD20), and filled histograms represent cells stained with an isotype control antibody Ab.

Figure 10 shows the absolute cytokine concentrations from T cell supernatants from the experiment in Fig. 5 are shown.

Figures 11A-11E show proliferation, cytokine secretion, and cytotoxicity of CAR T cells with fully human anti-CD20 scFv. Healthy donor CD14$^-$CD45RA$^-$CD62L$^+$ central memory T cells were stimulated using anti-CD3/28 beads, followed by transduction with lentiviral vector encoding either the 1.5.3-NQ-28-z or 1.5.3-NQ-28-BB-z CAR. CAR T cells were labeled with CFSE and restimulated with irradiated Raji-ffLuc, rituximab-refractory Raji-ffLuc (RR-Raji), or Rec-1 target cells. (A) Proliferation of 1.5.3-NQ-28-z T cells was assessed by analyzing the cells 4 days later by flow cytometry for CFSE dilution. The percent divided CD3$^+$ T cells relative to unstimulated T cells are shown. (B) Cytokine secretion by 1.5.3-NQ-28-z T cells was determined by harvesting supernatant samples from the above cultures at 24 hours after restimulation and analyzing the indicated cytokine concentrations by Luminex assay. IL-2 and TNF-α concentrations are shown on the left y-axis and IFN-γ concentrations are plotted on the right y-axis. (C) Cytokine secretion by 1.5.3-NQ-28-BB-z T cells determined as in part B above. (D) Cytotoxicity of 1.5.3-NQ-28-BB-z CAR T cells was determined using a standard 5-hour Chromium^{51}-release assay with the indicated target cell lines. (E) Cytokine secretion by 1.5.3-NQ-28-BB-z T cells as in part (A) above and stimulated with Granta, Rec-1, FL-18, or K80-20 cells.

Figure 12 shows that rituximab-refractory Raji-ffLuc have the same CD20 expression as parental Raji-ffLuc cells. Raji-ffLuc (solid-line histogram) or rituximab-refractory Raji-ffLuc (dashed-line histogram) cells were stained with anti-CD20-PE or anti-CD20-APC antibodies and then analyzed by flow cytometry. CD20 expression relative to isotype control antibody (filled histogram) is shown for each cell line.

Figures 13A and 13B show bioluminescent traces and images from a xenograft tumor mouse model from Fig. 7 treated with anti-CD20 CAR T cells. (A) Individual mouse bioluminescent tumor burden traces over time. Each line represents an individual mouse. The grey line represents a mouse with no tumor, which defines the baseline autofluorescence. (B) Representative mouse bioluminescence images.

Figures 14A-14C show presence of circulating T cells in mice. Peripheral blood mononuclear cells (PBMC) were isolated from retroorbital blood samples taken at day 28 after tumor injection and analyzed by flow cytometry for human CD3, mouse CD45, and human CD19 (as a marker of transduced T cells). (A) Representative dot plots of circulating human T cells (gated on viable lymphocytes) are shown in left panels and CAR^+ cells (based on CD19 expression), gated on human $CD3^+$ T cells are shown in right panels. (B) Summary of T cell persistence at day 28. (C) Summary CAR expression on persisting T cells. For both Fig. 14B and Fig. 14C, the difference between CAR only and CAR + rituximab groups were not statistically significant, based on unpaired two-tailed t test.

Figures 15A-15D show cytokine secretion by various CAR constructs *in vitro*. Central memory ($CD14^-CD45RA^-CD62L^+$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vivo. At day 14, the cells were re-stimulated with either irradiated Raji-ffLuc cells (Fig. 15A and Fig. 15C), Granta-519 cells (Fig. 15B), and Jeko cells (Fig. 15D). The "19-BB-z" construct is a clinical-grade CD19-targeted CAR being used in clinical trials and is provided as a positive control. Supernatants were harvested 24 hours later and analyzed by Luminex assay for interferon (IFN)-γ, IL-2, and tumor necrosis factor-α levels.

Figures 16A and 16B show cytokine secretion by CD20 CAR T cells. (A) $CD4^+$ and $CD8^+$ T cells transduced with the 1.5.3-NQ-28-BB-z lentiviral vector and expanded ex vivo were restimulated with irradiated Raji-ffLuc $CD20^+$ lymphoma cells. Secretion of the indicated cytokines was measured in cell supernatants after 24 hours by Luminex assay. (B) Cryopreserved $CD4^+$ and $CD8^+$ CD20 CAR T cells were thawed and restimulated with K562 cells or K562 cells expressing CD20 and at 24 hours were analyzed by intracellular staining for IFN-γ by flow cytometry.

Figures 17A and 17B shows *in vitro* cytotoxicity of various CAR constructs. Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vivo. At day 14, the cells were used as effectors in a standard 4-hour ^{51}Cr-release assay, using (Figs. 17A and 17B) Raji-ffLuc, and (Fig. 17B) Jeko cells as targets. The "19-BB-z" construct is a clinical-grade CD19-targeted CAR being used in clinical trials and is provided as a positive control. The specific target cell lysis of each CAR T cell population is shown.

Figures 18A and 18B show proliferation of CD20 CAR T cells. $CD8^{+}$ T cells were transduced with the 1.5.3-NQ-28-BB-z lentiviral vector (or were mock-transduced) and expanded ex vivo, and then cryopreserved. The cells were then thawed, stained with carboxyfluorescein succinamidyl ester (CFSE), and restimulated with irradiated $CD20^{+}$ Raji-ffLuc lymphoma cells, K562 cells, or K562 cells expressing CD20. Cells were analyzed by flow cytometry 4 days later. (A) CFSE dilution of CAR^{+} cells (gated on $CD3^{+}$/$tCD19^{+}$) is shown. The dashed-line histogram shows CFSE fluorescence of T cells in culture medium only, and solid-line histograms are T cells co-incubated with target cells. (B) The percentage of divided cells is shown for each group.

Figures 19A and 19B show *in vivo* anti-tumor activity of various CAR constructs. Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vitro. The "19-BB-z" construct is a clinical-grade CD19-targeted CAR being used in clinical trials at our center and provided as a benchmark control. NSG mice were injected i.v. with Raji-ffLuc tumor cells, followed 2 days later by i.v. injection of expanded central memory ($CD14^{-}$ $CD45RA^{-}CD62L^{+}$) T cells transduced with the 1.5.3-NQ-28-BB-z CAR, 1.5.3-NQ-28-z CAR, JCAR-014 (anti-CD19-41BB-ζ), or an empty vector. (A) Tumor burden over time as assessed by bioluminescence imaging; and (B) Kaplan-Meier plot of overall survival.

Figure 20 shows *in vivo* activity of CD20 CAR T cells against mantle cell lymphoma. $CD4^{+}$ and $CD8^{+}$ CD20 CAR T cells were transduced with the 1.5.3-NQ-28-BBz CAR and used to treat NSG mice that had been inoculated 7 days earlier with

Granta-ffLuc mantle cell lymphoma cells by tail vein. Kaplan-Meier plot of overall survival.

Figures 21A and 21B show *in vivo* CAR T cell persistence. Retroorbital blood samples were obtained at serial time points after infusion of either CD20 CAR T cells or empty vector tCD19-expressing T cells in NSG mice bearing Raji-ffLuc disseminated tumors. CD20 CAR T cells expressing the tCD19 transduction marker were quantified by flow cytometry at each time point as human $CD3^+$/mouse CD45-negative/human $CD19^+$ cells. (Fig. 21A) $tCD19^+$ T cells at 3 post-infusion time points as a percentage of total nucleated cells in the blood are shown (n=9 initially in CAR T cell group). Truncated $CD19^+$ cells from an empty vector mouse are shown for reference. (Fig. 21B) In a separate experiment, the $tCD19^+$ cells from 2 mice in each group (empty vector vs CAR T cells) are shown longitudinally with weekly measurements.

Figures 22A and 22B show comparative data for various constructs having spacers of varying lengths. Central memory ($CD14^-CD45RA^-CD62L^+$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vitro. The 1F5-28-BB-z, IgG1mut have full-length spacers. The No Linker is nearly full length but missing a 6 amino acid linker (junction amino acid), and the CH3 has a truncated spacer, missing the junction amino acids and CH2 domain. (A) On day 20, the cells were re-stimulated with Granta or Rec-1 lymphoma cells, and 24 hours later supernatants were harvested and analyzed by Luminex assay for IL-2 (right) and IFN-γ (left) concentrations. (B) Central memory T cells ($CD14^-CD45RA^-CD62L^+$) were stimulated with anti-CD3/anti-CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded *in vitro*. The "IgG1mut NQ" has a full length CH2CH3 spacer, CH3 only is intermediate length as discussed above, and Leu16 short lacks both CH2 and CH3 domains. On day 20, cells were used as effector cells in a standard 4-hour ^{51}Cr-release assay, using Raji cells as targets.

Figures 23A-23F show comparative data for various constructs having spacers with various modifications. A schematic diagram of a CAR with IgG2 junction amino acids (denoted "IgG1mut") and N297Q (denoted "NQ") mutations is shown (Fig. 23A). T cells expressing CARs with a wild-type IgG1 spacer, IgG1 mutant spacer (IgG1 junction amino acids replaced with IgG2 junction amino acids), or no junction amino acids (IgG1 junction amino acids deleted) were stained with biotinylated soluble CD64 (FcγRI) followed by streptavidin-PE and then analyzed by flow cytometry, demonstrating Fc receptor binding to wild-type but not modified spacers (Fig. 23B). T cells expressing the indicated CAR constructs were co-incubated with K562 cells expressing CD64 (FcγRI) or parental K562 lacking Fc receptors. At 24 hours after co-incubation the T cells were evaluated for CD25 and CD69 expression by flow cytometry as an indication of activation. Dot plots represent $CD3^{+}CD19^{+}$ cells (CAR^{+} T cells). Binding of wild type spacers to Fc receptors led to T cell activation whereas modified spacers did not (Fig. 23C). Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, expanded in vitro, and injected into NSG mice 2 days after i.v. administration of Raji-ffLuc cells. (D and E) Tumor burden data by bioluminescence for two different experiments. (F) Kaplan-Meier survival curve from experiment in part (E) above.

Figure 24 shows a diagram of a treatment schema for a clinical trial involving immunotherapy methods and compositions of the present disclosure.

Figures 25A and 25B show a diagram of a method of formulation and model of administration of anti-CD20 CAR T cells in a clinical trial.

DETAILED DESCRIPTION

The instant disclosure provides compositions and methods for reducing the number of CD20-expressing cells or treating a disease or disorder associated with CD20 expression (*e.g.*, reducing the number of B-cells or treating a disease or disorder associated with aberrant B cell activity), comprising treating a subject with a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected to the extracellular component by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain. Optionally, the method may further comprise a therapeutically effective amount of a CD20-specific binding molecule in combination with the host cell expressing the fusion protein specific for CD20. In certain embodiments, the fusion protein is a chimeric antigen receptor (CAR). In still further embodiments, the CAR comprises a scFv from an anti-CD20 antibody or a scTCR from a TCR specific for a CD20 antigen.

By way of background, it is generally believed that residual anti-CD20 antibody levels might represent a major constraint for CD20-targeted CAR T cells. For example, previous studies with other targets have demonstrated that cytokine secretion and cytotoxicity of CAR T cells targeting carcinoembryonic antigen, Lewis-Y antigen, or CD30 are largely unimpaired in the presence of levels of soluble cognate antigen of up to 10 µg/ml (Hombach *et al.*, *Gene Ther* 2000;7(12):1067-75; Hombach *et al.*, *Gene Ther* 1999;6(2):300-4; Nolan *et al.*, *Clin Cancer Res* 1999;5(12):3928-41; Westwood *et al.*, *J Immunother* 2009;32(3):292-301); it was observed that levels higher than this are potentially inhibitory (Hombach *et al.*, *Gene Ther* 2000;7(12):1067-75). In this disclosure, it was surprisingly found that various anti-CD20 antibodies (*e.g.*, rituximab) in clinically relevant concentrations largely did not affect the activity of T cells expressing anti-CD20 CARs either *in vitro* or *in vivo* (*see*, *also*, Gall *et al.*, *Exp. Hematol.* 33:452, 2005). Moreover, mouse experiments of this disclosure demonstrate groups receiving a combination therapy had outcomes as good as or better than mice treated with CAR T cells alone.

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein. Additional definitions are set forth throughout this disclosure.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. Also, any number range recited herein relating to any physical feature, such as polymer subunits, size or thickness, are to be understood to include any integer within the recited range, unless otherwise indicated. As used herein, the term "about" means ± 20% of the indicated range, value, or structure, unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

"Optional" or "optionally" means that the subsequently described element, component, event, or circumstance may or may not occur, and that the description includes instances in which the element, component, event, or circumstance occurs and instances in which they do not.

In addition, it should be understood that the individual constructs, or groups of constructs, derived from the various combinations of the structures and subunits described herein, are disclosed by the present application to the same extent as if each construct or group of constructs was set forth individually. Thus, selection of particular structures or particular subunit is within the scope of the present disclosure.

The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, or to those that do not materially affect the basic characteristics of a claimed invention. For example, a protein domain, region, module, or fragment (*e.g.*, a binding domain, hinge region, linker module, or tag) or a protein (which may have one or more domains, regions, or modules) "consists essentially of" a particular amino acid sequence when the amino acid sequence of a domain, region, module, fragment, or protein includes insertions, deletions, substitutions, or a combination thereof (*e.g.*, addition of amino acids at the amino- or carboxy-terminus, or between domains) that, in combination, contribute to at most 20% (*e.g.*, at most 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% or 1%) of the length of a domain, region, module, cassette or protein and do not substantially affect (*i.e.*, do not reduce the activity by more than 50%, such as no more than 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 1%) the activity of the domain(s), region(s), module(s), cassette(s), or protein (*e.g.*, the target binding affinity of a binding domain).

As used herein, "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

A "conservative substitution" refers to amino acid substitutions that do not significantly affect or alter binding characteristics of a particular protein. Generally, conservative substitutions are ones in which a substituted amino acid residue is replaced with an amino acid residue having a similar side chain. Conservative substitutions include a substitution found in one of the following groups: Group 1: Alanine (Ala or A), Glycine (Gly or G), Serine (Ser or S), Threonine (Thr or T); Group 2: Aspartic acid (Asp or D), Glutamic acid (Glu or Z); Group 3: Asparagine (Asn or N), Glutamine (Gln or Q); Group 4: Arginine (Arg or R), Lysine (Lys or K), Histidine (His or H); Group 5: Isoleucine (Ile or I), Leucine (Leu or L), Methionine (Met or M), Valine (Val or V); and Group 6: Phenylalanine (Phe or F), Tyrosine (Tyr or Y), Tryptophan (Trp or W). Additionally or alternatively, amino acids can be grouped into conservative substitution groups by similar function, chemical structure, or composition (*e.g.,* acidic, basic, aliphatic, aromatic, or sulfur-containing). For example, an aliphatic grouping may include, for purposes of substitution, Gly, Ala, Val, Leu, and Ile. Other conservative substitutions groups include: sulfur-containing: Met and Cysteine (Cys or C); acidic: Asp, Glu, Asn, and Gln; small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro, and Gly; polar, negatively charged residues and their amides: Asp, Asn, Glu, and Gln; polar, positively charged residues: His, Arg, and Lys; large aliphatic, nonpolar residues: Met, Leu, Ile, Val, and Cys; and large aromatic residues: Phe, Tyr, and Trp. Additional information can be found in Creighton (1984) Proteins, W.H. Freeman and Company.

As used herein, "protein" or "polypeptide refers to a polymer of amino acid residues. Proteins apply to naturally occurring amino acid polymers, as well as to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid and non-naturally occurring amino acid polymers.

"Percent sequence identity" refers to a relationship between two or more sequences, as determined by comparing the sequences. Preferred methods to determine sequence identity are designed to give the best match between the sequences being compared. For example, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment). Further, non-homologous sequences may be disregarded for comparison purposes. The percent sequence identity referenced herein is calculated over the length of the reference sequence, unless indicated otherwise. Methods to determine sequence identity and similarity can be found in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using a BLAST program (e.g., BLAST 2.0, BLASTP, BLASTN, or BLASTX). The mathematical algorithm used in the BLAST programs can be found in Altschul *et al.*, Nucleic Acids Res. 25:3389-3402, 1997. Within the context of this disclosure, it will be understood that where sequence analysis software is used for analysis, the results of the analysis are based on the "default values" of the program referenced. "Default values" mean any set of values or parameters which originally load with the software when first initialized.

"Nucleic acid molecule" or "polynucleotide" refers to a polymeric compound including covalently linked nucleotides, which can be made up of natural subunits (*e.g.*, purine or pyrimidine bases) or non-natural subunits (*e.g.*, morpholine ring). Purine bases include adenine, guanine, hypoxanthine, and xanthine, and pyrimidine bases include uracil, thymine, and cytosine. Nucleic acid molecules include polyribonucleic acid (RNA), polydeoxyribonucleic acid (DNA), which includes cDNA, genomic DNA, and synthetic DNA, either of which may be single or double stranded. If single stranded, the nucleic acid molecule may be the coding strand or non-coding (anti-sense strand). A nucleic acid molecule encoding an amino acid sequence includes all nucleotide sequences that encode the same amino acid sequence. Some versions of the nucleotide sequences may also include intron(s) to the extent that the intron(s) would be removed through co- or post-transcriptional mechanisms. In other words, different nucleotide sequences may encode the same amino acid sequence as the result of the redundancy or degeneracy of the genetic code, or by splicing.

Variants of nucleic acid molecules of this disclosure are also contemplated. Variant nucleic acid molecules are at least 70%, 75%, 80%, 85%, 90%, and preferably 95%, 96%, 97%, 98%, 99%, or 99.9% identical a nucleic acid molecule of a defined or reference polynucleotide as described herein, or that hybridizes to a polynucleotide under stringent hybridization conditions of 0.015M sodium chloride, 0.0015M sodium citrate at about 65-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C. Nucleic acid molecule variants retain the capacity to encode a fusion protein or a binding domain thereof having a functionality described herein, such as specifically binding a target molecule (*e.g.*, CD20).

A "functional variant" refers to a polypeptide or polynucleotide that is structurally similar or substantially structurally similar to a parent or reference compound of this disclosure, but differs slightly in composition (*e.g.*, one base, atom or functional group is different, added, or removed), such that the polypeptide or encoded polypeptide is capable of performing at least one function of the encoded parent polypeptide with at least 50% efficiency, preferably at least 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 100% level of activity of the parent polypeptide. In other words, a functional variant of a polypeptide or encoded polypeptide of this disclosure has "similar binding," "similar affinity" or "similar activity" when the functional variant displays no more than a 50% reduction in performance in a selected assay as compared to the parent or reference polypeptide, such as an assay for measuring binding affinity (*e.g.*, Biacore® or tetramer staining measuring an association (K_a) or a dissociation (K_D) constant).

As used herein, a "functional portion" or "functional fragment" refers to a polypeptide or polynucleotide that comprises only a domain, portion or fragment of a parent or reference compound, and the polypeptide or encoded polypeptide retains at least 50% activity associated with the domain, portion or fragment of the parent or reference compound, preferably at least 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 100% level of activity of the parent polypeptide, or provides a biological benefit (*e.g.*, effector function). A "functional portion" or "functional fragment" of a polypeptide or encoded polypeptide of this disclosure has "similar binding" or "similar activity" when the functional portion or fragment displays no more than a 50% reduction in performance in a selected assay as compared to the parent or reference polypeptide (preferably no more than 20% or 10%, or no more than a log difference as compared to the parent or reference with regard to affinity), such as an assay for measuring binding affinity or measuring effector function (*e.g.*, cytokine release).

As used herein, "heterologous" or "non-endogenous" or "exogenous" refers to any gene, protein, compound, nucleic acid molecule, or activity that is not native to a host cell or a subject, or any gene, protein, compound, nucleic acid molecule, or activity native to a host cell or a subject that has been altered. Heterologous, non-endogenous, or exogenous includes genes, proteins, compounds, or nucleic acid molecules that have been mutated or otherwise altered such that the structure, activity, or both is different as between the native and altered genes, proteins, compounds, or nucleic acid molecules. In certain embodiments, heterologous, non-endogenous, or exogenous genes, proteins, or nucleic acid molecules (*e.g.*, receptors, ligands, etc.) may not be endogenous to a host cell or a subject, but instead nucleic acids encoding such genes, proteins, or nucleic acid molecules may have been added to a host cell by conjugation, transformation, transfection, electroporation, or the like, wherein the added nucleic acid molecule may integrate into a host cell genome or can exist as extra-chromosomal genetic material (*e.g.*, as a plasmid or other self-replicating vector). The term "homologous" or "homolog" refers to a gene, protein, compound, nucleic acid molecule, or activity found in or derived from a host cell, species, or strain. For example, a heterologous or exogenous polynucleotide orgene encoding a polypeptide may be homologous to a native polynucleotide or gene and encode a homologous polypeptide or activity, but the polynucleotide or polypeptide may have an altered structure, sequence, expression level, or any combination thereof. A non-endogenous polynucleotide or gene, as well as the encoded polypeptide or activity, may be from the same species, a different species, or a combination thereof.

As used herein, the term "endogenous" or "native" refers to a polynucleotide, gene, protein, compound, molecule, or activity that is normally present in a host cell or a subject.

"Expression" refers to transcription or translation of a nucleic acid molecule that is operably linked to an expression control sequence (*e.g.*, promoter).

As used herein, the term "engineered," "recombinant" or "non-natural" refers to an organism, microorganism, cell, nucleic acid molecule, or vector that includes at least one genetic alteration or has been modified by introduction of an exogenous nucleic acid molecule, wherein such alterations or modifications are introduced by genetic engineering (*i.e.*, human intervention). Genetic alterations include, for example, modifications introducing expressible nucleic acid molecules encoding proteins, fusion proteins or enzymes, or other nucleic acid molecule additions, deletions, substitutions or other functional disruption of a cell's genetic material. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a polynucleotide, gene or operon.

As used herein, a "fusion protein" refers to a protein that, in a single chain, has at least two distinct domains, wherein the domains are not naturally found together in a protein. A polynucleotide encoding a fusion protein may be constructed using PCR, recombinantly engineered, or the like, or such fusion proteins can be synthesized. A fusion protein may further contain other components, such as a tag, a linker module or a transduction marker. In certain embodiments, a fusion protein expressed or produced by a host cell (*e.g.*, a T cell) locates to a cell surface, where the fusion protein is anchored to the cell membrane (*e.g.*, via a transmembrane domain) and comprises an extracellular portion (*e.g.*, containing a binding domain) and an intracellular portion (*e.g.*, containing a signaling domain, effector domain, co-stimulatory domain or combinations thereof).

Terms understood by those in the art of antibody technology are each given the meaning acquired in the art, unless expressly defined differently herein. The term "antibody" refers to an intact antibody comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as an antigen-binding portion of an intact antibody that has or retains the capacity to bind a target molecule. Antibodies include polyclonal and monoclonal antibodies. An antibody may be naturally occurring, recombinantly produced, genetically engineered, or modified, and includes modified forms of immunoglobulins, such as, for example intrabodies, peptibodies, nanobodies, single domain antibodies, multispecific antibodies (*e.g.*, bispecific antibodies, diabodies, triabodies, tetrabodies, tandem di-scFV, tandem tri-scFv). "Antigen-binding portion," "antigen-binding fragment" or "antigen-binding domain" from an antibody refers to an "antibody fragment" that comprises a portion of an intact antibody and contains the antigenic determining variable regions or complementary determining regions of an antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments, Fab'-SH, $F(ab')_2$, diabodies, linear antibodies, single chain antibodies, scFv (*i.e.*, a fusion protein of the variable heavy (VH) and variable light (VL) regions of an Ig molecule, connected with a short linked peptide of generally about 10 to about 25 amino acids), VHH, single domain antibodies (*e.g.*, sdAb, sdFv, nanobody), and multispecific antibodies comprising antibody fragments. A monoclonal antibody or antigen-binding portion thereof may be non-human, chimeric, humanized, or human, preferably humanized or human. Immunoglobulin structure and function are reviewed, for example, in Harlow *et al.*, Eds., Antibodies: A Laboratory Manual, Chapter 14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988). An antibody may be of any class or subclass, including IgG and subclasses thereof (IgG_1, IgG_2, IgG_3, IgG_4), IgM, IgE, IgA, and IgD.

The terms "V_L" and "V_H" refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of discrete, well-defined sub-regions known as "complementarity determining regions" (CDRs) and "framework regions" (FRs).

The terms "complementarity determining region" (CDR) or "hypervariable region" (HVR) are known in the art to refer to non-contiguous sequences of amino acids within antibody variable regions, which confer antigen specificity or binding affinity. In general, there are three CDRs in each heavy chain variable region (HCDR1, HCDR2, and HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, and LCDR3).

"Framework regions" (FR) as used herein refer to the non-CDR portions of the variable regions of the heavy and light chains. In general, there are four FRs in each full-length heavy chain variable region (FR-Hl, FR-H2, FR-H3, and FR-H4), and four FRs in each full-length light chain variable region (FR-L1, FR-L2, FR-L3, and FR-L4).

The term "CL" refers to an "immunoglobulin light chain constant region" or a "light chain constant region," *i.e.*, a constant region from an antibody light chain. The term "CH" refers to an "immunoglobulin heavy chain constant region" or a "heavy chain constant region," which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM).

A "fragment antigen binding" (Fab) region is a part of an antibody that binds to antigens, and includes the variable region and CH1 of the heavy chain linked to the light chain via an inter-chain disulfide bond. A "fragment crystallizable" (Fc) region is a part of an antibody that is not a Fab region, and includes the CH regions other than CH1 (*e.g.*, CH2 and CH3 of an IgG, IgA, or IgD antibody, or CH2, CH3, and CH4 of an IgE antibody). By way of background, an Fc region is responsible for the effector functions of an immunoglobulin, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and complement fixation, binding to Fc receptors (*e.g.*, CD16, CD32, FcRn), greater half-life *in vivo* relative to a polypeptide lacking an Fc region, protein A binding, and perhaps even placental transfer (*see* Capon *et al.*, *Nature 337*:525, 1989).

As used herein, "Fc region portion" refers to the heavy chain constant region segment of an Fc fragment from an antibody, which can include one or more constant domains, such as CH2, CH3, CH4, or any combination thereof. In certain embodiments, an Fc region portion includes the CH2 and CH3 domains of an IgG, IgA, or IgD antibody or any combination thereof, or the CH2, CH3, and CH4 domains of an IgM or IgE antibody and any combination thereof. In other embodiments, a CH2CH3 or a CH3CH4 structure has sub-region domains from the same antibody isotype and are human, such as human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM (*e.g.*, CH2CH3 from human IgG1 or IgG4). In certain embodiments, an Fc region portion found in fusion proteins of the present disclosure will be capable of mediating one or more of effector functions of an immunoglobulin, will be capable of mediating one or more enhanced effector functions, or will lack one or more or all of these activities by way of, for example, one or more mutations known in the art.

In addition, antibodies have a hinge sequence that is typically situated between the Fab and Fc region (but a lower section of the hinge may include an amino-terminal portion of the Fc region). By way of background, an immunoglobulin hinge acts as a flexible spacer to allow the Fab region to move freely in space. In contrast to the constant regions, hinges are structurally diverse, varying in both sequence and length between immunoglobulin classes and even among subclasses. For example, a human IgG1 hinge region is freely flexible, which allows the Fab regions to rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. By comparison, a human IgG2 hinge is relatively short and contains a rigid poly-proline double helix stabilized by four inter-heavy chain disulfide bridges, which restricts the flexibility. A human IgG3 hinge differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix and providing greater flexibility because the Fab regions are relatively far away from the Fc region. A human IgG4 hinge is shorter than IgG1 but has the same length as IgG2, and its flexibility is intermediate between that of IgG1 and IgG2.

A "T cell" is an immune system cell that matures in the thymus and produces T cell receptors (TCRs). T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cells) and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced $CD8^+$ cytotoxic T lymphocytes that have decreased expression of CD62L,CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. T helper cells (T_H) release cytokines to aid in antigen signaling and, when mature, express the surface protein CD4 (are $CD4^+$)As used herein, "T cells" or "T lymphocytes" are from any mammal, including primates, dogs, or horses, preferably humans. In some embodiments, T cells are autologous, allogeneic, or syngeneic.

"T cell receptor" (TCR) refers to a molecule found on the surface of T cells (or T lymphocytes) that, in association with CD3, is generally responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. The TCR has a disulfide-linked heterodimer of the highly variable α and β chains (also known as TCRα and TCRβ, respectively) in most T cells. In a small subset of T cells, the TCR is made up of a heterodimer of variable γ and δ chains (also known as TCRγ and TCRδ, respectively). Each chain of the TCR is a member of the immunoglobulin superfamily and possesses one N-terminal immunoglobulin variable domain, one immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail at the C-terminal end (*see,* Janeway *et al.*, *Immunobiology: The Immune System in Health and Disease*, 3rd Ed., Current Biology Publications, p. 4:33, 1997). TCR, as used in the present disclosure, may be from various animal species, including human, mouse, rat, cat, dog, goat, horse, or other mammals. TCRs may be cell-bound (*i.e.*, have a transmembrane region or domain) or in soluble form. As discussed herein, a binding domain according to the present disclosure may comprise a single-chain TCR (scTCR), which is analogous to an scFv derived from an immunoglobulin and comprises the variable domains from TCRα and TCRβ chains linked together using, *e.g.*, a peptide or non-peptide linker and optionally through disulfide bonding.

"Major histocompatibility complex molecules" (MHC molecules) refer to glycoproteins that deliver peptide antigens to a cell surface. MHC class I molecules are heterodimers consisting of a membrane spanning α chain (with three α domains) and a non-covalently associated β2 microglobulin. MHC class II molecules are composed of two transmembrane glycoproteins, α and β, both of which span the membrane. Each chain has two domains. MHC class I molecules deliver peptides originating in the cytosol to the cell surface, where a peptide:MHC complex is recognized by $CD8^+$ T cells. MHC class II molecules deliver peptides originating in the vesicular system to the cell surface, where they are recognized by $CD4^+$ T cells. An MHC molecule may be from various animal species, including human, mouse, rat, cat, dog, goat, horse, or other mammals.

"Cells of T cell lineage" refer to cells that show at least one phenotypic characteristic of a T cell, or a precursor or progenitor thereof that distinguishes the cells from other lymphoid cells, and cells of the erythroid or myeloid lineages. Such phenotypic characteristics can include expression of one or more proteins specific for T cells (*e.g.*, $CD3^+$, $CD4^+$, $CD8^+$), or a physiological, morphological, functional, or immunological feature specific for a T cell. For example, cells of the T cell lineage may be progenitor or precursor cells committed to the T cell lineage; $CD25^+$ immature and inactivated T cells; cells that have undergone CD4 or CD8 linage commitment; thymocyte progenitor cells that are $CD4^+CD8^+$ double positive; single positive $CD4^+$ or $CD8^+$; TCRαβ or TCR γδ; or mature and functional or activated T cells.

As used herein, "enriched" or "depleted" with respect to amounts of cell types in a mixture refers to an increase in the number of the "enriched" type, a decrease in the number of the "depleted" cells, or both, in a mixture of cells resulting from one or more enriching or depleting processes or steps. Thus, depending upon the source of an original population of cells subjected to an enriching process, a mixture or composition may contain 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more (in number or count) of the "enriched" cells. Cells subjected to a depleting process can result in a mixture or composition containing 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% percent or less (in number or count) of the "depleted" cells. In certain embodiments, amounts of a certain cell type in a mixture will be enriched and amounts of a different cell type will be depleted, such as enriching for $CD4^+$ cells while depleting $CD8^+$ cells, or enriching for $CD62L^+$ cells while depleting $CD62L^-$ cells, or combinations thereof.

"Treat" or "treatment" or "ameliorate" refers to medical management of a disease, disorder, or condition of a subject (*e.g.*, a human or non-human mammal, such as a primate, horse, cat, dog, goat, mouse, or rat). In general, an appropriate dose or treatment regimen comprising a host cell expressing a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain of this disclosure, and optionally an adjuvant, is administered in an amount sufficient to elicit a therapeutic or prophylactic benefit. Therapeutic or prophylactic/preventive benefit includes improved clinical outcome; lessening or alleviation of symptoms associated with a disease; decreased occurrence of symptoms; improved quality of life; longer disease-free status; diminishment of extent of disease, stabilization of disease state;

delay of disease progression; remission; survival; prolonged survival; or any combination thereof. As further described herein, a treatment regimen may comprise a combination therapy in which one or more CD20-specific binding molecules, such as, for example, anti-CD20 antibodies are administered prior to, simultaneous with, contemporaneous with, or subsequent to administration of one or more second or adjunctive therapeutic. Exemplary anti-CD20 antibodies suitable for use in the therapeutic methods described herein include 1.5.3, 1F5, Leu16, rituximab, ofatumumab, veltuzumab, ublituximab, and ocrelizumab.

A "therapeutically effective amount" or "effective amount" of a CD20-specific binding molecule, a fusion protein, or host cell expressing a fusion protein of this disclosure (*e.g.*, CD20 CAR) refers to an amount of CD20-specific binding molecules, fusion proteins, or host cells sufficient to result in a therapeutic effect, including improved clinical outcome; lessening or alleviation of symptoms associated with a disease; decreased occurrence of symptoms; improved quality of life; longer disease-free status; diminishment of extent of disease, stabilization of disease state; delay of disease progression; remission; survival; or prolonged survival in a statistically significant manner. When referring to an individual active ingredient or a cell expressing a single active ingredient, administered alone, a therapeutically effective amount refers to the effects of that ingredient or cell expressing that ingredient alone. When referring to a combination, a therapeutically effective amount refers to the combined amounts of active ingredients or combined adjunctive active ingredient with a cell expressing an active ingredient that results in a therapeutic effect, whether administered serially or simultaneously. A combination may also be a cell expressing more than one active ingredient, such as two different CD20 CARs, or one CD20 CAR and CD20 TCR, or CD20 CAR and another relevant therapeutic.

The term "pharmaceutically acceptable excipient or carrier" or "physiologically acceptable excipient or carrier" refer to biologically compatible vehicles, *e.g.*, physiological saline, which are described in greater detail herein, that are suitable for administration to a human or other non-human mammalian subject and generally recognized as safe or not causing a serious adverse event.

As used herein, "statistically significant" refers to a p value of 0.050 or less when calculated using the Students t-test and indicates that it is unlikely that a particular event or result being measured has arisen by chance.

As used herein, the term "adoptive immune therapy" or "adoptive immunotherapy" refers to administration of naturally occurring or genetically engineered, disease antigen-specific immune cells (e.g., T cells). Adoptive cellular immunotherapy may be autologous (immune cells are from the recipient), allogeneic (immune cells are from a donor of the same species) or syngeneic (immune cells are from a donor genetically identical to the recipient).

Fusion Proteins

In certain aspects, the present disclosure provides fusion proteins comprising an extracellular component and an intracellular component connected by a hydrophobic portion.

An "extracellular component" comprises a binding domain that specifically binds CD20. A "binding domain" (also referred to as a "binding region" or "binding moiety"), as used herein, refers to a molecule, such as a peptide, oligopeptide, polypeptide, or protein that possesses the ability to specifically and non-covalently associate, unite, or combine with a target molecule (*e.g.*, CD20). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced binding partner for a biological molecule or other target of interest. In some embodiments, a binding domain is an antigen-binding domain, such as an antibody or TCR, or functional binding domain or antigen-binding fragment thereof.

In certain embodiments, a binding domain comprises a variable region linker (*e.g.*, scFv). A "variable region linker" specifically refers to a five amino acid to about 35 amino acid sequence that connects a heavy chain immunoglobulin variable region (VH) to a light chain immunoglobulin variable region (VL), or connects TCR $V_{\alpha/\beta}$ and $C_{\alpha/\beta}$ chains (*e.g.*, V_α-C_α, V_β-C_β, V_α-V_β) or connects each V_α-C_α, V_β-C_β, or V_α-V_β pair to a hinge or hydrophobic domain, which provides a spacer function and flexibility sufficient for interaction of the two sub-binding domains so that the resulting single chain polypeptide retains a specific binding affinity to the same target molecule as an antibody or TCR.

In certain embodiments, a variable region linker comprises from about ten amino acids to about 30 amino acids or from about 15 amino acids to about 25 amino acids. In particular embodiments, a variable region linker peptide comprises from one to ten repeats of Gly_xSer_y, wherein x and y are independently an integer from 0 to 10, provided that x and y are not both 0 (*e.g.*, Gly_4Ser), Gly_3Ser, Gly_2Ser, or $(Gly_3Ser)_n(Gly_4Ser)_1$, $(Gly_3Ser)_n(Gly_2Ser)_n$, $(Gly_3Ser)_n(Gly_4Ser)_n$, or $(Gly_4Ser)_n$, wherein n is an integer of 1, 2, 3, 4, 5, or 6) and wherein linked variable regions form a functional immunoglobulin-like binding domain (*e.g.*, scFv or scTCR).

Exemplary binding domains include single chain antibody variable regions (*e.g.*, domain antibodies, sFv, scFv, or Fab), antigen-binding regions of TCRs, such as single chain TCRs (scTCRs), or synthetic polypeptides selected for the specific ability to bind to a biological molecule.

As used herein, "specifically binds" refers to an association or union of a binding domain, or a fusion protein thereof, to a target molecule with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1}, while not significantly associating or uniting with any other molecules or components in a sample. Binding domains (or fusion proteins thereof) may be classified as "high affinity" binding domains (or fusion proteins thereof) or "low affinity" binding domains (or fusion proteins thereof). "High affinity" binding domains (or fusion proteins thereof) refer to those binding domains (or fusion proteins thereof) with a K_a of at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 10^{12} M^{-1}, or at least 10^{13} M^{-1}. "Low affinity" binding domains (or fusion proteins thereof) refer to those binding domains (or fusion proteins thereof) with a K_a of up to 10^7 M^{-1}, up to 10^6 M^{-1}, up to 10^5 M^{-1}.

Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, 10^{-5} M to 10^{-13} M). In certain embodiments, a binding domain may have "enhanced affinity," which refers to a selected or engineered binding domain with stronger binding to a target antigen than a wild type (or parent) binding domain. For example, enhanced affinity may be due to a K_a (equilibrium association constant) for the target antigen that is greater than the wild type binding domain, due to a K_d (dissociation constant) for the target antigen that is less than that of the wild type binding domain, or due to an off-rate (K_{off}) for the target antigen that is less than that of the wild type binding domain. A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, as well as determining binding domain or fusion protein affinities, such as Western blot, ELISA, and Biacore® analysis (*see also*, *e.g.*, Scatchard *et al*., *Ann. N.Y. Acad. Sci. 51*:660, 1949; and U.S. Patent Nos. 5,283,173, 5,468,614, or an equivalent).

Analysis or computer modeling of the primary and secondary amino acid structure of a binding domain to analyze the tertiary structure of a protein may aid in identifying specific amino acid residues that can be substituted, added, or deleted without significantly altering the structure and as a consequence, potentially significantly reducing the binding specificity and affinity of a binding domain.

In certain embodiments, a binding domain comprises a V_H region. For example, a V_H region in a binding domain of the present disclosure can be derived from or based on a V_H of a known monoclonal antibody and may contain one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) insertions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) deletions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared with the V_H of a known monoclonal antibody. An insertion, deletion, or substitution may be anywhere in the V_H region, including at the amino-terminus, carboxy-terminus, or both ends of the region, provided that each CDR comprises zero changes or at most one, two, three or four changes from a CDR of the V_H region of a known monoclonal antibody, and provided a binding domain containing the modified V_H region specifically binds its target with an affinity similar to the wild type binding domain.

In certain embodiments, a binding domain comprises a V_L region. For example, a V_L region in a binding domain of the present disclosure is derived from or based on a V_L of a known monoclonal antibody and may contain one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) insertions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) deletions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions), or a combination of the above-noted changes, when compared with the V_L of a known monoclonal antibody. An insertion, deletion, or substitution may be anywhere in the V_L region, including at the amino-terminus, carboxy-terminus, or both ends of the region, provided that each CDR comprises zero changes or at most one, two, three or four changes from a CDR of the V_L region of a known monoclonal antibody, and provided a binding domain containing the modified V_L region specifically binds its target with an affinity similar to the wild type binding domain.

In certain embodiments, a binding domain comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a light chain variable region (V_L); *e.g.*, to a V_L from 1.5.3 (SEQ ID NO.:1), 1F5 (SEQ ID NO.:3), Leu16 (SEQ ID NO.:2), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab.

In further embodiments, a binding domain comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a heavy chain variable region (V_H); *e.g.*, to a V_H from 1.5.3 (SEQ ID NO.:4), 1F5 (SEQ ID NO.:6), Leu16 (SEQ ID NO.:5), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab.

In still further embodiments, a binding domain comprises (a) an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a V_L; *e.g.*, to a V_L from 1.5.3 (SEQ ID NO.:1), 1F5 (SEQ ID NO.:3), Leu16 (SEQ ID NO.:2), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab; and (b) an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a V_H; *e.g.*, to a V_H from 1.5.3 (SEQ ID NO.:4), 1F5 (SEQ ID NO.:6), Leu16 (SEQ ID NO.:5), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab. In any of the aforementioned embodiments, each CDR of the V_L, V_H, or both comprises zero changes or at most one, two, three, four, five or six changes, as compared to a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that a binding domain containing the modified V_L, V_H, or both region specifically binds CD20 with an affinity similar to the wild type binding domain.

In certain embodiments, a binding domain comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a scFv, *e.g.*, a scFv from an antibody of 1.5.3 (SEQ ID NO.:64), 1F5 (SEQ ID NO.:66), Leu16 (SEQ ID NO.:65), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes, as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody.

In certain embodiments, a binding domain is encoded by a polynucleotide that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a polynucleotide sequence encoding a light chain variable region (V_L); *e.g.*, to a V_L–encoding polynucleotide from 1.5.3 (SEQ ID NO.:70), 1F5 (SEQ ID NO.:72), Leu16 (SEQ ID NO.:71), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab.

In further embodiments, a binding domain comprises a polynucleotide that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a polynucleotide sequence encoding a heavy chain variable region (V_H); *e.g.*, to a V_H–encoding polynucleotide from 1.5.3 (SEQ ID NO.:73), 1F5 (SEQ ID NO.:75), Leu16 (SEQ ID NO.:74), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab.

In still further embodiments, a binding domain comprises (a) a polynucleotide that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a polynucleotide sequence encoding a V_L; *e.g.*, to a V_L-encoding polynucleotide from 1.5.3 (SEQ ID NO.:70), 1F5 (SEQ ID NO.:72), Leu16 (SEQ ID NO.:71), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab; and (b) a polynucleotide that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a polynucleotide sequence encoding a V_H; *e.g.*, to a V_H-encoding polynucleotide from 1.5.3 (SEQ ID NO.:73), 1F5 (SEQ ID NO.:75), Leu16 (SEQ ID NO.:74), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab. In any of the aforementioned embodiments, polynucleotides encoding each CDR of the V_L, V_H, or both comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that a binding domain containing the modified V_L, V_H, or both regions specifically binds CD20 with an affinity similar to the wild type binding domain.

In certain embodiments, a binding domain comprises a polynucleotide that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a polynucleotide sequence encoding a scFv, *e.g.*, an encoded scFv comprising variable domains from an antibody of 1.5.3 (SEQ ID NO.:67), 1F5 (SEQ ID NO.:69), Leu16 (SEQ ID NO.:68), rituximab, ofatumumab, veltuzumab, ublituximab, or ocrelizumab. In each of the aforementioned embodiments, polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody.

In any of the embodiments described herein, a binding domain may consist, comprise, be based on or be derived from a VH, a VL, or both, from ublituximab (*see, e.g.*, US 2015/0290317), rituximab (*see*, *e.g.*, US 2014/0004037), ocrelizumab (*see*, *e.g.*, US 8,679,767), ofatumumab (*see*, *e.g.*, US 2009/0169550), or veltuzumab (*see*, *e.g.*, US 2009/0169550), the nucleotide and amino acid sequences of which are herein incorporated by reference in their entirety. Additionally, in any of the methods described herein, a CD20 binding molecule may comprise rituximab, ofatumumab, veltuzumab, or ocrelizumab, ublituximab, or any combination thereof.

A fusion protein of the present disclosure comprises an intracellular component that comprises an effector domain. As used herein, an "effector domain" is an intracellular portion or domain of a fusion protein or receptor that can directly or indirectly promote a biological or physiological response in a cell when receiving an appropriate signal. In certain embodiments, an effector domain is from or a portion of a protein or protein complex that receives a signal when bound, or when the protein or portion thereof or protein complex binds directly to a target molecule, and triggers a signal from the effector domain. An effector domain may directly promote a cellular response when it contains one or more signaling domains or motifs, such as an immunoreceptor tyrosine-based activation motif (ITAM), as found in costimulatory molecules. A costimulatory molecule or portion thereof comprising ITAMs are generally known to be capable of initiating T cell activation signaling following ligand engagement. In further embodiments, an effector domain will indirectly promote a cellular response by associating with one or more other proteins that directly promote a cellular response.

In certain embodiments, an effector domain comprises a lymphocyte receptor signaling domain (*e.g.*, CD3ζ), comprises a polypeptide having one or more ITAMs from a costimulatory molecule (*e.g.*, CD28, 4-1BB (CD137), OX40 (CD134)), or combinations thereof. In still further embodiments, an effector domain comprises a cytoplasmic portion that associates with a cytoplasmic signaling protein, wherein the cytoplasmic signaling protein is a lymphocyte receptor or signaling domain thereof, a protein comprising a plurality of ITAMs, a costimulatory factor, or any combination thereof.

Exemplary effector domains include those from 4-1BB (CD137), CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD79A, CD79B, CARD11, DAP10, FcRα, FcRβ, FcRγ, Fyn, HVEM, ICOS, Lck, LAG3, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, Wnt, OX40 (CD134), ROR2, Ryk, SLAMF1, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

In certain embodiments, an effector domain comprises a portion or domain from costimulatory molecule CD28, which may optionally include a LL→GG mutation at positions 186-187 of the native CD28 protein (SEQ ID NO.:15; *see* Nguyen *et al.*, *Blood 102*:4320, 2003). In further embodiments, an effector domain comprises CD3ζ or a functional portion thereof (SEQ ID NO.:17) and one or more portions or domains from a costimulatory molecule, such as CD28 (SEQ ID NO.:15), 4-1BB (SEQ ID NO.:16), CD27, or OX40. In particular embodiments, an effector domain of a fusion protein of the instant disclosure comprises an effector domains or a functional portion thereof from CD3ζ (SEQ ID NO.:17) and CD28 (SEQ ID NO.:15); CD3ζ (SEQ ID NO.:17) and 4-1BB

(SEQ ID NO.:16); or CD3ζ (SEQ ID NO.:17), CD28 (SEQ ID NO.:15), and 4-1BB (SEQ ID NO.:16).

In certain embodiments, an effector domain comprises CD3ζ or a functional portion thereof, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity with SEQ ID NO.:86. In further embodiments, an effector domain comprises a portion or a domain from costimulatory molecule CD28, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity to SEQ ID NO.:84. In still further embodiments, an effector domain comprises a portion or a domain from costimulatory molecule 4-1BB, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity to SEQ ID NO.:85.

An extracellular domain and an intracellular domain of the present disclosure are connected by a hydrophobic portion. A "hydrophobic portion," as used herein, means any amino acid sequence having a three-dimensional structure that is thermodynamically stable in a cell membrane, and generally ranges in length from about 15 amino acids to about 30 amino acids. The structure of a hydrophobic portion may comprise an alpha helix, a beta barrel, a beta sheet, a beta helix, or any combination thereof. In certain embodiments, a hydrophobic portion is comprised of a "transmembrane domain" from a known transmembrane protein, which is a portion of the transmembrane protein that can insert into or span a cell membrane. In some embodiments, a hydrophobic portion is a transmembrane domain, such as a CD4 transmembrane domain, CD8 transmembrane domain, CD28 (*e.g.*, SEQ ID NO.:14), CD27 transmembrane domain, and 4-1BB transmembrane domain. In certain embodiments, a hydrophobic portion is a CD28 transmembrane domain (SEQ ID NO.:14). In further embodiments, a hydrophobic portion is a CD28 transmembrane domain, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity to SEQ ID NO.:83.

A fusion protein of the present disclosure may further comprise a linker module. A "linker module" may be an amino acid sequence having from about two amino acids to about 500 amino acids, which can provide flexibility and room for conformational movement between two regions, domains, motifs, fragments, or modules connected by a linker. In certain embodiments, a linker module may be located between a binding domain and a hydrophobic region. In such embodiments, a linker module can position a binding domain away from the cell surface to enable proper cell/cell contact, antigen binding, and activation (Patel *et al.*, *Gene Therapy 6*: 412-419, 1999). Linker module length may be varied to maximize tumor recognition based on the selected target molecule, selected binding epitope, or antigen binding domain size and affinity (*see*, *e.g.*, Guest *et al.*, *J. Immunother. 28*:203-11, 2005; PCT Publication No. WO 2014/031687). Exemplary linker modules include those having a glycine-serine (Gly-Ser) linker having from one to about ten repeats of Gly_xSer_y, wherein x and y are independently an integer from 0 to 10, provided that x and y are not both 0 (*e.g.*, $(Gly_4Ser)_2$, $(Gly_3Ser)_2$, Gly_2Ser, or a combination thereof, such as $(Gly_3Ser)_2Gly_2Ser$). In certain embodiments, a linker module comprises one or more immunoglobulin heavy chain constant regions, such as a CH3 alone, or a CH2CH3 structure, a CH3CH4 structure, an immunoglobulin hinge, or any combination thereof (*e.g.*, a CH2CH3 structure together with a hinge). In further embodiments, a linker module comprises all or a portion of an Fc domain selected from: a CH1 domain, a CH2 domain, a CH3 domain, or combinations thereof (*see, e.g.*, PCT Publication WO 2014/031687).

Exemplary linker modules can vary in length, for instance, from about five amino acids to about 500 amino acids, from about ten amino acids to about 350 amino acids, from about 15 amino acids to about 100 amino acids, from about 20 amino acids to about 75 amino acids, or from about 25 amino acids to about 35 amino acids. In further embodiments, a linker module may further comprise a hinge region, a tag or both. Each such component of the linker module is not mutually exclusive.

In certain embodiments, a linker module of a fusion protein of this disclosure may include an IgG1 CH2 region with a N297Q mutation (SEQ ID NO.:10); an IgG4 CH2 region (SEQ ID NO.:11); an IgG1 CH3 region (SEQ ID NO.:12); or an IgG4 CH3 region (SEQ ID NO.:13). In certain embodiments, a linker module may include a glycine-serine linker (SEQ ID NO.:20, which may be encoded by SEQ ID NO.:89, or SEQ ID NO.:21, which may be encoded by SEQ ID NO.:90).

In further embodiments, a linker module of a fusion protein of this disclosure may include an IgG1 CH2 region with a N297Q mutation, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity with SEQ ID NO.:79. In other embodiments, a linker module of a fusion protein of this disclosure may include an IgG4 CH2 region, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity with SEQ ID NO.:80. In still other embodiments, a linker module of a fusion protein of this disclosure may include an IgG1 CH3 region, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity with SEQ ID NO.:81. In yet other embodiments, a linker module of a fusion protein of this disclosure may include an IgG4 CH3 region, which is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity with SEQ ID NO.:82.

In certain embodiments, a linker module further comprises a hinge region. As used herein, a "hinge region" or a "hinge" refers to (a) an immunoglobulin hinge sequence (made up of, for example, upper and core regions), or a functional fragment or variant thereof, (b) a type II C-lectin interdomain (stalk) region, or a functional fragment or variant thereof, or (c) a cluster of differentiation (CD) molecule stalk region, or a functional variant thereof. As used herein, a "wild type immunoglobulin hinge region" refers to naturally occurring upper and middle hinge amino acid sequences interposed between and connecting the CH1 and CH2 domains found in the heavy chain of an antibody. In certain embodiments, a hinge region is human, and in particular embodiments, comprises a human IgG hinge region. In further embodiments, a hinge region is an altered IgG4 hinge region as described in PCT Publication No. WO 2014/031687. In particular embodiments, a hinge region of a fusion protein of this disclosure may be an IgG1 hinge (SEQ ID NO.:7). In related embodiments, a hinge region of a fusion protein of this disclosure may be an IgG1 hinge, which is encoded by a polynucleotide as set forth in SEQ ID NO.:76.

A fusion protein of the present disclosure may further comprise junction amino acids. "Junction amino acids" or "junction amino acid residues" refer to one or more (*e.g.*, about 2-20) amino acid residues between two adjacent domains, motifs, regions, modules, or fragments of a protein, such as between a binding domain and an adjacent linker module, between a hydrophobic domain and an adjacent effector domain, or on one or both ends of a linker module that links two domains, motifs, regions, modules, or fragments (*e.g.*, between a linker module and an adjacent binding domain or between a linker module and an adjacent hinge). Junction amino acids may result from the construct design of a fusion protein (*e.g.*, amino acid residues resulting from the use of a restriction enzyme site during the construction of a nucleic acid molecule encoding a fusion protein). For example, a hydrophobic portion of a fusion protein may have one or more junction amino acids at the amino-terminal end, carboxy-terminal end, or both. Examples of junction amino acids include junction amino acids from IgG2 (*e.g.*, SEQ ID NO.:9, which may be encoded by SEQ ID NO.:78). In some embodiments where a hinge region is from IgG4, the hinge region can include junction amino acids (*e.g.*, SEQ ID NO.:8, which may be encoded by SEQ ID NO.:77). In certain embodiments, a hydrophobic portion is a CD28 transmembrane domain having an amino acid of SEQ ID NO.:14 wherein the CD28 transmembrane domain comprises an amino-terminal junction amino acid of, for example, methionine (*see*, *e.g.*, fusion proteins of SEQ ID NO.:30, 31, 39, and 40). Thus, in certain embodiments, a linker module comprises an IgG4 hinge, IgG4 junction amino acids, and IgG4 CH2-CH3. In certain other embodiments, a linker module comprises an IgG1 hinge, IgG2 junction amino acids, and IgG1 CH2-CH3.

In some embodiments, a fusion protein of the present disclosure may further comprise a tag. As used herein, "tag" refers to a unique peptide sequence affixed to, fused to, or that is part of a protein of interest, to which a heterologous or non-endogenous cognate binding molecule (*e.g.*, receptor, ligand, antibody, or other binding partner) is capable of specifically binding, where the binding property can be used to detect, identify, isolate or purify, track, enrich for, or target a tagged protein or cells expressing a tagged protein, particularly when a tagged protein is part of a heterogeneous population of proteins or other material, or when cells expressing a tagged protein are part of a heterogeneous population of cells (*e.g.*, a biological sample like peripheral blood).

(*See, e.g.*, WO 2015/095895.) In the provided fusion proteins, the ability of the tag(s) to be specifically bound by the cognate binding molecule(s) is distinct from, or in addition to, the ability of the binding domain(s) to specifically bind to the target molecule(s). A tag generally is not an antigen-binding molecule, for example, is not an antibody or TCR or an antigen-binding portion thereof. Examples of tags include Strep tag, His tag, Flag tag, Xpress tag, Avi tag, Calmodulin tag, Polyglutamate tag, HA tag, Myc tag, Nus tag, S tag, X tag, SBP tag, Softag, V5 tag, CBP, GST, MBP, GFP, Thioredoxin tag. In particular embodiments, a Strep tag has an amino acid sequence of SEQ ID NO.:62 or SEQ ID NO.:63.

A fusion protein of the present disclosure may comprise a signal peptide. A "signal peptide" is a short (*e.g.*, 5-30 amino acids) sequence that is used to target the fusion protein for cell surface expression. Exemplary signal peptides include Granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling peptide (SEQ ID NO.:18, which may be encoded by SEQ ID NO.:87) and murine kappa signal peptide (SEQ ID NO.:19, which may be encoded by SEQ ID NO.:88).

In certain embodiments, a fusion protein is a chimeric antigen receptor. "Chimeric antigen receptor" (CAR) refers to a fusion protein of the present disclosure engineered to contain two or more naturally-occurring amino acid sequences linked together in a way that does not occur naturally or does not occur naturally in a host cell, which fusion protein can function as a receptor when present on a surface of a cell.

In some embodiments, a CAR is fully human or humanized. In certain embodiments, a CAR has a scFv from an anti-CD20 antibody or a scTCR from a TCR specific for a CD20 antigen. In particular embodiments, a CAR comprises a scFv from 1.5.3, 1F5, Leu16, rituximab, ofatumumab, veltuzumab, ocrelizumab, ublituximab, or any combination thereof. In particular embodiments, a CAR comprises a linker module comprising an IgG1 hinge, an IgG4 hinge, or any combination thereof. In further embodiments, a CAR comprises a linker module comprising an IgG1 CH2 region with a N297Q mutation, an IgG4 CH2 region, an IgG1 CH3 region, an IgG4 CH3 region, or any combination thereof. In still further embodiments, a hydrophobic portion of a CAR comprises a CD28 transmembrane domain. In some embodiments, a CAR comprises an intracellular domain comprising a portion or domain from CD3ζ, 4-1BB, CD28, or any combination thereof. In any of the above embodiments, a CAR comprises junction amino acids between two adjacent domains, motifs, regions, modules, or fragments.

In certain embodiments, a CAR may be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to 1.5.3-NQ-28-BB-z (SEQ ID NO.:26); 1.5.3-NQ-28-z (SEQ ID NO.:27); 1.5.3-NQ-BB-z (SEQ ID NO.:28); 1.5.3-NQ-z (SEQ ID NO.:29); Leu16-28-BB-z (SEQ ID NO.:30); Leu16-28-z (SEQ ID NO.:31); 1F5-NQ-28-BB-z (SEQ ID NO.:32); 1F5-NQ-28-z (SEQ ID NO.:33); or 1F5-NQ-BB-z (SEQ ID NO.:34). In particular embodiments, a CAR comprise or consists of an amino acid sequence of any one of SEQ ID NOS.:26-34.

In certain embodiments, a CAR may be at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a nucleic acid molecule sequence of any one of SEQ ID NOS.:44-52. In particular embodiments, a CAR is encoded by a polynucleotide comprising or consisting of a sequence of any one of SEQ ID NOS.:44-52.

Methods of making fusion proteins, including CARs, are well known in the art and are described, for example, in U.S. Patent No. 6,410,319; U.S. Patent No. 7,446,191; U.S. Patent Publication No. 2010/065818; U.S. Patent No. 8,822,647; PCT Publication No. WO 2014/031687; U.S. Patent No. 7,514,537; and Brentjens *et al.*, 2007, *Clin. Cancer Res.* 13:5426.

Host Cells, Nucleic Acids and Vectors

In certain aspects, the present disclosure provides nucleic acid molecules that encode any one or more of the fusion proteins described herein. A polynucleotide encoding a desired fusion protein can be obtained or produced using recombinant methods known in the art using standard techniques, such as screening libraries from cells expressing a desired sequence or a portion thereof, by deriving a sequence from a vector known to include the same, or by isolating a sequence or a portion thereof directly from cells or tissues containing the same. Alternatively, a sequence of interest can be produced synthetically. Such nucleic acid molecules can be inserted into an appropriate vector (*e.g.*, viral vector or non-viral plasmid vector) for introduction into a host cell of interest (*e.g.*, an immune cell, such as a T cell).

A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid. In some embodiments, vectors contain transcription or translation terminators, initiation sequences, or promoters for regulation of expression of a desired nucleic acid sequence. Vectors may be, for example, plasmids, cosmids, viruses, or phage, or a transposon system (*e.g.*, Sleeping Beauty, *see, e.g.,* Geurts *et al., Mol. Ther.* 8:108, 2003; Mátés *et al., Nat. Genet.* 41:753, 2009). An "expression vector" is a vector that is capable of directing expression of a protein encoded by one or more genes carried by a vector when it is present in the appropriate environment.

A vector that encodes a core virus is referred to herein as a "viral vector." There are a large number of available viral vectors suitable for use with compositions of the instant disclosure, including those identified for human gene therapy applications (*see* Pfeifer and Verma, *Ann. Rev. Genomics Hum. Genet. 2*:177, 2001). Suitable viral vectors include vectors based on RNA viruses, such as retrovirus-derived vectors.

"Retroviruses" are viruses having an RNA genome, which is reverse-transcribed into DNA using a reverse transcriptase enzyme, the reverse-transcribed DNA is then incorporated into the host cell genome. "Gammaretrovirus" refers to a genus of the retroviridae family. Examples of gammaretroviruses include mouse stem cell virus, murine leukemia virus, feline leukemia virus, feline sarcoma virus, and avian reticuloendotheliosis viruses. "Lentivirus" refers to another genus of retroviruses that are capable of infecting dividing and non-dividing cells. Several examples of lentiviruses include human immunodeficiency virus (HIV; including HIV type 1, and HIV type 2); equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

In certain embodiments, the viral vector can be a gammaretrovirus, *e.g.*, Moloney murine leukemia virus (MLV)-derived vectors. In other embodiments, the viral vector can be a more complex retrovirus-derived vector, *e.g.*, a lentivirus-derived vector. HIV-1-derived vectors belong to this category. Other examples include lentivirus vectors derived from HIV-2, FIV, equine infectious anemia virus, SIV, and Maedi-Visna virus (ovine lentivirus). Methods of using retroviral and lentiviral viral vectors and packaging cells for transducing mammalian host cells with viral particles containing CAR transgenes are known in the art and have been previous described, for example, in U.S.

Patent 8,119,772; Walchli *et al.*, *PLoS One 6*:327930, 2011; Zhao *et al.*, *J. Immunol. 174*:4415, 2005; Engels *et al.*, *Hum. Gene Ther. 14*:1155, 2003; Frecha *et al.*, *Mol. Ther. 18*:1748, 2010; Verhoeyen *et al.*, *Methods Mol. Biol. 506*:97, 2009. Retroviral and lentiviral vector constructs and expression systems are also commercially available. Other viral vectors also can be used for polynucleotide delivery including DNA viral vectors, including, for example adenovirus-based vectors and adeno-associated virus (AAV)-based vectors; vectors derived from herpes simplex viruses (HSVs), including amplicon vectors, replication-defective HSV and attenuated HSV (Krisky *et al.*, *Gene Ther. 5*: 1517, 1998).

Other vectors recently developed for gene therapy uses can also be used with the compositions and methods of this disclosure. Such vectors include those derived from baculoviruses and α-viruses. (Jolly, D J. 1999. Emerging Viral Vectors. pp 209-40 in Friedmann T. ed. The Development of Human Gene Therapy. New York: Cold Spring Harbor Lab), or plasmid vectors (such as sleeping beauty or other transposon vectors).

In certain embodiments, a viral vector is used to introduce a non-endogenous polynucleotide encoding a fusion protein specific for a target, such as CD20. In such embodiments, a viral vector may be a retroviral vector or a lentiviral vector. A viral vector may also include nucleic acid sequences encoding a marker for transduction. Transduction markers for viral vectors are known in the art and include selection markers, which may confer drug resistance, detectable markers, such as fluorescent markers or cell surface proteins that can be detected by methods such as flow cytometry.

In certain embodiments, a viral vector comprises a transduction marker. As used herein, a "transduction marker" can be included in any of the constructs as a way to monitor transfection efficiency or to detect cells expressing a fusion protein of interest. Exemplary transduction markers green fluorescent protein, an extracellular domain of human CD2, a truncated human EGFR (huEGFRt; SEQ ID NO.:25, which may be encoded by SEQ ID NO.:94; *see* Wang *et al.*, *Blood 118*:1255, 2011), or a truncated CD19 (SEQ ID NO.:24, which may be encoded by SEQ ID NO.:93). In certain embodiments, a viral vector comprises a suicide gene, such as iCasp9 (*see*, *e.g.*, Gargett and Brown, *Front. Pharmacol. 5*:235, 2104), or HSV-TK (*see*, *e.g.*, Fillat *et al.*, *Curr. Gene Ther. 3*:13, 2003).

When a viral vector genome comprises a plurality of polynucleotides to be expressed in a host cell as separate transcripts, the viral vector may also comprise additional sequences between the two (or more) transcripts allowing for bicistronic or multicistronic expression. Examples of such sequences used in viral vectors include internal ribosome entry sites (IRES), furin cleavage sites, viral 2A peptide, or any combination thereof. In certain embodiments, a vector construct may comprise a polynucleotide encoding a self-cleaving peptide (*e.g.*, E2A (SEQ ID NO.:22, which may be encoded by SEQ ID NO.:91),T2A (SEQ ID NO.:23, which may be encoded by SEQ ID NO.:92), P2A (SEQ ID NO.:95, which may be encoded by SEQ ID NO.:97) , or F2A (SEQ ID NO.:96, which may be encoded by SEQ ID NO.:98)) such that the mature fusion protein does not contain a transduction marker or a suicide gene. In certain embodiments, a nucleic acid vector may encode a fusion peptide of the present disclosure, optionally containing a transduction marker (such as tCD19 or tEGFR). In further embodiments, nucleic acid molecules encoding a fusion protein of this disclosure may be codon optimized to enhance or maximize expression in certain types of cells, such as T cells (Scholten *et al.*, *Clin. Immunol. 119*: 135-145, 2006), and may optionally contain a transduction marker (such as tCD19 or tEGFR).

In any of the embodiments described herein, a vector containing a polynucleotide encoding a fusion protein of this disclosure may also contain a polynucleotide encoding a transduction marker, which may be used to target a host cell expressing the transduction marker for ablation or death. It has been shown that the persistence of functional antigen-targeting CAR T cells may cause sustained depletion of healthy cells that endogenously express the antigen (*see, e.g.*, Paskiewicz *et al.*, *J. Clin. Invest.*, *126*(11):4262-4272 (2016). Thus, control mechanisms that permit regulation (*e.g.*, ablation, killing, or producing another cytotoxic effect) of the transferred T cells after a achieving a desired antitumor affect are desirable. As used herein, the term "cytotoxic effect" encompasses ablating, killing, or otherwise impairing or reducing the ability of a cell to grow, divide, or survive. Non-limiting examples of cytotoxic effects include necrosis, lysis, apoptosis, swelling, loss of membrane integrity, reduced levels or rates of transcription, reduced levels or rates of translation, reduced levels or rates of ATP production, increased levels or rates of reactive oxygen species, reduced mitochondrial function, nuclear condensation, increased cleavage of the cell's DNA, reduced rates of division or proliferation, and reduction or loss of specific cell function (*e.g.*, the ability of a B lymphocyte to produce immunoglobulins). One exemplary approach is to use a marker (*e.g.*, tEGFR) recognizable by an antibody (*e.g.*, cetuximab) or antibody-drug conjugate that, upon binding the marker, facilitates antibody-dependent cell-mediated cytotoxic (ADCC) or complement-dependent cytotoxic (CDC) responses, or delivers a cytotoxic molecule, to ablate, kill, or otherwise cause a cytotoxic effect on the the transferred T cells. Thus, in certain embodiments, a vector comprises a polynucleotide encoding a fusion protein and comprises a polynucleotide encoding a transduction marker. A transduction marker that can be specifically bound by a cytotoxic antibody, antibody-drug conjugate or other cytotoxic agent is referred to herein as "a suicide transduction marker." In certain embodiments, a method of treating a disease or disorder associated with CD20 expression comprises administering a therapeutically effective amount of a transformed host cell to a subject according to the present disclosure, wherein the transformed host cell comprises a heterologous polynucleotide encoding a fusion protein and a heterologous polynucleotide encoding a suicide transduction marker, wherein the method optionally comprises administering a cytotoxic antibody, antibody-drug conjugate or other cytotoxic agent that specifically associates with, binds to or forms a complex with the suicide transduction marker. In some embodiments, a suicide transduction marker comprises or consists of a truncated EGFR (*e.g.*, SEQ ID NO.: 25), which is specifically bound by an anti-EGFR antibody, such as, for example, cetuximab. In further embodiments, a suicide transduction marker comprises or consists of a truncated CD19 (*e.g.*, SEQ ID NO.: 24), which is specifically bound by a cytotoxic anti-CD19 antibody or antibody-drug conjugate, such as, for example, blinatumomab, coltuximabravtansine, MOR208, MEDI-551, denintuzumabmafodotin, Merck patent anti-CD19, taplutumomabpaptox, XmAb 5871, MDX-1342, SAR3419, SGN-19A, or AFM11 (*see, e.g.*, Naddafi and Davami, *Int. J. Mol. Cell. Med.*, *4*(3):143-151 (2015)).

In any of the embodiments described herein, an encoded fusion protein of this disclosure may be a CAR, such as a CD20 specific CAR. In certain embodiments, a CAR or binding domain thereof encoded by a polynucleotide contained in a vector of this disclosure is fully human or humanized. In further embodiments, a CAR encoded by a vector of this disclosure has a scFv from an anti-CD20 antibody or a scTCR from a TCR specific for a CD20 antigen. In still further embodiments, a CAR encoded by a vector of this disclosure comprises a scFv from 1.5.3, 1F5, Leu16, rituximab, ofatumumab, veltuzumab, ocrelizumab, ublituximab, or any combination thereof. In particular embodiments, a CAR encoded by a polynucleotide contained in a vector of this disclosure comprises a linker module comprising an IgG1 hinge, an IgG4 hinge, or any combination thereof. In further embodiments, a CAR encoded by a polynucleotide contained in a vector of this disclosure comprises a linker module comprising an IgG1 CH2 region with a N297Q mutation, an IgG4 CH2 region, an IgG1 CH3 region, an IgG4 CH3 region, or any combination thereof. In particular embodiments, a linker module or a variable region linker of a CAR encoded by a vector of this disclosure comprises a glycine-serine linker. In still further embodiments, a hydrophobic portion of a CAR encoded by a polynucleotide contained in a vector of this disclosure comprises a CD28 transmembrane domain. In some embodiments, a CAR encoded by a polynucleotide contained in a vector of this disclosure comprises an intracellular domain comprising a portion or domain from CD3ζ, 4-1BB, CD28, or any combination thereof. In any of the embodiments described herein, a CAR encoded by a polynucleotide contained in a vector of this disclosure comprises junction amino acids between adjacent domains, motifs, regions, modules, or fragments.

In any of the embodiments described herein, a vector may comprise a polynucleotide that encodes a CAR that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to 1.5.3-NQ-28-BB-z (SEQ ID NO.:26); 1.5.3-NQ-28-z (SEQ ID NO.:27); 1.5.3-NQ-BB-z (SEQ ID NO.:28); 1.5.3-NQ-z (SEQ ID NO.:29); Leu16-28-BB-z (SEQ ID NO.:30); Leu16-28-z (SEQ ID NO.:31); 1F5-NQ-28-BB-z (SEQ ID NO.:32); 1F5-NQ-28-z (SEQ ID NO.:33); or 1F5-NQ-BB-z (SEQ ID NO.:34). In further embodiments, a vector may comprise a polynucleotide that encodes a CAR that is comprised of or consists of an amino acid sequence of any one of SEQ ID NOS.:26-34.

In still further embodiments, a CD20-specific CAR is encoded by a polynucleotide contained in a vector, wherein the polynucleotide has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to a nucleic acid molecule sequence of any one of SEQ ID NOS.:44-52. In related embodiments, a CD20-specific CAR is encoded by a polynucleotide contained in a vector, wherein the polynucleotide comprises or consists of a sequence of any one of SEQ ID NOS.:44-52.

Optionally, any vector of this disclosure containing a polynucleotide that encodes a CAR of this disclosure can also encode a transduction marker (*e.g.*, tCD19), which may also include a self-cleaving peptide so that the transduction marker and CAR are separated into separate molecules – a CAR and a transduction marker. In certain embodiments, a vector may comprise a polynucleotide encodes a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker, which polynucleotide is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61. In further embodiments, a vector may comprise a polynucleotide encoding a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker and that comprises or consists of a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61.

In any of the embodiments disclosed herein, an isolated polynucleotide encodes a fusion protein capable of specifically binding CD20, wherein the polynucleotide: (a) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (b) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (c) comprises a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (d) comprises a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (e) consists of a polynucleotide sequence of any one of SEQ ID NOS.:53-56; or (f) consists of a polynucleotide sequence of any one of SEQ ID NOS.:44-47.

In any of the embodiments disclosed herein, a fusion protein is encoded by an isolated polynucleotide as disclosed herein. In certain embodiments, the fusion protein consists of or comprises an amino acid sequence wherein the fusion protein: (a) is at least 90% identical to a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.:26-29 and 35-38 and 32-34 with the tCD19 transduction marker removed; (b) is comprised of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.:

35-38 with the tCD19 transduction marker removed; (c) consists of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed; (d) is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26 29; (e) is comprised of an amino acid sequence of any one of SEQ ID NOS.:26 29; (f) consists of an amino acid sequence of any one of SEQ ID NOS.:26 29.

In certain embodiments, a host cell is provided that comprises a heterologous polynucleotide as disclosed herein and is capable of expressing the fusion protein encoded by the heterologous polynucleotide.

In any of the embodiments disclosed herein, a host cell comprises an isolated polynucleotide encoding a fusion protein capable of specifically binding CD20, wherein the polynucleotide: (a) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (b) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (c) comprises a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (d) comprises a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (e) consists of a polynucleotide sequence of any one of SEQ ID NOS.:53-56; or (f) consists of a polynucleotide sequence of any one of SEQ ID NOS.:44-47.

In certain embodiments, a host cell comprises a fusion protein that consists of or comprises an amino acid sequence wherein the fusion protein: (a) is at least 90% identical to a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.:26-29 and 35-38 and 32-34 with the tCD19 transduction marker removed; (b) is comprised of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed; (c) consists of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed; (d) is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26 29; (e) is comprised of an amino acid sequence of any one of SEQ ID NOS.:26 29; (f) consists of an amino acid sequence of any one of SEQ ID NOS.:26 29.

In certain embodiments, a host cell comprises a heterologous polynucleotide as disclosed herein and is capable of expressing the fusion protein encoded by the heterologous polynucleotide.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein comprising a binding domain, wherein the binding domain is: (a) a 1.5.3 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:64, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (b) a 1.5.3 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:64; (c) a 1F5 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:66, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (d) a 1F5 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:66; (e)a Leu16 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:65, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or (f) a Leu16 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:65.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein comprising an scFv, wherein the scFv is encoded by: (a) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:67, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (b) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:67; (c) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:69, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (d) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:69; (e) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:68, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or (f) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:68.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein, wherein the fusion protein is a chimeric antigen receptor and comprises or consists of an amino acid sequence that is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26-3443.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein comprising a hydrophobic portion, wherein the hydrophobic portion is a transmembrane domain. In certain embodiments, the hydrophobic portion is a CD4, CD8, CD28 or CD27 transmembrane domain.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein comprising an effector domain or functional portion thereof, wherein the effector domain or functional portion thereof is a 4-1BB (CD137), CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD79A, CD79B, CARD11, DAP10, FcRα, FcRβ, FcRγ, Fyn, HVEM, ICOS, Lck, LAG3, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, OX40 (CD134), ROR2, Ryk, SLAMF1, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

In certain embodiments, a host cell comprises a heterologous polynucleotide encoding a fusion protein comprising an intracellular component, wherein the intracellular component comprises: (a) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof; (b) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof; (c) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof; (d)a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof; (e) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a CD28 costimulatory domain or portion thereof; or (f)a CD3ζ effector domain or functional portion thereof, a 4-1BB (CD137) costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof.

In any of the embodiments described herein, a vector containing a fusion protein of this disclosure is transduced into a host cell. "Transduction" refers to introduction of a nucleic acid molecule (*e.g.*, a vector encoding a fusion protein of the present disclosure) into a host cell. After transduction, a host cell may carry a vector extra-chromosomally or integrated into a chromosome. Integration into a host cell genome or self-replicating vectors generally result in genetically stable inheritance of a transformed vector. Any suitable transduction method can be utilized. A vector can be transferred into a host cell by physical, chemical, or biological means. A host cell containing a transformed nucleic acid molecule is referred to as "engineered," "recombinant," or "non-natural."

In certain embodiments, a cell, such as a T cell, obtained from a subject may be converted into an engineered, non-natural, or recombinant cell (*e.g.*, an engineered, non-natural, or recombinant T cell) by introducing a nucleic acid molecule encoding a cell surface located fusion protein as described herein, where the cell expresses the fusion protein.

In certain embodiments, a host cell transfected to express a fusion protein of this disclosure is a functional T cell, such as a virus-specific T cell, a tumor antigen specific cytotoxic T cell, a naïve T cell, a memory stem T cell, a central or effector memory T cell, γδ T cells, or a CD4+ CD25+ regulatory T cell. In further embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into bulk CD8+ T cells, naïve CD8+ T cells, CD8+ T_{CM} cells, CD8+ T_{EM} cells, or any combination thereof. In still further embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into bulk CD4+ T cells, naïve CD4+ T cells, CD4+ T_{CM} cells, CD4+ T_{EM} cells, or any combination thereof. In other embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into a population of T cells enriched for naïve CD8+ T cells and CD8+ T_{CM} cells. In still other embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into a population of T cells enriched for naïve CD4+ T cells and CD4+ T_{CM} cells. In any of the aforementioned embodiments, the T cells further contain a nucleic acid molecule encoding an engineered CD20-specific TCR, an engineered CD20-specific high affinity TCR, a CD20-specific CAR, or any combination thereof.

In certain embodiments, prior to expansion and genetic modification of the T cells with a fusion protein construct of this disclosure, a source of T cells is obtained from a subject (*e.g.*, peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, or spleen tissue), from which T cells are isolated using methods known in the art. Specific T cell subsets can be collected in accordance with known techniques and enriched or depleted by known techniques, such as affinity binding to antibodies, flow cytometry, or immunomagnetic selection. After enrichment or depletion steps and introduction of a fusion protein, *in vitro* expansion of the desired modified T cells can be carried out in accordance with known techniques (including those described in U.S. Patent No. 6,040,177), or variations thereof that will be apparent to those skilled in the art.

For example, a desired T cell population or subpopulation may be expanded by adding an initial T cell population to a culture medium *in vitro*, and then adding feeder cells, such as non-dividing PBMCs to the culture medium, (*e.g.*, such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T cell in the initial population to be expanded); and incubating the culture (*e.g.* for a time sufficient to expand the numbers of T cells). Non-dividing feeder cells can comprise gamma-irradiated PBMC feeder cells. In some embodiments, PBMCs are irradiated with gamma rays in the range of about 3000 to 3600 rads. The order of addition of T cells and feeder cells to the culture media can be reversed if desired. A culture can typically be incubated under conditions of temperature and the like that are suitable for the growth of T cells. For the growth of human T lymphocytes, for example, the temperature will generally be at least about 25°C, preferably at least about 30°C, more preferably about 37°C.

Optionally, expansion methods may further comprise adding non-dividing Epstein-Barr Virus (EBV)-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of about 6000 to 10,000 rads. The LCL feeder cells may be provided in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least about 10:1.

After isolation of T lymphocytes, both CD8+ cytotoxic and CD4+ helper T lymphocytes can be sorted into naïve, memory, and effector T cell subpopulations before genetically modifying with a fusion protein and expanding. In certain embodiments, T cells that are modified to express fusion proteins of this disclosure are bulk T cells (*e.g.*, bulk CD4+ T cells or bulk CD8+ T cells), or are a subpopulation of T cells, such as central memory T cells (*e.g.*, CD8+ central memory T cells) or a combination of central memory (T_{CM}) and naïve (T_N) T cells (*e.g.*, CD4+ T_{CM} + T_N cells).

In any of the embodiments described herein, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide that encodes a CAR that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to 1.5.3-NQ-28-BB-z (SEQ ID NO.:26); 1.5.3-NQ-28-z (SEQ ID NO.:27); 1.5.3-NQ-BB-z (SEQ ID NO.:28); 1.5.3-NQ-z (SEQ ID NO.:29); Leu16-28-BB-z (SEQ ID NO.:30); Leu16-28-z (SEQ ID NO.:31);

1F5-NQ-28-BB-z (SEQ ID NO.:32); 1F5-NQ-28-z (SEQ ID NO.:33); or 1F5-NQ-BB-z (SEQ ID NO.:34). In further embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide that encodes a CAR that is comprised of or consists of an amino acid sequence of any one of SEQ ID NOS.:26-34. In any of these embodiments, the host cell is a T cell, wherein the T cells bulk CD4+ T cells, bulk CD8+ T cells, CD4+ central memory T cells, CD8+ central memory T cells or a combination of CD4+ central memory (T_{CM}) and CD4+ naïve (T_N) T cells. The CAR-modified CD4+ T cells and CAR-modified CD8+ T cells can be mixed in a ratio of 3:1 to 1:1 to 1:3 before administration to a subject, or can be administered to a subject separately at the same or similar ratios.

In still further embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide that encodes a CD20-specific CAR, wherein the polynucleotide has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to a nucleic acid molecule sequence of any one of SEQ ID NOS.:44-52. In related embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide that encodes a CD20-specific CAR, wherein the polynucleotide comprises or consists of a sequence of any one of SEQ ID NOS.:44-52. In any of these embodiments, the host cell is a T cell, wherein the T cells bulk CD4+ T cells, bulk CD8+ T cells, CD4+ central memory T cells, CD8+ central memory T cells or a combination of CD4+ central memory (T_{CM}) and CD4+ naïve (T_N) T cells. The CAR-modified CD4+ T cells and CAR-modified CD8+ T cells can be mixed in a ratio of 3:1 to 1:1 to 1:3 before administration to a subject, or can be administered to a subject separately at the same or similar ratios.

Optionally, a host cell comprising any vector of this disclosure that contains a polynucleotide that encodes a CAR of this disclosure can also encode a transduction marker (*e.g.*, tCD19), which may also include a self-cleaving peptide so that the transduction marker and CAR are separated into separate molecules – a CAR and a transduction marker. In certain embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide encoding a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker, which polynucleotide is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or

100% identical to a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61. In further embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide encoding a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker and comprises or consists of a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61.

Whether a T cell or T cell population is positive for a particular cell surface marker can be determined by flow cytometry using staining with a specific antibody for the surface marker and an isotype matched control antibody. A cell population being "negative" for a marker refers to the absence of significant staining of the cell population with a specific antibody above an isotype control, and "positive" refers to uniform staining of the cell population above the levels found on an isotype control. In some embodiments, a decrease in expression of one or more markers refers to a loss of 1 log10 in the MFI or a percentage decrease of T cells that exhibit the marker of at least 20% of the cells, 25% of the cells, 30% of the cells, 35% of the cells, 40% of the cells, 45% of the cells, 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of the cell, 95% of the cells, or 100% of the cells, or any percentage between 20% and 100% when compared to a reference T cell population. In some embodiments, a T cell population positive for a marker refers to a percentage of cells that exhibit the marker, which may be at least 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of the cell, 95% of the cells, or 100% of the cells, or any percentage between 50% and 100% when compared to a reference T cell population.

Immunomagnetic selection methods may also be used to purify T cell subpopulations using commercially available clinical grade antibody bead conjugates using a CliniMACS device (*see*, *e.g.*, Terakura *et al.*, 2012, *Blood* 119:72-82; Wang *et al.*, 2012, *J. Immunother*. 35:689-701). For example, to isolate human CD8+ T_{CM} cells, CD4+, CD14+, and CD45RA+ cells are removed from peripheral blood mononuclear cells by depletion with antibody conjugated paramagnetic beads, and then the CD62L+ fraction from the remaining cells is positively selected with an anti-CD62L labeled bead to enrich for the CD45RO+, CD62L+, CD8+ T_{CM} subpopulation. The enriched CD8+

T_{CM} subpopulation can be activated with anti-CD3/CD28 beads or with antigen, modified with tumor-specific CAR using retroviral or lentiviral vectors, and expanded for use in cellular immunotherapy (*see*, *e.g.*, Terakura *et al*., *supra*; Wang *et al*., *supra*).

Alternatively, T cell subsets may be selected using low-affinity Fab fragments fused to Strep-tag II. A Fab monomers do not have sufficient binding affinity for stable binding to a target antigen on the cell surface. However, when multimerized on a StrepTactin bead, these reagents stably bind a target cell and enable selection based on cell surface marker specificity. A Fab multimer binding can be rapidly reversed by the addition of excess D-biotin, which has a higher affinity for StrepTactin and disrupts the binding between a Strep-tag on a Fab-fragment and a Strep-Tactin "backbone." Fab monomers cannot maintain stable binding a the cell. This "Fab-Streptamers" technology allows for serial positive enrichment of T cells based on multiple cell surface markers and can be used to select any desired T cell subset (*see*, *e.g.*, Stemberger *et al*., *PloS One* 7:e35798, 2012).

Bulk CD8+ T cells can be obtained by using standard methods. In some embodiments, bulk CD8+ T cells are further sorted into naïve, central memory, and effector T cells by identifying certain cell surface markers that are associated with each of those types of CD8+ T cells. In certain embodiments, memory T cells are present in both CD62L+ and CD62L− subsets of CD8+ peripheral blood lymphocytes. For example, PBMCs can be sorted into CD62L−CD8+ and CD62L+CD8+ fractions after staining with anti-CD8 and anti-CD62L antibodies. In some embodiments, expression of phenotypic markers of CD8+ central memory T cells include CD45RO, CD62L, CCR7, CD28, CD3, or CD127 or are negative for granzyme B. In some embodiments, central memory T cells are CD45RO+, CD62L+, CD8+ T cells. In some embodiments, CD8+ effector T cells are negative for or have reduced expression of CD62L, CCR7, CD28, or CD127, or are positive for or have increased expression of granzyme B or perforin, as compared to CD8+ central memory T cells. In some embodiments, naïve CD8+ T cells are characterized by the expression of phenotypic markers of naïve T cells including CD62L, CCR7, CD28, CD3, CD127, or CD45RA.

Bulk CD4+ lymphocytes can be obtained by standard methods. In some embodiments, bulk CD4+ T cells are further sorted into naïve, central memory, and effector cells by identifying cell populations that have certain cell surface markers. In some embodiments, naïve CD4+ T lymphocytes are CD45RO−, CD45RA+, CD62L+, CD4+ T cell. In some embodiments, central memory CD4+ cells are CD62L positive and CD45RO positive. In some embodiments, effector CD4+ cells are CD62L or CD45RO negative or have reduced expression of CD62L or CD45RO as compared to central memory CD4+ cells.

Populations of CD4+ and CD8+ having TCRs that are antigen specific can be obtained by stimulating naïve or antigen-specific T lymphocytes with antigen. For example, T cell clones having antigen-specific TCRs can be generated against, for example, Cytomegalovirus antigens by isolating T cells from infected subjects and stimulating the cells *in vitro* with the same antigen. Naïve T cells may also be used by exposing them to peptide antigens presented in the context of an antigen presenting cell or a peptide-MHC complex. Any number of antigens from tumor cells, cancer cells, or pathogenic agents may be utilized. Examples of such antigens include HIV antigens, Hepatitis C Virus (HCV) antigens, Hepatitis B Virus (HBV) antigens, Cytomegalovirus (CMV) antigens, EBV antigens, parasitic antigens, and tumor antigens, such as orphan tyrosine kinase receptor ROR1, EGFR, EGFRvIII, GD2, GD3, HPV E6, HPV E7, Her2, L1-CAM, Lewis A, Lewis Y, MUC1, MUC16, PSMA, CD19, CD20, CD22, CD56, CD23, CD24, CD37, CD30, CD33, CD38, CD56, CD123, CA125, c-MET, FcRH5, WT1, folate receptor α, VEGF-α, VEGFR1, VEGFR2, IL-13Rα2, IL-11Rα, MAGE-A1, PSA, ephrin A2, ephrin B2, NKG2D ligands, NY-ESO-1, TAG-72, mesothelin, CEA, or the like. Such T cells having antigen-specific TCRs may be further modified to contain a fusion protein as described herein, wherein the fusion protein is specific for the same antigen, specific for a different epitope on the same antigen, or specific for a different antigen. In any of these embodiments, the CD4+ T cells and the CD8+ T cells will contain different CARs, and in particular the intracellular signaling components of the CARs will be distinct.

Methods of preparing and modifying T cells to express fusion proteins of this disclosure, confirming fusion protein modified T cell activity, expanding fusion protein modified T cell populations are known in the art and are described, for example, in Hollyman *et al.*, 2009, *J. Immunother.* 32:169-180; PCT Publication No.

WO 2012/079000; U.S. Patent No. 8,802,374; Brentjens *et al.*, *Blood* 118:4817-4828, 2011; U.S. Patent Publication No. US 2014/0271635.

Uses

The present disclosure provides methods of treating a disease, condition, or disorder in a subject comprising: administering any of the fusion proteins described herein to the subject. In embodiments, methods of the present disclosure include methods of reducing the number of B-cells or treating a disease or disorder associated with aberrant B-cell activity in a subject. Another embodiment provides a method of treating a disease, condition, or disorder a subject comprising analyzing a biological sample of the subject for the presence of an antigen associated with the disease, condition, or disorder and administering a fusion protein described herein, wherein the fusion protein specifically binds to the antigen. In some embodiments, the antigen associated with the disease, condition, or disorder is a tumor associated antigen.

Diseases, conditions, or disorders that may be treated with compositions and methods as described in the present disclosure include cancer and immune diseases (*e.g.*, autoimmune). For example, in certain embodiments, a CD20-expressing cell comprises B-cells. In further embodiments, the disease or disorder associated with CD20 expression is in B-cells or aberrant B cell activity, such as B-cell-related cancers. Adoptive immune and gene therapy are promising treatments for various types of cancer (Morgan *et al.*, *Science 314*:126, 2006; Schmitt *et al.*, *Hum. Gene Ther. 20*:1240, 2009; June, *J. Clin. Invest. 117*:1466, 2007).

A wide variety of cancers, including solid tumors and leukemias are amenable to the compositions and methods disclosed herein. Exemplary types of cancer that may be treated include adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid leukemia; melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; and carcinoma (*e.g.*, Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell). Additional types of cancers that may be treated include histiocytic disorders; malignant histiocytosis; leukemia; Hodgkin's disease;

immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma; liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor. Further, the following types of cancers are also contemplated as amenable to treatment: adenoma; cholangioma; cholesteatoma; cyclindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor; gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; Leydig cell tumor; papilloma; sertoli cell tumor; theca cell tumor; leimyoma; leiomyosarcoma; myoblastoma; myomma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma; neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma; paraganglioma; paraganglioma nonchromaffin. The types of cancers that may be treated also include angiokeratoma; angiolymphoid hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma; lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma; chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma; neoplasms; nerofibromatosis; and cervical dysplasia.

Exemplifying the variety of hyperproliferative disorders amenable to the compositions and methods disclosed herein described herein are disorders or diseases associated with CD20 expression, such as aberrant B-cell activity, including B-cell cancers, such as B-cell lymphomas (such as various forms of Hodgkin's disease, non-Hodgkins lymphoma (NHL) or central nervous system lymphomas), leukemias (such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), hairy cell leukemia, B cell blast transformation of chronic myeloid leukemia) and myelomas (such as multiple myeloma). Additional B cell cancers include small lymphocytic lymphoma (SLL), Waldenström's macroglobulinemia, CD37+ dendritic cell lymphoma, B-cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B-cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, precursor B-lymphoblastic lymphoma, immunoblastic large cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, B-cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder. In certain embodiments, the compositions and methods of this disclosure can be used treat non-B-cell disorders or diseases associated with CD20 expression, including multiple myeloma, melanoma, multiple myeloma of stem cells and melanoma of stem cells.

Inflammatory and autoimmune diseases amenable to the compositions and methods disclosed herein include arthritis, rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, polychondritis, psoriatic arthritis, psoriasis, dermatitis, idiopathic inflammatory myopathy, polymyositis/dermatomyositis, inclusion body myositis, inflammatory myositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, CREST syndrome, inflammatory bowel disease, Crohn's disease, Grave’s disease, ulcerative colitis, respiratory distress syndrome, adult respiratory distress syndrome (ARDS), meningitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE), subacute cutaneous lupus erythematosus, discoid lupus, lupus myelitis, lupus cerebritis, juvenile onset diabetes, multiple sclerosis, allergic encephalomyelitis, neuromyelitis optica, rheumatic fever, Sydenham's chorea, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis and Churg-Strauss disease, agranulocytosis, vasculitis (including hypersensitivity vasculitis/angiitis, ANCA and rheumatoid vasculitis), aplastic anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, central nervous system (CNS) inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Behcet disease, Castleman's syndrome, Goodpasture's syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection, graft versus host disease (GVHD), bullous pemphigoid, pemphigus, autoimmune polyendocrinopathies, seronegative spondyloarthropathies, Reiter's disease, stiff-man syndrome, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), Henoch-Schonlein purpura, autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes mellitus, also referred to as insulin-dependent diabetes mellitus (IDDM), and Sheehan's syndrome; autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), non-specific interstitial pneumonia (NSIP), Guillain-BarréSyndrome, large vessel vasculitis (including polymyalgia rheumatica and giant cell (Takayasu's) arteritis), medium vessel vasculitis (including Kawasaki's disease and polyarteritis nodosa), polyarteritis nodosa (PAN) ankylosing spondylitis, Berger's disease (IgA nephropathy), rapidly progressive glomerulonephritis, primary biliary cirrhosis, Celiac sprue (gluten enteropathy), cryoglobulinemia, cryoglobulinemia associated with hepatitis, amyotrophic lateral sclerosis (ALS), coronary artery disease, familial Mediterranean fever, microscopic polyangiitis, Cogan's syndrome, Whiskott-Aldrich syndrome and thromboangiitis obliterans.

Subjects that can be treated by the present invention are, in general, human and other primate subjects, such as monkeys and apes for veterinary medicine purposes. The subjects can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects.

Fusion proteins of the present disclosure may be formulated for administration in any suitable manner, as understood by persons skilled in the art. A CD20-specific fusion protein (*e.g.*, a CAR) of this disclosure (or fusion protein specific for a different target) may be administered to a subject in cell-bound form (*e.g.*, *ex vivo* modification of a target cell population (mature T cells (*e.g.*, $CD8^+$ or $CD4^+$ T cells) or other cells of T cell lineage)). In a particular embodiment, cells of T cell lineage expressing CD20-specific fusion proteins of this disclosure (or fusion protein specific for a different target) administered to a subject are syngeneic, allogeneic, or autologous cells to the subject. In some embodiments, cells comprising fusion proteins of this disclosure are prepared by harvesting cells (from a biological sample, tissue, or culture medium), washing, concentrating, and formulating in a medium and container system suitable for administration.

The present disclosure provides compositions comprising cells expressing fusion proteins as disclosed herein and a pharmaceutically acceptable carrier, diluents, or excipient. Suitable excipients include water, saline, dextrose, glycerol, or the like and combinations thereof. In embodiments, compositions comprising cells expressing fusion proteins as disclosed herein further comprise a suitable infusion media. Suitable infusion media can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), 5% dextrose in water, Ringer's lactate can be utilized. An infusion medium can be supplemented with human serum albumin or other human serum components.

In other embodiments, CD20-specific fusion proteins of this disclosure (or fusion protein specific for a different target) may be administered to a subject in soluble form. For example, soluble TCRs are known in the art (*see*, *e.g.*, Molloy *et al.*, *Curr. Opin. Pharmacol.* *5*:438, 2005; U.S. Patent No. 6,759,243).

Fusion proteins of this disclosure, or cells including the same, may be administered in a manner appropriate to the disease, condition, or disorder to be treated as determined by persons skilled in the medical art. In any of the above embodiments, a cell comprising a fusion protein as described herein is administered intravenously, intraperitoneally, intratumorly, into the bone marrow, into a lymph node, or into cerebrospinal fluid. In some embodiments, cells comprising a fusion protein of the present disclosure are delivered to the site of a tumor.

An appropriate dose, suitable duration, and frequency of administration of the compositions will be determined by such factors as a condition of the patient; size, type, and severity of the disease, condition, or disorder; particular form of the active ingredient; and the method of administration.

In any of the above embodiments, methods of the present disclosure comprise administering a therapeutically effective amount of a host cell expressing a fusion protein of the present disclosure or a host cell expressing a fusion of this disclosure. A therapeutically effective amount of cells in a composition is at least one cell (for example, one fusion protein modified CD8+ T cell subpopulation; one fusion protein modified CD4+ T cell subpopulation) or is more typically greater than 10^2 cells, for example, up to 10^6, up to 10^7, up to 10^8 cells, up to 10^9 cells, or more than 10^{10} cells. In certain embodiments, the cells are administered in a range from about 10^6 to about 10^{10} cells/m^2, preferably in a range of about 10^5 to about 10^9 cells/m^2. The number of cells will depend upon the ultimate use for which the composition is intended as well the type of cells included therein. For example, cells modified to contain a fusion protein specific for a particular antigen will comprise a cell population containing at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of such cells. For uses provided herein, cells are generally in a volume of a liter or less, 500 mls or less, 250 mls or less, or 100 mls or less. In embodiments, the density of the desired cells is typically greater than 10^4 cells/ml and generally is greater than 10^7 cells/ml, generally 10^8 cells/ml or greater. The cells may be administered as a single infusion or in multiple infusions over a range of time. A clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cells.

In some embodiments, methods of the present disclosure comprise administering a host cell expressing a CAR of this disclosure that is fully human or humanized. In any of the embodiments described herein, methods of the present disclosure comprise administering a host cell expressing a CAR that has a scFv from an anti-CD20 antibody or a scTCR from a TCR specific for a CD20 antigen. In any of the embodiments described herein, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises a scFv from 1.5.3, 1F5, Leu16, rituximab, ofatumumab, veltuzumab, ocrelizumab, ublituximab, or any combination thereof. In any of the above embodiments, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises a linker module comprising an IgG1 hinge, an IgG4 hinge, or any combination thereof. In any of the embodiments described herein, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises a linker module comprising an IgG1 CH2 region with a N297Q mutation, an IgG4 CH2 region, an IgG1 CH3 region, an IgG4 CH3 region, or any combination thereof. In any of the embodiments of this disclosure, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises a glycine-serine linker module or glycine-serine variable region linker. In any of the embodiments described herein, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises a hydrophobic portion comprised of a CD28 transmembrane domain. In any of the embodiments described herein, methods of the present disclosure comprise administering a host cell expressing a CAR that comprises an intracellular domain comprising a domain from CD3ζ, 4-1BB, CD28, or any combination thereof. In any of the above embodiments, methods of the present disclosure comprise administering a CAR that comprises junction amino acids between adjacent domains, motifs, regions, modules, or fragments.

In any of the embodiments described herein, methods of this disclosure comprise administering to a subject a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain, wherein the encoded fusion protein (*e.g.*, CAR) is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to 1.5.3-NQ-28-BB-z (SEQ ID NO.:26); 1.5.3-NQ-28-z (SEQ ID NO.:27); 1.5.3-NQ-BB-z (SEQ ID NO.:28); 1.5.3-NQ-z (SEQ ID NO.:29); Leu16-28-BB-z (SEQ ID NO.:30); Leu16-28-z (SEQ ID NO.:31); 1F5-NQ-28-BB-z (SEQ ID NO.:32); 1F5-NQ-28-z (SEQ ID

NO.:33); or 1F5-NQ-BB-z (SEQ ID NO.:34). In further embodiments, methods of the present disclosure comprise administering to a subject a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain, wherein the encoded fusion protein (*e.g.*, CAR) comprises or consists of an amino acid sequence of any one of SEQ ID NOS.:26-34. In any of these embodiments, the host cell is a T cell, wherein the T cells bulk CD4+ T cells, bulk CD8+ T cells, CD4+ central memory T cells, CD8+ central memory T cells or a combination of CD4+ central memory (T_{CM}) and CD4+ naïve (T_N) T cells. The CAR-modified CD4+ T cells and CAR-modified CD8+ T cells can be mixed in a ratio of 3:1 to 1:1 to 1:3 before administration to a subject, or can be administered to a subject separately at the same or similar ratios.

In still further embodiments, methods of this disclosure comprise administering to a subject a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain, wherein the fusion protein (*e.g.*, CAR) is encoded by a polynucleotide having at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to a nucleic acid molecule sequence of any one of SEQ ID NOS.:44-52. In related embodiments, methods comprise administering to a subject a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain, wherein the fusion protein (*e.g.*, CAR) is encoded by a polynucleotide comprising or consisting of a sequence of any one of SEQ ID NOS.:44-52. In any of these embodiments, the host cell is a T cell, wherein the T cells bulk CD4+ T cells, bulk CD8+ T cells, CD4+ central memory T cells, CD8+ central memory T cells or a combination of CD4+ central memory (T_{CM}) and CD4+ naïve (T_N) T cells. The CAR-modified CD4+ T cells and CAR-modified CD8+ T cells can be mixed in a ratio of 3:1 to 1:1 to 1:3 before administration to a subject, or can be administered to a subject separately at the same or similar ratios.

Optionally, a host cell comprising any vector of this disclosure that contains a polynucleotide that encodes a fusion protein of this disclosure, for use in the methods described herein, can also encode a transduction marker (*e.g.*, tCD19), which may also include a self-cleaving peptide so that the transduction marker and CAR are separated into separate molecules – a CAR and a transduction marker. In certain embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide encoding a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker, which polynucleotide is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61. In further embodiments, a host cell (*e.g.*, T cell) comprises a vector that contains a polynucleotide encoding a self-cleaving peptide disposed between a CD20-specific CAR and a tCD19 transduction marker and comprises or consists of a nucleic acid molecule sequence of any one of SEQ ID NOS.:53-61.

Accordingly, in any of the methods disclosed herein a host cell comprises an isolated polynucleotide encoding a fusion protein capable of specifically binding CD20, wherein the polynucleotide: (a) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (b) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (c) comprises a polynucleotide sequence of any one of SEQ ID NOS.:53-56; (d) comprises a polynucleotide sequence of any one of SEQ ID NOS.:44-47; (e) consists of a polynucleotide sequence of any one of SEQ ID NOS.:53-56; or (f) consists of a polynucleotide sequence of any one of SEQ ID NOS.:44-47.

In certain embodiments, the host cell used in the methods comprises a fusion protein that consists of or comprises an amino acid sequence wherein the fusion protein: (a) is at least 90% identical to a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.:26-29 and 35-38 and 32-34 with the tCD19 transduction marker removed; (b) is comprised of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed; (c) consists of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed; (d) is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26 29; (e) is comprised of an amino acid sequence of any one of SEQ ID NOS.:26 29; (f) consists of an amino acid sequence of any one of SEQ ID NOS.:26 29.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide as disclosed herein and is capable of expressing the fusion protein.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein comprising a binding domain, wherein the binding domain is: (a) a 1.5.3 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:64, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (b) a 1.5.3 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:64; (c) a 1F5 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:66, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (d) a 1F5 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:66; (e) a Leu16 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:65, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or (f) a Leu16 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:65.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein comprising an scFv, wherein the scFv is encoded by: (a) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:67, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (b) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:67; (c) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:69, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; (d) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:69; (e) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:68, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or (f) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:68.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein, wherein the fusion protein is a chimeric antigen receptor and comprises or consists of an amino acid sequence that is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26-43.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein comprising a hydrophobic portion, wherein the hydrophobic portion is a transmembrane domain. In certain embodiments, the hydrophobic portion is a CD4, CD8, CD28 or CD27 transmembrane domain.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein comprising an effector domain or functional portion thereof, wherein the effector domain or functional portion thereof is a 4-1BB (CD137), CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD79A, CD79B, CARD11, DAP10, FcRα, FcRβ, FcRγ, Fyn, HVEM, ICOS, Lck, LAG3, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, OX40 (CD134), ROR2, Ryk, SLAMF1, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

In certain embodiments, a host cell used in the methods comprises a heterologous polynucleotide encoding a fusion protein comprising an intracellular component, wherein the intracellular component comprises: (a) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof; (b) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof; (c) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof; (d)a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof; (e) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a CD28 costimulatory domain or portion thereof; or (f)a CD3ζ effector domain or functional portion thereof, a 4-1BB (CD137) costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof.

Compositions of this disclosure may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single dosage formulation which contains a fusion protein of this disclosure and one or more additional active agents, as well as administration of a fusion protein of this disclosure and each active agent in its own separate dosage formulation. For example, a fusion protein of this disclosure and another active agent can be administered to a subject together in a single infusion dosage composition, or each agent can be administered in separate infusion dosage formulations. Where separate dosage formulations are used, a fusion protein of this disclosure and one or more additional active agents can be administered at the same time, *i.e.*, simultaneously, at essentially the same time, *i.e.*, concurrently, or at separately staggered times, *i.e.*, sequentially; combination therapy is understood to include all these regimens.

The present disclosure provides pharmaceutical compositions comprising CD20-specific binding molecules, cells expressing fusion proteins as disclosed herein or both, and a pharmaceutically acceptable carrier, diluents, or excipient. In certain embodiments, the CD20-specific binding molecule is an antibody. In such embodiments, a CD20-specific antibody can be rituximab, ofatumumab, ocrelizumab, obinutuzumab, ublituximab, veltuzumab, ibritumomab tiuxetan, tositumomab, or any combination thereof.

In certain embodiments, a method of treating a disease or disorder associated with CD20 expression comprises administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain, and optionally administering a therapeutically effective amount of a CD20-specific binding molecule, chemotherapeutic or inhibitor of an immunosuppression component. In further embodiments, the method reduces the number of B-cells or treats a disease or disorder associated with aberrant B-cell activity.

Thus, in certain embodiments, provided are methods of treating a disease or disorder associated with CD20 expression, comprising administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein comprised of an amino acid sequence that is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.: 26-29 and 32-38, and 41-43, and optionally administering a CD20-specific binding molecule, a chemotherapeutic, an inhibitor of an immunosuppression component, or combinations thereof. In further embodiments, the method reduces the number of B-cells or treats a disease or disorder associated with aberrant B-cell activity.

In some embodiments, compositions as described herein are administered with chemotherapeutic agents or immune modulators (*e.g.*, immunosuppressants, or inhibitors of immunosuppression components, such as immune checkpoint inhibitors). Immune checkpoint inhibitors include inhibitors of CTLA-4, A2AR, B7-H3, B7-H4, BTLA, HVEM, GAL9, IDO, KIR, LAG-3, PD-1, PD-L1, PD-L2, Tim-3, VISTA, TIGIT, LAIR1, CD160, 2B4, TGFR beta, CEACAM-1, CEACAM-3, CEACAM-5, CD244, or any combination thereof. An inhibitor of an immune checkpoint molecule can be an antibody or antigen binding fragment thereof, a fusion protein, a small molecule, an RNAi molecule, (*e.g.*, siRNA, shRNA, or miRNA), a ribozyme, an aptamer, or an antisense oligonucleotide. A chemotherapeutic can be a B-Raf inhibitor, a MEK inhibitor, a VEGF inhibitor, a VEGFR inhibitor, a tyrosine kinase inhibitor, an anti-mitotic agent, or any combination thereof.

In any of the embodiments herein, a method of treating a disease or disorder associated with CD20 expression comprises administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein as disclosed herein, and a therapeutically effective amount of an inhibitor of an immunosuppression component, such as an immune checkpoint inhibitor. In some embodiments, an immune checkpoint inhibitor is an inhibitor of CTLA-4, A2AR, B7-H3, B7-H4, BTLA, HVEM, GAL9, IDO, KIR, LAG-3, PD-1, PD-L1, PD-L2, Tim-3, VISTA, TIGIT, LAIR1, CD160, 2B4, TGFR beta, CEACAM-1, CEACAM-3, CEACAM-5, CD244, or any combination thereof.

Accoridngly, in certain embodiments, this disclosure provides methods of treating a disease or disorder associated with CD20 expression, comprising administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein having an amino acid sequence that is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.: 26-29 and 32-38, and 41-43, and a therapeutically effective amount of an inhibitor of an immunosuppression component, such as an immune checkpoint inhibitor. In some embodiments, an immune checkpoint inhibitor is an inhibitor of CTLA-4, A2AR, B7-H3, B7-H4, BTLA, HVEM, GAL9, IDO, KIR, LAG-3, PD-1, PD-L1, PD-L2, Tim-3, VISTA, TIGIT, LAIR1, CD160, 2B4, TGFR beta, CEACAM-1, CEACAM-3, CEACAM-5, CD244, or any combination thereof. In some embodiments, an immune checkpoint inhibitor is selected from (a) an antibody specific for PD-1, such as pidilizumab, nivolumab, or pembrolizumab; (b) an antibody specific for PD-L1, such as MDX-1105, BMS-936559, MEDI4736, MPDL3280A, or MSB0010718C; or (c) an antibody specific for CTLA4, such as tremelimumab or ipilimumab.

In further embodiments, this disclosure provides methods of treating a disease or disorder associated with CD20 expression, comprising administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a host cell comprising a heterologous nucleic acid molecule encoding a fusion protein that comprises or consists of an amino acid sequence of any one of SEQ ID NOS.:26-29 , 32-38, and 41-43, and a therapeutically effective amount of an immune checkpoint inhibitor, optionally wherein the immune checkpoint inhibitor is selected from (a) an antibody specific for PD-1, such as pidilizumab, nivolumab, or pembrolizumab; (b) an antibody specific for PD-L1, such as MDX-1105, BMS-936559, MEDI4736, MPDL3280A, or MSB0010718C; or (c) an antibody specific for CTLA4, such as tremelimumab or ipilimumab.

Exemplary chemotherapeutic agents include alkylating agents (e.g., cisplatin, oxaliplatin, carboplatin, busulfan, nitrosoureas, nitrogen mustards such as bendamustine, uramustine, temozolomide), antimetabolites (*e.g.*, aminopterin, methotrexate, mercaptopurine, fluorouracil, cytarabine, gemcitabine), taxanes (*e.g.*, paclitaxel, nab-paclitaxel, docetaxel), anthracyclines (*e.g.*, doxorubicin, daunorubicin, epirubicin, idaruicin, mitoxantrone, valrubicin), bleomycin, mytomycin, actinomycin, hydroxyurea, topoisomerase inhibitors (*e.g.*, camptothecin, topotecan, irinotecan, etoposide, teniposide), monoclonal antibodies (*e.g.*, ipilimumab, pembrolizumab, nivolumab, avelumab, alemtuzumab, bevacizumab, cetuximab, gemtuzumab, panitumumab, rituximab, tositumomab, trastuzumab), vinca alkaloids (*e.g.*, vincristine, vinblastine, vindesine, vinorelbine), cyclophosphamide, prednisone, leucovorin, oxaliplatin, hyalurodinases, or any combination thereof. In certain embodiments, a chemotherapeutic is vemurafenib, dabrafenib, trametinib, cobimetinib, sunitinib, erlotinib, paclitaxel, docetaxel, or any combination thereof. In some embodiments, a patient is first treated with a chemotherapeutic agent that inhibits or destroys other immune cells followed by a pharmaceutical composition described herein. In some cases, chemotherapy may be avoided entirely.

In any of the embodiments described herein, the methods of this disclosure are applied to a subject that has been pre-treated with a CD20-specific binding molecule, optionally wherein the CD20-specific binding molecule is rituximab, ofatumumab, ocrelizumab, ublituximab, veltuzumab, or any combination thereof; or a chemotherapeutic (*e.g.*, a CHOP [Cyclophosphamide – Hydroxydaunorubicin – Oncovin - Prednisone], CHOP-R [R is rituximab], or CHOEP or CHOEP-R [E is etoposide] regimen); or an inhibitor of an immune suppression component (*e.g.*, an antibody against PD-1, PD-L1, CTLA4, or the like).

Administration of certain compounds of this disclosure (*e.g.* antibodies, chemotherapeutic agents or immune modulators), or their pharmaceutically acceptable salts, in pure form or in an appropriate pharmaceutical composition, can be carried out using any mode of administration for agents serving similar utilities. The pharmaceutical compositions of this disclosure can be prepared by combining a compound of this disclosure with an appropriate pharmaceutically acceptable carrier, diluent or excipient, and may be formulated into preparations in solid, semi solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Exemplary routes of administering such pharmaceutical compositions include oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal.

The term "parenteral" as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Pharmaceutical compositions of this disclosure (*e.g.*, chemotherapeutic agents or immune modulators) are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of this disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art (*see, e.g.*, Remington: The Science and Practice of Pharmacy, 22nd Edition (Pharmaceutical Press, 2012). The composition to be administered will, in any event, contain a therapeutically effective amount of a compound of this disclosure, or a pharmaceutically acceptable salt thereof, for therapeutic methods in accordance with the teachings of this disclosure.

As a solid composition for oral administration, the pharmaceutical composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Exemplary solid compositions can contain one or more inert diluents or edible carriers. In addition, one or more additives may be present, including binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, gum tragacanth or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; or a coloring agent. When a pharmaceutical composition is in the form of a capsule, such as a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or oil or combinations thereof.

The pharmaceutical composition may be in the form of a liquid, such as an elixir, syrup, solution, emulsion, or suspension. In certain embodiments, a liquid composition may be formulated for oral administration or for delivery by injection, as two examples. When intended for oral administration, exemplary compositions may further contain, in addition to one or more compounds of this disclosure, a sweetening agent, preservative, dye/colorant, flavor enhancer, or any combination thereof. Exemplary compositions intended for administration by injection may further contain a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer, isotonic agent, or any combination thereof.

Liquid pharmaceutical compositions of this disclosure, whether they are solutions, suspensions or other like forms, may further comprise adjuvants, including sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

A pharmaceutical composition of this disclosure may be intended for topical administration, in which case the carrier may comprise a suitable solution, emulsion, ointment, gel base, or any combination thereof. The base, for example, may comprise petrolatum, lanolin, polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, emulsifiers, stabilizers, or any combination thereof. Thickening agents may be present in a pharmaceutical composition of this disclosure for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device.

A pharmaceutical composition of this disclosure may be intended for rectal administration, in the form, for example, of a suppository, which will melt in the rectum and release the active compound(s). A composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Exemplary bases include lanolin, cocoa butter, polyethylene glycol, or any combination thereof.

A pharmaceutical composition of this disclosure may include various materials that modify the physical form of a solid or liquid dosage unit. For example, a composition may include materials that form a coating shell around the active ingredient(s). Exemplary materials for forming a coating shell may be inert, such as sugar, shellac, or other enteric coating agents. Alternatively, active ingredient(s) may be encased in a gelatin capsule.

In certain embodiments, compounds and compositions of this disclosure may be in the form of a solid or liquid. Exemplary solid or liquid formulations include semi solid, semi liquid, suspension, and gel forms. A pharmaceutical composition of this disclosure in solid or liquid form may further include an agent that binds to the compound of this disclosure and thereby assists in the delivery of the compound. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, a protein, or a liposome.

A pharmaceutical composition of this disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of compounds of this disclosure may be delivered in single phase, bi phasic, or tri phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, and the like, which together may form a kit.

Pharmaceutical compositions of this disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining a compound of this disclosure with sterile, distilled water to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non covalently interact with the compound of this disclosure to facilitate dissolution or homogeneous suspension of a compound in an aqueous delivery system.

Compounds of this disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific compound employed; the metabolic stability and length of action of the compound; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy. Following administration of therapies according to the formulations and methods of this disclosure, test subjects will exhibit about a 10% up to about a 99% reduction in one or more symptoms associated with the disease or disorder being treated, as compared to placebo-treated or other suitable control subjects.

Compounds of this disclosure, or pharmaceutically acceptable derivatives thereof, may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation which contains a compound of this disclosure and one or more additional active agents, as well as administration of the compound of this disclosure and each active agent in its own separate pharmaceutical dosage formulation. For example, a compound of this disclosure and the other active agent can be administered to the patient together in a single oral dosage composition such as a tablet or capsule, or each agent administered in separate oral dosage formulations. Where separate dosage formulations are used, the compounds of this disclosure and one or more additional active agents can be administered at essentially the same time, i.e., concurrently, or at separately staggered times, i.e., sequentially; combination therapy is understood to include all these regimens.

It will also be appreciated by those skilled in the art that in the process described herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto, and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, t-butyldimethylsilyl, t-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include t-butoxycarbonyl, benzyloxycarbonyl, or the like. Suitable protecting groups for mercapto include C(O) R" (where R" is alkyl, aryl or arylalkyl), p methoxybenzyl, trityl or the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and P.G.M. Wutz, Protective Groups in Organic Synthesis (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

It will also be appreciated by those of skill in the art, although such protected derivatives of compounds of this disclosure may not possess pharmacological activity as such, they may be administered to a mammal and thereafter metabolized in the body to form compounds of this disclosure which are pharmacologically active. Such derivatives may, therefore, be described as "prodrugs". In certain embodiments, compounds of this disclosure are in the form of a prodrug.

Furthermore, all compounds of this disclosure that exist in free base or acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to those skilled in the art. Salts of the compounds of this disclosure can be converted to their free base or acid form by standard techniques.

In the case of transformed host cells expressing a fusion protein according to this disclosure, administration may be performed using individual aliquots of the cells. In certain embodiments, transformed host cells comprise T cells, which may comprise $CD4^+$ T cells, $CD8^+$ T cells, or both. In certain embodiments, T cells comprise a heterologous nucleic acid encoding a chimeric antigen receptor (CAR). In certain embodiments, T cells are sorted to provide for a 1:1 ratio of $CD4^+$ and $CD8^+$ CD20 CAR T cells for administration to the subject. Cells may be administered intravenously over approximately 20-30 minutes at the specified cell dose for each subject. Specified cell doses may be determined by the expression level of a transduction marker that is expressed coordinately with the fusion protein in the vector. For example, in certain embodiments, a T cell is transformed using one or more vectors that coordinately express a truncated CD19 transduction marker and a CAR. Exemplary CD20 CAR T cell dosage levels for use in various embodiments of the present disclosure are set forth in Table 1 below.

Table 1. CD20 CAR T cell formulation and infusion

Dose Level	$tCD19^+$ $CD4^+$ / $tCD19^+$ $CD8^+$ ratio	Total $tCD19^+$ T cell dose*,**
0	1:1	1 x 10^5/kg
1	1:1	3.3 x 10^5/kg
2	1:1	1 x 10^6/kg
3	1:1	3.3 x 10^6/kg
4	1:1	1 x 10^7/kg

* per kg recipient weight; **upper limit per dosing level ±15%

In certain embodiments, cells are manufactured from an autologous peripheral blood mononuclear cell (PBMC) product obtained by standard non-mobilized leukapheresis from the subject. Immunomagnetic selection may be performed to enrich $CD8^+$ cells or $CD4^+$ T cells. In certain embodiments, $CD8^+$ cells and $CD4^+$ T cells are enriched separately, and each subset is separately stimulated with, *e.g.*, anti-CD3 / CD28 paramagnetic beads, followed by transduction with a vector (*e.g.*, a lentiviral vector) encoding the fusion protein and, optionally, a transduction marker such as, for example, a tCD19 transduction marker. The transduced T cells may be expanded, then re-stimulated with a CD20-expressing target cell line to boost growth, further expanded ex vivo, and then formulated to achieve the specified cell dose for infusion. For example, in certain embodiments, anti-CD20 CAR T cells (*e.g.*, 1.5.3-NQ-28-BB-z) according to the present disclosure may be manufactured in accordance with a method comprising:

1. Enrichment of $CD4^+$ T cells from a fraction of leukapheresis product or peripheral blood mononuclear cells (PBMC) from whole blood.

2. In parallel with $CD4^+$ T cell enrichment, enrichment of $CD8^+$ T cells from the remaining leukapheresis product or PBMC.

3. Stimulation of the enriched $CD4^+$ and $CD8^+$ cells in separate cultures with clinical grade anti-CD3 and anti-CD28 coated paramagnetic beads (anti-CD3/CD28 beads) in RPMI 1640 medium supplemented with glutamine, β mercaptoethanol, and fetal bovine serum (CTL Media + 10% FBS), 50 IU/mL IL-2.

4. Transduction of the $CD4^+$ and $CD8^+$ cells with 1.5.3-NQ-28-BB-z CAR lentiviral vector on day 1 after anti-CD3/CD28 bead stimulation.

5. Expansion of transduced $CD4^+$ and $CD8^+$ T cells in CTL Media + 10% FBS and 50 IU/mL IL-2.

6. 2× removal of the anti-CD3/CD28 beads by magnetic depletion on day 4 after CD3/CD28 stimulation.

7. Stimulation with an irradiated, clinically qualified, transformed CD20+ B cell line (TM-LCL) on day 7 after anti-CD3/CD28 stimulation. This step may be omitted

if in-process cell counts on day 7 predict sufficient cell expansion without the TM-LCL stimulation.

8. Expansion of $CD4^+$ and $CD8^+$ cells in G-Rex flasks with CTL Media + 10% FBS and 50 IU/mL IL-2.

9. Cell harvest of each subset on day 15 (range 13-17) after anti-CD3/CD28 stimulation, and formulation of a combined $CD4^+/CD8^+$ T cell product for cryopreservation or infusion.

10. Sample collection at appropriate points during the manufacturing procedure from each of the $CD8^+$ and $CD4^+$ T cells for in-process and final release testing.

11. Administration to the patient by intravenous infusion at the indicated dose in 1:1 ratio of $tCD19^+$ $CD4^+$ and $tCD19^+$ $CD8^+$ T cells. Subjects will be pre-treated with lymphodepletion chemotherapy and receive the T cell infusions at least 36 hours after completing chemotherapy.

In certain embodiments, cells generated for a subject may be given as fresh cells immediately after manufacture, or may be first cryopreserved and stored in a liquid nitrogen freezer, and then the thawed cells washed to remove residual cryoprotectant and then formulated for infusion. The total number of cells will be sufficient to account for cell loss during recovery from thaw and to achieve the cell dose level specified in the clinical protocol. In certain embodiments comprising both $CD4^+$ and $CD8^+$ T cells, the total ratio of $CD4^+$ and $CD8^+$ T cells may differ from 1:1 due to differences in transduction of the individual subsets in individual subjects. For this reason, the subsets may be transduced separately to achieve a desired formulation of the transduced T cells. CD4 and CD8 CAR T cells have demonstrated synergistic effects in animal models (Sommermeyer *et al.*, *Leukemia* 2015).

Transformed cells may be suspended in an appropriate cryopreservation medium (*e.g.*, CryoStor CS10®) for cryopreservation in a controlled rate freezer. Cryopreserved cells may be stored in the vapor phase of a liquid nitrogen freezer. The fresh or thawed cells may then be resuspended in Normosol + 1% HSA and transferred to a transfer pack at the total cell dose level specified in the clinical protocol. The formulated product may be stored at 2-8°C and then transferred under appropriate conditions to the clinical site for administration.

Following leukapharesis, subjects may receive cytoreductive chemotherapy to control disease during production of the transformed cells. For example, in certain embodiments, a subject may receive may receive low-intensity chemotherapy (e.g. lenalidomide, ibrutinib) after leukapheresis. Prior to administering transformed cells according to the present disclosure, chemotherapy or immune modulatory therapy may be appropriate in order to provide lymphodepletion to facilitate survival of transferred T cells, and to reduce the tumor burden prior to infusion of the cells. For example, subjects may receive lymphodepleting chemotherapy for a predetermined time prior to (*e.g.*, 36-96 hours) the infusion of the cells. In certain embodiments, a subject may initially be treated with a single dose of a chemotherapy agent such as cyclophosphamide (CY) i.v. (*e.g.*, at 1 g/m^2) initially. However, if the subject response rate is determined to be inadequate, the lymphodepletion regimen may be changed so that subsequent patients receive a second, further chemotherapeutic or immunomodulatory agent (*e.g.*, CY + fludarabine). Additionally, a subject may, but need not, receive a premedication prior to administration of the cells cells.

One or more intravenous infusions of the cells described herein may be administered to the subject following completion of lymphodepleting chemotherapy (*e.g.*, 36-96 hours thereafter). The dose of cells administered to the subject may be determined according to the dose levels shown in Table 1, and may be adjusted thereafter to increase, decrease, or otherwise change the amount, composition, ratio, or rate of the cells administered. In certain embodiments, a single infusion is administered to the subject. In further embodiments, a second infusion may be given if the first infusion does not produce a complete response (CR), or if the disease relapses after a CR. In still further embodiments, a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, or further infusion may be given. In certain embodiments, a cell infusion may be administered intravenously over a selected period of time (*e.g.*, approximately 20-30 minutes), adjusted as needed to comply with guidelines for endotoxin limits for parenteral drugs (£ 5 EU/kg/hour). The infusion rate may also be adjusted if subjects experience mild infusion-related adverse events (grade 2 or lower).

EXAMPLES

Example 1

Materials and Methods

Cell lines

Raji, Daudi, and Ramos (Burkitt lymphoma), Rec-1 (mantle cell lymphoma), and K562 (CD20-negative erythroid leukemia) tumor cell lines were obtained from ATCC. Granta-519 (mantle cell lymphoma) was obtained from DSMZ, and FL-18 (transformed follicular lymphoma) was obtained from Dr. David Maloney (Fred Hutchinson Cancer Research Center). CD20 expression was authenticated by flow cytometry on all cell lines prior to experiments. Cell lines were cultured in RPMI 1640 with 25 mM HEPES, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine and incubated at 37°C in 5% CO_2. K562 cells were transduced with a retroviral vector to express CD20, and some cells were again transduced with a lentiviral vector to express human CD80. Low, medium, and high CD20-expressing K562-CD80 cell lines were obtained by selection after limiting dilution cloning. Raji-ffLuc cells were produced by transduction of Raji cells with retrovirus encoding firefly luciferase-Thy1.1-Neo and selected with G418 as previously described (James *et al.*, *Blood* 2009;114(27):5454-63). Rituximab-refractory Raji-ffLuc cells were generated with repeated, intermittent cycles of escalating rituximab concentrations as previously described (Czuczman *et al.*, *Clin Cancer Res* 2008;14(5):1561-70).

Flow Cytometry

Ramos cell lines were incubated with rituximab concentrations ranging from 0 to 200 μg/ml at room temperature for 30 minutes. Following CD20 blocking, anti-CD20-PE antibody (clone L27 [Leu16], BD Biosciences) was added, and cells were incubated at either 4°C or 37°C for 30 minutes. Cells were washed with cold FACS buffer (0.5% fetal bovine serum and 2.5 mM EDTA in PBS) and analyzed on a BD Canto 2 flow cytometer. Data were analyzed using FlowJo version 7.6.1 (TreeStar). In a separate experiment, FL-18 cells were blocked with varying concentrations of rituximab, washed once with FACS buffer, and then anti-CD20-FITC antibody (clone 1F5, produced in-house from a hybridoma; Press *et al.*, *Blood* 1987;69(2):584-91) was added and incubated with blocked cells for 15 minutes at 4°C. Cells were then washed and analyzed as described above. Similar experiments were also conducted using ofatumumab instead of rituximab.

Vector constructs

The CD20-specific Leu16-28-BB-z-tEGFR construct (SEQ ID NO.:57) was generated by amplifying the Leu16 scFv (Wang *et al.*, *Hum Gene Ther* 2007;18(8):712-25; Wang *et al.*, *Mol Ther* 2004;9(4):577-86) by PCR and cloning into *Nhe*I and *Rsr*II sites of an epHIV7 lentiviral vector encoding an IgG4-Fc, CD28, and 41BB domains, and CD3ζ domain (Hudecek *et al.*, *Clin Cancer Res* 2013; 19(12):3153-64). The Leu16-28-z construct (SEQ ID NO.:49 or 58) was generated by splice overlap PCR of the Leu16-28-BB-z-tEGFR vector to remove the 41BB domain and truncated EGFR. The lentiviral vector encoding the CD20-specific 1F5-28-BB-z CAR has been previously described (Budde *et al.*, *PLoS One* 2013;8(12):e82742), but was transferred to the HIV-1-based RRL.sin.cPPT.PGK.GFP.wpre self-inactivating 3rd generation lentiviral vector backbone (Becker *et al.*, *Gene Ther* 2010;17(10):1244-52; from Dr. Hans-Peter Kiem, FHCRC). The Fc spacer region of this construct was modified to abrogate binding to Fcγ receptors by substituting the IgG1 junction amino acids with the IgG2 junction amino acids (SEQ ID NO.:9) and adding an N297Q mutation (SEQ ID NO.:10) as previously described (Hudecek *et al.*, *Cancer immunology research* 2014; 3(2):125-35; Hombach *et al.*, *Gene Ther* 2010;17(10):1206-13), to create the 1F5-NQ-28-BB-z construct (SEQ ID NO.:50 or 59). To generate the 1.5.3-NQ-28-BB-z CAR construct (SEQ ID NO.:44 or 53), a novel scFv sequence was produced by synthesizing the V_L and V_H sequences from the 1.5.3 fully human anti-CD20 antibody (*see*, *e.g.*, Bornstein *et al.*, *Invest New Drugs* 2010;28(5):561-74; PCT Publication No. WO 2006/130458) using a codon optimization algorithm (GenScript), separated by a 15 amino acid glycine-serine linker (SEQ ID NO.:20), preceded by the GM-CSF signal peptide (SEQ ID NO.:18). An overlapping fragment produced by splice overlap PCR was used to replace the scFv domain of the

1F5-NQ-28-BB-z construct, cloning it into *Age*I/*Sac*II restriction sites. The inducible caspase 9 suicide gene and downstream 2A sequence (SEQ ID NO.:22 or 23) were removed from this construct by splice overlap PCR. The 1.5.3-NQ-28-z construct (SEQ ID NO.:27) was generated by removing the 41BB domain from 1.5.3-NQ-28-BB-z by splice overlap PCR. All constructs were confirmed by Sanger sequencing. Lentivirus was produced using 293T cells transiently transfected with the described backbone vectors as well as the packaging vectors pCGHP-2, pCMV-Rev2, and pCMV-G, and supernatants containing packaged lentivirus were concentrated 100-fold by centrifugation.

<u>T cell Isolation and Transduction</u>

Peripheral blood mononuclear cells (PBMC) were obtained either by apheresis from healthy donors consented under Institutional Review Board (IRB)-approved research protocols at the FHCRC or from used Pall leukocyte filters purchased from the Puget Sound Blood Center. PBMC isolated by centrifugation with Ficoll-Paque density gradient medium underwent red blood cell lysis with ammonium-chloride-potassium (ACK) buffer and were cryopreserved in 10% DMSO and 90% FBS. For *in vitro* experiments, T cells were negatively selected from thawed PMBC by MACS using a Pan T cell Isolation Kit II (Miltenyi Biotec). For cytotoxicity experiments, $CD8^+$ T cells were positively selected from healthy donor apheresis PBMC by MACS using anti-CD8 antibody coated beads (Miltenyi Biotec) prior to cryopreservation. For some experiments, central memory T cells (T_{CM}) were isolated from healthy donor apheresis PBMC prior to cryopreservation by negative selection using an AutoMACS device after incubation with CliniMACS anti-CD14 and anti-CD45RA beads (Miltenyi Biotec), followed by positive selection with CliniMACS CD62L beads. In other experiments, CD4 and CD8 cells were enriched by positive immunomagnetic selection using anti-CD4 or anti-CD8 beads (Miltenyi Biotec). Selected T cells were stimulated with αCD3/αCD28 Ab-coated Human T-Expander Beads (Invitrogen) at a 3:1 bead:T-cell ratio. Activated T cells were spin-transduced (2100 rpm for 60 minutes at 32°C) the next day with lentiviral vector encoding one of the CD20 CAR constructs (multiplicity of infection of 2-6) plus 4-8 μg/ml polybrene. Transduced T cells were cultured in media containing 50 IU/ml recombinant human interleukin 2 (rhIL-2) with or without 10 ng/ml rhIL-15 (Miltenyi Biotec), incubated for 4-5 days after stimulation before magnetic removal of αCD3/αCD28 beads, and analyzed by flow cytometry to confirm CAR expression. CAR^+ T cells were used in functional assays.

For *in vivo* mouse experiments, T_{CM} or CD4 and CD8-enriched T cells were thawed, activated, and transduced the next day with concentrated lentiviral supernatant encoding the construct indicated in each experiment. CD3/CD28 beads were removed on day 5, cells were expanded in 50 IU/mL rhIL-2, restimulated on day 7-10 with irradiated $CD20^+$ LCL at a 1:1 responder:stimulator ratio, and injected into mice 8-11 days after restimulation with LCL.

Proliferation and Cytokine Secretion Assays

T cells (2 x 10^5 total cells) stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) were then co-cultured at 1:1 ratios with tumor target lines that had been irradiated with 8000-10000 cGy. In rituximab blocking experiments, irradiated target cells were incubated for 30 minutes at room temperature with various rituximab concentrations prior to co-incubation with T cells. Supernatant was collected 24 hours after plating and stored at -20°C until subsequent cytokine analysis by Luminex assay as previously described (Till *et al.*, *Blood* 2012;119(17):3940-50) to quantify interferon-gamma (IFN-γ), interleukin-2 (IL-2), and TNF-α. After 4-5 days, cells were stained with anti-CD3-APC (BioLegend), and CFSE dilution of CD3-gated lymphocytes as a measure of proliferation was determined by flow cytometry. Cell size as another measure of activation was determined by flow cytometry using the geometric mean of the forward scatter (FSC-A) parameter, and subtracting the cell size of resting T cells. Flow cytometry data were analyzed using FlowJo software (v7.6.1; Treestar, Ashland, OR). In some experiments, ofatumumab was substituted for rituximab.

Assessment of cytokine secretion was also determined by intracellular staining of IFN-γ. CD20 CAR^+ $CD4^+$ or $CD8^+$ T cells were were co-cultured with irradiated K562 or K562-CD20 cells for 24 hours. For intracellular staining, cells were fixed, permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences) for 15 minutes on ice. Cells were then stained with anti- IFN-γ (Biolegend) for 1 hour on ice after fixation and permeabilization. Data were analyzed on BD FACSCanto (BD Biosciences). FlowJo Software was used to analyze the data.

Cytotoxicity Assays

Standard ^{51}Cr-release assays were performed by co-incubating CD20 CAR $CD8^+$ T cells with ^{51}Cr-labeled target cell lines for 4-5 hours as previously described. (*See,* Wang, et al. Hum Gene Ther. 2007;18:712-725). Maximal ^{51}Cr release was determined by directly measuring the ^{51}Cr content of supernatants of labeled cells lysed with 5% IGEPAL CA-630. Supernatants were harvested into 96-well Lumaplates, air-dried overnight, and counts were assayed with a TopCount (PerkinElmer). Percent cytotoxicity was calculated by the equation: $[Sample - Min_{avg}] / [Max_{avg} - Min_{avg}]*100$.

For rituximab blocking experiments, ^{51}Cr-labeled target cell lines were incubated at various rituximab concentrations (ranging from 0 to 200 μg/mL) for 30 minutes (at double the final concentration during the initial incubation to yield final concentrations of 10, 25, 50, 100, and 200 μg/ml) before addition of CAR^+CD8^+ T cells at various effector to target (E:T) ratios. Cells were cultured in duplicate at 37°C for 5 hours in medium containing heat-inactivated FBS, with ^{51}Cr-labeled rituximab-blocked target cells in U-bottom 96-well plates. Control wells contained target cells incubated in rituximab-containing medium without T cells (denoted in figures as "0:1" E:T ratio) to exclude the possibility of rituximab-induced CDC. In some experiments ofatumumab was used in place of rituximab.

In vivo Assessment of Rituximab Effect on CAR T cell Efficacy

Groups of 5-10 NOD.Cg-$Prkdc^{scid}Il2rg^{tm1Wjl}$/SzJ (NOD/SCID/$\gamma^{-/-}$ [NSG]) mice 6-10 weeks of age (Jackson Laboratory) were inoculated with 5 x 10^5 rituximab-resistant Raji-ffLuc or Granta-519 lymphoma cells 2-7 days by tail vein. 2-7 days later (as indicated in each experiment), 10^7 CD20 CAR T cells ($tCD19^+$) or empty vector T cells were injected by tail vein. In the rituximab blocking experiment, 25 or 200 μg of rituximab was administered intraperitoneally (i.p.) 5 days after tumor inoculation and 1 day before administration of CAR T cells. Bioluminescence imaging to determine tumor growth was performed using known methods (*see,* James *et al.*, *Blood* 2009;114(27):5454-63; Rufener *et al., Cancer Immunol Res.* 2016;4:509-519). Binning and exposure were adjusted to achieve maximum sensitivity without leading to image saturation. Survival curves were generated using the Kaplan-Meier method with GraphPad Prism 6 software.

To test for persistence of adoptively transferred T cells, whole blood collected at various timepoints by retro-orbital bleeding was lysed by ACK lysing buffer (Quality Biological). Mouse serum was obtained by centrifugation of clotted blood specimens from the retro-orbital plexus on days 6 and 13 after tumor inoculation, and serum rituximab levels were measured using an ELISA assay to determine rituximab concentrations as previously described (*see,* Gopal AK, *et al.*, *Blood* 2008;112(3):830-5; Maloney DG, *et al.*, *Blood* 1997;90(6):2188-95). Fc receptors of isolated cells were blocked with intravenous immunoglobulin (IVIG), and cells were stained with monoclonal antibodies (mAbs) to mCD45 (30-F11, Biolegend), hCD3 (HTT3a, Biolegend), and hCD19 (HIB19, BD Bioscience). Data were collected with a BD Canto 2 and analyzed on FlowJo Software (Treestar). Mouse studies were approved by the FHCRC Institutional Animal Care and Use Committee.

Patient Serum Samples

Human serum samples were provided by B-cell lymphoma patients following IRB approval and informed consent obtained in accordance with the Declaration of Helsinki. Serum samples were collected within 4 months after rituximab-containing salvage chemoimmunotherapy. Serum rituximab concentrations were determined as previously published (Maloney *et al.*, *Blood* 1997;90(6):2188-95).

EXAMPLE 2

EFFECT OF RITUXIMAB ON CD20 BINDING BY CAR CONTAINING ANTI-CD20 SCFV

CD20-directed CARs using scFvs derived from two different murine monoclonal antibodies, either the Leu16 (L27; *see,* Till *et al.*, *Blood* 2008;112(6):2261-71; Till *et al.*, *Blood* 2012;119(17):3940-50) or 1F5 antibodies (*see,* Wang J, *et al.*, *Hum Gene Ther* 2007;18(8):712-25; Budde *et al.*, *PLoS One* 2013;8(12):e82742), each of which bind to epitopes on the large extracellular loop of the CD20 molecule, were previously tested (*see,* Polyak *et al.*, *Blood* 2002;99(9):3256-62). These CD20 epitopes overlap with the rituximab epitope (*see,* Polyak *et al.*, *Blood* 2002;99(9):3256-62) and, thus, rituximab would be expected to block the binding of these CARs. Using flow cytometry, the ability of varying concentrations of rituximab to block binding of the Leu16 anti-CD20 antibody to CD20 expressed on Ramos lymphoma cells was assessed by pre-incubating these cells with rituximab prior to incubation with the Leu16 Ab. A dose-dependent blockade of CD20 was observed, with near complete blockade at 50 μg/ml rituximab at 4°C. But, when anti-CD20-PE (Leu16) was incubated at the physiologically relevant temperature of 37°C, low-level CD20 binding occurred even at 200 μg/ml of rituximab (Figs. 2A-2F). Similar findings were observed in experiments using the 1F5 anti-CD20 antibody on FL-18 cells (data not shown). Thus, rituximab binds to overlapping epitopes with the anti-CD20 CARs of this disclosure and has the potential to interfere with CAR T cell activity against $CD20^{+}$ target cells.

EXAMPLE 3

EFFECT OF RITUXIMAB ON *IN VITRO* FUNCTION OF CAR T CELLS

The impact of CD20 blocking by rituximab on the function of CD20 CAR T cells was assessed by measuring proliferation, cytokine secretion, and cytotoxicity using five different CD20 CAR lentiviral constructs after incubation with a variety of $CD20^{+}$ B cell NHL cell lines. The CAR constructs (Figs. 1A and 1B) were the 3rd-generation Leu16-28-BB-z-tEGFR and 1F5-28-BBz constructs (*see,* Budde *et al.*, *PLoS One* 2013;8(12):e82742), the 2nd-generation Leu16-28-z construct, and two CD20 CARs (1.5.3-NQ-28-BB-z and 1.5.3-NQ-28-z) derived from the fully human 1.5.3 anti-CD20 Ab, which also binds to an overlapping epitope with rituximab (*see,* Bornstein *et al.*, *Invest New Drugs* 2010;28(5):561-74). CAR expression was typically achieved in 40-80% of the T cells (data not shown).

Proliferation of CFSE-labeled CAR T cells was largely unimpaired when cultured with various NHL target cell lines (Raji, Daudi, Rec-1, and FL-18) in the presence of rituximab. CAR T cells stimulated with target cells in the presence of rituximab at concentrations up to 200 μg/ml exhibited >96% of the proliferation observed after stimulation in the absence of rituximab (Fig. 3A). Cell size is another measure of T cell activation (*see,* Grumont *et al.*, *Immunity* 2004;21(1):19-30). CAR^{+} T cells were analyzed by flow cytometry for forward scatter as an estimate of cell size and found that following stimulation with Raji, Daudi, or Rec-1 tumor cells pre-incubated with rituximab, CAR T cells exhibited a median size >85% of the size of control cells not exposed to rituximab (Fig. 3A). T cells incubated with FL-18 cells exhibited a slightly more pronounced, but still modest, reduction in cell size following incubation with rituximab (73% of control cell size at 200 μg/ml).

In contrast to proliferation, cytokine secretion by CAR T cells was found to be decreased in the presence of increasing rituximab levels (Fig. 3B). However, even at 100 μg/ml of rituximab, the cytokines IFN-γ, IL-2, and TNF-α were produced at 34-51%, 70-92%, and 79-108% of baseline levels, respectively. Similar findings were observed using K562 cells genetically modified to express CD80 and CD20 as targets, with CD20-negative K562-CD80 cells as a control to demonstrate antigen specificity of CD20 CAR T cell activity (Figs. 6A and 6B).

The impact of rituximab on the cytolytic activity of CAR^+ T cells against various $CD20^+$ NHL target cell lines was also examined. Using standard ^{51}Cr-release assays with $CAR^+/CD8^+$ T cells as effectors and Raji, FL-18, Granta, or Rec-1 as targets, cytotoxicity was found to be minimally impaired at rituximab concentrations up to 50 μg/ml (Fig. 4), and >65% of baseline cytolytic activity was retained in rituximab concentrations of 100 μg/ml against all target cell lines tested.

The *in vitro* functionality of the fully human 1.5.3-NQ-28-z and 1.5.3-NQ-28-BB-z CAR T cells were tested in the presence of rituximab. As with the Leu16 and 1F5 CARs, a modest dose-dependent decrease in cytokine secretion and cytotoxicity against rituximab pre-treated target cells was observed, but not proliferation.

EXAMPLE 4

EFFECT OF EXPRESSION LEVELS OF CD20 ON CAR T CELL SENSITIVITY TO ANTI-CD20

To examine whether the level of CD20 expression on tumor cells might impact sensitivity to rituximab blockade, K562-CD80 cell lines with low, medium, and high levels of CD20 expression after limiting dilution cloning (Fig. 10) were selected for testing. The *in vitro* CAR T cell function was again assessed in the presence of varying concentrations of rituximab. As with the NHL cell lines, proliferation of CAR T cells was completely intact regardless of the expression level of CD20 on target cells (Fig. 5A). Cell size was undiminished when CD20^{high} cells were used as targets, although a modest reduction in cell size was found for cells expressing lower levels of CD20. In contrast to proliferation and cell size, cytokine secretion was significantly impaired upon stimulation with CD20^{low} target cells, with IFN-γ, IL-2, and TNF-α levels as low as 5%, 17%, and 22% of baseline values, respectively, at 100-200 μg/ml of rituximab (Fig. 5B; Fig. 11A-11E), whereas T cells stimulated with CD20^{high} targets retained >75% of baseline activity at rituximab concentrations of 100 μg/ml.

The impact of CD20 antigen density on the rituximab-mediated inhibition of CAR T cell cytolytic activity is shown in Fig. 5C. T cell killing of target cells expressing high levels of CD20 was minimally impacted by rituximab, even at low E:T ratios. However, there was a dose-dependent decrease in T cell cytotoxicity against CD20^{low} and CD20^{medium} K562-CD80 targets, which was most pronounced at lower effector to target (E:T) ratios. Cytolytic activity against CD20^{low} targets was retained at 47% of baseline at a 50:1 E:T ratio at 200 μg/ml rituximab, but was only 16% of baseline at a 2:1 E:T ratio.

Example 5

In vivo Anti-Tumor Activity of CD20 CAR T cells in the Presence of Residual Rituximab

The *in vitro* experiments above indicated that CD20 CAR T cells retain significant functionality against CD20^{+} tumors despite the presence of moderate levels of rituximab. To evaluate how these observations would translate to the *in vivo* setting, the impact of residual rituximab on CAR T cell activity in a mouse lymphoma model was examined.

By way of background, rituximab as a single agent has significant anti-tumor activity against Raji cells in immunocompromised mouse xenograft models (*see,* Hernandez-Ilizaliturri FJ, *et al.*, *Clin Cancer Res* 2003;9(16 Pt 1):5866-73). To overcome a potential confounding therapeutic effect from rituximab in combination therapy experiments, a rituximab-refractory Raji cell line (RR-Raji) was generated using previously described methods (*see,* Czuczman MS, *et al.*, *Clin Cancer Res*

2008;14(5):1561-70), and CD20 expression was found to be retained in this cell line (Fig. 12).

NSG mice were inoculated i.v. with RR-Raji cells and some groups were treated with high or low-dose rituximab once tumors were established 5 days after inoculation, and then CD20 CAR^+ T cells were administered i.v. the following day (Fig. 7A). Mice that received rituximab alone demonstrated a modest, transient anti-tumor effect, but all died of tumor progression by day 24, whereas mice treated with CAR T cells alone had significant tumor regression, with tumor eradication in 40% of mice and a doubling of median survival (52 days). Mice that received rituximab the day prior to T cell infusion did not have impaired *in vivo* CAR T cell activity as compared to mice receiving CAR T cells alone; all but one mouse in the 25 μg/ml rituximab group and all mice in the 200 μg/ml rituximab group demonstrated tumor eradication (Figs. 7B and 7C; Figs. 13A and 13B).

To confirm that these tumor remissions occurred in the presence of physiologically relevant serum levels of rituximab, serum from rituximab-treated mice was collected on the day of T cell infusion and one week later and serum rituximab levels were measured. Mice receiving 200 μg/ml rituximab had an initial median serum rituximab concentration of 138.5 μg/ml (range 54.5-173.6) and 39.7 μg/ml (range 1.6-51.9) a week later, and mice receiving 25 μg/ml rituximab had a median concentration of 11.7 μg/ml (range 2.8-17.8) at baseline and 0 μg/ml at 1 week after T cell infusion (Fig. 7D).

In addtion, circulating CAR T cell levels were quantified by flow cytometry 28 days after tumor injection. There was no significant difference CAR T cell levels between mice receiving CAR T cells alone or rituximab plus CAR T cells, indicating that the presence of rituximab did not impair the *in vivo* persistence of CAR T cells (Figs. 14A-14C).

EXAMPLE 6

SERUM RITUXIMAB CONCENTRATIONS OF PATIENTS TREATED WITH SALVAGE RITUXIMAB-CONTAINING REGIMENS

To place the above-noted results into a clinical context, a clinically relevant range of residual serum rituximab levels in the intended patient population was queried in a database of patients with B-cell NHL who underwent autologous stem cell transplantation on investigational protocols and had a pre-transplant serum rituximab measurement available (*see,* Gopal *et al.*, *Blood* 2008;112(3):830-5). A total of 103 patients who received a rituximab-containing chemotherapy regimen within 4 months of the serum blood draw (range 0.5–3.8 months, median 1.8) were identified, and the median rituximab concentration in these patients was 38.3 μg/ml, with an interquartile range of 19.1–71.7 μg/ml (Figs. 7E). The rituximab concentration was 100 μg/ml or lower in 86% of patients.

EXAMPLE 7

EFFECT OF OFATUMUMAB ON CD20 CAR T CELL FUNCTION

To determine the importance of epitope location on the effect of anti-CD20 antibodies on CAR function, the *in vitro* assays were repeated with ofatumumab, an anti-CD20 antibody that binds to a distinct epitope from rituximab, which involves a smaller extracellular loop of CD20 as well as a different area of the large loop (*see,* Du *et al.*, *Mol Immunol* 2009;46(11-12):2419-23; Teeling *et al.*, *J Immunol* 2006;177(1):362-71).The ability of ofatumumab to block binding of the Leu16 anti-CD20 antibody was first evaluated by flow cytometry, which showed that despite the different epitope, binding of the second antibody was profoundly blocked by ofatumumab. Moreover, the blocking of binding was at even lower concentrations than rituximab (Figs. 2D-F). Then *in vitro* functional assays were performed on Rec-1 and Raji-ffLuc lymphoma cells that had been pre-incubated with varying concentrations of ofatumumab (Figs.8A-8C). The results were similar to those with rituximab, in that proliferation and cell size were minimally affected, but cytokine production was more impacted, in a dose-dependent manner. Compared with rituximab, cytotoxicity was more profoundly impaired in the presence of ofatumumab. These findings indicated that the inhibitory effect of anti-CD20 antibody is due to steric inhibition and not to direct blocking of the CAR binding epitope. Hence, the stronger inhibitory effect of ofatumumab resulted from a slower off-rate compared with rituximab. This was supported by competitive cell-binding flow cytometry studies at 4°C or 37°C (Figs. 2D-F), which confirmed a much lower dissociation of ofatumumab, consistent with previously reported data (*see*, Teeling *et al.*, *Blood* 2004;104(6):1793-800).

EXAMPLE 8

CYTOKINE SECRETION BY VARIOUS CAR CONSTRUCTS *IN VITRO*

Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vivo. At day 14, the cells were re-stimulated with either irradiated Raji-ffLuc cells (Fig. 15A and Fig. 15C), Granta-519 cells (Fig. 15B), and Jeko cells (Fig. 15D). The “19-BB-z” construct is a clinical-grade CD19-targeted CAR being used in clinical trials and is provided as a positive control. Supernatants were harvested 24 hours later and analyzed by Luminex assay for interferon (IFN)-γ, IL-2, and tumor necrosis factor-α levels.

EXAMPLE 9

CYTOKINE SECRETION BY CD20 CAR T CELLS

$CD4^{+}$ and $CD8^{+}$ T cells transduced with the 1.5.3-NQ-28-BB-z lentiviral vector and expanded ex vivo were restimulated with irradiated Raji-ffLuc $CD20^{+}$ lymphoma cells. Secretion of the indicated cytokines was measured in cell supernatants after 24 hours by Luminex assay. (Fig. 16A). Cryopreserved $CD4^{+}$ and $CD8^{+}$ CD20 CAR T cells were thawed and restimulated with K562 cells or K562 cells expressing CD20 and at 24 hours were analyzed by intracellular staining for IFN-γ by flow cytometry. (Fig. 16B).

EXAMPLE 10

IN VITRO CYTOTOXICITY OF VARIOUS CAR CONSTRUCTS

Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vivo. At day 14, the cells were used as effectors in a standard 4-hour ^{51}Cr-release assay, using (Figs. 17A and 17B) Raji-ffLuc, and (Fig. 17B) Jeko cells as targets. The "19-BB-z" construct is a clinical-grade CD19-targeted CAR being used in clinical trials and is provided as a positive control. The specific target cell lysis of each CAR T cell population is shown.

EXAMPLE 11

PROLIFERATION OF CD20 CAR T CELLS

$CD8^{+}$ T cells were transduced with the 1.5.3-NQ-28-BB-z lentiviral vector (or were mock-transduced) and expanded ex vivo, and then cryopreserved. The cells were then thawed, stained with carboxyfluorescein succinamidyl ester (CFSE), and restimulated with irradiated $CD20^{+}$ Raji-ffLuc lymphoma cells, K562 cells, or K562 cells expressing CD20. Cells were analyzed by flow cytometry 4 days later. (Fig. 18A) CFSE dilution of CAR^{+} cells (gated on $CD3^{+}$/$tCD19^{+}$) is shown. The dashed-line histogram shows CFSE fluorescence of T cells in culture medium only, and solid line- histograms are T cells co-incubated with target cells. (Fig. 18B) The percentage of divided cells is shown for each group.

EXAMPLE 12

IN VIVO ANTI-TUMOR ACTIVITY OF VARIOUS CAR CONSTRUCTS

Central memory ($CD14^{-}CD45RA^{-}CD62L^{+}$) T cells were stimulated with anti-CD3/CD28 antibody coated beads, transduced 24 hours later with lentiviral vectors encoding the indicated CAR constructs, and expanded in vivo. The "19-BB-z" construct is a clinical-grade CD19-targeted CAR being used in clinical trials at our center and provided as a benchmark control. NSG mice were injected i.v. with Raji-ffLuc tumor cells, followed 2 days later by i.v. injection of expanded central memory (CD14^{-} CD45RA^{-}CD62L^{+}) T cells transduced with the 1.5.3-NQ-28-BB-z CAR, 1.5.3-NQ-28-z CAR, JCAR-014 (anti-CD19-41BB-ζ), or an empty vector. (Fig. 19A) Tumor burden over time as assessed by bioluminescence imaging; and (FIG. 19B) Kaplan-Meier plot of overall survival.

EXAMPLE 13

IN VIVO ACTIVITY OF CD20 CAR T CELLS AGAINST MANTLE CELL LYMPHOMA

CD4^{+} and CD8^{+} CD20 CAR T cells were transduced with the 1.5.3-NQ-28-BBz CAR and used to treat NSG mice that had been inoculated 7 days earlier with Granta-ffLuc mantle cell lymphoma cells by tail vein. A Kaplan-Meier plot of overall survival is shown in Fig. 20.

EXAMPLE 14

IN VIVO CAR T CELL PERSISTENCE AND RELATED PHYSIOLOGICAL EFFECTS

Retroorbital blood samples were obtained at serial time points after infusion of either CD20 CAR T cells or empty vector tCD19-expressing T cells in NSG mice bearing Raji-ffLuc disseminated tumors. CD20 CAR T cells expressing the tCD19 transduction marker were quantified by flow cytometry at each time point as human CD3^{+}/mouse CD45-negative/human CD19^{+} cells. (Fig. 21A) tCD19^{+} T cells at 3 post-infusion time points as a percentage of total nucleated cells in the blood are shown (n=9 initially in CAR T cell group). Truncated CD19^{+} cells from an empty vector mouse are shown for reference. (Fig. 21B) In a separate experiment, the tCD19^{+} cells from 2 mice in each group (empty vector vs CAR T cells) are shown longitudinally with weekly measurements.

Additionally, mice treated with CD20 CAR T cells were monitored for signs of toxicity based on weight, general behavior and appearance, physical activity, posture, grooming habits, skin color, presence of diarrhea, signs of eye, mouth, or skin inflammation, lethargy, or signs of severe anemia (pale ear pinnae or feet or mucous membranes). No signs of T-cell-related toxicity were observed in any mice over 11 experiments using CD20 CAR T cells, with the exception of the finding of xenogeneic graft-versus-host disease that developed in some mice at late time-points. This finding occurred both in mice receiving CD20 CAR T cells as well as in mice receiving empty vector cells and, thus, is not associated with the CAR vector but rather is a known consequence of xenogeneic T cell transfer. In each experiment, the weight of each mouse was recorded at least 3 times per week, and was generally stable except in mice that experienced terminal tumor progression, in which weight loss occurred over the last few days of life (data not shown).

Finally, blood samples were taken from a subset of mice in two experiments to determine physiological function of the animals. In the first experiment, mice bearing Granta mantle cell lymphoma tumors were treated with either untransduced T cells, CD20 CAR T cells that had not been restimulated with $CD20^{+}$ TM-LCL cells, or CD20 CAR T cells that had been restimulated with TM-LCL cells (either freshly infused or first cryopreserved, then thawed and infused). Renal function was measured using blood urea nitrogen (BUN) and creatinine, hepatic function was measured using alanine and aspartate aminotransferases (ALT and AST), and marrow function was measured by white blood cell count (WBC), hemoglobin, and platelet count in retroorbital blood samples in treated mice (data not shown). Compared with untreated mice, no increases in BUN or creatinine or significant changes in hepatic function were seen in mice treated with CAR T cells. Mice treated with CAR T cells that had not been restimulated with TM-LCL cells had a drop in WBC compared with untreated mice, but this was not observed in mice treated with T cells that had been restimulated with TM-LCLs. A small drop in hematocrit, but not hemoglobin, was observed in mice treated with TM-LCL-restimulated CAR T cells, though this was not seen in mice receiving non-restimulated CAR T cells. The platelet count increased in mice receiving non-restimulated CAR T cells, but no significant changes were seen in mice receiving restimulated CAR T cells.

In the second experiment, mice bearing Raji-ffLuc tumors were treated with either low-dose (1×10^{6} CAR+ cells/mouse) or high dose (5×10^{6} CAR+ cells/mouse), and renal and hepatic function was assessed. A trend towards higher BUN was seen in the mice receiving T cells, but there was no change in serum creatinine (data not shown). No elevation of hepatic transaminases was observed.

EXAMPLE 15

CLINICAL STUDY OF ANTI-CD20 CAR THERAPY

A phase I/II study was designed to assess the safety and maximum tolerated dose (MTD) of adoptive T cell therapy with a 1:1 mixture of autologous CD4+ and CD8+ T cells transduced to express a CD20-specific CAR, 1.5.3-NQ-28-BB-z. The self-inactivating (SIN) lentiviral vector carrying this construct used to transduce T cells in this study is a 3rd-generation HIV-1-derived lentivirus, which encodes an scFv from the 1.5.3 fully human monoclonal antibody that recognizes an epitope in the large extracellular loop of human CD20, and which is linked to a modified human IgG1 hinge/spacer region, human CD28 transmembrane and intracellular domains, and the human 4-1BB and CD3ζ signaling domains (Figure 1A). The vector also encodes a non-functional, truncated cell surface human CD19 (tCD19) separated from the CAR cassette by a self-cleavable E2A element, which facilitates tracking of the CAR T cells *in vivo*. The truncation of CD19 shortens the intracellular domain to 19 amino acids, removing all tyrosine residues that serve as phosphorylation sites, but retains the extracellular epitopes recognized by anti-CD19 antibodies. The tCD19 can also be used as a target for CD19-targeted antibodies or antibody-drug conjugates to eliminate the CAR T cells, for example, in the case of prolonged B cell aplasia.

Previous efforts investigating anti-CD20 CAR T therapies showed some success, but low transfection efficiency (< 0.1%) required antibiotic selection and prolonged *ex vivo* growth, resulting in low CAR expression and T cell exhaustion (*see*, *e.g.*, Till *et al.*, *Blood 119*(17):3940-3950, 2012; *see also* Till *et al.*, *Blood 112*(6):2261-2271, 2008; Wang *et al.*, *J. Clin. Immunol. 155*(2):160-75, 2014; da Silva *et al.*, Blood ASH Annual Meeting Abstracts:Abstract #1851, 2016).

The clinical trial will enroll 30 subjects with B-cell non-Hodgkin lymphoma, including mantle cell, follicular, lymphoplasmacytic, marginal zone, transformed indolent B cell lymphoma (including transformed CLL), or diffuse large B cell lymphoma that has relapsed after a response to at least one prior therapy regimen or is refractory to prior therapy. Critical eligibility criteria include: age 18 years or older (of any gender, race, or ethnicity); measurable disease with evidence of CD20 expression; female participants may not be pregnant or breastfeeding; adequate hepatic, renal, pulmonary, cardiac, and hematologic function as defined in clinical protocol; no active central nervous system metastases or past/current clinically relevant central nervous system pathology; no HIV, active uncontrolled infection, or active autoimmune disease requiring systemic immunosuppressive therapy.

Patients with *de novo* DLBCL must meet one of the following criteria:

- Biopsy-proven refractory disease after a frontline regimen containing both an anthracycline and rituximab or other anti-CD20 antibody (i.e. “primary refractory”), where any disease recurring within 3 months of completion of the regimen is considered refractory.
- Relapsed or refractory disease after at least one of the following:
    - At least 2 lines of therapy (including at least one with an anthracycline and anti-CD20 antibody) Autologous stem cell transplant
    - Allogeneic stem cell transplant

A diagram of the general treatment schema is provided in Figure 24, and diagrammatic representation of the formulation and model of administration of the CAR T cells is provided in Figures 25A and 25 B.

Leukapheresis will be performed on each patient to obtain peripheral blood mononuclear cells. Patients ineligible for a vein-to-vein apheresis may elect to have a percutaneous central venous catheter placed to permit this collection. Patients ineligible for apheresis who have a hematocrit of at least 38% and a total non-malignant (normal) lymphocyte count of > 2000/mcl may undergo phlebotomy of 400 ml of blood to obtain PBMCs necessary for generation of the CAR T cells. This approach would only be taken in patients that would be enrolled at dose levels 0 (1 x 10^5 tCD19+ cells/kg), 1 (3.3 x 10^5 $tCD19^+$ cells/kg) and 2 (1 x 10^6 $tCD19^+$ cells/kg). Participants will undergo tumor biopsy prior to leukapheresis. PET CT may be performed before or after tumor biopsy and leukapheresis, depending on accessibility of lymph node.

CAR T cells are manufactured from an autologous peripheral blood mononuclear cell (PBMC) product obtained by standard non-mobilized leukapheresis for each patient. PBMC undergo immunomagnetic selection to enrich CD8+ and CD4+ T cells separately, and each subset is separately stimulated with anti-CD3/CD28 paramagnetic beads, followed by transduction with the 1.5.3-NQ-28-BB-ζ lentiviral vector encoding the fully human 3rd-generation CD20-specific CAR and tCD19 transduction marker. The transduced T cells are expanded, then re-stimulated with a CD20-expressing target cell line to boost growth, further expanded ex vivo, and then formulated in a 1:1 CD4/CD8 ratio to achieve the specified cell dose for infusion. Cell products may either be infused fresh, or cryopreserved and then thawed, washed, and infused.

The CD20 CAR T cell product will consist of a 1:1 ratio of $tCD19^+$ $CD4^+$ and $tCD19^+$ $CD8^+$ T cells, where tCD19 is a transduction marker that is co-expressed with the CAR and identifies CAR^+ cells. The CD20 CAR T-cell product generated for each patient may be given either as fresh cells immediately after manufacture, or may be first cryopreserved and stored in a liquid nitrogen freezer, and then the thawed cells washed to remove residual cryoprotectant and then formulated for infusion. The total number of cells will be sufficient to account for cell loss during recovery from thaw and to achieve the cell dose level specified in the clinical protocol. The total ratio of $CD4^+$ and $CD8^+$ T cells may differ from 1:1, because transduction of the individual subsets is similar but not identical in individual patients. For this reason, the subsets are transduced separately enabling precise formulation of transduced T cells. The rationale for this ratio is based on published work demonstrating synergy between CD4 and CD8 CAR T cells in animal models (Sommermeyer *et al.*, *Leukemia* 2015) and on our objective of providing a uniform cell product to all patients to assist in evaluating toxicity and efficacy, which is difficult if every patient receives a different composition.

The CD20 CAR T cells will be suspended in CryoStor CS10® or other appropriate cryopreservation medium for cryopreservation in a controlled rate freezer. Cryopreserved cells will be stored in the vapor phase of a liquid nitrogen freezer. The fresh or thawed CD20 CAR T cells will be resuspended in Normosol + 1% HSA and transferred to a transfer pack at the total cell dose level specified in the clinical protocol. The formulated product will be stored at 2-8°C and then transferred on refrigerated gel packs to the clinical site at either the University of Washington or Seattle Cancer Care Alliance for administration. The product will be released by the FHCRC Cell Processing Facility. The cell product should be infused into the research participant within 6 hours of formulation. The FHCRC Cell Processing Facility will be responsible for documenting the dispensation and return (when applicable) of the investigational product.

Patients will receive lymphodepleting chemotherapy 36-96 hours prior to the infusion of CD20 CAR T cells. There must be at least a 36-hour interval between the last dose of chemotherapy and the T cell infusion. The goals of administering chemotherapy are to provide lymphodepletion to facilitate survival of transferred T cells, and to reduce the tumor burden prior to infusion of CD20 CAR T cells. As outlined in the statistical considerations of the protocol, patients will initially be treated with a single dose of cyclophosphamide (CY) i.v. 1 g/m^2 initially. However, if the response rate is inadequate, the lymphodepletion regimen will be changed so that subsequent patients receive CY + fludarabine.

Prior to receiving CD20 CAR T cells, participants will be assessed to ensure they have not developed any pulmonary, cardiovascular, hepatic, renal, or neurologic toxicities prohibited by the protocol; have not developed uncontrolled, active, and serious infection; and have not received treatment with other investigational agents within 30 days of T cell infusion.

Premedications are not required prior to the administration of the CD20 CAR T cell product. Standard premedications may be used at the discretion of investigator.

Each patient will receive a single intravenous infusion of CD20 CAR T cells 36-96 hours following completion of lymphodepleting chemotherapy. The dose of CD20 CAR T cells administered to each patient will be determined according to the statistical design described in the clinical protocol. The dose levels are shown in Table 2 below. A second infusion of CD20 CAR T cells may be given if the first infusion does not produce a CR, or if the disease relapses after a CR. For this purpose, patients must meet criteria specified in the clinical protocol below.

Cell administration: A single cell product, combined from individual aliquots of CD4+ and CD8+ CD20 CAR T cells in a 1:1 ratio, will be administered intravenously over approximately 20-30 minutes at the specified cell dose for each subject. The specified T cell dose refers to CAR+ T cells determined by the expression of the truncated CD19 transduction marker, which is expressed coordinately with the CAR in the vector. Dose levels planned for administration under the proposed protocol are as follows:

Table 2. CD20 CAR T Cell Formulation and Infusion

Dose Level	$tCD19^+ CD4^+$ / $tCD19^+ CD8^+$ ratio	Total $tCD19^+$ T cell dose*, **
0	1:1	1×10^5/kg
1	1:1	3.3×10^5/kg
2	1:1	1×10^6/kg
3	1:1	3.3×10^6/kg
4	1:1	1×10^7/kg

* per kg recipient weight

**upper limit per dosing level, ±15%; Dose level 1 is the starting dose level

All patients will be monitored during each T cell infusion. Vital signs (including oxygen saturation) should be recorded before and during the infusion and approximately hourly for 2 hours after the infusion. Oxygen saturation should be monitored with continuous pulse oximetry during the T cell infusion and for 2 hours following T cell infusion. Subjects will remain on the cell infusion unit for a minimum of 2 hours following infusion, or until resolution of any infusion-related toxicity deemed to pose a significant risk to the study subject as an outpatient.

Infusion Rate: Each cell infusion should be administered intravenously over approximately 20-30 minutes, adjusted as needed to comply with guidelines for endotoxin limits for parenteral drugs (£ 5 EU/kg/hour). The infusion rate can also be adjusted if subjects experience mild infusion-related adverse events (grade 2 or lower).

The primary objective of this study is to estimate the maximum tolerated dose (MTD) of CAR T cells. The MTD for these purposes will be defined as a true dose limiting toxicity rate of 25%, where DLT is defined as Grade 3 or higher non-hematologic toxicity attributable to the CAR T cell infusion occurring within 28 days of the infusion, lasting at least 4 days, and not responsive to tociluzimab, dexamethasone, or other anti-inflammatory drugs. A modification of the continual reassessment method

(CRM) will be used to estimate the MTD. The modifications include treating patients in groups of two (rather than one), and allowing a maximum increase of one dose level between groups. Patients will receive a single intravenous infusion of CD20 CAR T cells at one of four escalating dose levels beginning with dose level 1 for the first group of two patients. Dose escalation or de-escalation is determined by the CRM algorithm, taking into account the number of patients experiencing a serious toxicity at each dose level (see above).

Treatment of patients in the dose-escalation/de-escalation groups will be staggered such that a minimum of a 28-day interval following infusion is required between each set of 2 patients before escalating to the next dose level. These dose levels will be initially evaluated in combination with CY alone, evaluating the CR rate to determine if CY alone has sufficient activity or if fludarabine will be added (CY/flu). If any criteria are met to switch to CY/flu, the CRM will be reinitiated starting at one dose level below the interim recommended dose (with CY alone) in combination with CY/flu. The interim recommended dose will be defined as lower of either the maximum dose evaluated to date or the next dose that would have been selected based on the mCRM following the 8th, 16th, or 20th patient for the 1st, 2nd and 3rd interim analyses. This evaluation will continue to a total of 30 patients. If none of these criteria are met, the CRM approach will continue with CY alone in an additional 10 patients (to reach a total of 30 patients). For patients who receive a second infusion, DLT and efficacy outcomes will be evaluated based on the dose of their primary infusion.

Patients receiving CD20 CAR T cells may develop serious toxicity due to T cell activation, proliferation, and cytokine secretion after encounter with tumor antigen. Cytokine release syndrome, macrophage activation, and neurotoxicity may occur and require intensive care support, and will not be considered DLTs if they are considered due to T cell recognition of the tumor unless these toxicities are not reversible after 4 consecutive days of treatment with corticosteroids and/or tocilizumab.

If there ever exists sufficient evidence to suggest that the true probability of treatment-related death by day 100 exceeds 20% (regardless of dose), enrollment of patients will be suspended pending a detailed review by the PI, study monitor, statistician, and DSMB. Sufficient evidence for this purpose will be defined as any observed outcome whose lower 80% confidence limit exceeds 20%.

Evaluations will also be performed to provide a preliminary assessment of efficacy. Secondary objectives of the study include an examination of efficacy (in terms of rate of remissions, progression-free survival, and in vivo persistence of T cells). These analyses will be performed using patients treated with all doses combined with the final lymphodepletion regimen (either CY alone or CY/fludarabine), modeling outcomes as a function of dose. A logistic regression model will be used to evaluate binary outcomes (CR and CR/PR). A Cox proportional hazards model will be used to evaluate time-to-event outcomes (PFS, OS). No formal statistical hypotheses will be tested with respect to these endpoints; rather, estimates and associated confidence intervals will be provided descriptively.

Additional secondary objectives are to evaluate of the duration of persistence of adoptively transferred CD20 CAR T cells and the migration of adoptively transferred CD20 CAR T cells. To evaluate the persistence of the CAR T cells, the patient-level area under the curve (AUC) will be estimated and the summary statistics of the AUCs will be evaluated. Migration (if CAR T cells are present post treatment), is defined as the presence of CAR T cells in the tumor at day 10-16 and, if applicable, the BM at day 28. The association between AUC and migration with clinical outcomes will mostly descriptive in nature including graphical presentation.

To evaluate the secondary objectives associated with evaluating biological causes of treatment resistance, the following analyses will be performed. A paired t-test will be used to compare the biomarker profiles between baseline tumors and post-treatment tumors with appropriate transformation if needed. A logistic regression model will be used to evaluate the association between baseline biomarker values and response. A landmark analysis among patients achieving a CR or PR at 1 month, measuring survival times (PFS and OS) from the landmark time, using a Cox proportional hazard regression model to evaluate the association of correlates measured at the time of CR/PR for patients in whom a biopsy is acquired at that time. Models will include values for all patients/dose levels and include a variable for dose level.

The development of endogenous anti-tumor responses and epitope spreading will also be assessed in a largely exploratory fashion. Data at each time point will be summarized, and with sufficient data, a mixed effect model will be used to model time-varying outcomes. Differential gene expression analysis will be conducted between patients with and without demonstrated epitope spreading to identify the biomarker associated with immune response.

Patients in the study who failed to achieve a CR, or who achieve a complete response (CR) but later relapse, who wish to receive a second infusion of CD20 CAR T cells may be eligible to do so, provided that a sufficient number of CD20 CAR T cells can be produced and the criteria listed below are met:

a. There is evidence of persistent disease after the first T cell infusion, or the tumor relapses after a CR.

b. There were no toxicities attributed to the first infusion that were dose-limiting or required dose de-escalation

c. The patient is $\geq$ 30 days from the first T cell infusion.

d. There are no clinical and/or laboratory exclusion criteria (Patients who achieved a CR and later relapsed must have a post-relapse biopsy demonstrating ongoing CD20 expression on the tumor cells.

Participants will undergo evaluations at screening, prior to lymphodepleting chemotherapy, during T cell infusions, and at intervals following each T cell infusion. The following data will be obtained for safety and toxicity assessment, according to the clinical protocol:

- History and physical exam before and at intervals after T cell infusions.
- Pulse oximetry before and during the infusion
- Hematologic, hepatic, renal, and electrolyte blood tests before and at intervals after the T cell infusion
- Lab tests evaluating for tumor lysis syndrome, coagulopathy, and cytokine release syndrome before and at intervals after the T cell infusion.
- Toxicity grading according to NCI CTCAE Version 4.0
- Serum cytokine levels

- B cell reconstitution
- Serum immunoglobulin levels
- Replication competent lentivirus testing
- Persistence of genetically modified T cells
- Adverse event reporting

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. An isolated polynucleotide encoding a fusion protein capable of specifically binding CD20, wherein the polynucleotide:

(a) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:53-56;

(b) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:44-47;

(c) comprises a polynucleotide sequence of any one of SEQ ID NOS.:53-56;

(d) comprises a polynucleotide sequence of any one of SEQ ID NOS.:44-47;

(e) consists of a polynucleotide sequence of any one of SEQ ID NOS.:53-56; or

(f) consists of a polynucleotide sequence of any one of SEQ ID NOS.:44-47.

2. A fusion protein encoded by the polynucleotide according to claim 1.

3. The fusion protein according to claim 2, wherein the fusion protein:

(a) is at least 90% identical to a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed;

(b) is comprised of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed;

(c) consists of a mature fusion protein, wherein the mature fusion protein comprises an amino acid sequence of any one of SEQ ID NOS.: 35-38 with the tCD19 transduction marker removed;

(d) is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26-29;

(e) is comprised of an amino acid sequence of any one of SEQ ID NOS.:26-29; or

(f) consists of an amino acid sequence of any one of SEQ ID NOS.:26-29.

4. A host cell, the host comprising the polynucleotide according to claim 1 and capable of expressing the fusion protein according to claim 2 or claim 3.

5. The host cell according to claim 4, wherein the polynucleotide is codon optimized.

6. The host cell according to claim 4 or 5, wherein the host cell is a T cell or a T cell autologous to a subject.

7. The host cell according to claim 6, wherein the T cell is a $CD8^+$ T cell, a $CD4^+$ T cell, or both.

8. The host cell according to claim 7, wherein the T cell is a bulk population or a subpopulation of T cells, optionally a subpopulation of central memory cells or central memory and naïve cells.

9. A composition comprising the host cell according to any one of claims 4-8 and a CD20-specific binding molecule.

10. The composition according to claim 9, wherein the CD20-specific binding molecule is an antibody.

11. The composition according to claim 10, wherein the antibody is 1.5.3, 1F5, Leu16, rituximab, ofatumumab, ocrelizumab, veltuzumab, ublituximab, or any combination thereof.

12. A method of treating a disease or disorder associated with CD20 expression in a subject having or suspected of having a disease or disorder associated with CD20 expression, comprising administering a therapeutically effective amount of a host cell according to any one of claims 4-8.

13. The method according to claim 12, wherein the host cell is a T cell or a T cell autologous to the subject.

14. The method according to claim 13, wherein the T cell is a $CD8^+$ T cell, a $CD4^+$ T cell, or both.

15. The method according to claim 13 or 14, wherein the T cell is a bulk population or a subpopulation of T cells, optionally a subpopulation of central memory cells or central memory and naïve cells.

16. The method according to any one of claims 12-15, wherein the method reduces the number of B-cells or treats a disease or disorder associated with aberrant B-cell activity.

17. The method according to any one of claims 12-16, wherein the disorder or disease associated with CD20 expression is a B-cell lymphoma or leukemia such as B-cell non-Hodgkins lymphoma (NHL) (including Burkitt's lymphoma, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma), hairy cell leukemia, Waldenström's macroglobulinemia, B-cell pro-lymphocytic leukemia, CD37+ dendritic cell lymphoma, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, extra-nodal marginal zone B-cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, and primary effusion lymphoma; a disease characterized by autoantibody production such as idiopathic inflammatory

myopathy, rheumatoid arthritis, juvenile rheumatoid arthritis, myasthenia gravis, Grave's disease, type I diabetes mellitus, anti-glomerular basement membrane disease, rapidly progressive glomerulonephritis, Berger's disease (IgA nephropathy), systemic lupus erythematosus (SLE), Crohn's disease, ulcerative colitis, idiopathic thrombocytopenic purpura (ITP), anti-phospholipid antibody syndrome, neuromyelitis optica, multiple sclerosis, an autoimmune disease, dermatomyositis, polymyositis, or Waldenstrom's macroglobinemia; a disease characterized by inappropriate T-cell stimulation associated with a B-cell pathway; multiple myeloma; or melanoma.

18. The method according to any one of claims 12-17, wherein the subject has been pre-treated with a CD20-specific binding molecule, optionally wherein the CD20-specific binding molecule is 1.5.3, 1F5, Leu16, rituximab, ofatumumab, ocrelizumab, veltuzumab, ublituximab, or any combination thereof.

19. The method according to any one of claims 12-18, wherein the subject is administered a therapeutically effective amount of a CD20-specific binding molecule concurrent to, simultaneous with, or subsequent to the host cell.

20. The method according to claim 19, wherein the CD20-specific binding molecule is an antibody.

21. The method according to claim 20, wherein the antibody is selected from the group consisting of 1.5.3, 1F5, Leu16, rituximab, ofatumumab, ocrelizumab, veltuzumab, ublituximab, or any combination thereof.

22. A method of treating a disease or disorder associated with CD20 expression, comprising administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a CD20-specific binding molecule and a therapeutically effective amount of a host cell comprising a heterologous polynucleotide encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component

connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain.

23. The method according to claim 22, wherein the CD20-specific binding molecule is an antibody.

24. The method according to claim 23, wherein the antibody is1.5.3, 1F5, Leu16, rituximab, ofatumumab, ocrelizumab, veltuzumab, ublituximab, or any combination thereof.

25. The method according to any one of claims 22-24, wherein the fusion protein is a chimeric antigen receptor.

26. The method according to any one of claims 22-25, wherein the CD20-specific binding domain is a scFv or scTCR.

27. The method according to any one of claims 22-26, wherein the fusion protein comprises an scFv from 1.5.3, 1F5, Leu16, rituximab, ofatumumab, ocrelizumab, veltuzumab, ublituximab, or any combination thereof.

28. The method according to claim 27, wherein the scFv is:

(a) a 1.5.3 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:64, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody;

(b) a 1.5.3 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:64;

(c) a 1F5 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:66, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody;

(d) a 1F5 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:66;

(e) a Leu16 scFv comprising an amino acid sequence that is at least 90% identical to an amino acid sequence of SEQ ID NO.:65, wherein each CDR of the scFv comprises zero changes or at most one, two, three, four, five or six changes as compared to the corresponding CDR of a parent monoclonal antibody or fragment or derivative thereof that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or

(f) a Leu16 scFv comprising or consisting of an amino acid sequence of SEQ ID NO.:65.

29. The method according to claim 27 or 28, wherein the scFv is encoded by:

(a) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:67, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody;

(b) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:67;

(c) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:69, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody;

(d) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:69;

(e) a polynucleotide having at least 80% identity to a nucleic acid molecule sequence of SEQ ID NO.:68, wherein polynucleotide sequences encoding each CDR of a scFv comprises zero changes or at most one to six nucleotide changes, as compared to a polynucleotide encoding a parent scFv from a monoclonal antibody that specifically binds to CD20, provided that scFv containing one or more modified CDRs specifically binds CD20 with an affinity similar to the wild type scFv or corresponding antibody; or

(f) a polynucleotide comprising or consisting of a nucleic acid molecule sequence of SEQ ID NO.:68.

30. The method according to any one of claims 22-29, wherein the fusion protein is a chimeric antigen receptor that is at least 90% identical to an amino acid sequence of any one of SEQ ID NOS.:26-43.

31. The method according to any one of claims 22-30, wherein the hydrophobic portion is a transmembrane domain.

32. The method according to claim 31, wherein the transmembrane domain is a CD4, CD8, CD28 or CD27 transmembrane domain.

33. The method according to any one of claims 22-32, wherein the effector domain or functional portion thereof is a 4-1BB (CD137), CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD79A, CD79B, CARD11, DAP10, FcRα, FcRβ, FcRγ, Fyn, HVEM, ICOS, Lck, LAG3, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, OX40 (CD134), ROR2, Ryk, SLAMF1, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

34. The method according to any one of claims 22-33, wherein the intracellular component comprises:

(a) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof;

(b) a CD3ζ effector domain or functional portion thereof, a CD28 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof;

(c) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a 4-1BB (CD137) costimulatory domain or portion thereof;

(d) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof;

(e) a CD3ζ effector domain or functional portion thereof, a CD27 costimulatory domain or functional portion thereof and a CD28 costimulatory domain or portion thereof; or

(f) a CD3ζ effector domain or functional portion thereof, a 4-1BB (CD137) costimulatory domain or functional portion thereof and a OX40 (CD134) costimulatory domain or portion thereof .

35. The method according to any one of claims 22-34, wherein the CD20-specific binding molecule and the host cell comprising the heterologous nucleic acid molecule encoding the fusion protein are administered simultaneously, sequentially, or concurrently.

36. The method according to any one of claims 22-35, wherein the host cell is a T cell or a T cell autologous to the subject.

37. The method according to claim 36, wherein the T cell is a $CD8^+$ T cell, a $CD4^+$ T cell, or both.

38. The method according to claim 36 or 37, wherein the T cell is a bulk population or a subpopulation of T cells, optionally a subpopulation of central memory cells or central memory and naïve cells.

39. The method according to any one of claims 22-38, wherein the method reduces the number of B-cells or treats a disease or disorder associated with aberrant B-cell activity.

40. The method according to any one of claims22-39, wherein the disorder or disease associated with CD20 expression is a B-cell lymphoma or leukemia such as B-cell non-Hodgkins lymphoma (NHL) (including Burkitt's lymphoma, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma), hairy cell leukemia, Waldenström's macroglobulinemia, B-cell pro-lymphocytic leukemia, CD37+ dendritic cell lymphoma, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, extra-nodal marginal zone B-cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, and primary effusion lymphoma; a disease characterized by autoantibody production such as idiopathic inflammatory myopathy, rheumatoid arthritis, juvenile rheumatoid arthritis, myasthenia gravis, Grave’s disease, type I diabetes mellitus, anti-glomerular basement membrane disease, rapidly progressive glomerulonephritis, Berger's disease (IgA nephropathy), systemic lupus erythematosus (SLE), Crohn's disease, ulcerative colitis, idiopathic thrombocytopenic purpura (ITP), anti-phospholipid antibody syndrome, neuromyelitis optica, multiple sclerosis, an autoimmune disease, dermatomyositis, polymyositis, or Waldenstrom’s macroglobinemia; a disease characterized by inappropriate T-cell stimulation associated with a B-cell pathway; multiple myeloma; or melanoma.

41. The method according to any one of claims 12-40, wherein the host cell comprises a heterologous polynucleotide encoding a suicide transduction marker.

42, The method according to claim 41, further comprising administering a cytotoxic antibody, antibody-drug conjugate, or other cytotoxic agent capable of specifically binding the transduction marker and thereby causing a cytotoxic effect on the host cell expressing the fusion protein.

43. The method according to claim 41 or 42, wherein the suicide transduction marker comprises a truncated EGFR polypeptide.

44. The method according to claim 43, wherein the truncated EGFR polypeptide comprises an amino acid sequence of SEQ ID NO.: 25.

45. The method according to claim 43 or 44, wherein the cytotoxic agent comprises an anti-EGFR antibody.

46. The method according to claim 45, wherein the anti-EGFR antibody is cetuximab.

47. The method according to claim 41 or 42, wherein the suicide transduction marker comprises a truncated CD19 polypeptide.

48. The method according to claim 47, wherein the truncated CD19 polypeptide comprises an amino acid sequence of SEQ ID NO.: 24.

49. The method according to claim 47 or 48, wherein the cytotoxic agent is selected from the group consisting of blinatumomab, coltuximabravtansine, MOR208, MEDI-551, denintuzumabmafodotin, Merck patent anti-CD19, taplutumomabpaptox, XmAb 5871, MDX-1342, SAR3419, SGN-19A, or AFM11.

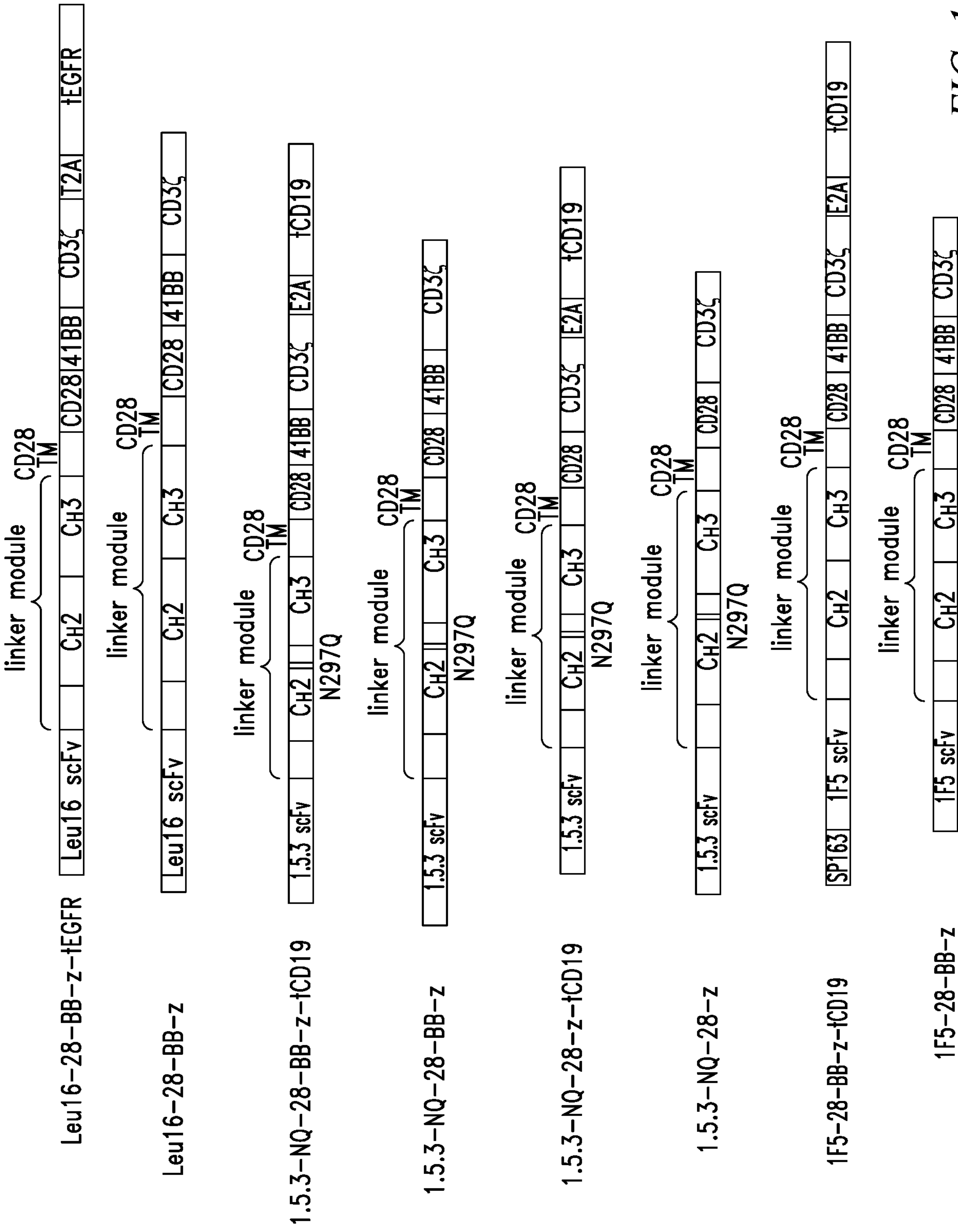

Leu16-28-BB-z-tEGFR
Leu16 scFv
linker module
CH2
CH3
CD28 TM
CD28
41BB
CD3ζ
T2A
tEGFR
Leu16-28-BB-z
1.5.3-NQ-28-BB-z-tCD19
1.5.3 scFv
N297Q
E2A
tCD19
1.5.3-NQ-28-BB-z
1.5.3-NQ-28-z-tCD19
1.5.3-NQ-28-z
1F5-28-BB-z-tCD19
SP163
1F5 scFv
1F5-28-BB-z

FIG. 1A

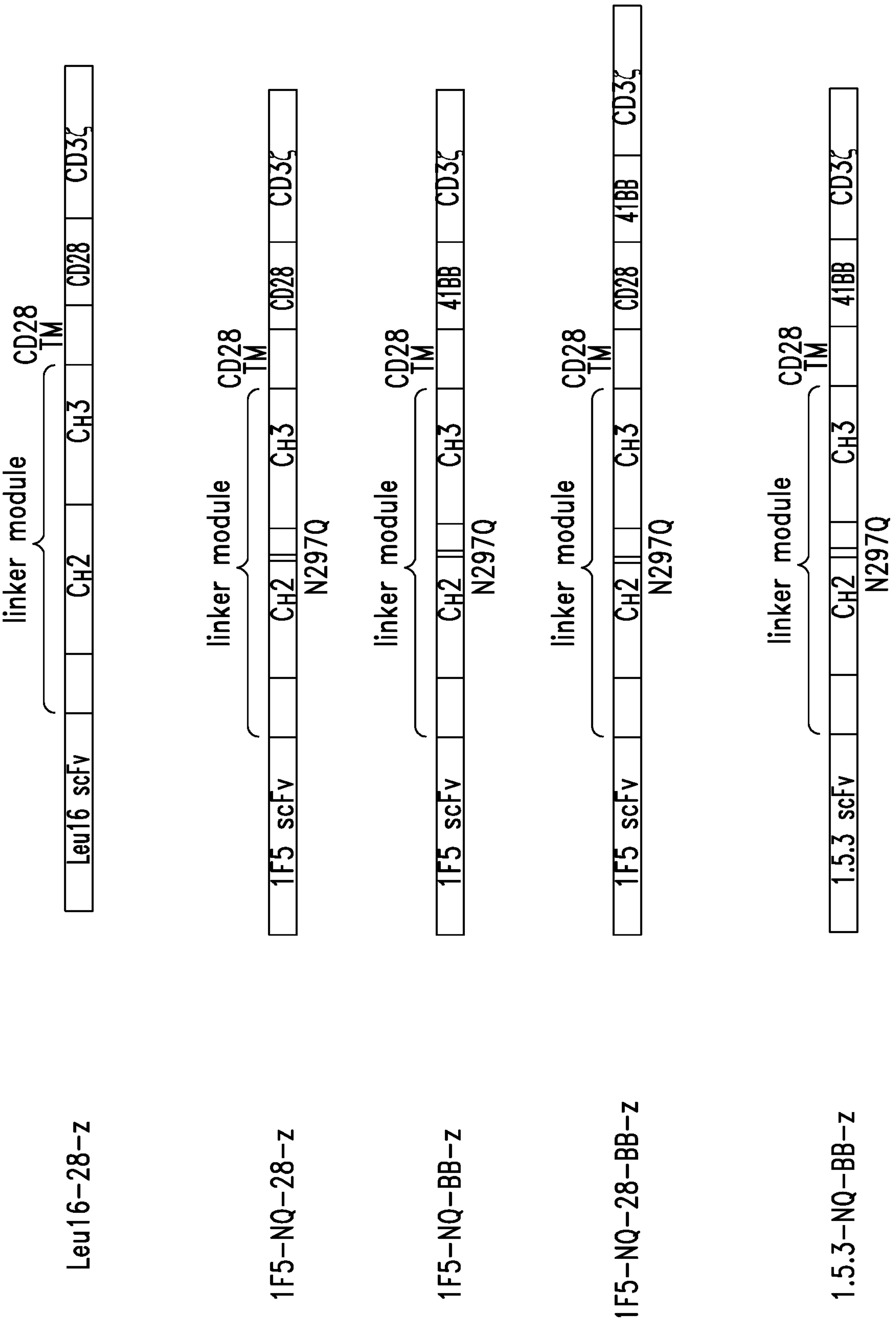

Leu16-28-z
Leu16 scFv
CH2
CH3
CD28
CD3ζ
linker module
CD28 TM
1F5-NQ-28-z
1F5 scFv
N297Q
1F5-NQ-BB-z
41BB
1F5-NQ-28-BB-z
1.5.3-NQ-BB-z
1.5.3 scFv

FIG. 1B

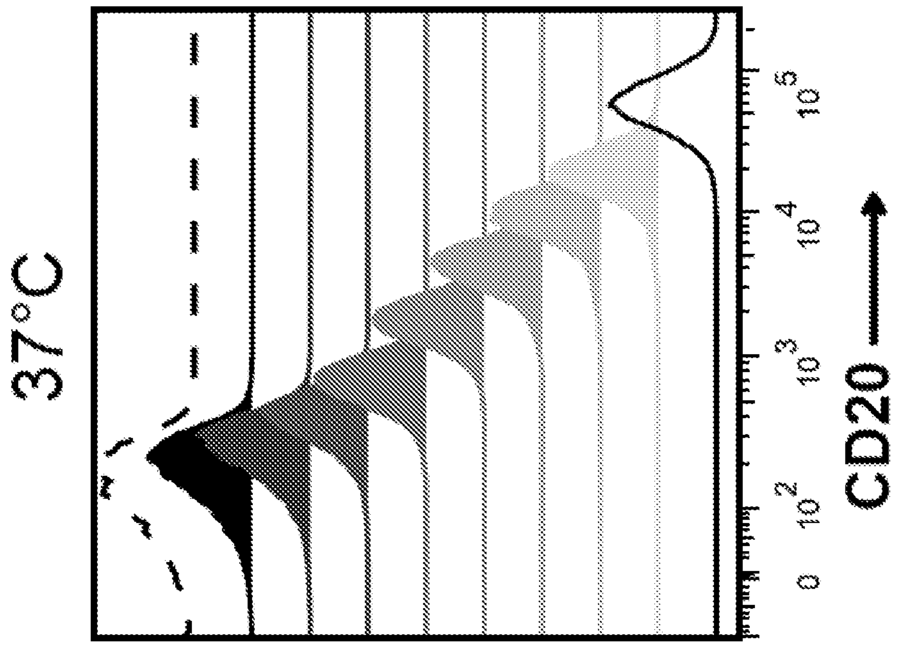

37°C
CD20
0
10^2
10^3
10^4
10^5

FIG. 2B

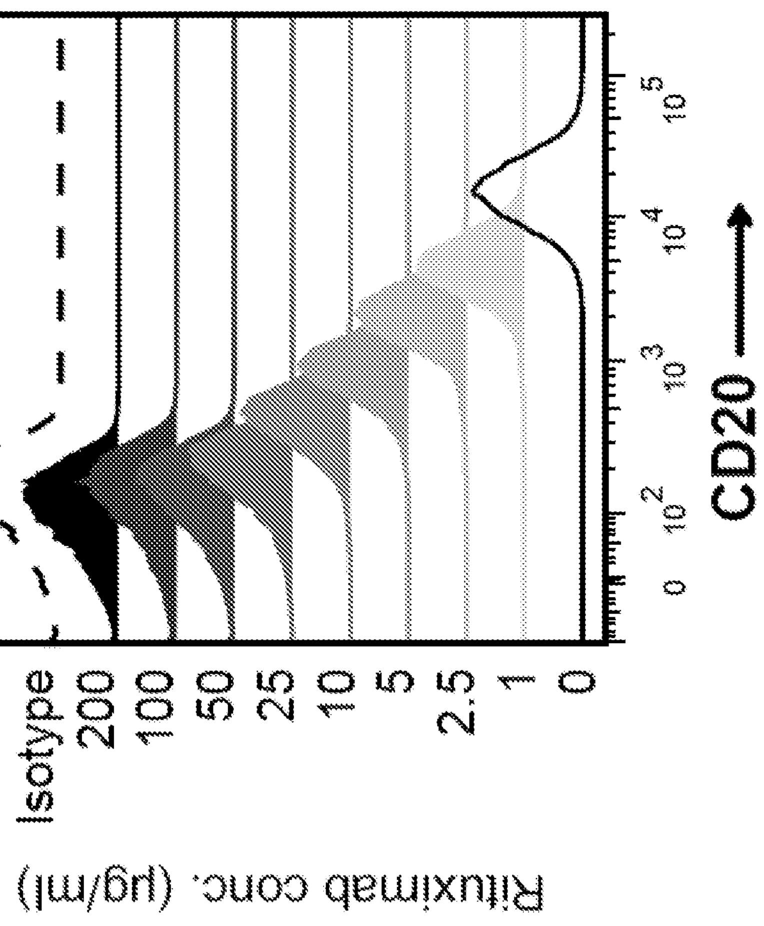

4°C
Rituximab conc. (μg/ml)
Isotype
200
100
50
25
10
5
2.5
1
0
CD20
0
10^2
10^3
10^4
10^5

FIG. 2A

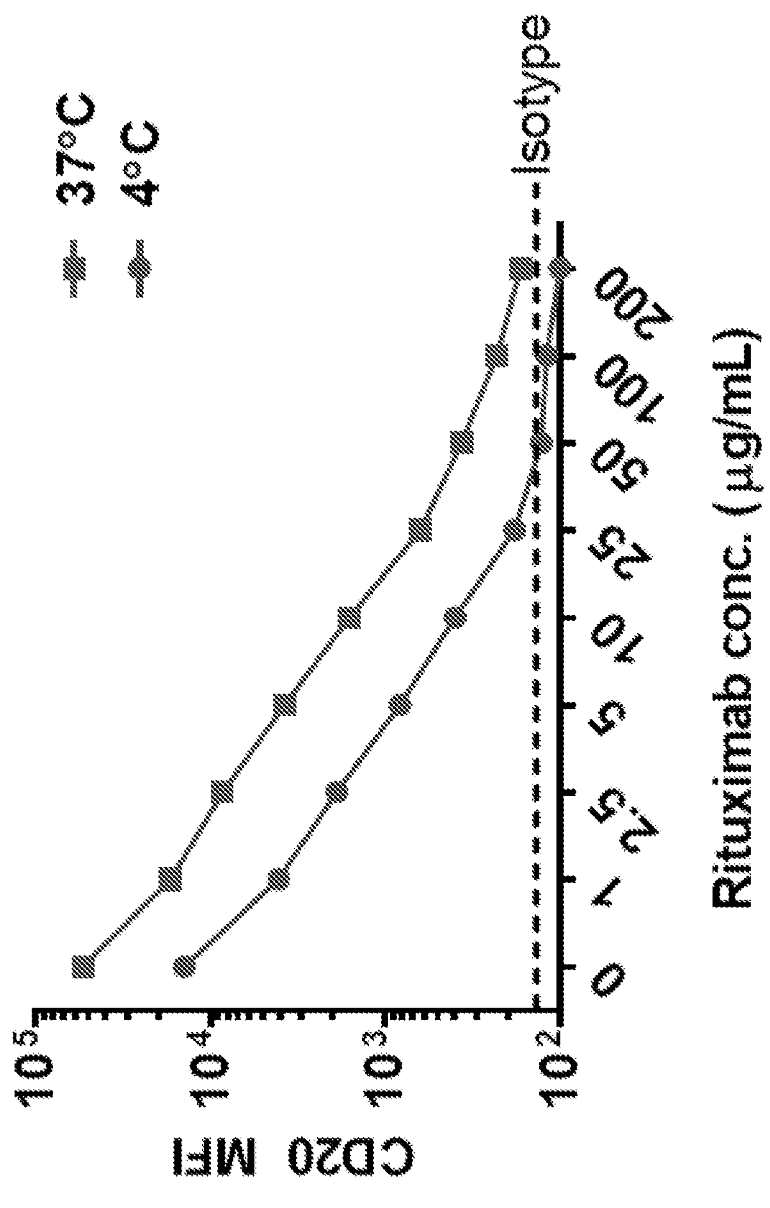

37°C
4°C
Isotype
CD20 MFI
10^5
10^4
10^3
10^2
0
1
2.5
5
10
25
50
100
200
Rituximab conc. (µg/mL)

FIG. 2C

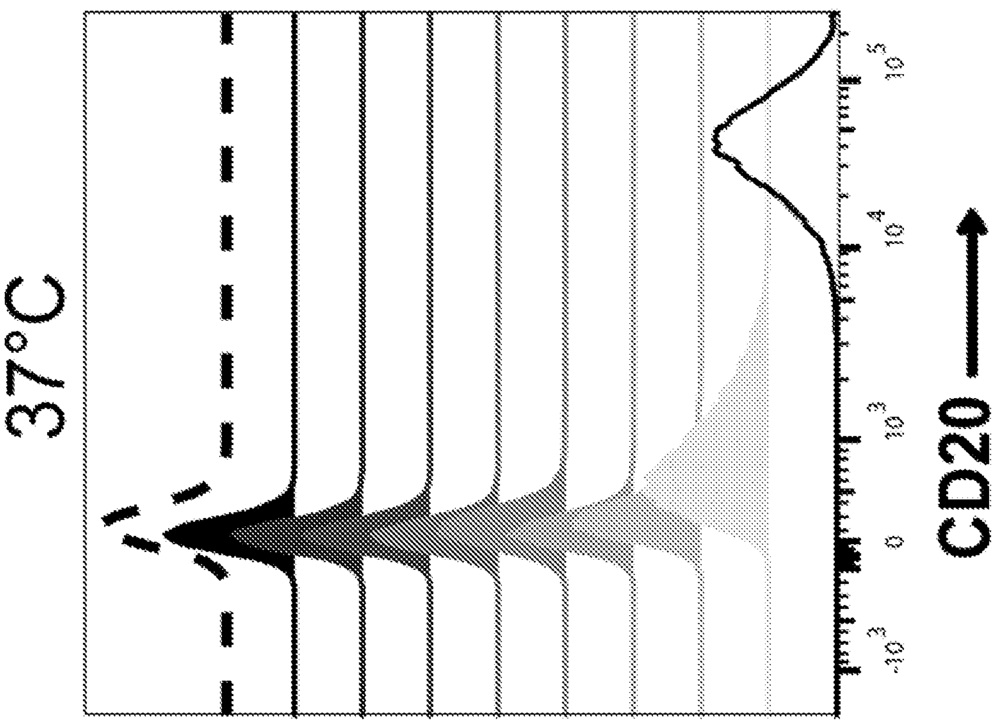

37°C
-10^3
0
10^3
10^4
10^5
CD20

FIG. 2E

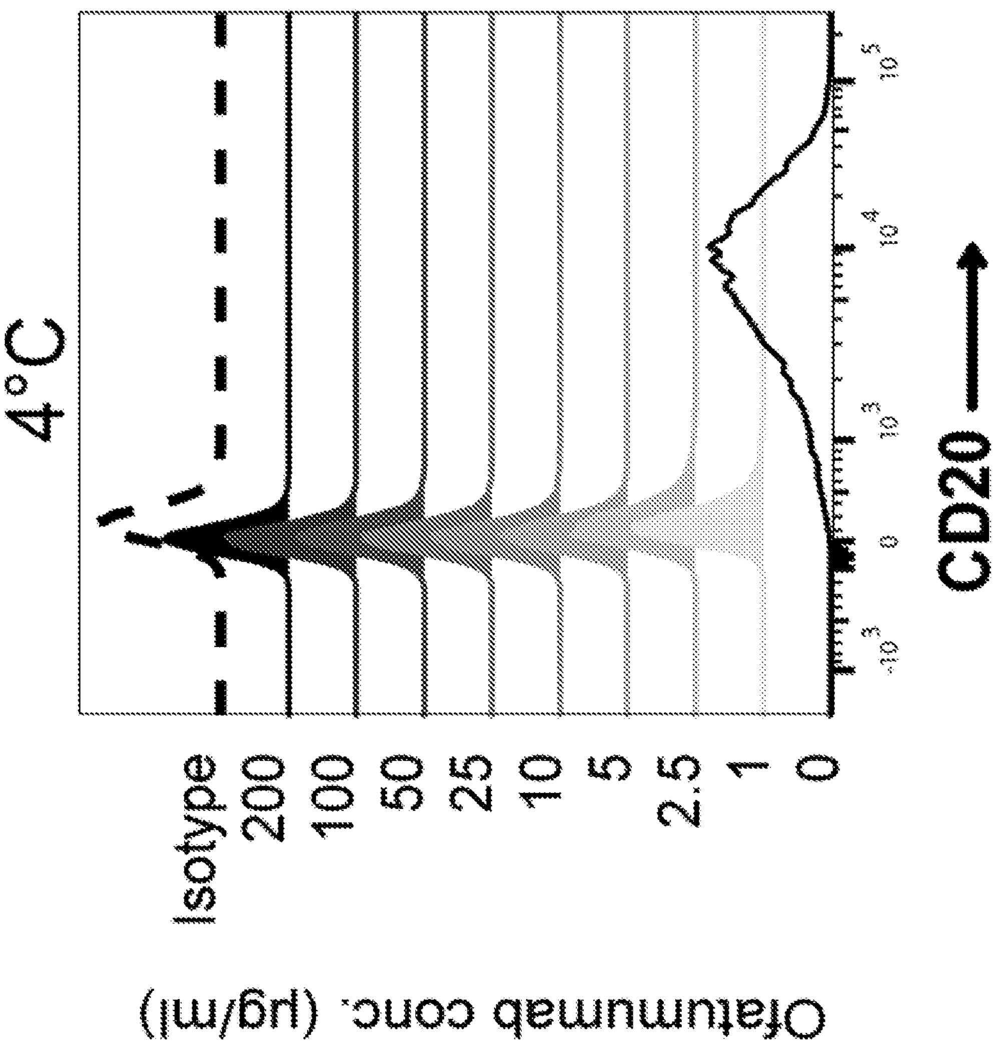

4°C
Ofatumumab conc. (µg/ml)
Isotype
200
100
50
25
10
5
2.5
1
0
-10^3
0
10^3
10^4
10^5
CD20

FIG. 2D

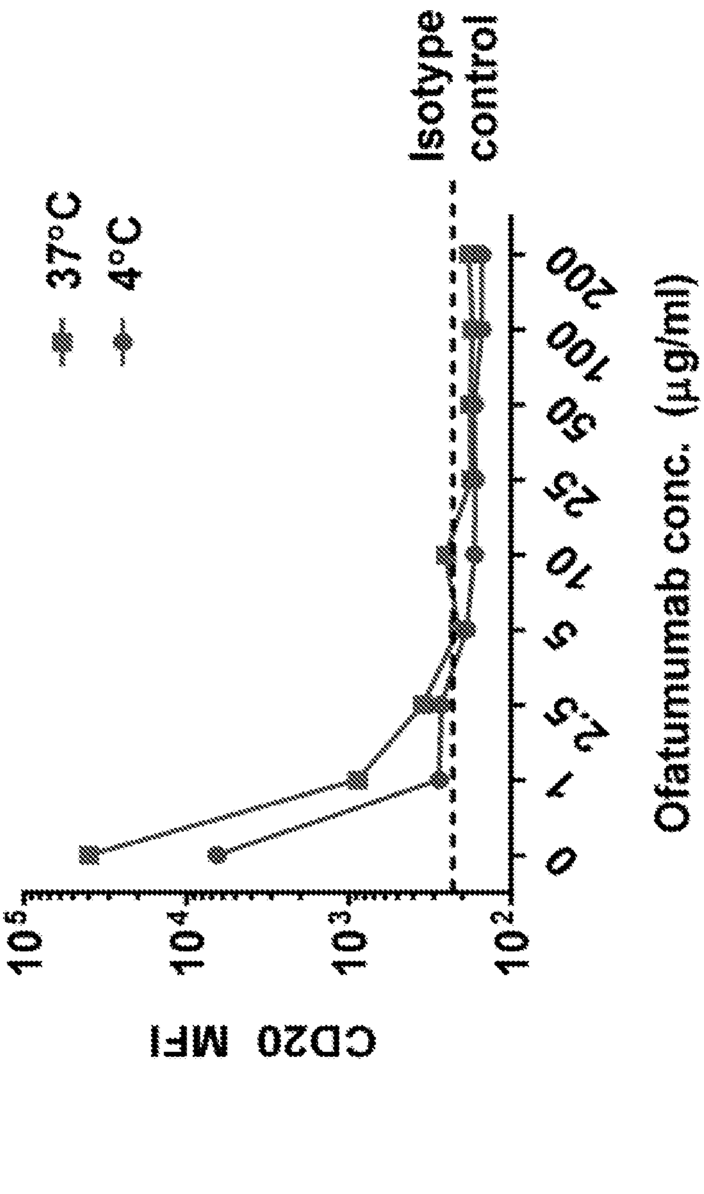

CD20 MFI
10^5
10^4
10^3
10^2
37°C
4°C
Isotype control
0
1
2.5
5
10
25
50
100
200
Ofatumumab conc. (µg/ml)

FIG. 2F

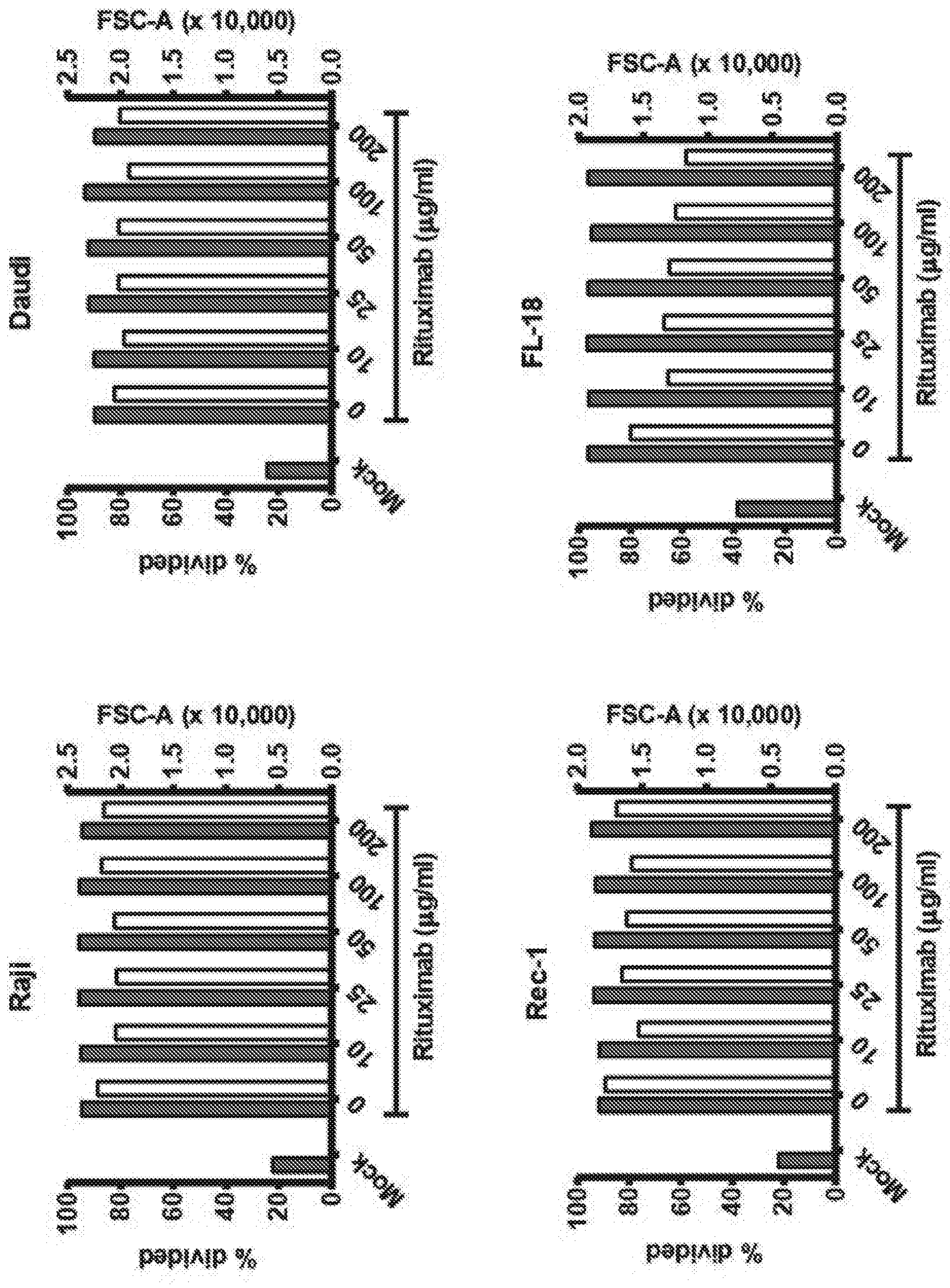
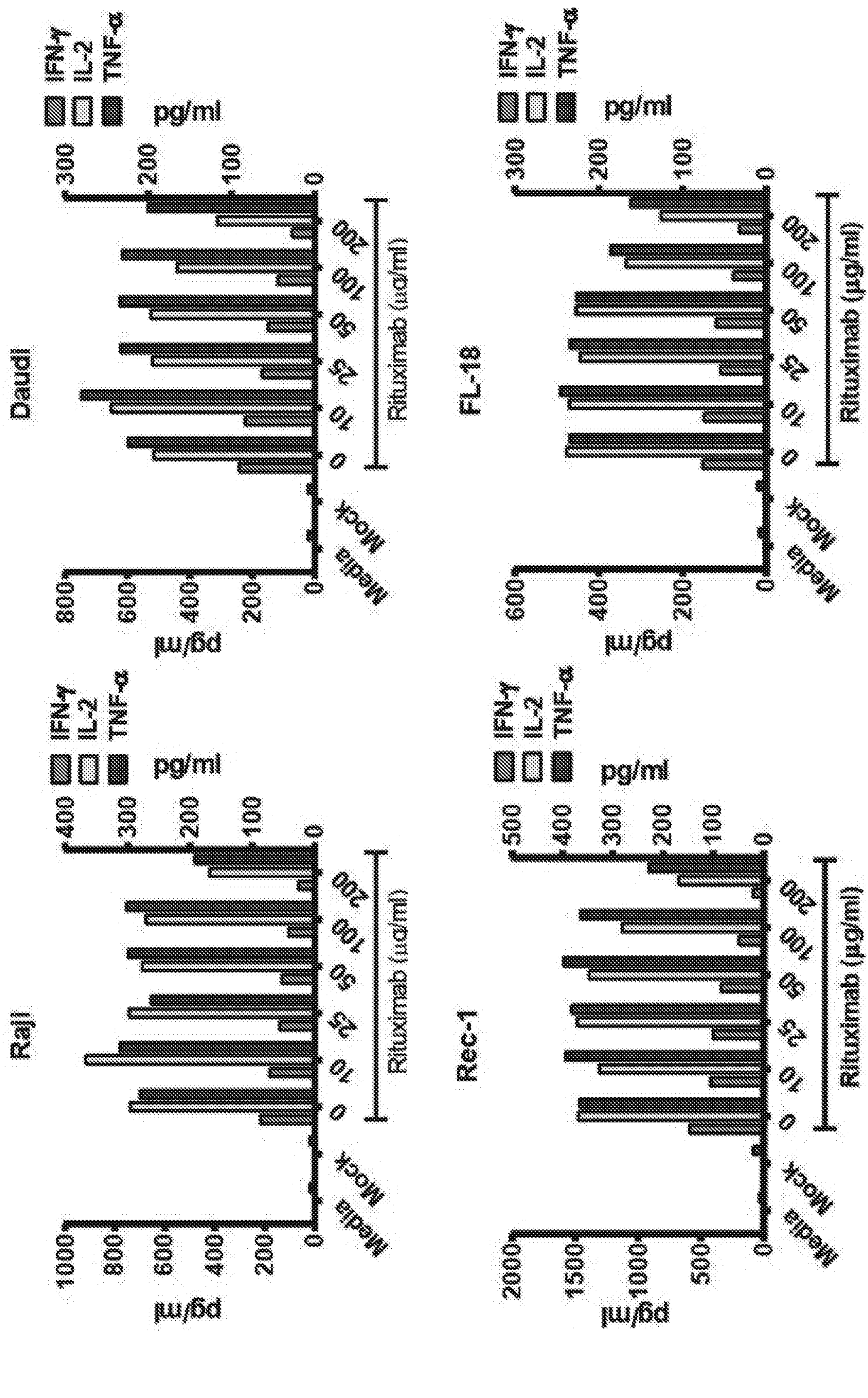

Daudi
FL-18
Raji
Rec-1
% divided
FSC-A (x 10,000)
Rituximab (µg/ml)
Mock
0
10
25
50
100
200

FIG. 3A



Raji
Daudi
Rec-1
FL-18
IFN-γ
IL-2
TNF-α
pg/ml
Media
Mock
Rituximab (μg/ml)

FIG. 3B

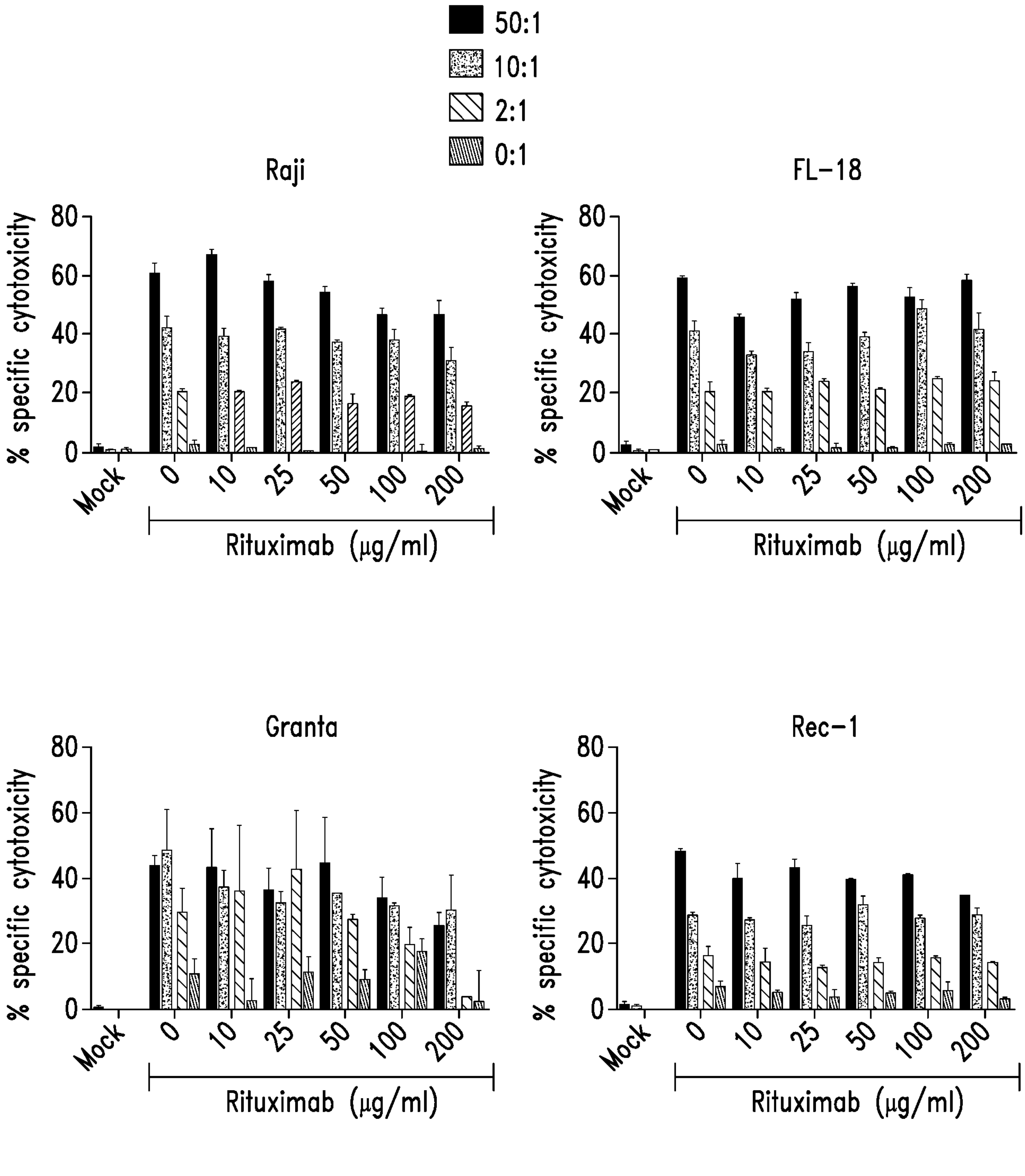

50:1
10:1
2:1
0:1
Raji
FL-18
Granta
Rec-1
% specific cytotoxicity
80
60
40
20
0
Mock
0
10
25
50
100
200
Rituximab (μg/ml)

FIG. 4

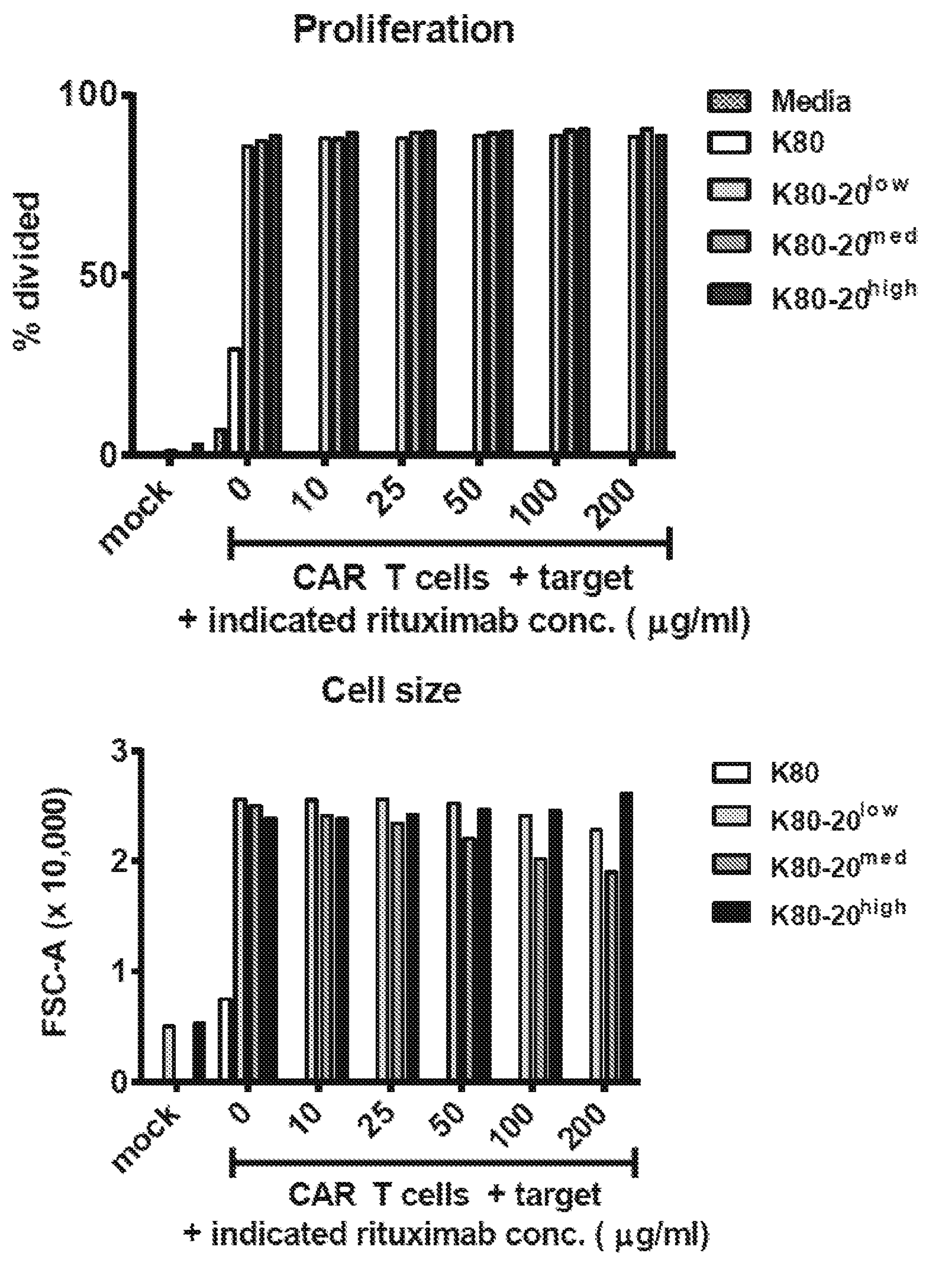

Proliferation
100
50
0
% divided
Media
K80
K80-20low
K80-20med
K80-20high
mock
0
10
25
50
100
200
CAR T cells + target
+ indicated rituximab conc. (µg/ml)
Cell size
3
2
1
0
FSC-A (x 10,000)
K80
K80-20low
K80-20med
K80-20high
mock
0
10
25
50
100
200
CAR T cells + target
+ indicated rituximab conc. (µg/ml)

FIG. 5A

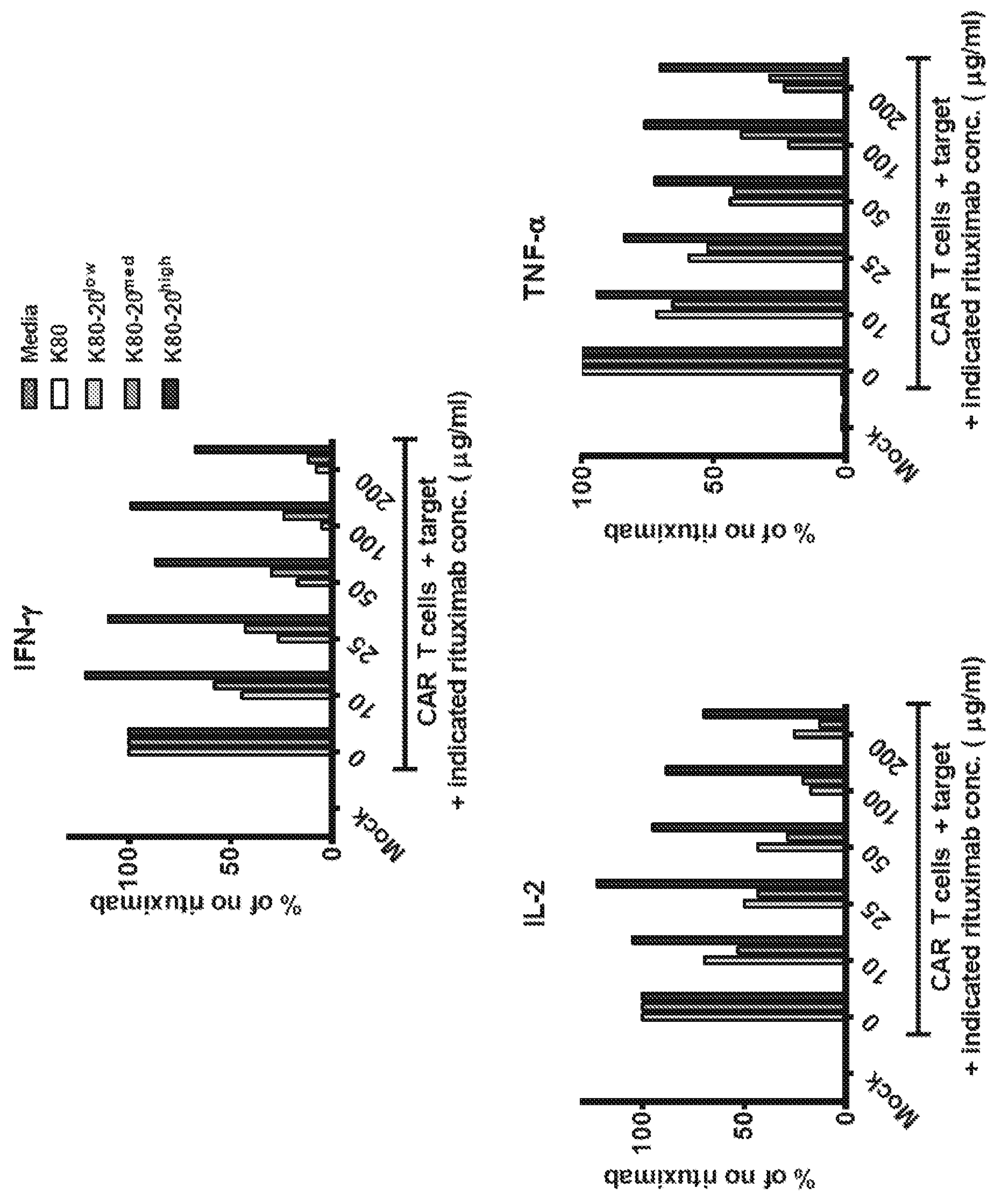

IFN-γ
TNF-α
IL-2
Media
K80
K80-20low
K80-20med
K80-20high
% of no rituximab
100
50
0
Mock
0
10
25
50
100
200
CAR T cells + target
+ indicated rituximab conc. (μg/ml)

FIG. 5B

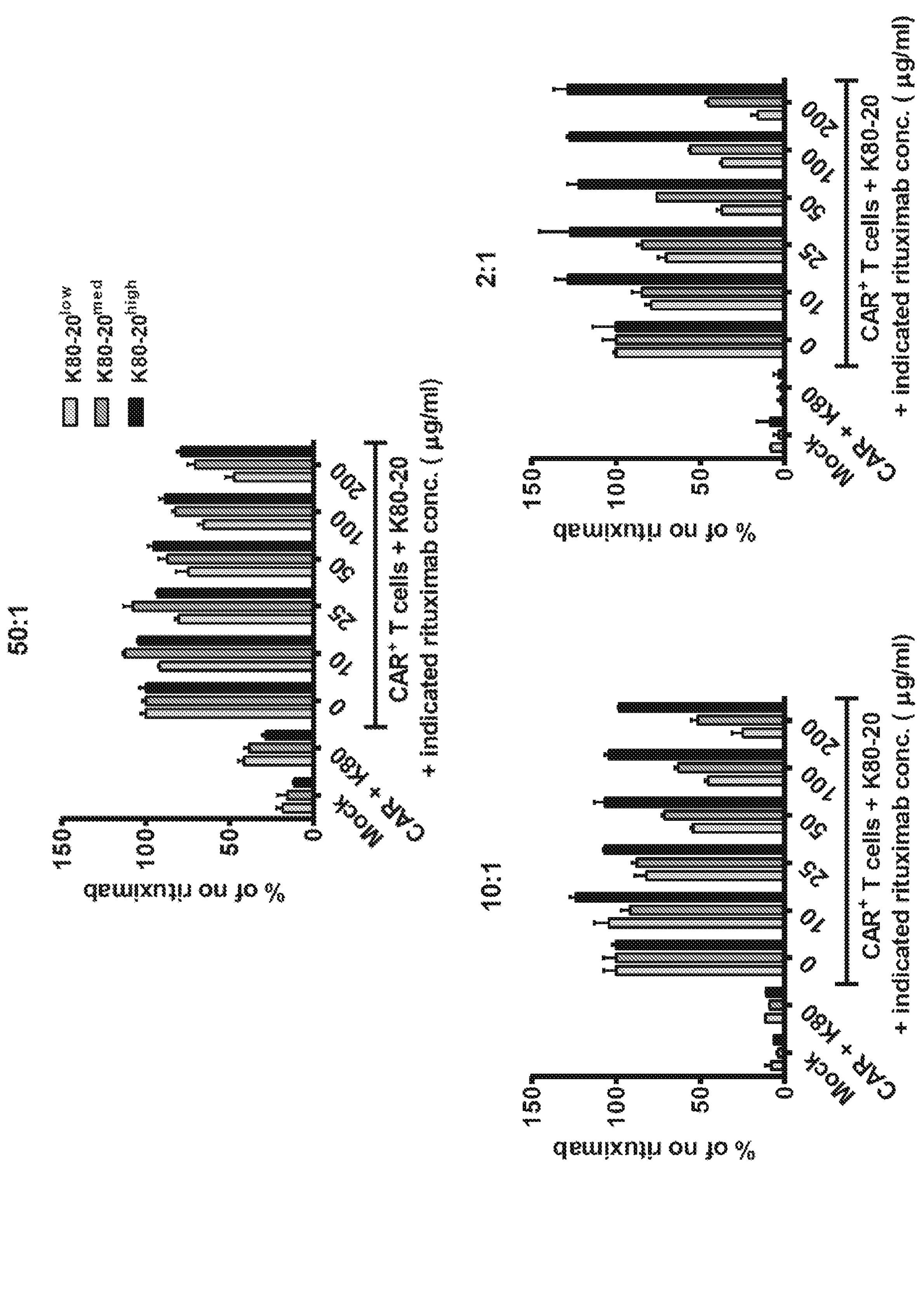

50:1
10:1
2:1
% of no rituximab
150
100
50
0
Mock
CAR + K80
0
10
25
50
100
200
CAR⁺ T cells + K80-20
+ indicated rituximab conc. (µg/ml)
K80-20low
K80-20med
K80-20high

FIG. 5C

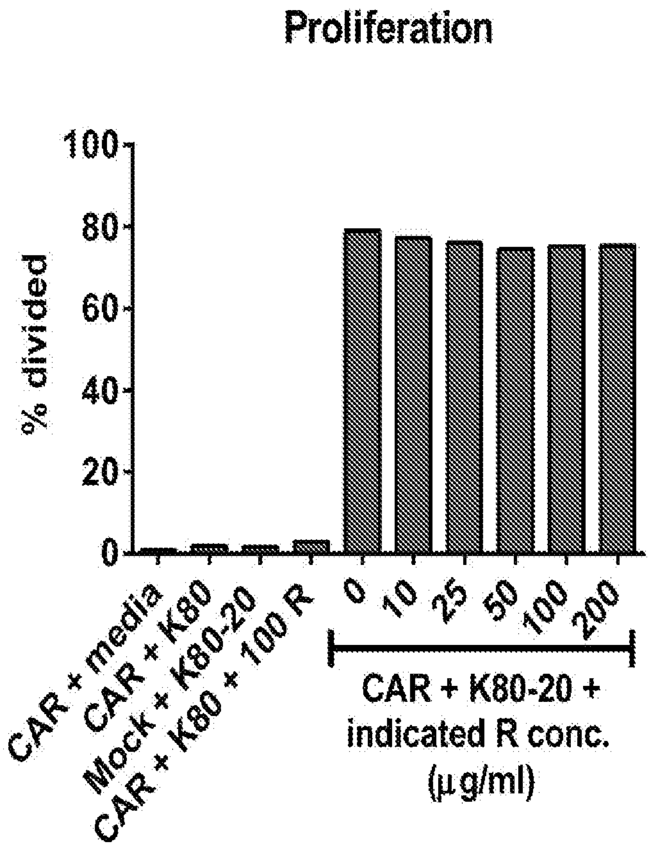

Proliferation
% divided
100
80
60
40
20
0
CAR + media
CAR + K80
Mock + K80-20
CAR + K80 + 100 R
0
10
25
50
100
200
CAR + K80-20 +
indicated R conc.
(μg/ml)

FIG. 6A

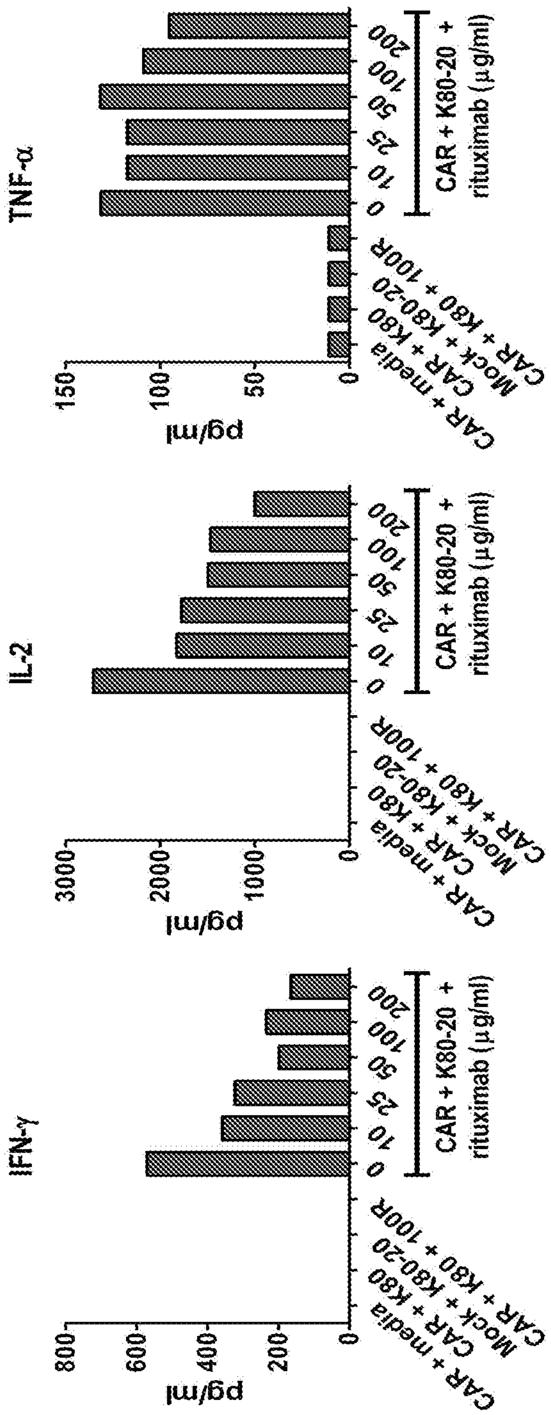

TNF-α
IL-2
IFN-γ
pg/ml
CAR + media
Mock + K80
CAR + K80
CAR + K80-20
CAR + K80 + 100R
0
10
25
50
100
200
CAR + K80-20 + rituximab (μg/ml)

FIG. 6B

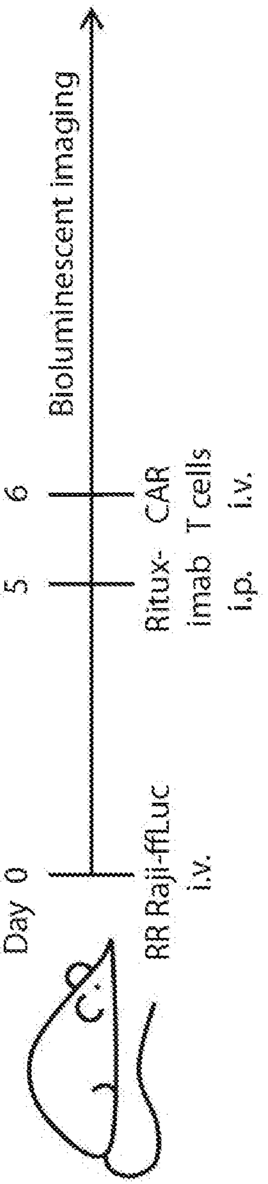
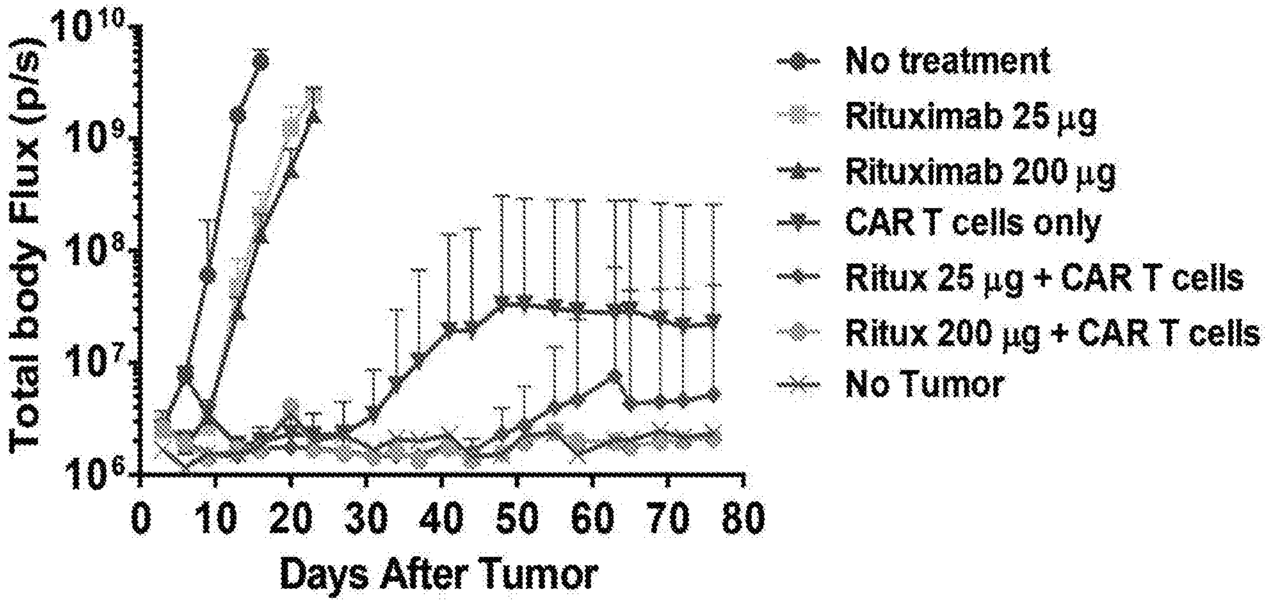
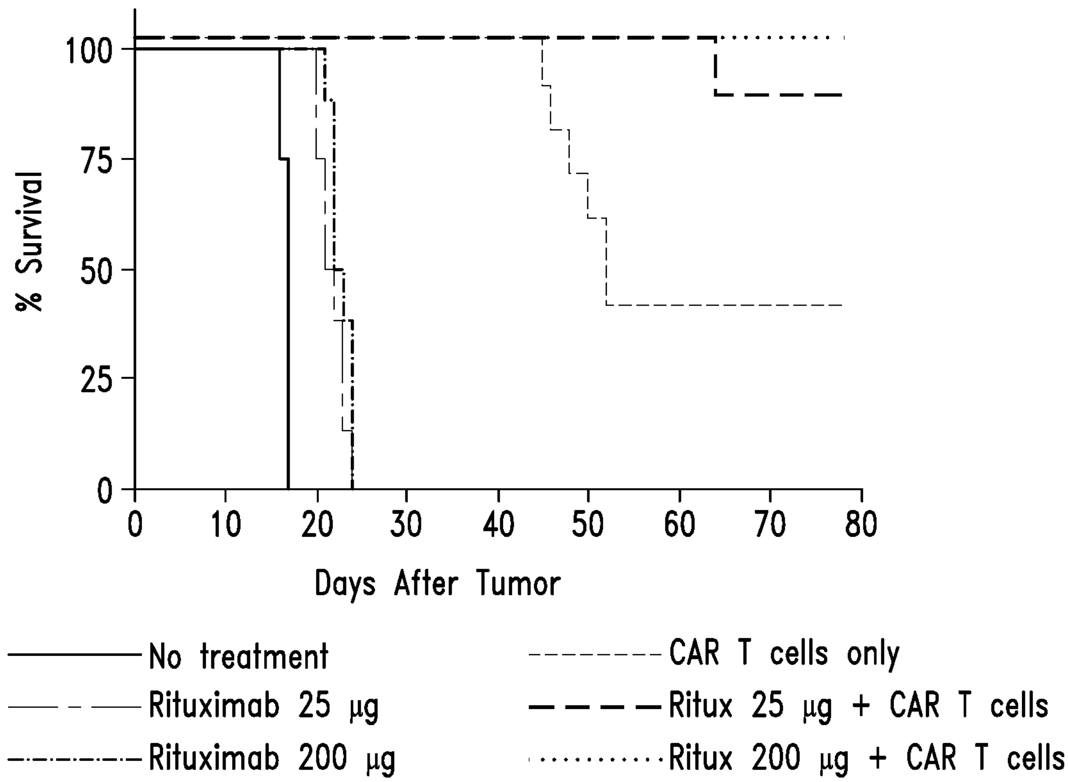

Day 0
RR Raji-ffLuc
i.v.
5
Ritux-
imab
i.p.
6
CAR
T cells
i.v.
Bioluminescent imaging

FIG. 7A



Total body Flux (p/s)
10^{10}
10^9
10^8
10^7
10^6
0 10 20 30 40 50 60 70 80
Days After Tumor
No treatment
Rituximab 25 µg
Rituximab 200 µg
CAR T cells only
Ritux 25 µg + CAR T cells
Ritux 200 µg + CAR T cells
No Tumor

FIG. 7B



% Survival
100
75
50
25
0
0 10 20 30 40 50 60 70 80
Days After Tumor
No treatment
Rituximab 25 µg
Rituximab 200 µg
CAR T cells only
Ritux 25 µg + CAR T cells
Ritux 200 µg + CAR T cells

FIG. 7C

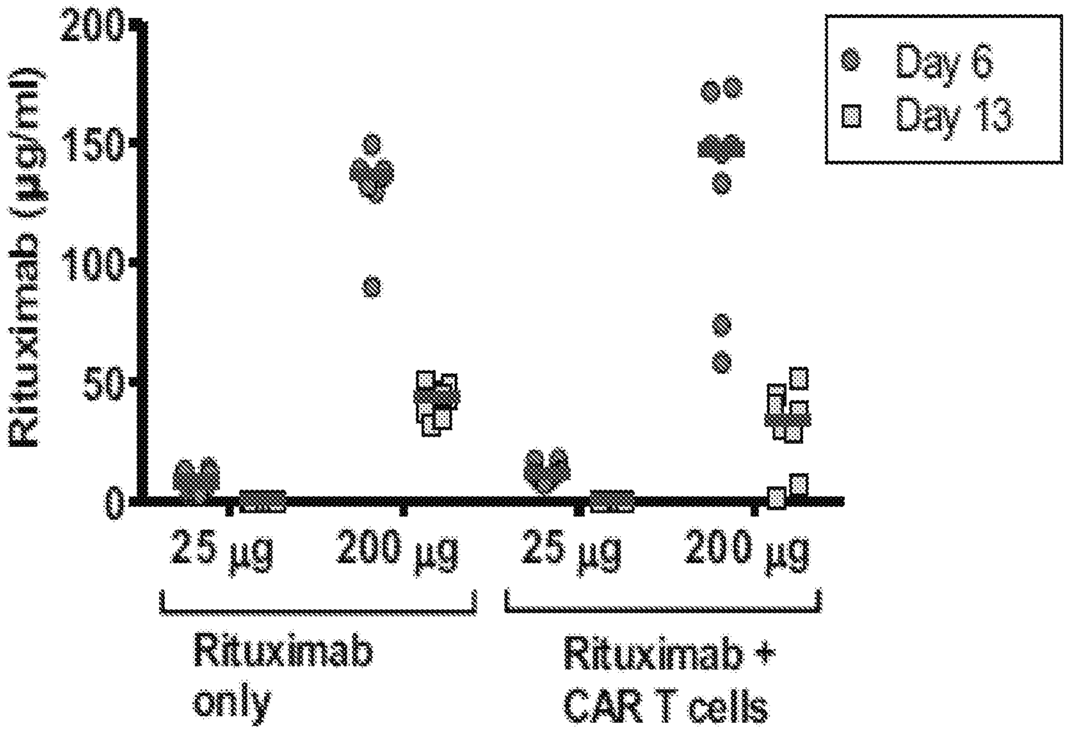

200
150
100
50
0
Rituximab (µg/ml)
Day 6
Day 13
25 µg
200 µg
25 µg
200 µg
Rituximab only
Rituximab + CAR T cells

FIG. 7D

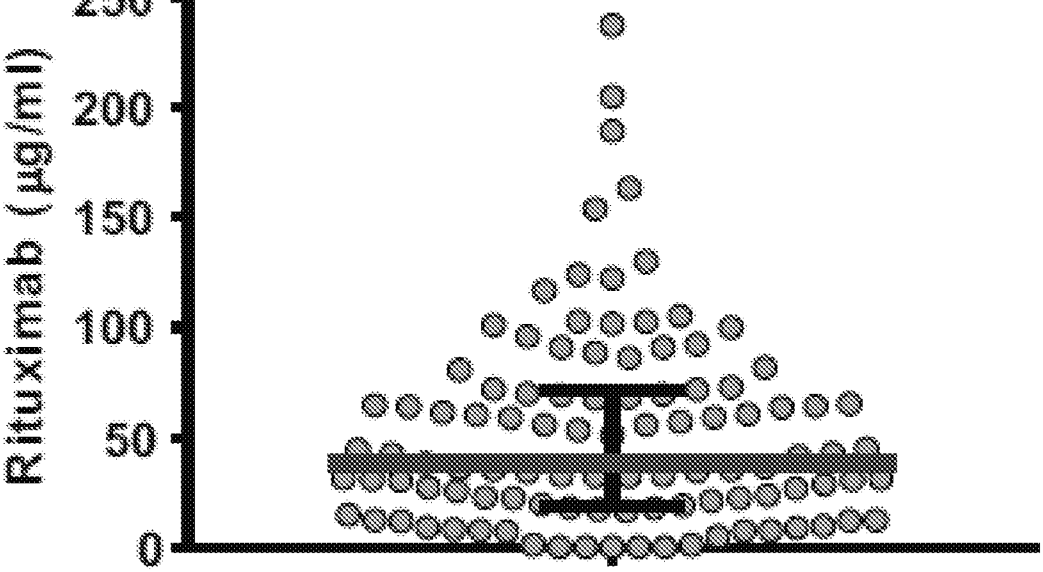
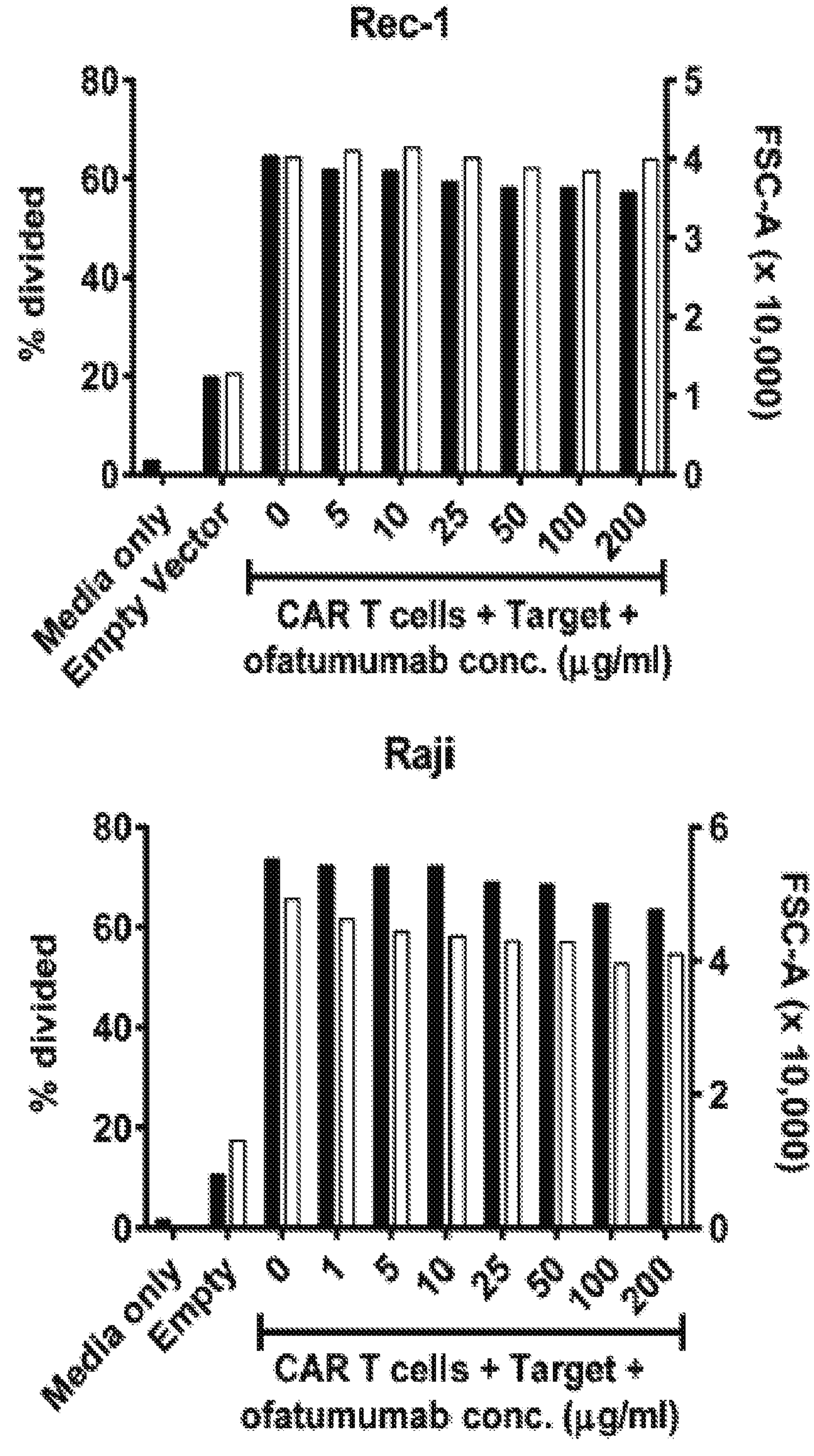

250
200
150
100
50
0
Rituximab (µg/ml)

FIG. 7E



Rec-1
% divided
80
60
40
20
0
FSC-A (x 10,000)
5
4
3
2
1
0
Media only
Empty Vector
0
5
10
25
50
100
200
CAR T cells + Target +
ofatumumab conc. (µg/ml)
Raji
% divided
80
60
40
20
0
FSC-A (x 10,000)
6
4
2
0
Media only
Empty
0
1
5
10
25
50
100
200
CAR T cells + Target +
ofatumumab conc. (µg/ml)

FIG. 8A

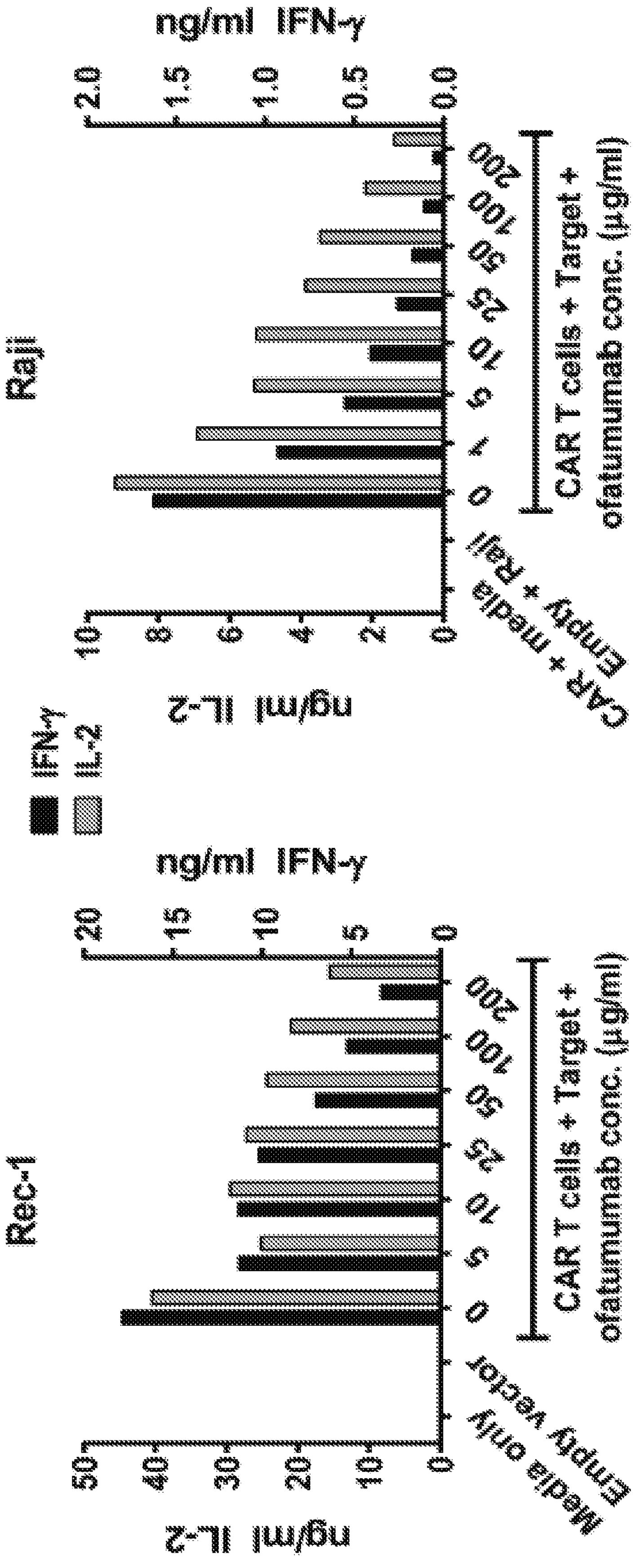

Raji
ng/ml IFN-γ
ng/ml IL-2
CAR + media
Empty + Raji
CAR T cells + Target +
ofatumumab conc. (μg/ml)
IFN-γ
IL-2
Rec-1
ng/ml IFN-γ
ng/ml IL-2
Media only
Empty vector
CAR T cells + Target +
ofatumumab conc. (μg/ml)

FIG. 8B

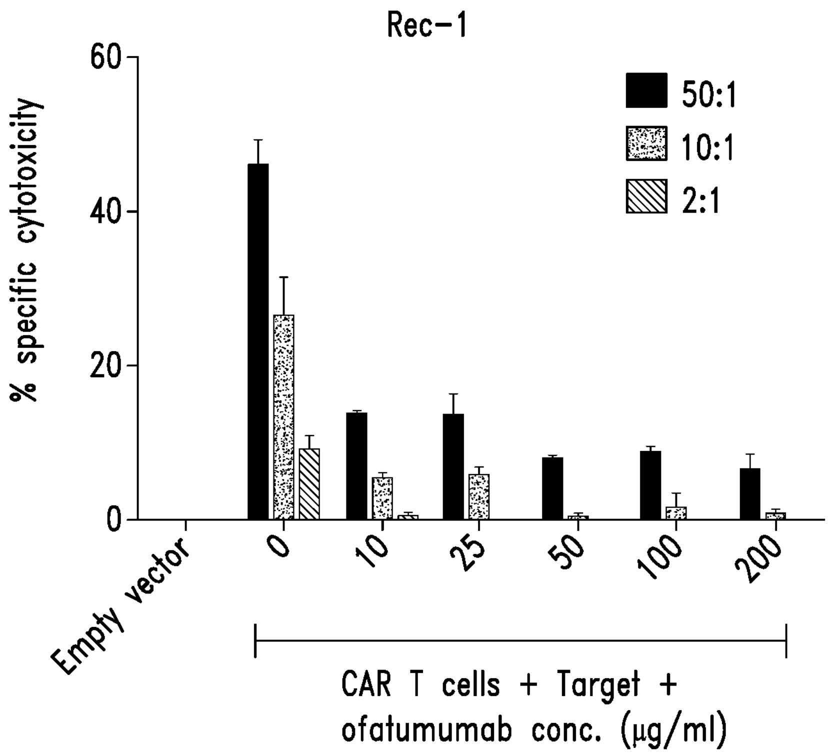

Rec-1
60
40
20
0
% specific cytotoxicity
50:1
10:1
2:1
Empty vector
0
10
25
50
100
200
CAR T cells + Target +
ofatumumab conc. (µg/ml)

FIG. 8C

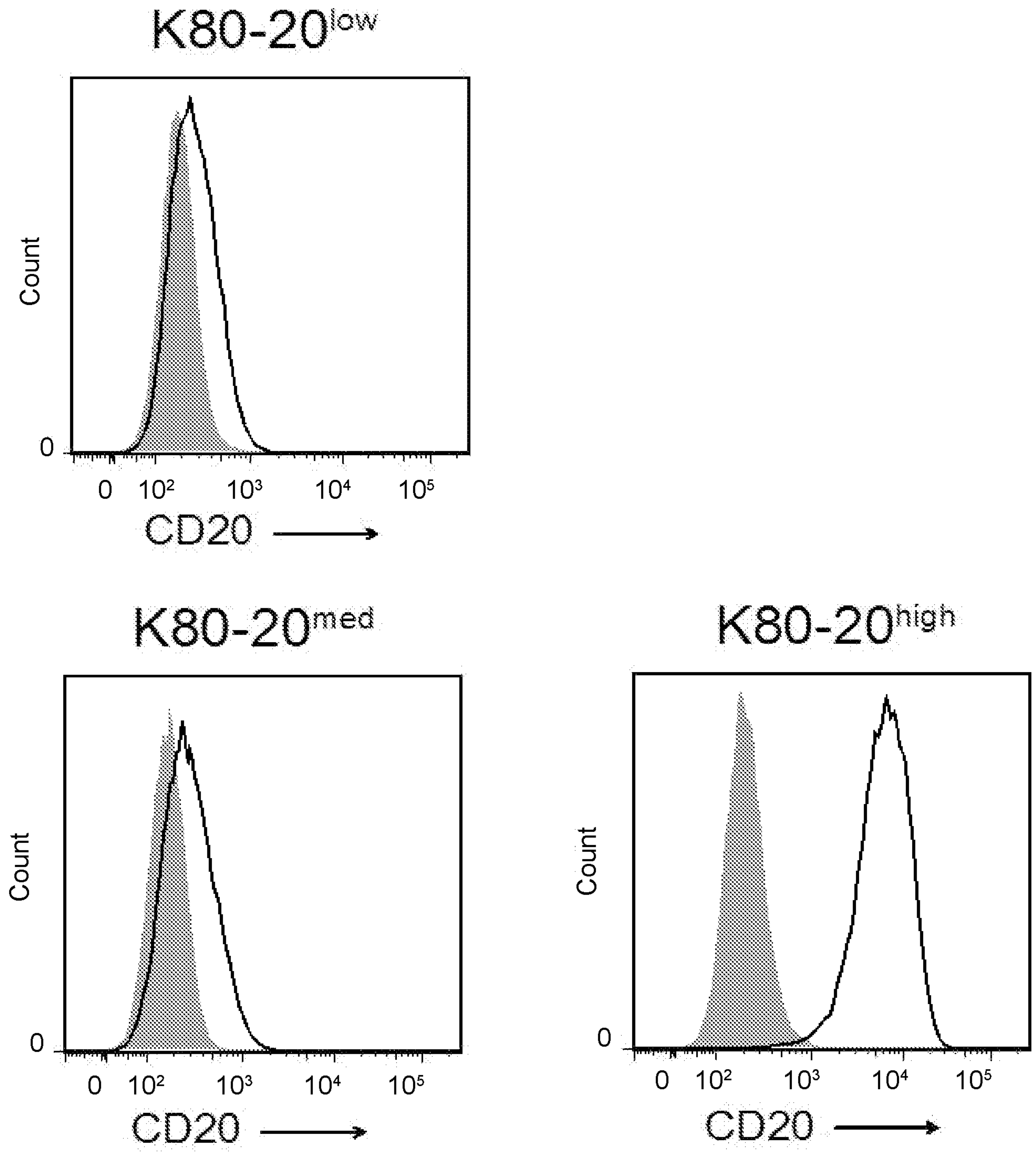

K80-20low
Count
0
0 10² 10³ 10⁴ 10⁵
CD20
K80-20med
Count
0
0 10² 10³ 10⁴ 10⁵
CD20
K80-20high
Count
0
0 10² 10³ 10⁴ 10⁵
CD20

FIG. 9

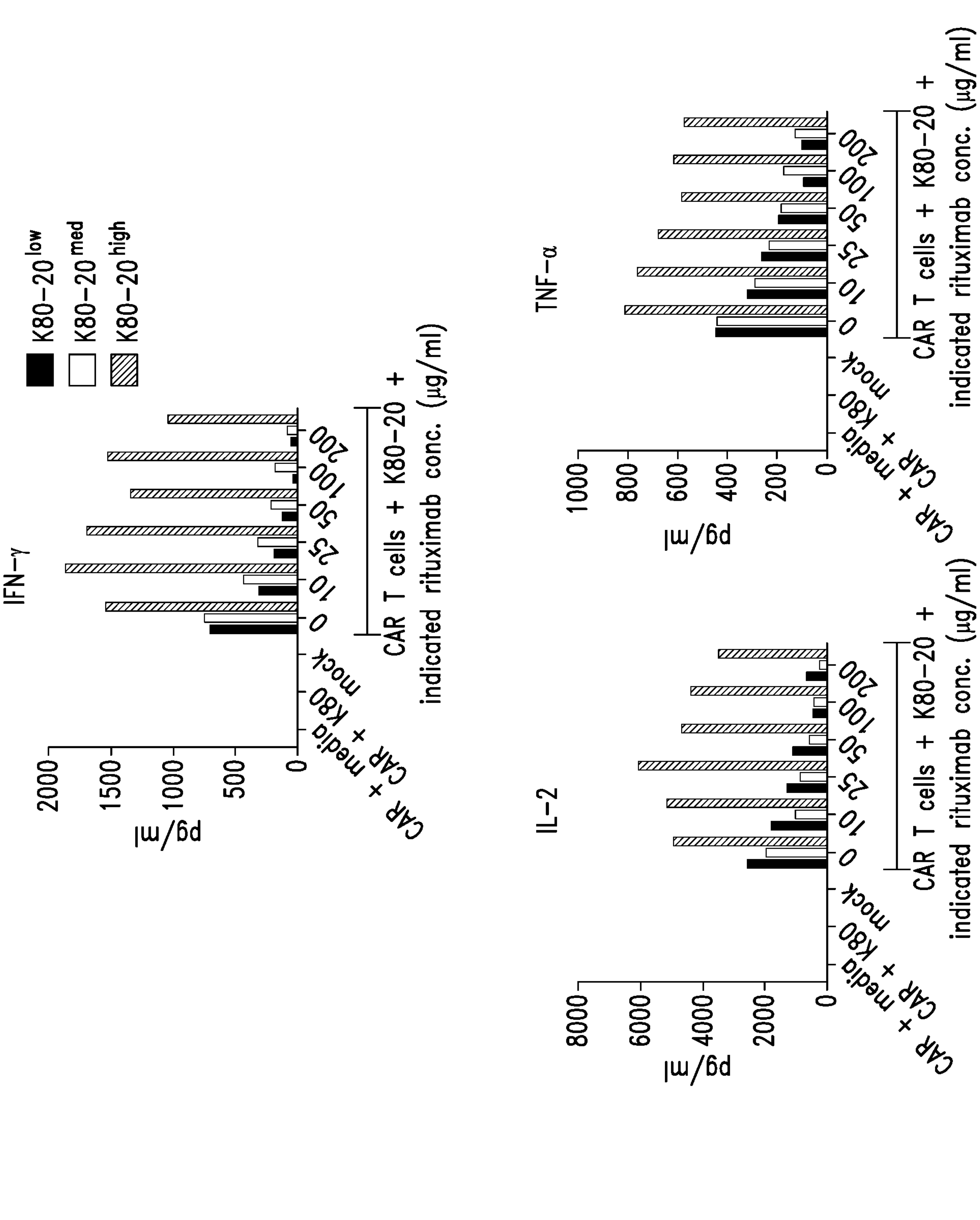

IFN-γ
pg/ml
2000
1500
1000
500
0
CAR + media
CAR + K80
mock
0
10
25
50
100
200
CAR T cells + K80-20 +
indicated rituximab conc. (μg/ml)
K80-20^low
K80-20^med
K80-20^high
TNF-α
pg/ml
1000
800
600
400
200
0
IL-2
pg/ml
8000
6000
4000
2000
0

FIG. 10

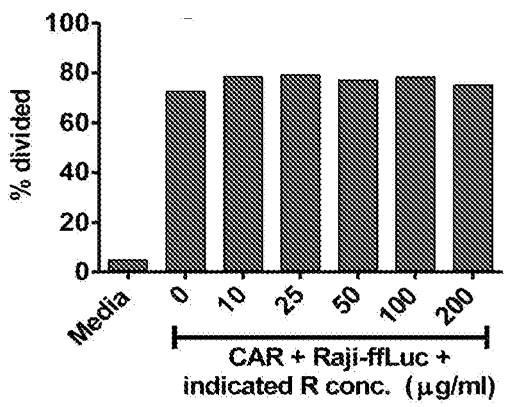


FIG. 11A

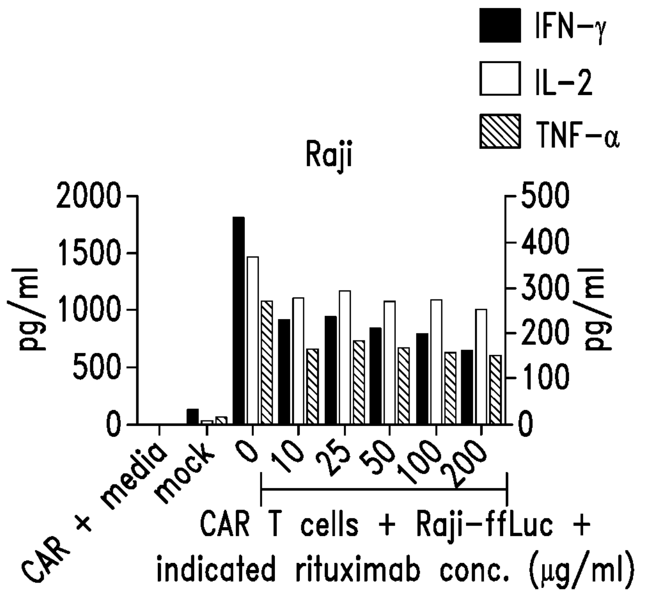


FIG. 11B

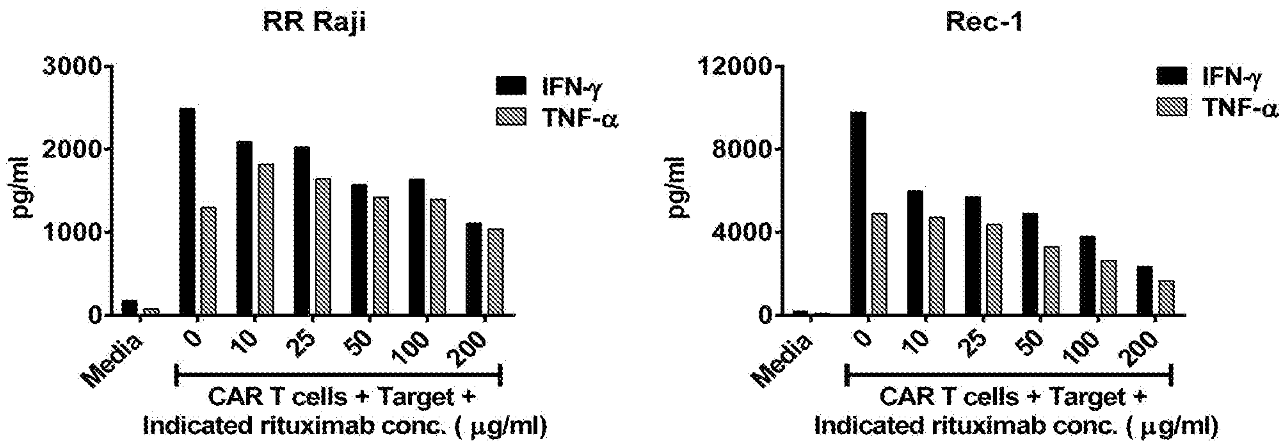


FIG. 11C

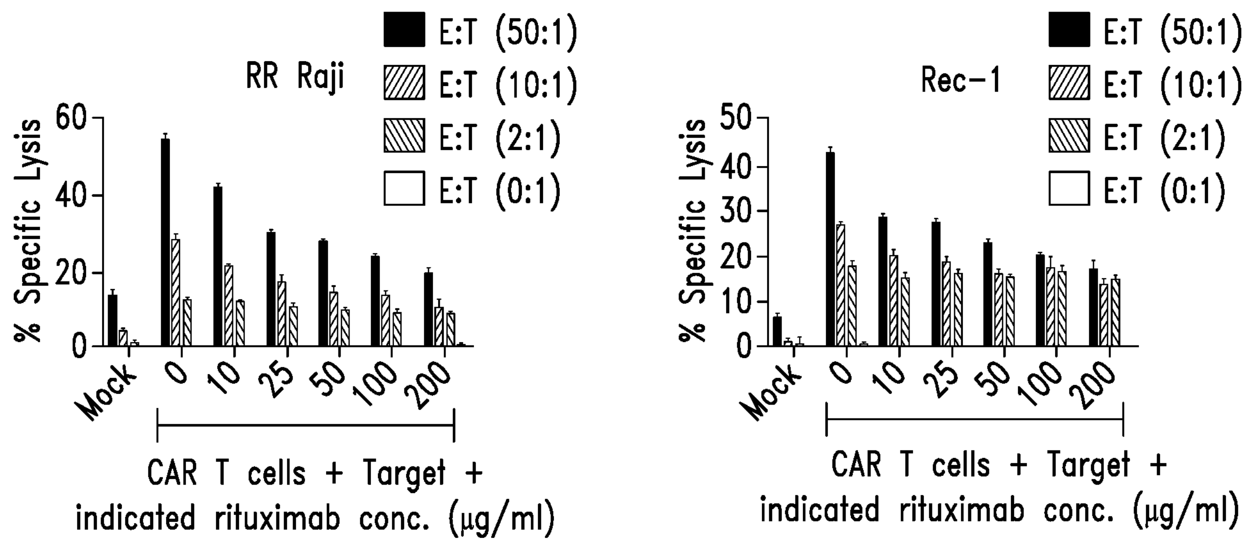


FIG. 11D

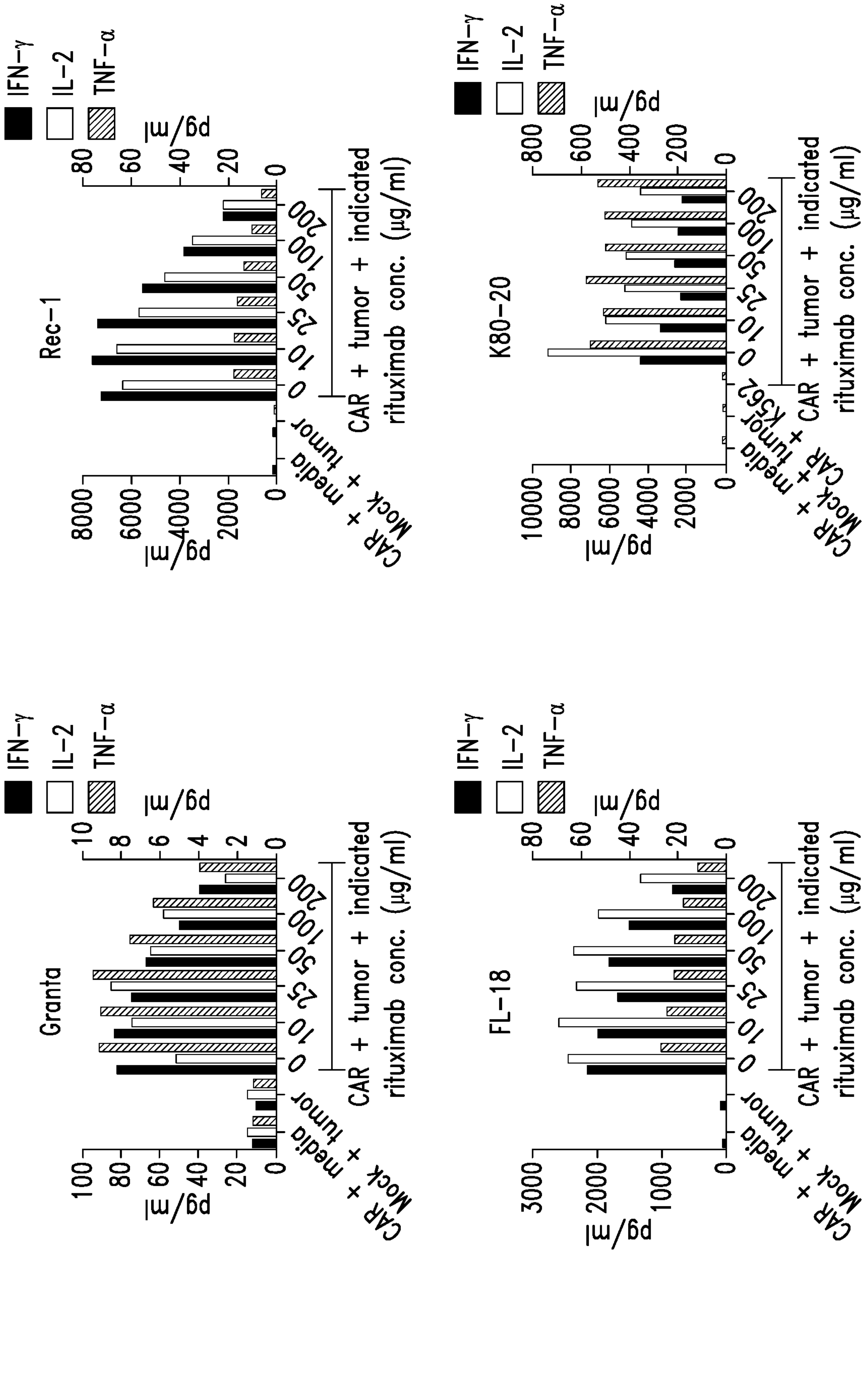

Granta
Rec-1
FL-18
K80-20
IFN-γ
IL-2
TNF-α
pg/ml
CAR + media
Mock + tumor
CAR + K562
CAR + tumor + indicated rituximab conc. (μg/ml)
0
10
25
50
100
200

FIG. 11E

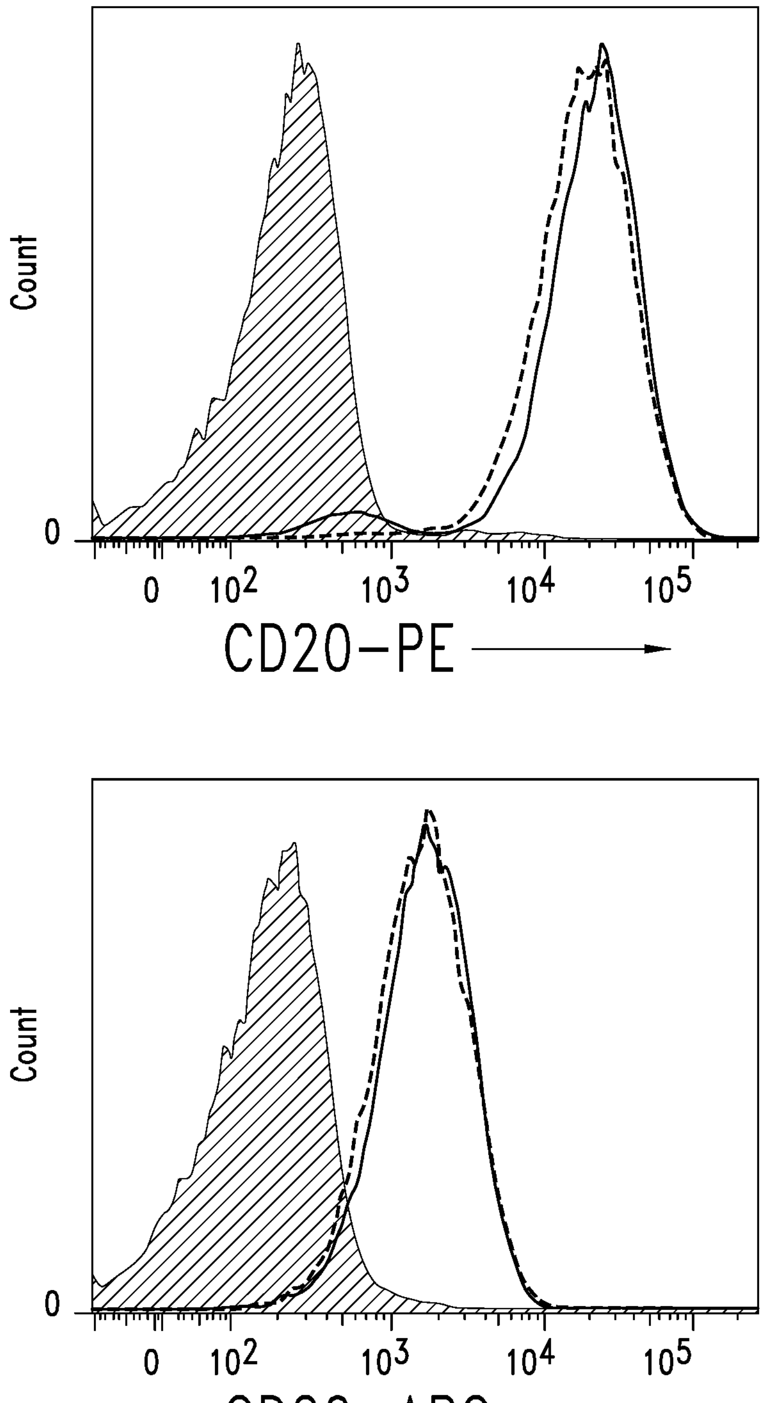

Count
0
0
10^2
10^3
10^4
10^5
CD20-PE
Count
0
0
10^2
10^3
10^4
10^5
CD20-APC

FIG. 12

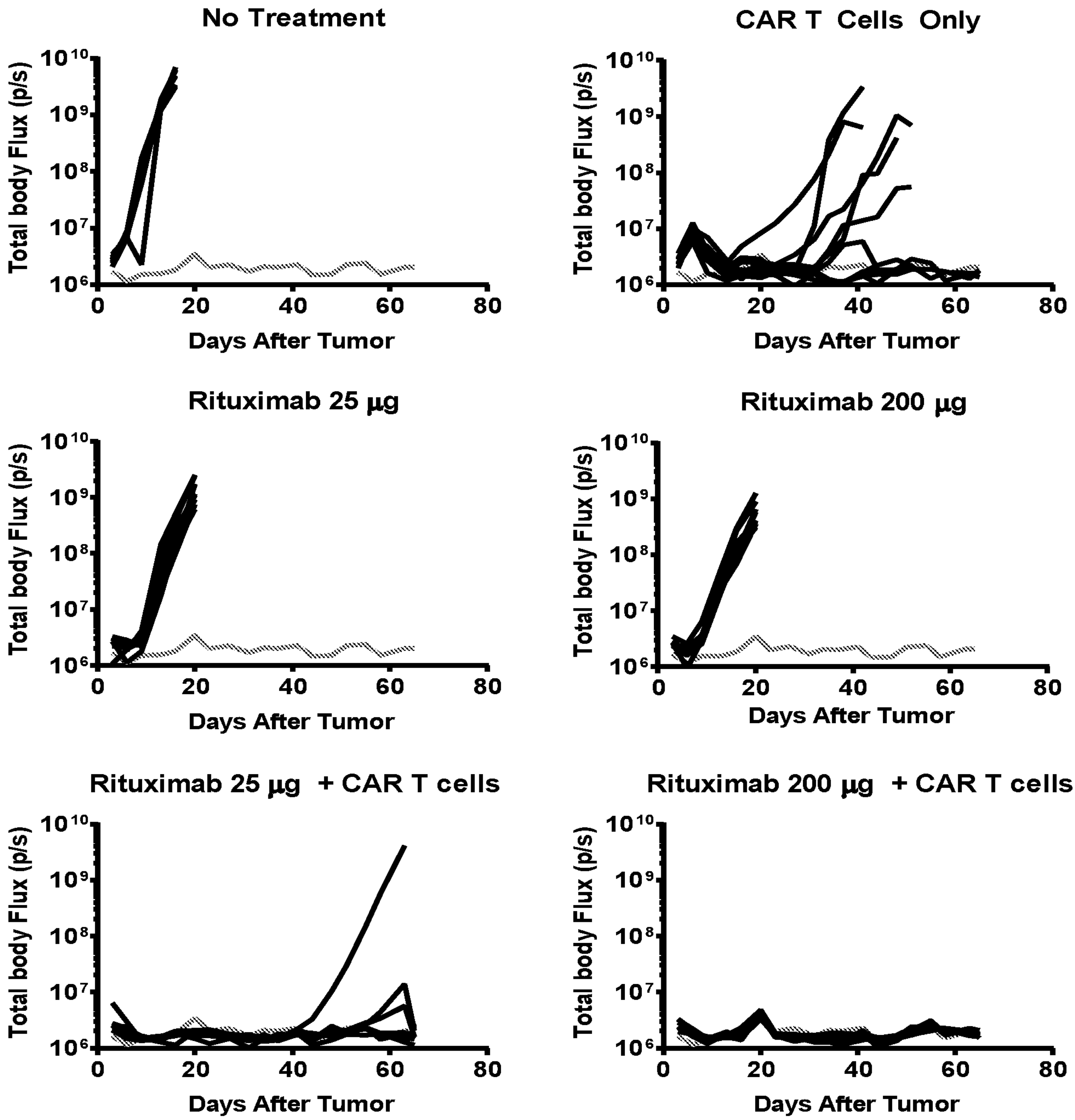

No Treatment
CAR T Cells Only
Rituximab 25 μg
Rituximab 200 μg
Rituximab 25 μg + CAR T cells
Rituximab 200 μg + CAR T cells
Total body Flux (p/s)
Days After Tumor
10^10
10^9
10^8
10^7
10^6
0
20
40
60
80

FIG. 13A

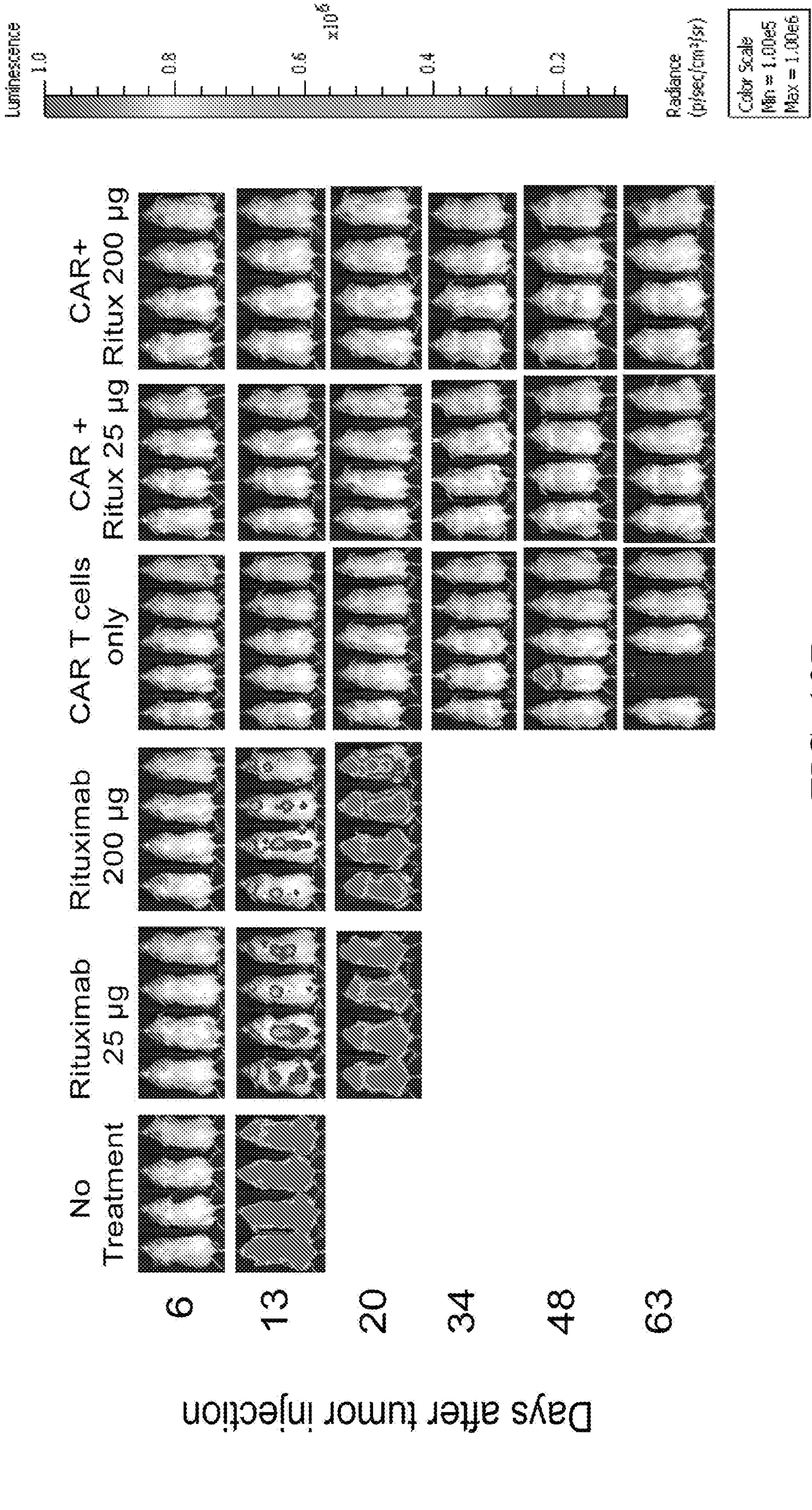

Luminescence
1.0
0.8
0.6
0.4
0.2
x10^6
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.00e5
Max = 1.00e6
No Treatment
Rituximab 25 μg
Rituximab 200 μg
CAR T cells only
CAR + Ritux 25 μg
CAR+ Ritux 200 μg
6
13
20
34
48
63
Days after tumor injection

FIG. 13B

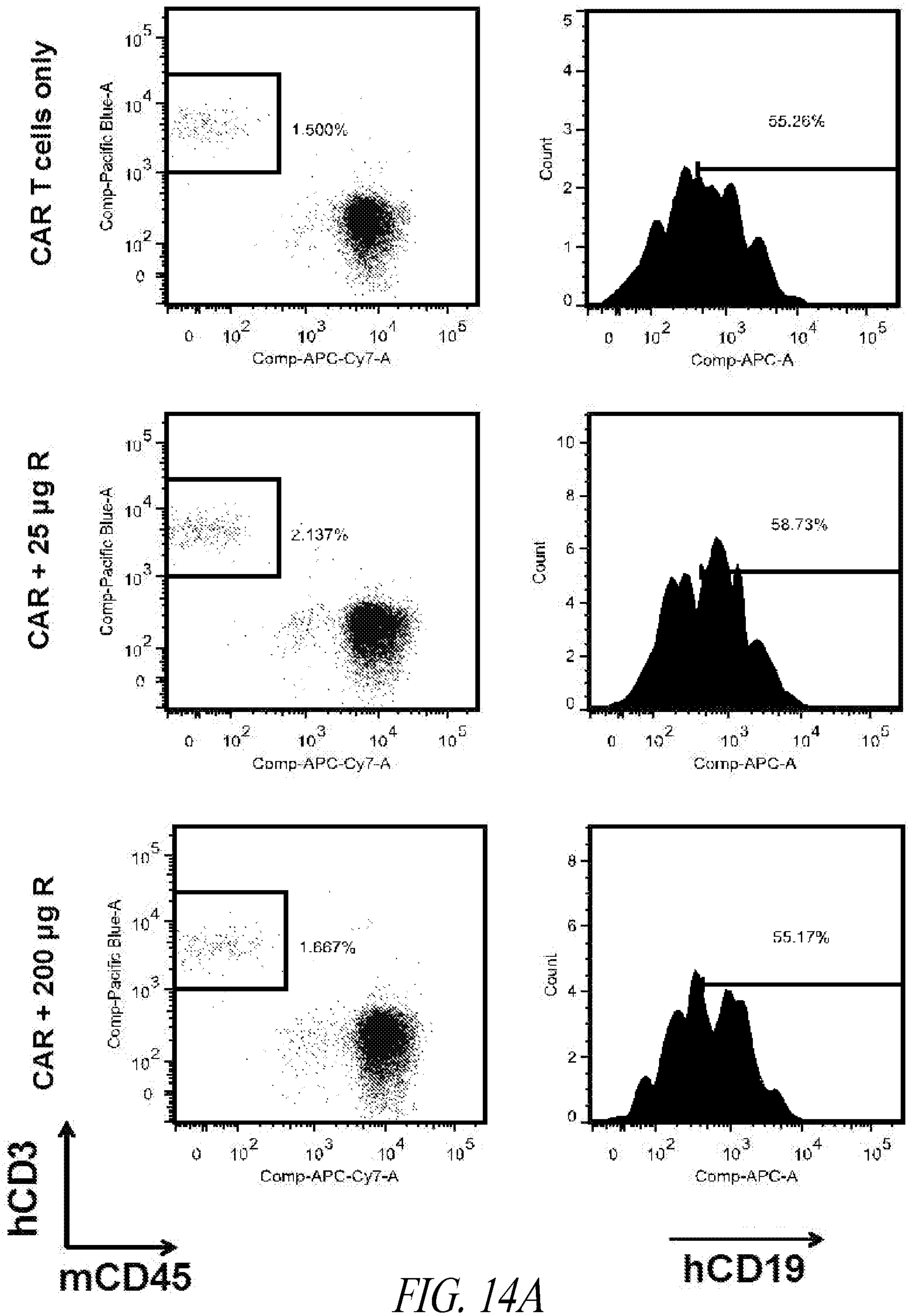

CAR T cells only
1.500%
55.26%
CAR + 25 µg R
2.137%
58.73%
CAR + 200 µg R
1.667%
55.17%
Comp-Pacific Blue-A
Comp-APC-Cy7-A
Count
Comp-APC-A
hCD3
mCD45
hCD19

FIG. 14A

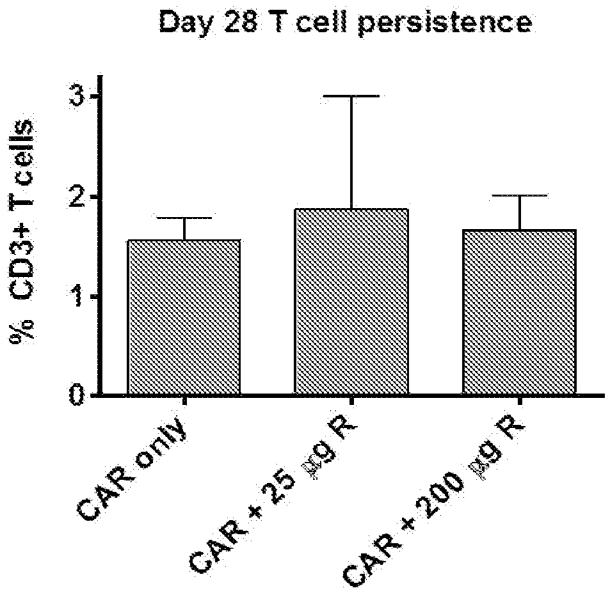


FIG. 14B

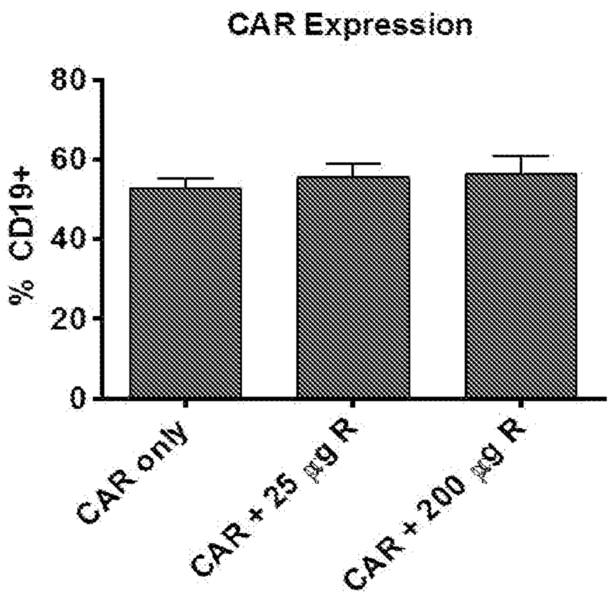


FIG. 14C

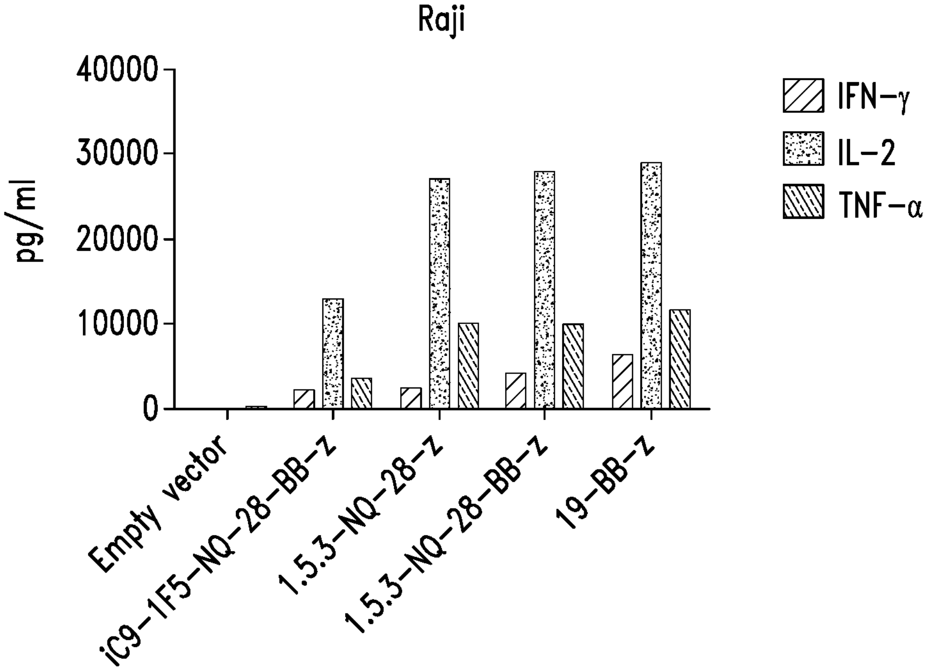


FIG. 15A

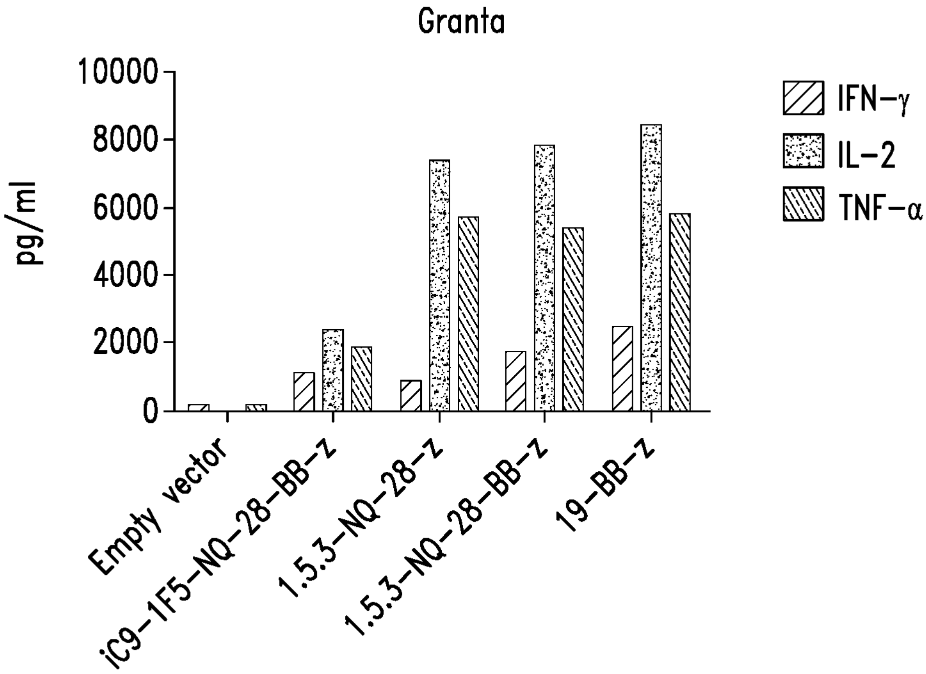


FIG. 15B

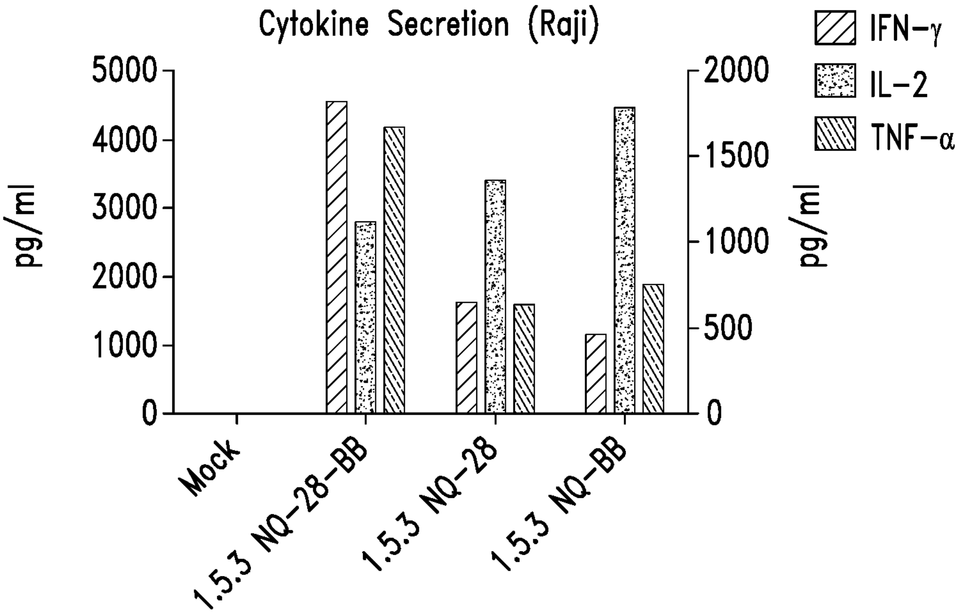


FIG. 15C

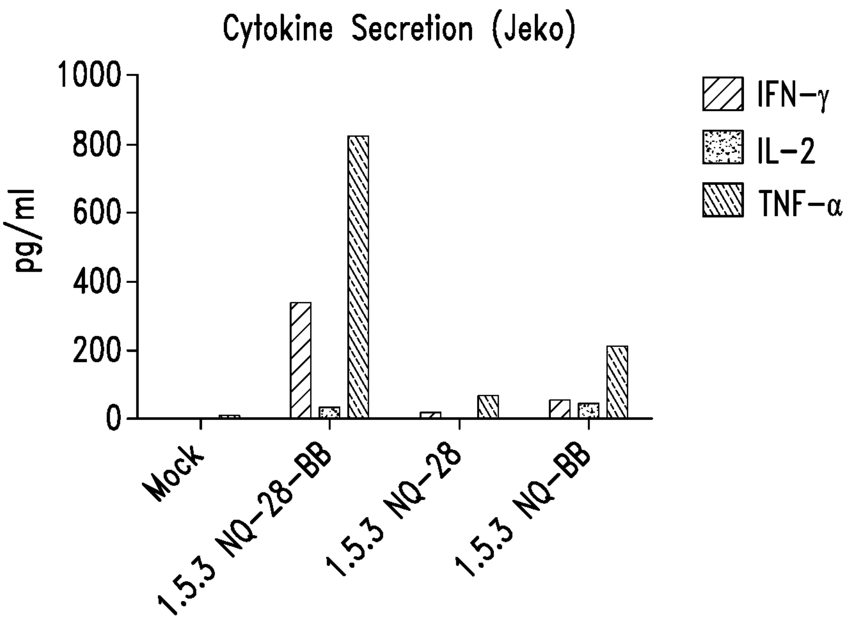
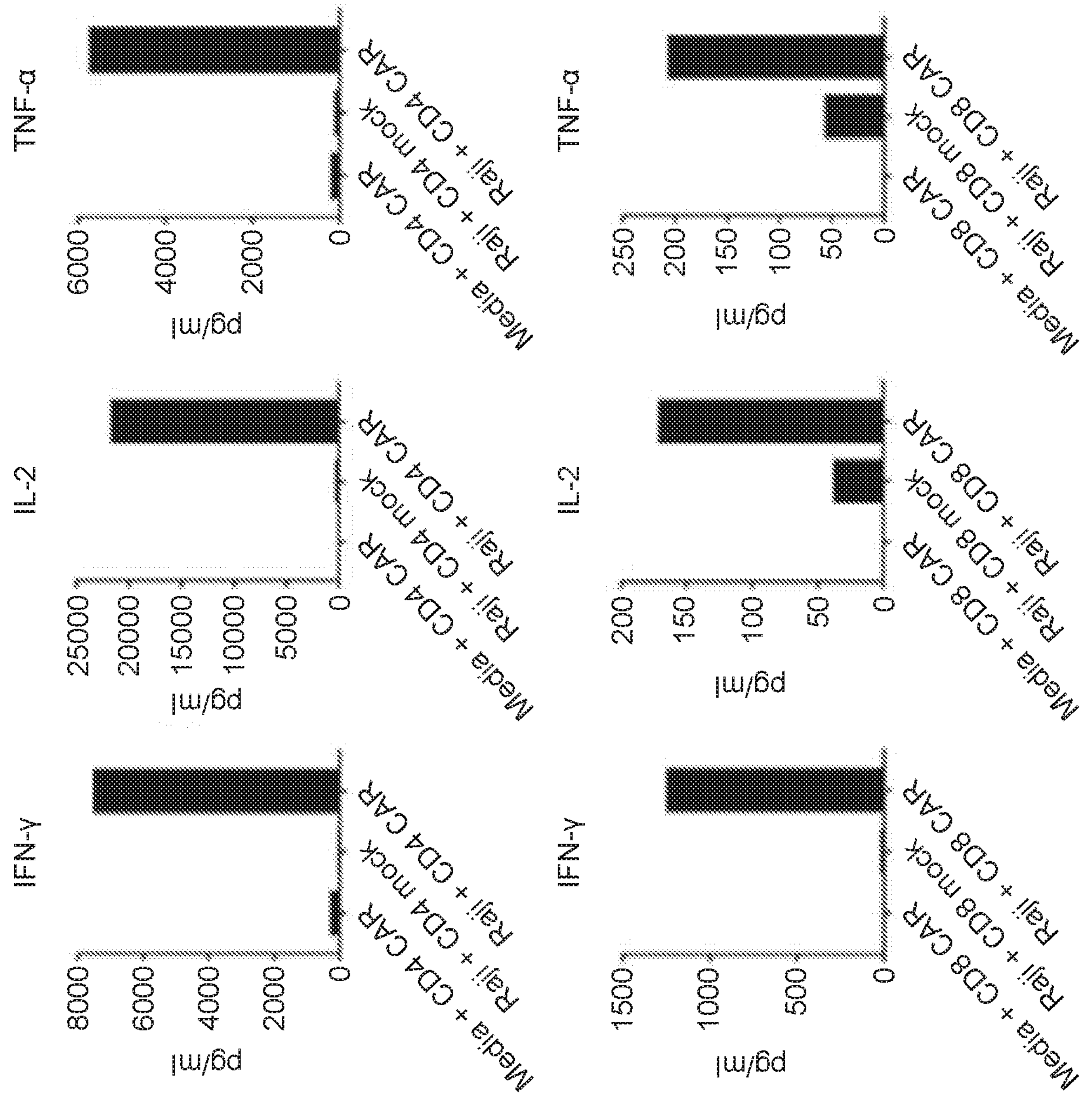


FIG. 15D



IFN-γ
IL-2
TNF-α
pg/ml
8000
6000
4000
2000
0
25000
20000
15000
10000
5000
1500
1000
500
250
200
150
100
50
Media + CD4 CAR
Raji + CD4 mock
Raji + CD4 CAR
Media + CD8 CAR
Raji + CD8 mock
Raji + CD8 CAR

FIG. 16A

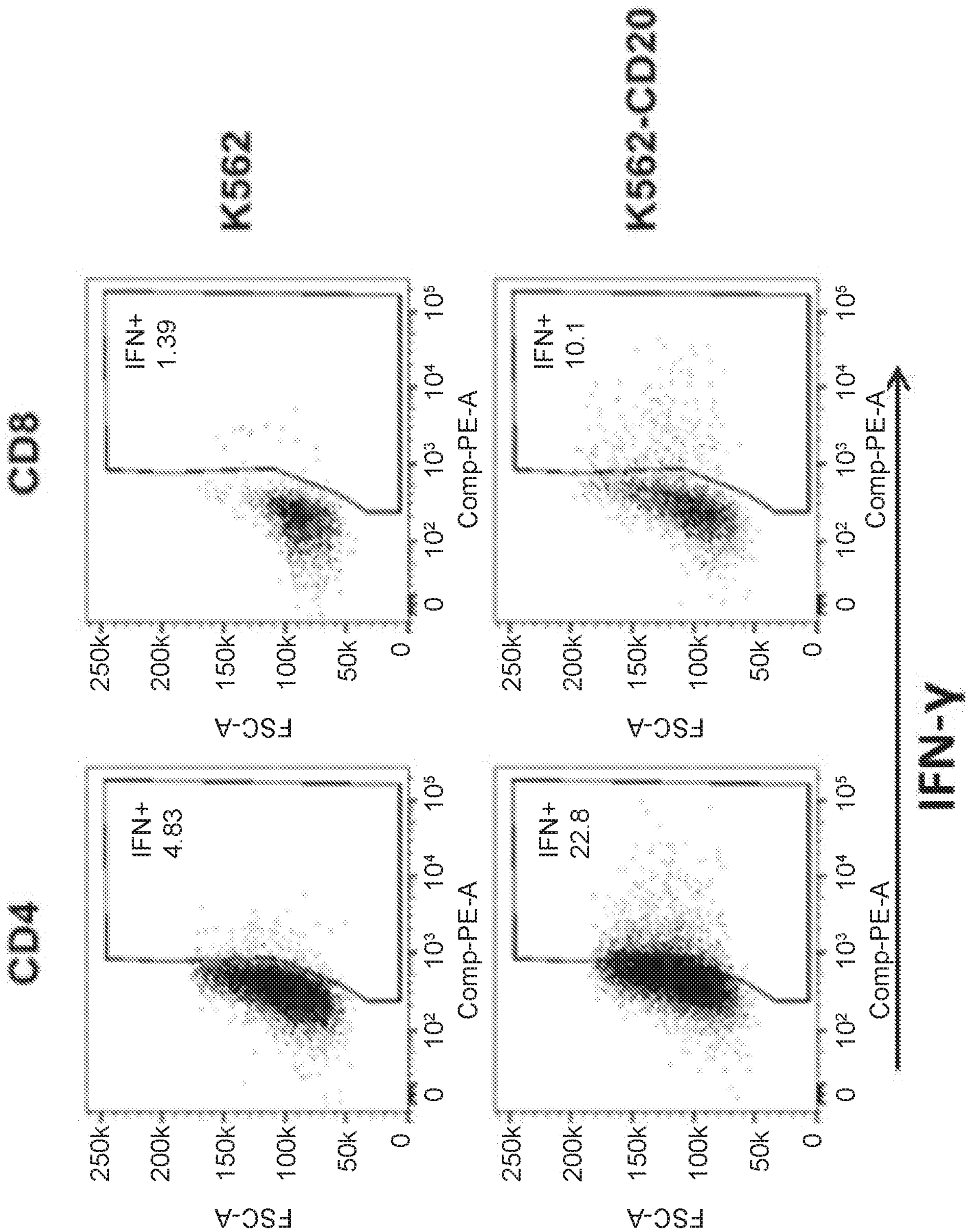

CD4
CD8
K562
K562-CD20
IFN+
4.83
IFN+
1.39
IFN+
22.8
IFN+
10.1
FSC-A
250k
200k
150k
100k
50k
0
0
10^2
10^3
10^4
10^5
Comp-PE-A
IFN-γ

FIG. 16B

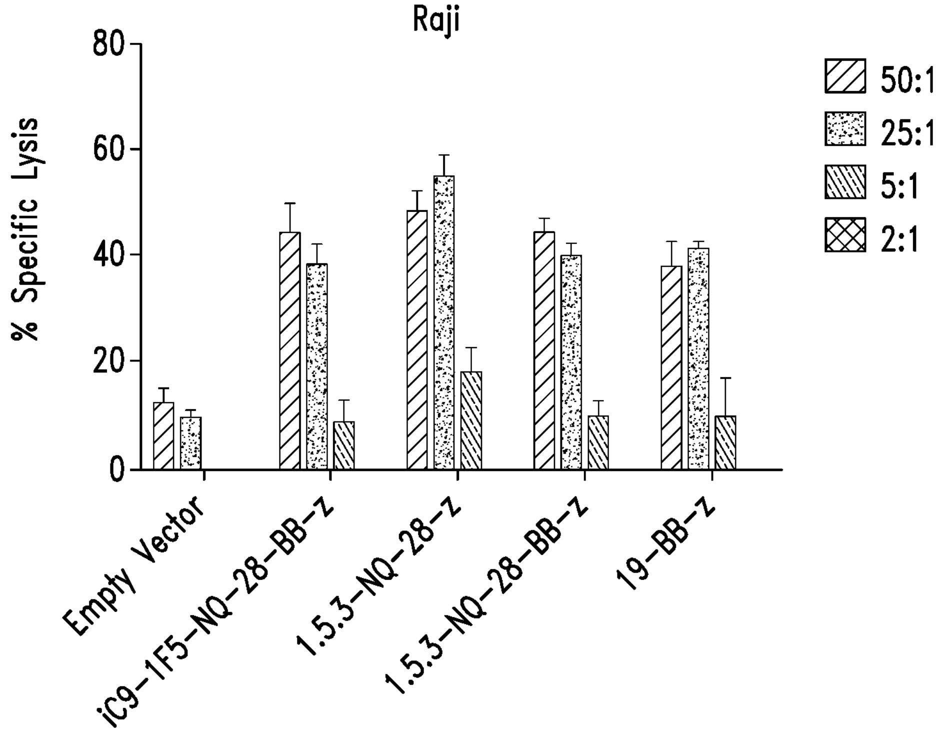

Raji
80
60
40
20
0
% Specific Lysis
50:1
25:1
5:1
2:1
Empty Vector
iC9-1F5-NQ-28-BB-z
1.5.3-NQ-28-z
1.5.3-NQ-28-BB-z
19-BB-z

FIG. 17A

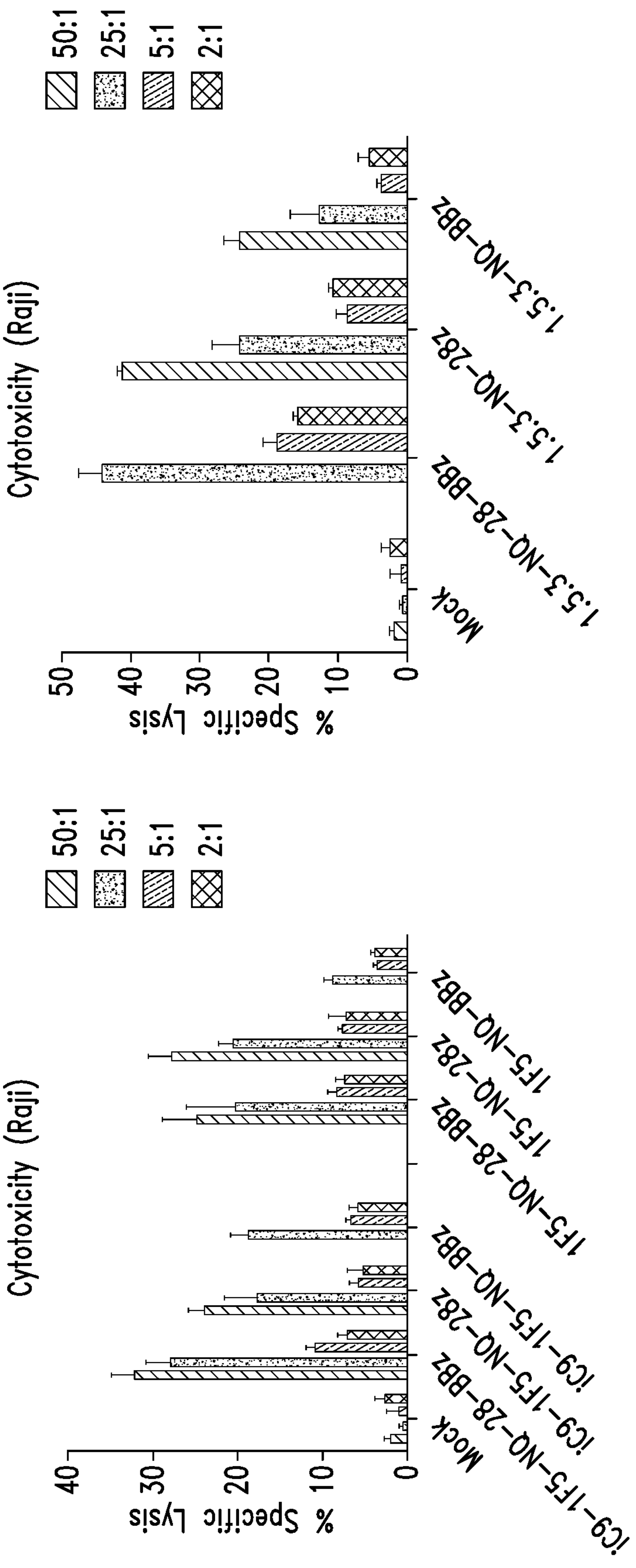

Cytotoxicity (Raji)
% Specific Lysis
0
10
20
30
40
50
Mock
1.5.3-NQ-28-BBz
1.5.3-NQ-28z
1.5.3-NQ-BBz
50:1
25:1
5:1
2:1
Cytotoxicity (Raji)
% Specific Lysis
0
10
20
30
40
Mock
iC9-1F5-NQ-28-BBz
iC9-1F5-NQ-28z
iC9-1F5-NQ-BBz
1F5-NQ-28-BBz
1F5-NQ-28z
1F5-NQ-BBz
50:1
25:1
5:1
2:1

FIG. 17B

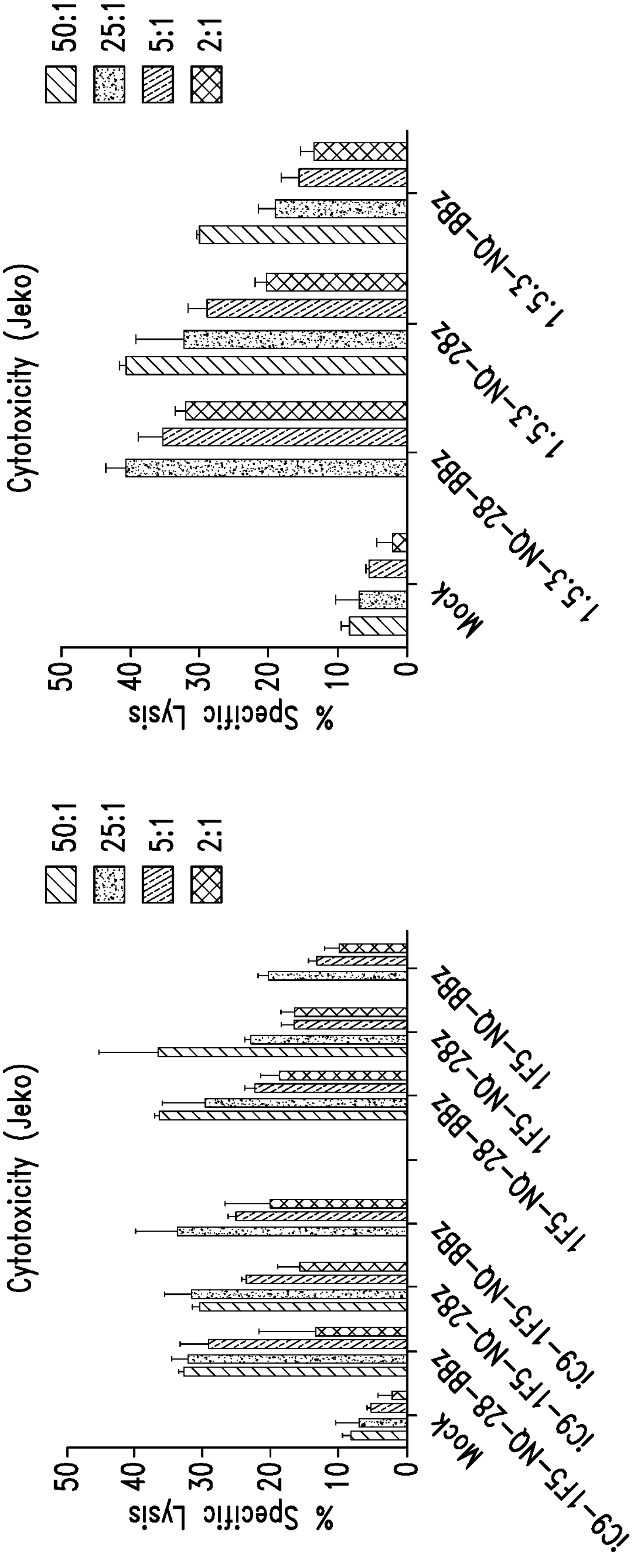


FIG. 17B (Continued)

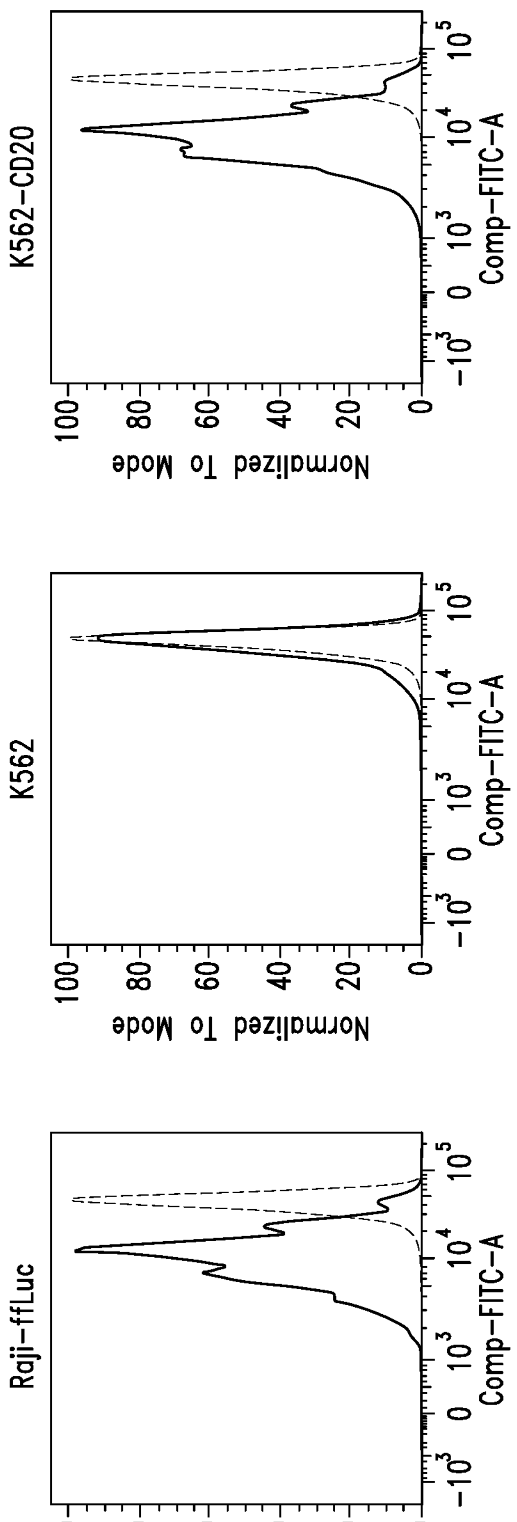

Raji-ffLuc
K562
K562-CD20
Normalized To Mode
100
80
60
40
20
0
Comp-FITC-A
-10^3
0
10^3
10^4
10^5

FIG. 18A

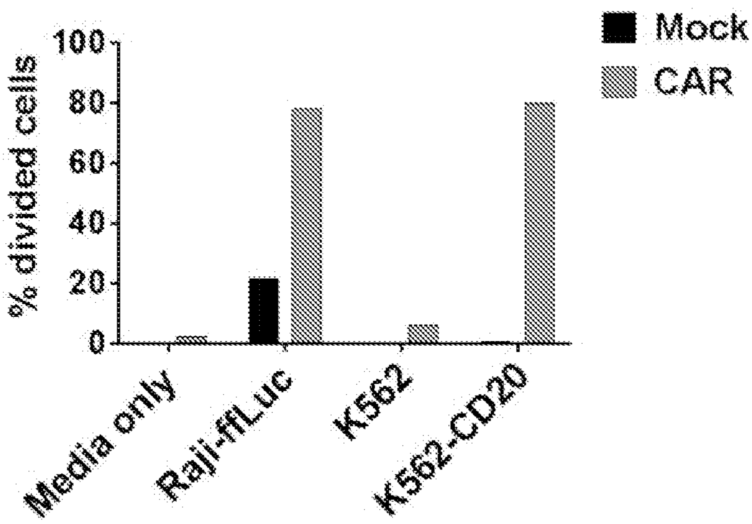

Mock
CAR
% divided cells
100
80
60
40
20
0
Media only
Raji-ffLuc
K562
K562-CD20

FIG. 18B

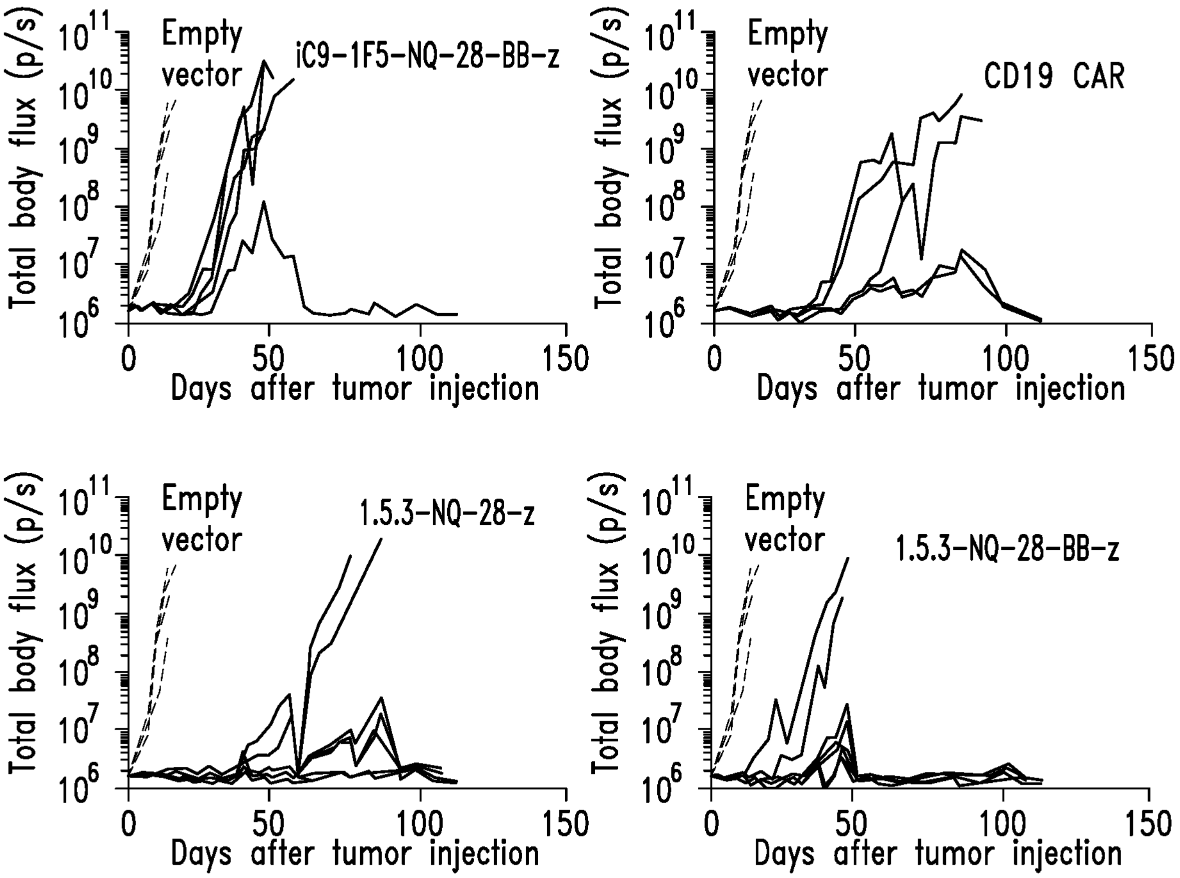


FIG. 19A

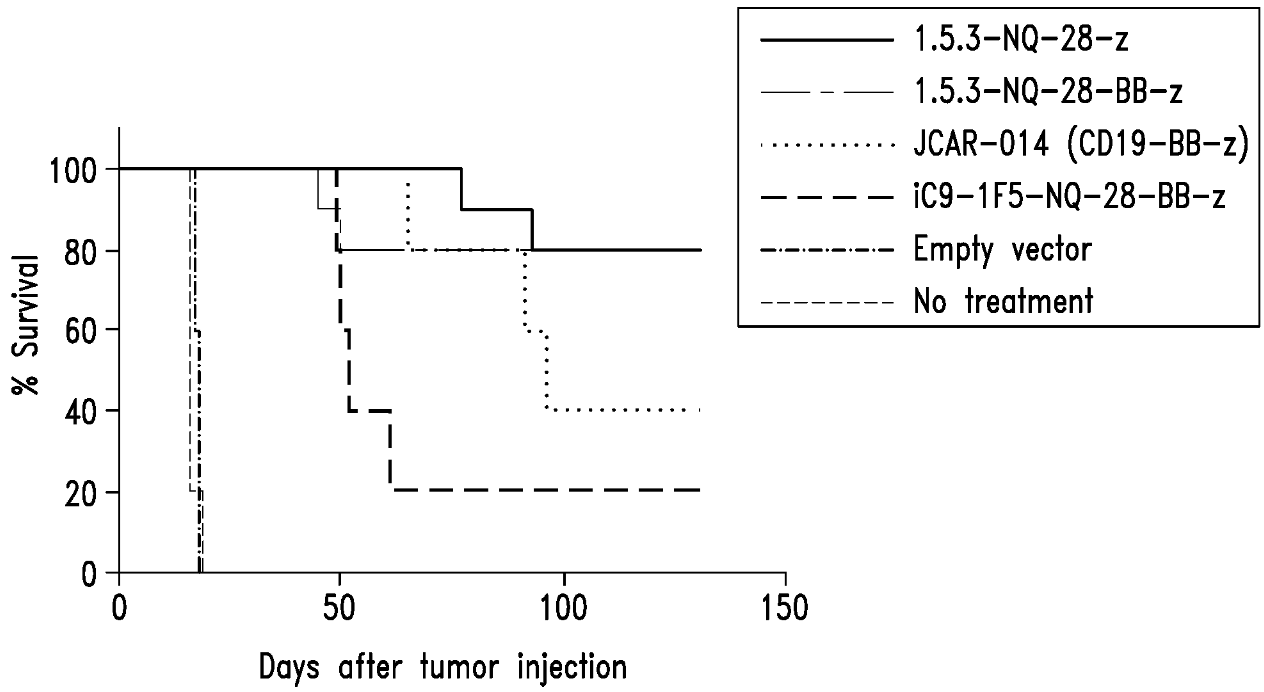


FIG. 19B

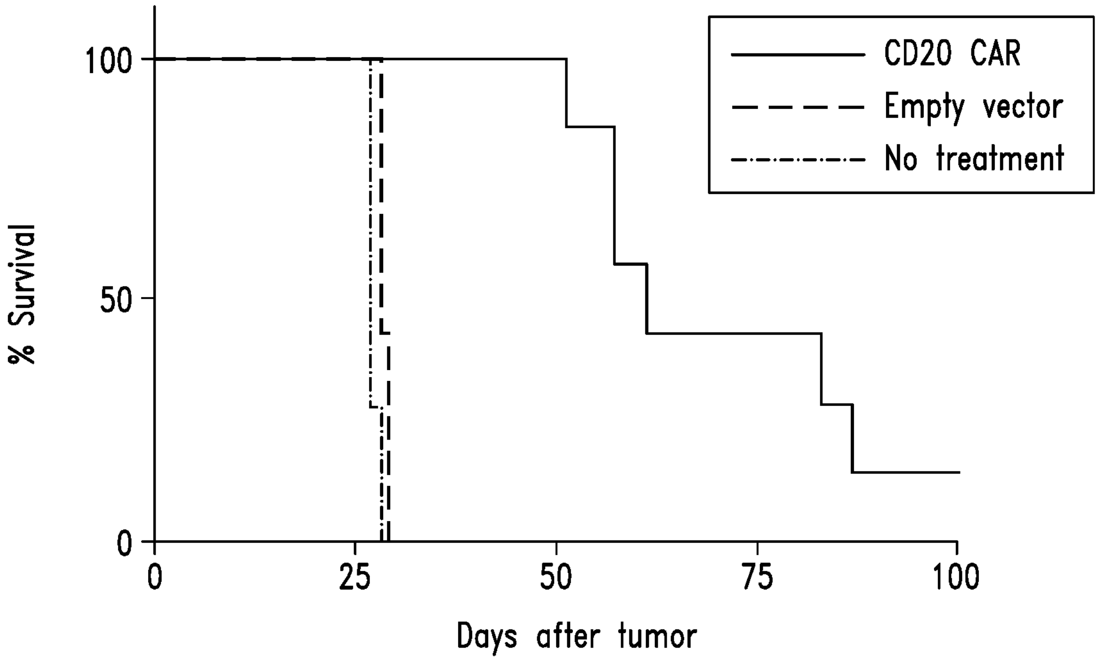

CD20 CAR
Empty vector
No treatment
% Survival
100
50
0
0
25
50
75
100
Days after tumor

FIG. 20

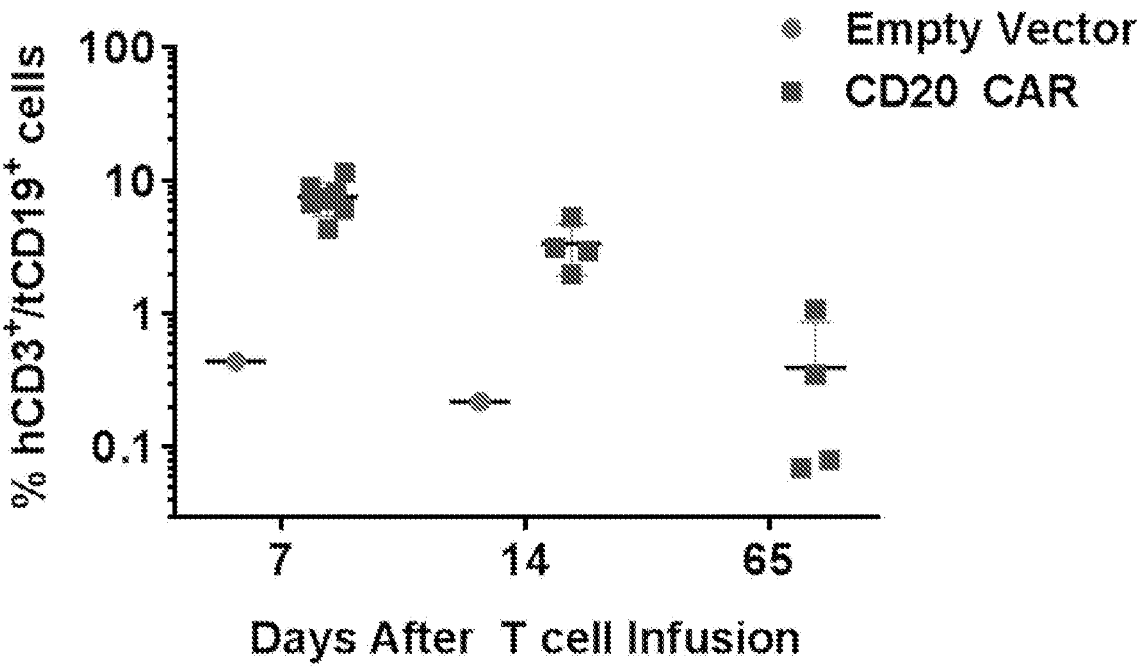


FIG. 21A

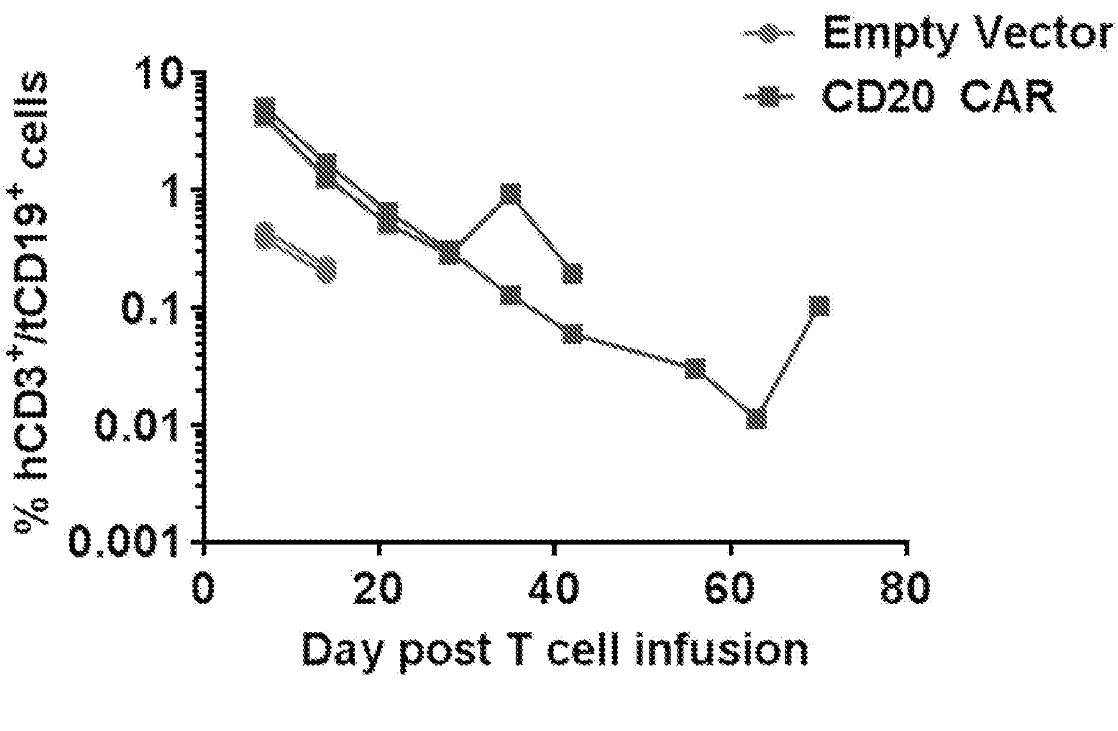


FIG. 21B

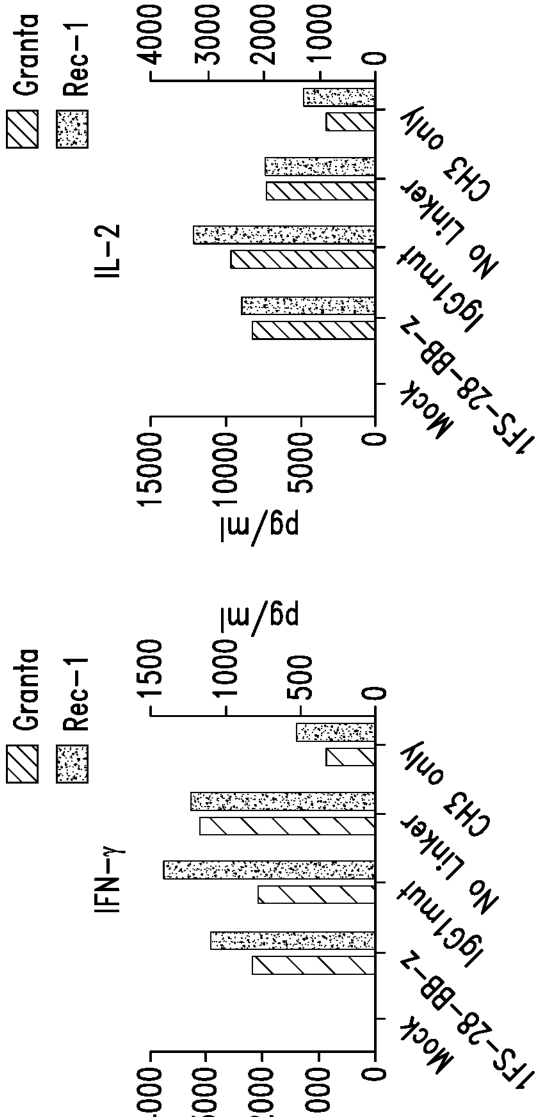


FIG. 22A

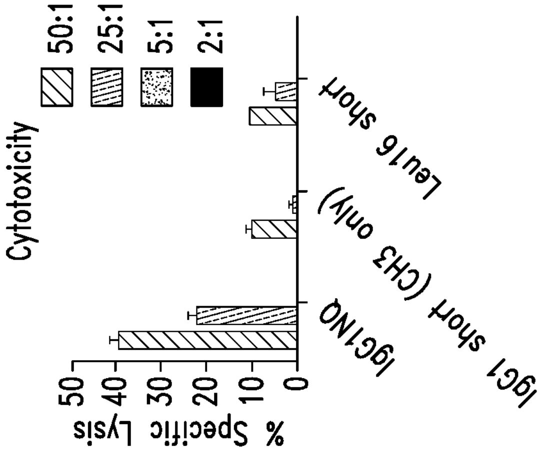


FIG. 22B

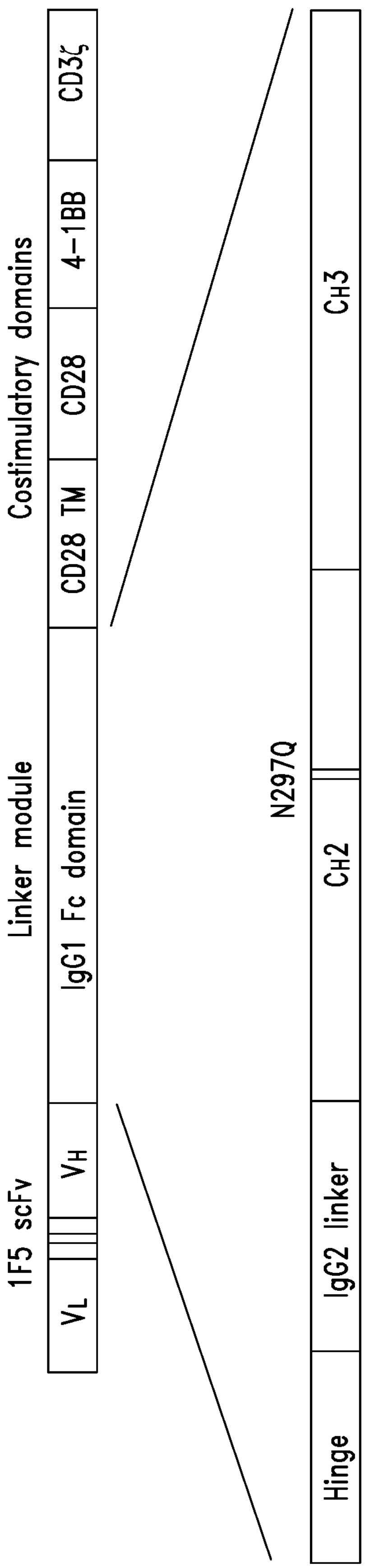

1F5 scFv
Linker module
Costimulatory domains
V_L
V_H
IgG1 Fc domain
CD28 TM
CD28
4-1BB
CD3ζ
N297Q
Hinge
IgG2 linker
C_H2
C_H3

FIG. 23A

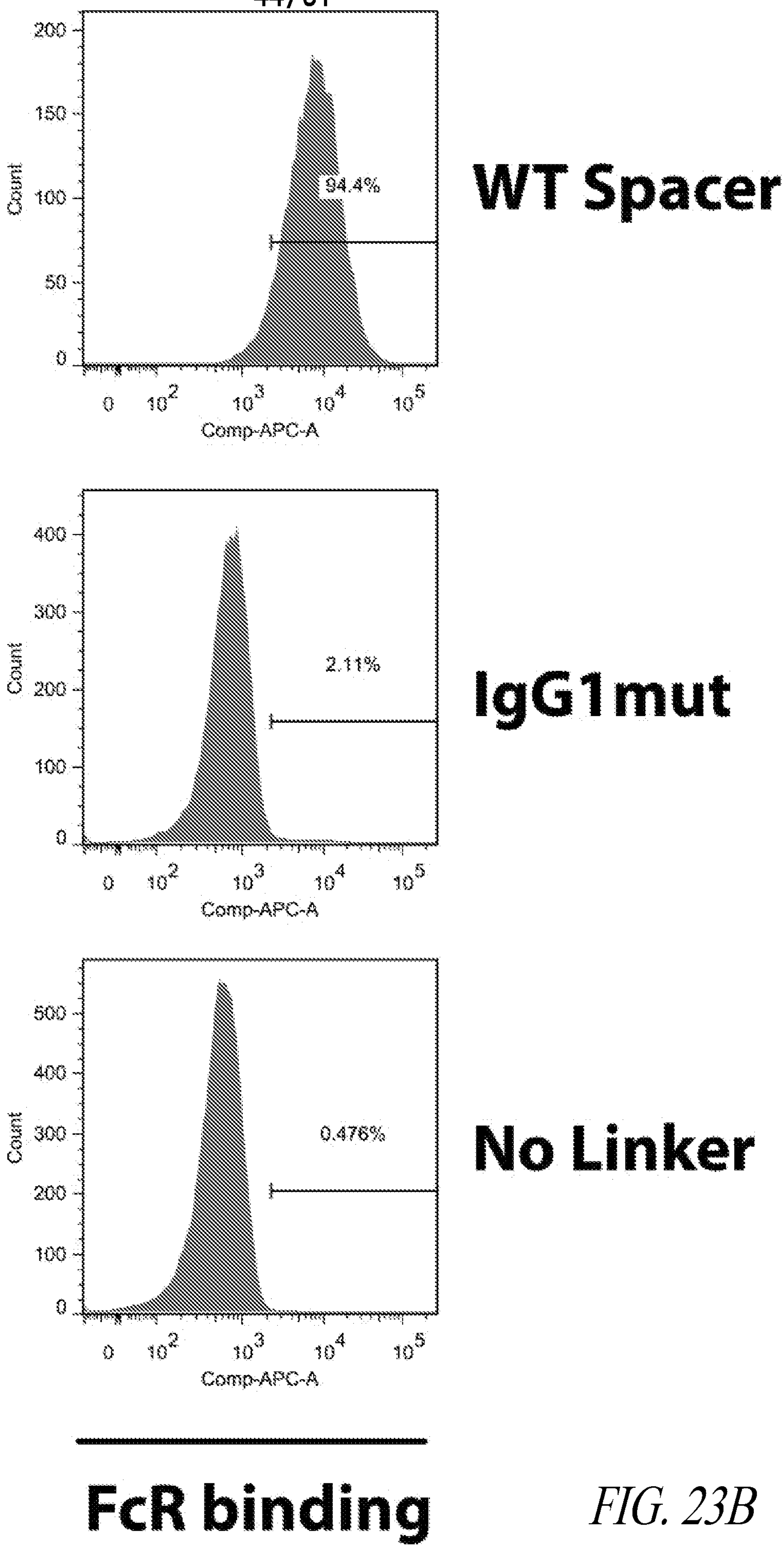

Count
Comp-APC-A
94.4%
WT Spacer
2.11%
IgG1mut
0.476%
No Linker
FcR binding

FIG. 23B

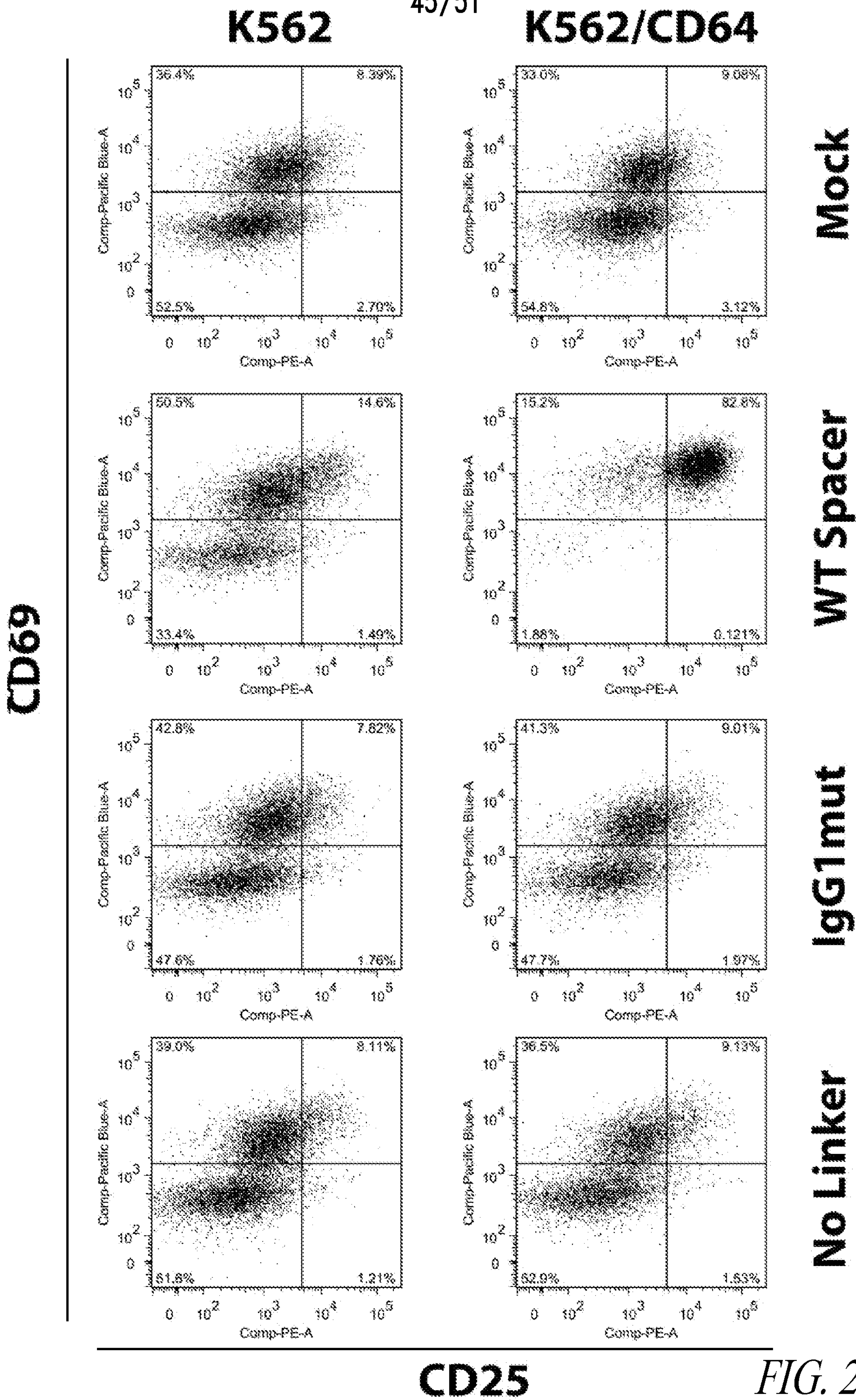

K562
K562/CD64
CD69
CD25
Mock
WT Spacer
IgG1mut
No Linker
Comp-Pacific Blue-A
Comp-PE-A
36.4%
8.39%
52.5%
2.70%
33.0%
9.08%
54.8%
3.12%
50.5%
14.6%
33.4%
1.49%
15.2%
82.8%
1.88%
0.121%
42.8%
7.82%
47.6%
1.76%
41.3%
9.01%
47.7%
1.97%
39.0%
8.11%
51.6%
1.21%
36.5%
9.13%
52.9%
1.53%

FIG. 23C

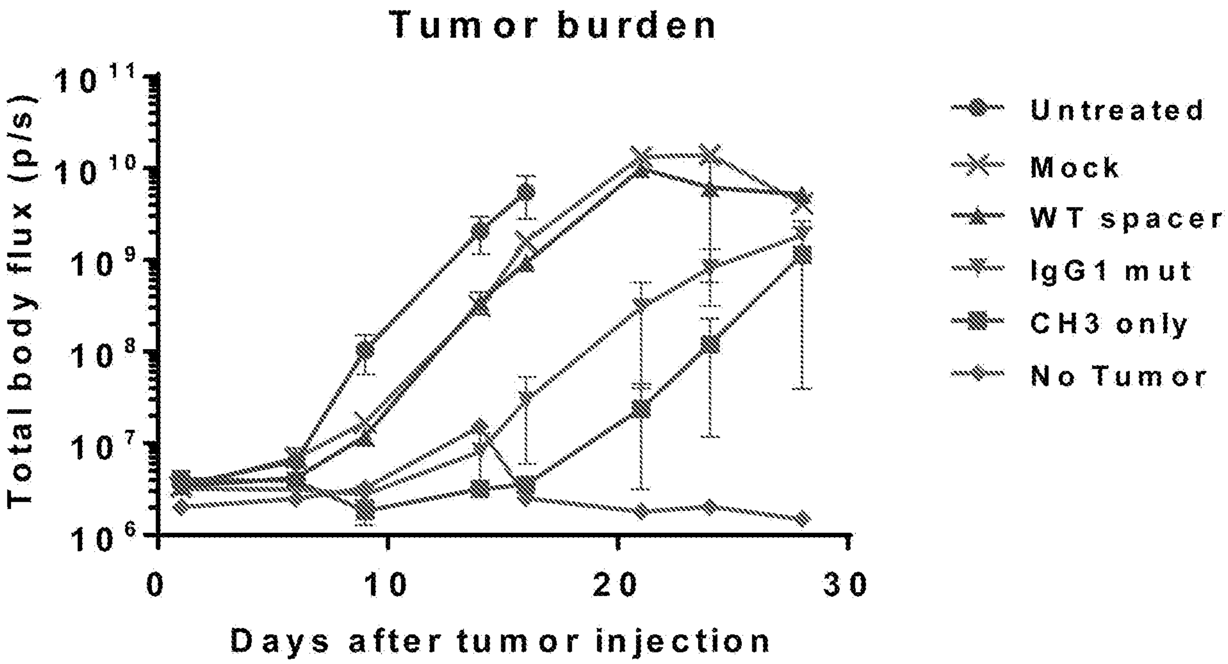

Tumor burden
Total body flux (p/s)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
10
20
30
Days after tumor injection
Untreated
Mock
WT spacer
IgG1 mut
CH3 only
No Tumor

FIG. 23D

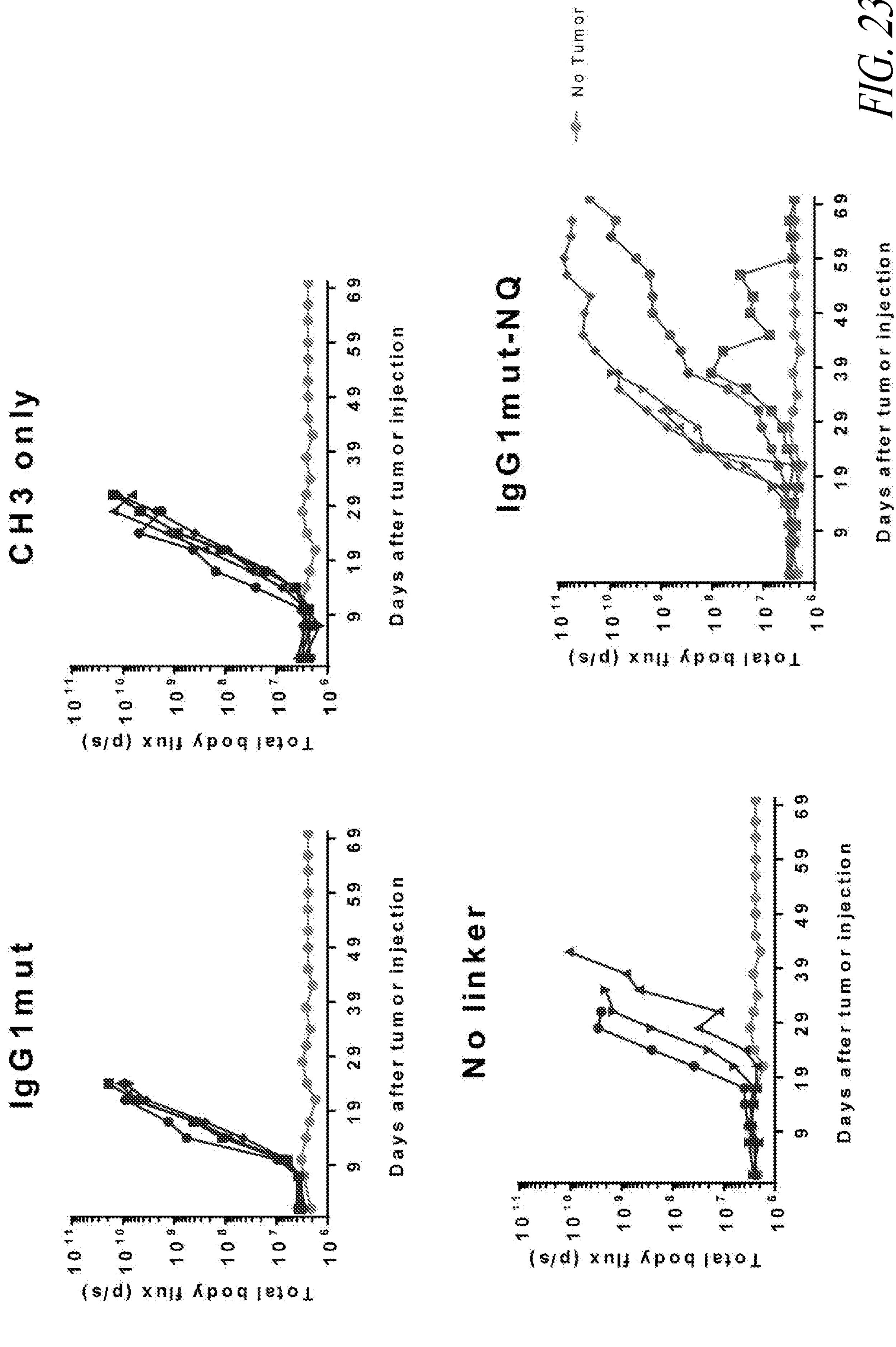

IgG1mut
CH3 only
No linker
IgG1mut-NQ
Total body flux (p/s)
Days after tumor injection
No Tumor

FIG. 23E

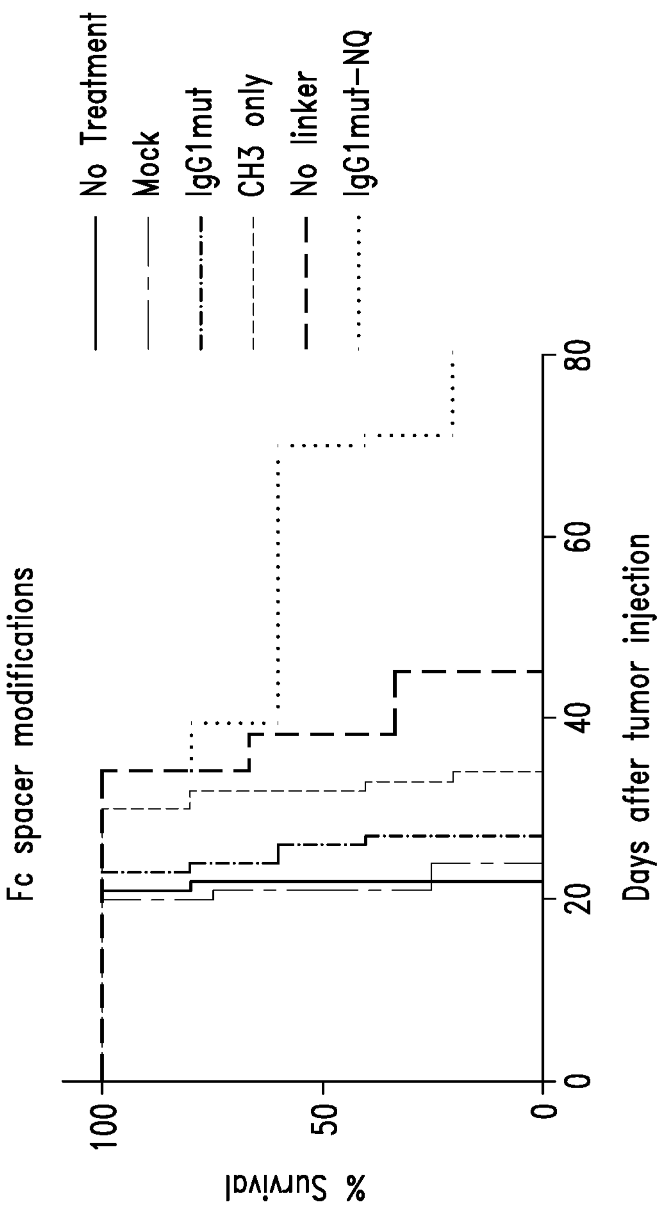
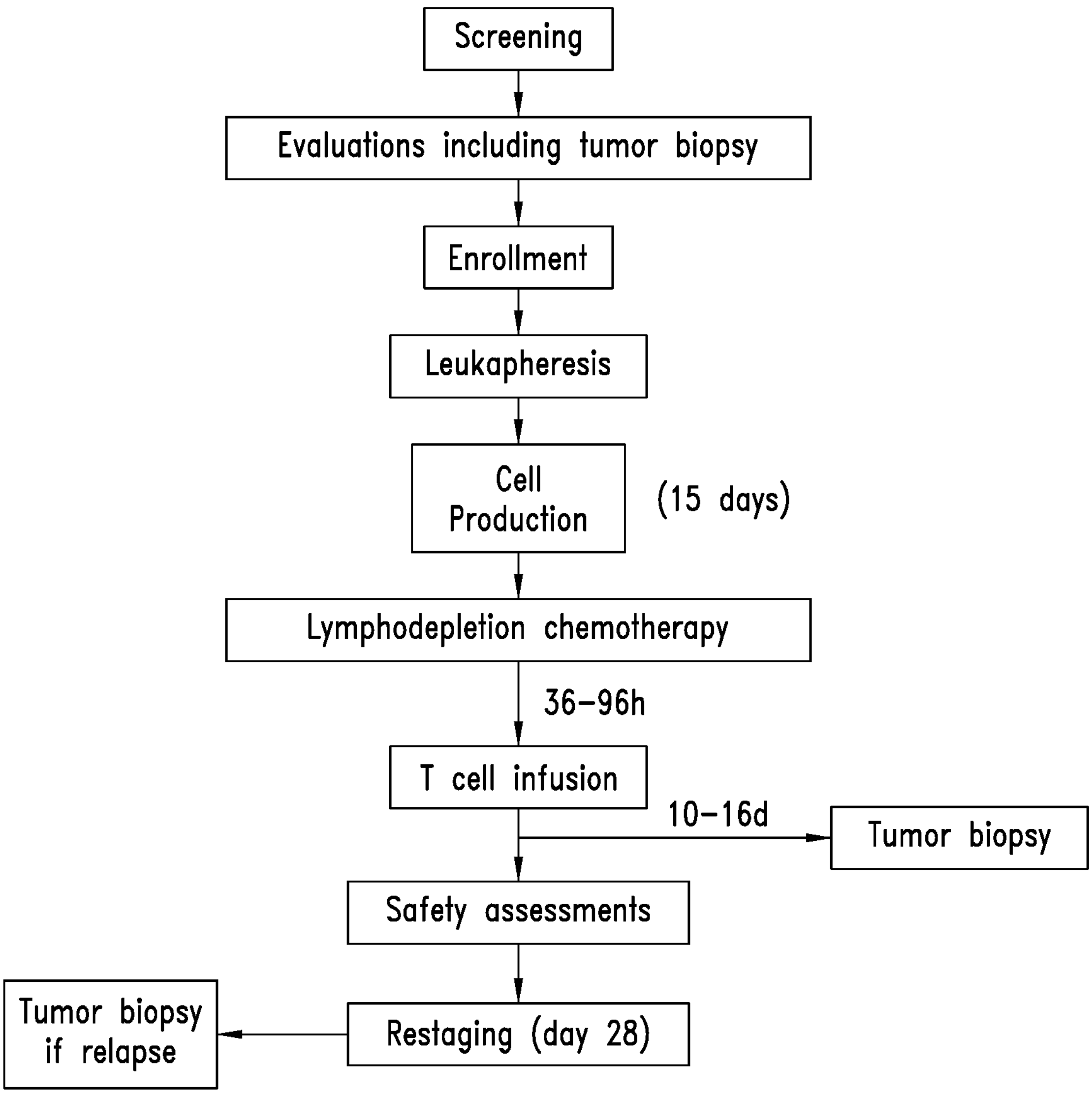

Fc spacer modifications
% Survival
100
50
0
0
20
40
60
80
Days after tumor injection
No Treatment
Mock
IgG1mut
CH3 only
No linker
IgG1mut-NQ

FIG. 23F



Screening
Evaluations including tumor biopsy
Enrollment
Leukapheresis
Cell Production
(15 days)
Lymphodepletion chemotherapy
36-96h
T cell infusion
10-16d
Tumor biopsy
Safety assessments
Restaging (day 28)
Tumor biopsy if relapse

FIG. 24

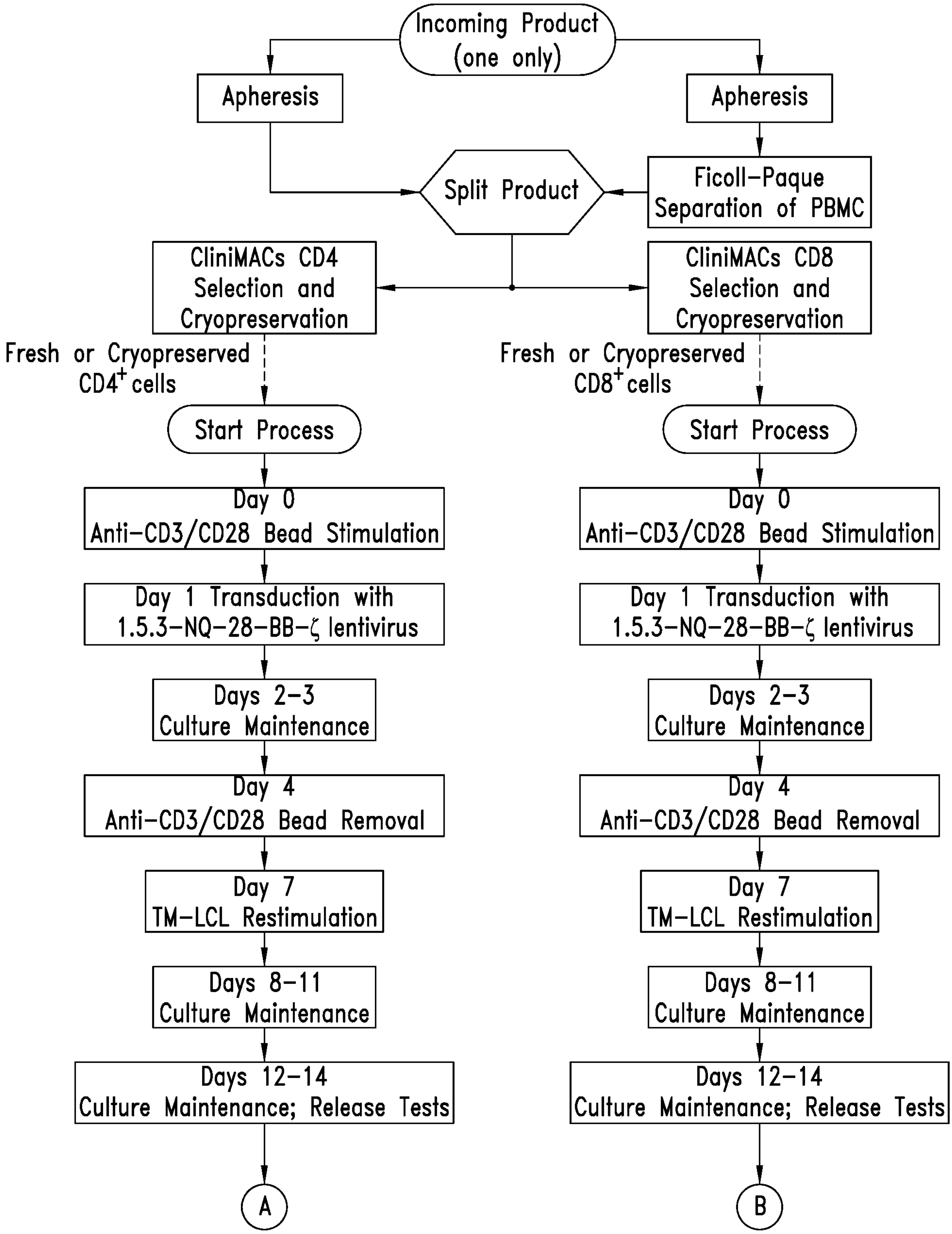

Incoming Product (one only)
Apheresis
Apheresis
Split Product
Ficoll–Paque Separation of PBMC
CliniMACs CD4 Selection and Cryopreservation
CliniMACs CD8 Selection and Cryopreservation
Fresh or Cryopreserved CD4+ cells
Fresh or Cryopreserved CD8+ cells
Start Process
Start Process
Day 0 Anti–CD3/CD28 Bead Stimulation
Day 0 Anti–CD3/CD28 Bead Stimulation
Day 1 Transduction with 1.5.3–NQ–28–BB–ζ lentivirus
Day 1 Transduction with 1.5.3–NQ–28–BB–ζ lentivirus
Days 2–3 Culture Maintenance
Days 2–3 Culture Maintenance
Day 4 Anti–CD3/CD28 Bead Removal
Day 4 Anti–CD3/CD28 Bead Removal
Day 7 TM–LCL Restimulation
Day 7 TM–LCL Restimulation
Days 8–11 Culture Maintenance
Days 8–11 Culture Maintenance
Days 12–14 Culture Maintenance; Release Tests
Days 12–14 Culture Maintenance; Release Tests
A
B

FIG. 25A

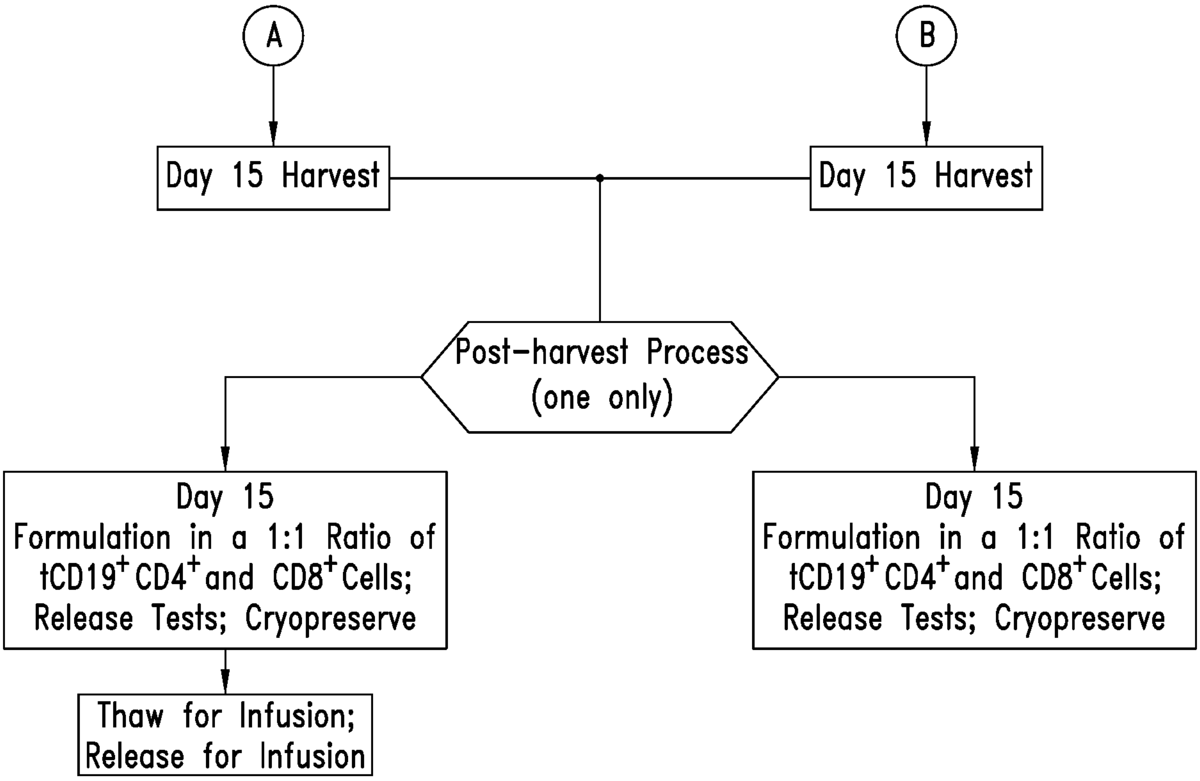

A
B
Day 15 Harvest
Day 15 Harvest
Post-harvest Process (one only)
Day 15 Formulation in a 1:1 Ratio of tCD19+ CD4+ and CD8+ Cells; Release Tests; Cryopreserve
Day 15 Formulation in a 1:1 Ratio of tCD19+ CD4+ and CD8+ Cells; Release Tests; Cryopreserve
Thaw for Infusion; Release for Infusion

FIG. 25B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/023098

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/725 A61K38/17 A61K39/395 C07K14/705 C07K16/28
C12N15/62
ADD.

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)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/23573 A2 (HOPE CITY [US]) 27 April 2000 (2000-04-27) the whole document sequences 1,2 -----	1,2, 4-10, 12-17
Y	LIHUA E. BUDDE ET AL: "Combining a CD20 Chimeric Antigen Receptor and an Inducible Caspase 9 Suicide Switch to Improve the Efficacy and Safety of T Cell Adoptive Immunotherapy for Lymphoma", PLOS ONE, vol. 8, no. 12, 17 December 2013 (2013-12-17), page e82742, XP055213511, DOI: 10.1371/journal.pone.0082742 abstract; figure 1 ----- -/--	3

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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)

"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

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 2017	24/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wiame, Ilse

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/130458 A2 (AMGEN FREMONT INC [US]; ASTRAZENECA AB [SE]; ASTRAZENECA UK LTD [GB];) 7 December 2006 (2006-12-07) cited in the application mAb 1.5.3 -----	3,11, 18-21
Y	RUFENER GREGORY A ET AL: "4428: Preservation of CD20-Specific Chimeric Antigen Receptor T Cell Function in the Presence of Residual Rituximab", BLOOD; 57TH ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY, THE AMERICAN SOCIETY OF HEMATOLOGY, US; ORLANDO, FL, USA , vol. 26, no. 23 3 December 2015 (2015-12-03), page 4428, XP008184699, ISSN: 0006-4971 Retrieved from the Internet: URL:http://www.bloodjournal.org/content/126/23/4428 the whole document -----	11,18-21
A	M. HUDECEK ET AL: "The Nonsignaling Extracellular Spacer Domain of Chimeric Antigen Receptors Is Decisive for In Vivo Antitumor Activity", CANCER IMMUNOLOGY RESEARCH, vol. 3, no. 2, 11 September 2014 (2014-09-11), pages 125-135, XP055177300, ISSN: 2326-6066, DOI: 10.1158/2326-6066.CIR-14-0127 page 133, column 1 -----	3
X,P	G. A. RUFENER ET AL: "Preserved Activity of CD20-Specific Chimeric Antigen Receptor-Expressing T Cells in the Presence of Rituximab", CANCER IMMUNOLOGY RESEARCH, vol. 4, no. 6, 21 April 2016 (2016-04-21), pages 509-519, XP055289850, US ISSN: 2326-6066, DOI: 10.1158/2326-6066.CIR-15-0276 the whole document -----	1-21

1

International application No.
PCT/US2017/023098

INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims 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. ☐ Claims 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 additional sheet

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 an 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 claims 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 claims Nos.:

1-21

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-21

An isolated polynucleotide encoding a fusion protein capable of specifically binding CD20, wherein the polynucleotide:
(a) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:53-56;
(b) is at least 80% identical to a polynucleotide sequence of any one of SEQ ID NOS.:44-47;
(c) comprises a polynucleotide sequence of any one of SEQ ID NOS.:53-56;
(d) comprises a polynucleotide sequence of any one of SEQ ID NOS. :44-47;
(e) consists of a polynucleotide sequence of any one of SEQ ID NOS.:53-56; or
(f) consists of a polynucleotide sequence of any one of SEQ ID NOS.:44-47.
A fusion protein encoded by said polynucleotide.
A host cell comprising said polynucleotide and capable of expressing said fusion protein.
A composition comprising said host cell and a CD20-specific binding molecule.
A method of treating a disease or disorder associated with CD20 expression in a subject having or suspected of having a disease or disorder associated with CD20 expression, comprising administering a therapeutically effective amount of said host cell.

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2. claims: 22-49

A method of treating a disease or disorder associated with CD20 expression, comprising administering to a subject having or suspected of having a disease or disorder associated with CD20 expression a therapeutically effective amount of a CD20-specific binding molecule and a therapeutically effective amount of a host cell comprising a heterologous polynucleotide encoding a fusion protein, the fusion protein comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds CD20 and the intracellular component comprises an effector domain.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/023098

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0023573 A2	27-04-2000	AU 2472400 A	08-05-2000
		US 6410319 B1	25-06-2002
		WO 0023573 A2	27-04-2000
WO 2006130458 A2	07-12-2006	AR 053514 A1	09-05-2007
		AU 2006252733 A1	07-12-2006
		BR PI0611220 A2	24-08-2010
		CA 2610234 A1	07-12-2006
		CN 101282993 A	08-10-2008
		EP 1891113 A2	27-02-2008
		JP 2008541758 A	27-11-2008
		KR 20080031001 A	07-04-2008
		TW 200716182 A	01-05-2007
		US 2007014720 A1	18-01-2007
		US 2011129412 A1	02-06-2011
		UY 29573 A1	29-12-2006
		WO 2006130458 A2	07-12-2006
		ZA 200710496 B	29-04-2009