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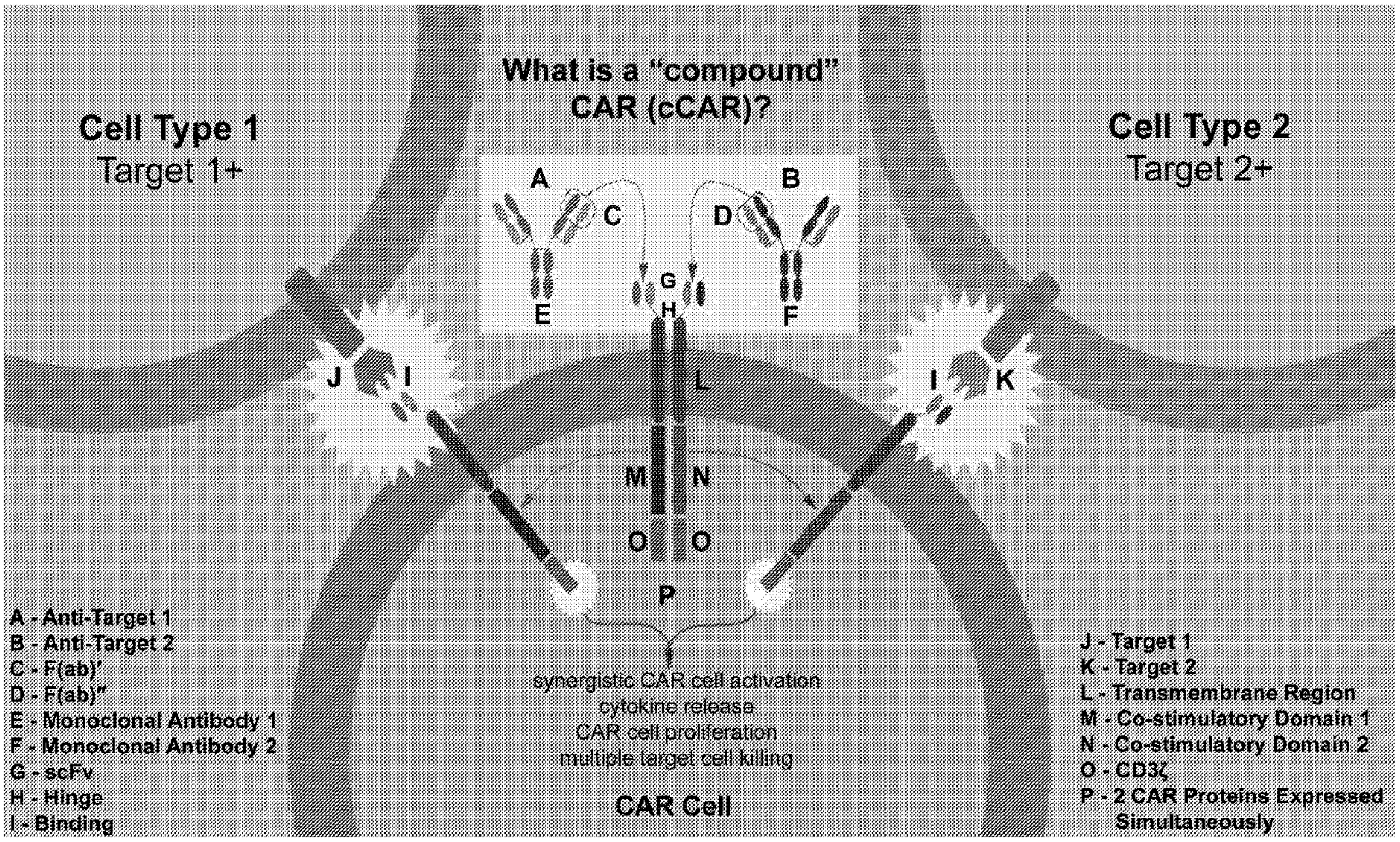

FIGURE 1

(57) Abrégé/Abstract:
The present disclosure relates to compositions and methods relating to chimeric antigen receptor (CAR) polypeptides and methods relating thereto. In one embodiment, the present disclosure relates to engineered cells having chimeric antigen receptor polypeptides directed to at least two targets. In another embodiment, the present disclosure relates to engineered cells having chimeric antigen receptor polypeptides and an enhancer moiety.

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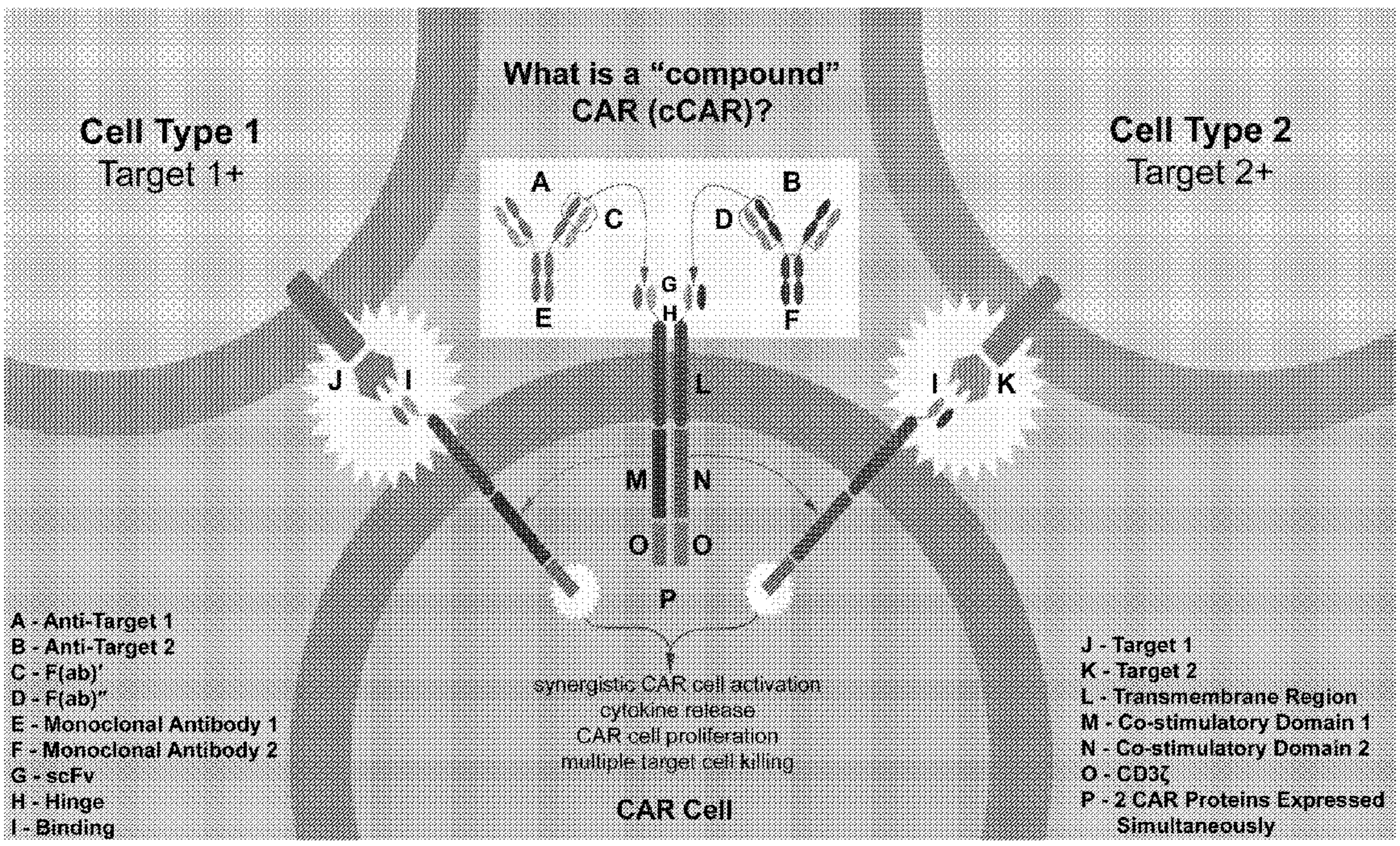

FIGURE 1

(57) Abstract: The present disclosure relates to compositions and methods relating to chimeric antigen receptor (CAR) polypeptides and methods relating thereto. In one embodiment, the present disclosure relates to engineered cells having chimeric antigen receptor polypeptides directed to at least two targets. In another embodiment, the present disclosure relates to engineered cells having chimeric antigen receptor polypeptides and an enhancer moiety.

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CHIMERIC ANTIGEN RECEPTORS (CARs), COMPOSITIONS AND METHODS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is an International PCT Application claiming priority from International PCT Application No. PCT/US16/39306, filed on June 24, 2016, and US Provisional Application No. 62/369,004, filed on July 29, 2016, the contents of which are incorporated herein by reference in its entirety.

BACKGROUND

T cells, a type of lymphocyte, play a central role in cell-mediated immunity. They are distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. T helper cells, also called CD4+ T or CD4 T cells, express CD4 glycoprotein on their surface. Helper T cells are activated when exposed to peptide antigens presented by MHC (major histocompatibility complex) class II molecules. Once activated, these cells proliferate rapidly and secrete cytokines that regulate immune response. Cytotoxic T cells, also known as CD8+ T cells or CD8 T cells, express CD8 glycoprotein on the cell surface. The CD8+ T cells are activated when exposed to peptide antigens presented by MHC class I molecules. Memory T cells, a subset of T cells, persist long term and respond to their cognate antigen, thus providing the immune system with “memory” against past infections and/or tumor cells.

T cells can be genetically engineered to produce special receptors on their surface called chimeric antigen receptors (CARs). CARs are proteins that allow the T cells to recognize a specific protein (antigen) on tumor cells. These engineered CAR T cells are then grown in the laboratory until they number in the billions. The expanded population of CAR T cells is then infused into the patient.

Clinical trials to date have shown chimeric antigen receptor (CAR) T cells to have great promise in hematologic malignancies resistant to standard chemotherapies. Most notably, CD19-specific CAR (CD19CAR) T-cell therapies have had remarkable results including long-term remissions in B-cell malignancies (Kochenderfer, Wilson et al. 2010, Kalos, Levine et al. 2011, Porter, Levine et al. 2011, Davila, Riviere et al. 2013, Grupp, Frey et al. 2013, Grupp, Kalos et al. 2013, Kalos, Nazimuddin et al. 2013, Kochenderfer, Dudley et al. 2013, Kochenderfer, Dudley et al. 2013, Lee, Shah et al. 2013, Park, Riviere et al. 2013, Maude, Frey et al. 2014).

Despite the success of CAR therapy in B-cell leukemia and lymphoma, the application of CAR therapy to T-cell malignancies has not yet been well established. Given that T-cell malignancies are associated with dramatically poorer outcomes compared to those of B-cell malignancies (Abramson, Feldman et al. 2014), CAR therapy in this respect has the potential to further address a great clinical need.

To date, current efforts have focused on CAR T-cells demonstrating efficacy in various B-cell malignancies. While initial remission rates of approximately 90% are common in B-ALL using CD19CAR, most of these relapse within a year. The relapse is at least in part due to the antigen escape. Thus, more effective CAR T cell treatments in order to prevent the relapse is urgently needed. Target discovery and selection are the initial step as there are no general rules to ensure or guide CAR design that are efficacious.

There are some roadblocks that hinder the broader adoption of CAR therapeutic approach. Among the most general challenges are: (1) selection of antigen target and chimeric antigen receptor(s); (2)CAR design; (3)tumor heterogeneity, particularly the variance in the surface expression of tumor antigens. Targeting single antigen carries the risk of immune escape and this could be overcome by targeting multiple desired antigens; (4) immunosuppressive microenvironment. CAR T cells may be suppressed and de-activated on arrival at the tumor site.

Most CAR chimeric antigen receptors are scFvs derived from monoclonal antibodies and some of these monoclonal antibodies have been used in the clinical trials or treatment for diseases. However, they have limited efficacy, which suggests that alternative and more potent targeting approaches, such as CARs are required. scFvs are the most commonly used chimeric antigen receptor for CARs. However, CAR affinity binding and locations of the recognized epitope on the antigen could affect the function. Additionally the level of the surface CAR expression on the T cells or NK cells is affected by an appropriate leader sequence and promoter. Furthermore, overexpressed CAR proteins can be toxic to cells.

Therefore, there remains a need for improved chimeric antigen receptor-based therapies that allow for more effective, safe, and efficient targeting of T-cell associated malignancies.

SUMMARY OF THE INVENTION

In one embodiment, the present disclosure provides an engineered cell having a first chimeric antigen receptor polypeptide including a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling domain; and a second chimeric antigen receptor polypeptide including a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain; wherein the first antigen recognition domain is different than the second antigen recognition domain.

In another embodiment, the present disclosure provides an engineered polypeptide including a chimeric antigen receptor and an enhancer.

In another embodiment, the present disclosure provides an engineered polypeptide including a chimeric antigen receptor polypeptide and an enhancer.

In another embodiment, the present disclosure provides an engineered chimeric antigen receptor polypeptide, the polypeptide including: a signal peptide, a CD45 antigen recognition domain, a hinge region, a transmembrane domain, at least one co-stimulatory domain, and a signaling domain. In another embodiment, the present disclosure provides a polynucleotide encoding for the aforementioned polypeptide.

In another embodiment, the present disclosure provides an engineered cell having the engineered polypeptide or polynucleotide described above.

In another embodiment, the present disclosure provides a method of reducing the number of target cells including the steps of (i.) contacting said target cells with an effective amount of an engineered cell having at least one chimeric antigen receptor polypeptide, for engineered cells having multiple chimeric antigen receptor polypeptides, each chimeric antigen receptor polypeptide is independent; and (ii.) optionally, assaying for the reduction in the number of said cells. The target cells include at least one cell surface antigen selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BAFF-R, BAFF, APRIL, BCMA, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD45, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1.

In another embodiment, the present disclosure provides methods for treating B-cell lymphoma, T-cell lymphoma, multiple myeloma, chronic myeloid leukemia, B-cell acute lymphoblastic leukemia (B-ALL), and cell proliferative diseases by administering any of the engineered cells described above to a patient in need thereof.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. A schematic representation of cCAR construct (hereinafter, “multiple CAR or compound CAR"). Multiple or compound CAR targets multiple antigens (e.g. cell type 1 or cell type 2 or the same cell type). Multiple or cCAR T cell immunotherapies comprises individual component CAR comprising a different or same antigen recognition domain, a hinge region, a transmembrane domain, various co-stimulatory domain(s) and an intracellular signaling domain.

Figure 2A. A schematic representation of cCAR-T construct. The construct comprises a SFFV promoter driving the expression of-multiple modular units of CARs linked by a P2A peptide. Upon cleavage of the linker, the cCARs split and engage upon targets expressing CD33 and/or CD123. As a novel cCAR construct, the activation domains of the construct may include, but is not limited to, 4-1BB on the CD33 CAR segment and a CD28 region on the CD123 CAR.

Figure 2B. A Western blot depicting the expression of transduced CD33CD123 cCAR-T cells. The figure depicts expression of two different CAR proteins, i.e., CD33 CAR and CD123 CARs. The cCAR-T cells expressing both CD33 and CD123 CARs upon cleavage of the linker generate two distinct and consistently intense protein bands. Green Fluroscent Protein (GFP) is included as negative control.

Figure 2C. Flow cytometry representing the efficiency of transduction. Upper panel shows the lentiviral titer for CD33CD123 cCARs (also referred to as CD33CD123-2G-CAR) tested on 293FT HEK (human embryonic kidney) cells to gauge maximum transduction efficiency before usage on UCB (umbilical cord blood) and PB (peripheral blood) T-cells. Lower panel shows CD33CD123 cCAR (also referred to as CD33CD123-2G-CAR) T-cells transduced with lentiviral vectors comprising CD33CD123 cCAR construct and GFP-transduced cells as control Percentages indicated by yellow circles are proxies for transduction efficiency.

Figure 3. Schematic showing a method of generating a high-efficiency compound CAR (cCAR). HEK-293-FT cells are transfected with compound CAR plasmid DNA and lipofectamine 2000; viral supernatant collected at about 36 hr and at about 60 h; filtered and stored at -80°C. T cells are activated with anti-mouse CD3 antibody and IL-2 for at least 2 days. Activated T cells are transduced at least once with thawed lentivirus on retronectin-coated plates; After at least one overnight transductions at 0.3 x 106 T cells/mL for about 2 days, the number of T cells was reduced in order to increase transduction efficiency. After transduction, cells are washed and expanded; Flow analysis (F(Ab')2 labeling) is done to confirm CAR efficiency on day 3; total 5-7 day expansion. cCAR T cells are co-cultured with target cells in vitro and cCAR T cells killing efficacy of cancer cells is assessed in vivo (mice).

Figure 4. A co-culture assay representing the incubation of CD33CD123-2G CAR-T cells (cCAR) with the promyelocytic leukemia cell line HL60. cCAR-T cell (lower panel) is compared to control GFP transduced T-cell (upper panel). The efficacy of the killing is measured by the population of CD33+ cells that is left over after incubation for about 24 hours (enclosed in yellow circles).

Figure 5. A co-culture assay representing incubation of cCAR-T cells with the myelogenous leukemia cell line KG-1a, which expresses about 100% CD33 and about 50-80% CD123. cCAR-T cell (lower panel) is compared to control GFP transduced T-cell (upper panel). The efficacy of the killing is measured by the population of CD33+ cells that is left over after incubation for about 24 hours.

Figure 6. CD33CD123 cCAR-T cells co-cultures with AML-9 at 5:1. A co-culture assay representing incubation of cCAR-T cells with AML patient samples (here referred to as AML-9). The patient cells include mixed populations of cells, such as for example, leukemia cells, monocytes, and other types of blasts. CD33 acts as a marker for CAR-T action as well as CD34, a specific marker for leukemia cells. The CAR-T panel (right) is compared to control GFP transduced T-cells (middle). The efficacy of the killing is measured by the population of CD33+/CD34+ cells that is left over after incubation for at least 24 hours.

Figure 7. CD33CD123 cCAR-T cells co-cultures with Sp-BM-B6 at 5:1. A co-culture assay representing incubation of cCAR-T cells with B-ALL patient samples (here referred to as Sp-BM-B6). The patient cells include mixed populations of cells, such as, for example, leukemia cells, monocytes, and other types of blasts. CD34 acts as a specific marker for leukemia cells. The CAR-T panel (right) is compared to control GFP transduced T-cells (middle). The efficacy of the killing is measured by the population of CD34+ cells left over after incubation for at least 24 hours.

Figure 8. CD33CD123 cCAR expression in NK-92 cells. The CD33CD123 cCAR expression are detected using goat-anti-mouse antibody, F(ab)2.

Figure 9. A co-culture assay representing incubation of CD33CD123 cCAR NK-92 cells with HL-60. The cCAR NK-92 cells are compared with GFP transduced NK-92 cells. The efficacy of the killing is measured by the population of CD33+ cells left over after incubation for about 24 hours.

Figure 10. A co-culture assay representing incubation of cCAR NK-92 cells with KG1a. The cCAR NK cell panel is compared with GFP transduced NK-92 cells. The efficacy of the killing is measured by the population of CD33+ cells left over after incubation for about 24 hours.

Figure 11. Dose response of CD33CD123 cCAR (CAR-CD33/123) NK-92 cells with HL-60 or KG1a. The efficacy of the killing is measured by the population of CD33+ cells left over after incubation for about 24 hours.

Figure 12. A comparison of CD33CD123 cCAR NK-92 cell killing ability with control in two populations of KG11 cells. Assays were performed at different ratios of CAR-CD33/123 (CD33CD123 cCAR NK-92 cells) and target cells, kG1a. The efficacy of the killing is measured by the population of CD33+CD123+ or CD33+CD123- cells left over after incubation for about 24 hours.

Figure 13A. Links by P2A and T2A schematic showing both cCAR-T and 4-1BBL in a single construct. The construct consists of a SFFV promoter driving the expression of two modular units of CARs A peptide and an enhancer, 4-1BBL. Upon cleavage of the linkers, the cCARs and 4-1BBL split and engage upon targets expressing CD33 and/or CD123 and 4-1BBL.

Compound CAR, CD33CD123 CAR T cells received not only costimulation through the CD28 but also 4-1BB ligand (4-1BBL or CD137L). The CD3-zeta signaling domain complete the assembly of this CAR-T.

Figure 13B. Expression the CD33CD123-41BBL-2G construct in T-cells. T-cells derived from peripheral blood from healthy donors were transduced with the CD33CD123-4-1BBL-2G construct in 6-well plates incubated with 2 ml of virus supernatant. CAR expression was assayed with F(ab)' labeling for surface expression of the CAR protein and subsequently underwent FACS analysis. Transduced cells were compared to control T-cells labeled at the same time. Expression was determined and transduced population encircled on plot 1 day after end of transduction period.

Figure 14. Links by P2A and T2A schematic showing both cCAR-T and IL-15/IL-15sushi in a single construct. The construct consists of a SFFV promoter driving the expression of two modular units of CARs and an enhancer, IL-15/IL-15sushi. Upon cleavage of the linkers, the cCARs and IL-15/IL-15sushi split and engage upon targets expressing CD33 and/or CD123. The CD3-zeta signaling domain completes the assembly of this CAR-T. The enhancers include, but not limited to, IL-15/IL-15sushi on cCAR.

Figure 15. A schematic representation of cCAR. The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs linked by a linker. Upon cleavage of the linker, the cCARs split and engage upon targets expressing combinations of various target antigens: CD19 and/or CD20, and/or CD22 and/or 138. Multiple cCARs utilize the same or different co-stimulatory domains, such as, without limiting 4-1BB (also labeled as 4-BB) and/or CD28.

Figure 16. Activated T cells transduced to make CD19CD20-2G, CD19CD22-2G CAR T cells (all are L8). (16A) Design of compound CARs. (16B) Western blot. HEK-293T cells were transfected with lentiviral plasmids for control vector (lane 1), CD19CD20-2G (lane 2), and CD19CD22-2G (lane 3). 48 hours after transfection, supernatant was removed, and cells were also harvested. Cells were lysed for Western blot and probed with mouse anti-human CD3z primary antibody, and goat anti-mouse HRP secondary antibody. (16C) PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), CD19CD20-2G (middle) , or CD19CD22-2G, (right) lentiviral supernatant. After 3 days of

incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2).

Figure 17. Expression of compound CD19CD22CAR T cells using different leader sequences. PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), L8-CD19CD22-2GCAR (middle left) , L45-CD19CD22-2GCAR, (middle right) or CSF-CD19CD22-2GCAR (right) lentiviral supernatant. The supernatants were each 3x concentrated. After 3 days of incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2).

Figure 18. Comparison of transduction efficiency using concentrated vs unconcentrated L8-CD19CD22-2G or L8-CD19CD20-2G lentiviral supernatant. A. PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), unconcentrated (middle) L8-CD19CD22-2GCAR or 3x concentrated L8-CD19CD22-2GCAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2). B. The same experiment was useed for constructs containing L8-CD19CD20-2G unconcentrated or 2.5x concentrated lentiviral vector.

Figure 19. L8-CD19CD22-2G CAR T cells lyse SP53 tumor cells in overnight co-culture. Activated PMBC T cells transduced with either control (top row), L8-CD19CD22-2G, or (bottom row) lentiviral supernatant were incubated with SP53 cells at the ratios of 1:1 (left) 2:1 (middle) and 5:1 (right), effector:target cells. After 24 hours of incubation at 37^{o}C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. SP53 cells alone are shown on the far upper right, and a summary of percent lysis at each ratio is on the lower right. (N=2).

Figure 20. L8-CD19CD22-2G CAR T cells lyse JeKo-1 tumor cells in overnight co-culture. Activated PMBC T cells transduced with either control (left), or L8-CD19CD22-2G, (middle) 3x concentrated lentiviral supernatant were incubated with JeKo-1 cells at the ratios of 2:1 (top) and 5:1 (bottom), effector:target cells. After 24 hours of incubation at 37°C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. JeKo-1 cells alone and a summary of cell lysis are shown on the right. (N=2).

Figure 21. L8-CD19CD22-2G CAR T cells lyse AML patient cells in overnight co-culture. Activated PMBC T cells transduced with either control (left), or L8-CD19CD22-2G, (middle) 3x concentrated lentiviral supernatant were incubated with CMTMR-stained cells from a patient diagnosed with AML (PT1) at the ratios of 2:1 (top) and 5:1 (bottom), effector:target cells. After 24 hours of incubation at 37 °C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. Patient cells alone and a summary of cell lysis are shown on the right. (N=2).

Figure 22A. L8-CD19CD22-2G CAR T cells deplete CD19+ B-ALL patient cells. Activated PMBC T cells transduced with either control (left), or L8-CD19CD22-2G, (middle) lentiviral supernatant were incubated with CMTMR-stained cells from a patient with B-ALL (PT2) at a 1:1 ratio for 4 days in the presence of 2.5% FBS and IL-2. Following this incubation at 37 °C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. Prestained patient cells cultured alone for 4 days are shown on the right.

Figure 22B. L-8-CD19CD22-2G cCAR T-cells show effect on $CD22^+$ K562 cells. An artificial K562 expressing CD22 cell line (K562xp22) via transduction into wild-type K562 cells was generated. Subsequently, we tested the anti-tumor properties of the CD19CD22 cCAR to target the minor $CD22^+$ population of the K562 cells. A co-culture experiment at 1:1 ratio (effective: target) show a modest significant cytotoxic effect on K562 expressing CD22 population compared to the control. Co-cultures were stained with CD3, CD19 and CD22 to separate effector and target populations by flow cytometry. The result was graphed. Cytotoxicity results remain consistent with other numbers reported for anti-tumor activity against artificial antigen presenting cell lines.

Figure 23. Various transduction schemes for BC1cCAR lentivirus. **(A)** Method 1 consisting of a 2x transduction for 24 hours each time is a baseline transduction scheme. Scheme proceeds according to the figure. **(B)** Method 2 possesses the same methodology as Method 1, however, the second transduction is replaced by continued incubation. **(C)** Method 2 revised uses viral supernatant incubated with cells directly for 48 hours.

Figures 24A-24C: CAR construct scheme and comparison of transduction methodologies. **(24A)** BC1cCAR's modular design consists of an anti-CD269 (BCMA) single-chain variable fragment (scFv) region fused to an anti-CD319 (CS1) scFv by a self-cleaving P2A peptide, CD8-derived hinge (H) and transmembrane (TM) regions, and tandem CD28 and 4-1BB co-activation domains linked to the CD3ζ signaling domain. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the CD3CAR molecule on the T-cell surface. **(24B)** BC1cCAR's expression was measured via flow cytometry against an isotype control. Population encircled represents transduced CAR cells. **(24C)** Transduction efficiency is improved by optimal methods.

Figure 24D. Protein expression of BC1cCAR and BCMA-CS1-2G in HEK-293FT cells. HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1), BC1cCAR (lane 2) 48 hours after transfection, supernatant was removed, and cells were also removed. Cells were lysed for Western blot and probe with mouse anti-human CD3z antibody. C. Transduction efficiency is improved by optimal methods.

Figures 25A-25C. in *vitro* evaluation of BC1cCAR T-cells against myeloma cell lines. **(25A)** BC1cCAR and control T-cells were cultured with highly BCMA positive MM1S and RPMI-8226 cells for 24 hours at E:T ratios of 2:1 and 5:1. Target MM1S and RPMI-8226 cells were stained by Cytotracker dye (CMTMR) to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319 antibodies) along with CMTMR-PE. Target U266 cells were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Encircled populations represent tumor cells. **(25B)** U266 target depletion. BC1cCAR and control T-cells were also incubated with U266 cells expressing BCMA and a subset of CS1. Target tumor cells were stained as described above and gating conditions applied similarly. Tumor populations are encircled. **(25C)** In vitro summary of BC1cCAR T activity against human myeloma cell lines. Graphical summary of BC1cCAR T-cell in *vitro* cytotoxicity against various myeloma cell lines at 2:1 and 5:1 E:T ratios.

Figures 26A-26D. Characterization of BC1cCAR T-cell anti-tumor activity against primary myeloma tumor cells. **(26A)** Dose dependent effect on MM7-G primary double phenotype tumors. BC1cCAR and control T-cells were cultured against $BCMA^{+}CS1^{+}$ primary myeloma cells MM7-G for 24 hours. Target cells were pre-stained with CMTMR and cultures were carried out in E:T ratios of 2:1, 5:1, and 10:1. Populations were gated by BCMA and CS1, along with CMTMR, and flow cytometry plots with populations encircled represent target tumor populations (left). Bar graph summarizing in *vitro* cytotoxicity is shown for clarity (right). **(26B)** Population specific depletion in MM10-G. Co-cultures with MM10-G primary tumor cells were carried out in similar conditions. When stained with anti-CS1 and anti-BCMA antibody, MM10-G reveal distinct populations. $BCMA^{+}CS1^{+}$ double positive populations are colored purple whilst $CS1^{+}$ only populations are colored dark blue. BC1cCAR T-cell cytotoxicity against each population is summarized in the bar graph below. **(26C)** Dose dependent effect on CS1dim BCMAneg. MM11-G primary tumor. A third experiment using $BCMA^{dim}CS1^{dim}$ primary cells (MM11-G) further shows BC1cCAR cytotoxicity effects over a range of E:T dosages summarized. **(26D)** Summary panel graph showing BC1cCAR T-cell cytotoxicity against myeloma cell lines and primary tumor cells with a variety of BCMA and CS1 compositions.

Figures 27A-27D. Functional validation of BC1cCAR antigenic specificity. **(27A)** We engineered a CML cell line, K562, to express either BCMA or CS1 independently. Wild-type K562 shows as a negative peak, while BCMA expressing K562 (BCMAxpK562) and CS1 expressing K562 (CS1xpK562) show population shifts in their respective antigen expression ranges. **(27B)** Short term (4 hour – 12 hour) cultures of BC1cCAR T-cells against either BCMAxpK562 or CS1xpK562 show antigen specific cytotoxicity correlating with E:T dosage increase. Experiments against wild-type K562 were performed as a negative control. A CS1-specific single CAR was generated to compare efficacy with BC1cCAR against CS1xpK562 cells and are delineated with red bars in the respective plot. Anti-CS1 specific activity was also seen against $CS1^{dim}$ NK-92 cells after 24 hours of culture. **(27C)** Comparison between single antigen CARs and BC1cCAR T in mixed cell assays. Long-term cultures were conducted over a 48 hour period with a 5:1 mixture of BCMAxpK562 cells and CS1xpK562 cells. BC1cCAR, CS1-CAR, BCMA-CAR, and control T-cells were added at a 5:1 E:T ratio to each treatment well and flow cytometry analyses acquired. Histogram plots showing residual populations of BCMA or CS1 cells are shown per treatment condition, with red lines demarcating T-cell or target tumor populations. Numerical values in histogram plots represent residual gated populations of target tumor cells. **(27D)** BC1cCAR T activity against CS1 subsets in primary bone-marrow aspirate.Further co-culture experiments were conducted using bone-marrow aspirate samples as CS1 expressing minority subsets. BC1cCAR or control T-cells were added at 2:1 (left panel), 5:1 (middle panel), or 10: 1 (right panel) ratios and encircled populations represent target CS1 expressing populations. Results are analyzed by flow cytometry (upper). Summary graph of anti-CS1 activity against bone marrow subsets (below).

Figures 28A-28C: Long-term sequential killing assay and tumor re-challenge. **(28A)** Scheme for construction of long-term sequential killing assay. Assay was conducted over a period of 168 hours with no exogenous cytokines where the initial culture was set-up with a 1:1 E:T ratio of CAR cells or control cells mixed with MM1S tumor cells. After 48 hours, flow cytometry analysis was acquired for a small sample collection and MM1S cells re-introduced into each treatment well. Repeated until the 168 hour time-point. **(28B)** T-cell proliferation and response after 48 hours. Images were taken on day of flow cytometry acquisition and cells were stained with anti-BCMA, anti-CS1, and anti-CD3 antibodies MM1S cells express as highly $BCMA^+$ with a large $CS1^+$ proportion. Encircled populations represent the MM1S tumor presence, colored blue. **(28C)** CAR cell proliferation and antigen depletion after 108h. Similar image acquisition and flow cytometry analysis was performed at the 108 hour time mark.

Figure 29A-29C. BC1cCAR T-cells demonstrate anti-leukemic effects *in vivo*. **(29A)** IVIS imaging of MM1S Luc+ injected mouse model. NSG mice were sublethally irradiated and intravenously injected with luciferase-expressing MM1S multiple myeloma cells to induce measurable tumor formation. After 3 days, the mice were intravenously injected with 5×10^6 BC1cCAR T-cells or control GFP T-cells. On days 3, 6, 8 and 11, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. **(29B)** BC1cCAR T-cells control MM1S tumor growth. Average light intensity measured for the BC1cCAR T-cells injected mice was compared to that of GFP control T-cell injected mice. **(29C)** BC1cCAR T-cells improve murine survival outlook. Percent survival of mice was measured and compared between the two groups and log-rank mantel-cox test was conducted to calculate significance of improved survival outlook.

Figure 29D. BCMA-CAR and BC1 cCAR T-cells demonstrate a profound anti-leukemic effect on a mixture of K562 cells expressing BCMA and CS1 in xenograft mouse model. Luciferase positive K562 cells expressing BCMA are mixed with luciferase positive K562 cells expressing CS1 at a ratio of 4:1 BCMA to CS1 K562 cells. The mixed K562 cells (0.5×10^6 cells) were then injected intravenously (day 1) at 24 h later after sub-lethal irradiation. After day 3, a course of BCMA CAR T-cells, BC1cCAR T-cells or control T-cells were intravenously injected into each mouse (n=5 for each group). Dorsal side of tumor burden was measured using IVIS imaging system at days 3, 7, 10 and 12. At day 7 BCMA mouse #3 has large tumor. At day10 Dorsal BCMA vs control=47.7% less tumor, cCAR vs control=53.8% less tumor. At day 12 RESULTS (ventral view only) Dorsal BCMA vs control=43.8% less tumor, cCAR vs control=60.7% less tumor

Figure 29E. BCMA and BC1 cCAR T-cells in vivo significant reduction of tumor burden. Percent reduction relative to control in mice treated with BCMA CAR T-cells or cCAR (BC1 cCAR) relative to control over time.

Figures 30A-30B: BC1cCAR transduction into NK-92 cells. **(30A)** BC1cCAR's modular design is comprised as shown and described previously. **(30B)** CAR expression on NK-92 cell surface. The construct was transduced into NK-92 cells by incubating with viral supernatant for 48 hours and labeling with F(ab)' antibody detection for CAR protein surface expression. Transduced populations are encircled and compared to control NK-92 cells.

Figures 31A-31B. Characterization of BC1cCAR NK-92 anti-tumor properties. **(31A)** BC1cCAR NK cells lyse myeloma cell lines and primary cells. BC1cCAR NK-92 cells were incubated against U266, RPMI-8226, and MM1S myeloma cell lines in addition to primary MM7-G tumor cells. Co-cultures were carried out over 2 hours at an E:T ratio of 5:1 and labeled with anti-CS1 and anti-BCMA antibodies to separate out populations. Tumor populations are encircled. MM7-G primary tumor cells were stained with cell cytotracker dye (CMTMR) to distinguish from NK-92 cells and are encircled. Summary bar graph of BC1cCAR NK-92 cytotoxic activity is presented as a visualization aid. **(31B)** BC1cCAR NK-92 cells were tested for antigen specific activity using artificially generated BCMA expressing K562 (BCMAxpK562) and CS1 expressing K562 (CS1xpK562) cells. Co-cultures were carried out over 4 hours at an E:T ratio of 5:1. K562 populations were previously stained with CMTMR and encircled in the flow cytometry plots. Bar graph summarizing anti-tumor activity to visualize.

Figures 32A-32C. Generation and characterization of different BAFF-CAR constructs. **(32A)** L45-BAFF-28 CAR expression T-cell surface. L45-BAFF-28 CAR was transduced into T-cells and evaluated for surface expression using F(ab)' antibody. Gating was compared to controls. **(32B)** CAR expression dependence on leader sequence. BAFF-CAR constructs using different leader sequences were tested to determine if efficiency in transduction could be improved. Surface detection was evaluated using F(ab)' antibody and transduced populations encircled. **(32C)** CAR expression dependence on construct design. Additional BAFF-CAR constructs containing different leader sequences and construct designs (additional units) were validated and used to determine if CAR transduction could be improved. Transduced populations are encircled and gating compared to control T-cells. CSF-BAFF-28 41BBL is a BAFF CAR co-expressing 4-1BBL (41BBL) with a CSF leader sequence. CSF-BAFF-28 IL-15RA is a BAFF CAR co-expressing IL-15/IL-15sushi (IL-15RA) with a CSF leader.

Figure 33: Characterization of L45-BAFF-28 CAR T anti-tumor properties. **(33A)** L45-BAFF-28 CAR T-cells possess anti-tumor activity against MM1S tumor cells. L45-BAFF-28 CAR T-cells were cultured for 48 hours at an E:T ratio of 3:1 against MM1S myeloma cells. Duplicate samples are shown. Cytotoxic activity is summarized in the bar graph.

Figures 34A-34B: Characterization of anti-tumor activity using different BAFF-CAR constructs and enhancements. **(34A)** BAFF-CAR constructs against MM1S cells. L8-BAFF-28IL-15/IL-15sushi and L8-BAFF-28-41BBL CARs were cultured for 24 hours against MM1S tumor cells at an E:T ratio of 5:1. Tumor populations are encircled. **(34B)** BAFF-CAR constructs against SP53 cells. Both CARs and L45-BAFF-28 CAR were cultured against Sp53 tumor cells (B-lineage) at an E:T ratio of 5:1 for 24 hours. **(34C)** Summary bar graph of cytotoxic activity.

Figure 35. A schematic showing cCAR construct. The construct consists a SFFV promoter driving the expression of two modular units of CAR linked by a P2A peptide. Upon cleavage of this P2A peptide, the cCARs split and engage upon targets expressing BCMA and /or CD19. Two unit CARs use different or same co-stimulatory domain. A co-stimulatory domain could be, but limited to, 4-1BB or CD28.

Figures 36A-36B. Characterization of the BCMA CAR unit. **(36A)** BCMA CAR effectively deplete BCMA+ MM1S cells. The BCMA CAR was transduced into T-cells and co-cultured with MM1S tumor cells. A CS1 CAR was also generated and used for robustness. MM1S cells are significantly dual positive for both BCMA and CS1. Co-cultures were conducted over 48 hours with BCMA and CS1 antibodies used to identify tumor centers. Encircled populations represent residual MM1S tumor cells after culture. **(36B)** BCMA CAR effectively lyses BCMA+ primary tumor cells. (36B) The BCMA CAR and CS1 CAR were also evaluated for its anti-tumor properties against primary MM7-G myeloma patient cells. The MM7-G population is a majority $BCMA^{+}$ $CS1^{+}$ population with minority but significant $CS1^{+}$ only populations as well. Both BCMA CAR and CS1 CAR were used in tandem to evaluate cytotoxicity with BCMA and cytotracker (CMTMR) used to differentiate tumor populations from CAR cells.

Figures 37A-37C. Characterization of CD19 CARs. **(37A)** Design of CD19CAR unit. **(37B)** Western blot. HEK-293T cells were transfected with lentiviral plasmids for control vector (lane 1) and CD19-2G (lane 2). 48 hours after transfection, supernatant was removed, and cells were also harvested. Cells were lysed for Western blot and probed with mouse anti-human CD3z primary antibody, and goat anti-mouse HRP secondary antibody. C. PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), L8-CD19-2G (middle left), L8-CD19CD20-2G, (middle right) or L8-CD19CD22-2GCAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2)

Figures 38A-38B. Expression of compound CD19CAR T cells using different leader sequences. **(38A)** CAR constructs were designed to express the fusion protein with different leader sequences. **(38B)** PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), HA-CD19-2G (top middle) , IL2-CD19-2G (top right), L8-CD19-2G (lower middle left) , L45-CD19-2G, (lower middle right) or CSF-CD19-2GCAR (lower right) lentiviral supernatant. After 3 days of incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2)

Figures 39A-39B. Expression of CD19CAR on T cells using different CD19 scFv sequences. **(39A)** CAR constructs were designed to express the fusion protein with different scFv sequences. **(39B)** PMBC buffy coat T cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), L8-CD19-2G (middle), IL2-CD19-2G (top right), or L8-CD19b-BB-2G (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and incubated with goat anti-mouse Fab2 or goat IgG antibodies conjugated with biotin for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE and mouse anti-human CD3-PerCp for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry to determine CAR efficiency. (N=2)

Figure 40. L8-CD19-2G and CD19b-BB CAR T cells lyse SP53 tumor cells in overnight co-culture. Activated PMBC T cells transduced with either control (left), L8-CD19-2G, (middle) or L8-CD19b-BB-2G (right) lentiviral supernatant were incubated with SP53 cells at the ratios of 2:1 (top) and 5:1 (bottom), effector:target cells. After 24 hours of incubation at 37^{o}C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. SP53 cells alone and a summary of cell lysis are shown on the far right. (N=2)

Figure 41. L8-CD19-2G and CD19b-BB CAR T cells lyse JeKo-1 tumor cells in overnight co-culture. Activated PMBC T cells transduced with either control (left), L8-CD19-2G, (middle) or L8-CD19b-BB-2G (right) lentiviral supernatant were incubated with JeKo-1 cells at the ratios of 2:1 (top) and 5:1 (bottom), effector:target cells. After 24 hours of incubation at 37^{o}C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. JeKo-1 cells alone and a summary of cell lysis are shown on the far right. (N=2).

Figure 42. L8-CD19-2G and L8-CD19b-BB-2G CAR T cells lyse AML patient cells in overnight co-culture. Activated PMBC T cells transduced with either control (left), L8-CD19-2G, (middle) or L8-CD19b-BB-2G (right) lentiviral supernatant were incubated with CMTMR-

stained cells from a patient with AML at the ratios of 2:1 (top) and 5:1 (bottom), effector:target cells. After 24 hours of incubation at 37^{o}C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. Prestained patient cells alone and a summary of cell lysis are shown on the far right. (N=2).

Figure 43. L8-CD19-2G and L8-CD19b-BB-2G CAR T cells deplete CD19+ patient cells. Activated PMBC T cells transduced with either control (left), L8-CD19-2G, (middle) or L8-CD19b-BB-2G (right) lentiviral supernatant were incubated with CMTMR-stained cells from a patient with B-ALL. L8-CD19-2G T cells were incubated with patient cells at a 1:1 ratio for overnight (top), while L8-CD19b-BB-2G T cells were incubated with patient cells at a 5:1 ratio for 40 hours (bottom). Following this incubation at 37^{o}C, samples were washed and stained with anti-human CD3-PerCp and anti-human CD19-APC, washed, and analyzed by flow cytometry. Prestained patient cells alone are shown on the far right. (N=2).

Figure 44. A schematic showing cCAR construct. The construct consists a SFFV promoter driving the expression of two modular units of CAR linked by a P2A peptide. Upon cleavage of this P2A peptide, the cCARs split and engage upon targets expressing BCMA and /or CD19b. Two unit CARs use different or same co-stimulatory domain. A co-stimulatory domain could be 4-1BB or CD28.

Figures 45A-45C. Generation and characterization of different BAFF-CAR constructs. **(45A)** L45-BAFF-28 CAR was transduced into T-cells and evaluated for surface expression using F(ab)' antibody. Gating was compared to controls. **(45B)** BAFF-CAR constructs using different leader sequences were tested to determine if efficiency in transduction could be improved. Surface detection was evaluated using F(ab)' antibody and transduced populations encircled. **(45C)** Additional BAFF-CAR constructs containing different leader sequences and construct designs (additional units) were validated and used to determine if CAR transduction could be improved. Transduced populations are encircled and gating compared to control T-cells. CSF-BAFF-28 41BBL is a BAFF CAR co-expressing 4-1BBL (41BBL) with a CSF leader sequence. CSF-BAFF-28IL-15/IL-15sushi - is a BAFF CAR co-expressing IL-15/IL-15sushi with a CSF leader.

Figures 46A-46B: L45-BAFF-28 CAR T-cells possess anti-tumor activity against MM1S tumor cells. Characterization of L45-BAFF-28 CAR T anti-tumor properties**. (46A)** BAFF CAR cytotoxic activity in vitro summarized from (46B). **(46B)** L45-BAFF-28 CAR T-cells possess anti-tumor activity against MM1S tumor cells. L45-BAFF-28 CAR T-cells were cultured for 48 hours at an E:T ratio of 3:1 against MM1S myeloma cells. Duplicate samples are shown.

Figures 47A-47B Characterization of anti-tumor activity using different BAFF-CAR constructs and enhancements. **(47A)** L8-BAFF-28IL-15/IL-15sushi and L8-BAFF-28 4-1BBL CARs were cultured for 24 hours against MM1S tumor cells at an E:T ratio of 5:1. Tumor populations are encircled. **(47B)** Both CARs and L45-BAFF-28 CAR were cultured against Sp53 tumor cells (B-lineage) at an E:T ratio of 5:1 for 24 hours.

Figure 48. CRISPR/Cas9 interference system. The expression of sgRNA and Cas9 puromycin is driven by the U6 and SFFV promoters, respectively. The Cas9 is linked with puromycin resistant gene by E2A self-cleaving sequences.

Figure 49A. Steps of generation of CAR T or NK cell targeting hematologic malignancies.

Figure 49B. Generation and cell sorting of stable CD45 knockdown NK-92 cells using CRISPR/Cas9 lentivirus system. Flow cytometry analysis indicated the CD45 expression levels on NK-92 cell surface (left panels). After transduced sgCD45B CRISPR into NK-92 cells, transduced cells were cultured in medium containing puromycin for a few weeks. CD45 negative NK-92 cells were determined using CD45 antibody and were sorted. The purity of stable NK^{45i}-92 (CD45 knockdown) NK-92 cells were determined by Flow cytometry analysis (right panel). This data showed that we we successfully generated and obtained **NK^{45i}-92** cells.

Figure 50. Cell growth curve of wild type, GFP transduced NK-92 or NK^{45i}-92NK cells. To evaluate the effect for cell proliferation caused by CD45-knockdown (KD) in NK-92 cells, the number of cells of NK-92(●), GFP-transduced NK-92(■) and NK^{45i}-92(▲) were counted at 48 h and 96 h after seeding into 24 well plates. IL-2 was added at 48 h time point. (n=3 independent experiments performed in duplicate). Data are mean $\pm$ S.D. These data indicated that knockdown of CD45 receptor on NK-92 show similar cell growth curve compared to non-transduced NK-92 or GFP-transduced NK-92 cells. 24 well, duplicate, n=3 IL-2 was added at 48hr time point.

Figures 51A-51B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92 or

GFP NK^{45i}-92 cells (effector: E) at 5:1 (E:T) ratio and 16 hour incubation. **(51A)** Flow cytometry analysis of CCRF-CEM only (blue dot in left panel), in co-culture with CCRF-CEM and control GFP transduced NK-92 cells (middle panel) or GFP NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target CCRF-CEM cells and red dots shows effector cells by co-culture assay. The majority of the blue dots are in the upper left square of each experiment. All of incubation time were 16 h and the ratio of effector T-cells: target cell is 5:1. All experiments were performed in duplicate. **(51B)** Bar graph indicates the percent of cell lysis by the GFP transduced NK^{45i}-92 cells compared to the control GFP transduced NK92 cells in co-culture assay with CCRF-CEM. These data suggest that knockdown of CD45 in NK-92 cells does not show a significant difference for killing activity against CCRF-CEM cells compared to GFP-control NK-92 cells in vitro co-culture assay.

Figures 52A-52B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92, CD5CAR NK-92 or CD5CAR NK^{45i}-92 cells (effector: E) at 5:1 (E:T) ratio and 16 hour incubation**.** **(52A)** Flow cytometry analysis of CCRF-CEM only (left panel), in co-culture with CCRF-CEM and control GFP NK-92 cells (middle left panel), CD5CAR NK-92 cells (middle right panel), CD5CAR NK^{45i}-92 cells (right panel) from right to left. Blue dots in all of panels indicates the leftover target CCRF-CEM cells and red dots shows effector cells by co-culture assay. All of incubation time were 16 h and the ratio of effector T-cells: target cell is 5:1. All experiments were performed in duplicate. **(52B)** Bar graph indicates the percent of cell lysis by the CD5CAR NK-92 cells or CD5CAR NK^{45i}-92 cells cells compared to the control GFP NK92 cells in co-culture assay with CCRF-CEM. Data are mean $\pm$ S.D. Both of CD5CAR NK-cells and CD5CAR NK^{45i}-92 cells shows near to 100 % cell killing activity against CD5-potitive CCRF-CEM compared to control GFP NK-92 cells. These data suggest that CD5CAR NK-cells and CD5CAR NK^{45i}-92 cells can effectively lyse CCRF-CEM cells that express CD5 compared to GFP-control NK-92 cells in vitro co-culture assay and prof that knockdown of CD45 does not affect cell function for killing activity in NK-92 cells.

Figures 53A-53B. Organization of the CD45CAR construct and its expression. **(53A)** *Schematic representation of the CD45CAR lentiviral vector.* The CD45CAR construct is a modularized signaling domain containing: a leader sequence, an anti-CD45scFv, a hinge domain (H), a transmembrane domain (TM), two co-stimulatory domains (CD28 and 4-1BB) that define the construct as a 3rd generation CAR, and the intracellular signaling domain CD3 zeta. **(53B)**, HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1) and CD45CAR (lane 2). 48 hours after transfection, supernatant was removed, and cells were also removed. Cells were lysed for Western blot and probe with mouse anti-human CD3z antibody.

Figure 54. Transduction of CD45CAR into NK^{45i}-92 cells and cell sorting of CD45CAR transduced cells**.** The expression levels of CD45CAR on NK^{45i}-92 were determined by flow cytometry analysis (circled in blue at middle panel) compared to NK^{45i}-92 cells (left panel) after CD45CAR lentviruses were transduced into NK^{45i}-92 cells. CD45CAR expressed NK^{45i}-92 cells were sorted and CD45 expression levels on cell surface were determined by Flow cytometry analysis (right panel). About 87% of CD45CAR expression on cell surface was detected by flow cytometry analysis.

Figures 55A-55B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92 or CD45CAR NK^{45i}-92 cells (effector: E) at 5:1 (E:T) ratio and 16 hour incubation**. (55A)** Flow cytometry analysis of in co-culture with CCRF-CEM and control GFP transduced NK-92 cells (left panel) or CD45CAR NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target CCRF-CEM cells and red dots shows effector NK-92 cells by co-culture assay. All of incubation time were 16 h and the ratio of effector T-cells: target cell is 5:1. All experiments were performed in duplicate. **(55B)** Bar graph indicates the percent of cell lysis by CD45CAR NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with CCRF-CEM. Data are mean $\pm$ S.D. CD45CAR NK^{45i}-92 cells shows about 70% cell lysis against CCRF-CEM cells compared to control GFP NK-92 cells. These data suggest that CD45CAR NK^{45i}-92 cells effectively lyse CCRF-CEM cells that express CD45 compared to GFP-control NK-92 cells in vitro co-culture assay.

Figures 56A-56C. Co-culture assay with Jurkat cells (target: T) and GFP-control or CD45CAR NK^{45i}-92 cells (effector: E) at 5:1 or 2:1 (E:T) ratio and 6 hour incubation. **(56A)** Flow cytometry analysis was carried out after Jurkat cells were stained by CMTMR cell tracker dye. These data show that Jurkat cells are CD45 positive (left panels) and mostly CD56 negative cells (right panel). **(56B)** Flow cytometry analysis of co-culture assay with Jurkat cells (target: T) and control or CD45CAR NK^{45i}-92 cells (effector: E). The ratio of co-culture assay was performed in 5:1 or 2:1 (E: T). Left panels showed that in co-culture with control GFP or

CD45CAR/CD45KD NK-92 cells in 5:1 (E:T) ratio and right panels indicated that in co-culture with control GFP or CD45CAR NK^{45i}-92 cells in 2:1 (E:T) ratio. Blue dots in panels indicate the leftover target Jurkat cells and red dots represent effector cells by co-culture assay. All of incubation time were 6 h. All experiments were performed in duplicate. **(56C)** Bar graph shows percent cell lysis by CD45CAR NK^{45i}-92 cells compared to control GFP NK92 cells at in 5:1 or 2:1 (E: T) ratio. Data are mean $\pm$ S.D. CD45CAR NK^{45i}-92 cells shows about 60% cell lysis against Jurkat cells compared to control GFP NK-92 cells in both conditions. This data suggests that CD45CAR NK^{45i}-92 cells effectively lyse Jurkat cells that express CD45 on cell surface compared to GFP-control NK-92 cells in vitro co-culture assay.

Figure 57A - 57C. Co-culture assay with GFP-NK-92 cells (target: T) and non-transduced NK-92 cells or CD45CAR NK^{45i}-92 cells (effector: E) at 5:1 or 2:1 (E:T) ratio, 6 hour incubation**.** **(57A)** Flow cytometry analysis was carried out using GFP control NK-92 cells. These data proof that GFP control NK-92 cells are about 99% GFP positive cells (green dots). (57B) Flow cytometry analysis of co-culture assay with GFP control NK-92 cells (target: T) and non-transduced or CD45CAR NK^{45i}-92 cells (effector: E). The ratio of co-culture assay was performed in 5:1 (57A) or 2:1 (E: T) (57C). Left panels showed that in co-culture with non-transduced or CD45CAR NK^{45i}-92 cells in 5:1 (E:T) ratio and right panels indicated that in co-culture with non-transduced or CD45CAR NK^{45i}-92 cells in 2:1 (E:T) ratio. Green dots in panels indicate the leftover target GFP NK-92 cells and red dots represent effector cells by co-culture assay. All of incubation times were 6 h. All experiments were performed in duplicate. (57C) Bar graph shows percent cell lysis of GFP NK-92 cells by CD45CAR NK^{45i}-92 cells compared to non-transduced NK-92 cells at in 5:1 or 2:1 (E: T) ratio. Data are mean $\pm$ S.D. CD45CAR NK^{45i}-92 cells shows about 20% cell lysis in 2:1 (E:T) ratio and about 55% cell lysis in 5:1 (E:T) ratio against GFP NK-92 cells compared to non-transduced NK-92 cells.This data suggests that CD45CAR NK^{45i}-92 cells effectively lyse GFP NK-92 cells that express CD45 on cell surface compared to non-transduced NK-92 cells in vitro co-culture assay.

Figure 57D. Transduction of CD45b-BB or CD45b-28 into NK^{45i}-92 cells and cell sorting of CD45b-BB or CD45b-28 transduced NK^{45i}-92 cells. The co-stimulatory domain for CDb-BB is 4-1BB while co-stimulatory domain for CD45b-28 is CD28. The expression levels of CD45b-BB or CD45b-28 on NK^{45i}-92 were determined by flow cytometry analysis (circled in blue at middle panel) compared to NK^{45i}-92 cells (left panel) after CD45b-BB or CD45b-28 on lentviruses were transduced into NK^{45i}-92 cells. CD45b-BB or CD45b-28 on expressed NK^{45i}-92 cells were sorted and CD45b-BB or CD45b-28 on expression levels on cell surface were determined by Flow cytometry analysis (right panel). About 74% of CD45b-BB or 82% of CD45b-28 on expression on cell surface was detected by flow cytometry analysis.

Figure 57E. Co-culture assay with REH cells (target: T) and GFP NK-92 cells, CD45CAR NK^{45i}-92 cells , CD45b-BB NK^{45i}-92 cells or CD45b-28 NK^{45i}-92 cells at 5:1 (E:T) ratio and 20 hour incubation. Upper**,** Flow cytometry analysis of CREH cells only (left panel), in co-culture with with REH cells and control GFP transduced NK-92 cells (2^{nd} left panel), CD45CAR NK^{45i}-92 cells (middle panel), CD45b-BB NK^{45i}-92 cells (4^{th} from left panel) or CD45b-28 NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target REH cells and red dots shows effector GFP or CARs-NK-92 cells by co-culture assay. All of incubation time were 20h and the ratio of effector NK-cells: target cell is 5:1. All experiments were performed in duplicate. Lower, Bar graph indicates the percent of cell lysis by CD45CAR NK^{45i}-92 cells, CD45b-BB NK^{45i}-92 cells or CD45b-28 NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with REH cells. Data are mean $\pm$ S.D. CD45CAR NK^{45i}-92 cells shows about 76% cell lysis, CD45b-BB NK^{45i}-92 cells shows about 79% cell lysis and CD45b-28 NK^{45i}-92 shows 100% cell lysis against REH cells compared to control GFP NK-92 cells. These data suggest that these 3 of CD45CARs NK^{45i}-92 cells effectively lyse REH cells which characterized as B-cells expressing CD45 compared to GFP-control NK-92 cells in vitro co-culture assay.

Figures 57FA-57FI. Co-culture assay with U937 cells (target: T) and GFP NK-92 cells or CD45b-28 NK^{45i}-92 cells.at 2:1 (E:T) ratio for 20 hours. **FA**, Flow cytometry analysis of U937 cells (monocytic leukemia cell line) only (left panel), in co-culture with U937 cells and control GFP transduced NK-92 cells (middle panel) or CD45b-28 NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target U937 cells and red dots shows effector GFP or CD45b-28 NK^{45i}-92 cells by co-culture assay. All of incubation time were 6h and the ratio of effector NK-cells: target cell is 2:1. **FB,** Bar graph indicates the percent of cell lysis by CD45b-28 NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with U937 cells. CD45b-28 NK^{45i}-92 shows about 81% cell lysis against U937 cells compared to control GFP NK-92 cells.

Figures 57GA-57GB. Co-culture assay with MOLM-13 cells (target: T) and GFP NK-92 cells or CD45b-28 NK^{45i}-92 cells at 5:1 (E:T) ratio for 20 hours. **GA**, Flow cytometry analysis of MOLM13 cells (monocytic leukemic cell line) only (left panel), in co-culture with Molm13 cells and control GFP transduced NK-92 cells (middle panel) or CD45b-28 NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target MOLM13 cells and red dots shows effector GFP or CD45b-28 NK^{45i}-92 cells by co-culture assay. All of incubation time were 20h and the ratio of effector NK-cells: target cell is 5:1. **GB,** Bar graph indicates the percent of cell lysis by CD45b-28 NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with MOLM13 cells. CD45b-28 NK^{45i}-92 shows about 91.6% cell lysis against Molm13 cells compared to control GFP NK-92 cells.

Figures 57HA-57HB. Co-culture assay with Jeko-1 cells (target: T) and GFP NK-92 cells or CD45b-28 NK^{45i}-92 cells at 2:1 (E:T) ratio for 6 hours**. HA,** Flow cytometry analysis of Jeko-1 cells (T cell acute lymphoblastic cell line) only (left panel), in co-culture with Jeko-1 cells and control GFP transduced NK-92 cells (middle panel) or CD45b-28 NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target Jeko-1 cells and red dots shows effector GFP or CD45b-28 NK^{45i}-92 cells by co-culture assay. All of incubation time were 6h and the ratio of effector NK-cells: target cell is 2:1. **HB.** Bar graph indicates the percent of cell lysis by CD45b-28 NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with Jeko-1 cells. CD45b-28 NK^{45i}-92 shows about 44.6% cell lysis against Jeko-1 cells compared to control GFP NK-92 cells.

Figures 57IA-57IB. Co-culture assay with SP53 cells (target: T) and GFP NK-92 cells or CD45b-28 NK^{45i}-92 cells at 2:1 (E:T) ratio for6 hour incubation. **IA**, Flow cytometry analysis of SP53 cells (mantle cell lymphoma cell line) only (left panel), in co-culture with Jeko-1 cells and control GFP transduced NK-92 cells (middle panel) or CD45b-28 NK^{45i}-92 cells (right panel). Blue dots in all of panels indicates the leftover target SP53 cells and red dots shows effector GFP or CD45b-28 NK^{45i}-92 cells by co-culture assay. All of incubation time were 6h and the ratio of effector NK-cells: target cell is 2:1. **IB,** Bar graph indicates the percent of cell lysis by CD45b-28 NK^{45i}-92 cells compared to the control GFP NK92 cells in co-culture assay with SP53 cells. CD45b-28 NK^{45i}-92 shows about 45% cell lysis against SP53 cells compared to control GFP NK-92 cells.

Figure 57J. Elimination of CD34(+) umbilical chord blood stem cells in 48hr co-culture. CD34(+) stem cells derived from human umbilical cord blood were co-cultured with either Control or CD45b-28 CAR NK cells for 48hr prior to labeling at a low ratio of 2:1 (effective: target). About 96% of CD34(+) cells were eliminated comparing to the control.

Figure 58A. A Link by P2A schematic showing both cCAR-T and 4-1BBL or IL-15/IL-15sushi in a single construct. The construct consists of a SFFV promoter driving the expression of CAR and an enhancer, 4-1BBL. Upon cleavage of the linkers, the CD45 CAR (or CD45b CAR) and 4-1BBL or IL-15/IL-15sush split and engage upon targets expressing CD45. CD45 CAR T cells received not only costimulation through the CD28 but also 4-1BB ligand (4-1BBL or CD137L) or IL-15/IL-15sushi. The CD3-zeta signaling domain completes the assembly of this CAR-T.

Figure 58B. Surface CD45b CAR expression levels on CD45b-28-2G-4-1BBL CAR transduced NK^{45i}-92 cells were determined using flow cytometry analysis. Left panel(NK92 cells) and middle panel (GFP-NK92) indicated negative control and right panel showed the surface expression of CD45b CAR which was labeled using goat anti-mouse F(AB')2-PE against ScFv region (circled in blue).Transduced cells expressed 86.99% of CD45b-CAR on the cell surface.

Figure 58C. Surface CD45b CAR expression levels on CD45b-28-2G-IL15/IL-15sushi CAR transduced NK45i-92 cells were determined using flow cytometry analysis. Left panel(NK92 cells) and middle panel (GFP-NK92) indicated negative control and right and right panel showed the surface expression of CD45b CAR which was labeled using goat anti-mouse F(AB')2-PE against ScFv region (circled in blue). CD45b-28-2G IL15RA virus transduced cells expressed 55.96% of CD45b-CAR on cells surface compared to negative control cells.

Figures 59A-59B. Schematic diagram to elucidate the construct and its expression in T or NK cells. **(59A)** a combination of CAR, (third generation), and IL-15/sushi domain of the IL-15 alpha receptor, is assembled on an expression vector and their expression is driven by the SFFV promoter. CAR with IL-15/sushi is linked with the P2A self-cleaving sequence. The IL-15/sushi portion is composed of IL-2 signal peptide fused to IL-15 and linked to sushi domain via a 26-amino acid poly-proline linker. **(59B)** CAR and IL-15/sushi are present on the T or NK cells.

Figure 59C. Surface CD45b CAR expression levels on CD45b-28-2G-IL-15/IL-15sushi CAR transduced NK^{45i}-92 cells were determined using flow cytometry analysis. Left panel(NK92 cells) and middle panel (GFP-NK92) indicated negative control and right and right panel showed the surface expression of CD45b CAR which was labeled using goat anti-mouse F(AB')2-PE against ScFv region (circled in blue). CD45b-28-2G IL-15/IL-15sushi virus transduced cells expressed 55.96% of CD45b-CAR on cells surface compared to negative control cells. CD45b-28-2G-IL-15/IL-15sushi NK cells showed a robust functional activity.

Figures 60A-60B. CD4IL-15/IL-15sushi expression**. (60A)** HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1) and CD4IL-15/IL-15sushi CAR (lane 2). 48 hours after transfection, supernatant was removed, and cells were also removed for a Western blot with mouse anti-human CD3z antibody. **(60B)** HEK-293 cells were transduced with either GFP (left) or CD4IL-15/IL-15sushi-CAR(right) viral supernatant from transfected HEK-293FT cells. After 3 days incubation, cells were harvested, stained with goat-anti-mouse F(Ab')2 and analyzed by flow cytometry.

Figure 61. Transduction of NK cells with CD4IL-15/IL-15sushi CAR. NK-92 cells were transduced with either GFP (left) or CD4 IL-15/IL-15sushi CAR (right) viral supernatant from transfected HEK-293FT cells. A second transduction was performed 24 hours after the first. 24 hours after the second transduction, cells were harvested, washed and moved to tissue culture plates with fresh media and IL-2. After 3 days incubation, cells were harvested and stained with goat-anti-mouse F(Ab')2 antibody or goat IgG (control) at 1:250 for 30 minutes. Cells were washed and stained with streptavidin-PE conjugate at 1:500, washed, suspended in 2% formalin, and analyzed by flow cytometry.

Figure 62. Transduction of T cells with CD4IL15RACAR. Left is the Western blot. HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1) and CD4IL15RA-CAR (lane 2). 48 hours after transfection, supernatant was removed, and cells were also collected for a Western blot with mouse anti-human CD3zeta antibody. Right is CD4IL15RACAR expression**.** Activated T cells from cord blood buffy coat were transduced with either GFP (left) or concentrated CD4IL15RACAR (right) viral supernatant from

transfected HEK-293FT cells. A second transduction was performed 24 hours after the first. 24 hours after the second transduction, cells were harvested, washed and moved to tissue culture plates with fresh media and IL-2. After 3 days incubation, cells were harvested and stained with goat-anti-mouse F(Ab') transduced with either GFP (left) or CD4IL15RA CAR (right). Cells were washed and stained with streptavidin-PE conjugate at 1:500, washed, suspended in 2% formalin, and analyzed by flow cytometry.

Figures 63A-63B. CD4CAR NK-92 cells and CD4IL-15/IL-15sushi CAR NK-92 cells eliminate KARPAS 299 T leukemic cells in co-culture. **(63A)** NK-92 cells transduced with either GFP control (upper right), CD4CAR (lower left), or CD4IL-15/IL-15sushi (lower right) lentiviral supernatant were incubated with KARPAS 299 cells at a ratio of 5:1. After 4 hours co-culture, cells were stained with mouse-anti-human CD4 (APC) and CD3 (PerCp) antibodies and analyzed by flow cytometry (N=2). The upper left panel shows labeled Karpas 299 cells alone. The percentage of target cells lysed is shown in the graph **(63B)**.

Figure 64. CD4CAR NK-92 cells and CD4IL-15/IL-15sushi CAR NK-92 cells eliminate MOLT4 T leukemic cells in co-culture. NK-92 cells transduced with either GFP control (left), CD4CAR (center), or CD4IL-15/IL-15sushi (second from right) lentiviral supernatant were incubated with MOLT4 cells at effector:target ratios of 1:1 or 2:1. After overnight co-culture, cells were stained with mouse-anti-human CD4 (APC) and CD56 (PerCp) antibodies and analyzed by flow cytometry (N=2). The upper right panel shows labeled MOLT4 cells alone. The percentage of target cells lysed is shown in the graph.

Figures 65A and 65B. CD4CAR and CD4IL-15/IL-15sushi CAR T cells demonstrate anti-leukemic effects *in vivo*. NSG mice were sublethally irradiated and intravenously (tail vein) injected the following day with luciferase-expressing MOLM13 cells to induce measurable tumor formation (65A). MOLM-13 cells are nearly 100% CD4+. After 3 days, the mice were intravenously injected with one course of 8 x 10^6 CD4CAR, or CD4IL-15/IL-15sushi CAR T cells, or vector control T control cells. On days 3, 6, 9, and 11, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging (65B).

Figures 65C and 65D. (65C)Average light intensity measured for the CD4CAR and CD4IL-15/IL-15sushi CAR T injected mice was compared to that of vector control T injected mice, and correlated with remaining tumor burden to determine a percent lysis. (65D) Percent survival of mice was measured and compared between the three groups.

Figure 66A and 66B. CD4IL15/IL-15sushi CAR NK cells demonstrate robust anti-leukemic activity under stressful condition *in vivo*. NSG mice were sublethally irradiated and intravenously (tail vein) injected the following day with luciferase-expressing Jurkat cells to induce measurable tumor formation (66A). Jurkat cells are less than 60% CD4+. After 3 days, the mice were intravenously injected with one course of 8 x 10^6 CD4CAR, or CD4IL-15/IL-

15sushi CAR NK cells, or vector control NK cells. On days 3, 7, 10, and 14, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging (66B).

Figure 66C. Average light intensity measured for the CD4CAR and CD4IL-15/IL-15sushi NK injected mice was compared to that of vector control NK injected mice, and correlated with remaining tumor burden to determine a percent lysis.

Figure 67. Repeat of the in vivo experiment demonstrating robust lysis of Jurkat tumor cells by CD4I-15/IL-15sushi CAR NK cells showing similar results to those described in Figure 66.

Figures 68A-68B. Effect of secreted IL-15/IL-15sushi on CAR and non-transduced neighboring cells. NK-92 cells stably expressing either CD4CAR or CD4IL15RA were mixed in a 50:50 ratio with NK-92 cells stably expressing GFP. These cells were co-cultured either with IL-2 added or no IL-2. (68A) Photographs taken on a fluorescent microscope at 20x on Day 0 (start of co-culture) and Day 7, without the addition of IL-2. (68B) Total cell counts calculated throughout the experiment (up to Day 14) for NK-92 cells co-cultured with or without IL-2.

Figure 69. Comparing the effect of secreted IL-15 and IL-15sushi on NK-92 cell growth. CD4IL-15/IL-15sushi, CD4 IL-15, and control transduced NK-92 cells were cultured from 250,000 cells in regular NK cell media but in the absence of IL-2 for up to 6 days. Both transduced cells had 10% surface CAR expression, while CD4IL15-IL15sushi transduced NK-92 cells were able to expand at a rate approximately 3-fold higher than the CD4 IL-15 transduced NK-92 cells on day 6. On day 4, the growth rate of CD4 IL-15 transduced NK-92 cells were slightly higher than the Control, but significantly below the CD4 IL-15/IL15sushi transduced NK-92 cells. This study pin-points the importance of co-expression functional complex of IL-15/IL-15sushi in promoting NK-92 cell growth.

Figure 70. A schematic showing the Treg CAR T construct targeting Tregs. The construct consists of a SFFV promoter driving the expression of two units of chimeric antigen receptors linked by a P2A peptide. Each unit contains a CD45 leader peptide sequence (signal peptide). Upon cleavage of the linker, two units of peptide are divided and engage upon targets expressing CD4 and CD25. The CD4 chimeric antigen receptor polypeptide unit comprises a signal peptide, a CD4 antigen recognition domain, a hinge region, a transmembrane domain and CD3 zeta chain; CD25 chimeric antigen receptor polypeptide unit comprises a signal peptide, a

CD25 antigen recognition domain, a hinge region, a transmembrane domain, a co-stimulatory domain (s). The Treg CAR can potentiate the lysis activity of a cell co-expressing CD4 and CD25 while minimizing a cell bearing CD4 or CD25 antigen.

Figures 71A-71B. Characterization of the CD4 zeta CD25 CAR. **(71A)** The CD4 zeta CD25 CAR was transduced into T-cells via viral incubation for 48 hours and stained with F(ab)' antibody to assay CAR surface expression. Encircled populations represent transduced cells. **(71B)** The C4-25z CAR was characterized using CD4 and CD25 antibodies to validate the construct function. Two most relevant populations are encircled: $CD4^{+}$ $CD25^{+}$ and $CD4^{-}$ $CD25^{+}$. The depletion of the double positive population and other phenotype groups are summarized in the bar graph adjacent.

Figure 72. CD4zetaCD25 CAR T cells target cells mainly co-expressing CD4 and CD25. 3 days after activation, PMBC buffy coat T cells transduced with either control vector (left), CD4CAR (middle) or CD4zetaCD25(right) lentiviral supernatant were harvested and incubated with mouse anti-human CD25-PE and mouse anti-human CD4-APC for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry.

Figure 73A. A schematic showing the CD52-52 construct. The construct consists of a SFFV promoter driving the co-expression of CD5CAR and CD52 surface antigen. Upon cleavage of the linker of P2A. The CD5 chimeric antigen receptor polypeptide unit comprises a signal peptide, a CD5 antigen recognition domain, a hinge region, a transmembrane domain and CD3 zeta chain; CD5 peptide comprises a signal peptide, a CD52 antigen recognition domain, a hinge region, a transmembrane domain (derived from CD28) .

Figure 73B. Experimental design to determine depletion of CD5CAR-52 T cells in blood. CD5CAR-52 T cells ($5x10^{6}$ cells) were injected intravenously into each NSG mouse after sublethally irradiation. After ~ 24 h later, PBS or 0.1mg/kg of CAMPATH was injected via I.P. (intraperitoneal injection). N=3. After 6 h and 24 h later, peripheral blood was collected from each mouse and labeled using CD3 and CD45 antibodies to determine the depletion of CAR-T cells as acute phase response by CAMPATH treatment. After 5 days later, whole blood was collected from each mouse and labeled using CD3 and CD45 antibodies to determine the persistency of CAR-T cells as well. CAR-T-cells were determined using Flow cytometry analysis.

Figure 73C. Depletion of CD5CAR-52 T in peripheral blood after 6 h and 24 h later with or without CAMPATH treatment. Flow cytometry analysis shows persistence of CD5CAR-52 T-cells (Blue dots) in peripheral blood of mouse with or without CAMPATH treatment. Blood samples were labeled with CD3 and CD45 antibodies to detect CD5CAR-52 T-cells. Blood samples from un-infused CAR-T cells (left panels) did not show CD3 and CD45 positive cells (negative control). 0.1 mg/kg of CAMPATH injected mice indicate elimination of CD5CAR-52 T-cells at 6h (middle panels) and 24 h (right panels) later compared to CAMPATH untreated mouse at 6h (second panels from left) and 24 h (second panels from right) in blood samples. N=3. These results suggest that CAMPAT treatment can delete CAR-T cells from blood during short time.

Figure 73D. Depletion of CD5CAR-52 T in whole blood after 5 days later with or without CAMPATH treatment. Flow cytometry analysis shows persistence of CD5CAR-52 T-cells (Blue dots) in whole blood samples from mouse with or without CAMPATH treatment. Blood samples were labeled with CD3 and CD45 antibodies to detect CD5CAR-52 T-cells persistence. Blood samples from uninfused CAR-T cells (left panel) did not show CD3 and CD45 positive cells (negative control). 0.1 mg/kg of CAMPATH treated mice eliminate CD5CAR-52 T-cells (right panels) compared to CAMPATH uninjected mouse (middle panels) after 5 days later in whole blood samples. These results also suppose CAMPAT treatment can delete CAR-T cells from blood.

Figure 74. HEK 293 cells were transduced with either EF1-GFP or SFFV-GFP viral supernatant, using the volumes indicated, in DMEM with 10% FBS in a 6 well tissue culture plate. Culture media was changed the following morning. Forty-eight hours later, transduced cells were visualized on an EVOS fluorescent microscope using GFP at 10x.

Figure 75. HEK 293 cells transduced with either EF1-GFP or SFFV-GFP viral supernatant, using the volumes from the previous figure, were trypsinized, suspended in formalin, and subjected to flow cytometry analysis, using the FITC channel to determine the percentage of GFP+ cells.

Figures 76A-76B. Activated cord blood buffy coat T cells transduced with either EF1-GFP or SFFV-GFP viral supernatant, with either low or high amounts of viral supernatant, were trypsinized, suspended in formalin, and subjected to flow cytometry analysis, using the FITC channel to determine the percentage of GFP+ cells, 7, 14, 21 and 28 days after transduction.

(76A) Percent GFP+ T cells for cells transduced with either low or high amounts of supernatant.

(76B) Percent of GFP+ T cells transduced with the high amount of EF1-GFP supernatant, relative to the percent GFP+ cells in the T cells transduced with the lower amount of SFFV-GFP supernatant. (50 μL of SFFV-GFP and 1 mL of EF1-GFP supernatant was used). (N=2).

Figure 77. Ligand receptor interactions in malignant plasma cells. The APRIL ligand binds TAC1 or BCMA. The BAFF ligand binds TAC1, BCMA, or BAFF-R.

Figure 78. Steps for elimination of tumor by CAR co-expressing secretory IL-15/IL-15sushi. I, tumor and its microenviroment. Macrophages, T cells, dendritic cells and NK cells are immune response cells against tumor in the tumor microenvironment and they secrete a low level of endogenous IL-15, which is unstable, which complexes with the soluble extracellular domain of IL-15RA. The complex forms a more stable molecule, which greatly enhances immune cell survival and expansion. In the tumor microenvironment, cancer cells express programmed death ligand 1 (PD-L1) as a transmembrane protein that has been considered to play a major role in suppressing the immune system during particular events including cancer. PD-L1 binds to its receptor, PD-1, found on activated T cells, B cells, and myeloid cells, to suppress these cell immune activities. II, CAR T or NK cells targeting tumor cells, could be a carrier to deliver an enhancer to the tumor microenvironment. CAR T or NK cells are engineered to co-express a secretory fusion protein, IL-15/IL-15sushi fusion. III, Engineered CAR T or NK cells bind to targeted tumor cells (either subset or all cells). IV, Engineered CAR T or NK cells in tumor microenvironment target tumor cells, binding to the CAR targeting antigen, and triggering lysis of tumor cells and massive secretion of soluble IL-15/IL-15sushi fusion from the expansion of CAR T or NK cells. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The secreted IL-15/IL-15sushi protein would be involved in trafficking of other T cells, dendritic cells, macrophages and NK cells to the tumor microenvironment, which then also: 1) lyse the tumor cells by supplementing the defect that CAR T or NK cells are unable to eliminate non-targeting cancer cells; 2) enhance CAR T/NK cell persistency and anti-tumor activity. The overexpression of IL-15/IL-15sushi overwhelms the PD-L1 ability to suppress the immune response. Preferably, this CAR therapy could be used synergistically with

administration of a checkpoint blockage including, but not limited to PD-L1, CTLA-4 inhibitor for even greater efficacy.

Figure 79. Surface markers during B cell and plasma cell development are shown. Both BAFF and APRIL binds to receptors, BCMA and TACI. BAFF also binds to BAFF-R receptor.

Figure 80. Protein sequence alignment of IL-2 signal peptide among different species.

Figure 81. Protein sequence alignment of BAFF extracellular domain among different species.

DETAILED DESCRIPTION

The disclosure provides chimeric antigen receptor (CAR) compositions, methods of making and using thereof.

A chimeric antigen receptor (CAR) polypeptide includes a signal peptide, an antigen recognition domain, a hinge region, a transmembrane domain, at least one co-stimulatory domain, and a signaling domain.

First-generation CARs include CD3z as an intracellular signaling domain, whereas second-generation CARs include at least one single co-stimulatory domain derived from various proteins. Examples of co-stimulatory domains include, but are not limited to, CD28, CD2, 4-1BB (CD137, also referred to as "4-BB"), and OX-40 (CD124). Third generation CARs include two co-stimulatory domains, such as, but not limited to, CD28, 4-1BB, CD134 (OX-40), CD2, and/or CD137 (4-1BB).

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound having amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can be included in a protein's or peptide's sequence. Polypeptides include any peptide or protein having two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides, and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, and fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

A “signal peptide” includes a peptide sequence that directs the transport and localization of the peptide and any attached polypeptide within a cell, e.g. to a certain cell organelle (such as the endoplasmic reticulum) and/or the cell surface. As used herein, “signal peptide” and “leader sequence” are used interchangeably.

The signal peptide is a peptide of any secreted or transmembrane protein that directs the transport of the polypeptide of the disclosure to the cell membrane and cell surface, and provides correct localization of the polypeptide of the present disclosure. In particular, the signal peptide of the present disclosure directs the polypeptide of the present disclosure to the cellular membrane, wherein the extracellular portion of the polypeptide is displayed on the cell surface, the transmembrane portion spans the plasma membrane, and the active domain is in the cytoplasmic portion, or interior of the cell.

In one embodiment, the signal peptide is cleaved after passage through the endoplasmic reticulum (ER), i.e. is a cleavable signal peptide. In an embodiment, the signal peptide is human protein of type I, II, III, or IV. In an embodiment, the signal peptide includes an immunoglobulin heavy chain signal peptide.

In one embodiment, the signal peptide includes the signal peptide from human CD45. (UniProtKB/Swiss-Prot Accession Number P08575). The CD45 signal peptide is 23 amino acids in length (MYLWLKLLAFGFAFLDTEVFVTG). In some embodiments, the signal peptide may be a functional fragment of the CD45 signal peptide. A functional fragment includes a fragment of at least 10 amino acids of the CD45 signal peptide that directs the appended polypeptide to the cell membrane and cell surface. Examples of fragments of the human CD45 signal peptide include: MYLWLKLLAFG, FAFLDTEVFVTG, and LKLLAFGFAFLDTE.

Functional equivalents of the human CD45 signal peptide have also been contemplated. As used herein, "functional equivalents" are to be understood as mutants that exhibit, in at least one of the abovementioned sequence positions, an amino acid substitution other than the one mentioned specifically, but still lead to a mutant which show the same or similar properties with respect to the wild -type CD45 signal peptide. Functional equivalents include polypeptides having at least 80%, at least 85%, at least 90%, or at least 95% identity to the human CD45 signal peptide, functional fragments thereof, or functional equivalents thereof.

Functional equivalents also include CD45 signal peptides from homologous proteins from other species. Examples of these signal peptides include signal peptide from mouse CD45 (MGLWLKLLAFGFALLDTEVFVTG); signal peptide from rat CD45 (MYLWLKLLAFSLALLGPEVFVTG); signal peptide from sheep CD45 (MTMYLWLKLLAFGFAFLDTAVSVAG); signal peptide from chimpanzee CD45 (MYLWLKLLAFGFAFLDTEVFVTG); and signal peptide from monkey CD45 (MTMYLWLKLLAFGFAFLDTEVFVAG.

In another embodiment, the signal peptide includes the following sequence: $MX^1LWLKLLAF\ X^2X^3AX^4LX^5X^6X^7VX^8\ VX^9G$; wherein X^1, X^2, X3, X^4, X^5, X^6, X^7, X^8, and X^9 are independently Y, G, S, F, L, D, P, T, E, or A. In one embodiment, X^1 is Y or G; X^2 is G or S; X^3 and X^4 are independently F or L; X^5 is D or G; X^6 is P or T; X^7 is E or A; X^8 is F or S; and X^9 is A or T.

In one embodiment, the signal peptide includes the signal peptide from human CD8a (MALPVTALLLPLALLLHAARP). In some embodiments, the signal peptide may be a functional fragment of the CD8a signal peptide. A functional fragment includes a fragment of at least 10 amino acids of the CD8a signal peptide that directs the appended polypeptide to the cell membrane and cell surface. Examples of fragments of the human CD8a signal peptide include: MALPVTALLLPLALLLHAA, MALPVTALLLP, PVTALLLPLALL, and LLLPLALLLHAARP.

In another embodiment, the signal peptide includes the signal peptide from human CD8b (MRPRLWLLLAAQLTVLHGNSV). In some embodiments, the signal peptide may be a functional fragment of the CD8b signal peptide. A functional fragment includes a fragment of at least 10 amino acids of the CD8b signal peptide that directs the appended polypeptide to the cell membrane and cell surface. Examples of fragments of the human CD8b signal peptide include: MRPRLWLLLAAQ, RLWLLLAAQLTVLHG, and LWLLLAAQLTVLHGNSV.

Functional equivalents of the human CD8a or CD8b signal peptide have also been contemplated. As used herein, "functional equivalents" are to be understood as mutants which exhibit, in at least one of the abovementioned sequence positions, an amino acid substitution other than the one mentioned specifically, but still lead to a mutant which show the same or similar properties with respect to the wild -type CD8a or CD8b signal peptide. Functional equivalents include polypeptides having at least 80%, at least 85%, at least 90%, or at least 95% identity to the human CD8 signal peptide, functional fragments thereof, or functional equivalents thereof.

Functional equivalents also include CD8a and CD8b signal peptides from homologous proteins from other species.

In one embodiment, the signal peptide includes the signal peptide from human IL-2. The IL-2 signal peptide is 23 amino acids in length (MYRMQLLSCIALSLALVTNS). In some embodiments, the signal peptide may be a functional fragment of the IL-2 signal peptide. A functional fragment includes a fragment of at least 10 amino acids of the IL-2 signal peptide that directs the appended polypeptide to the cell membrane and cell surface. Examples of fragments of the human IL-2 signal peptide include: MYRMQLLSCIAL, QLLSCIALSLAL, and SCIALSLALVTNS.

Functional equivalents of the human IL-2 signal peptide have also been contemplated. As used herein, "functional equivalents" are to be understood as mutants which exhibit, in at least one of the abovementioned sequence positions, an amino acid substitution other than the one mentioned specifically, but still lead to a mutant which show the same or similar properties with respect to the wild -type IL-2 signal peptide. Functional equivalents include polypeptides having at least 80%, at least 85%, at least 90%, or at least 95% identity to the human IL-2 signal peptide, functional fragments thereof, or functional equivalents thereof.

Functional equivalents also include IL-2 signal peptides from homologous proteins from other species. See for example Figure 80.

In another embodiment, the signal peptide includes the following sequence: $MYX^1X^2QLX^3SCX^4X^5LX^6LX^7LX^8X^9X^{10}X^{11}$; wherein X^1, X^2, X3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, and $X^{11}$are independently R, K, S, M, I, V, L, A, I, T, N, S, or G. In one embodiment, X^1 is R, K, or S; X^2 is M, I, or V; X^3 is L or A; X^4 and X^5 are independently I, A, V, or T; X^6 is S or T; X^7 is A or V; X^8 , X^9, X^{10}, and X^{11} are independently V, L, T, A, N, S, or G.

The "antigen recognition domain" includes a polypeptide that is selective for or targets an antigen, receptor, peptide ligand, or protein ligand of the target; or a polypeptide of the target.

The antigen recognition domain may be obtained from any of the wide variety of extracellular domains or secreted proteins associated with ligand binding and/or signal transduction. The antigen recognition domain may include a portion of Ig heavy chain linked with a portion of Ig light chain, constituting a single chain fragment variable (scFv) that binds specifically to a target antigen. The antibody may be monoclonal or polyclonal antibody or may be of any type that binds specifically to the target antigen. In another embodiment, the antigen recognition domain can be a receptor or ligand. In particular embodiments, the target antigen is specific for a specific disease condition and the disease condition may be of any kind as long as it has a cell surface antigen, which may be recognized by at least one of the chimeric receptor construct present in the compound CAR architecture. In a specific embodiment, the chimeric receptor may be for any cancer for which a specific monoclonal or polyclonal antibody exists or is capable of being generated. In particular, cancers such as neuroblastoma, small cell lung cancer, melanoma, ovarian cancer, renal cell carcinoma, colon cancer, Hodgkin's lymphoma, and childhood acute lymphoblastic leukemia have antigens specific for the chimeric receptors.

In some embodiments, antigen recognition domain can be non-antibody protein scaffolds, such as but not limited to, centyrins, non-antibody protein scaffolds that can be engineered to bind a variety of specific targets with high affinity. Centyrins are scaffold proteins based on human consensus tenascin FN3 domain, are usually smaller than scFv molecules CAR molecules.

The target specific antigen recognition domain preferably includes an antigen binding domain derived from an antibody against an antigen of the target, or a peptide binding an antigen of the target, or a peptide or protein binding an antibody that binds an antigen of the target, or a peptide or protein ligand (including but not limited to a growth factor, a cytokine, or a hormone) binding a receptor on the target, or a domain derived from a receptor (including but not limited to a growth factor receptor, a cytokine receptor or a hormone receptor) binding a peptide or protein ligand on the target.

In one embodiment, the antigen recognition domain includes the binding portion or variable region of a monoclonal or polyclonal antibody directed against (selective for) the target.

In another embodiment, the antigen recognition domain includes Camelid single domain antibody, or portions thereof. In one embodiment, Camelid single-domain antibodies include heavy-chain antibodies found in camelids, or VHH antibody. A VHH antibody of camelid (for example camel, dromedary, llama, and alpaca) refers to a variable fragment of a camelid single-chain antibody (See Nguyen et al, 2001; Muyldermans, 2001), and also includes an isolated VHH antibody of camelid, a recombinant VHH antibody of camelid, or a synthetic VHH antibody of camelid.

In another embodiment, the antigen recognition domain includes ligands that engage their cognate receptor. By way of example, APRIL is a ligand that binds the TAC1 receptor or the BCMA receptor. In accordance with the present disclosure, the antigen recognition domain includes APRIL, or a fragment thereof. By way of further example, BAFF is a ligand that binds the BAFF-R receptor or the BCMA receptor. In accordance with the present disclosure, the antigen recognition domain includes BAFF, or a fragment thereof. In another embodiment, the antigen recognition domain is humanized.

It is understood that the antigen recognition domain may include some variability within its sequence and still be selective for the targets disclosed herein. Therefore, it is contemplated that the polypeptide of the antigen recognition domain may be at least 95%, at least 90%, at least 80%, or at least 70% identical to the antigen recognition domain polypeptide disclosed herein and still be selective for the targets described herein and be within the scope of the disclosure.

The target includes interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, TACI, LeY, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, CS1, CD45, ROR1, PSMA, MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138

In another embodiment, the target includes any portion interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, TACI, LeY, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, CS1, CD45, TACI, ROR1, PSMA, MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

In one embodiment, the target includes surface exposed portions of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, TACI, LeY, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, CS1, CD45, TACI, ROR1, PSMA, MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138 polypeptides.

For example, the target includes the surface exposed regions of BAFF, as shown in Figure 81. The target may include a portion of the surface exposed regions of BAFF. For example, portions of BAFF include residues 1-200, 1-100, 50-150, or 100-200 of human BAFF.

In another embodiment, the target antigens include viral or fungal antigens, such as E6 and E7 from the human papillomavirus (HPV) or EBV (Epstein Barr virus) antigens; portions thereof; or surface exposed regions thereof.

In one embodiment, the TACI antigen recognition domain includes SEQ ID NO. 24.

In one embodiment, the BCMA antigen recognition domain includes SEQ ID NO. 25.

In one embodiment, the CS1 antigen recognition domain includes SEQ ID NO. 26.

In one embodiment, the BAFF-R antigen recognition domain includes SEQ ID NO. 27.

In one embodiment, the CD33 antigen recognition domain includes SEQ ID NO. 28.

In one embodiment, the CD123 antigen recognition domain includes SEQ ID NO. 29.

In one embodiment, the CD19 antigen recognition domain includes SEQ ID NO. 30.

In one embodiment, the CD20 antigen recognition domain includes SEQ ID NO. 31. In another embodiment, the CD20 antigen recognition domain includes SEQ ID NO. 32.

In one embodiment, the CD22 antigen recognition domain includes SEQ ID NO. 33.

In on embodiment, the CD45 antigen recognition domain includes SEQ ID NO. 34.

In on embodiment, the CD4 antigen recognition domain includes SEQ ID NO. 35

In on embodiment, the CD25 antigen recognition domain includes SEQ ID NO. 36

The hinge region is a sequence positioned between for example, including, but not limited to, the chimeric antigen receptor, and at least one co-stimulatory domain and a signaling domain. The hinge sequence may be obtained including, for example, from any suitable sequence from any genus, including human or a part thereof. Such hinge regions are known in the art. In one embodiment, the hinge region includes the hinge region of a human protein including CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, T cell receptor α or β chain, a CD3 zeta chain, CD28, CD3ε, CD45, CD4, CD5, CD8, CD8a, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, functional derivatives thereof, and combinations thereof.

In one embodiment the hinge region includes the CD8 a hinge region.

In some embodiments, the hinge region includes one selected from, but not limited to, immunoglobulin (e.g. IgG1, IgG2, IgG3, IgG4, and IgD).

The transmembrane domain includes a hydrophobic polypeptide that spans the cellular membrane. In particular, the transmembrane domain spans from one side of a cell membrane (extracellular) through to the other side of the cell membrane (intracellular or cytoplasmic).

The transmembrane domain may be in the form of an alpha helix or a beta barrel, or combinations thereof. The transmembrane domain may include a polytopic protein, which has many transmembrane segments, each alpha-helical, beta sheets, or combinations thereof.

In one embodiment, the transmembrane domain that is naturally associated with one of the domains in the CAR is used. In another embodiment, the transmembrane domain is selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

For example, a transmembrane domain includes a transmembrane domain of a T-cell receptor α or β chain, a CD3 zeta chain, CD28, CD3ε, CD45, CD4, CD5, CD7, CD8, CD9,

CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD68, CD134, CD137, ICOS, CD41, CD154, functional derivatives thereof, and combinations thereof.

In one embodiment, the transmembrane domain is artificially designed so that more than 25%, more than 50% or more than 75% of the amino acid residues of the domain are hydrophobic residues such as leucine and valine. In one embodiment, a triplet of phenylalanine, tryptophan and valine is found at each end of the synthetic transmembrane domain.

In one embodiment, the transmembrane domain is the CD8 transmembrane domain. In another embodiment, the transmembrane domain is the CD28 transmembrane domain. Such transmembrane domains are known in the art.

The signaling domain and co-stimulatory domain include polypeptides that provide activation of an immune cell to stimulate or activate at least some aspect of the immune cell signaling pathway.

In an embodiment, the signaling domain includes the polypeptide of a functional signaling domain of CD3 zeta, common FcR gamma (FCER1G), Fc gamma Rlla, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DNAX-activating protein 10 (DAP10), DNAX-activating protein 12 (DAP12), active fragments thereof, functional derivatives thereof, and combinations thereof. Such signaling domains are known in the art.

In an embodiment, the CAR polypeptide further includes one or more co-stimulatory domains. In an embodiment, the co-stimulatory domain is a functional signaling domain from a protein including OX40; CD27; CD28; CD30; CD40; PD-1; CD2; CD7; CD258; Natural killer Group 2 member C (NKG2C); Natural killer Group 2 member D (NKG2D), B7-H3; a ligand that binds to at least one of CD83, ICAM-1, LFA-1 (CDl la/CD18), ICOS, and 4-1BB (CD137); CDS; ICAM-1; LFA-1 (CD1a/CD18); CD40; CD27; CD7; B7-H3; NKG2C; PD-1; ICOS; active fragments thereof; functional derivatives thereof; and combinations thereof.

As used herein, the at least one co-stimulatory domain and signaling domain may be collectively referred to as the intracellular domain. As used herein, the hinge region and the antigen recognition domain may be collectively referred to as the extracellular domain.

The present disclosure further provides a polynucleotide encoding the chimeric antigen receptor polypeptide described above.

The term “polynucleotide” as used herein is defined as a chain of nucleotides. Polynucleotide includes DNA and RNA. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and polymerase chain reaction (PCR), and the like, and by synthetic means.

The polynucleotide encoding the CAR is easily prepared from an amino acid sequence of the specified CAR by any conventional method. A base sequence encoding an amino acid sequence can be obtained from the aforementioned NCBI RefSeq IDs or accession numbers of GenBenk for an amino acid sequence of each domain, and the nucleic acid of the present disclosure can be prepared using a standard molecular biological and/or chemical procedure. For example, based on the base sequence, a polynucleotide can be synthesized, and the polynucleotide of the present disclosure can be prepared by combining DNA fragments which are obtained from a cDNA library using a polymerase chain reaction (PCR).

In one embodiment, the polynucleotide disclosed herein is part of a gene, or an expression or cloning cassette.

The polynucleotide described above can be cloned into a vector. A “vector” is a composition of matter which includes an isolated polynucleotide and which can be used to deliver the isolated polynucleotide to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, phagemid, cosmid, and viruses. Viruses include phages, phage derivatives. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like. In one embodiment, vectors include cloning vectors, expression vectors, replication vectors, probe generation vectors, integration vectors, and sequencing vectors.

In an embodiment, the vector is a viral vector. In an embodiment, the viral vector is a retroviral vector or a lentiviral vector. In an embodiment, the engineered cell is virally transduced to express the polynucleotide sequence.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the patient either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

Viral vector technology is well known in the art and is described, for example, in Sambrook et al, (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient and unique restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

Lentiviral vectors have been well known for their capability of transferring genes into human T cells with high efficiency but expression of the vector-encoded genes is dependent on the internal promoter that drives their expression. A strong promoter is particularly important for the third or fourth generation of CARs that bear additional co-stimulatory domains or genes encoding proliferative cytokines as increased CAR body size does not guarantee equal levels of expression. There are a wide range of promoters with different strength and cell-type specificity. Gene therapies using CAR T cells rely on the ability of T cells to express adequate CAR body and maintain expression over a long period of time. The EF-1α promoter has been commonly selected for the CAR expression.

The present disclosure provides an expression vector containing a strong promoter for high level gene expression in T cells or NK cells. In further embodiment, the inventor discloses a strong promoter useful for high level expression of CARs in T cells or NK cells. In particular embodiments, a strong promoter relates to the SFFV promoter, which is selectively introduced in an expression vector to obtain high levels of expression and maintain expression over a long period of time in T cells or NK cells. Expressed genes prefer CARs, T cell co-stimulatory factors and cytokines used for immunotherapy.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor - 1 a (EF- 1 a). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the disclosure should not be limited to the use of constitutive promoters, inducible promoters are also contemplated as part of the disclosure. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence, which is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metalothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

Expression of chimeric antigen receptor polynucleotide may be achieved using, for example, expression vectors including, but not limited to, at least one of a SFFV (spleen-focus forming virus) (for example, SEQ ID NO. 23) or human elongation factor 11α (EF) promoter, CAG (chicken beta-actin promoter with CMV enhancer) promoter human elongation factor 1α (EF) promoter. Examples of less-strong/ lower-expressing promoters utilized may include, but is not limited to, the simian virus 40 (SV40) early promoter, cytomegalovirus (CMV) immediate-early promoter, Ubiquitin C (UBC) promoter, and the phosphoglycerate kinase 1 (PGK) promoter, or a part thereof. Inducible expression of chimeric antigen receptor may be achieved using, for example, a tetracycline responsive promoter, including, but not limited to, TRE3GV (Tet-response element, including all generations and preferably, the 3rd generation), inducible promoter (Clontech Laboratories, Mountain View, CA) or a part or a combination thereof.

In a preferred embodiment, the promoter is an SFFV promoter or a derivative thereof. It has been unexpectedly discovered that SFFV promoter provides stronger expression and greater persistence in the transduced cells in accordance with the present disclosure.

"Expression vector" refers to a vector including a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector includes sufficient cis- acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide. The expression vector may be a bicistronic or multicistronic expression vector. Bicistronic or multicistronic expression vectors may include (1) multiple promoters fused to each of the open reading frames; (2) insertion of splicing signals between genes; fusion of genes whose expressions are driven by a single promoter; (3) insertion of proteolytic cleavage sites between genes (self-cleavage peptide); and (iv) insertion of internal ribosomal entry sites (IRESs) between genes.

In one embodiment, the disclosure provides an engineered cell having at least one chimeric antigen receptor polypeptide or polynucleotide.

An "engineered cell" means any cell of any organism that is modified, transformed, or manipulated by addition or modification of a gene, a DNA or RNA sequence, or protein or polypeptide. Isolated cells, host cells, and genetically engineered cells of the present disclosure include isolated immune cells, such as NK cells and T cells that contain the DNA or RNA sequences encoding a chimeric antigen receptor or chimeric antigen receptor complex and express the chimeric receptor on the cell surface. Isolated host cells and engineered cells may be used, for example, for enhancing an NK cell activity or a T lymphocyte activity, treatment of cancer, and treatment of infectious diseases.

In an embodiment, the engineered cell includes immunoregulatory cells. Immunoregulatory cells include T-cells, such as CD4 T-cells (Helper T-cells), CD8 T-cells (Cytotoxic T-cells, CTLs), and memory T cells or memory stem cell T cells. In another embodiment, T-cells include Natural Killer T-cells (NK T-cells).

In an embodiment, the engineered cell includes Natural Killer cells. Natural killer cells are well known in the art. In one embodiment, natural killer cells include cell lines, such as NK-92 cells. Further examples of NK cell lines include NKG, YT, NK-YS, HANK-1, YTS cells, and NKL cells.

NK cells mediate anti-tumor effects without the risk of GvHD and are short-lived relative to T-cells. Accordingly, NK cells would be exhausted shortly after destroying cancer cells, decreasing the need for an inducible suicide gene on CAR constructs that would ablate the modified cells.

In accordance with the present disclosure, it was surprisingly found that NK cells provide a readily available cell to be engineered to contain and express the chimeric antigen receptor polypeptides disclosed herein.

Allogeneic or autologous NK cells induce a rapid immune response but disappear relatively rapidly from the circulation due to their limited lifespan. Thus, applicants surprisingly discovered that there is reduced concern of persisting side effects using CAR cell based therapy.

According to one aspect of the present disclosure, NK cells can be expanded and transfected with CAR polynucleotides in accordance to the present disclosure. NK cells can be derived from cord blood, peripheral blood, iPS cells and embryonic stem cells. According to one aspect of the present disclosure, NK-92 cells may be expanded and transfected with CAR. NK-92 is a continuously growing cell line that has features and characteristics of natural killer (NK) cells (Arai, Meagher et al. 2008). NK-92 cell line is IL-2 dependent and has been proven to be safe(Arai, Meagher et al. 2008) and feasible. CAR expressing NK-92 cells can be expanded in the serum free-medium with or without co-culturing with feeder cells. A pure population of NK-92 carrying the CAR of interest may be obtained by sorting.

In one embodiment, engineered cells include allogeneic T cells obtained from donors that are modified to inactivate components of TCR (T cell receptor) involved in MHC recognition. As a result, TCR deficient T cells would not cause graft versus host disease (GVHD).

In some embodiments, the engineered cell may be modified to prevent expression of cell surface antigens. For example, an engineered cell may be genetically modified to delete the native CD45 gene to prevent expression and cell surface display thereof.

In some embodiments, the engineered cell includes an inducible suicide gene ("safety switch") or a combination of safety switches, which may be assembled on a vector, such as, without limiting, a retroviral vector, lentiviral vector, adenoviral vector or plasmid. Introduction of a "safety switch" greatly increases safety profile and limits on-target or off-tumor toxicities of the compound CARs. The "safety switch" may be an inducible suicide gene, such as, without limiting, caspase 9 gene, thymidine kinase, cytosine deaminase (CD) or cytochrome P450. Other safety switches for elimination of unwanted modified T cells involve expression of CD20 or CD52 or CD19 or truncated epidermal growth factor receptor in T cells. All possible safety switches have been contemplated and are embodied in the present disclosure.

In some embodiments, the suicide gene is integrated into the engineered cell genome.

In one embodiment, the present disclosure provides an engineered cell having a CD45 chimeric antigen receptor polynucleotide. In one embodiment, the CD45 CAR polypeptide includes SEQ ID NO. 13 and corresponding polynucleotide sequence SEQ ID NO. 14. In another embodiment, the CD45 CAR polypeptide includes SEQ ID NO. 15, and corresponding polynucleotide sequence SEQ ID NO. 16. In another embodiment, the CD45 CAR polypeptide includes SEQ ID NO. 17, and corresponding polynucleotide sequence SEQ ID NO. 18.

In particular embodiments, the engineered cell includes CD45 CAR linked to IL15/IL-15sushi via the P2A cleavage sequence. A polypeptide providing this embodiment includes SEQ ID No. 43 and corresponding polynucleotide sequence SEQ ID No. 44.

In particular embodiments, the engineered cell includes CD45 CAR linked to 4-1BBL (CD137L) via the P2A cleavage sequence. A polypeptide providing this embodiment includes SEQ ID No. 41 and corresponding polynucleotide sequence SEQ ID No. 42.

Multiple CAR units

In one embodiment, the present disclosure provides an engineered cell having at least two distinct or separate CAR units. The two CAR units may be complete CAR units or incomplete CAR units. As used herein, "distinct CAR polypeptide" and "distinct CAR polypeptide unit" are used interchangeably.

The present disclosure provides chimeric antigen receptor polypeptides having a signal peptide, antigen recognition domain, a hinge region, a transmembrane domain, a signaling domain, and at least one co-stimulatory domain, defining a CAR unit or a complete CAR unit. As used herein, an incomplete CAR unit includes a polypeptide having a signal peptide, antigen recognition domain, a hinge region, a transmembrane domain, and a signaling domain or at least one co-stimulatory domain. An incomplete CAR unit will not contain a signaling domain and at least one co-stimulatory domain, but one or the other.

In one embodiment, the present disclosure provides an engineered cell having a first chimeric antigen receptor polypeptide having a first antigen recognition domain and a co-stimulatory domain (first incomplete CAR unit); and a second chimeric antigen receptor polypeptide having a second antigen recognition domain and a signaling domain (second incomplete CAR unit); wherein the first antigen recognition domain is different than the second antigen recognition domain.

Therefore, an engineered cell having two incomplete CAR units will only be fully activated when both target antigens are bound to the antigen recognition domain. This strategy provides added specificity in that the engineered cells are not fully activated until targets are bound at the antigen recognition domain of each incomplete CAR unit.

Furthermore, in embodiments wherein an engineered cell includes two incomplete CAR units, one of the antigen recognition domains may be specific for and bind streptavidin, biotin, HIS, MYC, HA, agarose, V5, Maltose, GST, GFP, CD52, CD20, 4-1BB, or CD28.

As used herein, compound CAR (cCAR) or multiple CAR refers to an engineered cell having at least two complete and distinct chimeric antigen receptor polypeptides. As used herein, a "distinct chimeric antigen receptor polypeptide" has a unique antigen recognition domain, a signal peptide, a hinge region, a transmembrane domain, at least one costimulatory domain, and a signaling domain. Therefore, two unique chimeric antigen receptor polypeptides will have different antigen recognition domains. The signal peptide, hinge region, transmembrane domain, at least one costimulatory domain, and signaling domain may be the same or different between the two distinct chimeric antigen receptor polypeptides. As used herein, a chimeric antigen receptor (CAR) unit refers to a distinct chimeric antigen receptor polypeptide, or a polynucleotide encoding for the same.

As used herein, a unique antigen recognition domain is one that is specific for or targets a single target, or a single epitope of a target.

In some embodiments, the compound CAR targets the same antigen. For example, cCAR targets different epitopes or parts of a single antigen. In some embodiments, each of the CAR units present in the compound CAR targets different antigen specific to the same or different disease condition or side effects caused by a disease condition.

In some embodiments, the compound CAR targets two different antigens.

Creation of compound CARs bearing different CAR units can be quite challenging: (1) CAR-CAR interactions might have a deleterious effect and an appropriate CAR design is a key to offset this effect; (2) a compound CAR in a single construct could increase the length of the expression cassette, which may cause the reduction of the viral titer and level of protein expression; (3) an appropriate design to include various CAR body elements particularly to select a strategy to express multiple CARs in a single vector is required; (4) A strong promoter is particularly important for a compound CAR that bears additional units of CAR; (5) The hinge region in the CAR needs to be designed so that interaction of the hinge region between each CAR unit is avoided preferably; (6) two or more units of CARs expressing in a cell may cause toxic effects (CAR-CAR interaction). Applicants herein provide novel and surprising CAR compositions and methods to overcome these hurdles.

In one embodiment, the present disclosure provides an engineered cell having multiple CAR units. This allows a single engineered cell to target multiple antigens. Targeting multiple surface markers or antigens simultaneously with a multiple CAR unit prevents selection of resistant clones and reduces tumor recurrence. Multiple CAR T cell immunotherapies, with each individual component CAR comprising various domains and activation sites has not yet been developed for any malignancies.

In one aspect of the present disclosure, cCAR includes multiple CAR units. In some embodiments, cCAR includes at least two CAR units. In another embodiment, the cCAR includes at least three CAR units. In another embodiment, the cCAR includes at least four units.

In one embodiment, the present disclosure provides an engineered cell having at least two distinct chimeric antigen receptor polypeptides, each having a different antigen recognition domain.

In one embodiment, the engineered cell having at least two distinct chimeric antigen receptor polypeptides is a T-cell. The T-cell may be engineered so that it does not express a cell surface antigen. For example, a T-cell may be engineered so that it does not express a CD45 cell surface antigen.

In a preferred embodiment, the engineered cell having at least two distinct chimeric antigen receptor polypeptides is a primary NK cell isolated from the peripheral blood or cord blood and NK-92 cells, such that it is administered "off-the-shelf" to any mammal with a disease or cancer.

In one embodiment, the engineered cell includes (i.) a first chimeric antigen receptor polypeptide comprising a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling domain; and (ii.) a second chimeric antigen receptor polypeptide comprising a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain. The first antigen recognition domain is different from the second antigen recognition domain.

In a preferred embodiment, each engineered CAR unit polynucleotide have different nucleotide sequences in order to avoid homologous recombination.

In one embodiment, the target of the first antigen recognition domain is selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BAFF-R, BCMA, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1; and the target of the second recognition domain is selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BAFF-R, BCMA, TACI, LeY, CD5, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD20 recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 3 and corresponding polynucleotide of SEQ ID NO. 4.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD22 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 5 and corresponding polynucleotide of SEQ ID NO. 6.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD123 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 7 and corresponding polynucleotide of SEQ ID NO. 8.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD33 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD123antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 9 and corresponding polynucleotide of SEQ ID NO. 10. In another embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 11 and corresponding polynucleotide of SEQ ID NO. 12.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a BAFF-R antigen recognition domain and second chimeric antigen receptor polypeptide having a CS1antigen recognition domain.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD269 antigen recognition domain and second chimeric antigen receptor polypeptide having a CS1 antigen recognition domain. In one embodiment, the engineered cell includes a polypeptide including SEQ ID NO. 19 and corresponding polynucleotide SEQ ID NO.

20. In one embodiment, the engineered cell includes a polypeptide including SEQ ID NO. 21 and corresponding polynucleotide SEQ ID NO. 22.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD33 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD123 antigen recognition domain.

In one embodiment, each CAR unit includes the same or different hinge region. In another embodiment, each CAR unit includes the same or different transmembrane region. In another embodiment, each CAR unit includes the same or different intracellular domain.

In one embodiment, each CAR unit includes the CD3 zeta chain signaling domain.

In one embodiment, each distinct CAR unit includes different co-stimulatory domains to avoid interaction. For example, the first chimeric antigen receptor polypeptide includes a 4-BB co-stimulatory domain; and the second chimeric antigen receptor polypeptide includes a CD28 co-stimulatory domain.

In another embodiment, the hinge region is designed to exclude amino acids that may cause undesired intra- or intermolecular interactions. For example, the hinge region may be designed to exclude or minimize cysteine residues to prevent formation of disulfide bonds. In another embodiment, the hinge region may be designed to exclude or minimize hydrophobic residues to prevent unwanted hydrophobic interactions.

Compound CAR can perform killing independently or in combination. Multiple or compound CAR comprises same or different hinge region, same or different transmembrane, same or different co-stimulatory and same or different intracellular domains. Preferably, the hinge region is selected to avoid the interaction site.

The compound CAR of the present disclosure may target same or different tumor populations in T or NK cells. The first CAR, for example, may target the bulky tumor population and the next or the second CAR, for example, may eradicate cancer or leukemic stem cells, to avoid cancer relapses.

In accordance with the present disclosure it was surprisingly found that the compound CAR in a T or NK cells targeting different or same tumor populations combat tumor factors causing cancer cells resistant to the CAR killing activity, thereby producing down regulation of the target antigen from the cancer cell surface. It was also surprisingly found that this enables the cancer cell to "hide" from the CAR therapy referred to as "antigen escape" and tumor heterogeneity, by which different tumor cells can exhibit distinct surface antigen expression profiles.

Engineered cell having CAR polypeptide and enhancer

In another embodiment, the present disclosure provides an engineered cell having at least one chimeric antigen receptor polypeptide and an enhancer.

In one embodiment, the present disclosure provides an engineered cell having at least two distinct chimeric antigen receptor polypeptides and an enhancer.

As used herein, an enhancer includes a biological molecule that promotes or enhances the activity of the engineered cell having the chimeric antigen receptor polypeptide. Enhancers include cytokines. In another embodiment, enhancers include IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, and TGFR beta, receptors for the same, and functional fragments thereof.

Enhancers may be expressed by the engineered cell described herein and displayed on the surface of the engineered cell or the enhancer may be secreted into the surrounding extracellular space by the engineered cell. Methods of surface display and secretion are well known in the art. For example, the enhancer may be a fusion protein with a peptide that provides surface display or secretion into the extracellular space.

The effect of the enhancer may be complemented by additional factors such as enhancer receptors and functional fragments thereof. The additional factors may be co-expressed with the enhancer as a fusion protein, or expressed as a separate polypeptide and secreted into the extracellular space.

Enhancers can be cytokines secreted from engineered CAR cells and are designed to co-express with the CAR polypeptide. A massive release occurs upon CAR engagement of cognate antigen. Inflammatory cells surrounding tumor cells have a significant correlation with cancer cell progression and metastasis. Inflammatory cells could include T cells and innate immune response cells, such as NK cells, macrophages, and dendritic cells and their proliferation and anti-tumor activity are regulated by cytokines. CAR cells such as CAR T or NK cells bind to targeted cancer cells and trigger massive secretion of enhancers from the expansion of CAR T/NK cells. The secreted enhancers efficiently promote survival, differentiation and activation of immune response cells against cancer cells. The co-expression of an enhancer(s) with CAR can supplement the defect that CAR T or NK cells are unable to eliminate non-targeting cancer cells (Figure 78).

CAR cells can be a carrier of cytokines, and cytokines can be delivered to targeted cancer sites by CAR cells to reduce systemic toxicity with high-dose exogenous cytokines(Figure 78).

To improve sustained survival or long-lived persistence of CAR cells, a membrane bound enhancer (s) can be co-expressed with CAR to improve CAR persistency.

In one embodiment, the enhancer is IL-15. In this instance, the additional factor described above is the IL-15 receptor, and functional fragments thereof. Functional fragments include the IL-15 receptor, IL-15RA, and the sushi domain of IL-15RA (IL-15sushi). Soluble IL-15RA or IL15sushi profoundly potentiates IL-15 functional activity by prevention of IL-15 degradation. Soluble IL-15/IL-15RA or IL-15/IL-15sushi complexes are stable and much more stimulatory than IL-15 alone in vivo.

In one embodiment, IL-15 is co-expressed as a fusion protein with at least one of IL-15 receptor, IL-15RA, and the sushi domain of IL-15RA (IL-15sushi). In one embodiment, the IL-15 receptor, IL-15RA, or the sushi domain of IL-15RA (IL-15sushi) is at the N-terminus of IL-15. In another embodiment, , the IL-15 receptor, IL-15RA, or the sushi domain of IL-15RA (IL-15sushi) is at the C-terminus of IL-15. As used herein, IL-15/IL-15 sushi denotes that IL-15 sushi is at the C-terminus of IL-15 in a fusion protein; and IL-15sushi/il-15 denotes that IL-15 sushi is at the N-terminus of IL-15 in a fusion protein.

In some embodiments, IL-15 and the IL-15 receptor or functional fragments thereof polypeptide is on a single polypeptide molecule and is separated by a peptide linker, the peptide linker may be 1-25 amino acid residues in length, 25-100 amino acid residues in length, or 50-200 amino acid residues in length. This linker may include a high efficiency cleavage site described herein.

An example of a suitable sushi domain includes a CAR construct, SEQ ID NO. 1. In accordance with the present disclosure, any chimeric antigen receptor polypeptide disclosed herein may be co-expressed with the Human Interleukin 15 with human interleukin 2 signal peptide SEQ ID NO. 2.

Interleukin (IL)-15 and its specific receptor chain, IL-15Rα (IL-15-RA) play a key functional role in various effector cells, including NK and CD8 T cells. CD8+ T cells can be modified to express autocrine growth factors including, but not limited to, IL-2, Il-7, IL21 or IL-15, to sustain survival following transfer in vivo. Without wishing to be bound by theory, it is believed that IL-15 overcomes the CD4 deficiency to induce primary and recall memory CD8T cells. Overexpression of IL-15-RA or an IL-15 IL-RA fusion on CD8 T cells significantly enhances its survival and proliferation in-vitro and in-vivo. In some embodiments, CD4CAR or any CAR is co-expressed with at least one of IL-15, IL15RA and IL-15/IL-15R or IL15-RA/IL-15, or a part or a combination thereof, to enhance survival or proliferation of CAR T or NK, and to improve expansion of memory CAR CD8+ T cells.

The present disclosure provides an engineered cell having a CAR polypeptide as described herein and at least one of IL-15, IL-15RA, IL-15sushi, IL-15/IL-15RA, IL15-RA/IL-15, IL-15/IL-15sushi, IL15sushi/IL-15, fragment thereof, a combination thereof, to enhance survival or persistence or proliferation of CAR T or NK for treating cancer in a patient.

In another embodiment, the present disclosure provides an engineered cell having at least one of recombinant IL-15, IL-15RA, IL-15sushi, IL-15/IL-15RA, IL15-RA/IL-15, IL-15/IL-15sushi, IL15sushi/IL-15, functional fragment thereof, and combination thereof; and at least one distinct CAR polypeptide wherein the antigen recognition domain includes NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, BCMA, TACI, LeY, CD5, CD7, CD2, CD3, CD4, CD45, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, ROR1, PSMA, MAGE A3, Glycolipid, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38 and CS1. The target antigens can also include viral or fungal antigens, such as E6 and E7 from the human papillomavirus (HPV) or EBV (Epstein Barr virus) antigens. In further embodiment, the antigen recognition polypeptides (scFv) and corresponding polynucleotides for CD2, CD3, CD5, CD7, and CD52 are described in more detail publications in PCT Application NO. PCT/US2016/39306, the contents of which are incorporated herein by reference.

Without wishing to be bound by theory, it is believed that IL-15/IL-15sushi and other types of IL-15 or IL-15RA proteins or protein fragments thereof provide synergistic efficacy of a CAR polypeptide when combined with checkpoint inhibitors or modulators (e.g. anti-PD-1).

In one embodiment, the disclosure provides a CD4 CAR engineered cell that includes IL-15/IL-15sushi (SEQ ID NO. 1), and corresponding polynucleotide (SEQ ID NO. 2). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD4 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD4 CAR polypeptide provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the present disclosure provides engineered cell having a CD45 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 44), and corresponding nucleotides (SEQ ID NO. 43).

In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD45 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD45 CAR polypeptide provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD45 chimeric antigen receptor polypeptide and 4-1BBL (SEQ ID NO. 74), and corresponding nucleotides (SEQ ID NO. 73).

In one embodiment, the present disclosure provides a method of providing long-term durable remission in patients suffering from cancer by administering a CD45 CAR engineered cell that co-expresses 4-1BBL to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of 4-1BBL with a CD45 CAR provides long-term durable remission in patients by increasing the persistence of CAR engineered cells.

In one embodiment, the engineered cell includes a CD19 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 59), and corresponding polynucleotide (SEQ ID NO. 60). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD19 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD19 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD20 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.58), and corresponding polynucleotide (SEQ ID NO. 57). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD20 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD20 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD22 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.62), and corresponding polynucleotide (SEQ ID NO. 61). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD22 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD22 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD269 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 44), and corresponding polynucleotide (SEQ ID NO. 45). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD269 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD269 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells as plasma cells or myeloma cells are usually dim CD269 (BCMA) positive.

In one embodiment, the engineered cell includes a CAR, CD4 polypeptide of SEQ ID NO. 90, and corresponding polynucleotide of SEQ ID NO. 89.

In one embodiment, the engineered cell includes a CD4 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 96), and corresponding polynucleotide (SEQ ID NO. 95). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD4 CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD4 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD4 chimeric antigen receptor polypeptide and IL-15/IL-15RA (membrane bound) (SEQ ID NO. 98), and corresponding polynucleotide (SEQ ID NO. 97). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD4 CAR engineered cell that includes IL-15/IL-15RA to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15RA (membrane bound) with a CD4 CAR provides long-term durable remission in patients by increasing the persistence of CAR engineered cells.

In one embodiment, the engineered cell includes a compound CAR, CD33CD123 polypeptide and IL-15/IL-15sushi (SEQ ID NO.40), and corresponding polynucleotide (SEQ ID NO. 39). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD33CD123 compound CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD33CD123 CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a compound CAR, CD33CD123 polypeptide and 4-1BBL(SEQ ID NO.38), and corresponding polynucleotide (SEQ ID NO. 37).

In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a CD33CD123 compound CAR engineered cell that co-expresses 4-1BBL to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of 4-1BBL with a CD33CD123 cCAR provides long-term durable remission in patients by increasing the persistency of cCAR engineered cells.

In one embodiment, the engineered cell includes a BAFF CAR polypeptide with a CD45 leader sequence (SEQ ID NO. 78) and corresponding polynucleotide sequence (SEQ ID NO. 77).

In one embodiment, the engineered cell includes BAFF CAR polypeptide with a CD8a leader sequence (includes SEQ ID NO. 80) and corresponding polynucleotide sequence (SEQ ID NO. 79).

In one embodiment, the engineered cell includes a BAFF CAR polypeptide and IL-15/IL-15sushi (SEQ ID NO. 84), and corresponding polynucleotide (SEQ ID NO. 83).

In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a BAFF CAR engineered cell that includes IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a BAFF CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells as BAFF receptor, CD269 (BCMA) is weakly expressed in plasma cells and myeloma cells.

In one embodiment, the engineered cell includes a BAFF CAR polypeptide and 4-1BBL (SEQ ID NO.82), and corresponding polynucleotide (SEQ ID NO. 81). In one embodiment, the present disclosure provides a method of providing long-term durable remission in cancer patients by administering a BAFF CAR engineered cell co-expresses 4-1BBL to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of 4-1BBLwith a BAFF CAR can provide long-term durable remission in patients by increasing the persistence of CAR engineered cells.

In one embodiment, the engineered cell includes a compound CAR, BAFF CD19b polypeptide of SEQ ID NO. 86 and corresponding polynucleotide of SEQ ID NO. 85.

In one embodiment, the present disclosure provides a method of treating an autoimmune disorder in a patients by administering a BAFF CD19b compound CAR engineered cell to a patient in need thereof. Without wishing to be bound by theory, it is believed that the BAFF CD19b compound CAR engineered cells provide a better therapeutic outcome for depletion of B-cells and plasma cells associated with autoimmune disorders.

In one embodiment, the engineered cell includes a APRIL CD19b compound CAR polypeptide of SEQ ID NO. 88 and corresponding polynucleotide of SEQ ID NO. 77.

In one embodiment, the present disclosure provides a method of depleting B-cells and plasma cells in a patient in need thereof by administering a APRIL CD19b compound CAR engineered cell to a patient in need thereof. Without wishing to be bound by theory, it is believed that the APRIL CD19b compound CAR engineered cell can provide a better therapeutic outcome for depletion of B-cells and plasma cells associated with autoimmune disorders.

In one embodiment, the engineered cell includes a compound CAR, CD269 CS1 polypeptide of SEQ ID NO. 48 and corresponding polynucleotide of SEQ ID NO. 47. In one embodiment, the present disclosure provides a method of treating myeloma in a patient by administering a CD269CS1 compound CAR engineered cell to a patient in need thereof.

Without wishing to be bound by theory, it is believed that CD269 CS1 compound CAR engineered cells provide a better therapeutic outcome for patients with myeloma, and prevent antigen escape or disease relapse.

In one embodiment, the engineered cell includes a compound CAR, CD269 CD19b polypeptide of SEQ ID NO. 50 and corresponding polynucleotide of SEQ ID NO. 49.

In one embodiment, the present disclosure provides a method of depleting B-cells and plasma cells in patients by administering a CD269 CD19b compound CAR engineered cell to a patient in need thereof. Without wishing to be bound by theory, it is believed that CD269 CD19b compound CAR engineered cells provide a better therapeutic outcomes for patients suffering from an autoimmune disorder by depletion of B-cells and plasma cells associated with autoimmune disorders.

In one embodiment, the engineered cell includes another compound CAR, CD269 CD19 polypeptide of SEQ ID NO. 52 and corresponding polynucleotide of SEQ ID NO. 51. In one embodiment, the present disclosure provides a method of depleting B-cells and plasma cells in patients by administering a CD269 CD19 compound CAR engineered cell to a patient in need thereof. Without wishing to be bound by theory, it is believed that CD269 CD19 compound CAR engineered cells provide a better therapeutic outcomes in patients suffering from an autoimmune disorder by depletion of B-cells and plasma cells associated with autoimmune disorders.

In one embodiment, the present disclosure provides an engineered cell having a CD19 chimeric antigen receptor polynucleotide. In one embodiment, the CD19 CAR polypeptide includes SEQ ID NO. 54 and corresponding polynucleotide sequence SEQ ID NO. 53. In another embodiment, the CD19 CAR polypeptide includes SEQ ID NO. 56, and corresponding polynucleotide sequence SEQ ID NO. 55

In one embodiment, the engineered cell includes a CD30 CAR polypeptide, and IL-15/IL-15sushi polypeptide (SEQ ID NO. 100), and corresponding polynucleotide (SEQ ID NO. 99). The targeted disease is malignant Hodgkin lymphoma with cancer cells expressing CD30.

In one embodiment, the present disclosure provides a method of re-activating T-cell and innate immune cells in the tumor microenvironment patients by administering a CD30CAR engineered cell that secretes IL-15/IL-15 complexes to a patient in need thereof. Without wishing to be bound by theory, it is believed that IL-15/IL-15 complexes (e.g. IL-15/IL-15sushi complexes) secreted from engineered cells can re-activate T-cell and innate immune cells in the tumor microenvironment and then restore or augment their anti-tumor immune responses for Hodgkin lymphoma or anaplastic large cell lymphoma.

In one embodiment, the present disclosure provides a method of restoring or augmenting T-cell or innate immune cell activation or expansion including coexpression of IL-15/IL-15sushi with a CAR polypeptide disclosed herein.

In another embodiment, the disclosure provides a chimeric antigen receptor polypeptide having an antigen recognition domain specific for a CD30 antigen.

In one embodiment, the CD30CAR includes at least one-costimulatory domain. In another embodiment, the CD30CAR includes at least two co-stimulatory domains.

In some embodiments, the disclosure includes a method of co-expressing IL-15/IL-15sushi with CD30CAR. In further embodiments, massive secretion of stable, functional IL-15/IL-15sushi complexes occurs upon binding of CAR to target cells.

In another embodiment, the present disclosure provides a method of treating a patient suffering from Hodgkin's lymphoma or a cancer associated with a malignant cell expressing CD30 antigen by administering a CD30 CAR engineered cell to a patient in need thereof. An example of a malignant cells expressing CD30 includes anaplastic large cell lymphoma.

Malignant Hodgkin lymphoma bears CD30+ Reed-Sternberg or Reed-Sternberg like cells, which are surrounded by an overwhelming numbers of T cells and innate immune cells. These T or innate immune cells are immunologically tolerant as they fail to eliminate cancer cells. Therefore, one of critical aspects for treating Hodgkin lymphoma is to re-activate T-cell and innate immune cells in the tumor microenvironment and then restore or augment their anti-tumor immune responses.

In some embodiments, the present disclosure comprises a method of co-expression of IL-15/IL-15sushi with a CD30CAR. Engineered CD30CAR T or NK cells bind to targeted cancer cells, trigger massive secretion of IL-15/IL-15sushi from the expansion of CD30CAR T or NK cells, whereby secreting IL-15/IL-15sushi efficiently restore or augment T or innate immune cells against cancer cells to overcome immunosuppressive tumor microenvironment.

In one embodiment, the present disclosure provides a method of providing long-term durable remission in a cancer patient by administering a CD30 CAR engineered cell that co-express IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD30CAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells against target cancer cells to overcome immunosuppressive tumor microenvironment.

In some embodiments, the present disclosure provides an engineered cell that co-expresses IL-15/IL-15sushi and a CD30CAR polypeptide.Without wishing to be bound by theory, it is believed that the combination of CD30CAR engineered cell with co-expression of

IL-15/IL-15sushi provides synergistic efficacy when combined with checkpoint inhibitors or modulators (e.g. anti-PD-1).

In some embodiments, the present disclosure provides a method of treating Hodgkin's lymphoma in a patient by administering a CD30 CAR engineered cell that co-expresses IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, co-expression of CD30CAR polypeptide and IL-15/IL-15sushi provides better outcomes for treatment of Hodgkin's lymphoma or anaplastic large cells than CD30CAR alone as CD30 is not expressed in all cancer cells.

Steps for elimination of tumor by CAR co-expressing secretory IL-15/IL-15sushi (Figure 78)

In some embodiments, the present disclosure provides a method of provide long-term durable remission in a cancer patient by administering a APRIL CAR engineered cell that co-expresses IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a APRIL CAR polypeptide provides long-term durable remissions in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate cells to cancer cells. APRIL receptor, CD269 (BCMA) is weakly expressed in plasma cells and myeloma cells.

In particular embodiments, the present disclosure provides a method for elimination of tumor cells including contacting said tumor cell with a CAR engineered cell that co-expresses IL-2 to destroy said tumor cell. .

IL-15 was originally considered as an interleukin-2 (IL-2)-like factor for T and NK cells. Unlike IL-2, IL-15 is a survival factor for memory T cells.

In particular embodiments, elimination of tumor can be achieved by combination of at least one or more of the following steps:

(1) binding of an CAR engineered T cell or NK cell disclosed herein to a portion of tumor cells by targeting CAR or NK antigen(s);

(2) Triggering massive secretion of IL-15/IL-15sushi or IL-2 with a prolonged half-life from expansion of CAR T/NK cells, which co-express this molecule;

(3) Recruiting and stimulating a variety of innate and adaptive immune cells against tumor;

(4) Reducing tumor suppression that is present in tumor by administration of a checkpoint blockage such as PD-L1 and CTLA-4 inhibitor.

Without wishing to be bound by theory, it is believed that the combination of steps described above provide potent anti-tumor effects via a concerted innate and adaptive immune response.

The engineered cells and methods described herein are suitable for the treatment of any cancer wherein specific monoclonal or polyclonal antibodies exist or are capable of being generated in accordance with the current state of the art. In particular, the following cancers have been contemplated and are considered within the scope of the present disclosure, neuroblastoma, lung cancer, melanoma, ovarian cancer, renal cell carcinoma, colon cancer, brain cancer, Hodgkin's lymphoma, B cell lymphoma/leukemia and T cell lymphoma/leukemia. All of which have cell surface antigens that may be targeted by the chimeric antigen receptor polypeptides and methods disclosed herein.

Many tumors escape the specific CAR T/NK killing due to the loss of targeted antigen(s) or CAR T or NK exhaustion. The present disclosure provides a method to overcome this escape. Without wishing to be bound by theory, the present disclosure provides a method of preventing tumor escape by administering a CAR engineered cell having an enhancer or cytokine as disclosed herein, in particular IL-15 or IL-2 to a tumor site by CAR engineered cell. It is believed that this directly stimulates innate and adaptive immune responses. Furthermore, it is believed that IL-15 and/or IL-2 secretion from CAR engineered cells promote the expansion of infused CAR T cells or CAR NK cells and infiltration of immune cells to the tumor site, which is believed to result in tumor destruction.

In embodiments, half-life extension and prolonged therapeutic activity can be established in the presence of the Fc domain, such IL-15Fc or IL-2Fc. For IL-15 cytokine, IL-15/IL-15sushi or IL-15/IL-15sushi Fc is preferred. Fc domain is referred to as the IgG Fc-domain fused to various effector molecules (so-called Fc-fusion proteins).

Single antigen-directed CAR immunotherapy, such as, but not limited to, CD19, CD20, CD22, CD2, CD3, CD4, CD5, CD7, CD33, CD30, CD123, CD45, BCMA, CS1, BAFF, TACI, and APRIL CAR, bears a risk of remission in patients due to the complete loss of target antigen or changes of target antigen expression. On this basis, the present disclosure provides a method of providing long-term durable remission in patients by administering an engineered cell having a CAR polypeptide disclosed herein and co-expression of IL-15/IL-15sushi to increase the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

The large volume of some solid tumors or lymphoma can be difficult for CAR T cells to eradicate the whole tumor. In addition, the immunosuppressive microenvironment needs to be overcome as CAR T cells may end up simply being inactivated or suppressed when contacting tumor.

In some embodiments, the present disclosure provides a method of co-expressing secretory IL-15/IL-15sushi and a chimeric antigen receptor polypeptide in an engineered cell.

In some embodiments, the present disclosure provides a method of increasing CAR engineered cell in vivo half life by co expressing secretory IL-15/IL-15sushi in said engineered cell. Without wishing to be bound by theory, it is believed that the secreted complexes of IL-15/IL-15sushi are functionally stable and efficiently promote survival of the CAR containing engineered cell.

In some embodiments, the present disclosure provides a method of delivering IL-15/IL-15sushi to targeted cancer sites using CAR as a carrier to promote the proliferation of innate immune response cells against cancer cells, prevent the tumor microenvironment suppression, and reduce systemic toxicity with high-dose exogenous cytokines.

In some embodiments, the present disclosure provides a method of delivering IL-15/IL-15sushi to targeted cancer sites using CAR as a carrier to recruit other effector immune cells to the site and help them to kill cancer cells.

In some embodiments, the present disclosure provides a method of delivering IL-15/IL-15sushi to targeted cancer sites using CAR as a carrier to activate bystander immunity to eradicate cancer cells that lose the antigen for CAR T/NK cells.

Methods of generating engineered cells

Any of the polynucleotides disclosed herein may be introduced into an engineered cell by any method known in the art.

In one embodiment, CAR polynucleotides are delivered to the engineered cell by any viral vector as disclosed herein.

In one embodiment, to achieve enhanced safety profile or therapeutic index, the any of the engineered cells disclosed herein be constructed as a transient RNA-modified "biodegradable" version or derivatives, or a combination thereof. The RNA-modified CARs of the present disclosure may be electroporated into T cells or NK cells. The expression of the compound CAR may be gradually diminished over few days.

In some embodiments of the present disclosure, any of the engineered cells disclosed herein may be constructed in a transponson system (also called a "Sleeping Beauty"), which integrates the CAR DNA into the host genome without a viral vector.

Methods of generating an engineered cell having multiple CAR units

In another embodiment, the present disclosure provides a method making an engineered cell having at least two CAR units.

In some embodiments, multiple units of CAR are expressed in a T or NK cell using bicistronic or multicistronic expression vectors. There are several strategies which can be employed to construct bicistronic or multicistronic vectors including, but not limited to, (1) multiple promoters fused to the CARs' open reading frames;(2) insertion of splicing signals between units of CAR; fusion of CARs whose expressions are driven by a single promoter;(3) insertion of proteolytic cleavage sites between units of CAR (self-cleavage peptide); and (iv) insertion of internal ribosomal entry sites (IRESs).

In a preferred embodiment, multiple CAR units are expressed in a single open reading frame (ORF), thereby creating a single polypeptide having multiple CAR units. In this embodiment, an amino acid sequence or linker containing a high efficiency cleavage site is disposed between each CAR unit.

As used herein, high cleavage efficiency is defined as more than 50 %, more than 70 %, more than 80%, or more than 90% of the translated protein is cleaved. Cleavage efficiency may be measured by Western Blot analysis, as described by Kim 2011.

Furthermore, in a preferred embodiment, there are equal amounts of cleavage product, as shown on a Western Blot analysis.

Examples of high efficiency cleavage sites include porcine teschovirus-1 2A (P2A), FMDV 2A (abbreviated herein as F2A); equine rhinitis A virus (ERAV) 2A (E2A); and Thoseaasigna virus 2A (T2A), cytoplasmic polyhedrosis virus 2A (BmCPV2A) and flacherie Virus 2A (BmIFV2A), or a combination thereof. In a preferred embodiment, the high efficiency cleavage site is P2A. High efficiency cleavage sites are described in Kim JH, Lee S-R, Li L-H, Park H-J, Park J-H, Lee KY, et al. (2011) High Cleavage Efficiency of a 2A Peptide Derived from Porcine Teschovirus-1 in Human Cell Lines, Zebrafish and Mice. PLoS ONE 6(4): e18556, the contents of which are incorporated herein by reference.

In embodiments wherein multiple CAR units are expressed in a single open reading frame (ORF), expression is under the control of a strong promoter. Examples of strong promoters include the SFFV promoter, and derivatives thereof.

Engineered cell having CAR polypeptide and enhancer

In another embodiment, the present disclosure provides a method making an engineered cell that expresses at least one CAR unit and an enhancer.

In some embodiments, at least one CAR unit and enhancer is expressed in a T or NK cell using bicistronic or multicistronic expression vectors. There are several strategies which can be employed to construct bicistronic or multicistronic vectors including, but not limited to, (1) multiple promoters fused to the CARs' open reading frames;(2) insertion of splicing signals between units of CAR; fusion of CARs whose expressions are driven by a single promoter;(3) insertion of proteolytic cleavage sites between units of CAR (self-cleavage peptide); and (4) insertion of internal ribosomal entry sites (IRESs).

In a preferred embodiment, at least one CAR unit and an enhancer are expressed in a single open reading frame (ORF), thereby creating a single polypeptide having at least one CAR unit and an enhancer. In this embodiment, an amino acid sequence or linker containing a high efficiency cleavage site is disposed between each CAR unit and between a CAR unit and enhancer. In this embodiment, the ORF is under the control of a strong promoter. Examples of strong promoters include the SFFV promoter, and derivatives thereof.

Furthermore, in a preferred embodiment, there are equal amounts of cleavage product, as shown on a Western Blot analysis.

Methods of treatment using the compositions disclosed herein

In another embodiment, the present disclosure provides a method of targeting CD45 for conditioning prior to allogenic transplantation in cancer treatment. CD45 is also known as leukocyte common antigen (LCA) and is a tyrosine phosphatase expressed on virtually all cells of hematopoietic origin except erythrocytes and platelets. Most hematologic malignancies express CD45. For instance, 85% to 90% acute lymphoid and myeloid leukemias express CD45. CD45 is not found in non-hematopoietic origin. In addition, CD45 is expressed at a high density of an average copy number of approximately 200,000 molecules per cells on malignant cells and leukocytes. CD45 presents an ideal target for a variety of hematologic malignancies. However, CAR T and NK cells also express CD45. Without inactivation of endogenous CD45, CAR T or NK cells armed with CARs targeting CD45 may result in self-killing.

The association of CD45 with TCR complexes is essential in regulation of T-cell activation in response to antigen. The inability of CD45-deficient T cells to present antigen is due to reduced signaling through the T cell receptors (TCRs). TCRs are cell surface receptors that play an essential role in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, alpha and beta, which are associated with the transducing subunits, the CD3, to form the T-cell receptor complex present on the cell surface.

It was surprisingly found that multiple CARs (Compound CARs, cCAR) of the present disclosure combat a key mechanism by which cancer cells resist CAR activity, i.e., the downregulation or heterogeneous expression of the target antigen from the cancer cell surface. This mechanism allows the cancer cell to “hide” from the CAR therapy, a phenomenon referred to as ‘antigen escape’. The present disclosure pre-empts cancer antigen escape by recognizing a combination of two or more antigens to rapidly eliminate the tumor.

The disclosure provides a method of simultaneous targeting of multi-antigens using a cCAR resulting in improved tumor control by minimizing the possibility of tumor selection on the basis of target antigen loss or down-regulation.

The disclosed disclosure includes compound (multiple or compound) cCAR in a T or NK cell targeting different or same surface antigens present in tumor cells. The compound chimeric antigen receptors of the present disclosure comprise at least multiple chimeric receptor constructs linked by a linker and target of the same or different antigens. For example, each of the CAR construct present in the compound CAR (cCAR) construct includes an antigen recognition domain, an extracellular domain, a transmembrane domain and/or a cytoplasmic domain. The extracellular domain and transmembrane domain can be derived from any desired source for such domains. The multiple CAR constructs are linked by a linker. The expression of the compound CAR construct is driven by a promoter. The linker may be a peptide or a part of a protein, which is self-cleaved after a protein or peptide is generated (also called as a self-cleaving peptide).

In one embodiments, the compound CARs of the present disclosure target Myelodysplastic Syndrome and acute myeloid leukemia (AML) populations. Myelodysplastic Syndrome (MDS) remains an incurable hematopoietic stem cell malignancy that occurs most frequently among the elderly, with about 14,000 new cases each year in the USA. About 30–40% of MDS cases progress to AML. The incidence of MDS continues to increase as our population ages. Although MDS and AML have been studied intensely, no satisfactory treatments have been developed.

The compositions and methods of this disclosure can be used to generate a population of T lymphocyte or NK cells that deliver both primary and co-stimulatory signals for use in immunotherapy in the treatment of cancer, in particular, the treatment of lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, brain cancer, sarcoma, leukemia and lymphoma.

Immunotherapeutics generally rely on the use of immune effector cells and molecules to target and destroy cancer cells. The effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells, NK cells, and NK-92 cells. The compositions and methods described in the present disclosure may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth. The compositions and methods described in the present disclosure may be utilized in other disease conditions that rely on immune responses such as inflammation, immune diseases, and infectious diseases.

In some embodiments, the compound CAR of the present disclosure may act as a bridge to bone marrow transplant, by achieving complete remission for patients who have minimal residual disease and are no longer responding to chemotherapy. In other embodiments, the compound CAR eliminates leukemic cells followed by bone marrow stem cell rescue to support leukopenia.

In some embodiments, the compound CAR of the present disclosure can combat a key mechanism by which cancer cells resist CAR activity by the down-regulation of the target antigen. In another embodiment, the invented compound CAR can also combat the heterogeneity of cancer cells, which creates significant challenges in a regular CAR T/NK cell therapy. In a further embodiment, the disclosed compound CAR is designed that the first CAR targets the bulky tumor population and another eradicates cancer or leukemic stem cells to avoid cancer relapses.

In one embodiment, the present disclosure provides a method of destroying cells having a CD33 antigen or a CD123 antigen, or both by contacting said cells with an engineered cell having at least one of chimeric antigen receptor polypeptide having a CD33 antigen recognition domain and chimeric antigen receptor polypeptide having a CD23 antigen recognition domain. The engineered cell may be a T or NK cell.

Cells having at least one of the CD33 antigen and the CD123 antigen include acute myeloid leukemia, precursor acute lymphoblastic leukemia, chronic myeloproliferative neoplasms, chronic myeloid leukemia, myelodysplasia syndromes, blastic plasmocytoid dendritic neoplasms (BPDCN), Hodgkin's lymphoma, mastocytosis, and hairy cell leukemia cells.

In another embodiment, the present disclosure provides a method of providing myeloblative conditioning regimens for hematopoietic stem cell transplantation. In this embodiment, a T or NK engineered cell having a CD33 unit and a CD123 unit is administered to a patient in need thereof.

In further embodiments, the present disclosure provides a method of eradicating or killing leukemic stem cells (LSCs) or bulk leukemic cells expressing CD123 or CD33, or both. In this embodiment, a T or NK engineered cell having a CD33 unit and a CD123 unit is administered to a patient in need thereof.

In further embodiments, the compound CAR in a T or NK cell may be used to eradicate or kill CD34+ CD38- leukemic stem cells or bulk leukemic cells expressing CD123 or CD33 or both.

In some embodiments, a compound CAR targets cells expressing CD19 or CD20 antigens or both. In another embodiment, a compound CAR targets cells expressing CD19 or CD22 antigens or both. The targeted cells may be cancer cells, such as, without limiting, B-cell lymphomas or leukemias. In further embodiments, the target antigens can include at least one of this group, but not limited to, ROR1, PSMA, MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138. The target antigens can also include viral or fungal antigens, such as E6 and E7 from the human papillomavirus (HPV) or EBV (Epstein Barr virus) antigens.

In some embodiments, the compound CAR engineered cells target cells having cell surface CD19 antigen or cell surface CD123 antigen or both. The targeted cells are cancer cells, such as, without limiting, B-cell lymphomas or leukemias.

Clinical trials of CD19 CAR T cell therapy have shown that 80-94% of patients with B-ALL achieve complete remission, but a substantial proportion of patients eventually relapse. The prevalence of CD123 expression in B-ALL is high, and can be used as a CAR target for B-ALL.

In some embodiments, the compound CAR targets cells expressing CD19 or CD123 antigen or both. Without wishing to be bound by theory, it is believed that CD19 and/or CD123 compound CAR engineered cells offset tumor escape due to the loss of CD19 or CD123 antigen or prevent B-ALL or other type B-cell lymphoma/leukemia relapse.

In further embodiments, the CD19 and/or CD20 compound CAR engineered cells target cells having cell surface CD19 antigens and/or CD20 cell surface antigens. In another embodiment, the targeted cells are malignant B cell lymphoma/leukemia such as, without limiting, B-ALL, high grade B cell lymphoma, low grade B-cell lymphoma, diffuse large B cell lymphoma, Burkett lymphoma, mantle cell lymphoma, CLL, marginal zone B cell lymphoma and follicular lymphoma.

Without wishing to be bound by theory, it is believed that the CD19 and/or CD20 CAR engineered cells provide an effective safeguard against antigen escape and prevent disease relapse in adoptive T/NK-cell therapy for B-cell malignancies.

CAR target cells having at least one of the antigens CD19, CD20, CD22, and CD123, include precursor acute lymphoblastic leukemia, B- cell lymphoma/leukemia, chronic lymphocytic leukemia/lymphoma, mantle lymphoma, follicular lymphoma, marginal zone B cell lymphoma, diffuse large B cell lymphoma, Burkett lymphoma, blastic plasmocytoid dendritic neoplasms (BPDCN), Hodgkin's lymphoma, and hairy cell leukemia cells.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD22 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 64 and corresponding polynucleotide of SEQ ID NO. 63.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD20 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 66 and corresponding polynucleotide of SEQ ID NO. 65.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD123 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 68 and corresponding polynucleotide of SEQ ID NO. 67.

Multiple myeloma is an incurable disease exhibiting uncontrollable proliferation of plasma cells in the bone marrow. CS1 and BCMA are widely expressed on myeloma cells, but is not expressed in hematopoietic stem/progenitor cells. Therefore, BCMA and CS1 are ideal targets for CAR T/NK cell therapy.

In further embodiments, the present disclosure provides compound CAR engineered cell having a CS1 (SLAM7) antigen recognition domain and/or an antigen recognition domain that targets B-cell maturation antigens (BCMA). In another embodiment, the targeted cells are malignant plasma cells, such as, but not limited to, multiple myeloma.

Without wishing to be bound by theory, it is believed that a compound CAR engineered cell having at least one of CS1 and BCMA antigen recognition domain enhances functionality against multiple myeloma and offset antigen escape.

In some embodiments, a CAR targets cells expressing multiple antigens including, but not limited to, CS1, BCMA, CD267, BAFF-R, CD38, CD138, CD52, CD19, TACI, CD20, interleukin 6 receptor, and NY-ESO-1 antigens. In another embodiment, the targeted cells are plasma cells, B-cells, malignant plasma cells such as, without limiting, multiple myeloma.

In some embodiments, the compound CAR targets cells expressing multiple antigens including, but not limited to, CS1, BCMA, CD267, BAFF-R, CD38, CD138, CD52, CD19, TACI, CD20, interleukin 6 receptor, and NY-ESO-1 antigens. In another embodiment, the targeted cells are malignant plasma cells such as, without limiting, multiple myeloma.

In some embodiments, the compound CAR targets cells expressing multiple antigens including but not limited to, alpha fetoprotein (AFP) and Glypican-3 (GPC3). In another embodiment, the targeting cells are hepatocellular carcinoma, fibrolamellar carcinoma, hepatoblastoma, undifferentiated embryonal sarcoma and mesenchymal hamartoma of liver, lung-squamous cell carcinoma, testicular nonseminomatous germ cell tumors, liposarcoma, ovarian and extragonadal yolk sac tumors, ovarian choriocarcinoma, teratomas, ovarian clear cell carcinoma, and placental site trophoblastic tumor.

Without wishing to be bound by theory, the present disclosure provides compound CAR engineered T cells or NK cells that target different or the same antigens offset tumor escape and provides simultaneous targeting of tumor cells.

The T or NK host cells comprising compound CAR disclosed herein is embodied in the present disclosure. The nucleotide and polypeptide constructs, sequences, host cells, and vectors of the compound CAR are considered to be part of the present disclosure and is embodied herein.

In some embodiments, the compound CAR engineered cell is administrated in combination with any chemotherapy agents currently being developed or available in the market. In some embodiments, the compound CAR engineered cell is administrated as a first line treatment for diseases including, but not limited to, hematologic malignancies, cancers, non-hematologic malignances, inflammatory diseases, infectious diseases such as HIV and HTLV and others. In one embodiment, T cells expressing the compound CAR engineered cells are co-administrated with NK cells expressing the same or different compound CAR as an adaptive immunotherapy. Compound CAR NK cells provide rapid, innate activity targeting cells while compound T cells provide relative long-lasting adaptive immune activity.

In one embodiment, the compound CAR engineered cells are administrated as a bridge to bone marrow stem transplantation for mammals, e.g. patients who are resistant to chemotherapies and are not qualified for bone marrow stem cell transplantation.

In some embodiments, the compound CAR co-expresses a transgene and releases a transgenic product, such as IL-12 in the targeted tumor lesion and further modulates the tumor microenvironment.

In one embodiment, compound CAR engineered cells are administrated to a mammal for bone marrow myeloid ablation as a part of the treatment to a disease.

In a specific embodiment, the cells expressing a compound CAR can be T cells or NK cells, administrated to a mammal, e.g. human. The presented disclosure includes a method of treating a mammal having a disorder or disease by administration of a compound CAR. The targeted cells may be cancer cells such as, or cells affected by any other disease condition, such as infectious diseases, inflammation, and autoimmune disorders.

The present disclosure is intended to include the use of fragments, mutants, or variants (e.g., modified forms) of the compound CAR or antigens that retain the ability to induce stimulation and proliferation of T/NK cells. A "form of the protein" is intended to mean a protein that shares a significant homology with at least one CAR or antigen and is capable of effecting stimulation and proliferation of T/NK cells. The terms "biologically active" or "biologically active form of the protein," as used herein, are meant to include forms of the proteins or variants that are capable of effecting anti-tumor activity of the cells.

The compositions and methods of this disclosure can be used to generate a population of T/NK cells that deliver both primary and co-stimulatory signals for use in immunotherapy in the treatment of cancer, in particular the treatment of lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, neuroblastoma, rhabdomyosarcoma, leukemia and lymphoma. The compositions and methods described in the present disclosure may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth.

1) In some embodiments, the disclosure provides a method of depleting B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with an autoimmune disease by administering to patients CAR or compound CAR T cells or NK cells. CAR targeted cells are B or plasma cells expressing one or two or all of the

antigens, BCMA, TACI and BAFF-R. The autoimmune diseases include systemic scleroderma, multiple sclerosis, psoriasis, dermatitis, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), systemic lupus erythematosus, vasculitis, rheumatoid arthritis, Sjorgen's syndrome, polymyositis, pulmonary alveolar proteinosis, granulomatosis and vasculitis, Addison's disease, antigen-antibody complex mediated diseases, and anti-glomerular basement membrane disease.

Multiple extracellular cell markers are now being studied for value as tumor-associated antigens and thus potential targets for CAR T/NK cell therapy. However, expression of these antigens on healthy tissue leading to on-target, off-tumor adverse events remains a major safety concern in addition to off-target toxicities. Furthermore, a major limitation of CAR T/NK cell therapy is in the possibility of selecting for antigen escape variants when targeting molecules non-essential to tumorigenesis. Thus, malignant cells that persist with little or no expression of the target antigens may evade CAR T/NK cells, despite their high-affinity action.

In accordance with the present disclosure, natural killer (NK) cells represent alternative cytotoxic effectors for CAR driven killing. Unlike T-cells, NK cells do not need pre-activation and constitutively exhibit cytolytic functions. Further expression of cCARs in NK cells allow NK cells to effectively kill cancers, particularly cancer cells that are resistant to NK cell treatment.

Further, NK cells are known to mediate anti-cancer effects without the risk of inducing graft-versus-host disease (GvHD).

Studies have shown an aberrant overexpression of CD123 on CD34+ CD38- AML cells, while the normal bone marrow counterpart CD34+ CD38- does not express CD123(Jordan, Upchurch et al. 2000). This population of CD123+, CD34+CD38- has been considered as LSCs as these cells are able to initiate and maintain the leukemic process in immunodeficient mice.

The number of CD34+ /CD38- /CD123+ LSCs can be used to predict the clinical outcome for AML patients. The CD34+ /CD38- /CD123+ cells, greater than 15% in AML patients, are associated with a lack of complete remission and unfavorable cytogenetic profiles. In addition, the presence of more than 1% of CD34+ /CD38- /CD123+ cells could also have a negative impact on disease-free survival and overall survival.

At the present, therapies for MDS and AML have focused on the leukemic blast cells because they are very abundant and clearly represent the most immediate problem for patients. Importantly, leukemic stem cells (LSCs) are quite different from most of the other leukemia cells ("blast" cells), and they constitute a rare subpopulation. While killing blast cells can provide short-term relief, LSCs, if not destroyed, will always re-grow, causing the patient to relapse. It is imperative that LSCs be destroyed in order to achieve durable cures for MDS disease. Unfortunately, standard drug regimens are not effective against MDS or AML LSCs. Therefore, it is critical to develop of new therapies that can specifically target both the leukemic stem cell population and the bulky leukemic population. The compound CAR disclosed in the present disclosure target both of these populations and is embodied herein.

In accordance to the present disclosure, it was surprisingly found that NK cells provide an off-the-shelf product that may be used as an allogeneic product for treatment. Thus, according to the present disclosure, cCAR cell therapy needs to be performed on a patient-specific basis as required by the current state of art. The applicants of the present disclosure have discovered a novel immunotherapy, where the patient's lymphocytes or tumor infiltrated lymphocytes need not be isolated for an effective CAR cell based therapy.

Allogeneic or autologous NK cells are expected to induce a rapid immune response but disappear relatively rapidly from the circulation due to their limited lifespan. Thus, applicants surprisingly discovered that there is reduced concern of persisting side effects using cCAR cell based therapy.

According to one aspect of the present disclosure, NK cells can be expanded and transfected with cCAR in accordance to the present disclosure. NK cells can be derived from cord blood, peripheral blood, iPS cells and embryonic stem cells. According to one aspect of the present disclosure, NK-92 cells may be expanded and transfected with cCAR. NK-92 is a continuously growing cell line that has features and characteristics of natural killer (NK) cells. NK-92 cell line is IL-2 dependent and has been proven to be safe and feasible. cCAR expressing NK-92 cells can be expanded in the serum free-medium with or without co-culturing with feeder cells. A pure population of NK-92 carrying the cCAR of interest may be obtained by sorting.

Identification of appropriate surface target antigens is a prerequisite for developing CAR T/NK cells in adaptive immune therapy.

In one aspect of the present disclosure, CD123 antigen is one of the targets for cCAR therapy. CD123, the alpha chain of the interleukin 3 receptor, is overexpressed on a variety of hematologic malignancies, including acute myeloid leukemia (AML), B-cell acute lymphoblastic leukemia (B-ALL), hairy cell leukemia, and blastic plasmocytoid dendritic neoplasms. CD123 is absent or minimally expressed on normal hematopoietic stem cells. More importantly, CD123 is expressed on a subset of leukemic cells related to leukemic stem cells (LSCs), the ablation of which is essential in preventing disease refractoriness and relapse.

In one aspect of the present disclosure, CD 33 antigen is one of the targets for cCAR therapy. CD33 is a transmembrane receptor expressed on 90% of malignant cells in acute myeloid leukemia. Thus, according to the present disclosure, CD123 and CD33 target antigens are particularly attractive from a safety standpoint.

In accordance with the present disclosure, the compound CD33CD123 CARs may be highly effective for therapeutic treatment of chronic myeloid leukemia (CML) population. In chronic myeloid leukemia (CML), there is a rare subset of cells that are CD34+CD38-. This population is considered as comprised of LSCs. Increased number of LSCs is associated with the progression of the disease. A small-molecule Bcr-Abl tyrosine kinase inhibitor (TKI) is shown to significantly improve the overall survival in CP-CML patients. However, LSCs are thought to be resistant to TKI therapy. A novel therapy targeting CML resistant LSCs is urgently needed for treatment of CML and the novel therapy is embodied in the compound CD33CD123 CAR disclosed in the present disclosure. CD123 expression is high in the CD34+CD38- population. In accordance with the present disclosure, the compound CD33CD123 CARs is highly effective for therapeutic treatment of this population.

In one embodiment of the present disclosure, leukemic cells expressing both CD123 and CD33 in the cCAR is used as a therapeutic treatment. CD33 is expressed on cells of myeloid lineage, myeloid leukemic blasts, and mature monocytes but not normal pluripotent hematopoietic stem cells. CD33 is widely expressed in leukemic cells in CML, myeloproliferative neoplasms, and MDS.

Since a significant number of patients with acute myeloid leukemia (AML) are refractory to standard chemotherapy regimens or experience disease relapse following treatment (Burnett 2012), the development of CAR T cell immunotherapy for AML has the potential to address a great clinical need. In the majority of these patients, leukemic cells express both CD123 and

CD33, giving broad clinical applicability to the compound CD33CD123 CAR disclosed herein. Thus, the present disclosure discloses a novel multiple cCAR T/NK cell construct comprising multiple CARs targeting multiple leukemia-associated antigens, thereby offsetting antigen escape mechanism, targeting leukemia cells, including leukemic stem cells, by synergistic effects of co-stimulatory domain activation,thereby providing a more potent, safe and effective therapy.

The present disclosure further discloses a compound CAR construct with enhanced potency of anti-tumor activity against cells co-expressing target antigens, and yet retains sensitivity to tumor cells only expressing one antigen. In addition, each CAR of the compound CAR includes one or two co-stimulatory domains and exhibits potent killing capability in the presence of the specific target.

In pre-clinical studies on dual specificity, trans-signaling CARs targeting solid tumors including breast cancer and epithelial ovarian cancer, a CD3ζ intracellular signaling domain, is separated from co-stimulatory domains from second generation of CARs. In other words, one CAR contains the first generation of CAR without any co-stimulatory domain, and another lacks a CD3 zeta intracellular domain. Therefore, the presence of both target antigens is required for T cell activation and potent killing. Thus, they were proposed as a way to decrease off-tumor toxicity caused by healthy tissue expression of one of the two target antigens, increasing target specificity, but at the expense of sensitivity. In one embodiment, the compound CAR is a compound CD123CD19 CAR. It has been shown that more than 90% of B-ALLs express CD123 in a subset of population. Like AML and MDS, it has been considered that a rare LSC population exists in B-ALL. Therefore, targeting both leukemic stem cell and bulky leukemic populations in accordance to the present disclosure, can be applied to B-ALLs. CD123 and CD19 surface antigens expressed in the B-ALLs may be targets as CD19 is widely expressed in different stages of B-cell lymphoid populations, in accordance with the present disclosure.

Multiple myeloma (MM) is the second most common hematologic malignancy in the US and is derived from clonal plasma cells accumulated in the bone marrow or extramedullary sites. MM is an incurable disease with a median survival of approximately 4.5 years. Anti-Myeloma CARs in Pre-clinical Development have been developed and CAR targets include CD38, CS1, and B cell maturation Antigen (BCMA). However, heterogeneity of surface antigen expression commonly occurs in malignant plasma cells, which makes it a difficult target for CARs. Malignant plasma cells also express low levels of CD19. Previously it has been shown that myeloma stem cells also express some B-cell markers including CD19. Targeting this population could be effective in the treatment of myeloma in conjunction with standard and other myeloma CAR therapies.

Multiple myeloma (MM) is a haematological malignancy with a clonal expansion of plasma cells. Despite important advances in the treatment, myeloma remains an incurable disease; thus novel therapeutic approaches are urgently needed.

CS1 (also called as CD319 or SLAMF7) is a protein encoded by the SLAMF7 gene. The surface antigen CS1 is a robust marker for normal plasma cells and myeloma cells (malignant plasma cells).

Tumour necrosis factor receptor superfamily, member 17 (TNFRSF17), also referred to as B-cell maturation antigen (BCMA) or CD269 is almost exclusively expressed at the terminal stages of plasma cells and malignant plasma cells. Its expression is absent other tissues, indicating the potential as a target for CAR T or NK cells.

Malignant plasma cells display variable degrees of antigenic heterogeneity for CD269 and CS1. A single CAR unit product targeting either CD269 or CS1 could target the majority of the cells in a bulk tumor resulting in an initial robust anti-tumor response. Subsequently residual rare non-targeted cells are expanded and cause a disease relapse. While multiple myeloma is particularly heterogeneous, this phenomena could certainty apply to other leukemias or tumors. A recent clinical trial at NIH using BCMA CAR T cells showed a promising result with a complete response in some patients with multiple myeloma. However, these patients relapsed after 17 weeks, which may be due to the antigen escape. The antigen escape is also seen in CD19 CAR and NY-ESO1 CAR T cell treatments. Thus, there is an urgent need for more effective CAR T cell treatment in order to prevent the relapse.

In one aspect of the present disclosure, BCMA and CS1 are the targets for BCMACS1 CAR therapy.

In some embodiments, a compound CAR targets cells expressing BCMA or CS1 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

In some embodiments, the present disclosure provides a compound CAR polypeptide engineered cell that targets cells expressing BCMA or CD19 antigens or both. The targeted cells may be cancer cells, such as, but not limited to, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms are selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

BAFF (B-cell-activation factor) and APRIL (a proliferation-induced ligand) are two TNF homologs that bind specifically TACI (also called as TNFRSF1 3B or CD267) and BCMA with high affinity. BAFF (also known as BLyS) binds BAFF-R and functionally involves in the enhancement of survival and proliferation of later stage of B cells. BAFF has been shown to involve some autoimmune disorders. APRIL plays an important role in the enhancement of antibody class switching. Both BAFF and APRIL have been implicated as growth and survival factors for malignant plasma cells.

Ligand-receptor interactions in the malignant plasma cells or normal plasma cells are described in Figures 77 and 79.

In some embodiments, the present disclosure provides a compound CAR engineered cell that targets cells expressing TACI or CS1 antigens or both. In another embodiment, a compound CAR engineered cell that targets cells expressing TACI or CS1 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma. The target cells may also be one or two or multiple different cell types of B cells, immature B cells, naïve B cells, centroblasts, centrocytes, memory B cells, plasmablasts, long lived plasma cells, plasma cells. These cells involve autoimmune diseases include systemic scleroderma, multiple sclerosis, psoriasis, dermatitis, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), systemic lupus erythematosus, vasculitis, rheumatoid arthritis, Sjorgen's syndrome, polymyositis, granulomatosis and vasculitis, Addison's disease, antigen-antibody complex mediated diseases, and anti-glomerular basement membrane disease.

In some embodiments, the present disclosure provides a compound CAR engineered cell that targets cells expressing BAFF-R or CS1 antigens or both. In another embodiment, a compound CAR engineered cell that targets cells expressing BAFF-R or CS1 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas,or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

Autoimmune disorders such as lupus erythematosus (SLE), pemphigus vulgaris and multiple sclerosis (MS) cause significant morbidity and disability. These diseases respond poorly to current therapies and frequent relapse during disease course is noted. B and plasma cells play a critical role in the pathogenesis of autoimmune disorders as they are the sources of autoantibody production. B and plasma cells may contribute to disease progression and relapse through antigen presentation, cytokine secretion, or antibody production. Deletion of B cells or plasma cells or both using CAR T/NK cell approaches can be a beneficial therapy.

An organ transplant represents a new life for a person and organs that can be transplanted could include the kidneys, heart, lungs, pancreas and intestine. However, many patients are unable to receive a potentially life-saving organ because of pre-existing or developing donor-specific antibody against the donor's antigens such human leukocyte antigens (HLA). Thus, patients may lose the donated organ. Currently there are few treatment options available for antibody mediated rejection, and an enormous unmet need in the field for efficacious treatment of antibody mediated rejection. Deletion of B cells or plasma cells or both using CAR T/NK cell provide a therapy for antibody-mediated rejection.

The disclosed disclosure provides compositions and methods relating to CAR engineered cells that target cells expressing CD19 or CD20 that result in the deletion of B cells but spare long-lived plasma cells in patients with antibody mediated organ rejection or autoimmune disorders including, but not limited to, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and pemphigus vulgaris and multiple sclerosis (MS). The deletion of B cells by CAR is beneficial to the reduction of disease activity.

The present disclosure also provides compositions and methods relating to CAR engineered cells that target cells expressing BCMA or BAFF-R, TACI which results in the deletion of plasma cells in patients with antibody mediated organ rejection or autoimmune disorders including, but not limited to, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and pemphigus vulgaris and multiple sclerosis (MS). The deletion of plasma cells can contribute to the reduction of disease activity.

In some embodiments, he present disclosure provides compositions and methods relating to CAR engineered cells for CARs depleting mature, memory B cells, and short, long lived plasma cells for treatment of autoimmune disorders and organ antibody-mediated organ rejection. In one embodiment, the present disclosure provides a method for depleting mature, memory B cells, and short, long lived plasma cells using one or more of the following strategies:

1) Depletion of mature, memory B cells and short, long lived plasma cells by a contacting said cells with an CAR engineered cell having a scFv against CD19 or CD20 or CD22;
2) Depletion of short- and long-lived plasma cells by contacting said cells with a CAR engineered cell having a scFv against BCMA or TACI or BAFF-R; or
3) Depletion of short- and long-lived plasma cells by contacting said cells with a CAR engineered cell having having an antigen recognition domain including BCMA or TACI or BAFF-R binding domain (BAFF or APRIL);
4) Deletion of mature, memory B cells, and short, long lived plasma cells contacting said cells with a compound CAR engineered cell targeting more than one different antigen to provide a reduction of disease activity for patients with antibody mediated organ rejection or autoimmune disorders.
5) Deletion of mature, memory B cells, and short, long lived plasma cells by contacting a CAR engineered cells that target more than one different antigen selecting from CD19, CD20, CD22, BCMA, TACI, APRIL and BAFF.

In some embodiments, a compound CAR (cCAR) targets cells expressing one or two or all of BAFF-R, BCMA, TACI and CS1 antigens.

In some embodiments, a unit of CAR in a cCAR can comprise: 1)a scFv against either BAFF-R, BCMA, TACI and CS1; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, BAFF CAR can be a unit of CAR in a cCAR comprises: 1) BCMA or TACI or BAFF-R binding domain; 2) a hinge region; 3) co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, APRIL CAR can be a unit of CAR in a cCAR comprises: 1) BCMA or TACI binding domain; 2) a hinge region; 3) co-stimulatory domain (s) and intracellular signaling domain.

In a further embodiment, BCMA or TAC1 or BAFF-R binding domain can be a part of or entire APRIL and BAFF molecules.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) a scFv against BCMA or CS1; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) a scFv against BCMA or CD19; 2) a hinge region; 3) co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) a scFv against BCMA or CD20; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) BAFF-R binding domain or a scFv against BCMA; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) BAFF-R binding domain or a scFv against CD19; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

In some embodiments, a unit of CAR in a cCAR can comprise: 1) BAFF-R binding domain or a scFv against CD20; 2) a hinge region; 3)co-stimulatory domain (s) and intracellular signaling domain.

It is unexpected that some myeloma cells are dim (weak) or negative for BCMA. To increase the sensitivity of CAR antigen recognition in myeloma cells, it is critical to target multiple antigens to cure this disease.

TACI, BCMA and BAFF-R are receptors for BAFF. It is also unexpected that some myeloma cells express TACI or BAFF-R over BCMA.

In some embodiments, the disclosure provides a method of comprising a BAFF CAR targeting a cell expressing at least one of receptors, BAFF-R, TACI and BCMA to improve therapeutic efficacy and reduce the risk of antigen disease escape.

The affinity for BAFF binding to BCMA is within the micromolar range, which is much lower than that of BAFF-R or TACI in the nanomolar range.

In some embodiments, the disclosure provides a method of generating a compound cCAR comprising BAFF and BCMA CARs to complement some of myeloma cells that cannot be eliminated by a BAFF CAR.

In further embodiments, cCAR can comprise one or two or multiple units of CAR. Each unit CAR could bear same or different hinge region and co-stimulatory domain.

In further embodiments, cCAR polypeptides two or more CAR polypeptide units. Each unit CAR could bear a different polynucleotide sequence to avoid a homologous recombination.

In some embodiments, targeting more than one different antigen can be achieved by pooled CAR engineered cells, which are generated by at least two separate CAR T or NK cells. As used herein, pooled CAR engineered cells include a population of engineered cells having more than one distinct CAR polypeptide unit. By way of example, pooled engineered cells include a population of engineered cells with a distinct CAR polypeptide and a population of engineered cells with a different and distinct CAR polypeptide. Furthermore, the pooled CAR engineered cells include engineered cells having cCAR polypeptides.

The pooled CAR T or NK cells can be completed by the following steps:

1) Generate at least two separate constructs of CARs targeting different antigens;
2) Transduce individual construct to T or NK cells and expand them ex vivo in a standard medium;
3) Pool different expanded T or NK cells at an appropriate ratio; and
4) Administer pooled CAR T or NK cells to a subject.

In the alternative, the different engineered cells may be individual expanded and independently or sequentially administered.

In further embodiments, the target antigens can include at least one of this group, but not limited to, ROR1, PSMA, MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-

ESO, FAP, ErbB, c-Met, MART-1, CD30, EGFRvIII, immunoglobin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138. The target antigens can also include viral or fungal antigens, such as E6 and E7 from the human papillomavirus (HPV) or EBV (Epstein Barr virus) antigens.

In some embodiments, a cCAR engineered cell targets a cell expressing either CD19 or CD20 antigens or both of them. In another embedment, a cCAR engineered cells target a cell expressing either CD19 or CD22 antigens or both of them. The targeted cells are normal B cells associated with autoimmune disorders or cancer cells such as B-cell lymphomas or leukemias.

Acute graft-versus-host disease (GVHD) remains the most important cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation. In the effector phase of GVHD, T cell receptor (TCR), a heterodimer of alpha and beta chains, is expressed on the surface of T cells, TCR recognizes some antigens on the HLA molecule on host cells, enhances T cell proliferation, and releases cytotoxic agents that cause the damage on host cells. TCR gene inactivation is efficient at preventing potential graft-versus-host reaction. The inactivation of TCRs can result in the prevention of the TCR recognition of alloantigen and thus GVHD.

The role of CD45 on NK cells is quite different from that of T cells. NK cells from CD45-defficient mice have normal cytotoxic activity against the prototypic tumor cell line, Yac-1. In addition, CD45-deficient NK cells proliferate normally and respond to IL-15 and IL-21. Therefore, CD45 disruption or deletion would not affect the NK cell killing and proliferation.

The present disclosure includes methods of permanent deletion of CD45 in a T or NK cell with subsequent stable introduction of CD45-specific CARs. As a result, the engineered T cells display the desired properties of redirected specificity for CD45 without causing self-killing and response to presentation of antigen. In a further embodiment, the engineered T cells may have efficacy as an off-the-shelf therapy for treating malignancies or other diseases.

The present disclosure relates to a method where T-cells are engineered to allow proliferation when TCR signaling is reduced or lost through the inactivation or deletion of endogenous CD45. The reduction or loss of TCR signaling could result in the prevention of GVHD.

In a further embodiment, T cells reducing or losing the TCR signaling by the inactivation of CD45 could be used as an “off the shelf “therapeutic product.

The present disclosure includes methods of modified T or NK cells, which comprises: (a) modifying T or NK cells by inactivating CD45; (b) expanding these modified cells; (c) sorting modified T or NK cells, which do not express CD45; (d) introducing CD45CAR.
In embodiments, the CD45CAR gene encodes a chimeric antigen receptor (CAR), wherein the CAR comprises at least one of an antigen recognition domain, a hinge region, a transmembrane domain, and T cell activation domains, and the antigen recognition domain is redirected against CD45 surface antigen present on a cell. The antigen recognition domain includes a monoclonal antibody or a polyclonal antibody directed against CD45 antigen. The antigen recognition domain includes the binding portion or a variable region of a monoclonal or a polyclonal antibody.

The present disclosure includes methods of improving CD45CAR T/NK cell expansion, persistency and anti-tumor activity by co-expressing secretory IL-15/IL-15sushi complexes. In a further embodiment, engineered CD45CAR T/NK cells comprise secretory IL-15/IL-15sushi complexes, which can promote expansion of specific CAR T/NK cells, and promote infiltrate of innate immune cells to the tumor sites resulting in tumor destruction.

The present disclosure provides an alternative strategy in which engineered CD45 CAR T cells receive not only costimulation through the CD28 pathway but also co-express the 4-1BB ligand (CD137L), which provide high therapeutic efficacy.

In some embodiments, the modified T cells are obtained from allogeneic donors and used as an “off-the-shelf product”.

Targeting CD45 using CAR T or NK cells may cause self-killing as T and NK cells express this surface antigen. To overcome this drawback, the present disclosure provides engineered cells that are deficient in CD45. As used herein, an engineered cell is deficient for a particular gene when expression of the gene is reduced or eliminated. Reduction or elimination of expression can be accomplished by any genetic method known in the art. In one example, the CD45 gene may be inactivated by using engineered CRISPR/Cas9 system, zinc finger nuclease (ZFNs) and TALE nucleases (TALENs) and meganucleases. The loss of CD45 in T or NK cells is further transduced with CARs targeting neoplasms expressing CD45.

The disclosure includes methods for eliminating or reducing abnormal or malignant cells in bone marrow, blood and organs. In, B and some embodiments, malignant cells expressing CD45 are present in patients with acute leukemia,, chronic leukemia T cell lymphomas, myeloid leukemia, Acute lymphoblastic lymphoma or leukemia, primary effusion lymphoma, Reticulohistiocytoma , transient myeloproliferative disorder of Down's syndrome, lymphocyte predominant Hodgkin's lymphoma, myeloid leukemia or sarcoma, dendrocytoma, histiocytic sarcoma , Giant cell tumor of tendon sheath, interdigitating dendritic cell sarcoma, post-transplant lymphoproliferative disorders, etc.

Hematopoietic stem cell transplantation (HSCT) has been widely used for the treatment of hematologic malignancies or non-hematologic diseases. Non-hematologic diseases include genetic disorders and immunodeficiencies and autoimmune disorders. Genetic disorders include, not limited to, sickle cell disease, thalassemia and lysosomal storage diseases. Before stem cell transplant, patients are required to undergo a conditional therapy to deplete hematopoietic stem/progenitor cells in the bone marrow niches to promote the donor stem cell engraftment and proliferation. High doses of chemotherapies and total body irradiation are used for conditional therapies, which cause severe toxicity and long-term complications particularly in non-hematopoietic organs such as severe intestinal mucositis. In addition, conventional conditional therapies could destruct bone marrow niches resulting hematopoietic cell recovery. The safety concerns represent a major obstacle in effort to expand HSCT to a variety of non-hematologic diseases. CD45 is expressed only on hematopoietic cells and targeting CD45 should minimize the toxicity to non-hematopoietic organs.

In some embodiments, CD45CAR cells are used to make space in the bone marrow for bone marrow stem cell transplant by removing hematopoietic cells, at the same time removing leukemic/lymphoma cells or immunologic cells capable of graft rejection.

In some embodiments, CD45CAR engineered cells are used to deplete hematopoietic stem/progenitor cells while the architecture and vasculature of the bone marrow are preserved, in contrast to the disruptive effects of total body irradiation on these tissues. Preservation of the architecture and vasculature of the bone marrow allows faster hematopoietic recovery after transient CD45CAR treatment.

In a further embodiment, CD45CAR cells may be used for pre-treatment of patients before their undergoing a bone marrow transplant to receive stem cells. In a further embodiment, CD45CAR can be used as myeloblative conditioning regimens for hematopoietic stem cell transplantation.

In a preferred embedment, CD45CAR engineered cell therapy is transient for allowing faster recovery of bone marrow and peripheral hematopoietic cells. Transient therapy may be accomplished by using short lived engineered cells or providing an engineered cell having the suicide system as described herein.

In some embodiments, the present disclosure comprises a method of selectively depleting or ablating an endogenous hematopoietic stem/progenitor population, where the endogenous hematopoietic stem/progenitor cells expressing CD45, by contacting said cells with CD45CAR engineered cell that specifically targets CD45 expressing hematopoietic stem/progenitor cells.

In some embodiment, CD45CAR cells are utilized for treating or preventing a residual disease after stem cell transplant and/or chemotherapy.

In some embodiments, the CD45CAR is part of an expressing gene or a cassette. In a preferred embodiment, the expressing gene or the cassette includes an accessory gene or a tag or a part thereof, in addition to the CD45CAR. The accessory gene may be an inducible suicide gene or a part thereof, including, but not limited to, caspase 9 gene, thymidine kinase, cytosine deaminase (CD) or cytochrome P450. The "suicide gene" ablation approach improves safety of the gene therapy and kills cells only when activated by a specific compound or a molecule. In some embodiments, the suicide gene is inducible and is activated using a specific chemical inducer of dimerization (CID).

In some embodiments, the safety switch can include the accessory tags are a c-myc tag, CD20, CD52 (Campath), truncated EGFR gene (EGFRt) or a part or a combination thereof. The accessory tag may be used as a nonimmunogenic selection tool or for tracking markers.
In some embodiments, safety switch can include a 24-residue peptide that corresponds to residues 254–277 of the RSV F glycoprotein A2 strain (NSELLSLINDMPITNDQKKLMSNN).
In some embodiments, a safety switch can include the amino acid sequence of TNF α bound by monoclonal anti-TNF α drugs.

IL-15 and its receptor in enhancing CAR T and NK cell functions

Recent studies have demonstrated that T cell persistence correlates well with CAR T cell therapeutic efficacy. Recent trials demonstrate that potent and persistent antitumor activity can be generated by an infused small number of CAR T cells indicative that quality rather than quantity of infused products is more important in contributing to the anti-tumor activity. Interleukin (IL)-15 is a cytokine that promotes the development and hemostasis of lymphocytes. Increased levels of IL-15 promote T-cell proliferation and enhance T cell effector response. Data from recent studies have shown that IL-15 is crucial for the generation and maintenance of memory CD8 T-cells, one of the key factors associated with anti-tumor activity. IL-15 binds the IL-15 receptor alpha chain (also called IL-15RA or RA) contributing to IL-15-mediated effects such as T-cell survival, proliferation and memory T cell generation.

IL-15RA binds the βγ complex in the surface of T cells and IL-15 signals by binding with this IL-15RA/ βγ complex on the cell surface of T cells and other types of cells.

Transfection of IL-15 alone does not significantly influence T-cell function, transfection of IL-15/1IL-15RA allows T cells to survive and proliferate autonomously.

The efficacy of administered IL-15 alone may be limited by the availability of free IL-15RA and its short half-life. Administration of soluble IL-15/RA complexes greatly enhanced Il-15 half-life and bioavailability in vivo. Therefore, treatment of mice with this complex, but not with IL-15 alone results in robust proliferation and maintenance of memory CD8 T cells and NK cells. A portion of the extracellular region of IL-15RA called sushi domain (IL-15sushi) is required for its binding of IL-15 (WEI et al., J. Immunol., vol.167(1), p:277-282, 2001). The IL-15/sushi fusion protein is also called IL-15/IL-15sushi containing the linker is more potent than IL-15 and soluble IL-15RA (IL-15sushi) alone. The combination of IL-15/RA (membrane bound form) or IL-15/sushi (soluble form) can maximize IL-15 activity. The membrane bound form, IL-15/RA would not release of free IL-15 by keeping IL-15 bound to IL-15RA on the surface of transduced cells.

In the present disclosure, it is unexpected to find that the soluble IL-15/IL-15sushi released from transduced cells are able to promote the expansion of transduced cells and their neighbor cells, and further stimulate them against tumor.

The present disclosure provides an engineered cell having both CAR and IL-15/IL-15sushi or IL-15/RA in a single construct. In some embodiments, the disclosure includes methods to generate higher virus titer and use a stronger promoter to drive both CAR and IL-15/RA or IL-15/IL-15sushi.

In some embodiments, the present disclosure provides an engineered cell having: (1) a CAR targeting an antigen including, but not limited to, CD4, CD2, CD3, CD7, CD5, CD45, CD20, CD19, CD33, CD123, CS1, and B-cell mature antigen (BCMA); and (2) IL-15; (3) IL-15RA (membrane bound) or sushi (IL-15/IL-15sushi. In further embodiments, CAR comprises chimeric antigen receptor, one or more of co-stimulatory endodomains including, but not limited to, CD28, CD2, 4-1BB, 4-1BBL (CD137L), B7-2/CD86, CTLA-4, B7-H1/PD-L1, ICOS, B7-H2, PD-1, B7-H3, PD-L2, B7-H4, CD40 Ligand/TNFSF5, DPPIV/CD26, DAP12 and OX40, and intracellular domain of CD3 zeta chain. In further embodiments, a strong promoter can be, but is not limited to, SFFV. CARs, IL-15/RA or sushi and inducible suicide gene ("safety switch"), or a combination can be assembled on a vector, such as a lentiviral vector, adenoviral vector and retroviral vector or a plasmid. The introduction of "safety switch" could significantly increase safety profile, and limit on-target or off-tumor toxicities of CARs.

In one embodiment, the engineered cell includes a CD2 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.102), and corresponding polynucleotide (SEQ ID NO. 101). Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD2 CAR provides long-term durable remissions in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD3 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.104), and corresponding polynucleotide (SEQ ID NO. 103). Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD3 CAR provides long-term durable remissions in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate cells against target cancer cells.

In one embodiment, the engineered cell includes a CD7 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.106), and corresponding polynucleotide (SEQ ID NO. 105). In some embodiments, the present disclosure provides a method of providing long-term durable remission in a cancer patient by administering a CD7 CAR engineered cell that co-expresses IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD7 CAR provides long-term durable remissions in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD5 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO.107), and corresponding polynucleotide (SEQ ID NO. 108). In some embodiments, the present disclosure provides a method of providing long-term durable remission in a cancer patient by administering a CD5 CAR engineered cell that co-expresses IL-15/IL-15sushi to a patient in need thereof. Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi with a CD5 CAR provides long-term durable remissions in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

CAR targeting CD4+CD25+ regulatory T cells

Regulatory T cells (Tregs), also called suppressor T cells, are a sub-population of T cells which regulate the immune system and maintain tolerance of self-antigens. Tregs constitute about 1-5% of total CD4+ T cells in blood with diverse clinical applications in transplantation, allergy, asthma, infectious diseases, graft versus host disease (GVHD), and autoimmunity. The biomarkers for Tregs are CD4, Foxp3 and CD25. Tregs are considered to be derived from Naïve CD4 cells.

In cancers, Tregs play an important role in suppressing tumor immunity and hindering the body's innate ability to control the growth of cancerous cells.

Tregs expand in patients with cancer and are often enriched in the tumor microenvironment. Tregs cab infiltrate tumors and limit antitumor immunity as well. Depletion of Treg can render mice capable of rejecting tumors that normally grow progressively.

Depletion of Tregs using antibodies targeting CD25 results in partial reduction of Tregs but anti-tumor activity is limited. A high-level of Treg depletion is required for a profound anti-tumor effect. In addition, there is a significant issue concerning specificity, as Tregs share CD25 expression with activated CD4+ and CD8+ lymphocytes as well as activated NK cells. In general, Tregs are very difficult to effectively discern from effector T cells and NK cells, making them difficult to study.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD4 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD25 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO.92 with a CD45 leader sequence and corresponding polynucleotide of SEQ ID NO. 91.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a CD4 antigen recognition domain and second chimeric antigen receptor polypeptide having a CD25 antigen recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO.94 with a CD8a leader sequence and corresponding polynucleotide of SEQ ID NO. 93.

Specific Embodiments for T-regulatory cells

T lymphocytes (T cells) are a subtype of white blood cells that play a key role in cell-mediated immunity. T cells are divided into CD4 and CD8 cells. Natural killer cells (NK cells) are a type of cytotoxic cells critical to the innate immunity.

T-regulatory cells (Tregs) are a type of CD4+ cells mediating immune tolerance and suppression and are distinguished from helper T cells. Tregs express CD4, CD25 and Foxp3 (CD4CD25+ regulatory T cells).

Tregs are enriched in the tumor microenvironment and considered to be important for hindering antitumor immune responses and promoting cancer cell tolerance. Increased numbers of infiltrating Tregs in tumors have been associated poor survival in a variety of cancers including hematologic malignancies and solid tumors.

Tregs appear to be preferentially trafficked to the tumor microenvironment and play a critical role of immunosuppression (Ethan M. Shevach et al, Annual Review of Immunology, Vol. 18: 423-449, 2000).

A number of different methods are employed to delete Tregs for cancer treatments by targeting CD25, resulting in a partial reduction of Tregs. However, this could be problematic as: (1) CD25 is also expressed in activated CD4, CD8 and NK cells. CD25 expression can be seen in activated B cells, macrophages, osteoblasts, some thymocytes, some myeloid precursors and some oligodendocytes. (2) a very high-level of Treg depletion is required for efficacy (Xingrui Li et al, Eur. J. Immunol. 2010. 40: 3325–3335).

The CAR design is to redirect patient or donor immune cells against a specific "target" antigen in a major-histocompatibility complex (MHC) – independent manner. The CAR protein construct usually includes a number of modular components or regions integral to function. The module for "target" recognition is the extracellular single-chain variable fragment (scFv). This component is derived from a monoclonal antibody with specific direction against a carefully selected target antigen. A hinge region of appropriate length tandem to the scFv conveys mobility of the scFv region to allow for optimal binding to the target protein. The transmembrane domain region serves as a conduit between the extracellular binding regions and co-activation domains' such as CD28 and/or 4-1BB. The final module includes the CD3 zeta intracellular signaling domain.

The present disclosure provides a method for a novel Treg CAR (also called CD4zetaCD25CAR or C4-25z CAR) construct targeting a cell co-expressing CD4 and CD25. The Treg CAR depletes Tregs specifically while sparing most of cells that do not co-express CD4 and CD25.

In some embodiments, T cell activation could be achieved upon simultaneous engagement of two scFv molecules against CD4 and CD25 in a Treg CAR. In a further embodiment, both T cell activation and co-stimulation signals are provided using two distinct/separate chimeric antigen receptor polypeptides.

In some embodiments, a TregCAR includes (1) a first chimeric antigen receptor polypeptide unit comprising a first signal peptide, a first antigen recognition domain, a first hinge region, a first transmembrane domain, and an intracellular signaling domain; and (2) a second chimeric antigen receptor polypeptide unit comprising a second signal peptide, a second antigen recognition domain, a second hinge region, a second transmembrane domain, and a co-stimulatory domain (s). Both the first chimeric antigen receptor polypeptide unit and the second chimeric engineered polypeptide unit are expressed on a single polypeptide molecule, and wherein an amino acid sequence comprising a high efficiency cleavage site is disposed between the first chimeric antigen receptor polypeptide unit and the second chimeric antigen receptor polypeptide unit.

In some embodiments, the Treg CAR potentiates the lysis activity of a cell co-expressing CD4 and CD25 while minimizing a cell bearing only CD4 or CD25 antigen.

In some embodiments, the nucleotide sequence of the first chimeric antigen receptor polypeptide unit is different from the second chimeric engineered polypeptide unit in order to avoid a homologous recombination

In some embodiments, the high efficiency cleavage site in Treg CAR is P2A.

In some embodiments, the target of the first antigen recognition domain is either CD4 or CD25 and the target of the second antigen recognition domain is either CD4 or CD25; wherein the first antigen recognition domain is different than the second antigen recognition domain.

In one embodiment, the antigen recognition domain includes the binding portion or variable region of a monoclonal or polyclonal antibody directed against (selective for) the target. In a further embodiment, the target antigen is CD4 or CD25.

In some embodiments, the T or NK cell comprising Treg CARs targeting different or same antigens.

In some embodiments, the T or NK cell comprises Treg CARs targeting Tregs expressing CD4 and CD25 while sparing most of cells, which do not co-express CD4 and CD25.

In some embodiments, the T or NK cell comprises Treg CARs depleting Tegs.

In some embodiments, the present disclosure provides a method of generation of Treg CAR useful for treating or preventing a CD4+CD25 +Foxp3+ T regulatory cell (Treg) related disease in a subject is provided. In a further embodiment, the diseases treated with Treg CAR include, but not limiting to, cancers.

In some embodiments, the present disclosure provides a method of generation of Treg CAR useful for treating or preventing a CD4+CD25 +Foxp3+ T regulatory cell (Treg) related Cancers including, but not limited, hepatocellular carcinoma, fibrolamellar carcinoma, hepatoblastoma, undifferentiated embryonal sarcoma and mesenchymal hamartoma of liver, lung-squamous cell carcinoma, testicular nonseminomatous germ cell tumors, liposarcoma, ovarian and extragonadal yolk sac tumors, ovarian choriocarcinoma, teratomas, ovarian clear cell carcinoma, placental site trophoblastic tumor, lymphoma and leukemia.

In some embodiments, the present disclosure provides a method of generation of Treg CAR useful for inhibiting the growth of a tumor in a subject is provided.

In some embodiments, the Treg CAR is administrated in combination with any chemotherapy agents currently being developed or available in the market. In some embodiments, the Treg CAR is administrated as a first line treatment for diseases including, but not limited to, hematologic malignancies and cancers.

In some embodiments, the cells expressing a Treg CAR are co-administrated with immunomodulatory drugs, such as, but not limited to, CTLA-4 and PD-1/PD-L1 blockades, or cytokines, such as IL-2, IL-15 or IL-15/IL-15sushi or IL-15/RA, and IL-12 or inhibitors of colony stimulating factor-1 receptor (CSF1R) for better therapeutic outcomes.

In some embodiments, the cells expressing a Treg CAR are co-administrated with other immunomodulatory drugs or CAR-expressing cells to provide synergistic effects in a subject.

In a specific embodiment, the cells expressing a Treg CAR can be T cells or NK cells, administrated to a mammal, e.g. human.

In some embodiments, the Treg CAR is used in immunotherapy in the treatment of cancers. The cancers may be selected from the group consisting of lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, cervical cancer, head or neck cancer, stomach cancer, liver cancer, neuroblastoma, rhabdomyosarcoma, leukemia and lymphoma. The compositions and methods described in the present disclosure may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth.

To achieve enhanced safety profile or therapeutic index, the Treg CAR of the present disclosure may be constructed as a transient RNA-modified "biodegradable" version or derivatives, or a combination thereof. The RNA-modified CARs of the present disclosure may be electroporated into T cells or NK cells. The expression of the Treg CAR may be gradually diminished over few days.

A method for treating cancers using Treg CAR in a subject is embodied in the present disclosure. The method comprises:

(1) Obtaining T/NK cells from a subject or donor(s).

(2) Culturing the lymphocytes/T cells or NK cells.

(3) Introducing an Treg CAR construct into the cultured T cells or NK cells.

(4) Expanding Treg CAR T cells or NK cells

(5) Treating the subject by administering a therapeutically effective amount thereto.

The *ex vivo* expansion of tumor-infiltrating lymphocytes (TILs) are successfully used in the current adoptive cell therapy. In one embodiment, TILs are harvested and successfully expanded *ex vivo*.

In some embodiments, TILs can be obtained from a tumor tissue sample and expanding the number of TILs. Treg CAR T or NK cells were used to co-culture with TILs to deplete Treg population to enhance TIL responses to cancers, which is valuable to the disease therapies.

In some embodiments, CD4CAR bear a set of CAR body including an antigen recognition domain, a hinge region, a co-stimulatory domain (s) and an intracellular domain (CD3 zeta chain). In a further embodiment, CD4CAR depletes Tregs, and then enhances T-cell responses to cancer cells and improves therapeutic outcomes of anti-tumor activity.

In some embodiments, CD25CAR bear a set of CAR body including an antigen recognition domain, a hinge region, a co-stimulatory domain (s) and an intracellular domain (CD3 zeta chain). In a further embodiment, CD25CAR depletes Tregs, and then enhances T-cell responses to cancer cells and improves therapeutic outcomes of anti-tumor activity.

The present disclosure may be better understood with reference to the examples, set forth below. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure

Administration of any of the engineered cells described herein may be supplemented with the co-administration of a CAR enhancing agent. Examples of CAR enhancing agents include immunomodulatory drugs that enhance CAR activities, such as, but not limited to agents that target immune-checkpoint pathways, inhibitors of colony stimulating factor-1 receptor (CSF1R) for better therapeutic outcomes. Agents that target immune-checkpoint pathways include small molecules, proteins, or antibodies that bind inhibitory immune receptors CTLA-4, PD-1, and PD-L1, and result in CTLA-4 and PD-1/PD-L1 blockades. As used herein, enhancing agent includes enhancer as described above.

As used herein, "patient" includes mammals. The mammal referred to herein can be any mammal. As used herein, the term "mammal" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order

Logomorpha, such as rabbits. The mammals may be from the order Carnivora, including Felines (cats) and Canines (dogs). The mammals may be from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). The mammals may be of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). Preferably, the mammal is a human. A patient includes subject.

In certain embodiments, the patient is a human 0 to 6 months old, 6 to 12 months old, 1 to 5 years old, 5 to 10 years old, 5 to 12 years old, 10 to 15 years old, 15 to 20 years old, 13 to 19 years old, 20 to 25 years old, 25 to 30 years old, 20 to 65 years old, 30 to 35 years old, 35 to 40 years old, 40 to 45 years old, 45 to 50 years old, 50 to 55 years old, 55 to 60 years old, 60 to 65 years old, 65 to 70 years old, 70 to 75 years old, 75 to 80 years old, 80 to 85 years old, 85 to 90 years old, 90 to 95 years old or 95 to 100 years old.

The terms "effective amount" and "therapeutically effective amount" of an engineered cell as used herein mean a sufficient amount of the engineered cell to provide the desired therapeutic or physiological or effect or outcome. Such, an effect or outcome includes reduction or amelioration of the symptoms of cellular disease. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what an appropriate "effective amount" is. The exact amount required will vary from patient to patient, depending on the species, age and general condition of the patient, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation. Generally, the engineered cell or engineered cells is/are given in an amount and under conditions sufficient to reduce proliferation of target cells.

Following administration of the delivery system for treating, inhibiting, or preventing a cancer, the efficacy of the therapeutic engineered cell can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a therapeutic engineered cell delivered in conjunction with the chemo-adjuvant is efficacious in treating or inhibiting a cancer in a patient by observing that the therapeutic engineered cell reduces the cancer cell load or prevents a further increase in cancer cell load. Cancer cell loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of certain cancer cell nucleic acids or identification of certain cancer cell markers in the blood using, for example, an antibody assay to detect the presence of the markers in a sample (e.g., but not limited to, blood) from a subject or patient, or by measuring the level of circulating cancer cell antibody levels in the patient.

Throughout this specification, quantities are defined by ranges, and by lower and upper boundaries of ranges. Each lower boundary can be combined with each upper boundary to define a range. The lower and upper boundaries should each be taken as a separate element.

Reference throughout this specification to "one embodiment," "an embodiment," "one example," or "an example" means that a particular feature, structure or characteristic described in connection with the embodiment or example is included in at least one embodiment of the present embodiments. Thus, appearances of the phrases "in one embodiment," "in an embodiment," "one example," or "an example" in various places throughout this specification are not necessarily all referring to the same embodiment or example. Furthermore, the particular features, structures or characteristics may be combined in any suitable combinations and/or sub-combinations in one or more embodiments or examples. In addition, it is appreciated that the figures provided herewith are for explanation purposes to persons ordinarily skilled in the art and that the drawings are not necessarily drawn to scale.

As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such process, article, or apparatus.

Further, unless expressly stated to the contrary, "or" refers to an inclusive "or" and not to an exclusive "or". For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

Additionally, any examples or illustrations given herein are not to be regarded in any way as restrictions on, limits to, or express definitions of any term or terms with which they are utilized. Instead, these examples or illustrations are to be regarded as being described with respect to one particular embodiment and as being illustrative only. Those of ordinary skill in the art will appreciate that any term or terms with which these examples or illustrations are utilized will encompass other embodiments which may or may not be given therewith or elsewhere in the specification and all such embodiments are intended to be included within the scope of that term or terms. Language designating such nonlimiting examples and illustrations includes, but is not limited to: "for example," "for instance," "e.g.," and "in one embodiment."

In this specification, groups of various parameters containing multiple members are described. Within a group of parameters, each member may be combined with any one or more of the other members to make additional sub-groups. For example, if the members of a group are a, b, c, d, and e, additional sub-groups specifically contemplated include any one, two, three, or four of the members, e.g., a and c; a, d, and e; b, c, d, and e; etc.

As used herein, a XXXX antigen recognition domain is a polypeptide that is selective for XXXX. "XXXX" denotes the target as discussed herein and above. For example, a CD38 antigen recognition domain is a polypeptide that is specific for CD38.

As used herein, CDXCAR refers to a chimeric antigen receptor having a CDX antigen recognition domain.

As used herein, a CAR engineered cell is an engineered cell as described herein that includes a chimeric antigen receptor polypeptide. By way of example, a CD45 engineered cell is an engineered cell having a CD45 chimeric antigen receptor polypeptide as disclosed herein.

As used herein, a compound CAR (cCAR) engineered cell is an engineered cell as described herein that includes at least two distinct chimeric antigen receptor polypeptides. By way of example, a CD19CD22 compound CAR engineered cell is an engineered cell as described herein that includes a first chimeric antigen receptor polypeptide having a CD19 antigen recognition domain, and a second chimeric antigen receptor polypeptide having a CD22 antigen recognition domain.

The present disclosure may be better understood with reference to the examples, set forth below. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure.

EXAMPLES

Engineered cCAR targets cells expressing CD33 or CD123 or both

Generation of compound CAR (cCAR)

The construction of the CD33CD123 cCAR follows the schematic in Figure 1 and 2A. It includes SFFV (spleen focus-forming virus) promoter that drives the expression of the functional compound CAR (cCAR) bearing two different units of CARs. The antigen receptor head, a scFv (single-chain variable fragment) nucleotide sequence of the anti-CD33 and anti-CD123. A P2A peptide derived from picornavirus is utilized due to the highly efficient mechanism of its self-cleaving dynamics for bicistronic genetic constructs. The self-cleaving P2A peptide serves to link the two independent units of CARs, CD33CAR, and CD123CAR together during expression. The advantages of this approach over an internal ribosomal entry site (IRES), which is commonly used in the literature, include its small size and high cleavage efficiency between two unit proteins upstream and downstream of the 2A peptide. In addition, the use of self-cleaving P2A peptide can avoid a problem of differences in expression levels between gene before and after IRES when IRES is applied.

The modular unit, CD33CAR includes the CD33 scFv domain, a CD8a hinge region, a CD8a transmembrane domain, 4-BB co-stimulatory domain and an intracellular domain of CD3 zeta chain. The second modular CAR, CD123CAR bears the same hinge, transmembrane and intracellular signaling domains as CD33CAR but different scFv, and co-stimulatory domains. The CD33 CAR recognizes its corresponding antigen and the CD123 CAR binds to its corresponding antigen. The hinge region was designed such that sequences where disulfide interactions are avoided. Different co-stimulatory domains, 4-BB and CD28 were used. The CD33CD123 compound CAR was subcloned into a lentiviral plasmid.

Generation of a high-efficiency compound CAR (cCAR)

Compound CAR lentivirus was generated by transfection of HEK-293 FT cells with Lipofectamine 2000 according to manufacturer's directions, except with 2x the vector DNA due to a large size of insert, in order to increase titer as shown in Figure 2. After about 12-16 hours incubation, media containing Lipofectamine was removed and replaced with DMEM containing 10% FBS, 20 mM HEPES, 1 mM sodium pyruvate and 1 mM sodium butyrate. After about 24 hours, the supernatant was harvested and refrigerated, and replaced with fresh media. After about another 24 hours, this was collected and combined with the previous supernatant, and filtered through a 0.45 μM filter disc. Supernatant was split into aliquots, flash frozen with liquid nitrogen and stored at -80°C. HEK-293 FT cells were harvested, stored frozen, and lysed for subsequent electrophoresis and Western blotting (Figure 2B).

PB (peripheral blood) or CB (human umbilical cord blood) buffy coat cells were activated 2 days with anti-CD3 antibody and IL-2. cCAR lentiviral supernatant was spinoculated onto retronectin-coated multiwell plates. Activated T cells were transduced in multiple wells with lentiviral supernatant at a low concentration of about 0.3 x 10^6 cells/mL to increase transduction efficiency (Figure 3).

Following the first overnight transduction, cells were added directly to a second virus-coated plate for a second transduction without washing, unless the cells did not look healthy. Following the second overnight transduction, cells were washed, combined and incubated in tissue culture treated plates. CAR T cells were allowed to expand for up to about 5 days prior to co-culture killing assays. After about 3 days of incubation, cells were incubated with goat anti-mouse F(Ab')2 or goat IgG (isotype) antibodies conjugated with biotin, washed and followed by incubation with streptavidin-PE and conjugated anti-human CD3. After washing and suspension in 2% formalin, cells were then analyzed by flow cytometry to determine percent transduction efficiency.

Characterization of the CD33CD123 cCAR

Transfected CD33CD123 cCAR HEK293T cells were subjected to Western blot analysis in order to confirm the compound construct. Immunoblot with an anti-CD3ζ monoclonal antibody showed bands of predicted size for the compoundCAR CD3ζ fusion protein (Figure 2B). Importantly, two distinct bands of similar intensity were observed on the blot signaling the successful high cleavage action of the P2A peptide as expected. No CD3ζ expression was seen for the GFP control vector as expected. The surface expression of scFv was also tested on HEK 293 cells (Figure 2C) and primary T cells (Figure 2C).

The compound CD33CD23CAR lentivirus was tested for transduction efficiency in the HEK293 cell line and analyzed by flow cytometry (Beckman Coulter) (Figure 2C). Flow cytometry showed that about 67% of HEK cells expressed CD33CD123 CARs. Human peripheral blood (PB) is often used for autologous T cell therapy. Human PB buffy coat cells were activated with anti-CD3 antibody and IL-2, and transduced with either CD4CAR or control (GFP) lentiviruses. After transduction, flow cytometry analysis showed that about 22% of T-cells expressed the CD33CD123CAR (Figure 2C).

CD33CD123 cCAR T-cells derived from Umbilical Cord Blood (UCB) and Peripheral Blood (PB) specifically kill CD33-expressing tumor cells

CD33CD123 cCAR T cells or GFP T cells (control) were incubated with target cells at ratios ranging from 0.5:1 from 50:1, preferably, at about 2:1, 5:1, 10:1, 20:1, 50:1, at about 100,000, 200,000, 500,000, about 1 million, or 2 million effector cells to about 50,000, 100,000, 200,000 target cells, respectively) in about 1-2 mL T cell culture media, without IL-2 for about 24h. Target cells were leukemic cell lines and leukemia cells from a patient with leukemia. After about 24 hours of co-culture, cells were stained with mouse anti-human CD33, CD123, CD34 and CD3 antibodies.

CD33CD123 cCAR T cells expressing the CD33CAR and CD123 CAR were generated and tested for anti-leukemic functions using the HL60 and KG-1a cell lines. The HL60 cell line is a promyelocytic leukemia cell line highly enriched for CD33. About100% of its cell population is CD33+ with a small subset (<10%) of it being dim CD123+. In culture, this cell line was tested to determine the effectiveness of the CD33CD123 CAR with an emphasis on targeting CD33-expressing leukemic cells. Additionally, due to the strong expression of CD33 in HL60, it is CD33CD123 cCAR action may be profound. Indeed, during 24h co-culture conditions with various ratios of effector to target cells, the CD33CD123 cCAR exhibited significant leukemic cell killing properties (Figure 4). CB-derived CD33CD123 CAR T-cells were first tested for their ability to kill HL60 cells. At about 24h incubation and low effector:target (E:T) ratios ranging from about 0.5:1 to 50:1, preferably, 1:1 to about 5:1, more preferably about 2:1 to 4:1, CD33CD123 CAR cells eliminated about 55% of the CD33 expressing HL60 cells when compared to GFP control. At a ratio of about 5:1, the killing action rose to about 82%.

CD33CD123 CAR derived from peripheral blood mononuclear cells (PBMCs) were co-cultured with the myelogenous leukemia cell line KG1a, which also expresses about 100% CD33 at moderate levels compared to HL60 and 50-80% CD123. KG1a is, therefore, a relatively dual target cell population that is double positive for the antigens targeted by the CD33CD123 CAR. At about 24 hours of incubation and low effector:target (E:T) ratios ranging from about 0.5:1 to

50:1 were used. While at a low E:T ratio of about 2:1, the CD33CD123 CAR exhibited modest anti-leukemic activity about 26%, an increase in E:T ratio to 10:1 resulted in a killing of KG1a of about 62% compared to GFP control (Figure 5), signaling that the intensity of the CD33 marker may be an indicator for the efficacy of killing with HL60 presenting strongly and harnessing more CAR action than KG1a . These experiments provide evidence for the function of the whole CD33CD123 CAR against its relevant antigen presenting cell populations.

Additional compound CAR, CD33CD123-BB cCAR has been generated (data not shown). This compound CAR comprises two independent units of CARs, CD33 and CD123. The first CAR comprises scFv binding to CD33 and the second CAR bears a different scFv recognizing CD123. Both CARs contain the same hinge region, transmembrane, co-stimulatory and intracellular domains. CD33CD123-BB cCAR lentiviruses were produced and their killing ability was tested in KG-1a cells. As shown in Figure 5, there was substantial killing at a ratio of about 10:1 but it is less potent than that of CD33CD123 cCAR.

CD33CD123 cCAR possesses activity against patient samples expressing CD33 and/or CD123

In addition to cell line experiments, studies were also conducted on patient samples in order to test the function of each individual CAR unit. An aggressive acute myeloid leukemia (AML), AML-9 was used for testing efficacy of the CD33CD123 cCAR. Due to the heterogeneity of the patient cell population, which includes multiple cell types in the AML-9 sample, leukemic blasts were gated with CD34 and CD33, as they were positive for these two markers. The depletion of this CD33+CD34+ population of leukemic cells was observed to be 48% over the GFP control at a ratio of CAR T cell:target cell (Figure 6).

Leukemic cells that were CD123 positive and CD33 negative were also tested. For this purpose, human B cell acute lympoblastic leukemia (B-ALL) sample, Sp-BM-B6 was chosen. All leukemic blasts in this sample were CD34+CD33 –, and more than about 50% positive for CD123. Depletion of the CD34+ leukemic cell population by CD33CD123 cCAR T cells was about 86% as compared to that of the GFP control (Figure 7). Based on the cell line and human sample studies, our data strongly suggest that the compound CD33CD123 CAR is able to target leukemic cells expressing CD33 or CD123 or both.

CD33CD123 cCAR NK cells targeting leukemia cells expressing CD33 or CD23 or both

Natural killer (NK) cells are CD56+ CD3- and can efficiently kill infected and tumor cells like CD8+ T cells. Unlike CD8+ T cells, NK cells launch cytotoxicity against tumors without the requirement of activation to kill cells. NK cells are safer effector cells, as they may avoid the potentially lethal complications of cytokine storms. However, the use of either CD33 or CD123 or both CAR NK cells in killing leukemias is entirely unexplored.

Production of CD33CD123 cCAR NK cells

NK-92 cells were transduced with CD33CD123 CAR lentiviral supernatant in two consecutive overnight transductions with a change of retronectin- and virus-coated plates in between. The transduced cells were expanded for 3 or 4 days and then analyzed by flow cytometry for CAR expression. Cells were harvested and incubated with goat anti-mouse F(Ab')2 at about 1:250 for about 30 minutes. Cells were washed, suspended and stained with streptavidin-PE for about 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry. NK-92 cells expressing CD33CD123 cCAR were then labeled as above and sorted on FACSAria, with the top 0.2% of F(Ab')2-expressing cells collected and cultured. Subsequent labeling of sorted, expanded cells showed about 89% of NK-92 cell positive for anti-mouse F(Ab')2 (Figure 8).

CD33CD123 cCAR NK cells efficiently lyse or eliminate leukemic cells

First, we tested the function of CD33CD123 cCAR NK-92 cells by assessing their ability to kill a HL-60 cancerous cell line in co-culture. Virtually all HL-60 cells highly express CD33 but CD123 expression in this cell line is only less than 10% (weak). Therefore, it is likely that the killing ability of CD33CD123cCAR is dependent on the ability for cCAR to properly targeting CD33.

CD33CD123 cCAR NK-92 cells were co-cultured with the HL-60 cells for about 24 hours in NK cell media without IL-2. After the incubation, the CD33CD123 cCAR NK-92 cells were labeled and compared to a control of non-CAR, GFP NK-92 cells. Dramatic killing of HL-60 cells by CD33CD123 cCAR NK-92 cells was observed as compared to the control, GFP NK-92 cells. Moreover, the killing ability of CD33CD123 cCAR NK-92 cells was dose-dependent, with a about 10 to 1 ratio of about 100% compared to the control (Figure 9 and 11).

A second co-culture experiment using the myeloid leukemia cell line was performed using KG1a, which expresses CD33 in all cells but at a moderate level compared to that of HL-

60. The CD123 antigen is expressed in about 50-80% of KG1a cells. The experimental design was similar to the first experiment of the HL-60 killing assay described above, with the same incubation time, effector:cancer cell ratios and GFP NK-92 cell controls. Results show a remarkable killing of KG1a cells by CD33CD123 cCAR NK-92 cells in a dose-dependent manner as compared to the GFP NK-92 cell control. At a ratio of effector: target of 10:1, killing of KG1a cells by CD33CD123 cCAR NK-92 cells was about 85% as compared to that of GFP control (Figure 10 and 11).

Analysis of KG1a cells showed two different populations, CD33+CD123- and CD33+CD123-. Figure 11 showed a dose dependent increase in cell killing seen in both populations. Surprisingly, the double positive population showed a higher efficient killing for each increased ratio, suggesting a possible synergistic effect of two modular CARs of CD33 and CD123 (Figure 12).

We also generated engineered CD33CD123 CAR T cells received not only costimulation through the CD28 but also co-express the 4-1BB ligand (4-1BBL or CD137L) in a single construct, which provide the better therapeutic efficacy (Figure 13A). T-cells derived from peripheral blood from healthy donors were transduced with the CD33CD123-4-1BBL-2G construct in 6-well plates incubated with 2 ml of virus supernatant. CAR expression was assayed with F(ab)' labeling for surface expression of the CAR protein and subsequently underwent FACS analysis. Transduced cells were compared to control T-cells labeled at the same time. Expression was determined and transduced population encircled on plot 1 day after the end of transduction period. The surface CD33CD123-41BBL-2G CAR expression on T cells was approximately 60% (Figure 13B). CD33CD123 CAR improves functional activates when 4-1BBL was included in the construct.

An enhancer, IL-15/IL-15sushi was also included in CD33CD123 CAR construct as an alternative approach. Both compound CAR, CD33CD123 and IL-15/IL-15sushi were in a single construct (Figure 14). IL-15/IL-15sushi is able to promote the expansion of CAR T/NK cells, and infiltrate of innate immune cells to the tumor site, which could result in better tumor destruction

Engineered cCAR targets cells expressing: 1)CD19 or CD20 or both; 2) CD19 or CD20 or both; 3)CD19 or CD138 or both

Generation of CD19CD20, CD19CD22, and CD19CD138 cCARs

The three cCARs have been generated (Figure 15) using the similar strategy to that of the CD33CD123 cCAR described above.

Generation of the second generation compound CARs (CD19CD20 and CD19CD22)

The construction of the compound CAR (cCAR) follows the schematic in Figure 16A. It comprises of SFFV (spleen focus-forming virus) promoter that drives the expression of the functional cCAR bearing two different units of CARs. The first CAR is the complete L8-CD19-2G CAR (using human CD8a leader sequence, called L-8), linked to the complete second CAR (either CD20-2G or CD22-2G) by a high efficient P2A self-cleaving peptide, derived from picornavirus. The entire sequence is in frame as to result in initially one large fusion protein which is cleaved in half prior to cell surface expression. This method ensures equal expression levels of both CARs. The cCAR DNA molecules were subsequently sub-cloned into the same lentiviral plasmid as above.

Transduced T cells efficiently express cCARs

Lentiviral vector supernatant was generated from HEK293T cells transfected with either CD19CD20-2G or CD19CD22-2G vector construct or control vector. After collection of lentiviral supernatant was collected, cells were harvested, lysed, and electrophoresed prior to Western blot transfer. Incubation of blot membrane with anti-human CD3zeta antibody resulted in two distinct bands representing each CAR unit after cleavage; the CD19CAR is slightly larger than the CD20 or CD22 CAR units (Figure 18B). Next, peripheral blood mononuclear buffy coat cells were activated for three days and transduced with concentrated CD19CD20-2G, CD19CD22-2G or control vector lentiviral supernatant on non-tissue culture plates coated with retronectin. The transduction procedure was repeated 24 hours after the first transduction. CAR expression on the T-cell surface was demonstrated three days after transduction by staining transduced T cells with goat anti-mouse F(Ab')2 antibody and mouse anti-human CD3.

Figure 16 shows that 26.9% of cells transduced with concentrated L8-CD19CD20-2G lentiviral supernatant and 35.6% of T cells transduced with concentrated CD19CD22-2G lentiviral supernatant were positive for both F(Ab')2 and CD3 as determined by flow cytometry, when compared to the control transduction.

Transduced T cells express CD19CD22-2G at different levels based on leader sequences

We then determined the leader sequence that would result in the highest level of cell surface expression of cCAR, three constructs were made that incorporated leader sequences for human CD8a (L8), CD45 (L45), and colony stimulating factor (CSF) (Figure 17). Following transduction of human peripheral blood T cells with lentiviral supernatant generated from each of these vectors, transduction efficiency for the T cells was determined using F(Ab')2 antibody as above. The L8 leader sequence again led to the highest transduction efficiency (43.8%), followed by L45 (9.8%) and CSF (1.3%). (Figure 17). This shows that the optimal design of a compound CAR, like a single CAR, depends in part on the leader sequence for surface CAR expression.

Concentration of lentiviral supernatant can lead to higher transduction efficiency for cCARs

To improve CAR efficiency in transduced T cells, lentiviral supernatant for CD19CD20-2G and CD19CD22-2G was centrifuged at 3,880 x g for 24 hours. The resulting viral pellets were suspended in media at one third their original volume, making them 3x concentrated. This concentrate was used to transduce activated T cells in the same volume as non-concentrated virus. Figure 10a shows that CAR efficiency for T cells transduced with 3x concentrated CD19CD22-2G lentiviral supernatant nearly tripled, while CAR efficiency for T cells transduced with 2.5x concentrated CD19CD20-2G lentiviral supernatant increased nearly 10-fold (Figure 18). This illustrates the importance of concentrating lentiviral vector for the longer cCAR constructs.

cCAR CAR T cells specifically target CD19-expressing tumor cell lines

T cell co-culture killing assays were performed to determine the ability of L8-CD19CD22-2G and L8-CD19CD20-2G CAR T cells to effectively lyse the CD19+ cell lines, SP53 and JeKo-1 (both mantle cell lymphoma lines). Briefly, each target cell line was pre-labeled with CMTMR membrane dye, and then co-cultured with either vector control, L8-CD19CD22-2G or L8-CD19CD20-2G CAR T cells at ratios of 2:1 and 5:1 effector:target cells (200,000 or 500,000 effector cells to 100,000 target cells, in 1 mL T cell media without serum or IL-2). After overnight incubation, cells were labeled with anti-human CD3-PerCp and CD19-

APC for 30 minutes, washed, and suspended in 2% formalin for analysis by flow cytometry. The L8-CD19CD22-2G CAR T cells demonstrated robust lysis of tumor cells (Figure 19), lysing 53.4% and 93% of the SP53 cells at 2:1 and 5:1 ratios, respectively. At the same ratios, the L8-CD19CD22-2G CAR T cells were able to lyse 69% and 97.3% of the JeKo-1 cells (Figure 20).

cCAR CAR T cells eliminate CD19+ cells from AML and B-ALL patient samples

Studies were again conducted using patient samples. Buffy coat fractions of these primary cells were pre-labeled with CMTMR and co-cultured with either vector control, or L8-CD19CD22-2G CAR T cells in the same manner as the tumor cell lines. L8-CD19CD22-2G CAR T cells lysed 54.3% and 77% of the AML patient cells with aberrant expression of CD19 at 2:1 and 5:1 ratios, respectively, in an overnight co-culture, and lysed 84.3% of the B-ALL tumor cells at a 1:1 ratio in a four day co-culture with 2.5% FBS and IL-2 added to the media (Figures 21, 22A). As these AML patient cells only comprised 65% blasts and 75% of them expressed CD19, it was likely that L8-CD19CD22-2G CAR T cells were able to eliminate the entire CD19 positive blast population.

cCAR CAR T cells lyse K562 cells expressing CD22.

An artificial K562 expressing CD22 cell line (K562xp22) via transduction into wild-type K562 cells was generated. Subsequently, we tested the anti-tumor properties of the CD19CD22 cCAR to target the minor CD22$^+$ population of the K562 cells. A co-culture experiment at 1:1 ratio (effective: target) show a modest significant cytotoxic effect on K562 expressing CD22 population compared to the control. Cytotoxicity results remain consistent with other numbers reported for anti-tumor activity against artificial antigen presenting cell lines (Figure 22B).

Engineered cCAR targets cells expressing BCMA and CS1

Generation of cCAR including BCMA CS1 cCAR and BCMA CD19 cCAR for treatment of multiple myeloma or autoimmune disorders

Pre-clinical studies have been developed for cCARs to target surface antigens including CD38, CS1, CD138, B cell maturation antigen (BCMA) and CD38. CD19 CAR has also demonstrated some efficacy for the treatment of multiple myeloma in a phase I clinical trial. However, given that the heterogeneity of surface antigen expression commonly occurs in malignant plasma cells, it is unlikely that a single target is sufficient to eliminate this disease. BCMA CS1 cCAR, BCMA CD19 cCAR, BCMA CD38 cCAR and BCMA CD138 cCAR were generated and the experimental design was similar to that of CD33CD123 cCAR as described above.

Generation of cCAR including BCMA CS1 cCAR (BC1cCAR) for treatment of multiple myeloma or autoimmune disorders

Generation and characterization of BCMA-CS1 cCAR (BC1cCAR) construct

We have observed that transduction of compound CAR constructs in general lack high efficiency gene transfer rates compared to single antigen CARs. Whether due to construct size or metabolic stress on effector cells or other factors, optimization of a transduction schema for compound CARs remain necessary. We compared 3 different protocols for transductions and major differences included whether incubation occurs within viral supernatant, transduction procedure frequency, and final cell density numbers per treatment. Method 1 was a 2x transduction for 24 hours each time and uses retronectin coated plates incubated with virus first, aspirated, then incubated with T-cells to a final concentration of 0.5 x 10^6 cells/ml. Method 2 used the same viral retronectin procedure, however, it exchanged the 2nd transduction period for continued incubation to a total of 48 hours of incubation with a final cell density of 0.3 x 10^6 cells/ml. Method 2 revised uses an incubation scheme where viral supernatant was directly incubated with cells for 48 hours on a retronectin coated plate at a cell density of 0.3 x 10^6 cells/ml (Figure 23).

Transduction protocol optimizations correlate to improved BC1cCAR surface expression

BC1cCAR's modular design consists of an anti-CD269 (BCMA) single-chain variable fragment (scFv) region fused to an anti-CD319 (CS1) scFv by a self-cleaving P2A peptide, CD8-derived hinge (H) and transmembrane (TM) regions, and 4-1BB co-activation domains linked to the CD3ζ signaling domain (Figure 24A). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BC1cCAR molecule on the T-cell surface. T-cells isolated from human peripheral blood buffy coats were transduced with BC1cCAR lentivirus after 2 days of activation. According to the different transduction schemas above, various transduction efficiencies are reported for each technique (Figure 24B). We find that, in general, cells incubated with viral supernatant for 48 hours at reduced cell densities (0.3 x 10^6 cells/ml) support the highest gene-transfer efficiencies (Figure 24B). Thus, as we improve our transduction schemes, we observe a correspondingly higher rate of gene transfer (Figure 24C).

Transfected BC1cCAR HEK293T cells were subjected to Western blot analysis in order to confirm the compound construct. Immunoblot with an anti-CD3ζ monoclonal antibody showed bands of predicted size for the compound CAR CD3ζ fusion protein (Figure 24D). Importantly, two distinct bands of similar intensity were observed on the blot signaling the successful high cleavage action of the P2A peptide as expected. No CD3ζ expression was seen for the GFP control vector as expected.

BC1cCAR T-cells specifically lyse BCMA^{+} and CS1^{+} myeloma cell lines

To assess the cytotoxicity ability of BC1cCAR T-cells, we conducted co-culture assays against myeloma cell lines: MM1S (BMCA^{+} CS1$^{+)}$, RPMI-8226 (BCMA^{+} CS1^{-}), and U266 (BCMA^{+} CS1^{dim}). The ability of the BC1cCAR T-cells to lyse the target cells was quantified by flow cytometry analysis, and target cells were stained with Cytotracker dye (CMTMR). In 24 hour co-cultures, the BC1cCAR exhibited virtually complete lysis of MM1S cells, with over 90% depletion of target cells at an E:T ratio of 2:1 and over 95% depletion at an E:T of 5:1 (Figures 25A and 25C). In RPMI-8226 cells, BC1cCAR lysed over 70% of BCMA^{+} target cells at an E:T ratio of 2:1, and over 75% at an E:T of 5:1(Figure 25A and 25C). In 24 hour co-culture with U266 target cells, BC1cCAR lysed 80% of BCMA^{+} U266 cells at an E:T ratio of 2:1, reaching saturation (Figure 25B and 25C). As the myeloma cell lines are all mostly BCMA^{+}, these results suggest that largely BCMA targeting by BC1cCAR T-cells promotes effective cell lysis.

BC1cCAR T-cells specifically target BCMA^{+} and CS1^{+} populations in primary patient myeloma samples

We conducted co-cultures using BC1cCAR T cells against primary tumor cells to evaluate their ability to kill diverse primary myeloma cell types. Flow cytometry analysis of the MM10-G primary sample reveal distinct and consistent BCMA^{+} and CS1^{+} population subsets. MM7-G sample shows a complete BCMA^{+} CS1^{+} phenotype while MM11-G exhibits a noisy BCMA^{dim}CS1^{dim} phenotype likely attributable to its property of being a bone-marrow aspirate.

BC1cCAR T-cells show robust dose-dependent ablation of the MM7-G primary patient sample, with over 75% lysis at an E:T ratio of 5:1, increasing to over 85% at 10:1 (Figure 26A).

BC1cCAR also show targeted and specific lysis ability, by significantly ablating both the BCMA^{+} CS1^{+} and the BCMA^{-}CS1^{+} population subsets in MM10-G co-cultures. At an E:T ratio of 2:1, BC1cCAR T-cells ablate over 60% of the BCMA^{+} CS1^{+} population, and 70% of the CS1^{+} only population (Figure 26B). At an E:T ratio of 5:1, the ablation of CS1^{+} only population increases to 80% (Figure 26B). Against the MM11-G (Figure 26C), BC1cCAR T-cells were also able to demonstrate cytotoxic activity in a dose-dependent manner as well (Figure 26C). In summary, BC1cCAR T cells exhibit robust anti-tumor activity against both myeloma cell lines and primary tumor cells presenting different combinations of BCMA and CS1 (Figure 26D)

Functional evaluation of BC1cCAR antigenic specific activity

To assess and characterize the biological properties of the BC1cCAR in terms of its antigenic targeting, we established a model that would allow us to test the BC1cCAR scFv functionality independently. Using a CML cell line negative for myeloma markers (K562), we established a stable CS1 expressing K562 cell line (CS1xpK562) by transducing CS1 cDNA into K562 cells and subsequently promoting stable expression through puromycin selection (Figure 27A). To test the BCMA scFv functionality, we obtained a BCMA expressing K562 cell line (BCMAxpK562) from the NIH (Kochenderfer Lab). After we confirmed the independent expression of each antigen for each antigen expressing cell line (Figure 27A), we used them in co-culture experiments to determine BC1cCAR T targeting functionality.

In short-term cultures (<24 hrs), BC1cCAR T-cells exhibited cytotoxic activity against BCMAxpK562 cells while showing no effect against wild-type K562 cells (Figure 27B). Next, short-term cultures against CS1xpK562 cells show similar responses against CS1 expressing target cells. Furthermore, BC1cCAR T-cells appeared to have a stronger cytotoxic effect than a CS1-specific CAR against CS1xpK562 cells (Figure 27B). Further validation of the anti-CS1 activity was performed on CS1^{dim} expressing NK-92 cells where cytotoxicity exhibited as a dose-dependent effect (Figure 27B).

To model antigen escape in potential clinical scenarios, we conducted combined co-culture experiments. We mixed BCMAxpK562 and CS1xpK562 in 1:1 ratios and looked for evidence of antigen residual populations that could lead to relapse in real world scenarios. Co-cultures were carried out over 48 hours to ensure antigen depletion. Next, histograms were constructed that represents populations of T-cells and target tumor cells. The numbers in each histogram plot represents the residual gated target tumor population. We found that compared to control T-cells, a BCMA-specific CAR and a CS1-specific CAR were able to deplete or have profound cytotoxic effects against their respective populations. However, a CS1-specific CAR left a significant residual $BCMA^{+}$ population, whereas a BCMA-specific CAR achieved a high degree of cytotoxicity but still left a small but definite $CS1^{+}$ population (Figure 27C). In contrast, the BC1cCAR T-cells effectively depleted both target populations (Figure 27C). We speculate that residual tumor populations possessing 1 antigen may lead to relapse in patients that have undergone treatment using only a single antigen-specific CAR.

Since normal bone marrow expresses a small subset of plasma cells that can express CS1, there are concerns that a CS1 directed CAR could be adversely cytotoxic. While the CS1 population in bone marrow is indeed affected by the BC1cCAR in a dose-dependent manner (Figure 27D), the CS1 subset itself is small.

BC1cCAR T-cells exhibit persistency and sequential killing ability even with tumor re-challenge

We next investigated the ability of BC1cCAR T-cells to kill tumor cells in a sequential manner under unfavorable microenvironments caused by cell lysis, debris, and tumor re-challenge. Using the scheme in Figure 28A, we conducted long-term co-cultures using MM1S cells as a model myeloma tumor and periodically re-challenged BC1cCAR T-cells and other CAR constructs with fresh MM1S to simulate tumor expansion or relapse. The initial co-culture condition was done at an E:T ratio of 1:1. With no exogenous cytokines, we find that depletion of target antigens is accomplished by all CAR cells after 48 hours, with significant clustering and T-cell proliferation (Figure 28B). In contrast, control T-cells show no response and proliferation yielding a tumor population that has now expanded by twice its initial number. After re-challenging all treatment wells with fresh MM1S cells we find that all CARs still retain a high degree of cytotoxicity even without exogenous cytokines. By 108 hours, the newly inputted MM1S cells have been virtually depleted by both BCMA-CAR and the BC1cCAR with significant cytotoxicity still observed from the CS1-CAR. However, at this stage, flow cytometry show a diminished CS1-CAR population and a relative growth in the tumor antigen population to ~17% (Figure 28C), suggesting that the CS1-CAR T-cells may be faltering. At this time point, the control T-cells have been completely overgrown by tumor cells. All CAR tumor-lysis and cytotoxicity stopped after 168 hours, however, BCMA-CAR and BC1cCAR still show detectable minority T-cell populations while control T-cells and CS1-CAR T-cells have all virtually disappeared (data not shown).

BC1cCAR T-cells exhibit significant control and reduction of tumor in *vivo*

In order to evaluate the *in vivo* anti-tumor activity of BC1cCAR T-cells, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with luciferase-expressing MM1S cells, a multiple myeloma cell line, to induce measurable tumor formation. Three days following tumor cell injection, mice were intravenously injected with 5 x 10^6 BC1cCAR T-cells or control GFP cells in a single dose. On days 3, 6, 8 and 11, mice were injected subcutaneously with RediJect D-Luciferin (Perkin Elmer) and subjected to IVIS imaging to measure tumor burden (Figure 29A). Average light intensity measured for the BC1cCAR T-cell injected mice was compared to that of GFP control mice in order to determine the control of tumor growth by BC1cCAR treatment (Figure 29B). Unpaired T test analysis revealed an extremely significant difference ($P<0.01$) between the two groups by Day 6 with less light intensity and thus less tumor burden in the BC1cCAR T-cell injected group compared to control (Figure 29B). Next, we compared mouse survival across the two groups (Figure 29C). All of the BC1cCAR T-cell injected mice survived past day 50 and over a quarter remained past day 65. P-value between control and treated mice is 0.0011 based on Long-Rank Mantel-Cox test. The percent survival of control T-cell injected mice started to decrease shortly by Day 50 and were deceased by Day 55. In summary, these *in vivo* data indicate that BC1cCAR T-cells significantly reduce tumor burden and prolong survival in MM1S-injected NSG mice when compared to control cells.

BC1cCAR T-cells exhibit improved cytotoxic effect in a mixed antigen xenogeneic mouse model.

To evaluate the dual targeting nature of the compound CAR that may preclude antigen escape, we designed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with luciferase-expressing K562 cells expressing either stably transduced BCMA or CS1. BCMA and CS1 expressing K562 cells were further sorted for expression following puromycin selection and established as stable homogenous single antigen populations. BCMA and CS1 expressing K562 cells were then mixed at a 4:1 ratio respectively before injection to model potential antigen escape. Three days following tumor cell injection, mice were intravenously injected with a course 15 x 10^6 control T-cells, BCMA-specific CAR, or BC1cCAR T-cells. Two control mice died as a result of injection procedure as a result of technical issues during T-cell infusion and cell aggregation. On days 3, 7, 10 and 12, mice were injected subcutaneously with RediJect D-Luciferin (Perkin Elmer) and subjected to IVIS imaging to visualize tumor burden (Figure 19D). Average light intensity (signifying tumor burden) measured for the BC1cCAR T-cell injected mice was compared to that of a BCMA-specific CAR and GFP control injected mice in order to determine the control of tumor growth by treatment (Figure 29D). By day 10, both the BCMA-specific CAR and BC1cCAR T-cells exhibited over 47% tumor reduction compared to control. However, there was a 6% difference in the tumor burden reduction in favor of the BC1cCAR as early as day 10 on the dorsal side of the mice. By day 12, there was a 17% difference in tumor reduction in favor of BC1cCAR (Figure 19D and E) on the dorsal side. This number approaches the percentage of CS1-K562 cells injected (20%) versus BCMA-K562 (80%). It is likely the result of CS1 expressing K562 cells surviving and proliferating as a model for antigen escape. In summary, these *in vivo* data indicate that BC1cCAR T-cells appeared to show improved tumor burden control for multiple antigen populations.

BC1cCAR transduction and validation of anti-tumor properties in NK cells

To further evaluate the robustness of BC1cCAR in different settings, we transduced the BC1cCAR construct into a model NK cell line, NK-92. The construct was successfully able to be transduced via lentiviral incubation for 48 hours into NK-92 cells and resulted in a surface expression profile of 62.1% after gene-transfer (Figure 30A and 30B). Maintenance of NK-92 cells at densities of 0.3-0.5 x 10^6 cells/ml resulted in stable populations. To test for BC1cCAR anti-tumor activity in *vitro,* we conducted co-cultures against myeloma cell lines and a primary patient sample. The BC1cCAR approached 80% lysis against MM1S, U266, and RPMI-8226 cell lines at E:T ratios of 5:1 in culture. It also successfully lysed over 60% of the primary MM7-G tumor (Figure 31A and 31B). These results are similar in terms of comparability with BC1cCAR T-cells. Next, we assayed the antigen specificity of the BC1cCAR in its ability to lyse $BCMA^+$ or $CS1^+$ cell independently. Similar assays were carried out for BC1cCAR T-cells (Figure 27). In 4 hour cultures with either BCMA expressing K562 (BCMAxpK562) or CS1 expressing K562 (CS1xpK562 cells), we find that the BC1cCAR NK cells are able to have cytotoxic effects against either population (Figure 31B).

Generation of cCAR including BCMA CD19 or BCMA CD19b for treatment of plasma cell myeloma or autoimmune disorders

Generation and characterization of BCMA-CD19 cCAR or BCMA-CD19b cCAR construct

BC1cCAR's modular design consists of an anti-CD269 (BCMA) single-chain variable fragment (scFv) region fused to an anti-CD319 (CS1) scFv by a self-cleaving P2A peptide, CD8-derived hinge (H) and transmembrane (TM) regions, and 4-1BB co-activation domains linked to the CD3ζ signaling domain (Figure 35). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BC1cCAR molecule on the T-cell surface and anti-tumor activities in vitro and in vivo using a similar approach described above.

Each of units of CAR in the BCMA CD19 CAR will be tested for its anti-plasma cell or anti-B cell activity. We found that the BCMA CAR unit was able to potently lyse any $BCMA^+$ population. We first conducted co-cultures against the dual BCMA CS1 positive plasma cell line MM1S and used a CS1 CAR as a secondary measure for robustness. We observed that both BCMA and CS1 specific CARs were able to lyse MM1S targets at high efficiency (Figure 36A). Next, we cultured the BCMA CAR and CS1 CAR against a majority $BCMA^+$ primary myeloma sample MM7-G. We find that, with regard to BCMA expression, the BCMA CAR was able to virtually deplete all $BCMA^+$ cells. In contrast, the CS1 CAR left a residual $BCMA^+$ population

(Figure 36B). These results suggest that a BCMA CAR achieves high potency and specificity in its cytotoxic effect.

We next tested the CD19 CAR unit for its anti-B cell activity. The single-chain variable fragment (scFv) nucleotide sequences of the anti-CD19 molecule was used for two different constructs, CD19-2G and CD19b-BB CAR. To improve signal transduction, the CD19CAR was designed with 4-1BB co-activation domain fused to the CD3zeta signaling domain, making it a second generation CAR (Figure 37A). CD19-targeting second generation CAR T-cells have previously been used in clinical trials. For efficient expression of the CD19CAR molecule on the T cell surface, a strong spleen focus-forming virus promoter (SFFV) was used and the leader sequence of CD8a was incorporated in the construct. For comparison, CD19CAR constructs using the leader sequences of CD45, CSF, human albumin (HA) or IL-2 were also made. The anti-CD19 scFv was separated from the intracellular signaling domains by CD-8 derived hinge (H) and transmembrane (TM) regions (Figure 37). The CD19CAR DNA molecules, with different leader sequences or different scFv sequences, were also subsequently sub-cloned into a lentiviral plasmid.

Transduced T cells efficiently express CD19CAR-Lentiviral vector supernatant was generated from HEK293T cells transfected with CD19-2G vector construct and control vector. After collection of lentiviral supernatant was collected, cells were harvested, lysed, and electrophoresed prior to Western blot transfer. Incubation of blot membrane with anti-human CD3zeta antibody resulted in a ~56 kDa band in the lane containing lysate from cells transfected with CD19-2G, the predicted size for the expressed fusion protein (Figure 37B). Next, peripheral blood mononuclear buffy coat cells were activated for three days and transduced with L8-CD19-2G, or control vector lentiviral supernatant on non-tissue culture plates coated with retronectin. The transduction procedure was repeated 24 hours after the first transduction. CAR expression on the T-cell surface was demonstrated three days after transduction by staining transduced T cells with goat anti-mouse Fab antibody and mouse anti-human CD3. Figure 37C shows that 19.8% of cells transduced with the L8-CD19-2G virus were positive for both F(Ab')2 and CD3 as determined by flow cytometry, when compared to the control transduction.

Transduced T cells express CD19-2G at different levels based on leader sequences-To determine the leader sequence that would result in the highest level of cell surface expression of CD19-2G CAR, several constructs were made that incorporated leader sequences for human CD8a (L8), CD45 (L45), colony stimulating factor (CSF), human albumin (HA), and IL2 (Figure 38A). Following transduction of human peripheral blood T cells with lentiviral supernatant generated from each of these vectors, transduction efficiency for the T cells was determined using F(Ab')2 antibody as above. Only the CD19-2G construct incorporating the L8 leader sequence resulted in any appreciable cell surface expression of CAR (32.5%), while the L45 leader sequence resulted in only 3.3% transduction efficiency, and CSF, HA and IL2 were below 1% (Figure 38B). This shows that the optimal design of CD19-2G CAR depends in part on the leader sequence used.

Transduced T cells express CD19-2G at different levels based on scFv sequences

To determine the scFv sequence of CD19 that would result in the highest level of cell surface expression of CD19-2G CAR, two different sequences were used in the design of CD19-2G CAR (Figure 39A), CD19 and CD19b. Both used the L8 leader sequence. Following transduction of human peripheral blood T cells with lentiviral supernatant generated from each of these vectors under the same condition, transduction efficiency for the T cells was determined using F(Ab')2 antibody as above. The CD19-2G construct resulted in 18.2% CAR cells, but the CD19b-BB-2G construct resulted in 54.7% CAR efficiency (Figure 39b). This shows that the optimal design of CD19-2G CAR also depends in part on the sequence of the scFv used.

CD19-2G and CD19b-BB-2G CAR T cells specifically target CD19-expressing cell lines

T cell co-culture killing assays were performed to determine the ability of CD19-2G and CD19b-BB-2G CAR T cells to effectively lyse the CD19+ cell lines, SP53 and JeKo-1 (both mantle cell lymphoma lines). Briefly, each target cell line was pre-labeled with CMTMR membrane dye, and then co-cultured with either vector control, L8-CD19-2G or L8-CD19b-BB-2G CAR T cells at ratios of 2:1 and 5:1 effector:target cells (200,000 or 500,000 effector cells to 100,000 target cells, in 1 mL T cell media without serum or IL-2). After overnight incubation, cells were labeled with anti-human CD3-PerCp and CD19-APC for 30 minutes, washed, and suspended in 2% formalin for analysis by flow cytometry. Both CD19-2G and CD19b 2G CAR T cells displayed robust lysis of B cell lines, SP53 and Jeko-1 (Figure 41).

CD19-2G and CD19b-BB-2G CAR T cells eliminate CD19+ cells from AML and B-ALL patient samples

Studies were also conducted using patient samples. Two patients with CD19+cells were used: one diagnosed as AML (aberrant expression of CD19), and one with B-ALL, were used in the study. The patients' blood contained 26.4% and 90% of CD19+ cells, respectively (Figures 41A, 42A). Buffy coat fractions of these primary cells were pre-labeled with CMTMR and co-cultured with either vector control, L8-CD19-2G or L8-CD19b-BB-2G T cells in the same manner and ratios as the tumor cell lines. Both L-8-CD19-2G CAR and L-8-CD19b-2G cells were able to complete eliminate the target cells expressing CD19 (Figure 42 and 43).

Viral titers generally decrease as the size of insert increases and the sequence of CD19b scFv provided a higher titer for CD19b CAR (Figure 39). Therefore, CD19b scFv was used to generate the compound BCMA CD19b CAR (Figure 44). BCMA CD19b CAR.

An alternative CAR design for myeloma and plasma cells

We designed a ligand expressing CAR that binds to various B-cell activation factor receptors. While it seems a logical leap to design CARs for any potential antigen or ligand factor that can be bound to a tumor population, technical troubleshooting in CAR technology is still a high and persistent barrier. Not all CAR constructs are able to achieve consistent or sufficient surface expression as a result of undefined molecular interactions or design problems. We were able to achieve surface expression of CD45 leader sequence BAFF-CAR with a CD28 intracellular signaling domain of around 21% (Figure 45A). However, BAFF-CARs with alternate leader sequences from CD8 or CSF did not achieve any meaningful expression (Figure 45B). Yet another factor was observed when CAR design was considered. We designed BAFF-CAR constructs using the 4-1BBL ligand binding domain as a supportive stimulatory pathway in one case. In another, we added an IL-15/IL-15sushi armor expressing arm to the construct. The CD8 leader sequence paired with the 4-1BBL or the IL-15/IL-15sushi both achieved higher surface expression than the CSF leader sequence in both cases (Figure 45C).

Anti-plasma cell properties of the BAFF-CARs

We characterized the biological properties of the various BAFF-CARs by culturing them with either plasma cell myeloma cells (MM1S) or mantle (MCL) cells (SP53) that all express a component of the plasma cell marker CD138 to which BAFF is a ligand bound complex. The L45-BAFF-28 CAR was able to lyse MM1S tumor cells after 48 hours at an E:T ratio of 3:1 approaching 60% (Figure 46). Furthermore, the L8-BAFF-28 IL-15/IL-15sushi and L8-BAFF-28 4-1BBL CARs were also able to achieve comparable degrees of cytotoxicity (Figure 47A, 47C). Co-culture with the B cell mantle cell line SP53 show a limited effect with around 25% cytotoxicity observed for the L8-BAFF-28 IL-15/IL-15 CAR only (Figure 47).

CD45 CAR therapy

Three pairs of sgRNA are designed with CHOPCHOP to target the gene of interest. Gene-specific sgRNAs are then cloned into the lentiviral vector (Lenti U6-sgRNA-SFFV-Cas9-puro-wpre) expressing a human Cas9 and puromycin resistance genes linked with an E2A self-cleaving linker. The U6-sgRNA cassette is in front of the Cas9 element. The expression of sgRNA and Cas9puro is driven by the U6 promoter and SFFV promoter, respectively (Figure 48).

The following gene-specific sgRNA sequences were used and constructed,

In a non-limiting embodiment of the disclosure, exemplary gene-specific sgRNAs have been designed and constructed as set forth below:

CD45 sgRNA construct::

Lenti-U6-sgCD45a-SFFV-Cas9-puro	GTGGTGTGAGTAGGTAA
Lenti-U6-sgCD45b-SFFV-Cas9-puro	GAGTTTTGCATTGGCGG
Lenti-U6-sgCD45c-SFFV-Cas9-puro	GAGGGTGGTTGTCAATG

Figure 49A shows steps of generation of CD45 CAR T or NK cell targeting hematologic malignancies.

CRISPR/Cas nucleases target to CD45 on NK cells

Lentiviruses carried gene-specific sgRNAs were used to transduce NK-92 cells. The loss of CD45 expression on NK-92 cells was determined by flow cytometry analysis. The CD45 negative population of NK-92 cells was sorted and expanded (Figure 49B). The sorted and expanded CD45 negative NK-92 cells were used to generate CD45CAR NK cells. The resulting CD45CAR NK cells were used to test their ability of killing CD45+ cells.

Functional characterization of CD45 inactivated NK-92 cells (NK^{45i} -92) after CRISPR/Cas nucleases target

We demonstrated that, following CRISPR/Cas nuclease inactivation of CD45, the growth of NK^{45i} -92 cells was similar to that of the wild NK-92 cells (Figure 50). Inactivation of CD45 did not significantly affect the cell proliferation of NK-92. In addition, we showed that the lysis ability of NK^{45i} -92 cells was compatible to that of wild type, NK-92 when cells were co-cultured with leukemic cells, CCRF (Figure 51).

To demonstrate that CD45 -inactivated NK-92 was compatible with CAR lysis, NK^{45i} -92 cells and their wild type, NK-92 were transduced with lentiviruses expressing CD5CAR or GFP. The resulting CD5CAR NK^{45i} -92 cells and GFP NK^{45i} -92 were sorted by FACS, and used to compare their ability of killing targeted cells. CD5CAR NK^{45i} -92 cells displayed the ability of robustly killing CD5 target leukemic cells at ratios (E:T), 2:1 and 5:1 when they were co-cultured with CCRF-CEM cells. We showed that there was a similar efficacy of elimination of CCRF-CEM cells in vitro between CD5CAR NK^{45i} -92 and CD5 CAR NK-92 cells (Figure 52). This suggests that the loss of CD45 expression does not diminish the anti-tumor activity of CAR NK-cells.

Generation of CD45CAR construct

We next investigate that CD45CAR in NK^{45i} -92 cells response to the CD45 antigen in leukemic cells. We generated CD45CAR. CD45CAR consists of an anti-CD45 single-chain variable fragment (scFv) region, CD8-derived hinge (H) and transmembrane (TM) regions, and tandem CD28 and 4-1BB co-activation domains linked to the CD3ζ signaling domain (Figure 53A). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used. CD45CAR protein was characterized by Western blot of HEK293-FT cells transfected with CD45CAR lentiviral plasmid with appropriate vector control. Additionally, anti-CD3zeta monoclonal antibody immunoblots revealed bands of predicted size for the CD45CAR protein with no bands observed in vector control (Figure 53B).

CD45CAR NK^{45i} -92 NK cells

Following fluorescence-activated cell sorting (FACS) to enrich for NK^{45i} -92 cells, CD45CAR NK-92 transduction efficiency was determined to be 87%, as determined by flow cytometry (Figure 54) after sorting. After FACS collection of NK^{45i} -92 cells, CD45CAR expression levels remained consistently stable for at least 10 passages.

CD45CAR NK^{45i} -92 cells specifically lyse CD45+ leukemic cells.

To assess CD45CAR NK^{45i} -92 anti-leukemic activity, we conducted co-culture assays using T-ALL cell lines, CCRF-CEM and Jurkat, and NK cell line and NK--92 cells since they all express CD45 (Figures 55, 56 and 57). We demonstrated that CD45CAR NK^{45i} -92 cells consistently displayed robust lysis of leukemic cells. Following 6-hour incubation at a low effective to target cell (E:T ratio 5:1), CD45CAR NK^{45i} -92 cells effectively lysed more than 60% of CCRF-CEMcells (Figure 55). After 6-hour co-culture, CD45CAR NK^{45i} -92 cells were also able to eliminate about 60% of Jurkat cells at a ratio of E:T, 2:1 or 5:1(Figure 56). After 6 hours of co-culture, CD45CAR NK^{45i} -92 cells efficiently lysed 20% CD45 positive NK-92 cells at an E:T ratio of 2:1, with close to 60% lysis at an E:T of 5:1 (Figures 57A-57C).

To further analyze the CD45 target for hematologic malignancies, we also generated additional two CARs: CD45-28 and CD45-BB, and the lentiviruses expressing CD45-28 or CD45-BB CAR were used to transduce NK45i -92 cells. CD45-28 and CD45-BB CARs contain a new anti-CD45 scFv, which is different from that of CD45CAR described above. CD45-28 CAR uses a CD28 co-stimulatory domain while the CD45-BB bears a 4-BB co-stimulatory domain. Both CARs use the CD8-derived hinge (H), transmembrane (TM) regions and CD3ζ signaling domain. CD45CARs displayed robust lysis of B acute lymphoblastic cell line, REH. CD45CAR NK45i-92 cells lysed about 76% REH cells. CD45b-BB CAR NK45i-92 cells and CD45b-28 CAR NK45i-92 cells showed about 79% and 100% lysis of REH cells, respectively compared to control GFP NK-92 cells (Figure 57D-57E). CD45b-28 CAR NK45i-92 cells exhibited the highest ability of lysis of REH cells (B-ALL cells).

We also investigated if CD4b-28CAR CD45b-28 CAR NK45i-92 cells could lyse other types of leukemic cells. As shown in Figure 57F, co-culture assay was performed with U937 cells (target: T) and GFP NK-92 cells or CD45b-28 NK^{45i}-92 cells (effector: E) at 2:1 (E:T) ratio for 20 hours, CD45b-28 NK^{45i}-92 cells exhibited a robust anti-leukemic activity with about 81% cell lysis against U937 cells compared to control GFP NK-92 cells. U937 is an acute myeloid leukemia cell line. A similar finding was seen when co-culture assay was done with MOLM-13 cells (target: T) and GFP NK-92 cells or CD45b-28 NK45i-92 cells (effector: E) at 5:1 (E:T) ratio for 20 hours (Figure 57G). MOLM-13 cells are derived from a patient with aggressive acute monocytic leukemia. The anti-leukemic activities were also examined in two mantle cell lines, SP53 and Jeko (Figure 57H and I). CD45b-28 NK^{45i}-92 with a low ratio of

2:1(E:T), were able to lyse more than 40% of SP53 cells or Jeko leukemic cells compared to control GFP NK-92 cells at a relative short co-culture period of time, 6 hours. These studies demonstrated that CD45b-28 NK^{45i}-92 had a remarkable anti-leukemic property against different types of malignant leukemias.

We further investigated if CD45b-28 NK^{45i}-92 cells could lyse CD34+ hematopoietic stem/progenitor cells. CD34(+) stem cells derived from human umbilical cord blood were co-cultured with either control or CD45b-28 CAR NK cells for 48hr at a low ratio of 2:1 (effective: target). CD45b-28 NK^{45i}-92 cells nearly eliminate CD34+ hematopoietic precursor cells (Figure 57J) compared to the control.

An alternative CAR design to enhance CD45 CAR activity

We also generated engineered CD45 CAR cells received not only costimulation through the CD28 but also co-express the 4-1BB ligand (4-1BBL or CD137L) in a single construct, which provide the better therapeutic efficacy (Figure 58A) and their example is described below:

Example: CD45b-28-2G-4-1BBL was generated and the generated CD45b CAR cells could receive both co-stimulatory pathways, CD28 and 4-1BB. CD45b-28-2G-4-1BBL viruses were concentrated by 4 fold and used to transduce NK^{45i}-92 cells. Its CAR surface expression was about 87%(Figure 58B). CD45b-28-2G-4-1BBL viruses were concentrated by 4 fold and used for transduction. Anti-tumor activity of CD45b-2G CAR cells was significantly improved when 4-1BBL was included in the construct.

An enhancer, IL-15/IL-15sushi was also included in CD45 CAR construct as an alternative approach to enhance CD45 CAR anti-tumor activity. Both CD45 CAR and IL-15/IL-15sushi were in a single construct (Figure 58). Anti-tumor activity of CD45b-2G CAR cells is significantly improved when IL-15/IL-15sushi is included in the construct.

Example: CD45b-28-2G-IL-15/IL-15sushi NK cells was generated. Surface CD45b CAR expression were about 60%.(Figure 58C). Anti-tumor activity of CD45b-2G CAR cells was significantly improved when IL-15/IL-15sushi was included in the construct.

Characterization of CD4IL-15/IL-15sushi CAR

The CD4IL-15/IL-15sushi -CAR has been generated and it contains the third generation of CD4CAR linked to IL-15/IL-15sushi (Figure 59). A combination of CAR, (third generation), sushi/IL-15 is assembled on an expression vector and their expression is driven by the SFFV promoter (Figure 59). CAR with IL-15/IL-15sushi is linked with the P2A cleaving sequence. The IL-15/IL-15sushi portion is composed of IL-2 signal peptide fused to IL-15 linked to IL-15susi via a 26-amino acid poly-proline linker (Figure 59). The IL-2 signal peptide provide a better secreting signal. The stable, functional complexes of IL-15/IL-15sushi can be secreted from the transduced cells and the secretion is directed by IL-2 signal peptide.

To verify the CD4IL-15/IL-15sushi construct, HEK293FT cells were transfected with lentiviral plasmids for either GFP (control) or. CD4IL-15/IL-15sushi. Approximately 60 hours after transfection, both HEK-293FT cells and supernatant were collected. Cells were lysed in RIPA buffer containing protease inhibitor cocktail and electrophoresed. The gel was transferred to Immobilon FL blotting membrane, blocked, and probed with mouse anti-human CD3z antibody at 1:500. After washes, membrane was probed with goat anti-mouse HRP conjugate, washed, and exposed to film following treatment with HyGlo HRP substrate. The CD4IL-15/IL-15sushi was successfully expressed in HEK 293 cells (Lane 2, Figure 60a). The CD4IL-15/IL-15sushi lentiviral supernatant was further examined by the transduction of fresh HEK-293 cells (Figure 60A). HEK-293 cells were transduced with either GFP or CD4IL-15/IL-15sushi CAR viral supernatant from transfected HEK-293FT cells. Polybrene was added to 4 μL/mL. Media was changed after 16 hours and replaced with media containing no viral supernatant or polybrene. Three days after transduction, cells were harvested and stained with goat-anti-mouse F(Ab')2 antibody at 1:250 for 30 minutes. Cells were washed and stained with streptavidin-PE conjugate at 1:500, washed, suspended in 2% formalin, and analyzed by flow cytometry. Figure 60b shows that HEK-293 cells that were transduced with the CD4IL-15/IL-15sushi CAR lentivirus were 80% positive for F(Ab)2-PE (circled, Figure 60B), while transduction with GFP control lentivirus was minimal for F(Ab)2-PE (Figure 60).

Production of CD4IL-15/IL-15sushi--CAR NK cells

NK-92 cells were transduced with concentrated CD4IL-15/IL-15sushi--CAR lentiviral supernatant. After 5 days incubation, cells were harvested and incubated with goat anti-mouse F(Ab')2 at 1:250 for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry, resulting in nearly 70% of the transduced cells expressing CD4IL-15/IL-15sushi-

CAR (circled, Figure 61. Further experimental tests for CD4IL-15/IL-15sushi-CAR included leukemia/lymphoma killing assays in vitro and vivo, and comparison of target killing and proliferation rates with cells transduced with CD4CAR. The inventor also used the same strategy described above to generate CD19IL-15/IL-15sush CAR, CD20IL-15/IL-15sush CAR and CD22IL-15/IL-15sush CAR.

Production of CD4IL-15/IL-15sushi -CAR T cells

Human umbilical cord buffy coat cells were transduced with concentrated CD4IL-15/IL-15sushi -CAR lentiviral supernatant. After 5 days incubation, cells were harvested and incubated with goat anti-mouse F(Ab')2 at 1:250 for 30 minutes. Cells were washed, suspended and stained with streptavidin-PE for 30 minutes. Cells were washed and suspended in 2% formalin, and analyzed by flow cytometry, resulting in 63% of the transduced cells expressing CD4IL-15/IL-15sushi-CAR(circled, Figure 62). Further experimental tests for CD4IL-15/IL-15sushi -CAR will include leukemia/lymphoma killing assays in vitro and vivo, and comparison of target killing and proliferation rates with cells transduced with CD4CAR.

CD4IL-15/IL-15sushi CAR NK cells were tested for anti-leukemic activity relative to CD4CAR NK cells in vitro by co-culturing them with the following CD4 positive cell lines: Karpas 299 and MOLT4.

The Karpas 299 cell line was derived from a patient with anaplastic large T cell lymphoma. The MOLT4 cell line expressing CD4 was established from the peripheral blood of a 19-year-old patient with acute lymphoblastic leukemia (T-ALL). During 4-hour co-culture experiments, CD4IL-15/IL-15sushi CAR NK cells showed profound killing (95%) of Karpas 299 cells at a 5:1 ratio of effector:target, at an even higher rate than that of CD4CAR NK cells (82%; Figure 63). Similarly, when co-cultured 1:1 with MOLT4 cells, CD4IL-15/IL-15sushi CAR NK cells lysed target cells at a higher rate (84% to 65%) than CD4CAR NK cells in an overnight assay (Figure 64). These results show that CD4IL-15/sushi CAR NK cells can ablate tumor cells.

Both CD4CAR and CD4IL-15/IL-15sushi CAR T cells exhibit significant anti-tumor activity *in vivo*

In order to evaluate the *in vivo* anti-tumor activity of CD4CAR and CD4IL-15/IL-15sushi CAR T cells, and to determine the possible increase in persistence of the CD4IL-15/IL-15sushi CAR T cells relative to the CD4CAR T cells, we developed a xenogeneic mouse model using

NSG mice sublethally irradiated and intravenously injected with luciferase-expressing MOLM13 cells, an acute myeloid leukemia cell line that is 100% CD4+, to induce measurable tumor formation (Figure 65). Three days following tumor cell injection, 6 mice each were intravenously injected with a course of 8 x 10^6 CD4CAR, CD4IL-15/IL-15sushi T cells or vector control T cells. On days 3, 6, 9 and 11, mice were injected subcutaneously with RediJect D-Luciferin (Perkin Elmer) and subjected to IVIS imaging to measure tumor burden (Figure 65B). Average light intensity measured for the CD4CAR and CD4IL-15/IL-15sushi CAR T cell injected mice was compared to that of vector control T cell injected mice in order to determine the percentage of tumor cells in treated versus control mice (Figure 65C). CD4CAR T cell-treated mice had a 52% lower tumor burden relative to control on Day 6, whereas CD4IL-15/IL-15sushi CAR T cell-treated mice had a 74% lower tumor burden. On Day 11, nearly all tumor cells had been lysed in both of these groups. Unpaired T test analysis revealed a very significant difference (P=0.0045) between control and the two groups by day 9 with less light intensity and thus less tumor burden in the CD4CAR and CD4IL-15/IL-15sushi CAR T cells treated group compared to control. In summary, these *in vivo* data indicate that CD4CAR and CD4IL-15/IL-15sushi CAR T cells both significantly reduce tumor burden and in MOLM13-injected NSG mice when compared to vector control T cells.

Next, we compared mouse survival across the two groups (Figure 65D). All leukemic mice injected with CD4IL-15/IL-15sushi CAR T cell survived longer than that of CD4CAR T cells. In summary, these *in vivo* data indicate that CD4IL-15/IL-15sushi CAR T cells significantly reduce tumor burden and prolong survival in CD4IL-15/IL-15sush CAR T-injected NSG mice when compared to control cells.

CD4IL-15/IL-15sushi CAR NK cells exhibit robust and persistent anti-tumor activity *in vivo*

In order to further evaluate the CD4IL-15/IL-15sushi CAR function, we created a stressful condition utilizing NK CAR cells and Jurkat tumor cells. The NK cells bear a short half-life property and leukemic Jurkat cells show less than 60% CD4+ phenotype (Figure 66A). In such a condition, it allows us to investigate how secretory soluble IL-15sushi affects the CAR functions in terms of its persistence and killing capability. We then used our xenogeneic NSG mouse model using NSG mice sublethally irradiated and intravenously injected with luciferase-expressing Jurkat cells to induce measurable tumor formation. In contrast with MOLM-13 cells, Jurkat cells show less than 60% CD4+ phenotype (Fig 66A5). Three days following Jurkat cell injection, mice were intravenously injected with a course of 10 x 10^6 either CD4CAR, CD4IL-15/IL-15sushi, or vector control NK cells. On day 3 (the day before treatment), 7, 10, and 14, mice were subjected to IVIS imaging to measure tumor burden (Figure 66B). Average light intensity measured for the CD4CAR and CD4IL-15/IL-15sushi NK injected mice was compared to that of vector control NK injected mice to determine percent lysis of Jurkat cells (Figure 66C). Although both conditions showed significant tumor cell lysis by Day 7, lysis percentage for CD4CAR NK cells stayed the same to Day 14 while CD4IL-15/IL-15sushi NK cells increased to over 97%. (Figure 66D). Unpaired T test analysis revealed an extremely significant difference (P<0.0001) between the two groups by Day 14. These results indicate that CD4CAR NK cell lysis of Jurkat tumor cells was not able to keep up with the expansion of CD4- Jurkat cells, whereas the continued expansion of NK CAR cells secreting IL-15/IL-15sushi effectively lysed. The co-expression of secretory IL-15/IL-15sushi with CAR could supplement the defect that CAR T or NK cells are unable to eliminate dim expressed cancer cells or non-targeting cancer cells. A repeat of experiments (Figure 67) showed similar results to those described in Figure 66.

Secreted IL-15/IL-15sushi substitutes for IL-2 in NK cell survival and expansion.

The effect of IL-15/IL-15sushi-secreting NK cells on cell survival was determined. NK-92 cells stably transduced with either CD4CAR or CD4IL-15/IL-15sushi were cultured in the presence or absence of IL-2 to determine if IL-15/IL-15sushi secretion alone could lead to survival and expansion. CD4CAR-expressing NK cells cultured without IL-2 died by Day 7, while CD4IL-15/IL-15sushi-expressing NK cells cultured without IL-2 expanded at approximately the same rate as either CD4CAR or CD4IL-15/IL-15sushi cells cultured with IL-2 (Figure 68B), showing that secreted IL-15/IL-15sushi could substitute for IL-2. Furthermore, we were able to demonstrate that NK cells secreting IL-15/IL-15sushi could aid in the survival and expansion of non-transduced NK-92 cells in a co-culture. In this experiment, an equal ratio of NK GFP-expressing cells were cultured with either CD4CAR- or CD4IL-15/IL-15sushi-expressing NK cells, in the presence or absence of IL-2. Cells were counted every 2-3 days

(Figure 8A). By Day 7, CD4CAR NK cells given no IL-2 had died, but CD4IL-15/IL-15sushi NK cells without IL-2 had survived and expanded at approximately the same rate as either CD4CAR or CD4IL-15/IL-15sushi cells cultured with IL-2. The number of GFP-expressing cells had risen along with the CD4IL-15/IL-15sushi NK cells (Figure 68B), indicating that the secreted IL-15/IL-15sushi had positively affected GFP NK cell survival. The percentage of GFP-positive cells had risen from 50% to over 70% over the course of the experiment (data not shown). In the second experiment (Figure 69), GFP NK and either control vector NK or CD4IL-15/IL-15sushi NK cells were mixed at a 10 to 1 ratio, with no IL-2. By Day 6, cells co-cultured with control cells had all died, but survival of cells cultured with NK cells secreting IL-15/IL-15sushi survived until Day 10.

To further determine if this effect was due to secreted protein alone, or an interaction between co-cultured cells, we devised an experiment in which the GFP NK cells were cultured in a chamber above the cultured CD4CAR or CD4IL-15/IL-15sushi NK cells, or non-transduced NK-92 cells. In this situation, only proteins and not cells could pass between the membrane separating the two cultures. Cells were incubated without IL-2, counted and split 1:1 every other day. While GFP NK cells in the upper chamber above NK-92 cells had died by Day 6, the GFP NK cells above the CD4IL-15/IL-15sushi NK cells had survived and expanded by Day 12 (Figure 70), thereby indicating that it was the IL-15/IL-15sushi protein secreted by the CD4IL-15/IL-15sushi NK cells which had kept them alive, and not direct cell-to-cell contact. In this model, the upper chamber represents the tumor microenvironment, in which the survival of T cells or NK cells is improved by the secretion of IL-15/IL-15sushi from the CD4IL-15/IL-15sushi NK cells.

Effect of secreted IL-15/IL-15sushi on CAR T and non-transduced neighboring cells.

We also compared the cell growth of CD4CAR and CD4IL-15/IL-15sushi transduced T cells in the presence or absence of IL-2. Total cell counts calculated throughout the experiment (up to Day 17) for transduced cells with or without IL-2. CD4IL-15/IL-15sushi transduced T cells appeared to be more tolerant to the absence of IL-2 than that of CD4CAR transduced T cells.

Examples

Generation of Treg CAR target Treg cells

Treg CAR (also called CD4zetaCD25CAR or C4-25z) followed the schematic in Figure 70. It comprises of SFFV (spleen focus-forming virus) promoter that drives the expression of two different units of incomplete CARs linked by a P2A cleavage peptide. The CD4 chimeric antigen receptor polypeptide unit comprises a CD45 signal peptide, a CD4 antigen recognition domain, a hinge region (derived CD8a), a transmembrane domain (CD8a) and CD3 zeta chain; CD25 chimeric antigen receptor polypeptide unit comprises a CD45 signal peptide, a CD25 antigen recognition domain, a hinge region (CD8a), a transmembrane domain (CD8a), a co-stimulatory domain (s), CD28. The Treg CAR can potentiate the lysis activity of a cell co-expressing CD4 and CD25 while minimizing a cell bearing CD4 or CD25 antigen alone.

The CD4zetaCD25CAR (C4-25z) (Treg CAR) was transduced in an assay. Compared to control T-cells, CD4zetaCD25CAR cells show ~15% surface expression and this was sufficient to observe the following phenotype validation of construct function (Figure 70A). CD4zetaCD25 CAR cells and control T-cells were both assayed with CD4 and CD25 antibody to look for logic gated behavior using flow cytometry analysis. Due to the construct design, the CD4zetaCD25CAR cell would potentiate the lysis activity for cells co-expressing both CD4 and CD25 antigens. Here, we showed depletion (~95%) of the CD4+ CD25+ double positive population with little impact of off-logic events in the other phenotype cases. A bar graph summary shows that the logic gated CAR construct design only significantly impacts the double positive population (Figure 70B).

We further characterized CD4zetaCD25 CAR by comparing it with CD4 CAR. As expected, CD4CAR T cells had a profound lysis ability of cells expressing CD4 only while CD4zetaCD25CAR T cells had a limited killing ability on this population (Figure 71). CD4zetaCD25CAR T cells also showed virtually complete depletion of cells expressing both CD4 and CD25 antigens (Figure 71). These studies demonstrate that the robust CD4zetaCD25CAR targeting cells co-expressing both CD4 and CD25, has been established. Due to human-specific CD4 or CD25scFv in the construct, the functional properties of CD4zetaCD25CAR are difficult to test in animals.

In some embodiments, the disclosed disclosure also comprises methods of improving the CD4zetaCD25CAR therapeutic activity. The example is described below.

EXAMPLE

An engineered CD4zetaCD25CAR cell was prepared in accordance with the present disclosure.

Cell killing assay is performed

Targeted Cells killing by CD4zetaCD25CAR is improved when co-expressed with 4-1BBL or IL-15/IL-15sushi or IL-15/IL-15RA.

Safety switch

Introduction of a “safety switch” greatly increases safety profile and the “safety switch” may be an inducible suicide gene, such as, without limiting, caspase 9 gene, thymidine kinase, cytosine deaminase (CD) or cytochrome P450. Other safety switches for elimination of unwanted modified T cells involve co-expression of CD20 or CD52 or CD19 or truncated epidermal growth factor receptor in T cells.

Example: Co-expression of CD52 with CARs using CD5CAR targeting T- cell malignancies as an example.

For clinical treatment using CAR T-cells against T-cell malignancies, establishment of safety methods to eliminate CAR T-cells from patients may be necessary after tumor depletion or in emergency cases due to unexpected side effects caused by CAR therapy. T-cells and B-cells express CD52 on the cell surface and a CD52 specific antibody, CAMPATH (alemtuzumab), can eliminate CD52+ cells from circulation. We thus incorporated a human CD52 sequence into the CD5CAR vector construct (Figure 72A). This additional CD52 construct mechanically separates the signaling from native CD52. The aim was to preempt the possibility of native CD52 antigen escape on CAR T-cell surface after CAMPATH treatment. CD5CAR-CD52 lentiviral protein and expression were confirmed via western blot and flow cytometry analysis using CD52 antibody on transduced HEK293 cells. We also found that co-expressing CD52 would affect the CAR T cell functions.

In *vivo* depletion of infused CD5CAR-CD52 T cells following treatment with CAMPATH

To assess the effect of CAR elimination by CAMPATH (alemtuzumab) treatment, we conducted in *vivo* procedures as described (Figure 72B and 72C). We intravenously injected 5 x 10^6 CD5CAR-52 T-cells into irradiated mice. Next day, we added 0.1mg/kg of CAMPATH or PBS via IP injection for 3 mice of each group. After 6 and 24 hours following CAMPATH treatment, we collected peripheral blood from the mouse tail and determined presence of CD5CAR-52 T-cells by FACS analysis. CAMPATH injection virtually completely deplete CD5CAR-CD52 T-cells in blood at both 6 h and 24 h (Figure 72C). Five days following CAMPATH administration, CD5CAR-CD52 cells were also completely depleted in both the bone marrow and spleen (Figure 72D). These findings support the use of CAMPATH as a useful strategy in acting as a safety trigger to deplete CAR-T cells from circulation and lymphoid organs.

In one embodiment, the engineered cell includes a CD5 chimeric antigen receptor polypeptide and an anchor CD52 (SEQ ID NO.70), and corresponding polynucleotide (SEQ ID NO. 69). In some embodiments, CD52 is incorporated into CD5 CAR engineered cell or any CAR engineered cell and can be used as a "safety switch" for CAR therapy.

Promoter testing using the GFP reporter

HEK293FT cells were transfected with lentiviral plasmids expressing GFP under the SFFV, EF1 or CAG promoters. Approximately 60 hours after transfection, supernatant was collected from each. Relative viral titer was determined by first transducing HEK293 cells with supernatant from each of the 3 promoters. HEK-293 cells were transduced with GFP viral supernatant from each of the 3 transfected HEK-293FT cells. Polybrene was added to 4 µL/mL. Media was changed after 16 hours and replaced with media containing no viral supernatant or polybrene. Three days after transduction, cells were harvested and washed, suspended in 2% formalin, and analyzed by flow cytometry for GFP expression (FITC). GFP expression was seen in each sample, but was highest for the cells transduced with virus made using the SFFV promoter.

Activated human umbilical cord buffy coat cells were transduced with GFP lentiviral supernatant (amount based on the results of the HEK293 transduction efficiency) from each of the promoters. After 5 days incubation, cells were harvested, washed and suspended in 2% formalin, and analyzed by flow cytometry for GFP expression. 43% of cells expressed GFP at high levels (>10^3) while GFP-expression for cells transduced with virus using promoters EF1 (15%) and CAG (3%) were considerably lower. Five days later, cells analyzed the same way showed nearly the same percentages for each (46%, 15% and 3%, respectively). These results indicate that SFFV promoter leads to stronger expression than EF1 or CAG promoters, and that the expression remains high for at least 10 days post-transduction. Further experimental tests will include longer incubation times for transduced cells beyond the 10-day window.

Functional titer of viral vector particles in supernatants (*The % GFP cells as determined by flow cytometry allows for proxy viral titer adjustments as higher titer virus infiltrates more cells, leading to higher %GFP cell populations).*

To determine functional titer of viral vector particles in each of our supernatants, HEK 293 cells were transduced with either EF1-GFP or SFFV-GFP viral supernatant, with either 30 μL (low), 125 μL (medium), or 500 μL (high) per well of a 12 well tissue-culture treated plate. Culture media was changed the following morning to DMEM plus 10% FBS (Figure 73).

Transduced cells were then trypsinized, washed, and suspended in formalin and subjected to flow analysis. The percentage of GFP+ cells in each of the conditions was determined by flow cytometry using the FITC channel (Figure 74). In each case, the percentage of GFP+ was higher in cells transduced with SFFV-GFP than the cells transduced with the corresponding volume of EF1-GFP viral supernatant (50% to 18% for low, 80% to 40% for medium, and 82% to 70% for high). From this, we determined that using the highest volume of EF1-promoter virus was comparable to using the lowest volume of SFFV-promoter virus in terms of titer, and would allow for comparison of relative promoter strengths for the following transduction experiments

Transduced cells were also visualized on an EVOS fluorescent microscope using GFP at 20x at the same exposure conditions for each well (Figure 73). Cells transduced with SFFV-GFP viral supernatant were dramatically brighter than cells transduced with EF1-GFP. Furthermore, comparing the image of the EF1-promoter under high viral volume loads with the image of the SFFV-promoter using low viral volume loads show similar fluorescent intensity. This suggests that the SFFV promoter is a stronger driver of gene expression.

Comparison of surface expression and persistence of different promoters in primary T-cells *(The % GFP cells as determined by flow cytometry for T-cell transductions show expected differences in GFP cell populations as expected from the prior experiments on HEK293 cells)*

To determine promoter transduction efficiency and persistence of surface expression in primary T cells, activated cord blood buffy coat T cells were transduced with either 50 μL of SFFV-GFP or 1 mL of EF1-GFP EF1-GFP viral supernatant, in 12-well tissue culture-treated plates pre-coated with retronectin (Clontech). Following two overnight transductions, cells were cultured on T cell media with 300 IU/mL IL-2 (Peprotech) and maintained at 1.0-4.0 x 10^6/mL. Cells were washed, suspended in formalin, and subjected to flow cytometry analysis, using the FITC channel to determine the percentage of GFP+ cells, on 7, 14, 21 and 28 days after transduction. The percentage of GFP+ cells was consistently higher for T cells transduced with SFFV-GFP compared to EF1-GFP-transduced T cells (Figure 75A), even as the percentage of total GFP+ cells decreased over this period. A further comparison showed that T cells transduced with the higher (1 mL) amount of EF1-GFP supernatant actually decreased in percentage relative to the percent of GFP+ cells transduced with the lower amount (50 μL, or 20-fold less) of SFFV-GFP, between Day 7 and Day 28, from over 60% to under 40% (Figure 75B). This suggests that transduction using the SFFV promoter led to greater persistence of transduced cells.

Methods of generating the CAR gene including at least one of a T antigen recognition moiety (at least one of CD4, CD8, CD3, CD5, CD7, and CD2, or a part or a combination thereof), a hinge region and T-cell activation domains is provided.

Methods of generating multiple units of CARs (cCAR) targeting antigen (s) including at least one of CD33, CD123, CD19, CD20, CD22, CD269, CS1, CD38, CD52, ROR1, PSMA, BAFF, TACI, CD138, and GPC3, or a part or a combination of a hinge region and T- cell activation domains is provided.

The provided methods also include: 1) generating of the CAR T or NK cells targeting leukemias and lymphomas expressing CD45 and avoiding self-killing; 2) generation of "armored" CAR T or NK cells designed to both overcome the inhibitory tumor microenvironment and exhibit enhanced anti-tumor activity and long-term persistence.

The present disclosure is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the disclosure and claims.

While there have been described what are presently believed to be the preferred embodiments of the present disclosure, those skilled in the art will realize that other and further changes and modifications may be made thereto without departing from the spirit of the disclosure, and it is intended to claim all such modifications and changes as come within the true scope of the disclosure.

Various terms relating to aspects of the disclosure are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art, unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definition provided herein.

INCORPORATION OF SEQUENCE LISTING

Incorporated herein by reference in its entirety is the Sequence Listing for the application. The Sequence Listing is disclosed on a computer-readable ASCII text file titled, "sequence_listing.txt", created on December 22, 2016. The sequence-listing.txt file is 508 KB in size.

CLAIMS

1. A engineered cell comprising:

a chimeric antigen receptor polypeptide comprising a signal peptide, an antigen recognition domain, a hinge region, a transmembrane domain, a signaling domain, and co-stimulatory domain.

2. A engineered cell comprising:

a first chimeric antigen receptor polypeptide comprising a signal peptide, first antigen recognition domain, a first transmembrane domain, first hinge region, and a co-stimulatory domain; and

a second chimeric antigen receptor polypeptide comprising a signal peptide, second antigen recognition domain, a second transmembrane domain, a second hinge region, and a signaling domain;

wherein the first antigen recognition domain is different than the second antigen recognition domain.

3. A engineered cell comprising:

a first chimeric antigen receptor polypeptide comprising a signal peptide, first antigen recognition domain, a first transmembrane domain, a first hinge region, a first signaling domain, and first co-stimulatory domain; and

a second chimeric antigen receptor polypeptide comprising a signal peptide, second antigen recognition domain, a second transmembrane domain, a second hinge region, a second signaling domain, and a second co-stimulatory domain;

wherein the first antigen recognition domain is different than the second antigen recognition domain.

4. The engineered cell according to any one of claims 1-3, wherein the signal peptide comprises a CD8 signal peptide, CD45 signal peptide, functional fragments thereof, and functional equivalents thereof.

5. The engineered cell according to claim 1, wherein the antigen recognition domain is selected from the group consisting of CD25, CD2, CD3, CD4, CD5, CD7, CD19, CD20, CD22, CD25, CD33, CD123, CD138, TACI, CD269, CS1, and CD52.

6. The engineered cell according to any one of claims 2-4, wherein the first and second antigen recognition domain are independently selected from the group consisting of CD25, CD2, CD3, CD4, CD5, CD7, CD19, CD20, CD22, CD25, CD33, CD123, CD138, TACI, CD269, CS1, and CD52.

7. The engineered cell according to any one of claims 2-4 and 6, wherein the first antigen recognition domain is selected from the group consisting of CD25, CD2, CD3, CD4, CD5, and CD7; and the second antigen recognition domain is selected from the group consisting of CD25, CD2, CD3, CD4, CD5, and CD7.

8. The engineered cell according to any one of claims 2-4, wherein the first antigen recognition domain comprises CD4; and the second antigen recognition domain comprises CD25.

9. The engineered cell according to any one of claims 1-8, wherein said hinge region comprises the hinge region of a human protein selected from the group consisting of CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, functional derivatives thereof, and combinations thereof.

10. The engineered cell according to any one of claims 1-9, wherein said transmembrane domain comprises the transmembrane region of a human protein selected from the group consisting of CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, functional derivatives thereof, and combinations thereof.

11. The engineered cell according to any one of claims 1-10, wherein said signaling domain comprises the signaling domain selected from the group consisting of CD45, CD3 zeta, common FcR gamma (FCER1G), Fc gamma Rlla, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DAP10, DAP12, active fragments thereof, and combinations thereof.

12. The engineered cell according to any one of claims 1-7, wherein said co-stimulatory domain comprises the co-stimulatory domain from a protein selected from the group consisting of OX40, CD27, CD28, CD30, CD40, PD-1, CD2, CD7, CD258, NKG2C, NKG2D, B7-H3,

CD83, ICAM-1, LFA-1 (CDl la/CD18), ICOS, and 4-1BB (CD137), active fragments thereof, and combinations thereof.

13. The engineered cell according to any one of claims 1-9, wherein the co-stimulatory domain is CD28; and the signaling domain is CD3 zeta.

14. The engineered cell according to any one of claims 1-9, wherein the co-stimulatory domain is 4-1BB; and the signaling domain is CD3 zeta.

15. The engineered cell according to claim 3, wherein the first co-stimulatory domain is different than the second co-stimulatory domain.

16. The engineered cell according to claim 3, wherein the first co-stimulatory domain is CD28, and the second co-stimulatory domain comprises 4-1BB

17. The engineered cell according to any one of claims 2-16, wherein the first chimeric antigen receptor polypeptide and the second chimeric engineered polypeptide are on a single polypeptide molecule, and wherein an amino acid sequence comprising a high efficiency cleavage site is disposed between the first chimeric antigen receptor polypeptide and the second chimeric antigen receptor polypeptide.

18. The engineered cell according to claim 17, wherein the high efficiency cleavage site is selected from the group consisting of P2A, T2A, E2A, and F2A.

19. The engineered cell according to any one of claims 1-18, wherein said engineered cell does not include a CD4 cell surface antigen.

20. The engineered cell according to 1-19, wherein said engineered cell does not include a CD25 antigen.

21. The engineered cell according to any one of claims 1-20, wherein said engineered cell comprises a T-Cell or NK cell.

22. An engineered polynucleotide encoding for a chimeric antigen receptor polypeptide according to any one of claims 1-21.

23. A method of reducing the number of cells having CD4 and CD25 cell surface antigens comprising contacting said cells with an engineered cell according to any one of claims 1-19; and optionally assaying for the reduction of cells having CD4 and CD25 cell surface antigens.

24. A method of reducing the number of cells having at least one cell surface antigen selected from the group consisting of CD25, CD2, CD3, CD4, CD5, CD7, CD19, CD20, CD22, CD25, CD30 CD33, CD123, CD138, TACI, CD269, and CS1 comprising contacting said cells with an engineered cell according to any one of claims 1-21; and optionally assaying for the reduction of cells having at least one cell surface antigens selected from the group consisting of CD25, CD2, CD3, CD4, CD5, CD7, CD19, CD20, CD22, CD25, CD30, CD33, CD123, CD138, TACI, CD269, and CS1.

25. A method of treating a cell proliferative disease comprising administering an engineered cell according to any one of claims 1-21 to a patient in need thereof.

26. A method of reducing the number of T-regulatory cells, said method comprising contacting T-regulatory cell with an engineered cell according to claim 8.

27. A engineered cell comprising:

(i.) a first chimeric antigen receptor polypeptide comprising a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling domain; and

(ii.) a second chimeric antigen receptor polypeptide comprising a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain;

wherein the first antigen recognition domain and the second antigen recognition domain are different.

28. The engineered cell according to claim 27, wherein the first chimeric antigen receptor polypeptide and the second chimeric engineered polypeptide are on a single polypeptide molecule, and wherein an amino acid sequence comprising a high efficiency cleavage site is disposed between the first chimeric antigen receptor polypeptide and the second chimeric antigen receptor polypeptide.

29. The engineered cell according to claim 27, wherein the high efficiency cleavage site is selected from the group consisting of P2A, T2A, E2A, and F2A.

30. The engineered cell according to any one of claims claim 27-29, wherein the first co-stimulatory domain and the second co-stimulatory domain are different.

31. The engineered cell according to any one of claims 27-30, wherein the first co-stimulatory domain comprises CD28, and the second co-stimulatory domain comprises 4-1BB.

32. The engineered cell according to any one of claims 27-31, wherein the target of the first antigen recognition domain is selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, BCMA, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1; and the target of the second antigen recognition domain is selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, BCMA, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1.

33. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises TACI or CD269; and the target of the second antigen recognition domain is selected from the group consisting of CD19, CD38, CD138, CD138, and CS1.

39. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the target of the second antigen recognition domain is selected from the group consisting of CD20, CD22, CD33, CD123, TACI, CD269, CD38, and CS1.

35. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the target of the second antigen recognition domain selected from the group consisting of CD20, CD22, and CD123.

36. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition domain comprises LeY or CD123.

37. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises BCMA; and the target of the second antigen recognition domain comprises CS1, CD19, CD20, CD22, CD38, CD138, or CS1.

38. The engineered cell according to any one of claims 27-32, wherein the engineered cell is a T-cell or Natural Killer cell.

39. The engineered cell according to any one of claims 38, wherein the T-cell is a CD4 T-cell or CD8 T-cell.

40. The engineered cell according to any one of claims 38, wherein the Natural Killer cell is a NKT cell or NK-92cell.

41. An engineered polypeptide comprising a chimeric antigen receptor polypeptide and an enhancer.

42. The engineered polypeptide according to claim 41, wherein the chimeric antigen receptor polypeptide comprises an antigen recognition domain selective for a target selected from the group consisting of: CD2, CD3, CD4, CD5, CD7, CD8, CD45, and CD52.

43. The engineered polypeptide according to claim 41, wherein the chimeric antigen receptor polypeptide comprises an antigen recognition domain selective for a target selected from the group consisting of: CD19, CD20, CD22, CD30, CD33, CD38, CD123, CD138, CD30, TACI, CD269, CD38, and CS1.

44. The engineered polypeptide according to claim 41, wherein the chimeric antigen receptor polypeptide comprises the CD45 antigen recognition domain.

45. The engineered polypeptide according to any one of claims 41-44, wherein said enhancer is selected from the group consisting of PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, TGFR beta, IL-2, IL-6, IL-7, IL-12, IL-15, IL-17, IL-18 IL-21, functional fragments thereof, and combinations thereof.

46. The engineered polypeptide according to any one of claims 41-44, wherein the engineered polypeptide further comprises an enhancer receptor or functional fragment thereof.

47. The engineered polypeptide of 46, wherein the enhancer receptor comprises IL-15RA or a functional fragment thereof.

48. The engineered polypeptide of 47, wherein the functional fragment comprises the sushi domain.

49. The engineered polypeptide according to claim 41-48, wherein chimeric antigen receptor polypeptide and enhancer are on a single polypeptide molecule.

50. The engineered polypeptide of claim 49, wherein a high efficiency cleavage site is disposed between the chimeric antigen receptor and enhancer.

51. The engineered polypeptide according to claim 50, wherein the high efficiency cleavage site is selected from the group consisting of P2A, T2A, E2A, and F2A.

52. The engineered polypeptide according to any one of claims 41-51, wherein the chimeric antigen receptor polypeptide comprises an antigen recognition domain selective for a target selected from the group consisting of CD2, CD4, and CD19; and the enhancer comprises IL-15, IL-15/IL-15 sushi, or IL-15sushi/IL-15.

53. An engineered polynucleotide, the polynucleotide encodes for any one of the polypeptides according to claims 41-52.

54. The engineered polynucleotide according to claim 53, wherein the polynucleotide is in a vector.

55. An engineered cell comprising the polypeptide according to any one of claims 41-52.

56. An engineered cell comprising the polynucleotide according to any one of claims 50-51.

57. The engineered cell according to any one of claims 55-56, wherein the engineered cell comprises T-cells or Natural Killer cells.

58. The engineered cell according to any one of claims 57, wherein the cell is a T-cell or Natural Killer cell.

59. The engineered cell according to any one of claims 59, wherein the T-cell is a CD4 T-cell or CD8 T-cell.

60. The engineered cell according to any one of claims 57, wherein the Natural Killer cell is a NKT cell or NK-92 cell.

61. A method of treating B-cell lymphoma comprising administering to a patient in need thereof an engineered cell according to any one of claims 27-41 and 55-60.

62. A method of treating T-cell lymphoma comprising administering to a patient in need thereof an engineered cell according to any one of claims 27-41 and 55-60.

63. A method of treating multiple myeloma comprising administering to a patient in need thereof an engineered cell according to any one of claims 27-41 and 55-60.

64. A method of treating chronic myeloid leukemia comprising administering to a patient in need thereof an engineered cell according to any one of claims 1-15 and 55-60, wherein the target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition domain comprises CD123.

65. A method of treating B-cell acute lymphoblastic leukemia (B-ALL) comprising administering to a patient in need thereof an engineered cell according to claim 27-41, wherein the target of the first antigen recognition domain comprises CD19; and the target of the second antigen recognition domain comprises CD123.

66. A method of treating multiple myeloma comprising administering to a patient in need thereof an engineered cell according to any one of claims 27-42, wherein the target of the first antigen recognition domain is selected from the group consisting of CD19, CS1, BCMA, and CD38; and the target of the second antigen recognition domain is selected from the group consisting of CS1, BCMA, and CD38.

67. A method of treating a cell proliferative disease comprising administering to a patient in need thereof an engineered cell according to any one of claims 27-41, wherein the target of the first antigen recognition domain is selected from the group consisting of BCMA, TAC1, CS1, and BAFF-R; and the target of the second antigen recognition domain is selected from the group consisting of BCMA, TAC1, CS1, and BAFF-R.

68. The method of treating a cell proliferative disease according to claim 67, wherein the cell proliferative disease is selected from the group consisting of lymphomas, leukemias, and plasma cell neoplasms.

69. The method of treating a cell proliferative disease according to claim 68, wherein plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma,

amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS), and smoldering multiple myeloma.

70. A method of treating a cell proliferative disease, comprising: administering to a patient in need thereof an engineered cell according to any one of claims 56-60.

71. A method of treating a cell proliferative disease, comprising: administering to a patient in need thereof an engineered cell comprising a chimeric antigen receptor polypeptide having an antigen recognition domain selective for a target selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, TACI, LeY, CD5, CD13, CD14, CD15, CD45, CD19, CD20, CD22, CD33, CD41, CD61, CD64, CD68, CD117, CD123, CD138, CD30, CD269, CD38, Flt3 receptor, and CS1; and CAR enhancing agent.

72. A method of treating a cell proliferative disease according to claim 71, wherein said CAR enhancing agent is selected from the group consisting of agents that target immune-checkpoint pathways, inhibitors of colony stimulating factor-1 receptor (CSF1R), PD-1, PD-L1, IL-2, IL-12, IL-15, CSF1R, CTAL-4, TIM-3, and TGFR beta.

73. A method of treating a cell proliferative disease comprising administering to a patient in need thereof an engineered cell comprising an engineered polypeptide according to any one of claims 39-49.

74. An engineered chimeric antigen receptor polypeptide, the polypeptide comprising: a signal peptide, a CD45 antigen recognition domain, a hinge region, a transmembrane domain, at least one co-stimulatory domain, and a signaling domain.

75. The engineered chimeric antigen receptor polypeptide according to claim 74, wherein said CD45 antigen recognition domain comprises the binding portion or variable region of a monoclonal antibody selective for CD45.

76. The engineered chimeric antigen receptor polypeptide of claim any one of claims 74-75, wherein said CD45 antigen recognition domain comprises the CD45 scFv.

77. The engineered chimeric antigen receptor polypeptide of any one of claims 76-77, wherein said hinge region comprises the hinge region of a human protein selected from the group

consisting of CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, functional derivatives thereof, and combinations thereof.

78. The engineered chimeric antigen receptor polypeptide of any one of claims 74-77, wherein said transmembrane domain comprises the transmembrane region of a human protein selected from the group consisting of CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, functional derivatives thereof, and combinations thereof.

79. The engineered chimeric antigen receptor polypeptide of any one of claims 74-78, wherein said signaling domain comprises the signaling domain selected from the group consisting of CD3 zeta, common FcR gamma (FCER1G), Fc gamma Rlla, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DAP10, DAP12, active fragments thereof, and combinations thereof.

80. The engineered chimeric antigen receptor polypeptide of any one of claims 74-79, wherein said co-stimulatory domain comprises the co-stimulatory domain from a protein selected from the group consisting of OX40, CD27, CD28, CD30, CD40, PD-1, CD2, CD7, CD258, NKG2C, NKG2D, B7-H3, a ligand that binds to CD83, ICAM-1, LFA-1 (CDl la/CD18), ICOS, and 4-1BB (CD137), active fragments thereof, and combinations thereof.

81. An engineered polynucleotide that encodes a polypeptide according to any one of claims 74-80.

82. The engineered polynucleotide according to claim 81, wherein the polynucleotide is in a vector.

83. An engineered cell comprising the polynucleotide according to any one of claims 81-82.

84. An engineered cell comprising the polypeptide according to any one of claims 74-80.

85. The engineered cell according to any one of claims 83-84, wherein the cell is a T-cell or Natural Killer cell.

86. The engineered cell according to claim 85, wherein the T-cell is a CD4 T-cell or CD8 T-cell.

87. The engineered cell according to claim 59, wherein the Natural Killer cell is a NKT cell or NK-92cell.

88. A method of reducing the number of target cells, the method comprising the steps of:

i. contacting said target cells with an effective amount of an engineered cell according to any one of claims 27-40, 55-60, and 83-87; and

ii. optionally, assaying for the reduction in the number of said cells;

wherein said target cells comprise at least one cell surface antigen selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BCMA, BAFF-R, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41, CD45, CD61, CD64, CD68, CD117, CD123, CD138, CD30, CD269, CD38, Flt3 receptor, and CS1.

89. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises BCMA; and the second antigen recognition domain comprises CS1.

90. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises BCMA.

91. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the target of the second antigen recognition domain comprises CD22.

92. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises CD20.

93. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises CD123.

94. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition domain comprises CD123.

95. The engineered cell according to any one of claims 27-32, wherein the target of the first antigen recognition domain comprises CD269; and the target of the second antigen recognition domain comprises CS1.

96. The engineered cell according to any one of claims 55-60, wherein the target of the antigen recognition domain comprises CD4 and the enhancer comprises IL-15/IL-15sushi.

97. The engineered cell according to any one of claims 27-32, wherein TAC1 antigen recognition domain comprises the APRIL ligand or the BAFF ligand or a portion thereof.

98. The engineered cell according to any one of claims 27-32, wherein the BCMA antigen recognition domain comprises APRIL ligand or BAFF ligand or a portion thereof.

99. The engineered cell according to any one of claims 27-32, wherein the BAFF-R antigen recognition domain comprises the BAFF ligand or a portion thereof.

100. The engineered cell according to any one of claims 27-32, wherein the first co-stimulatory domain and the second co-stimulatory domain are same.

101. The engineered cell according to any one of claims 27-32 and 37, wherein the first co-stimulatory domain and the second co-stimulatory domain comprise 4-1BB co-stimulatory domain.

102. A method of treating an autoimmune disorder or antibody mediated organ rejection comprising:

administering to a subject in need thereof, an effective amount of

a first engineered cell comprising a first chimeric antigen receptor polypeptide having an antigen recognition domain; and a second chimeric antigen receptor polypeptide having a second antigen recognition domain; and

a second engineered cell comprising a third chimeric antigen receptor polypeptide having a third antigen recognition domain; and a fourth chimeric antigen receptor polypeptide having a fourth antigen recognition domain;

wherein

the first antigen recognition domain and second antigen recognition domain are independently selected from the group consisting of CD19, CD20, and CD22; and

the third antigen recognition domain and fourth antigen recognition domain are independently selected from the group consisting of BCMA, CS1, BAFF, APRIL, BAFF-R, and TACI;

the first antigen recognition domain and second antigen recognition domain are different; and the third antigen recognition domain and fourth antigen recognition domain are different.

103. The method according to claim 102, wherein the first engineered cell and second engineered cell are NK cells or T cells.

104. A chimeric antigen receptor engineered cell comprising:

a first chimeric antigen receptor polypeptide comprising a signal peptide, CD4 or CD25 antigen recognition domain, a first transmembrane domain, first hinge region, and a co-stimulatory domain; and

a second chimeric antigen receptor polypeptide comprising a signal peptide, CD4 or CD25 antigen recognition domain, a second transmembrane domain, a second hinge region, and a signaling domain;

wherein the first antigen recognition domain and second antigen recognition domain are different.

105. The chimeric antigen receptor engineered cell according to claim 104, wherein said cell is at least one of CD4 deficient and CD25 deficient.

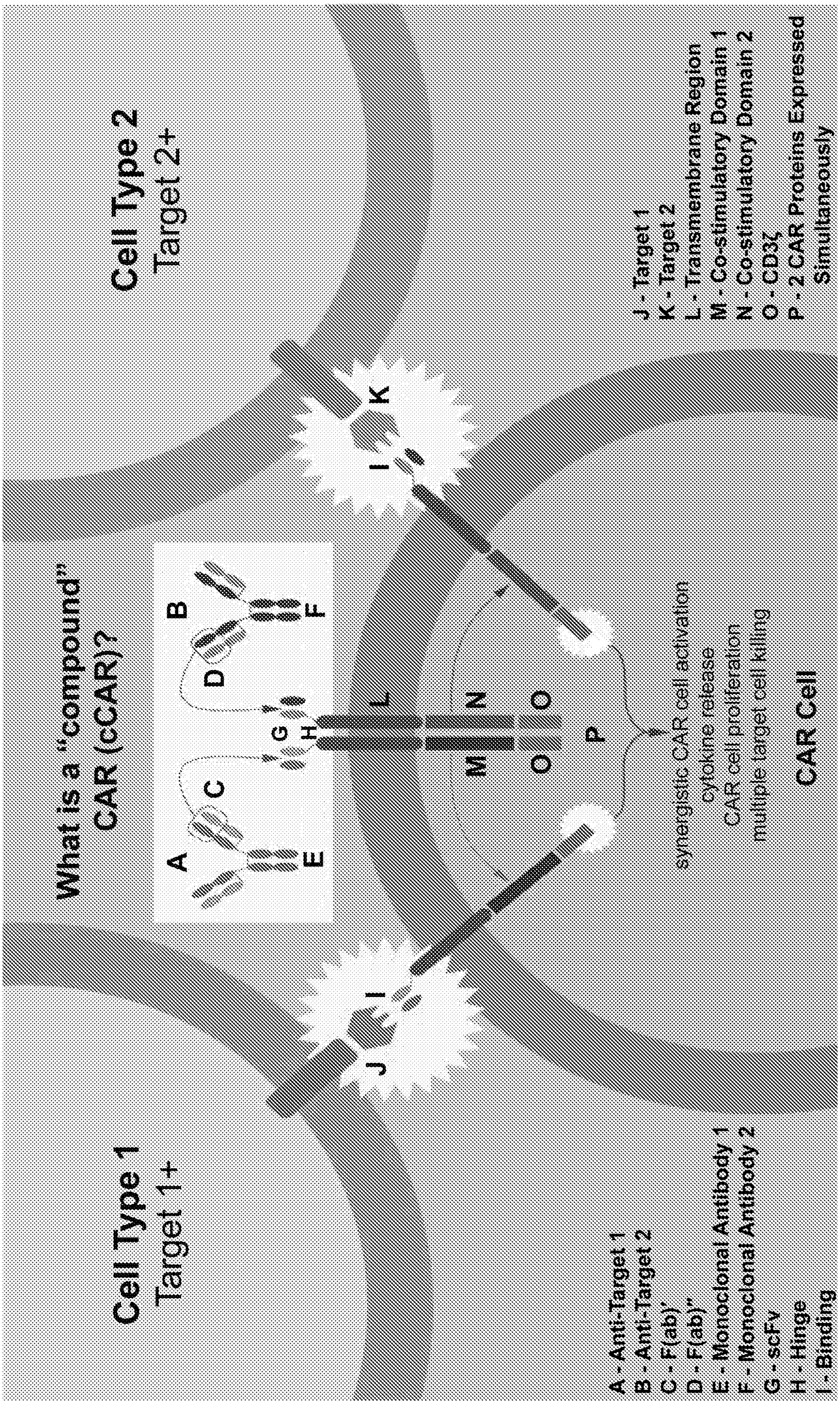

What is a "compound" CAR (cCAR)?
Cell Type 1
Target 1+
Cell Type 2
Target 2+
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
synergistic CAR cell activation
cytokine release
CAR cell proliferation
multiple target cell killing
CAR Cell
A - Anti-Target 1
B - Anti-Target 2
C - F(ab)'
D - F(ab)"
E - Monoclonal Antibody 1
F - Monoclonal Antibody 2
G - scFv
H - Hinge
I - Binding
J - Target 1
K - Target 2
L - Transmembrane Region
M - Co-stimulatory Domain 1
N - Co-stimulatory Domain 2
O - CD3ζ
P - 2 CAR Proteins Expressed Simultaneously

FIGURE 1

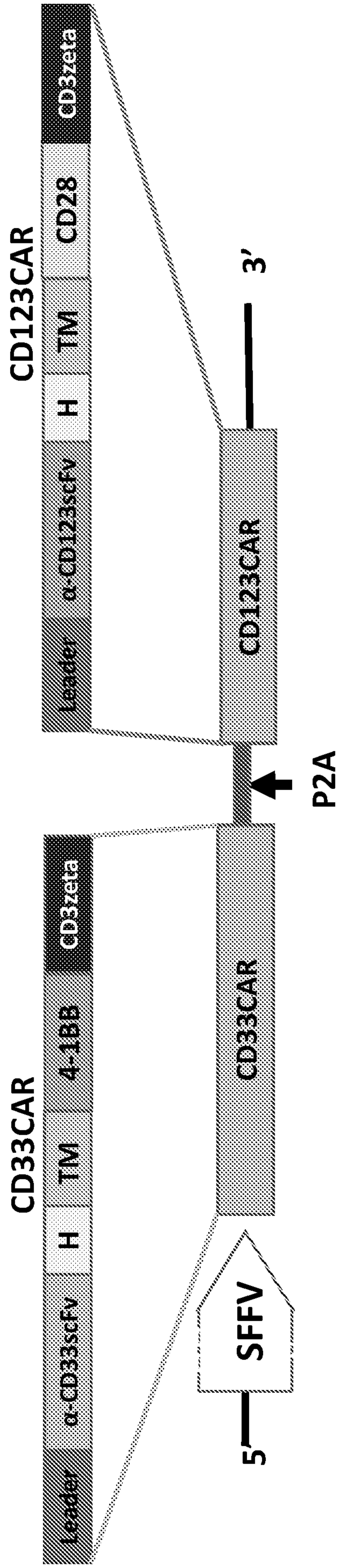

CD33CAR
Leader
α-CD33scFv
H
TM
4-1BB
CD3zeta
CD123CAR
Leader
α-CD123scFv
H
TM
CD28
CD3zeta
5'
SFFV
CD33CAR
P2A
CD123CAR
3'

FIGURE 2A

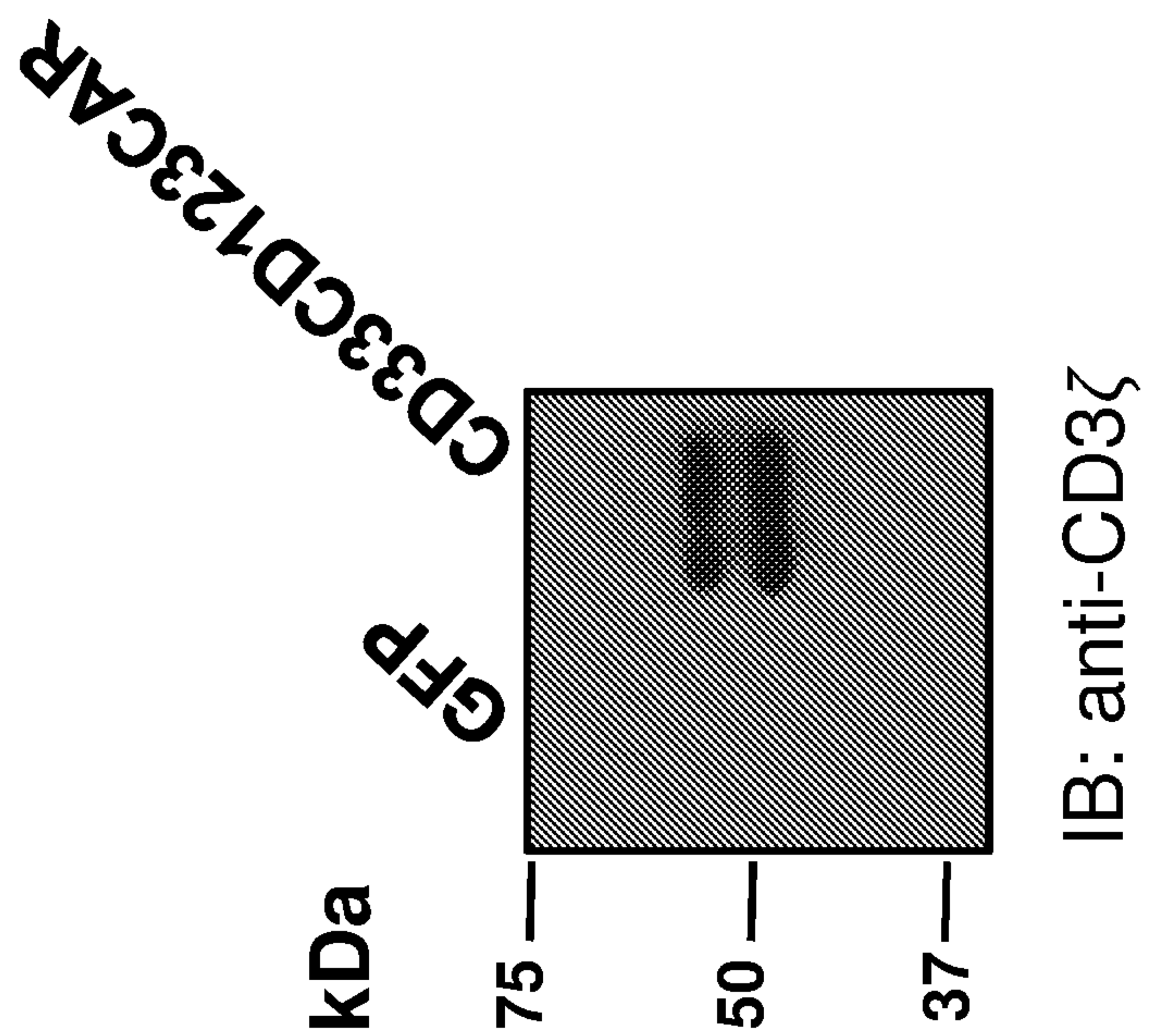


FIGURE 2B

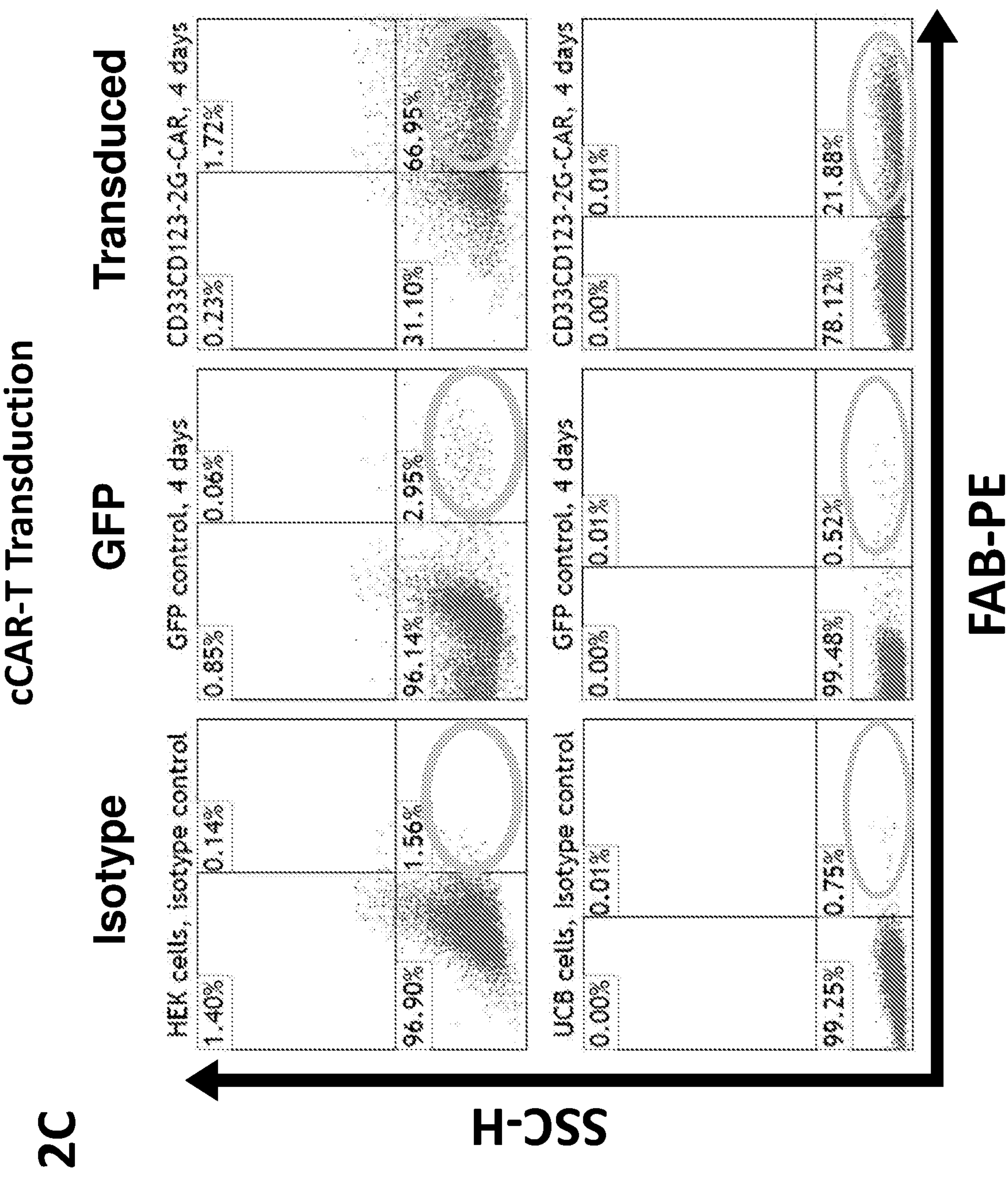

cCAR-T Transduction
Isotype
GFP
Transduced
HEK cells, isotype control
1.40%
0.14%
96.90%
1.56%
GFP control, 4 days
0.85%
0.06%
96.14%
2.95%
CD33CD123-2G-CAR, 4 days
0.23%
1.72%
31.10%
66.95%
UCB cells, isotype control
0.00%
0.01%
99.25%
0.75%
GFP control, 4 days
0.00%
0.01%
99.48%
0.52%
CD33CD123-2G-CAR, 4 days
0.00%
0.01%
78.12%
21.88%
SSC-H
FAB-PE

FIGURE 2C

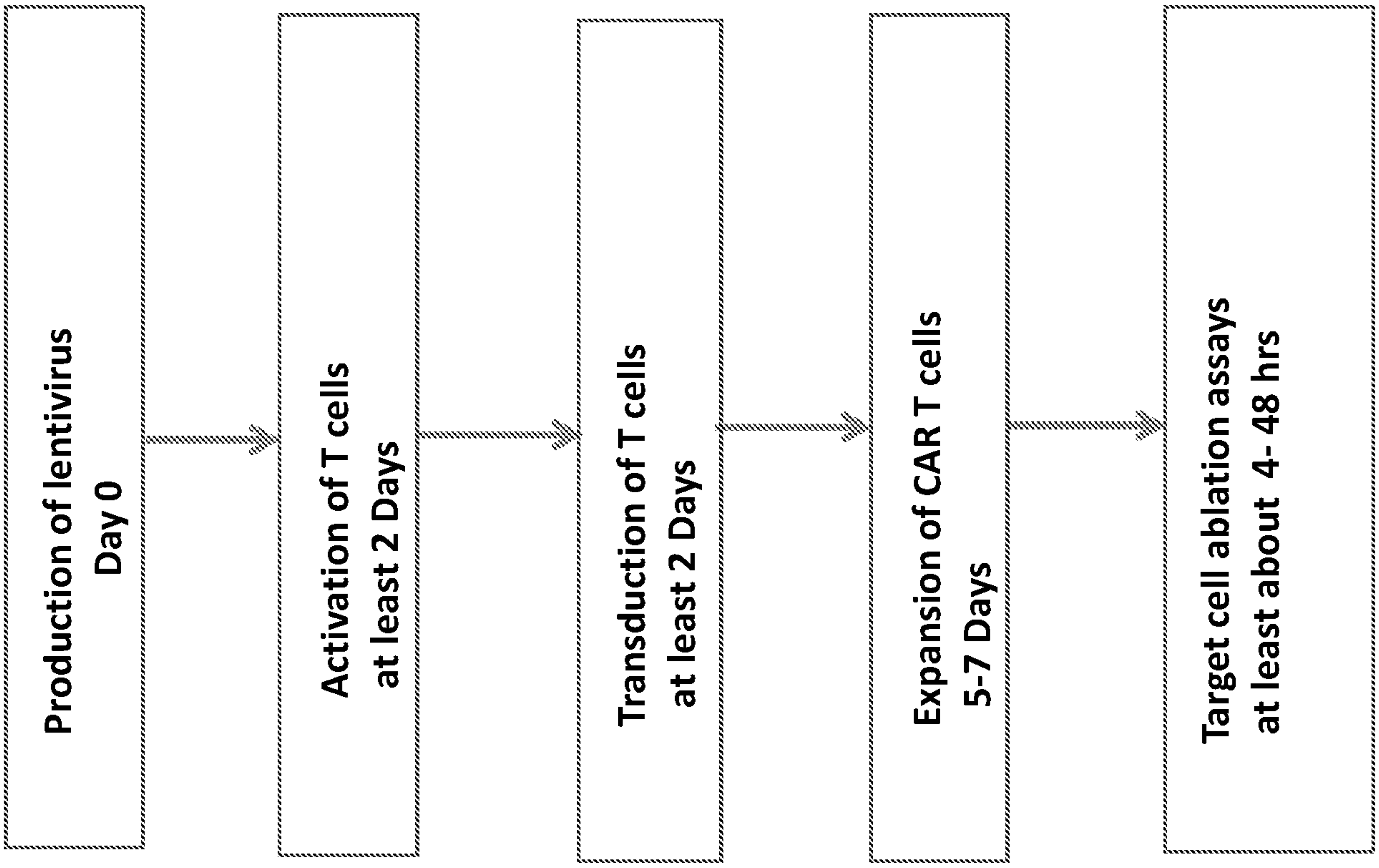

Production of lentivirus
Day 0
Activation of T cells
at least 2 Days
Transduction of T cells
at least 2 Days
Expansion of CAR T cells
5-7 Days
Target cell ablation assays
at least about 4- 48 hrs

FIGURE 3

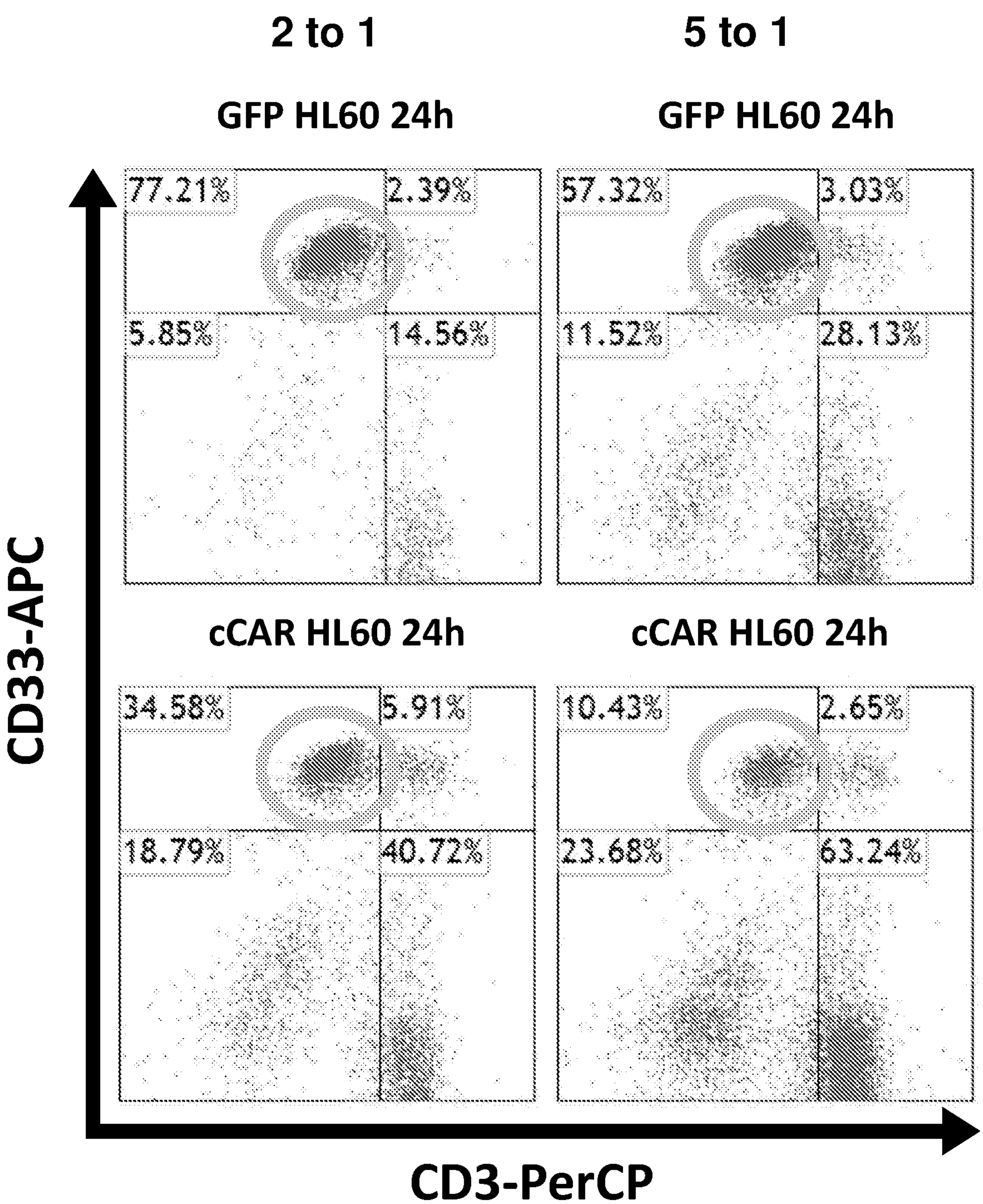

2 to 1
5 to 1
GFP HL60 24h
77.21%
2.39%
5.85%
14.56%
GFP HL60 24h
57.32%
3.03%
11.52%
28.13%
cCAR HL60 24h
34.58%
5.91%
18.79%
40.72%
cCAR HL60 24h
10.43%
2.65%
23.68%
63.24%
CD33-APC
CD3-PerCP

FIGURE 4

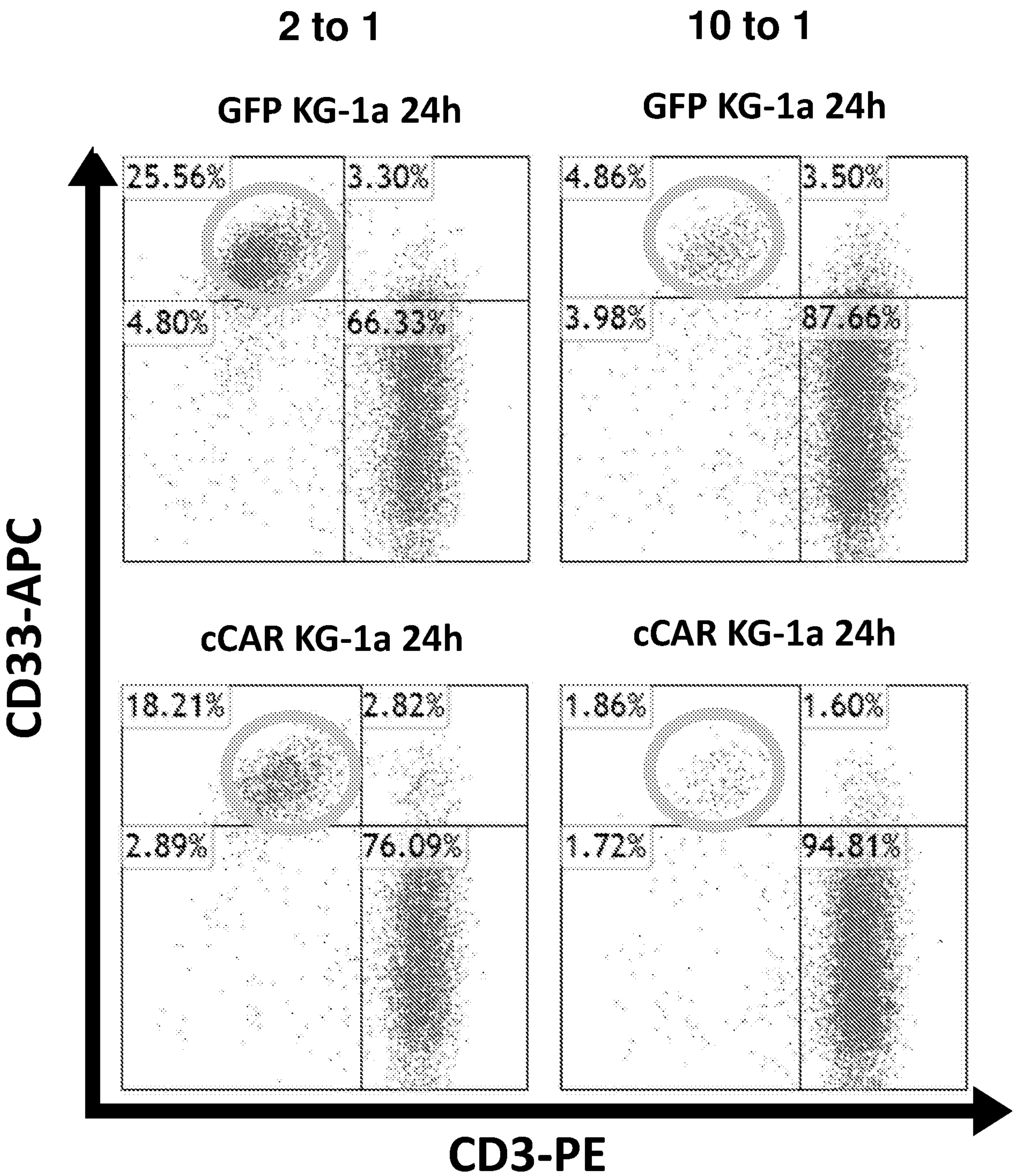
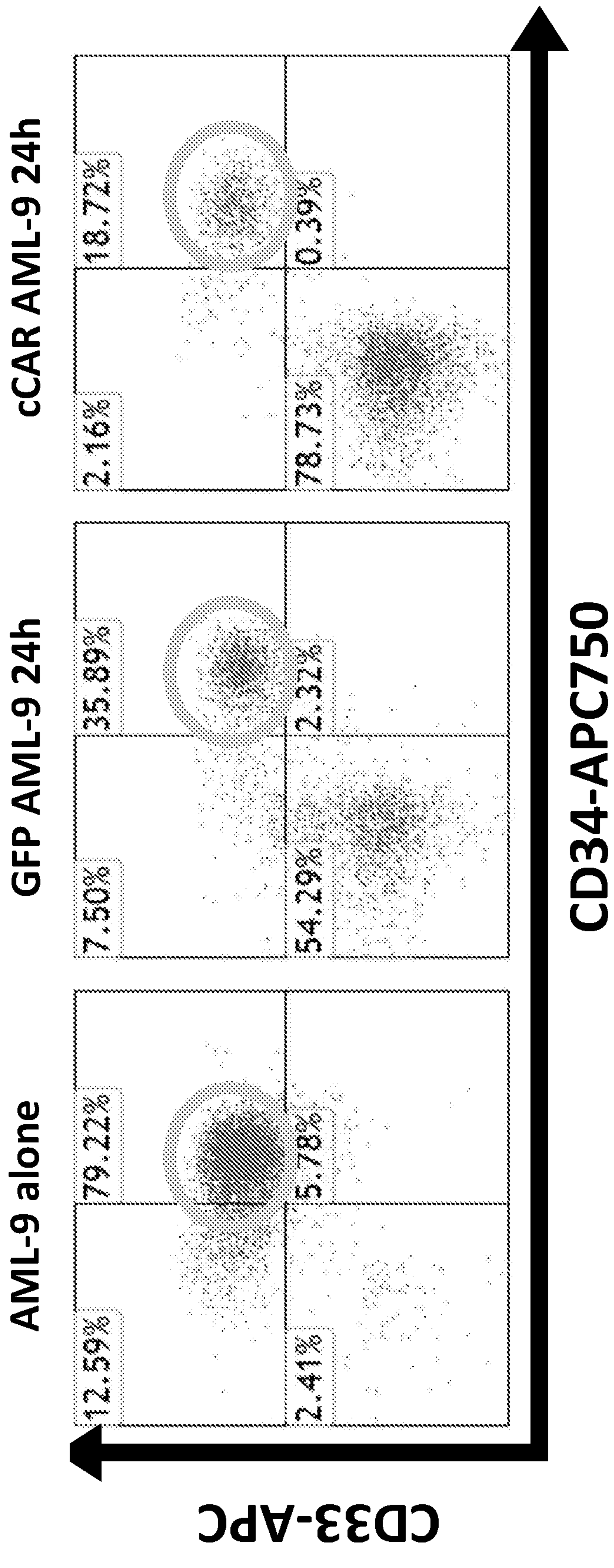

2 to 1
10 to 1
GFP KG-1a 24h
25.56%
3.30%
4.80%
66.33%
GFP KG-1a 24h
4.86%
3.50%
3.98%
87.66%
cCAR KG-1a 24h
18.21%
2.82%
2.89%
76.09%
cCAR KG-1a 24h
1.86%
1.60%
1.72%
94.81%
CD33-APC
CD3-PE

FIGURE 5

FIGURE 6


AML-9 alone
12.59%
79.22%
2.41%
5.78%
GFP AML-9 24h
7.50%
35.89%
54.29%
2.32%
cCAR AML-9 24h
2.16%
18.72%
78.73%
0.39%
CD34-APC750
CD33-APC

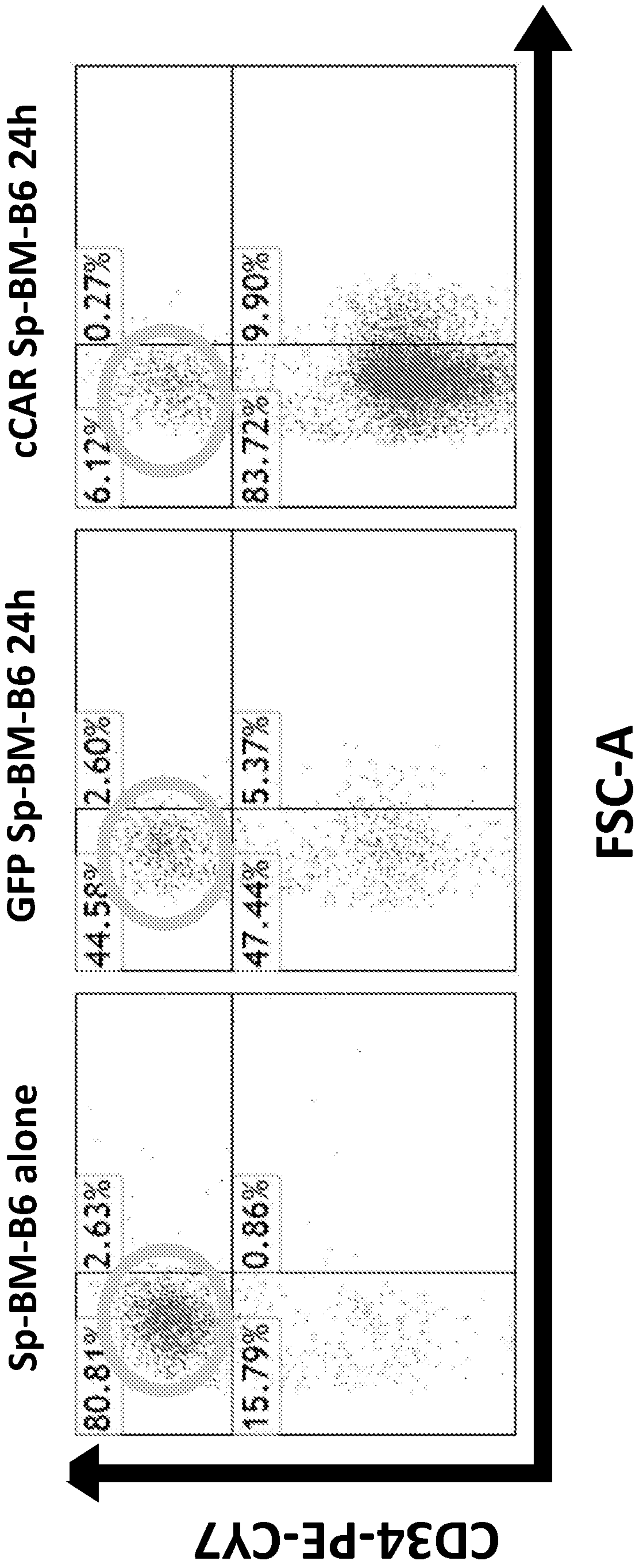

Sp-BM-B6 alone
80.81%
2.63%
15.79%
0.86%
GFP Sp-BM-B6 24h
44.58%
2.60%
47.44%
5.37%
cCAR Sp-BM-B6 24h
6.12%
0.27%
83.72%
9.90%
FSC-A
CD34-PE-CY7

FIGURE 7

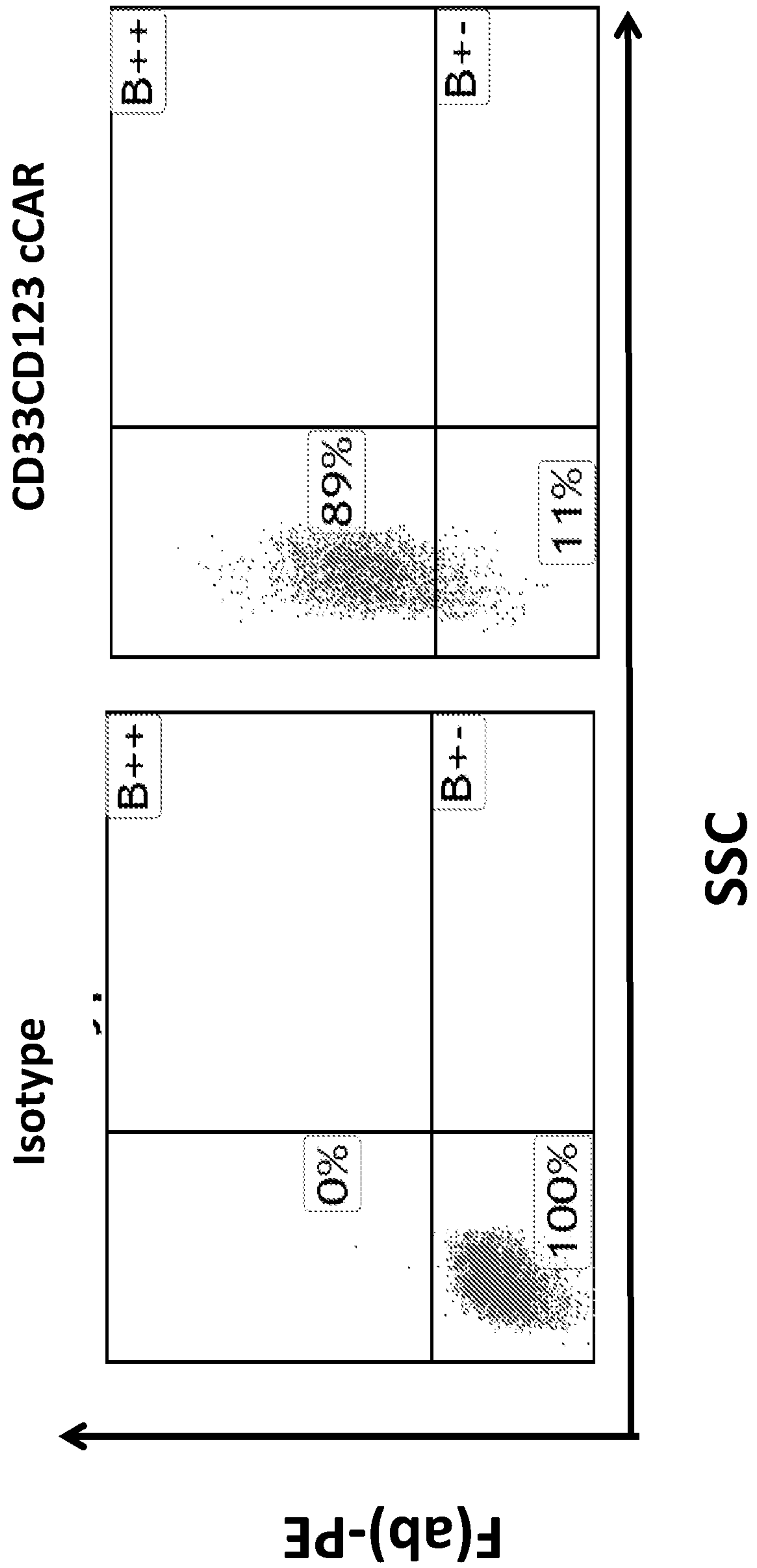


FIGURE 8

FIGURE 9

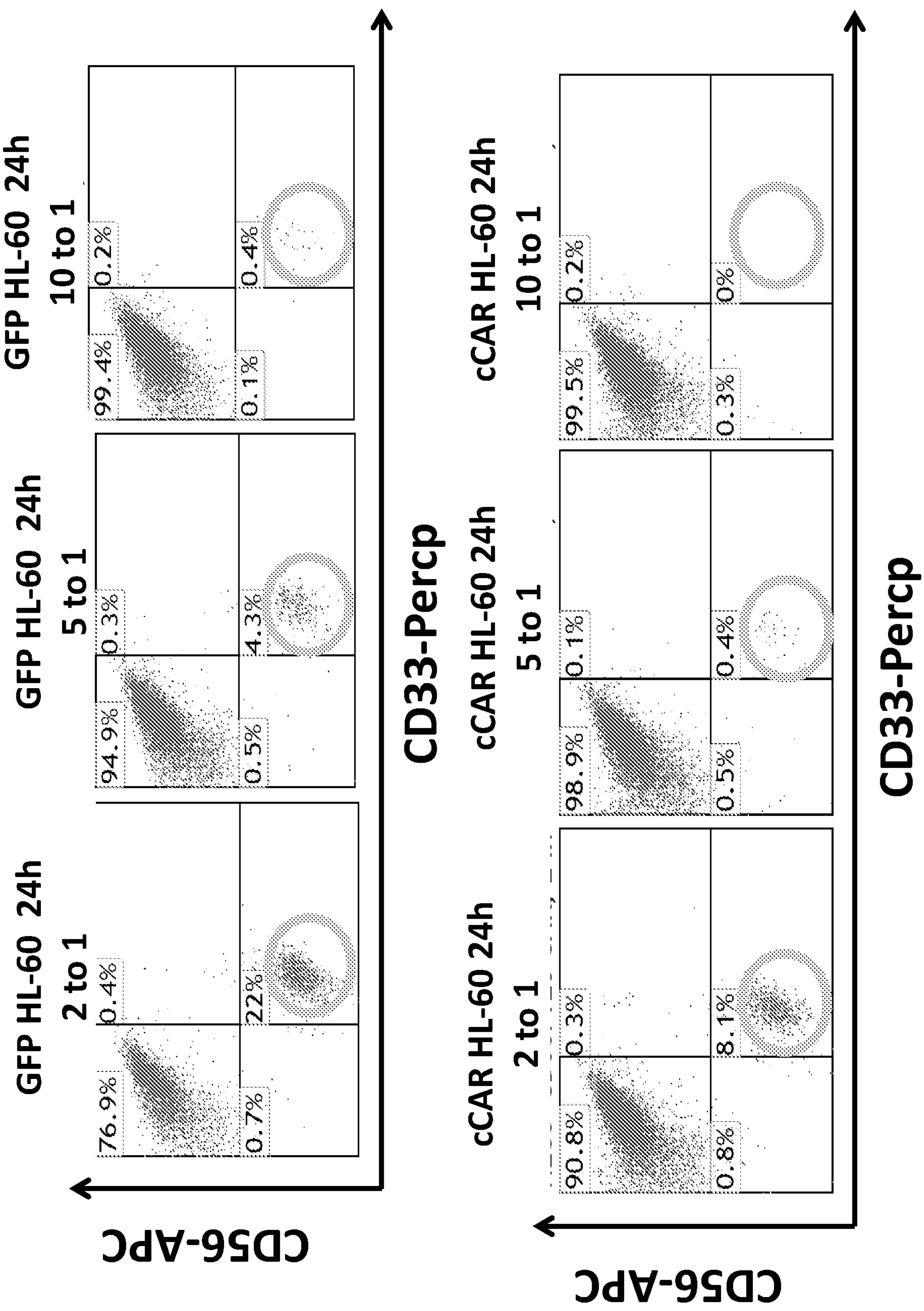

GFP HL-60 24h
2 to 1
76.9%
0.4%
0.7%
22%
GFP HL-60 24h
5 to 1
94.9%
0.3%
0.5%
4.3%
GFP HL-60 24h
10 to 1
99.4%
0.2%
0.1%
0.4%
CD33-Percp
CD56-APC
cCAR HL-60 24h
2 to 1
90.8%
0.3%
0.8%
8.1%
cCAR HL-60 24h
5 to 1
98.9%
0.1%
0.5%
0.4%
cCAR HL-60 24h
10 to 1
99.5%
0.2%
0.3%
0%
CD33-Percp
CD56-APC

FIGURE 10

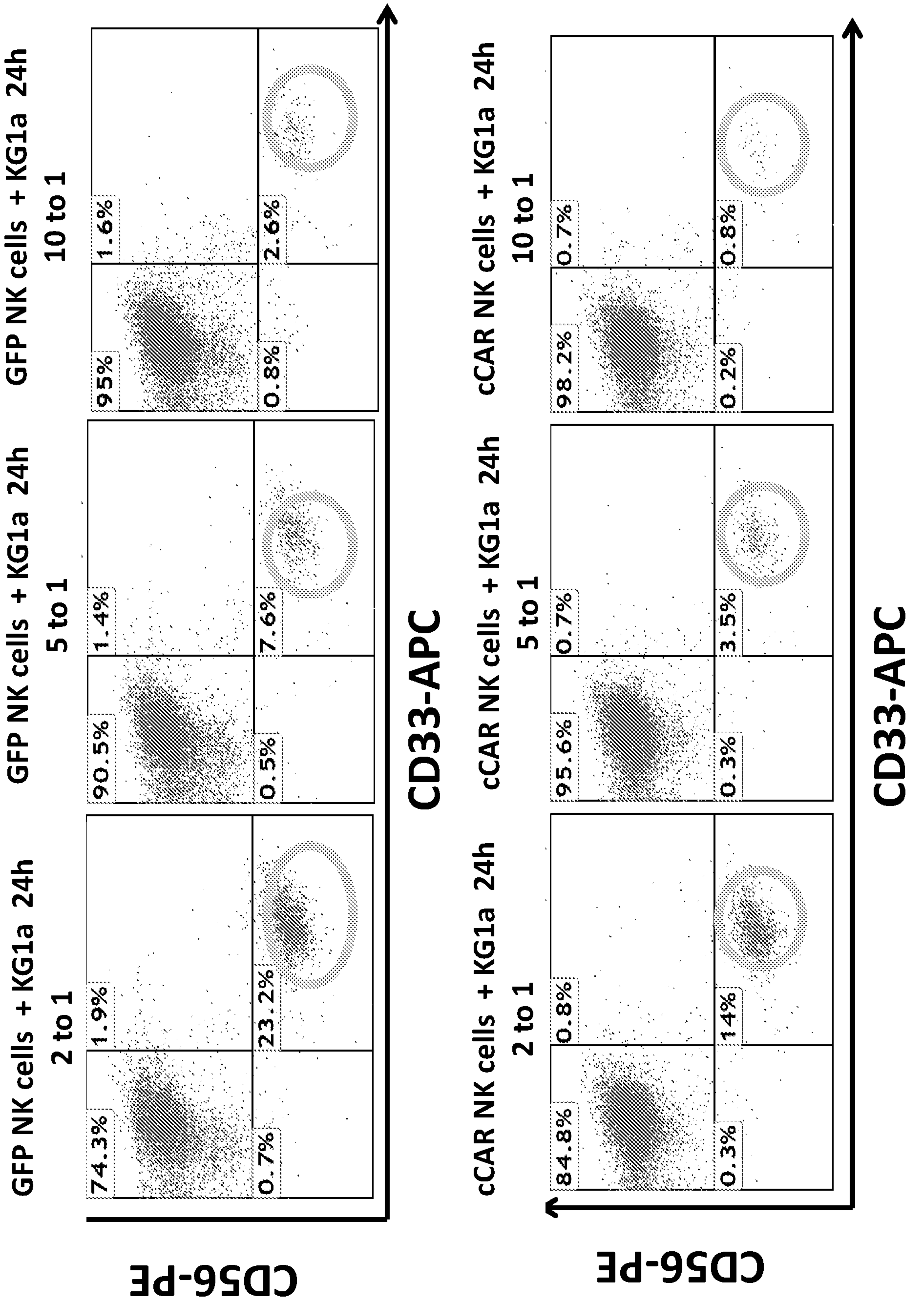

GFP NK cells + KG1a 24h
2 to 1
74.3%
1.9%
0.7%
23.2%
GFP NK cells + KG1a 24h
5 to 1
90.5%
1.4%
0.5%
7.6%
GFP NK cells + KG1a 24h
10 to 1
95%
1.6%
0.8%
2.6%
CD56-PE
CD33-APC
cCAR NK cells + KG1a 24h
2 to 1
84.8%
0.8%
0.3%
14%
cCAR NK cells + KG1a 24h
5 to 1
95.6%
0.7%
0.3%
3.5%
cCAR NK cells + KG1a 24h
10 to 1
98.2%
0.7%
0.2%
0.8%
CD56-PE
CD33-APC

FIGURE 11

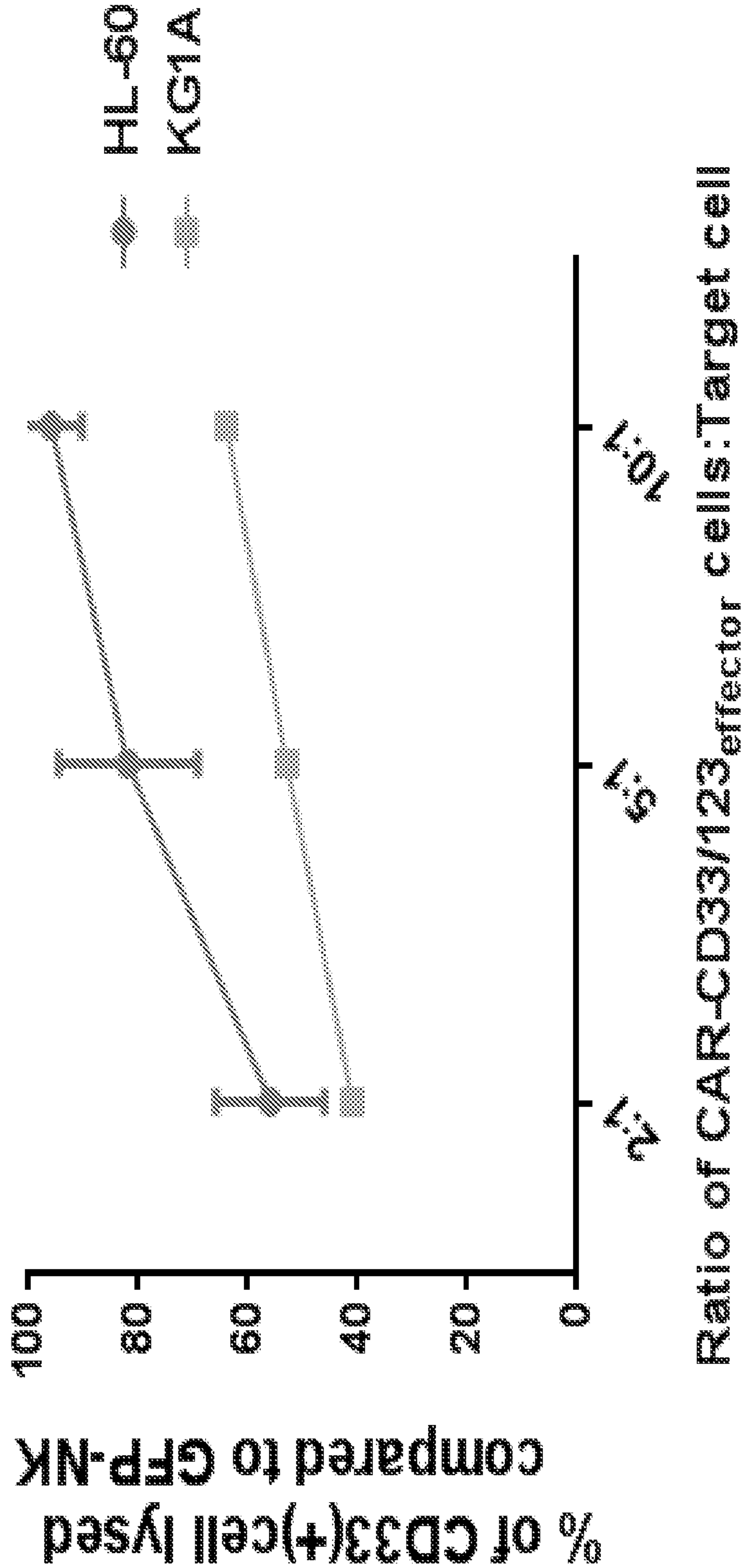
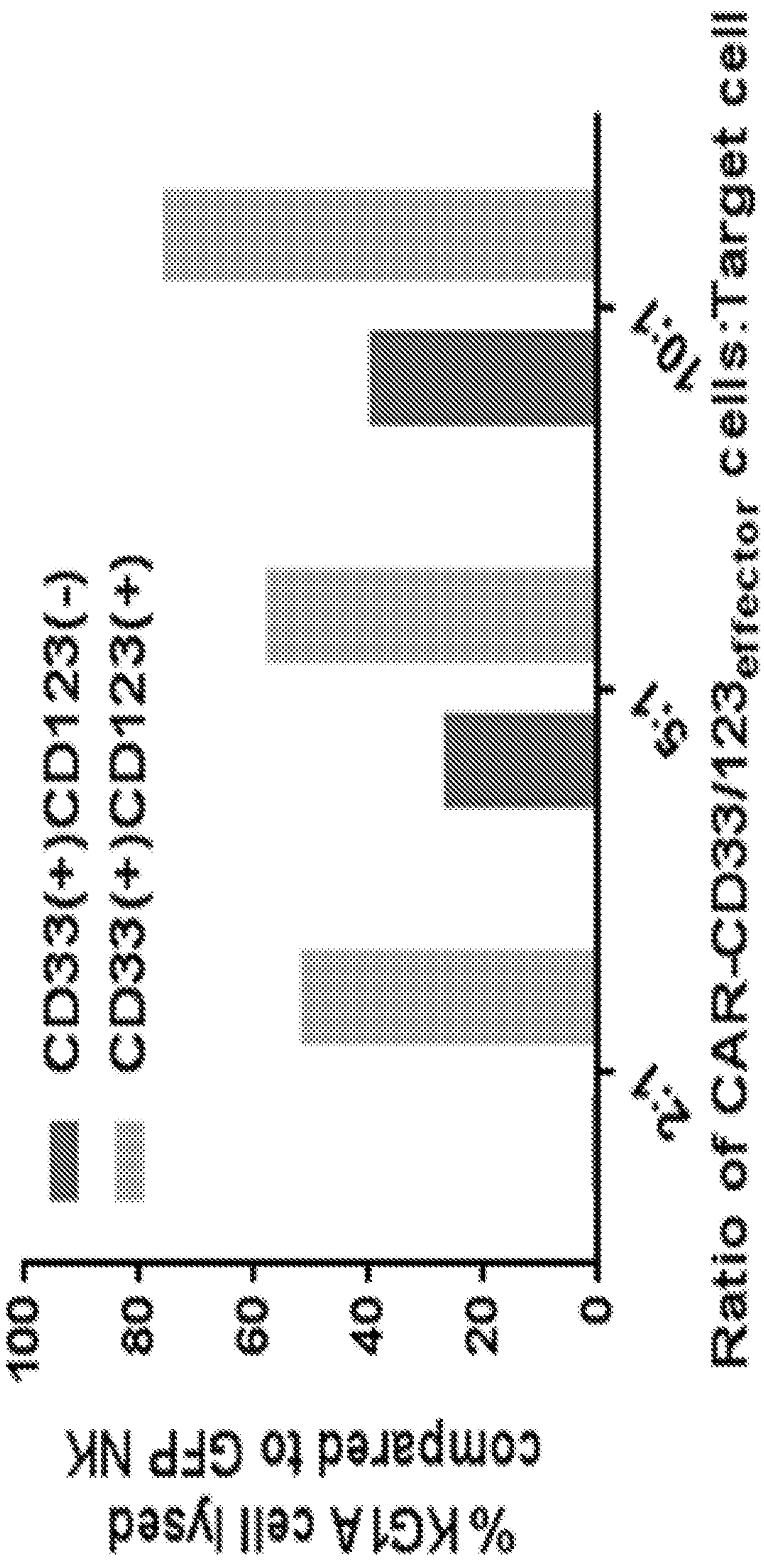

% of CD33(+)cell lysed
compared to GFP-NK
100
80
60
40
20
0
2:1
5:1
10:1
Ratio of CAR-CD33/123effector cells:Target cell
HL-60
KG1A

FIGURE 12


CD33(+)CD123(-)
CD33(+)CD123(+)
% KG1A cell lysed
compared to GFP NK
100
80
60
40
20
0
2:1
5:1
10:1
Ratio of CAR-CD33/123effector cells:Target cell

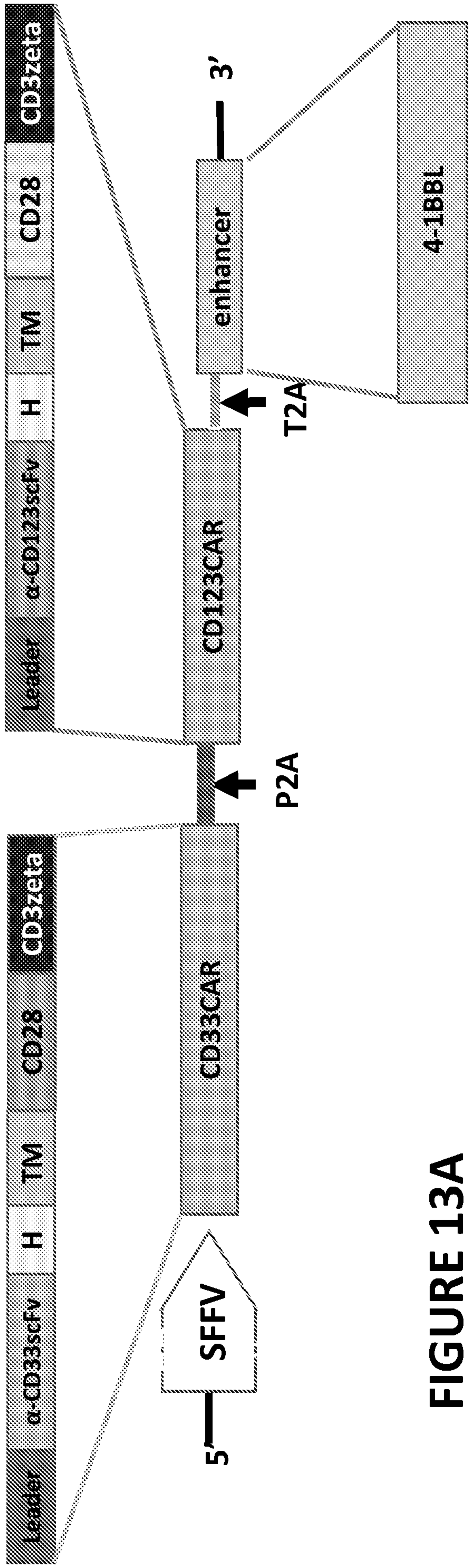

CD33CAR
Leader
α-CD33scFv
H
TM
CD28
CD3zeta
CD123CAR
Leader
α-CD123scFv
H
TM
CD28
CD3zeta
5'
SFFV
CD33CAR
P2A
CD123CAR
T2A
enhancer
3'
4-1BBL

FIGURE 13A

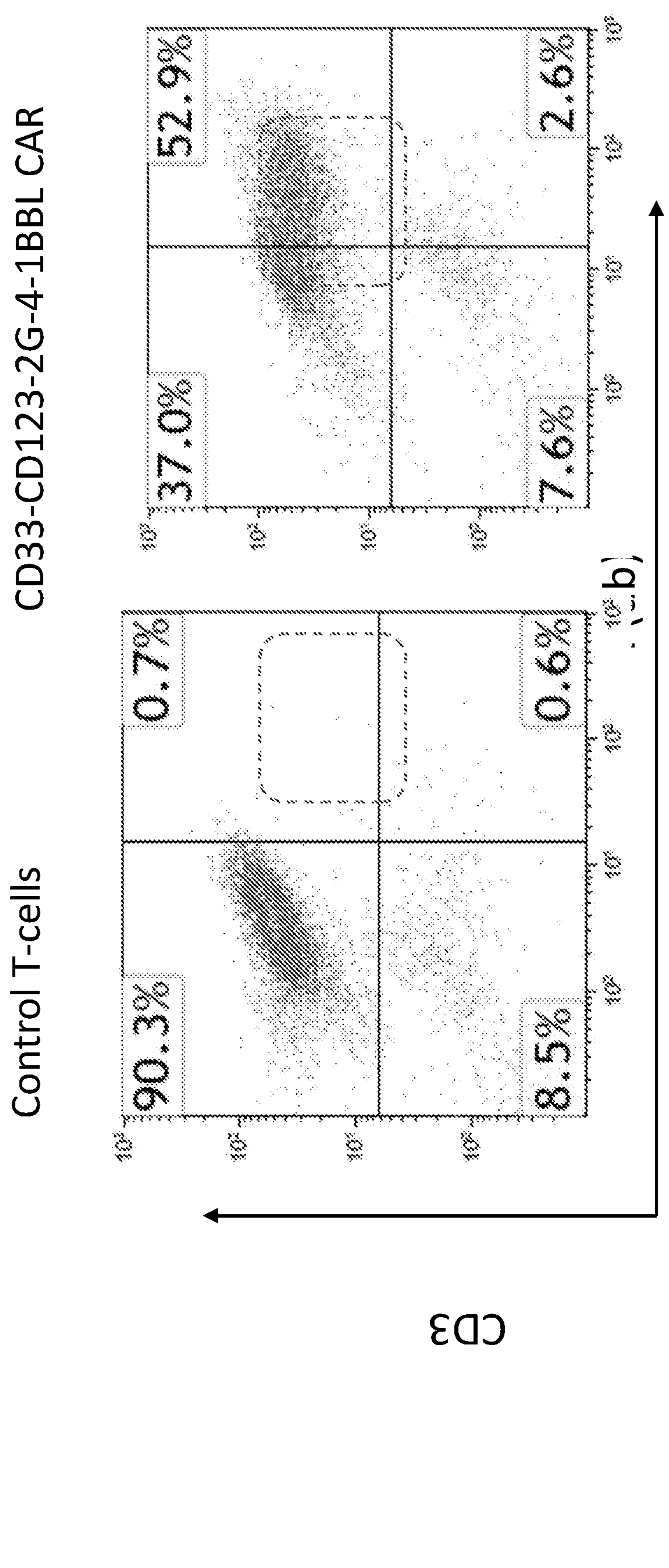

Control T-cells
CD33-CD123-2G-4-1BBL CAR
90.3%
0.7%
8.5%
0.6%
52.9%
37.0%
2.6%
7.6%
CD3

FIGURE 13B

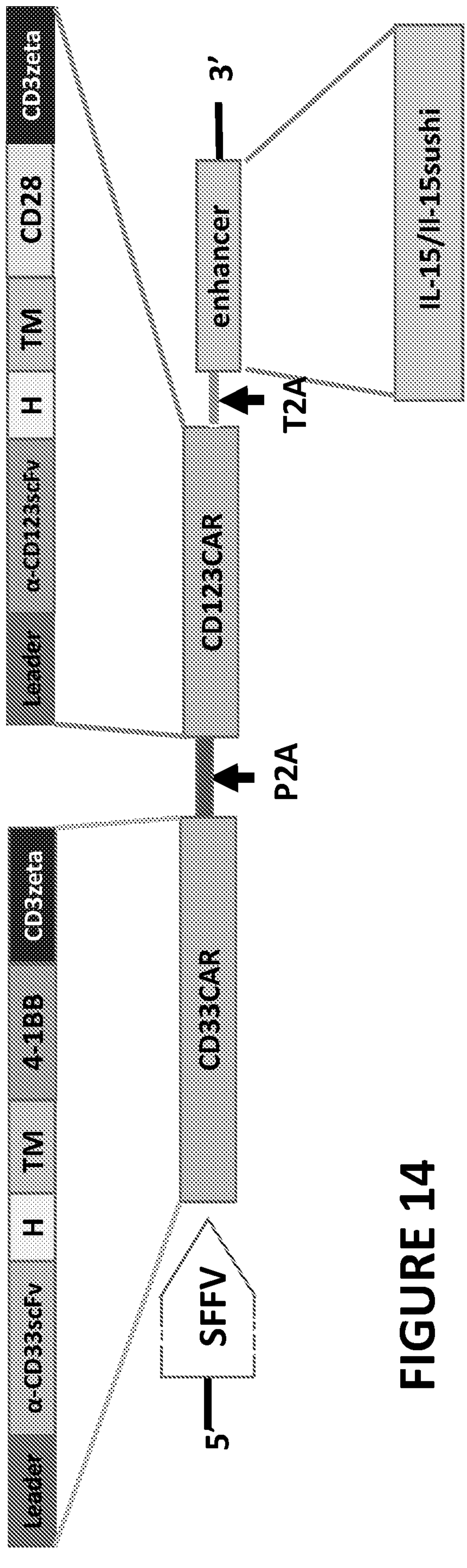

CD33CAR
Leader
α-CD33scFv
H
TM
4-1BB
CD3zeta
CD123CAR
Leader
α-CD123scFv
H
TM
CD28
CD3zeta
5'
SFFV
CD33CAR
P2A
CD123CAR
T2A
enhancer
3'
IL-15/Il-15sushi

FIGURE 14

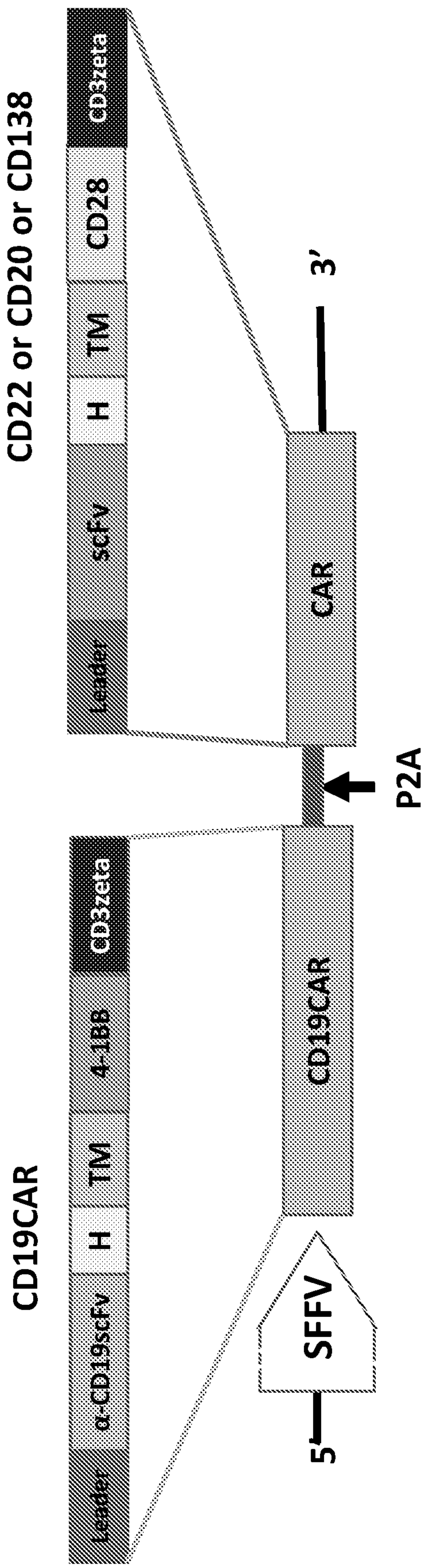
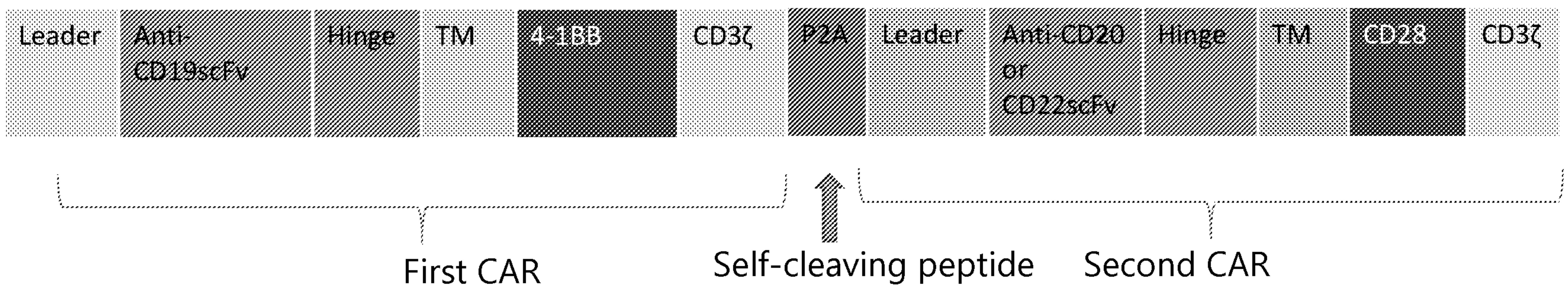

CD19CAR
Leader
α-CD19scFv
H
TM
4-1BB
CD3zeta
CD22 or CD20 or CD138
Leader
scFv
H
TM
CD28
CD3zeta
5'
SFFV
CD19CAR
P2A
CAR
3'

FIGURE 15

FIGURE 16A



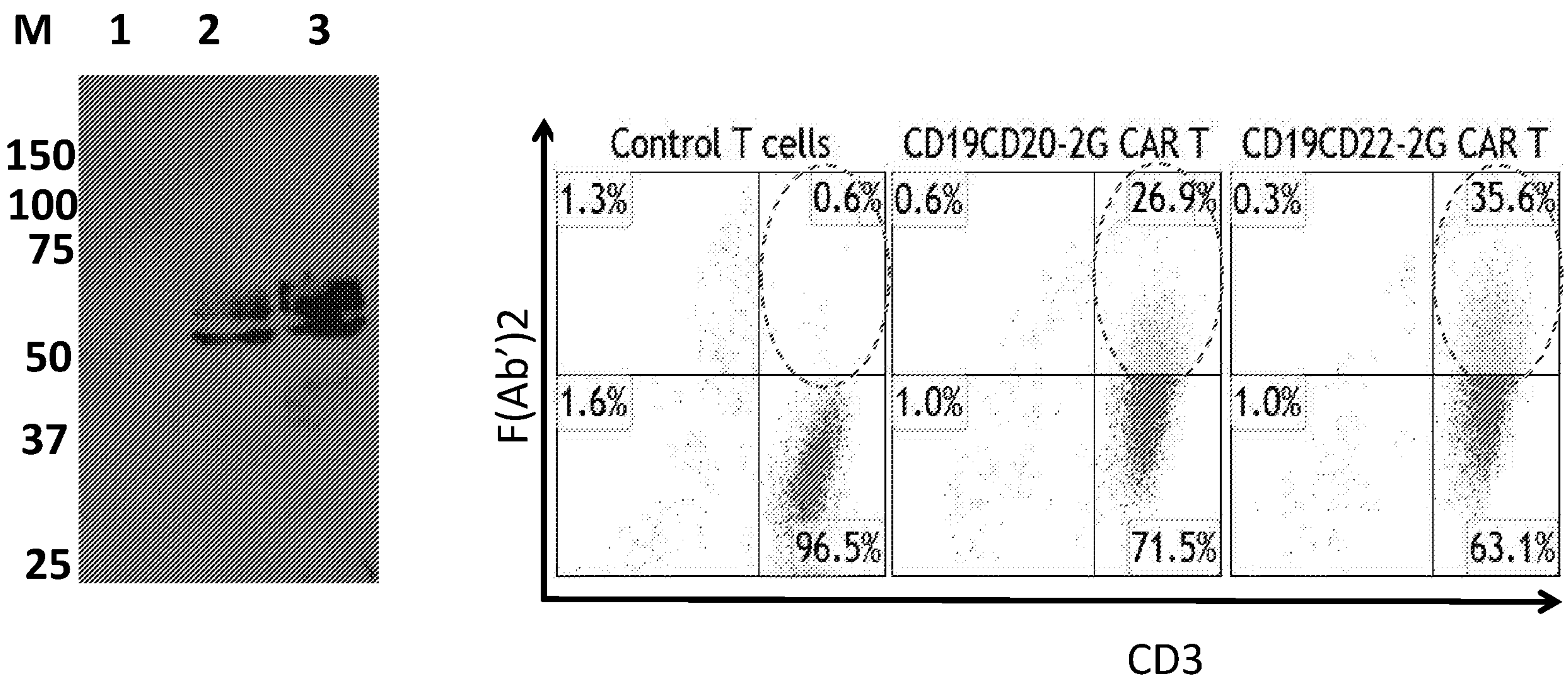


FIGURE 16B

FIGURE 17

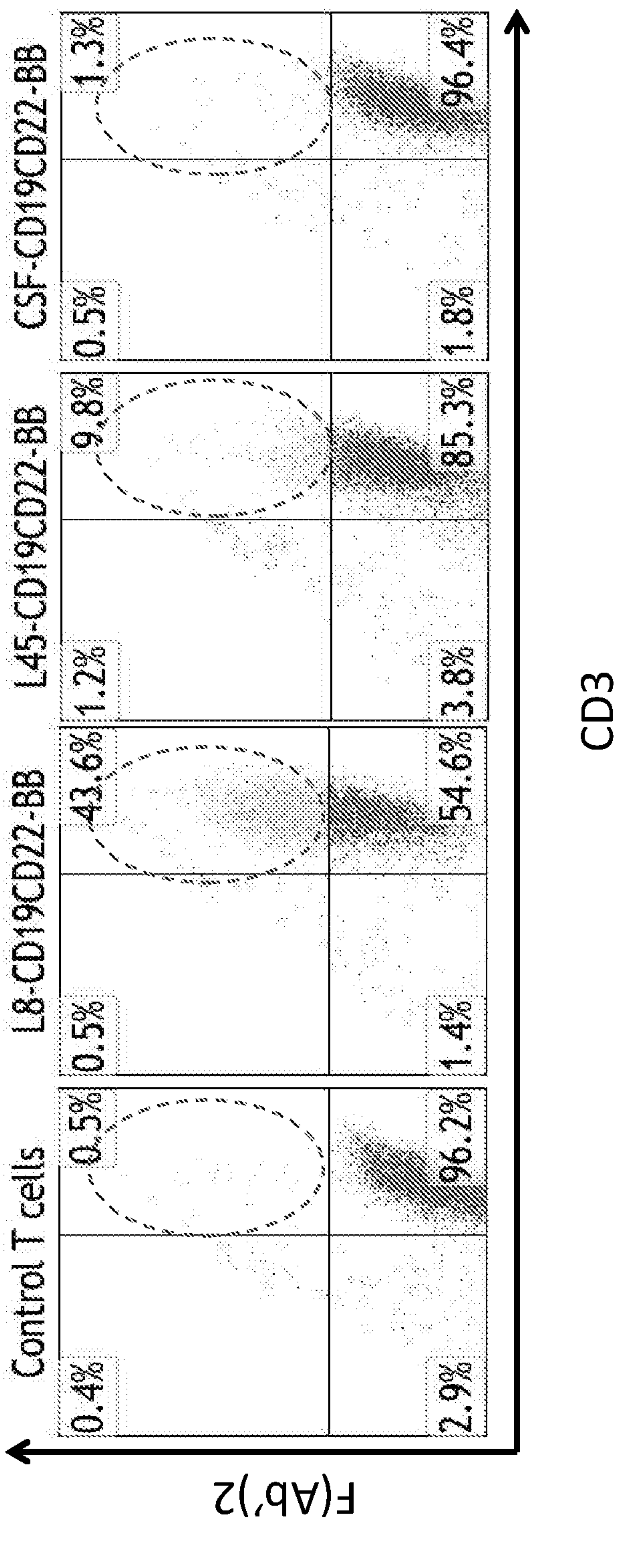

Control T cells
0.4%
0.5%
2.9%
96.2%
L8-CD19CD22-BB
0.5%
43.6%
1.4%
54.6%
L45-CD19CD22-BB
1.2%
9.8%
3.8%
85.3%
CSF-CD19CD22-BB
0.5%
1.3%
1.8%
96.4%
CD3
F(Ab')2

FIGURE 18

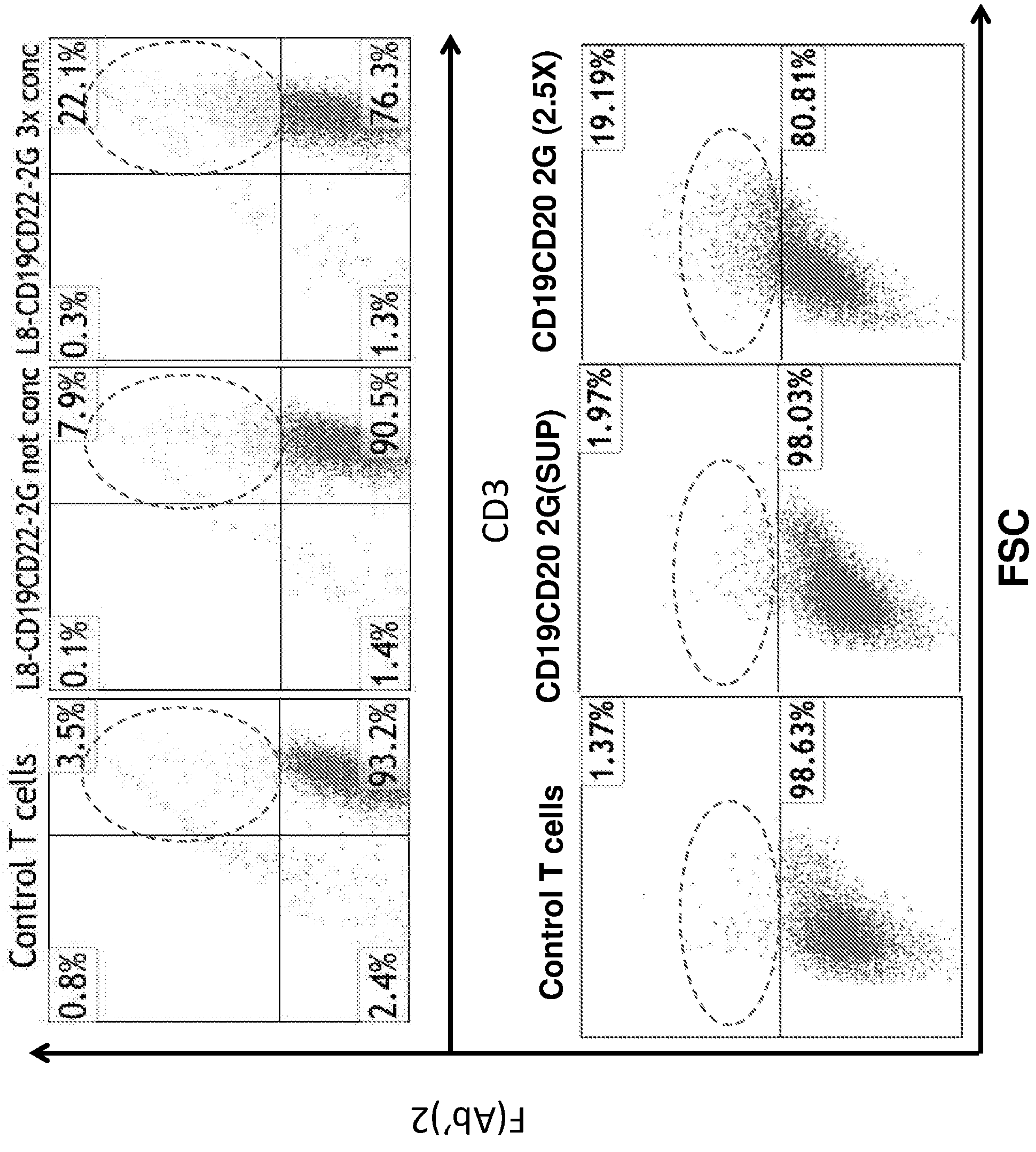
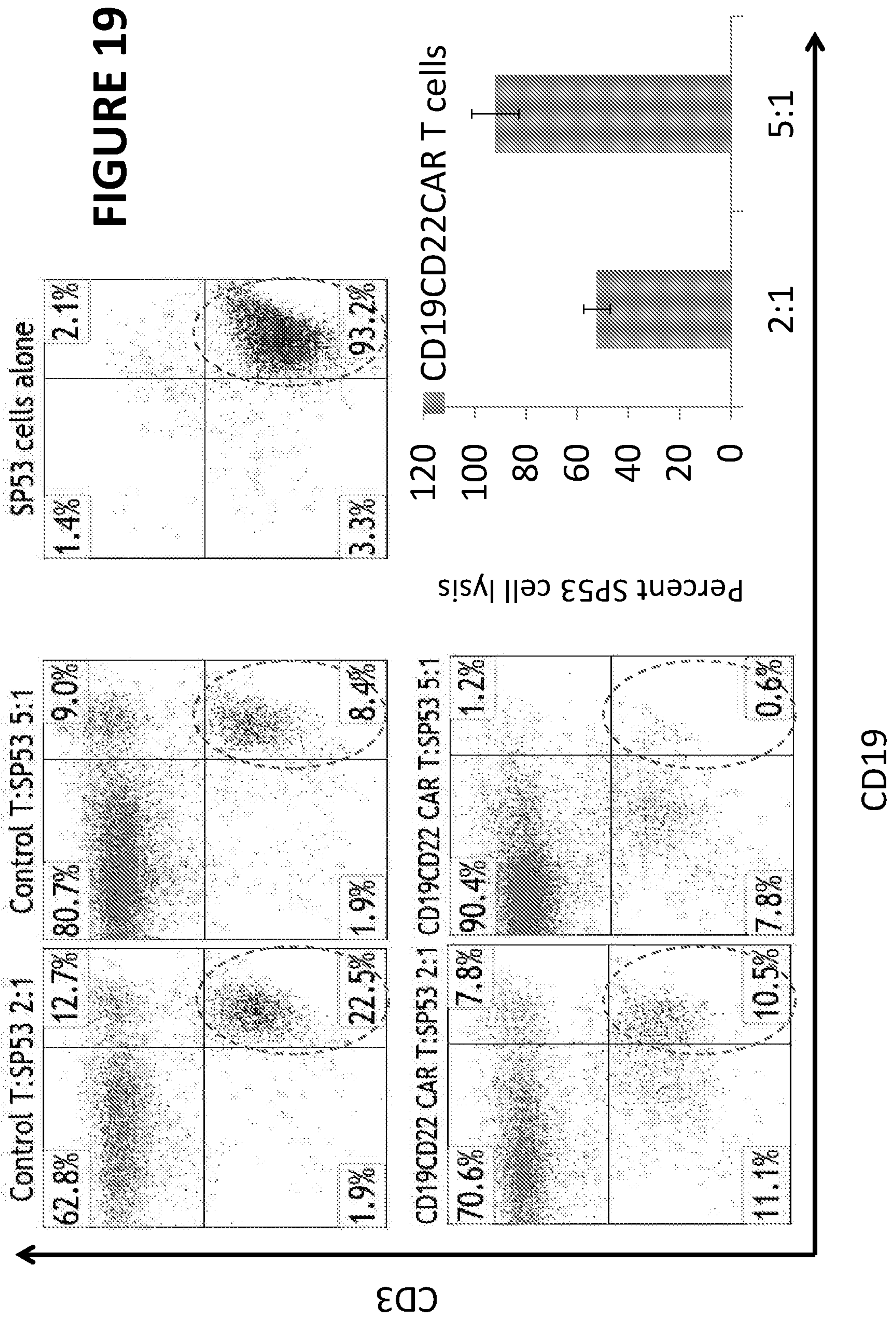

Control T cells
0.8%
3.5%
2.4%
93.2%
L8-CD19CD22-2G not conc
0.1%
7.9%
1.4%
90.5%
L8-CD19CD22-2G 3x conc
0.3%
22.1%
1.3%
76.3%
CD3
Control T cells
1.37%
98.63%
CD19CD20 2G(SUP)
1.97%
98.03%
CD19CD20 2G (2.5X)
19.19%
80.81%
FSC
F(Ab')2

FIGURE 19


Control T:SP53 2:1
62.8%
12.7%
1.9%
22.5%
Control T:SP53 5:1
80.7%
9.0%
1.9%
8.4%
SP53 cells alone
1.4%
2.1%
3.3%
93.2%
CD19CD22 CAR T:SP53 2:1
70.6%
7.8%
11.1%
10.5%
CD19CD22 CAR T:SP53 5:1
90.4%
1.2%
7.8%
0.6%
CD3
CD19
Percent SP53 cell lysis
120
100
80
60
40
20
0
CD19CD22CAR T cells
2:1
5:1

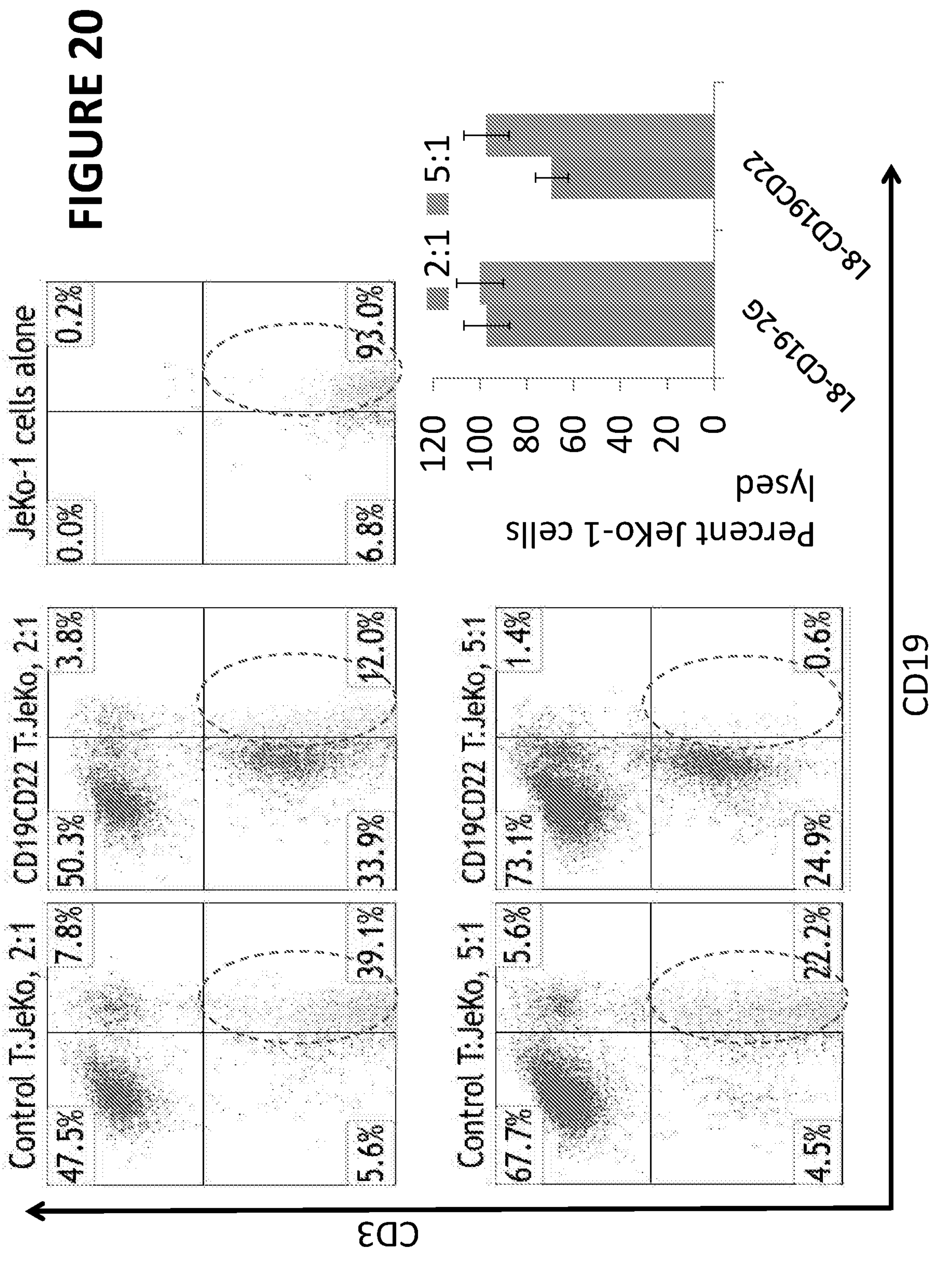

Control T:JeKo, 2:1
47.5%
7.8%
5.6%
39.1%
CD19CD22 T:JeKo, 2:1
50.3%
3.8%
33.9%
12.0%
JeKo-1 cells alone
0.0%
0.2%
6.8%
93.0%
Control T:JeKo, 5:1
67.7%
5.6%
4.5%
22.2%
CD19CD22 T:JeKo, 5:1
73.1%
1.4%
24.9%
0.6%
Percent JeKo-1 cells lysed
120
100
80
60
40
20
0
2:1
5:1
L8-CD19-2G
L8-CD19CD22
CD3
CD19

FIGURE 20

FIGURE 21

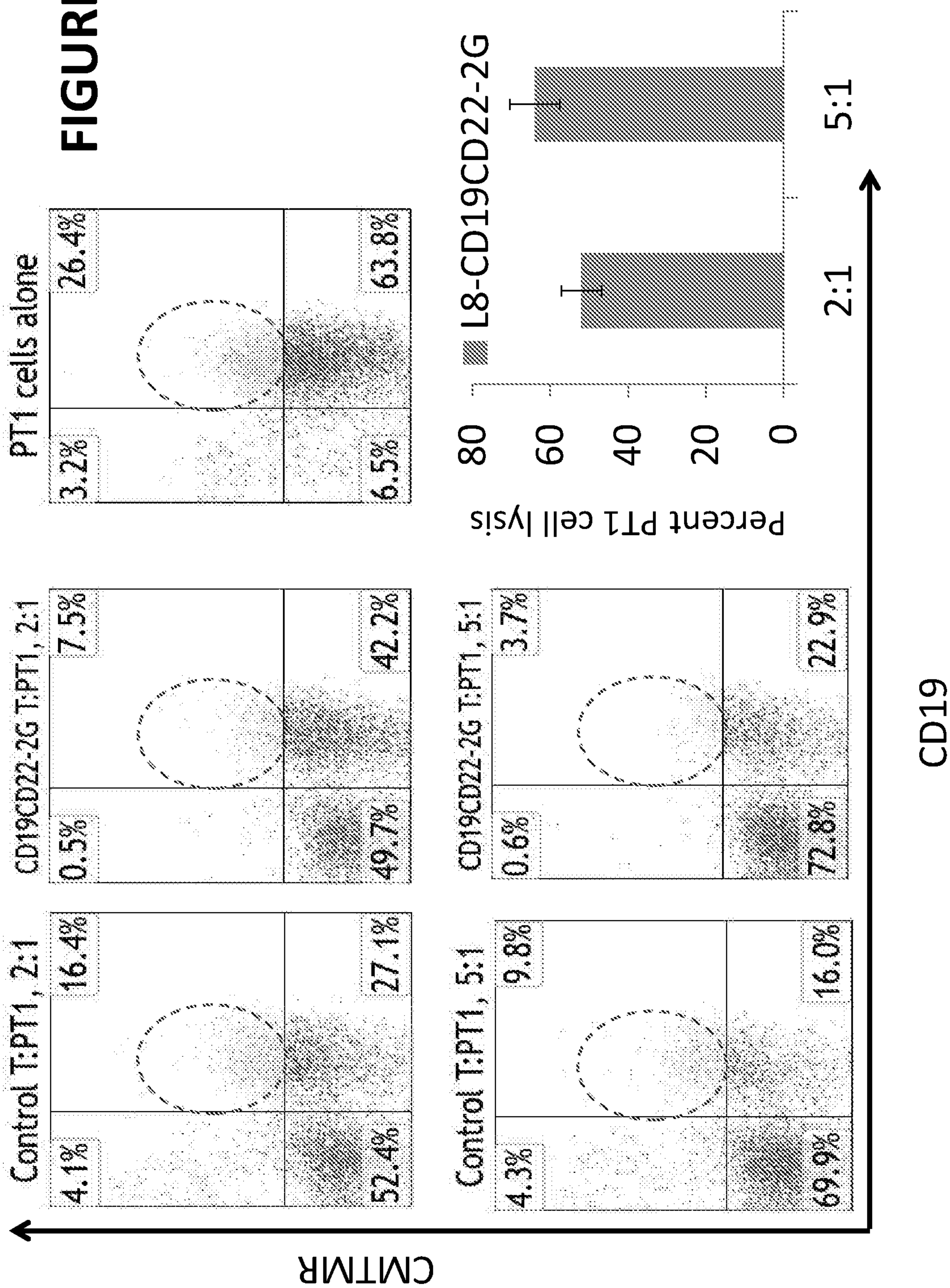
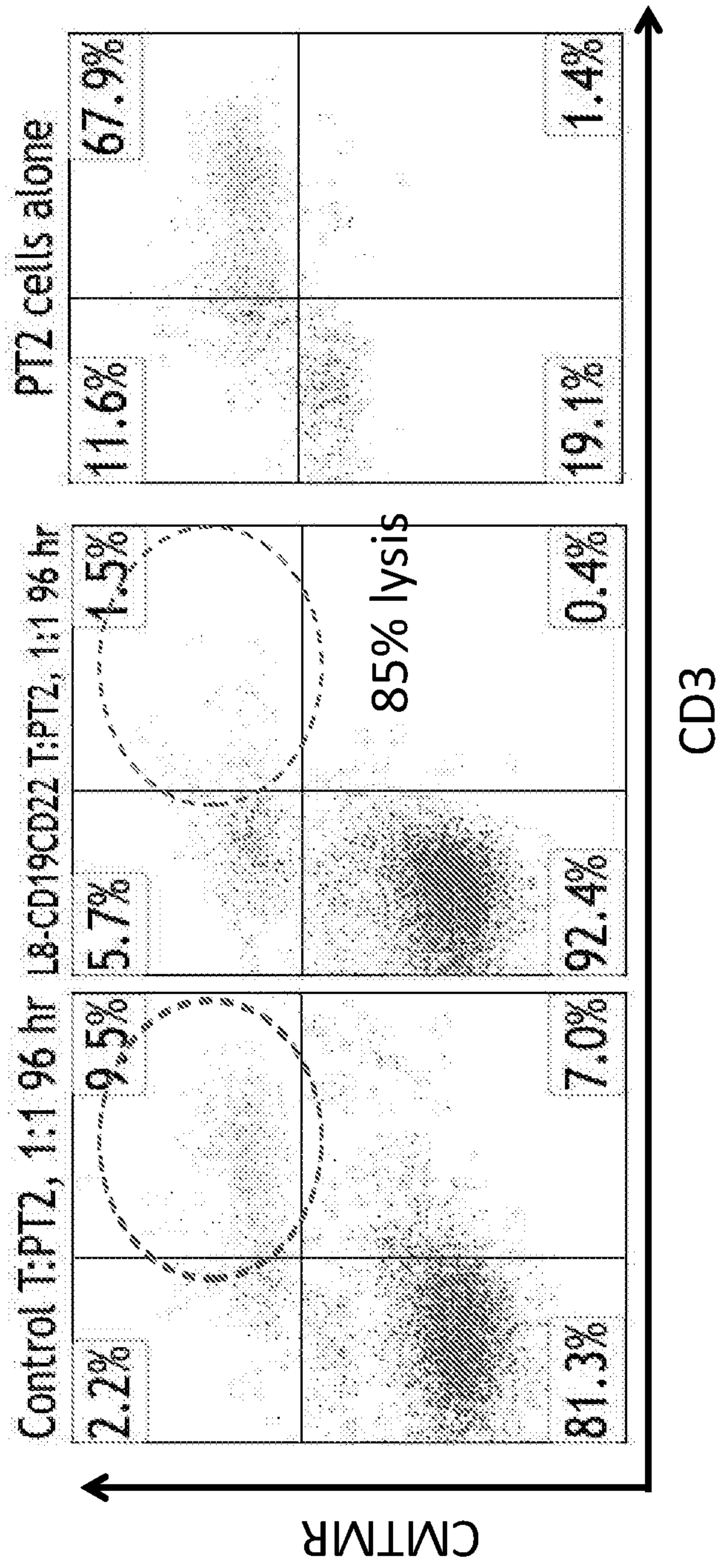

Control T:PT1, 2:1
4.1%
16.4%
52.4%
27.1%
CD19CD22-2G T:PT1, 2:1
0.5%
7.5%
49.7%
42.2%
PT1 cells alone
3.2%
26.4%
6.5%
63.8%
Control T:PT1, 5:1
4.3%
9.8%
69.9%
16.0%
CD19CD22-2G T:PT1, 5:1
0.6%
3.7%
72.8%
22.9%
L8-CD19CD22-2G
Percent PT1 cell lysis
80
60
40
20
0
2:1
5:1
CD19
CMTMR

FIGURE 22A


Control T:PT2, 1:1 96 hr
2.2%
9.5%
81.3%
7.0%
LB-CD19CD22 T:PT2, 1:1 96 hr
5.7%
1.5%
92.4%
0.4%
85% lysis
PT2 cells alone
11.6%
67.9%
19.1%
1.4%
CD3
CMTMR

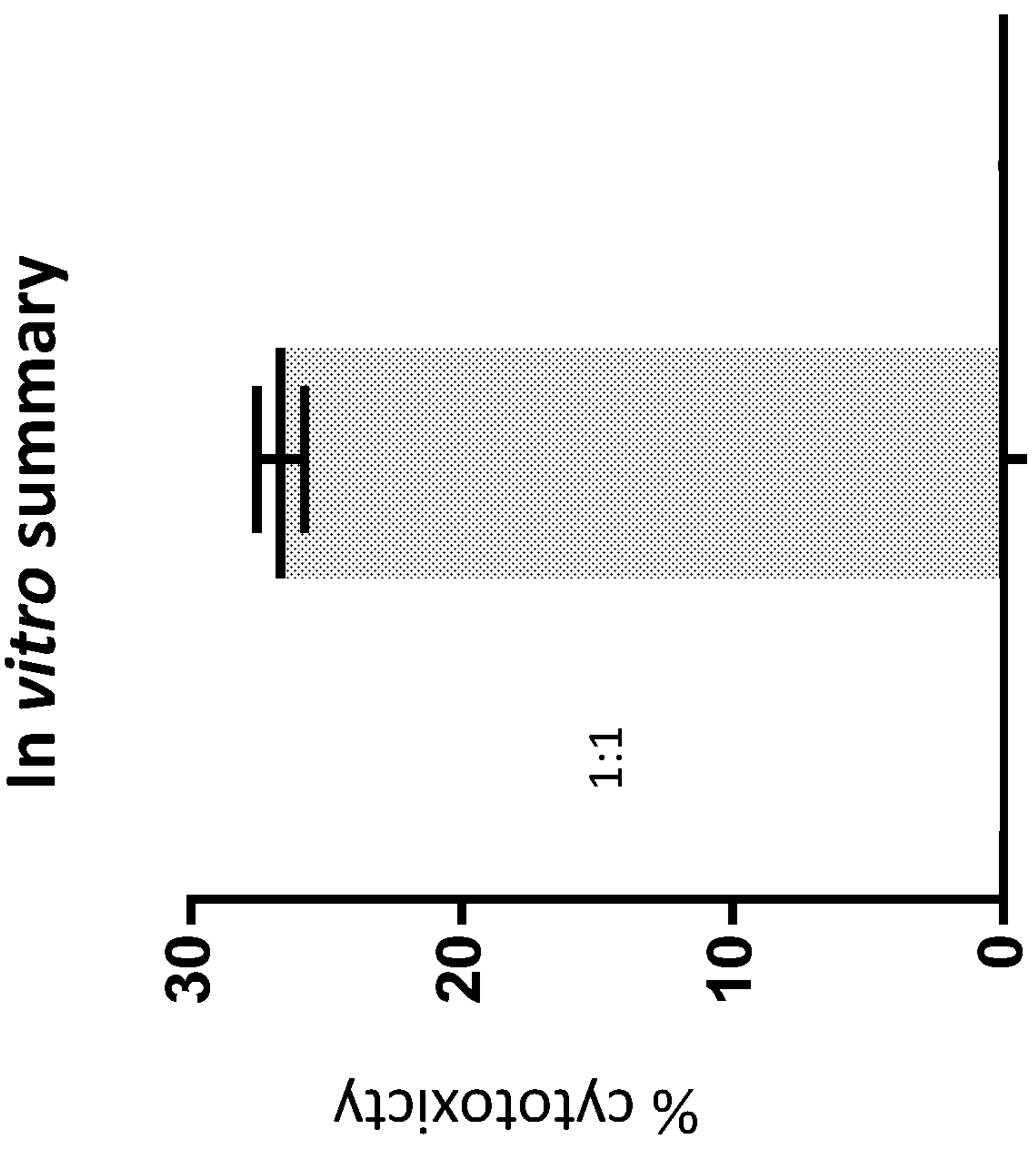


FIGURE 22B

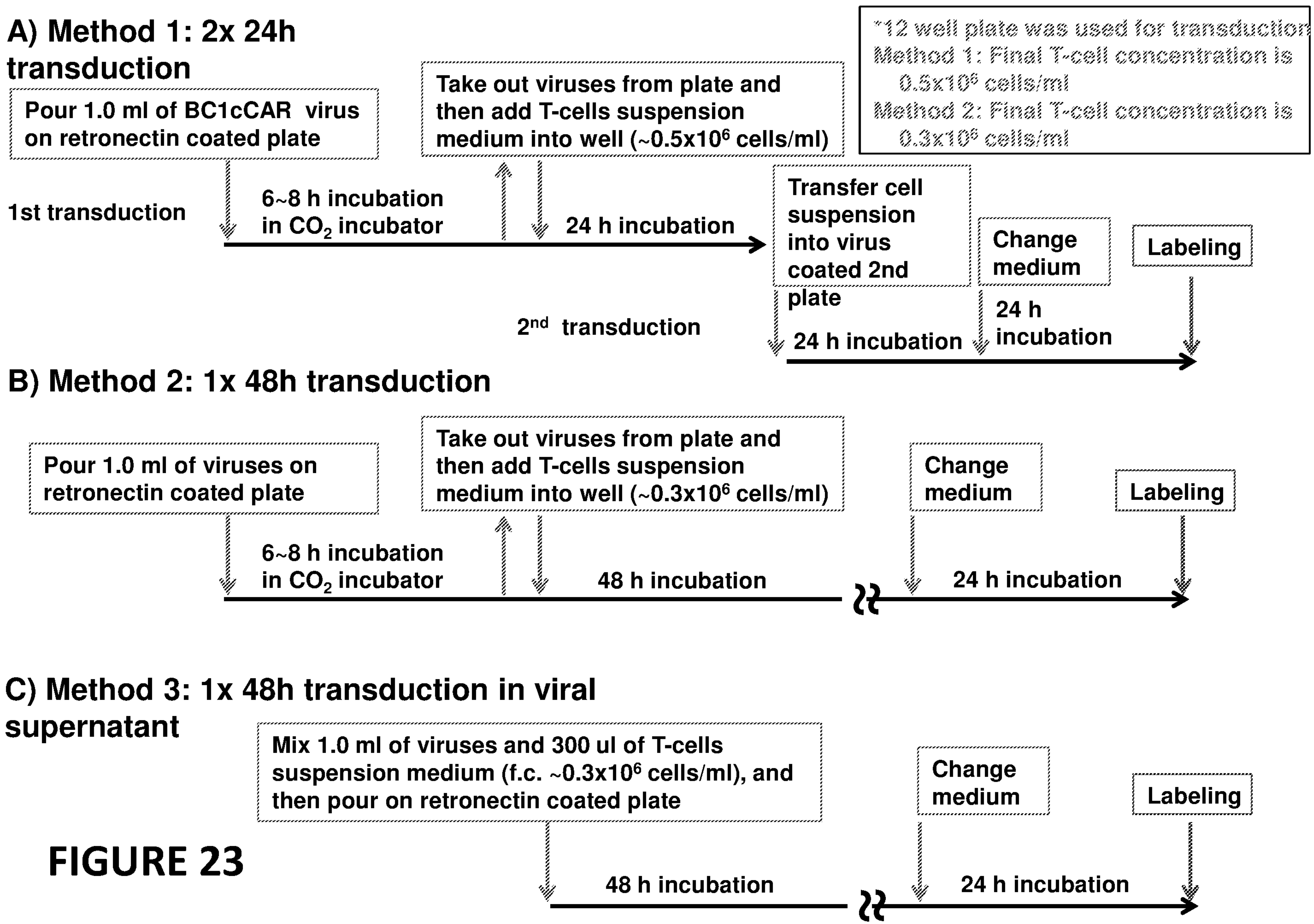

A) Method 1: 2x 24h transduction
Pour 1.0 ml of BC1cCAR virus on retronectin coated plate
1st transduction
6~8 h incubation in CO2 incubator
Take out viruses from plate and then add T-cells suspension medium into well (~0.5x10^6 cells/ml)
24 h incubation
Transfer cell suspension into virus coated 2nd plate
2nd transduction
24 h incubation
Change medium
24 h incubation
Labeling
*12 well plate was used for transduction
Method 1: Final T-cell concentration is 0.5x10^6 cells/ml
Method 2: Final T-cell concentration is 0.3x10^6 cells/ml
B) Method 2: 1x 48h transduction
Pour 1.0 ml of viruses on retronectin coated plate
6~8 h incubation in CO2 incubator
Take out viruses from plate and then add T-cells suspension medium into well (~0.3x10^6 cells/ml)
48 h incubation
Change medium
24 h incubation
Labeling
C) Method 3: 1x 48h transduction in viral supernatant
Mix 1.0 ml of viruses and 300 ul of T-cells suspension medium (f.c. ~0.3x10^6 cells/ml), and then pour on retronectin coated plate
48 h incubation
Change medium
24 h incubation
Labeling

FIGURE 23

FIGURE 24A

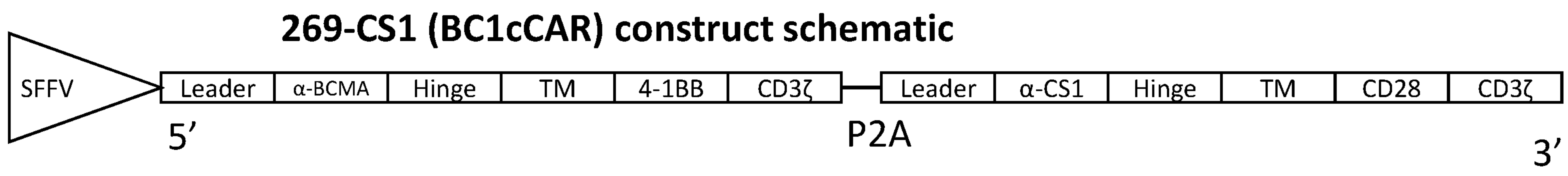
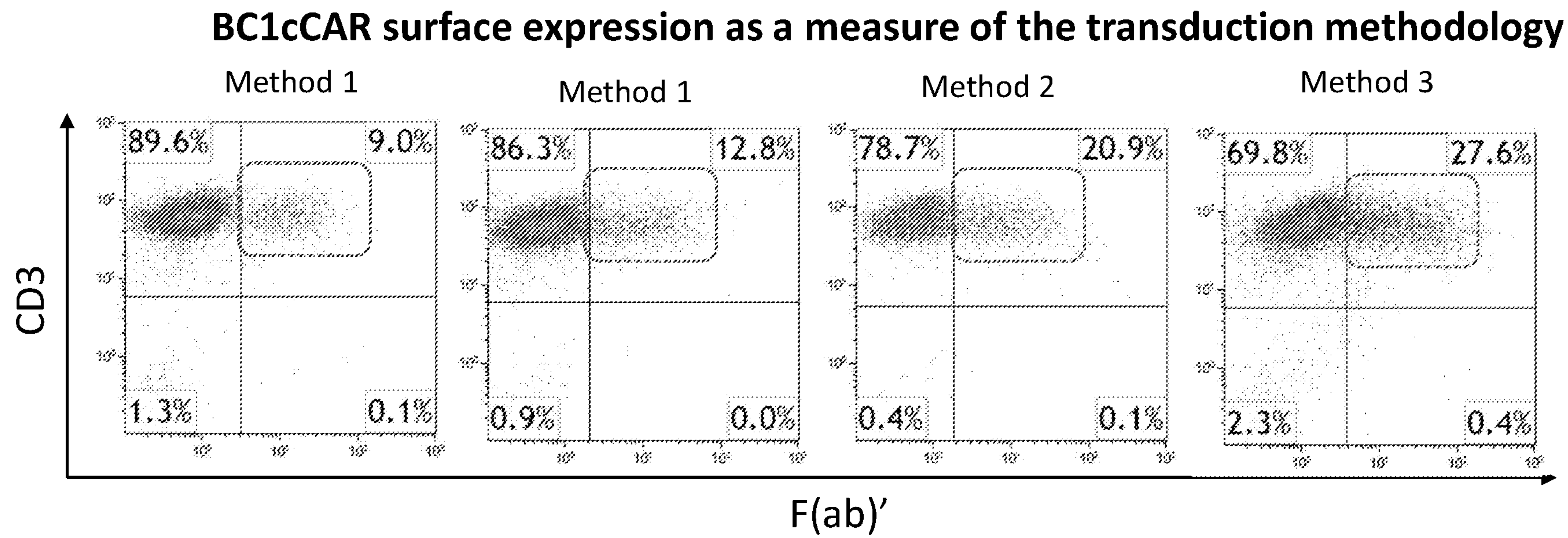


FIGURE 24B



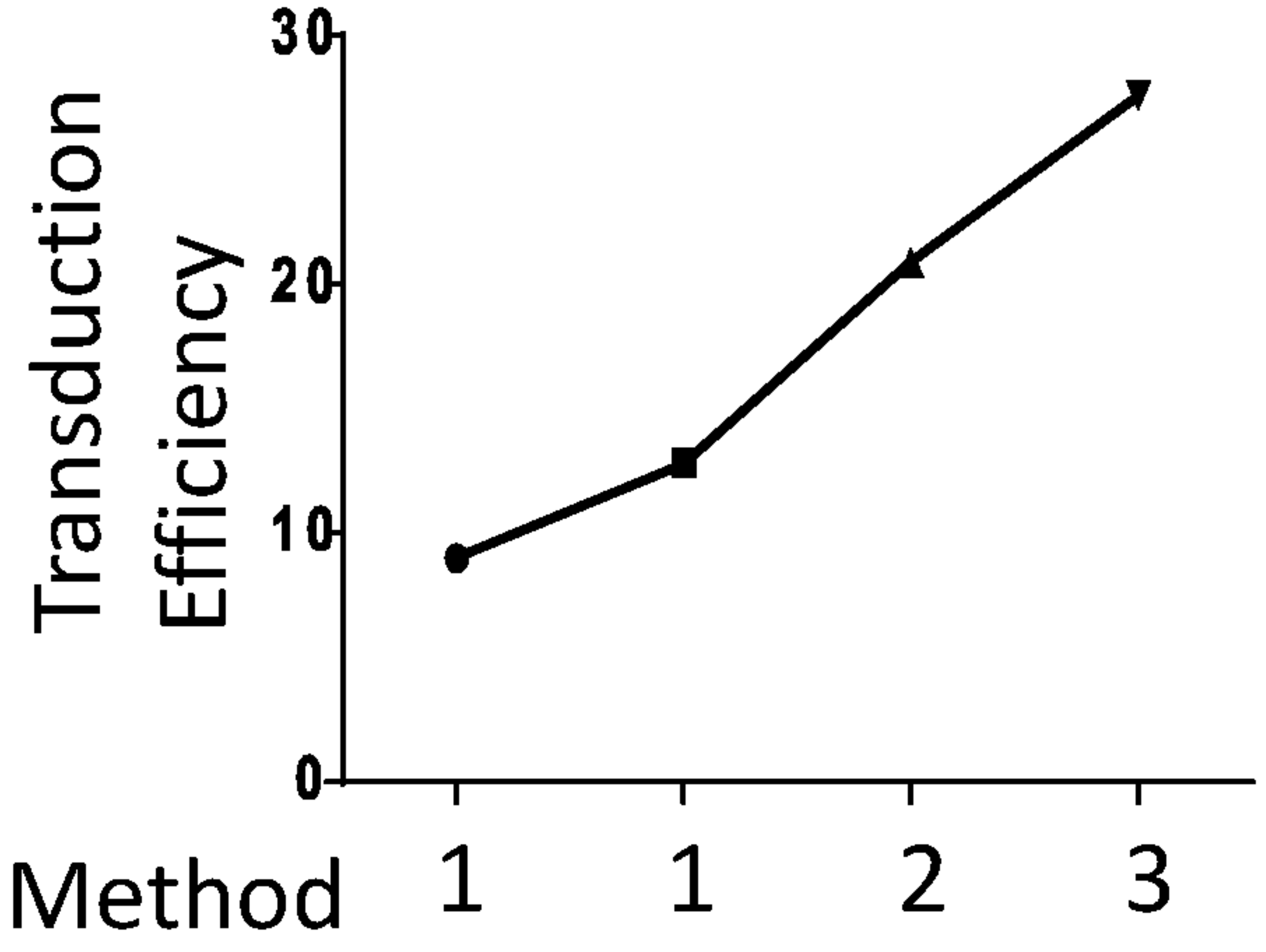


FIGURE 24C

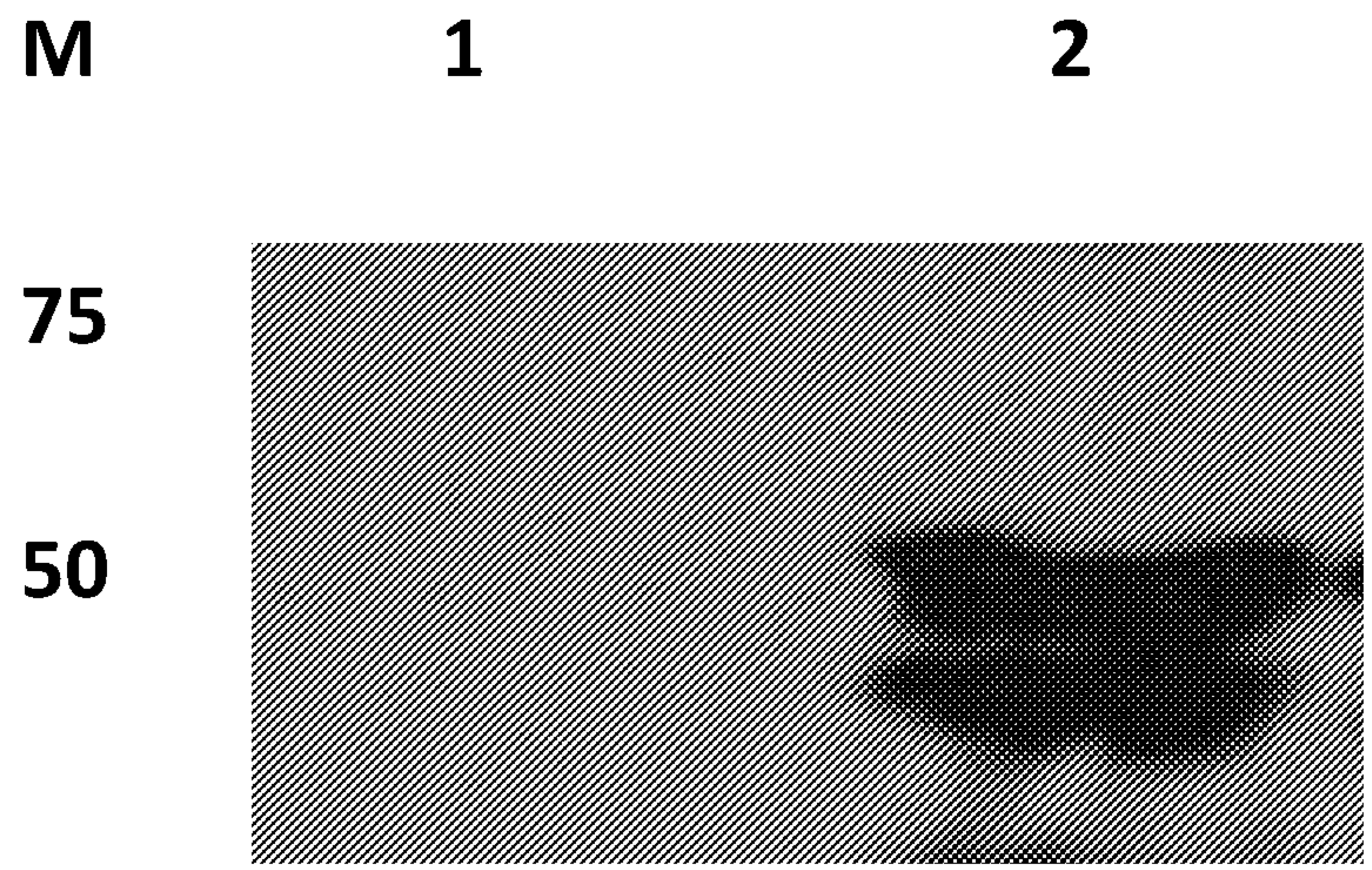


FIGURE 24D

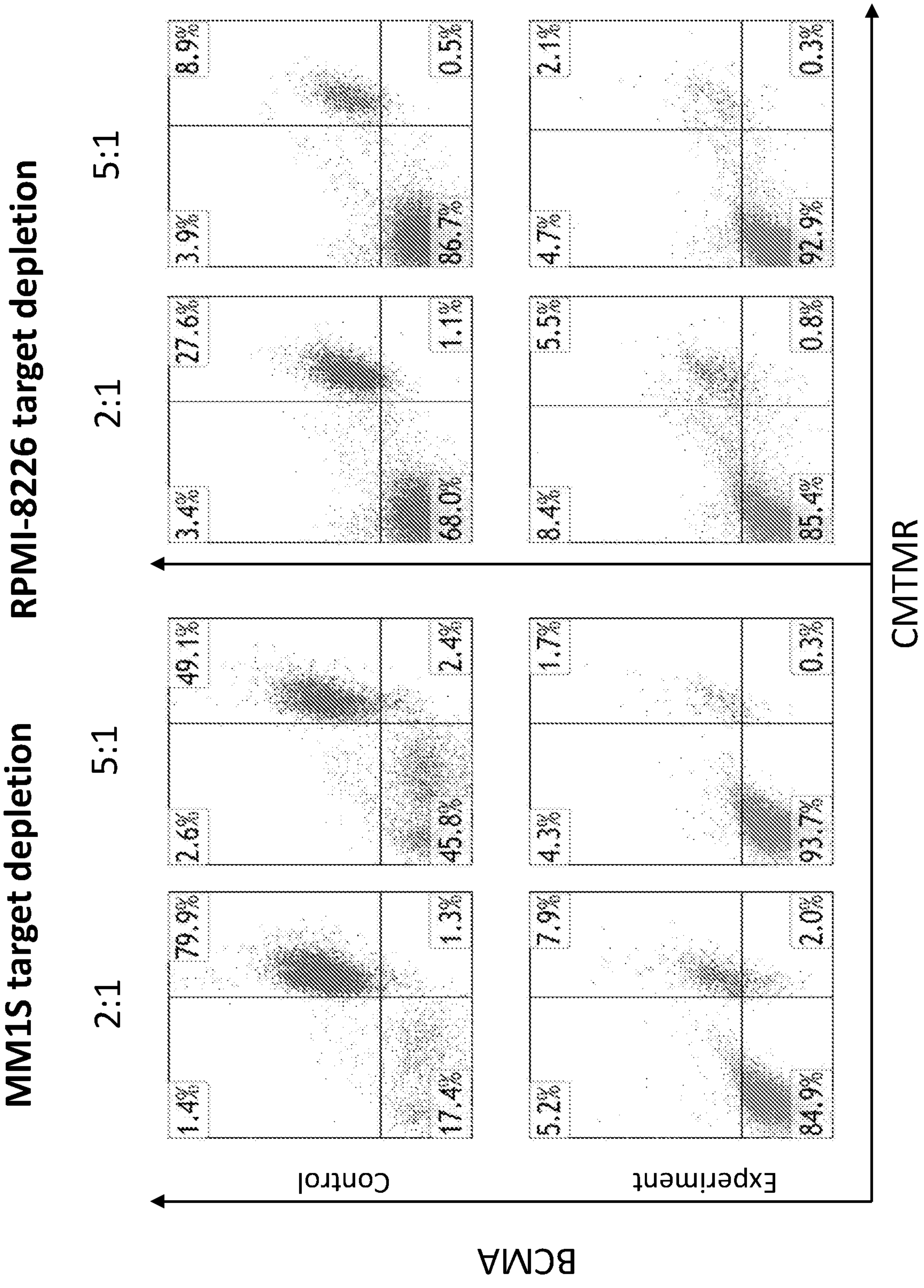

MM1S target depletion
RPMI-8226 target depletion
2:1
5:1
2:1
5:1
Control
Experiment
BCMA
CMTMR
1.4%
79.9%
17.4%
1.3%
2.6%
49.1%
45.8%
2.4%
3.4%
27.6%
68.0%
1.1%
3.9%
8.9%
86.7%
0.5%
5.2%
7.9%
84.9%
2.0%
4.3%
1.7%
93.7%
0.3%
8.4%
5.5%
85.4%
0.8%
4.7%
2.1%
92.9%
0.3%

FIGURE 25A

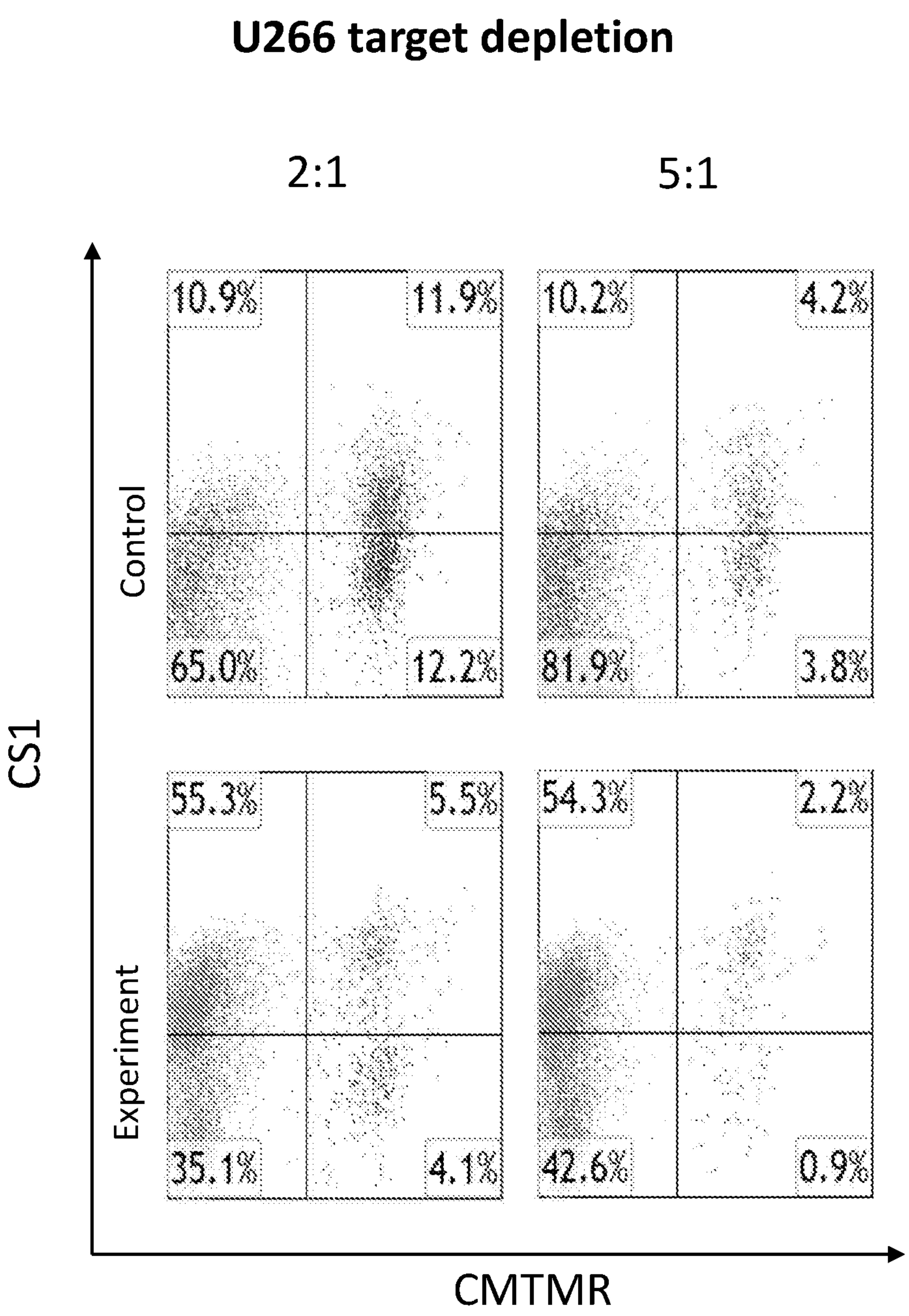

U266 target depletion
2:1
5:1
Control
Experiment
CS1
CMTMR
10.9%
11.9%
65.0%
12.2%
10.2%
4.2%
81.9%
3.8%
55.3%
5.5%
35.1%
4.1%
54.3%
2.2%
42.6%
0.9%

FIGURE 25B

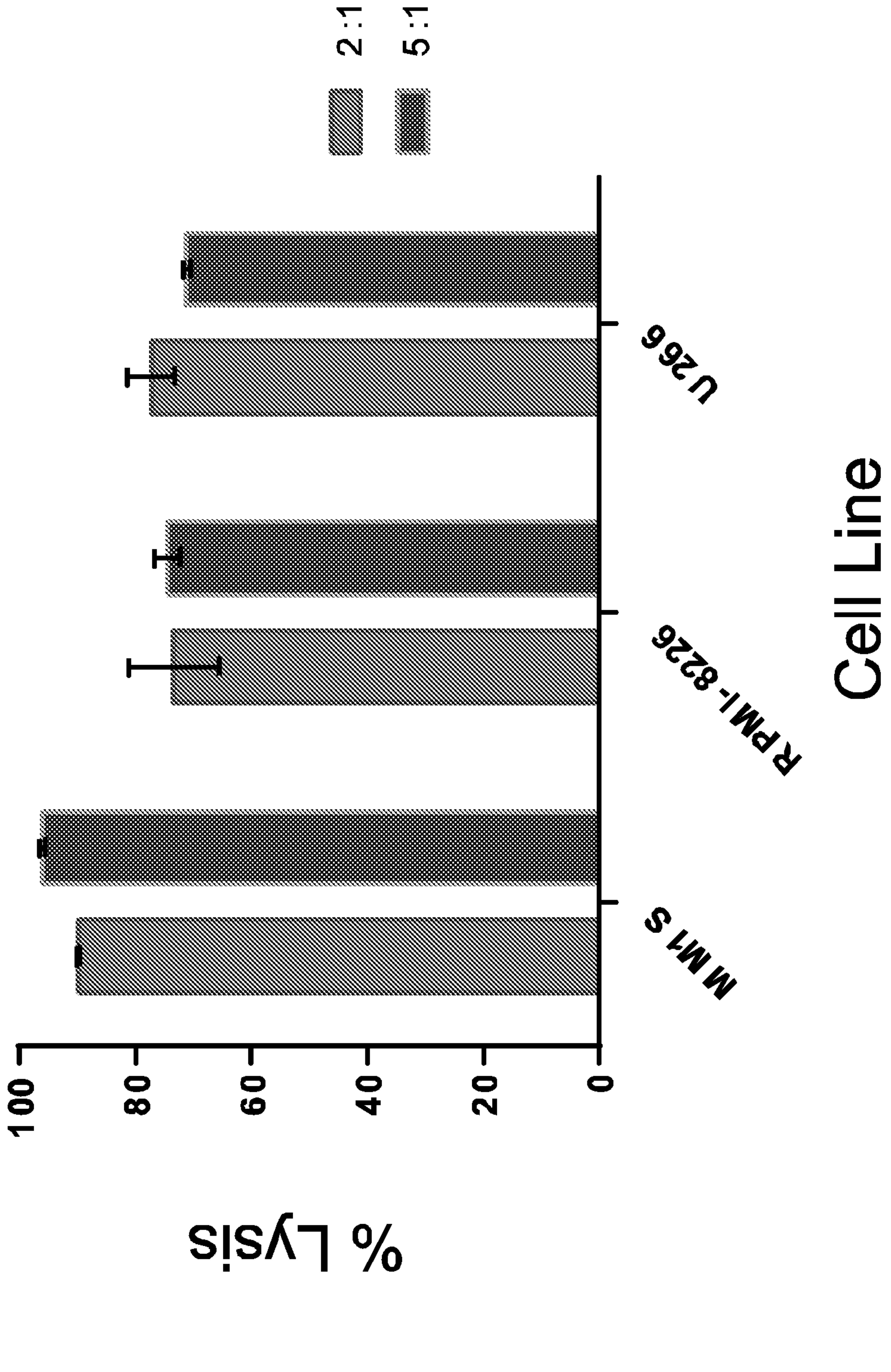


FIGURE 25C

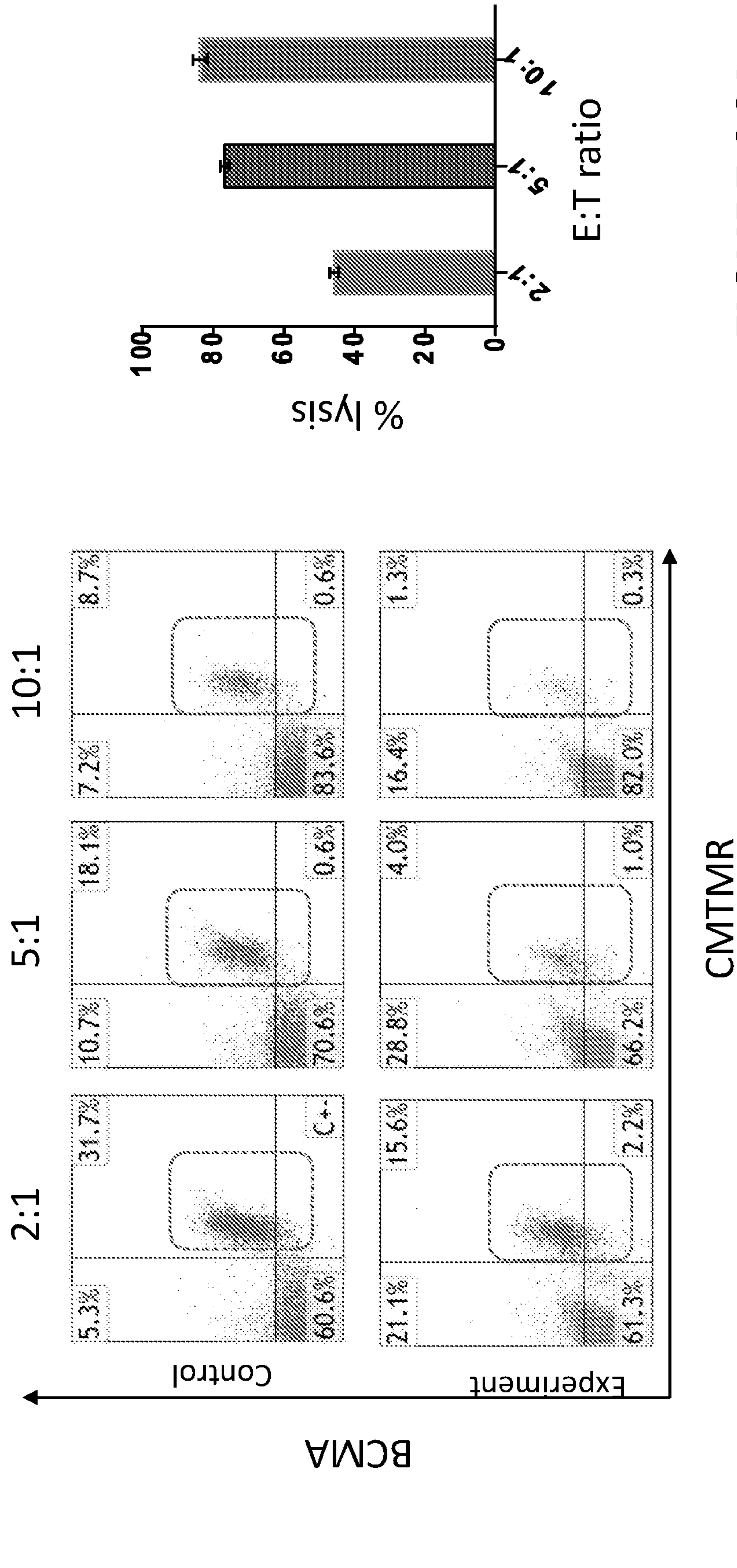

% lysis
100
80
60
40
20
0
2:1
5:1
10:1
E:T ratio
2:1
5:1
10:1
Control
Experiment
5.3%
31.7%
60.6%
C+-
10.7%
18.1%
70.6%
0.6%
7.2%
8.7%
83.6%
0.6%
21.1%
15.6%
61.3%
2.2%
28.8%
4.0%
66.2%
1.0%
16.4%
1.3%
82.0%
0.3%
BCMA
CMTMR

FIGURE 26A

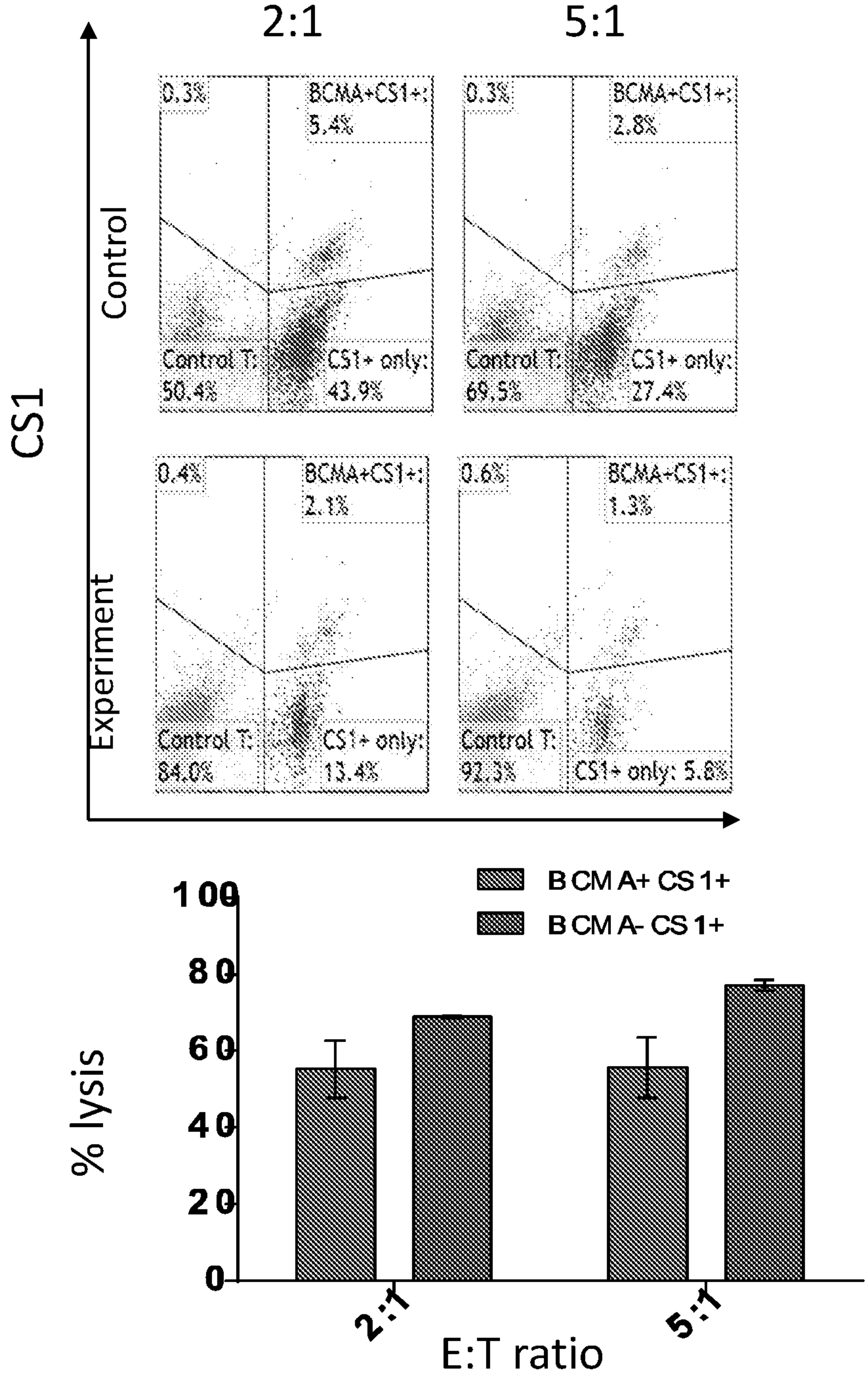

2:1
5:1
CS1
Control
Experiment
0.3%
BCMA+CS1+:
5.4%
Control T:
50.4%
CS1+ only:
43.9%
0.3%
BCMA+CS1+:
2.8%
Control T:
69.5%
CS1+ only:
27.4%
0.4%
BCMA+CS1+:
2.1%
Control T:
84.0%
CS1+ only:
13.4%
0.6%
BCMA+CS1+:
1.3%
Control T:
92.3%
CS1+ only: 5.8%
BCMA+ CS1+
BCMA- CS1+
% lysis
100
80
60
40
20
0
2:1
5:1
E:T ratio

FIGURE 26B

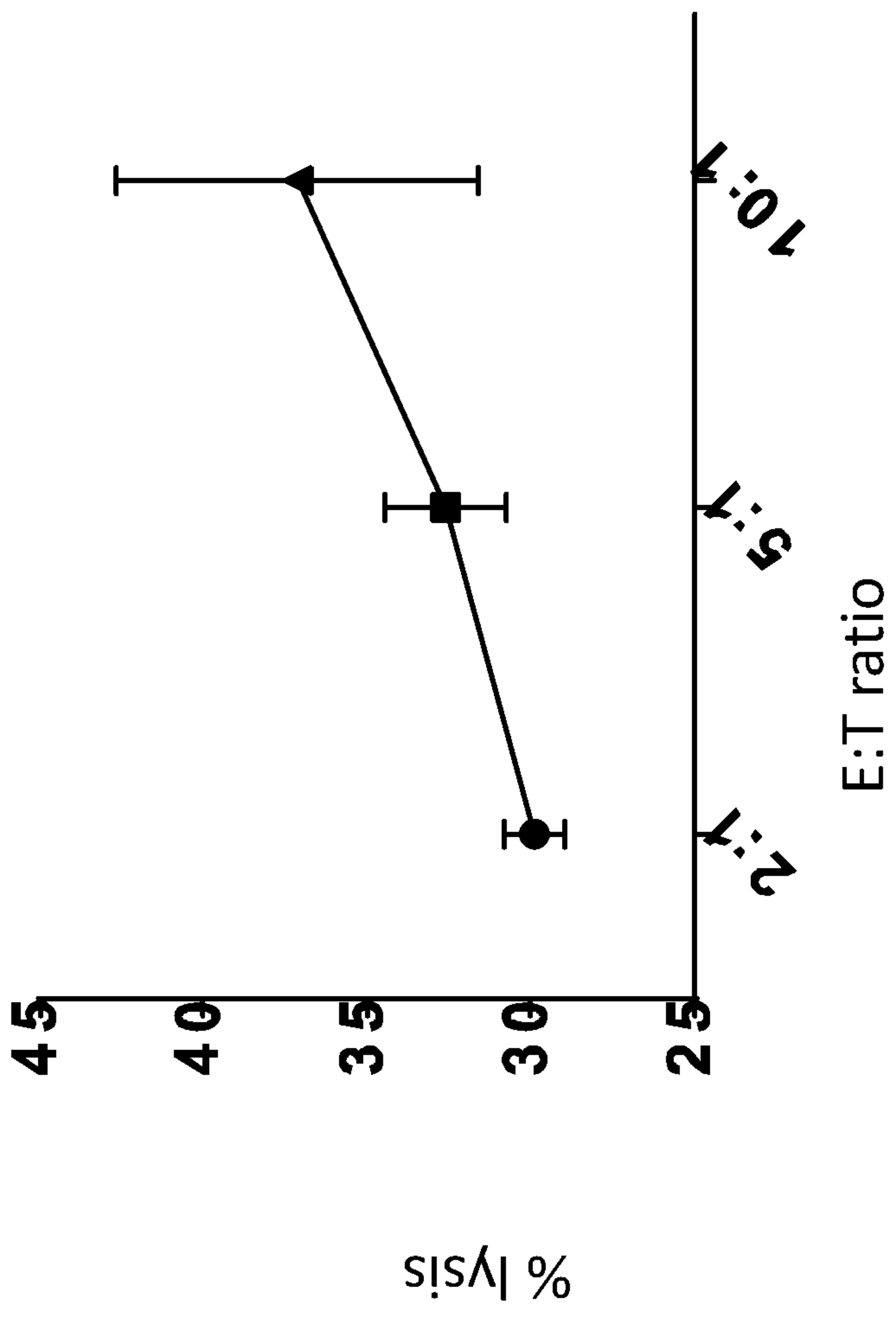


FIGURE 26C

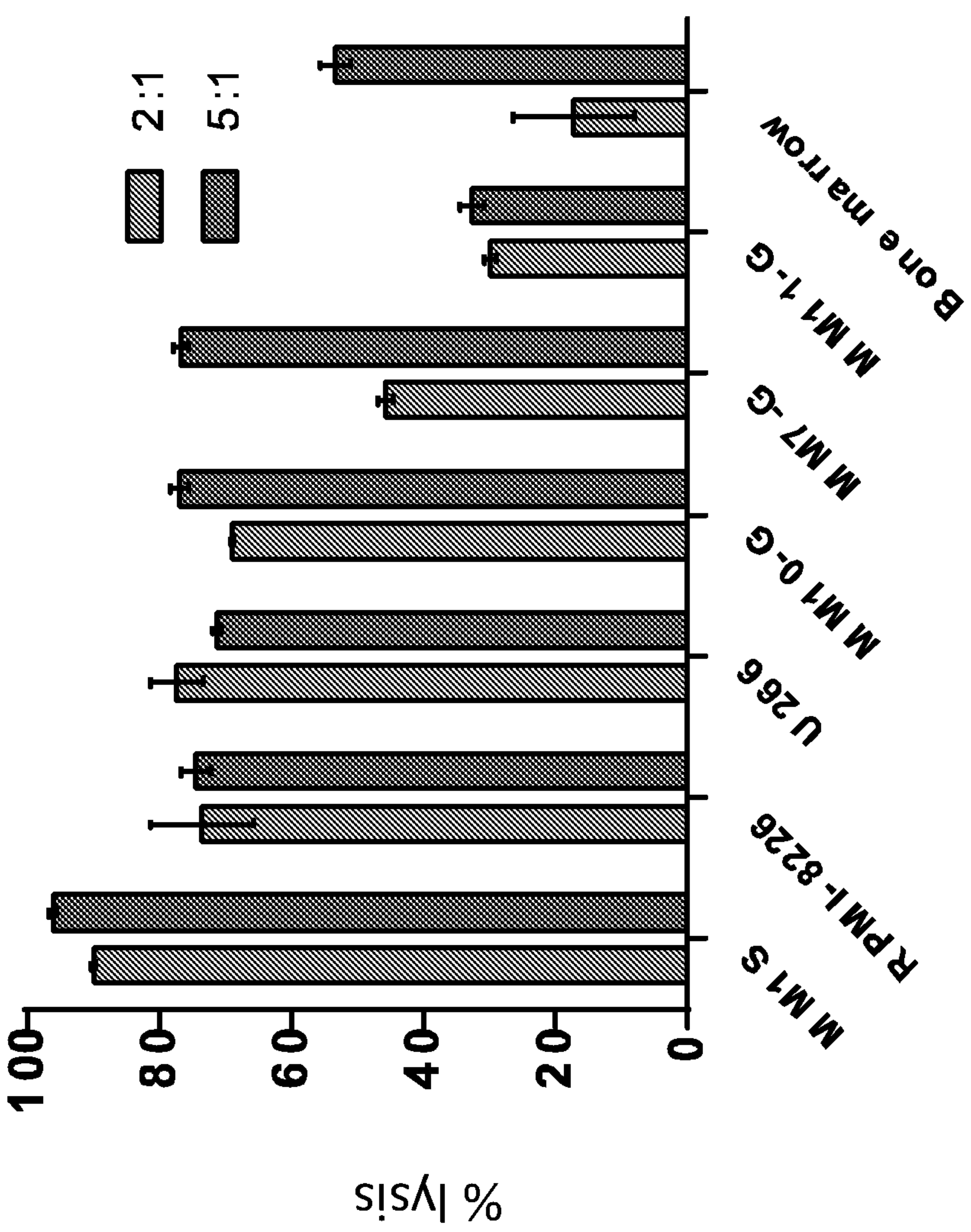

% lysis
100
80
60
40
20
0
MM1S
RPMI-8226
U266
MM10-G
MM7-G
MM11-G
Bone marrow
2:1
5:1

FIGURE 26D

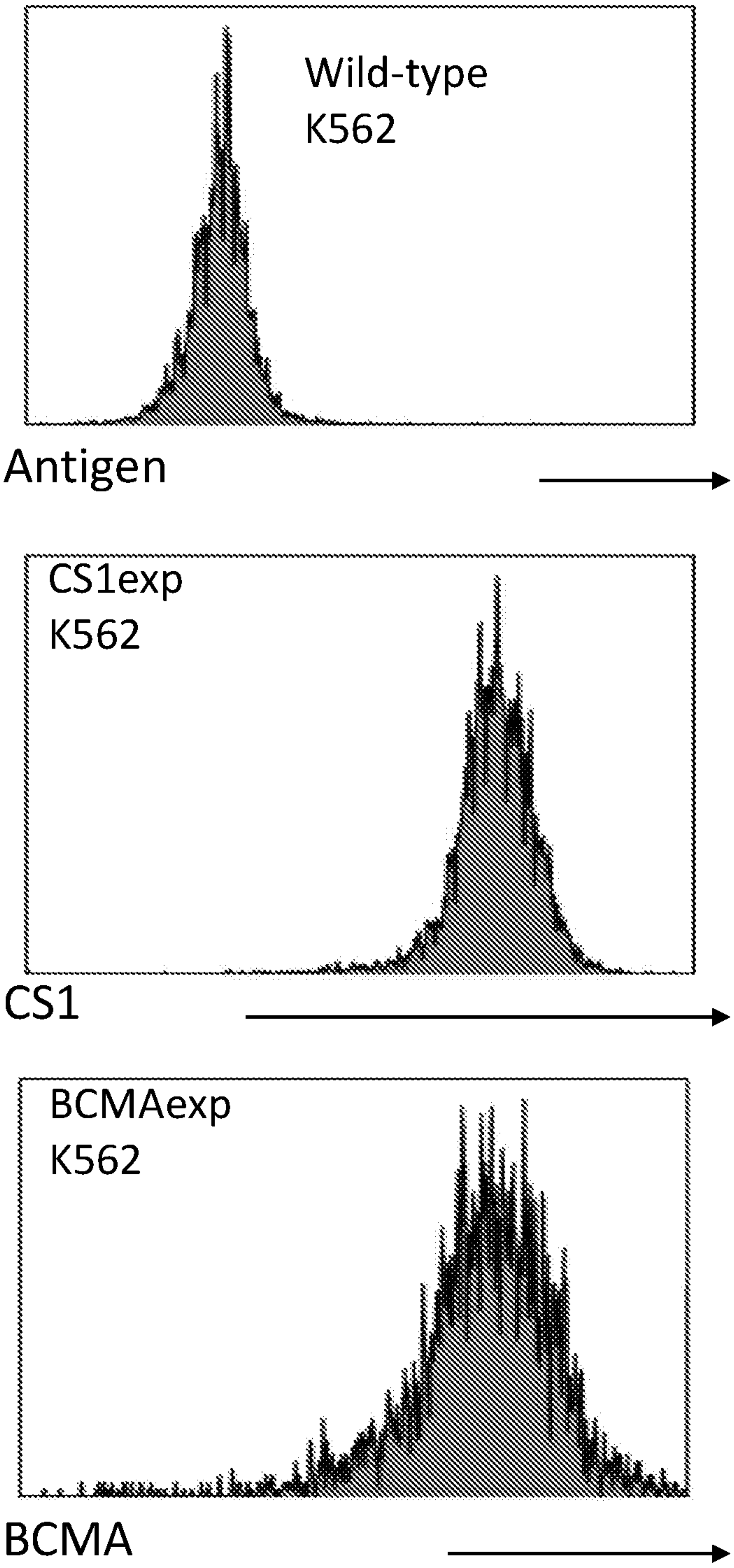
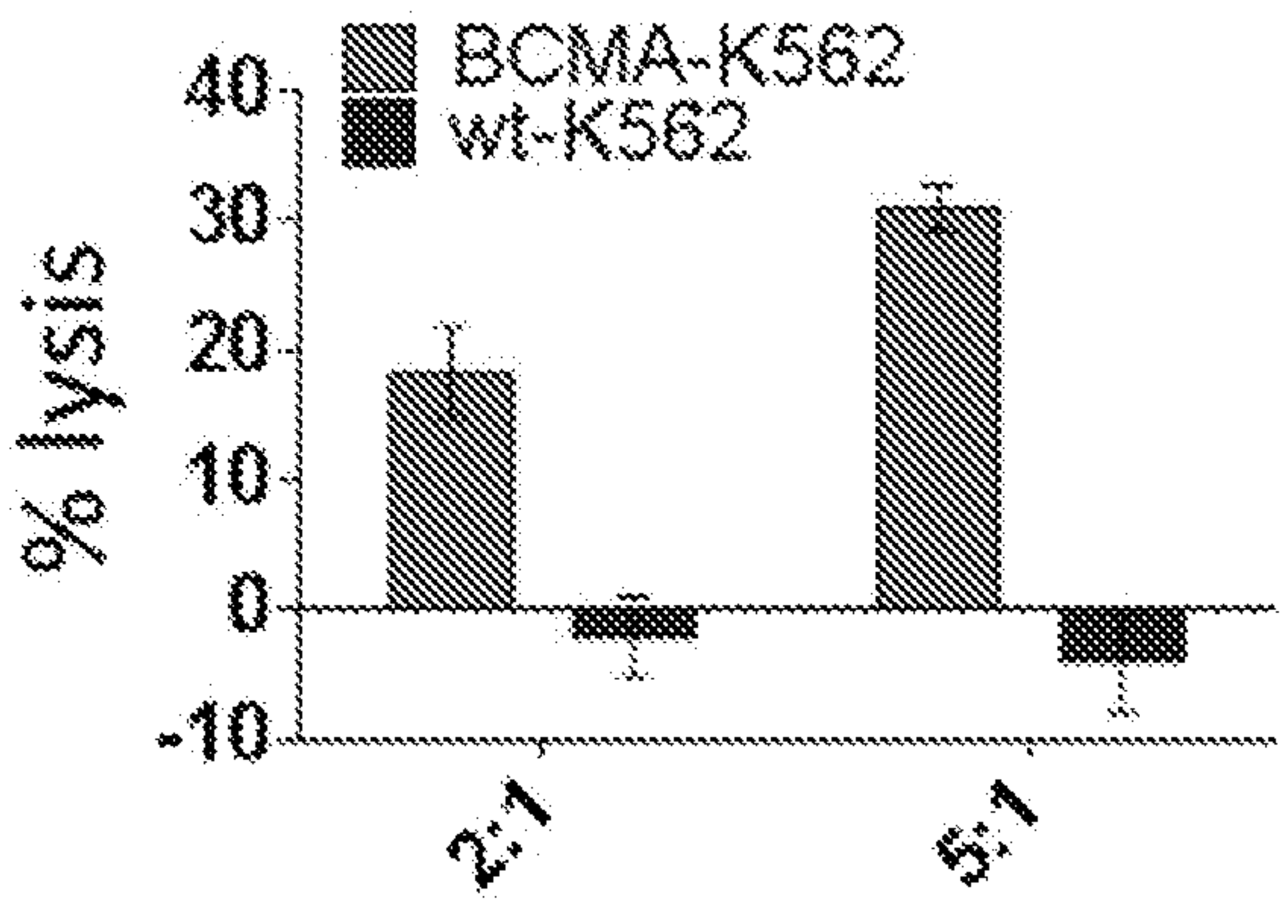
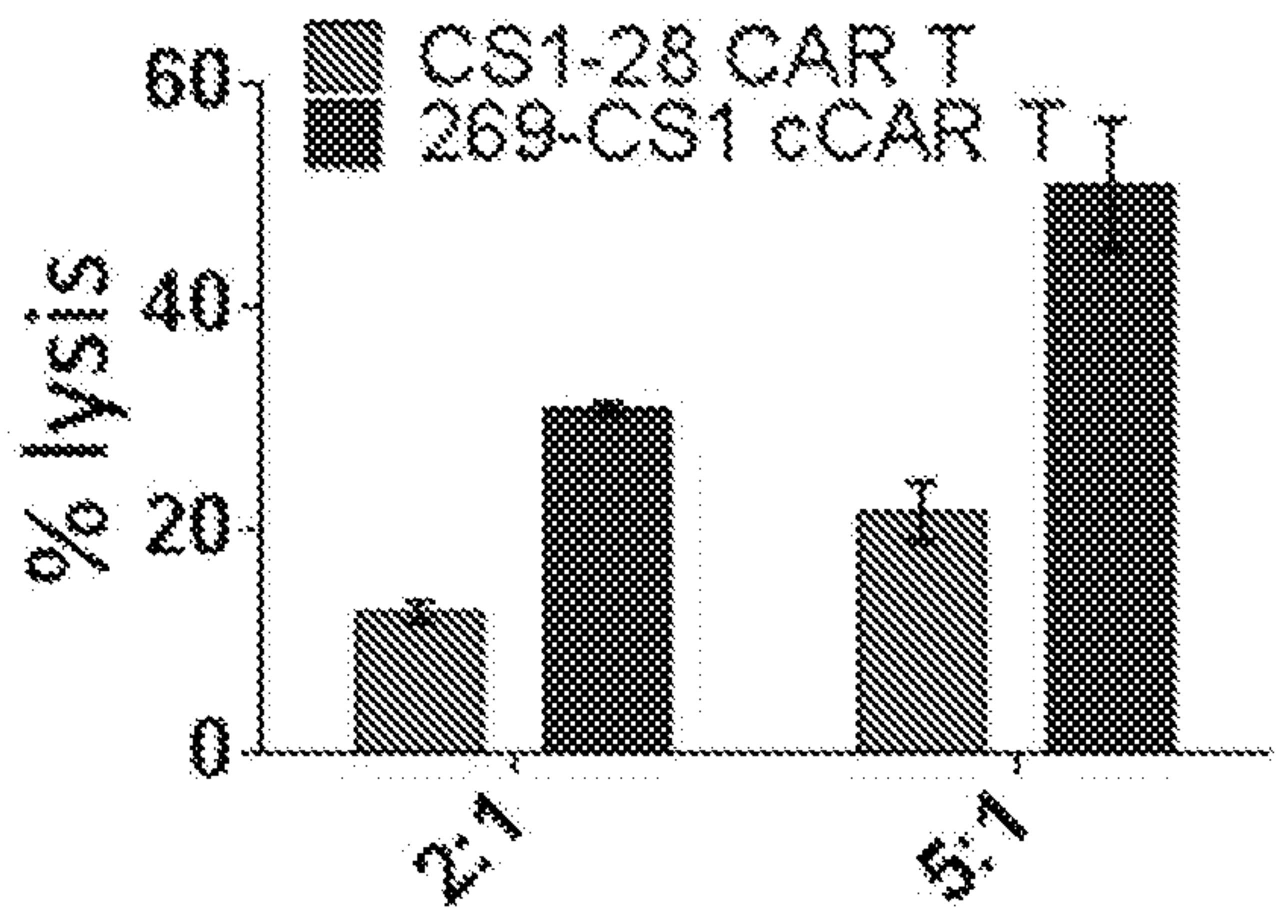


FIGURE 27A

Short-term BCMA depletion by BC1cCAR T-cells



Short-term CS1 depletion by BC1cCAR T vs. CS1 CAR T



Anti-CS1 activity of BC1cCAR T against CS1^{dim} NK-92 cells

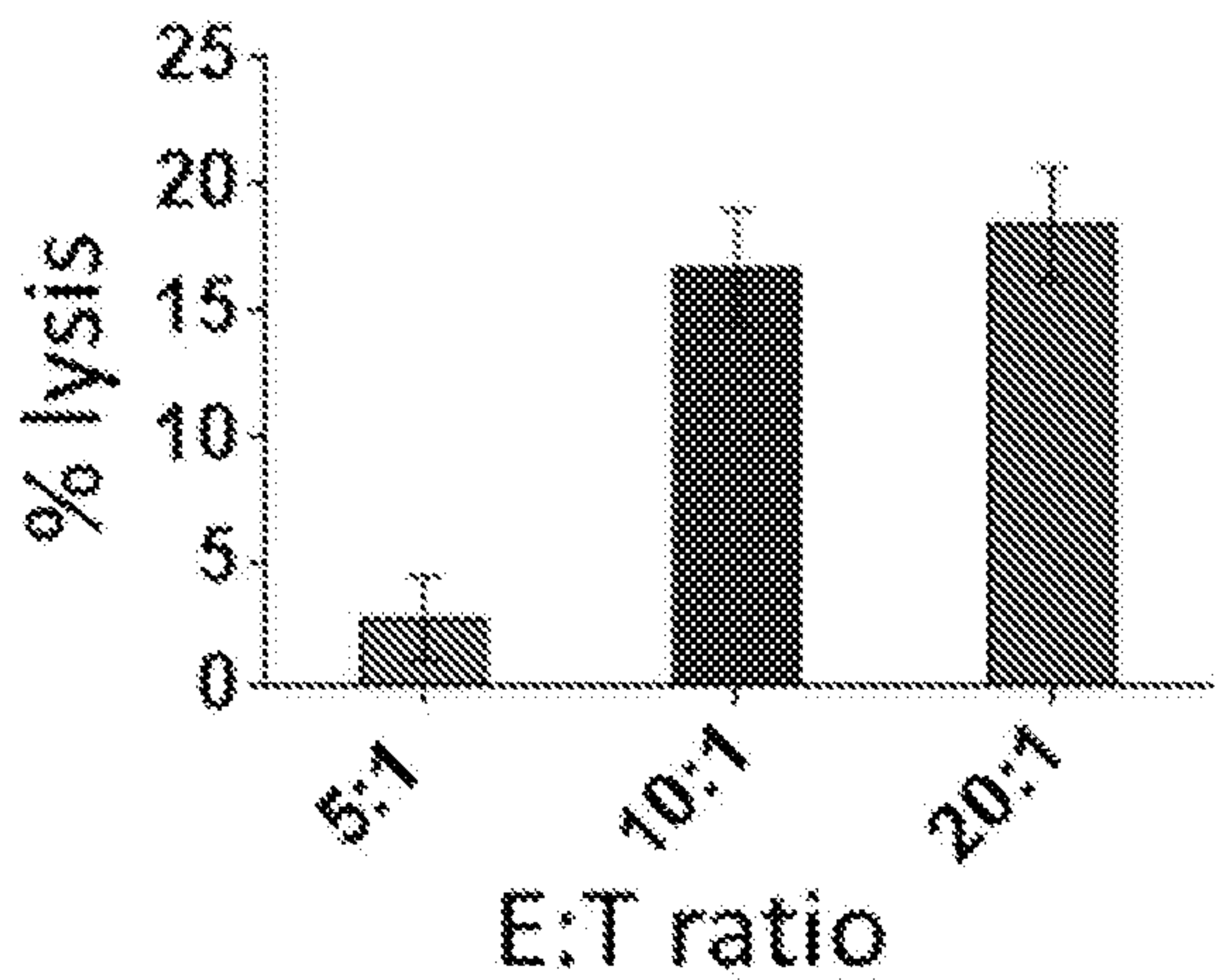


FIGURE 27B

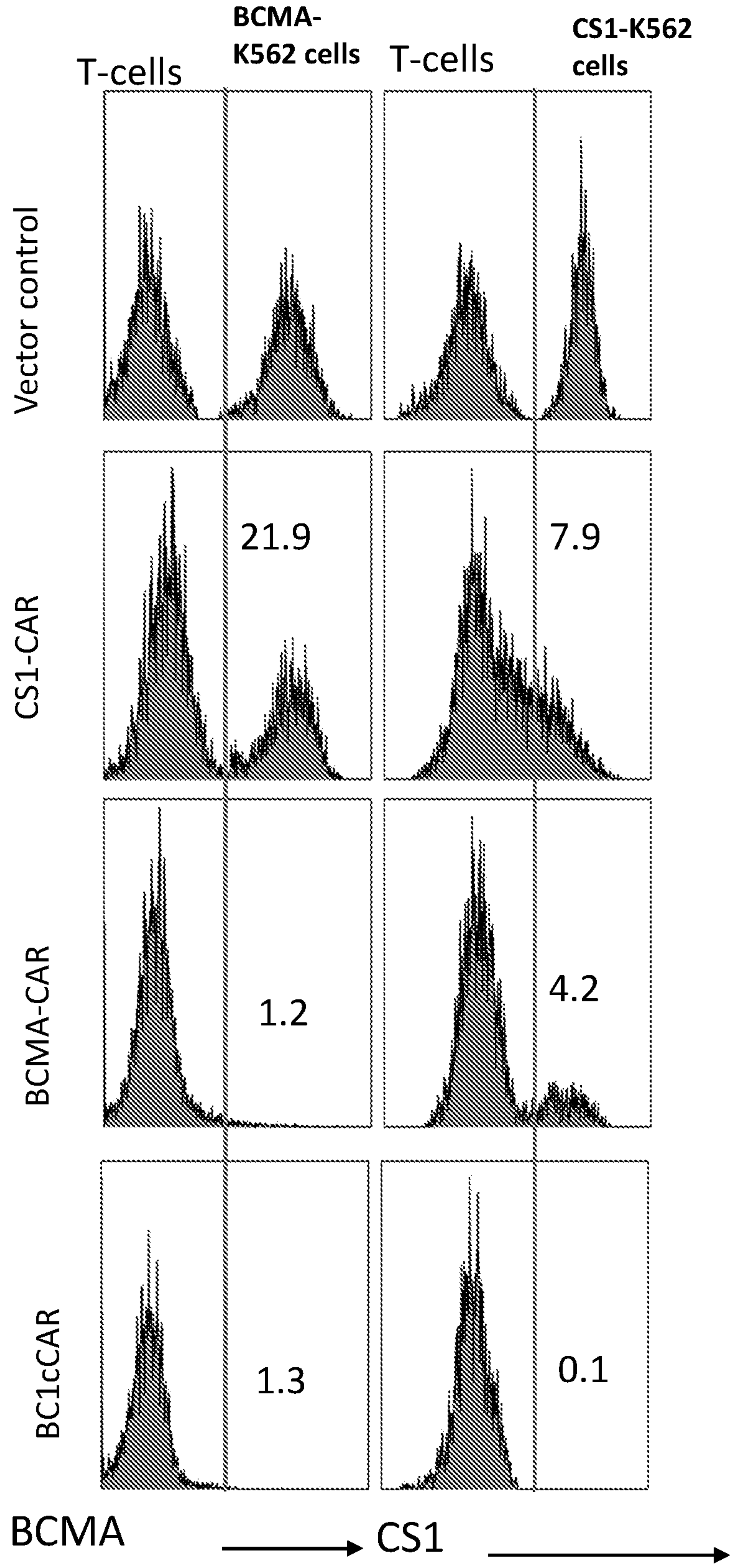
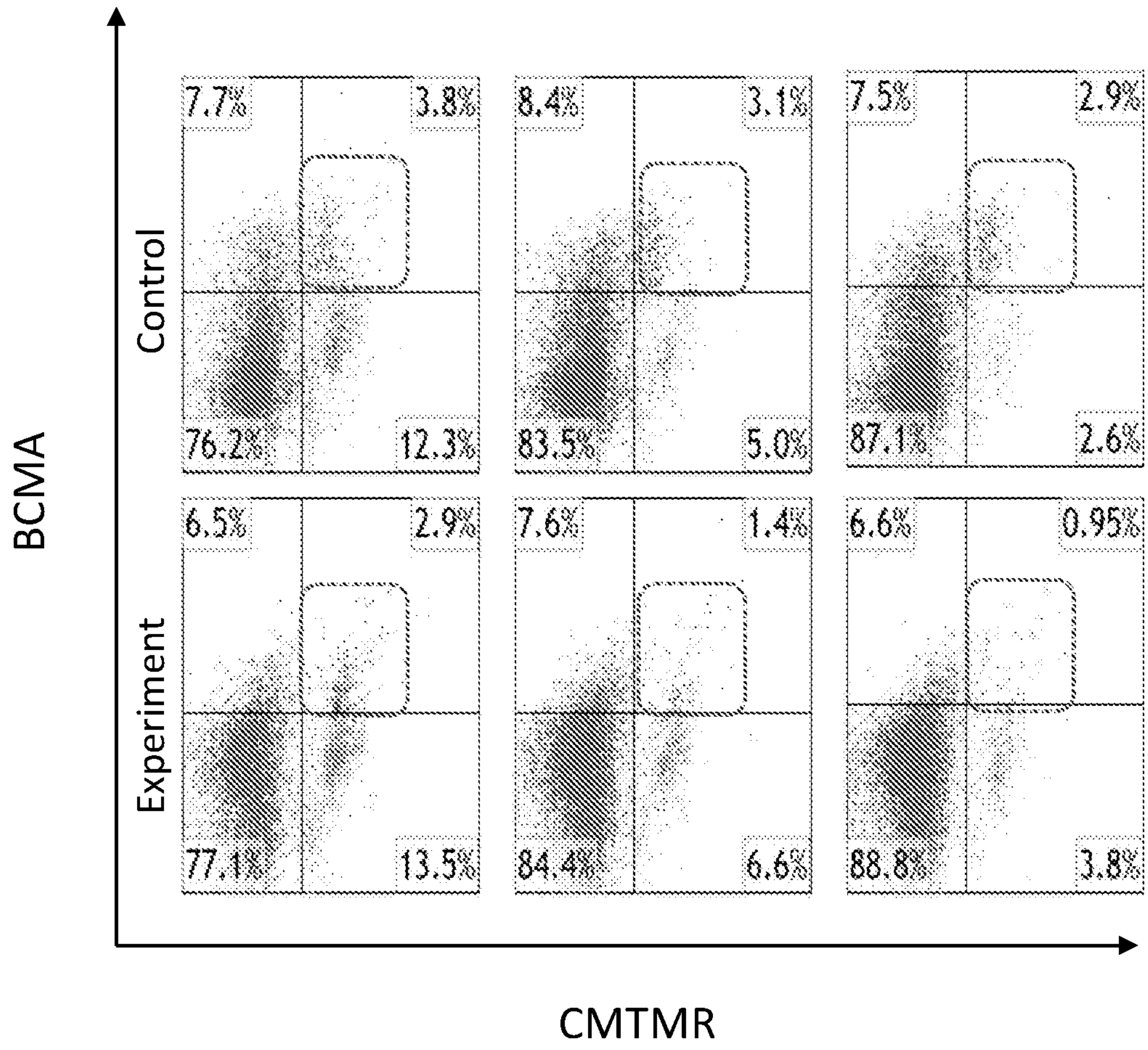

T-cells
BCMA-
K562 cells
T-cells
CS1-K562
cells
Vector control
CS1-CAR
21.9
7.9
BCMA-CAR
1.2
4.2
BC1cCAR
1.3
0.1
BCMA
CS1

FIGURE 27C


BCMA
Control
Experiment
7.7%
3.8%
76.2%
12.3%
8.4%
3.1%
83.5%
5.0%
7.5%
2.9%
87.1%
2.6%
6.5%
2.9%
77.1%
13.5%
7.6%
1.4%
84.4%
6.6%
6.6%
0.95%
88.8%
3.8%
CMTMR

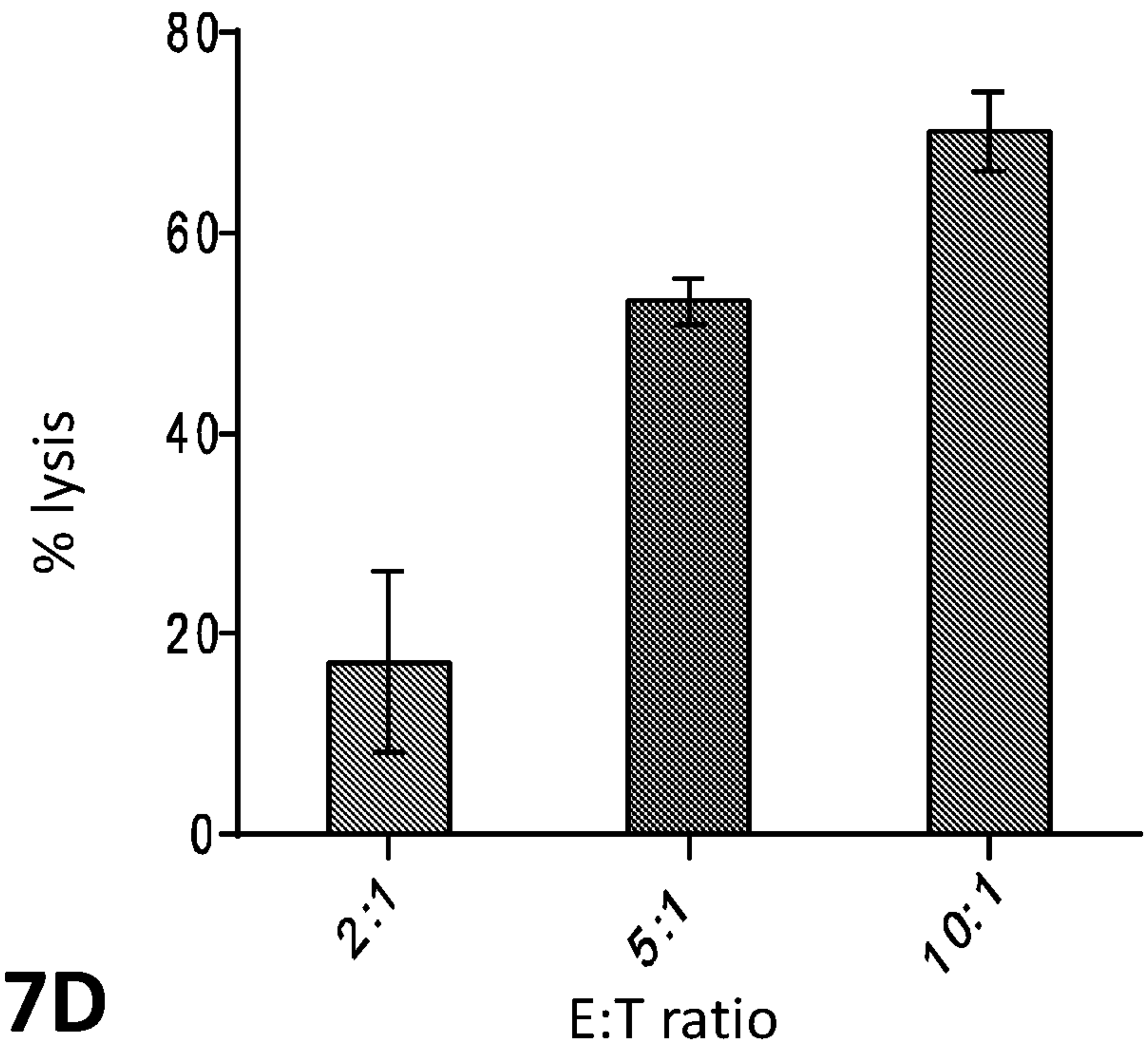

80
60
40
20
0
% lysis
2:1
5:1
10:1
E:T ratio

FIGURE 27D

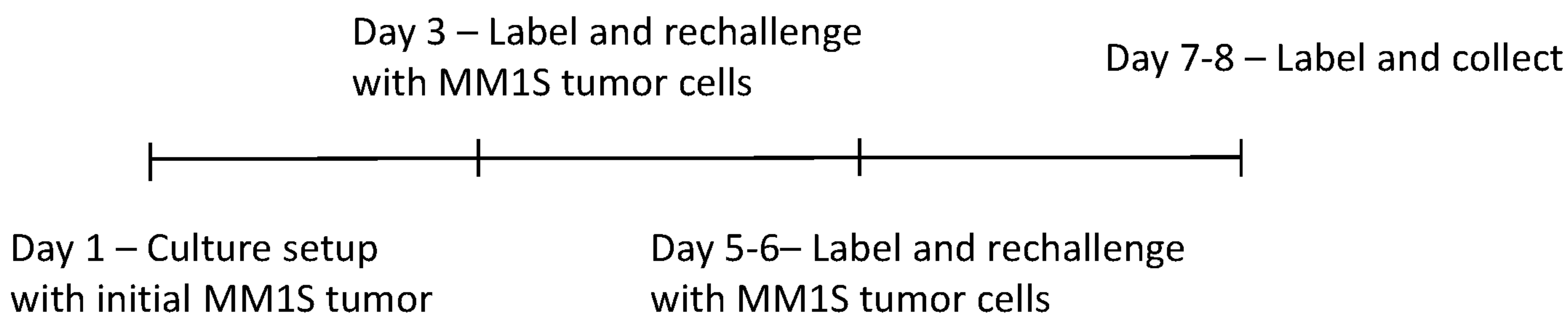

Day 3 – Label and rechallenge
with MM1S tumor cells
Day 7-8 – Label and collect
Day 1 – Culture setup
with initial MM1S tumor
Day 5-6– Label and rechallenge
with MM1S tumor cells

FIGURE 28A

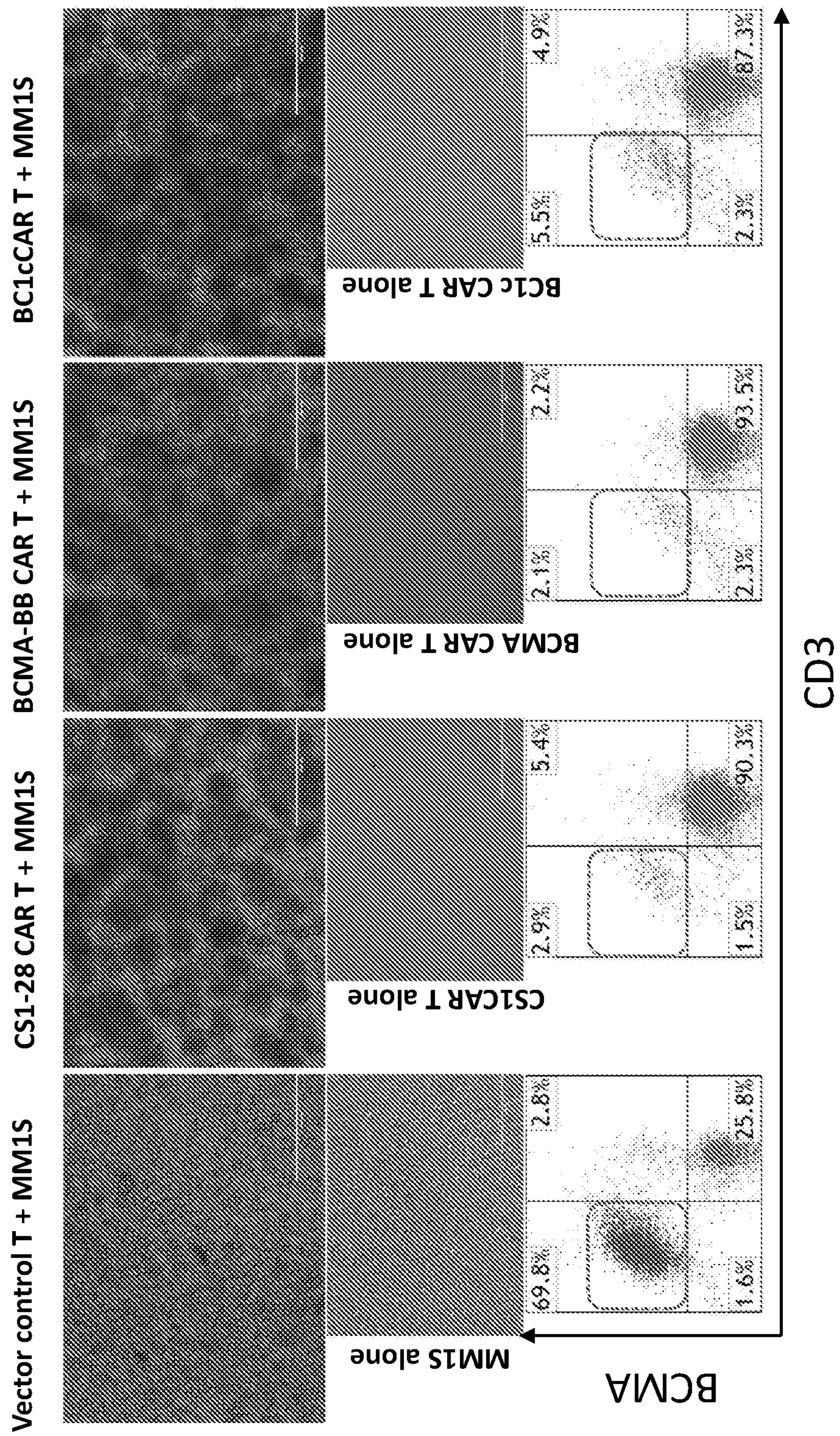

Vector control T + MM1S
CS1-28 CAR T + MM1S
BCMA-BB CAR T + MM1S
BC1cCAR T + MM1S
MM1S alone
CS1CAR T alone
BCMA CAR T alone
BC1c CAR T alone
69.8%
2.8%
1.6%
25.8%
2.9%
5.4%
1.5%
90.3%
2.1%
2.2%
2.3%
93.5%
5.5%
4.9%
2.3%
87.3%
BCMA
CD3

FIGURE 28B

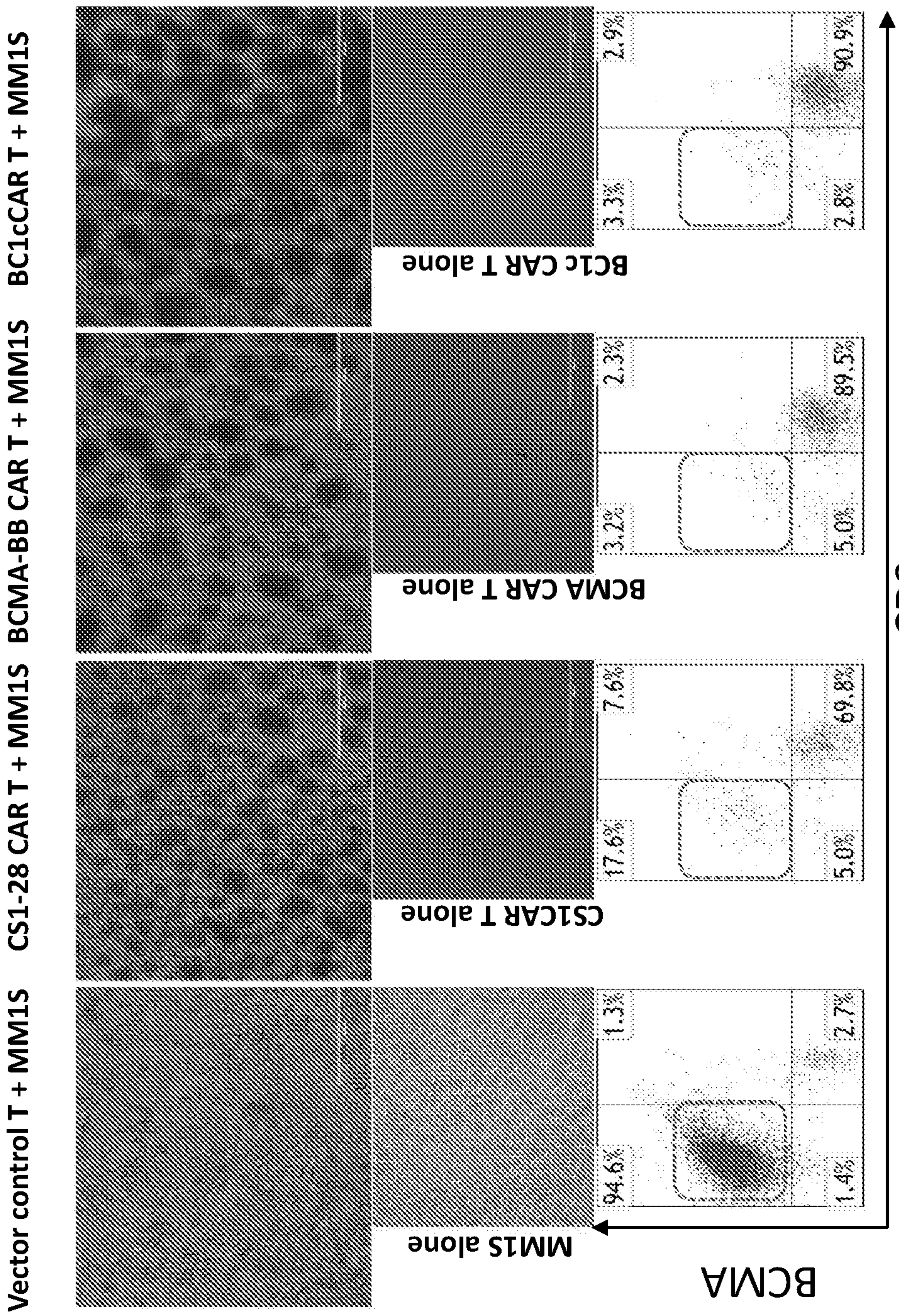

Vector control T + MM1S
CS1-28 CAR T + MM1S
BCMA-BB CAR T + MM1S
BC1cCAR T + MM1S
MM1S alone
CS1CAR T alone
BCMA CAR T alone
BC1c CAR T alone
94.6%
1.3%
1.4%
2.7%
17.6%
7.6%
5.0%
69.8%
3.2%
2.3%
5.0%
89.5%
3.3%
2.9%
2.8%
90.9%
BCMA
CD3

FIGURE 28C

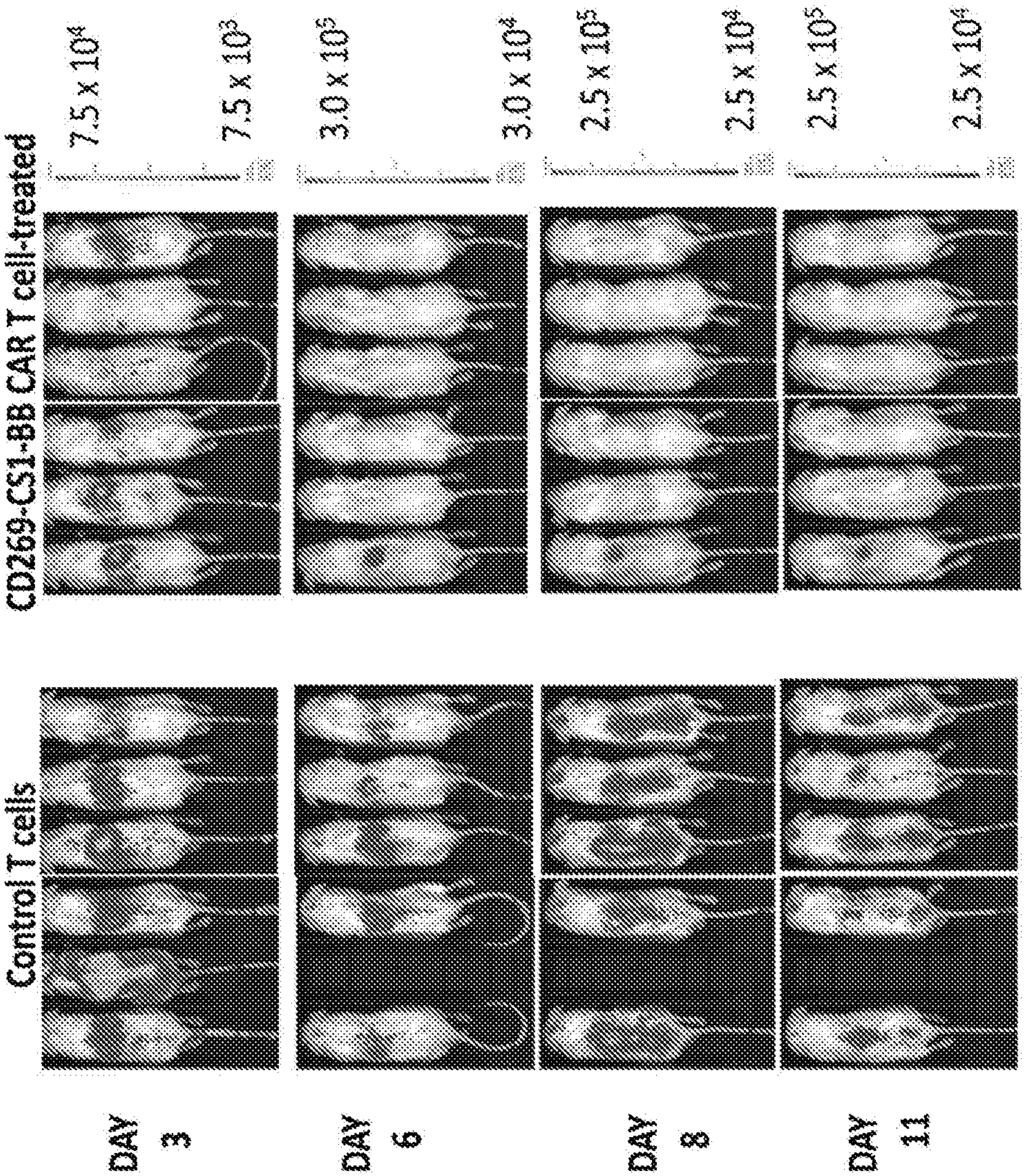

Control T cells
CD269-CS1-BB CAR T cell-treated
DAY 3
DAY 6
DAY 8
DAY 11
7.5 x 10^4
7.5 x 10^3
3.0 x 10^5
3.0 x 10^4
2.5 x 10^5
2.5 x 10^4
2.5 x 10^5
2.5 x 10^4

FIGURE 29A

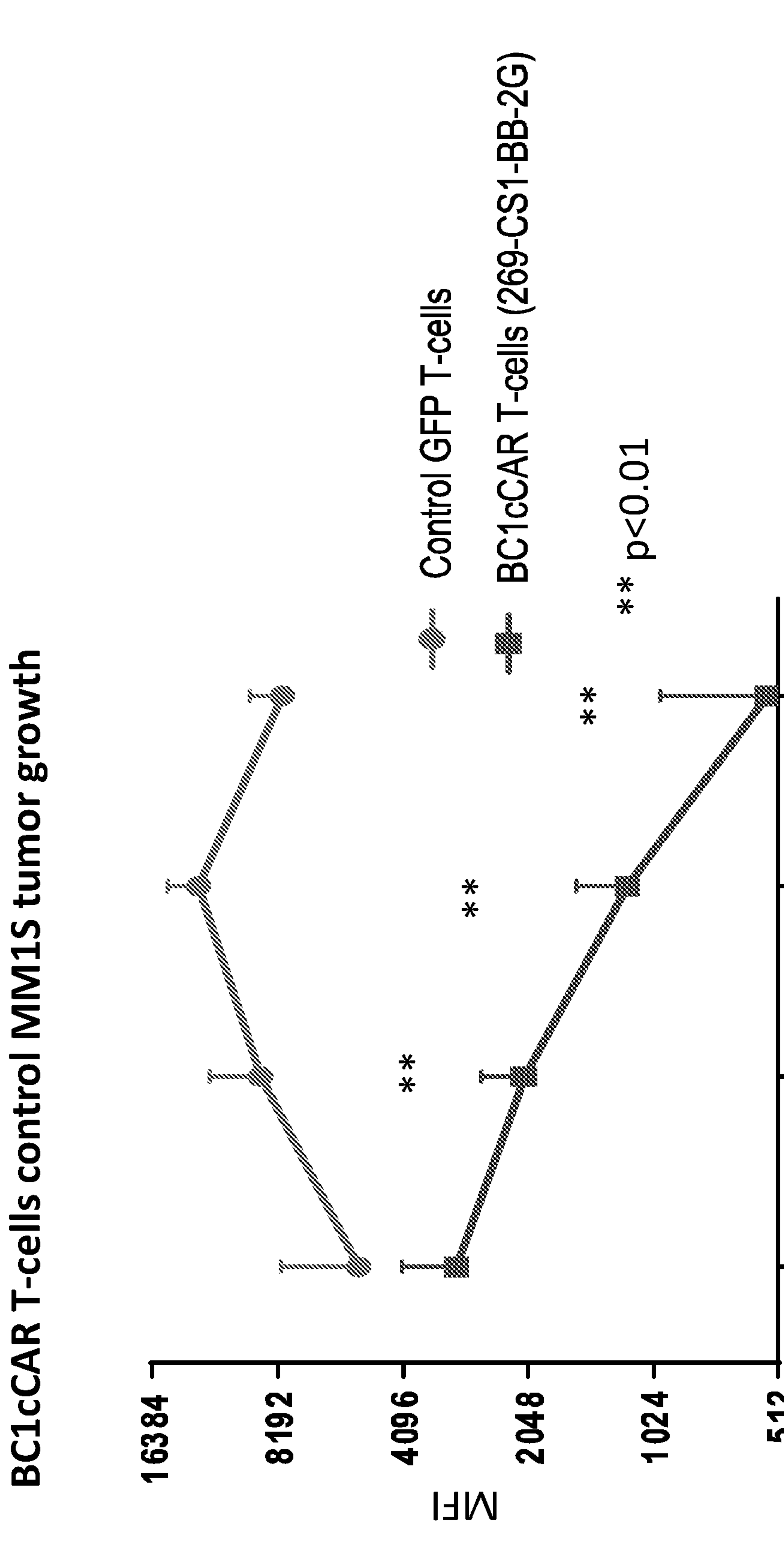

BC1cCAR T-cells control MM1S tumor growth
MFI
16384
8192
4096
2048
1024
512
Day 3
Day 6
Day 8
Day 11
**
**
**
Control GFP T-cells
BC1cCAR T-cells (269-CS1-BB-2G)
** p<0.01

FIGURE 29B

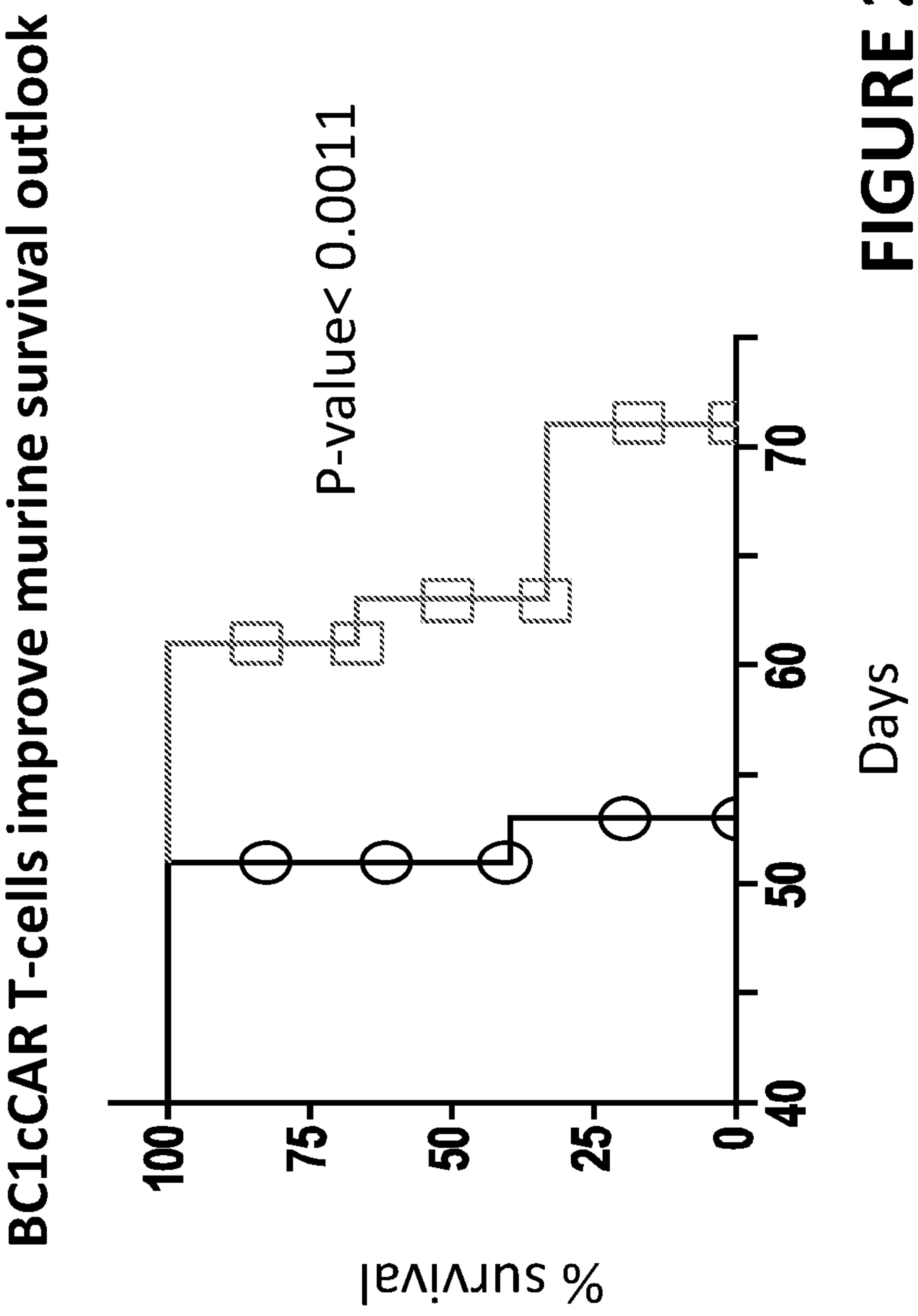

BC1cCAR T-cells improve murine survival outlook
P-value< 0.0011
% survival
100
75
50
25
0
40
50
60
70
Days

FIGURE 29C

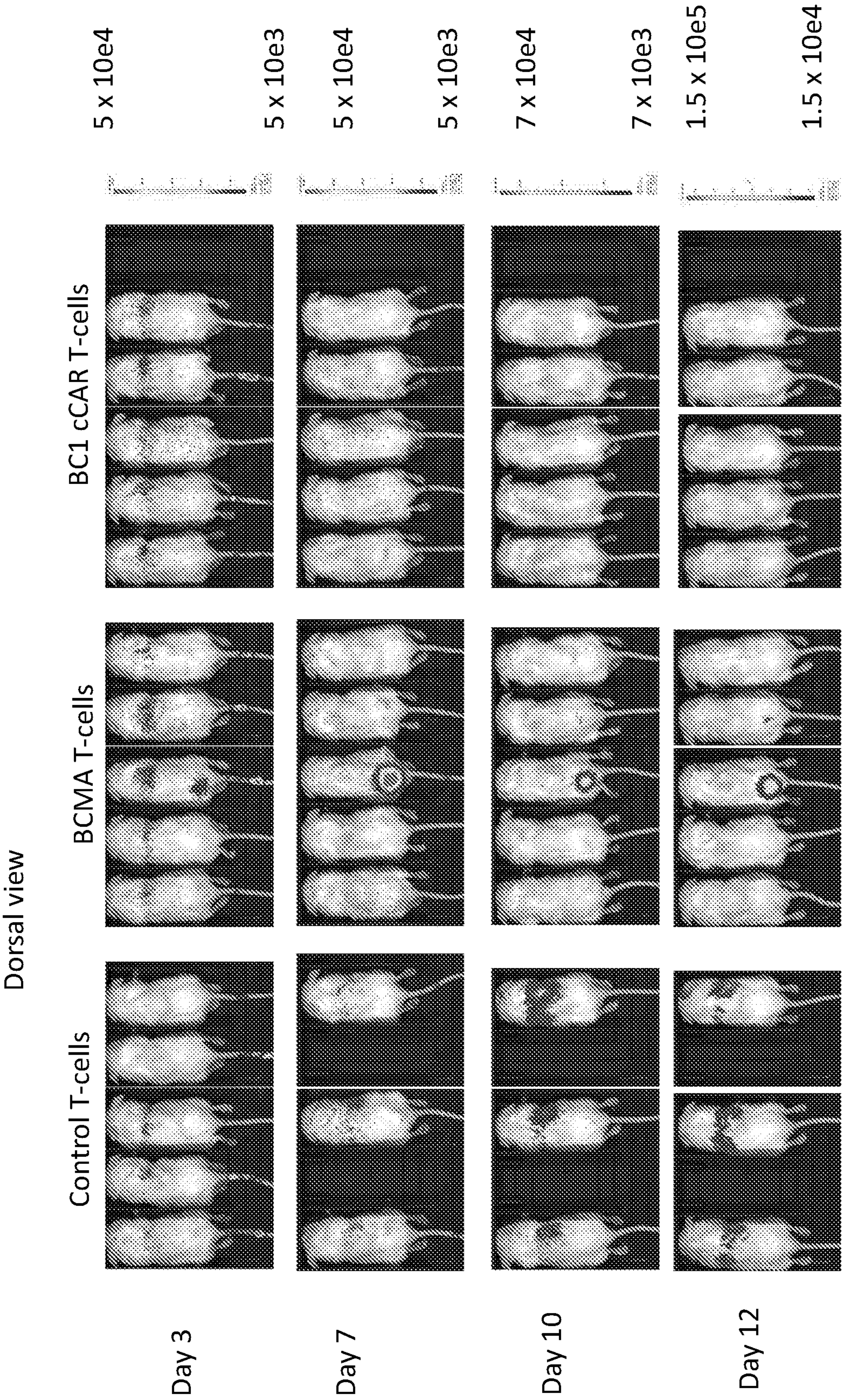

Dorsal view
Control T-cells
BCMA T-cells
BC1 cCAR T-cells
Day 3
Day 7
Day 10
Day 12
5 x 10e4
5 x 10e3
5 x 10e4
5 x 10e3
7 x 10e4
7 x 10e3
1.5 x 10e5
1.5 x 10e4

FIGURE 29D

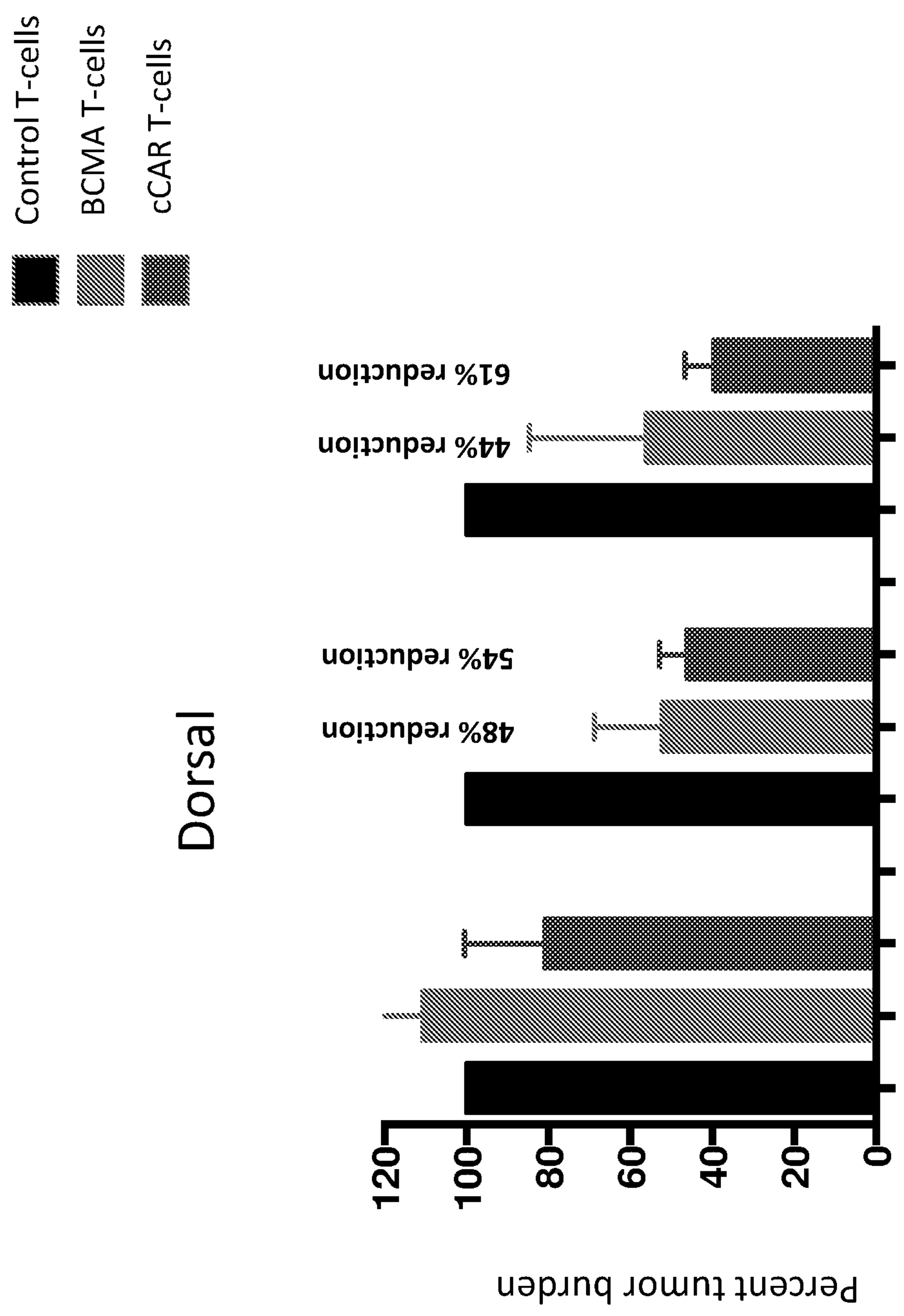

Dorsal
Control T-cells
BCMA T-cells
cCAR T-cells
Percent tumor burden
120
100
80
60
40
20
0
Day 7
Day 10
Day 12
48% reduction
54% reduction
44% reduction
61% reduction

FIGURE 29E

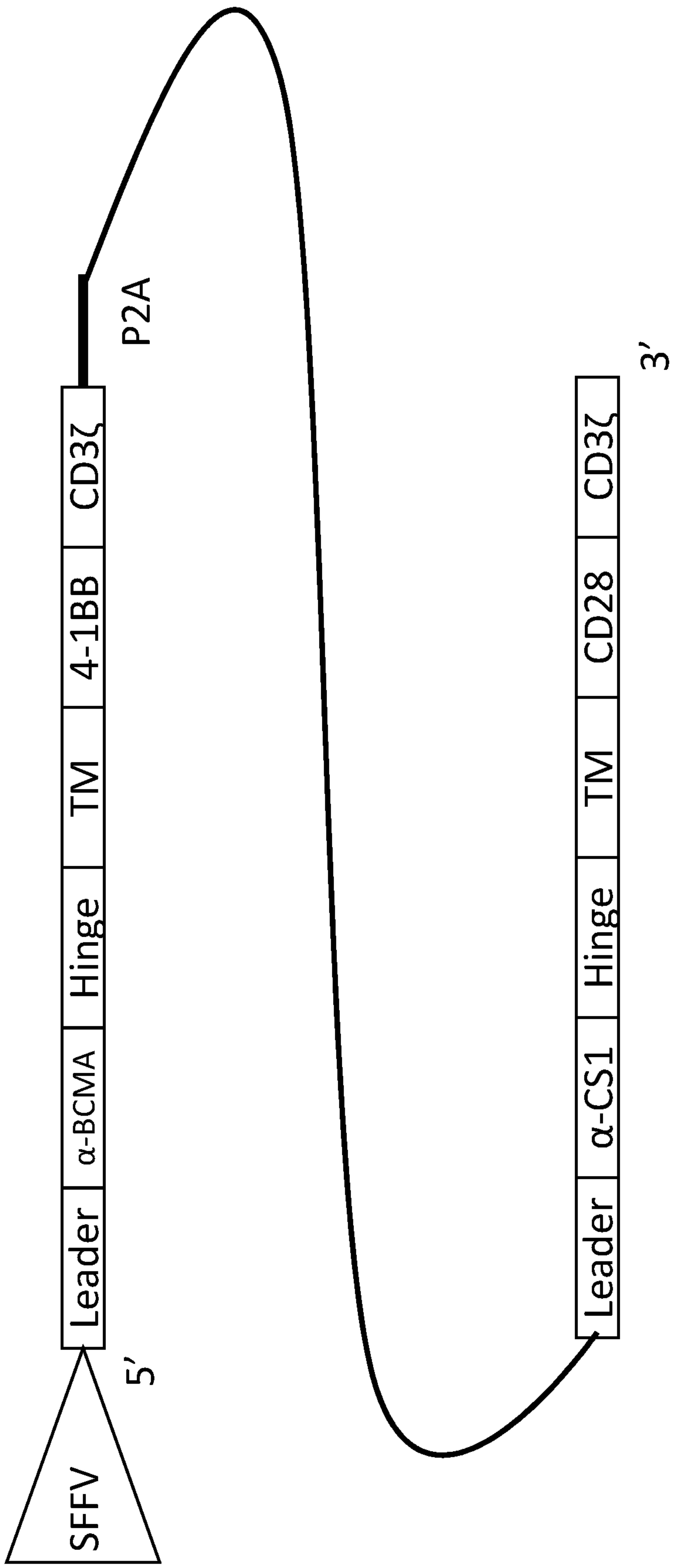
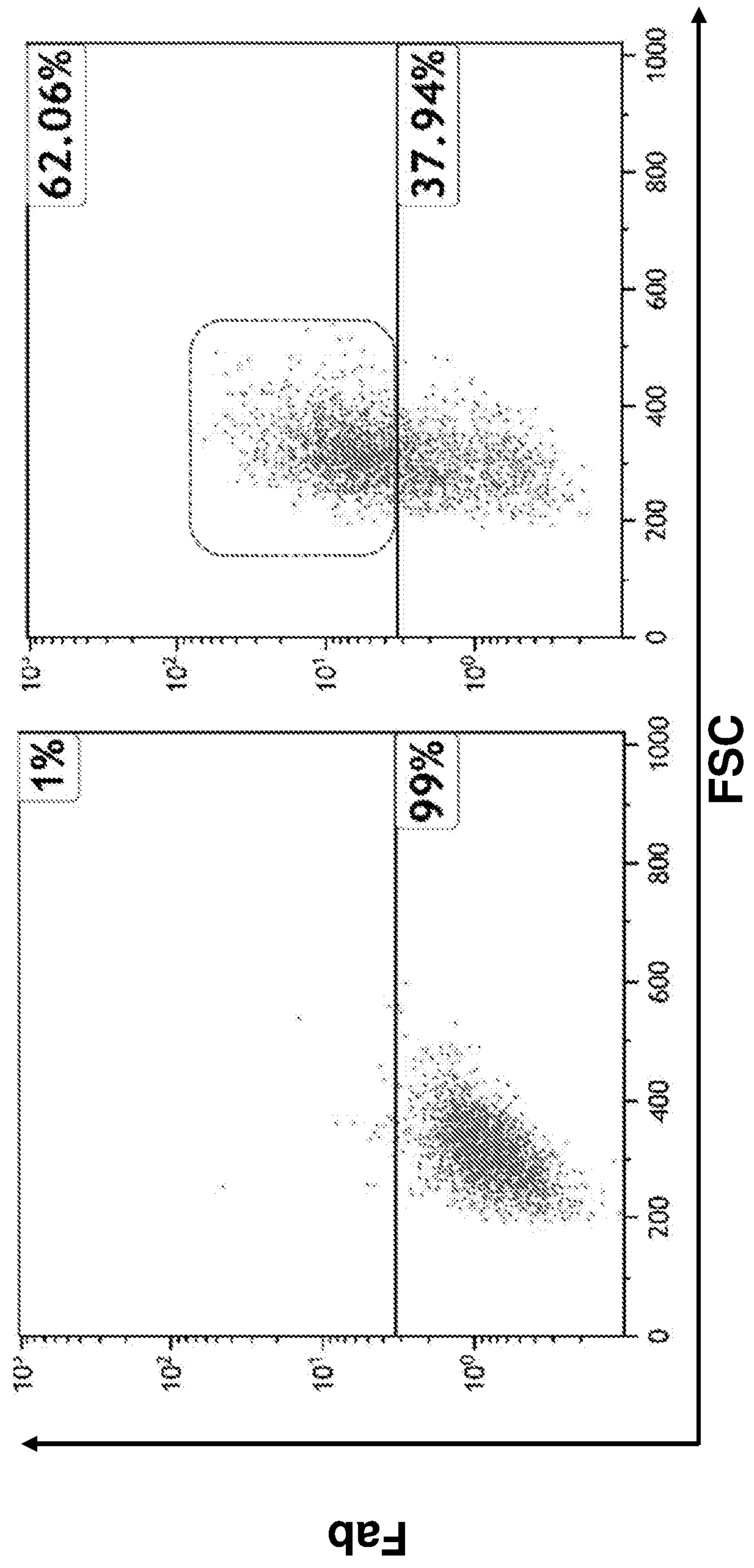

SFFV
5'
Leader
α-BCMA
Hinge
TM
4-1BB
CD3ζ
P2A
Leader
α-CS1
Hinge
TM
CD28
CD3ζ
3'

FIGURE 30A

FIGURE 30B



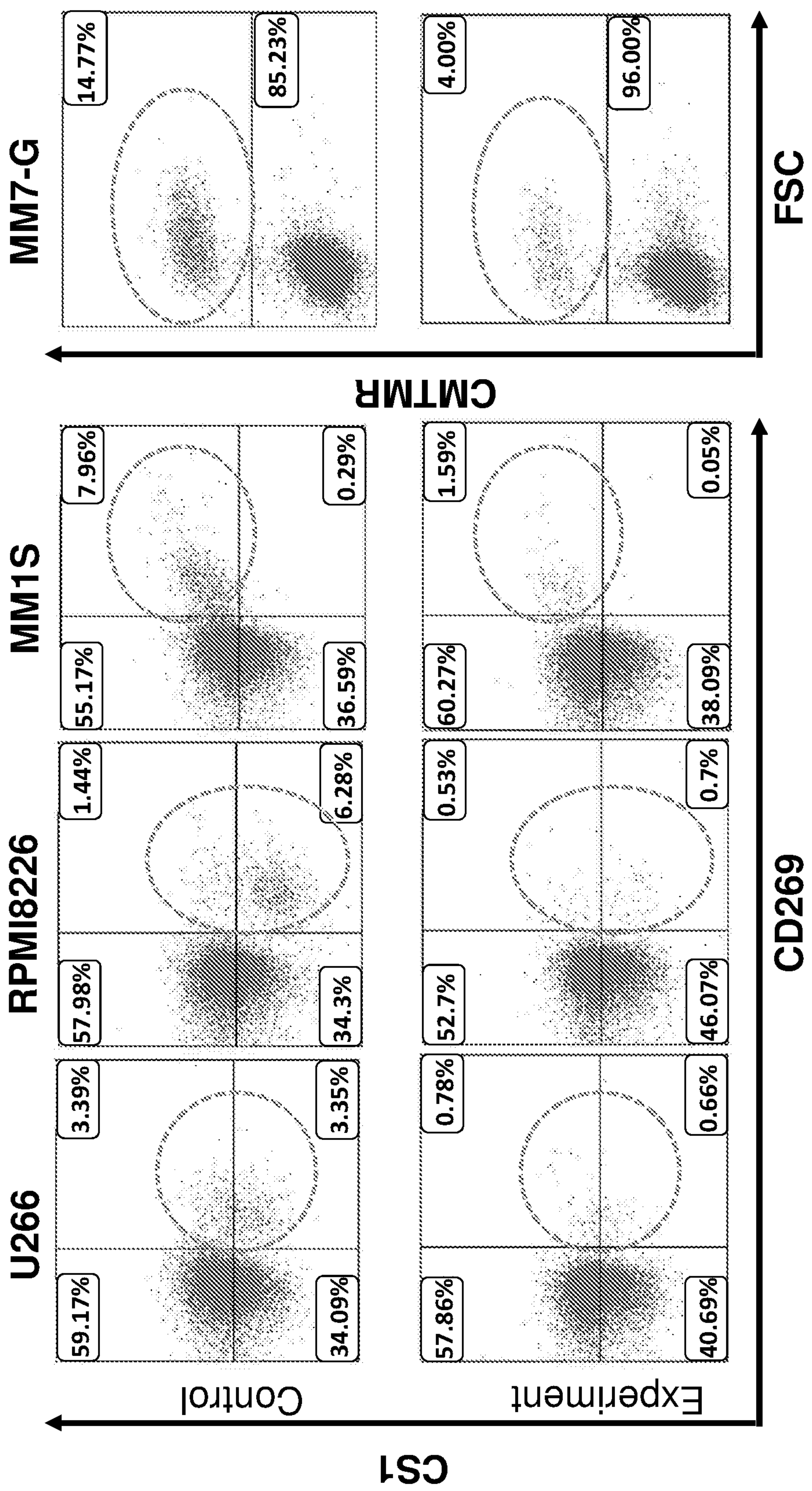

U266
RPMI8226
MM1S
MM7-G
Control
Experiment
CS1
CD269
CMTMR
FSC
59.17%
34.09%
3.39%
3.35%
57.86%
40.69%
0.78%
0.66%
57.98%
34.3%
1.44%
6.28%
52.7%
46.07%
0.53%
0.7%
55.17%
36.59%
7.96%
0.29%
60.27%
38.09%
1.59%
0.05%
14.77%
85.23%
4.00%
96.00%

FIGURE 31A

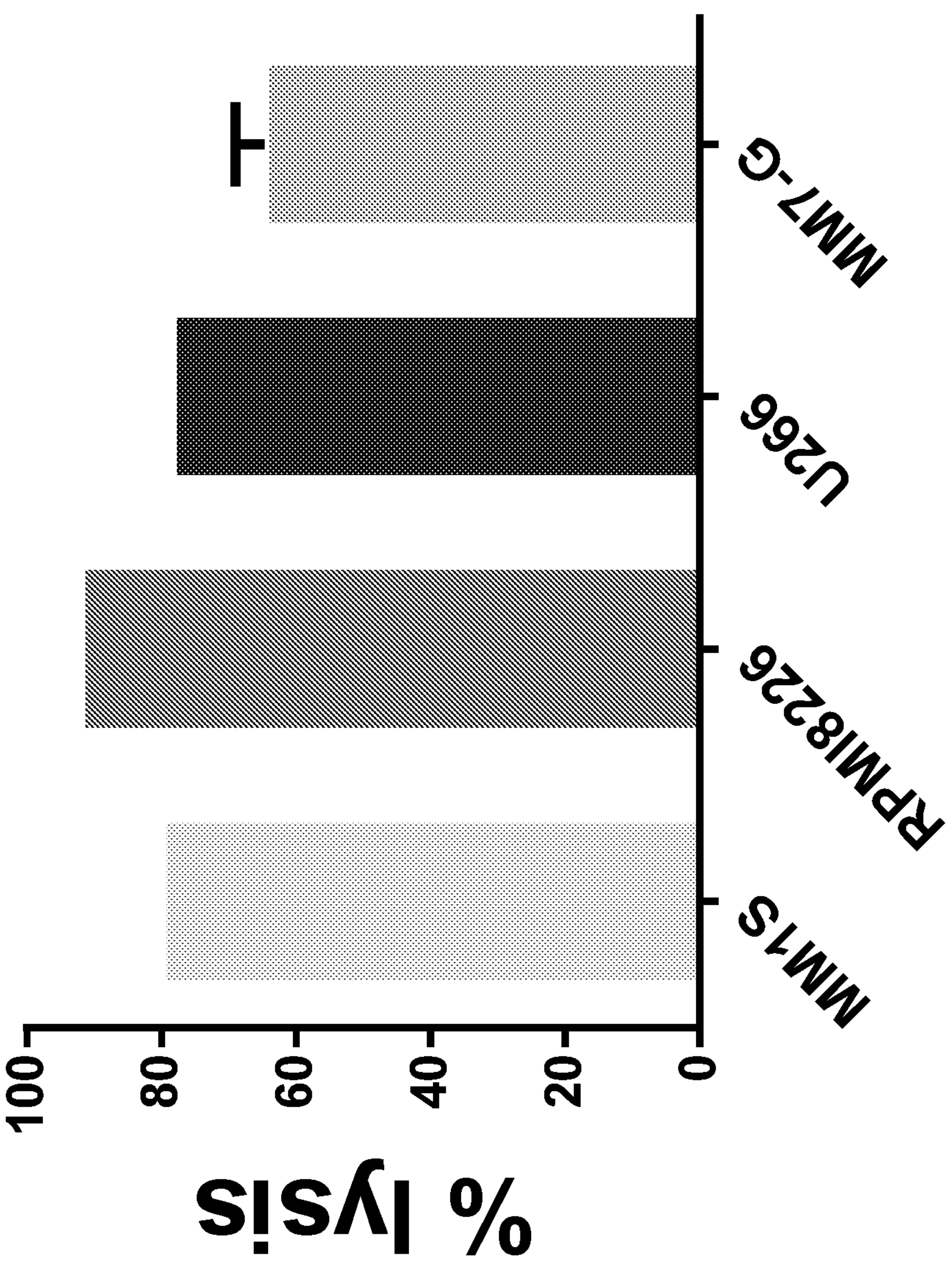


FIGURE 31B

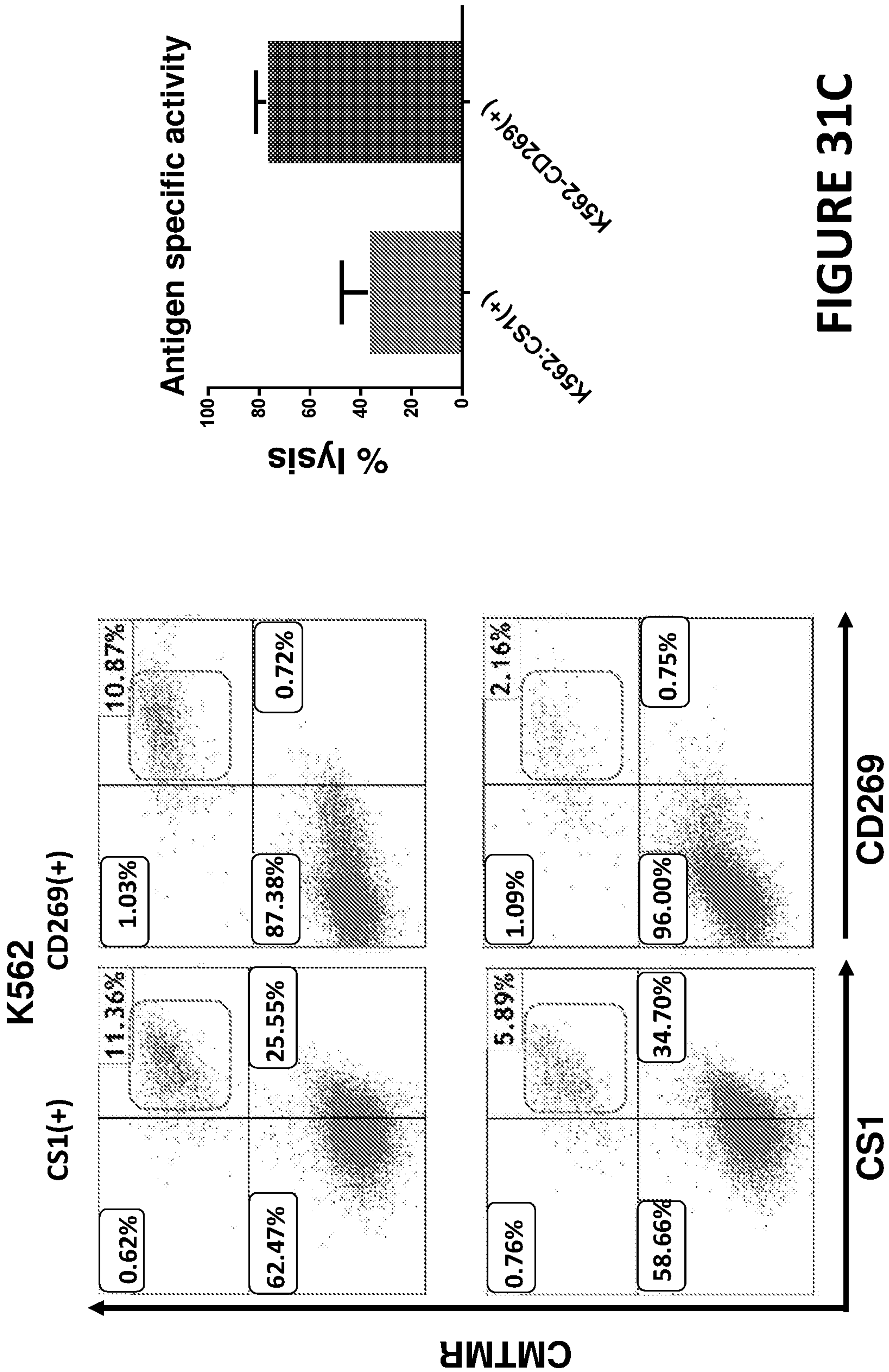

Antigen specific activity
% lysis
100
80
60
40
20
0
K562:CS1(+)
K562-CD269(+)
K562
CS1(+)
CD269(+)
0.62%
62.47%
11.36%
25.55%
1.03%
87.38%
10.87%
0.72%
0.76%
58.66%
5.89%
34.70%
1.09%
96.00%
2.16%
0.75%
CMTMR
CS1
CD269

FIGURE 31C

FIGURE 32A

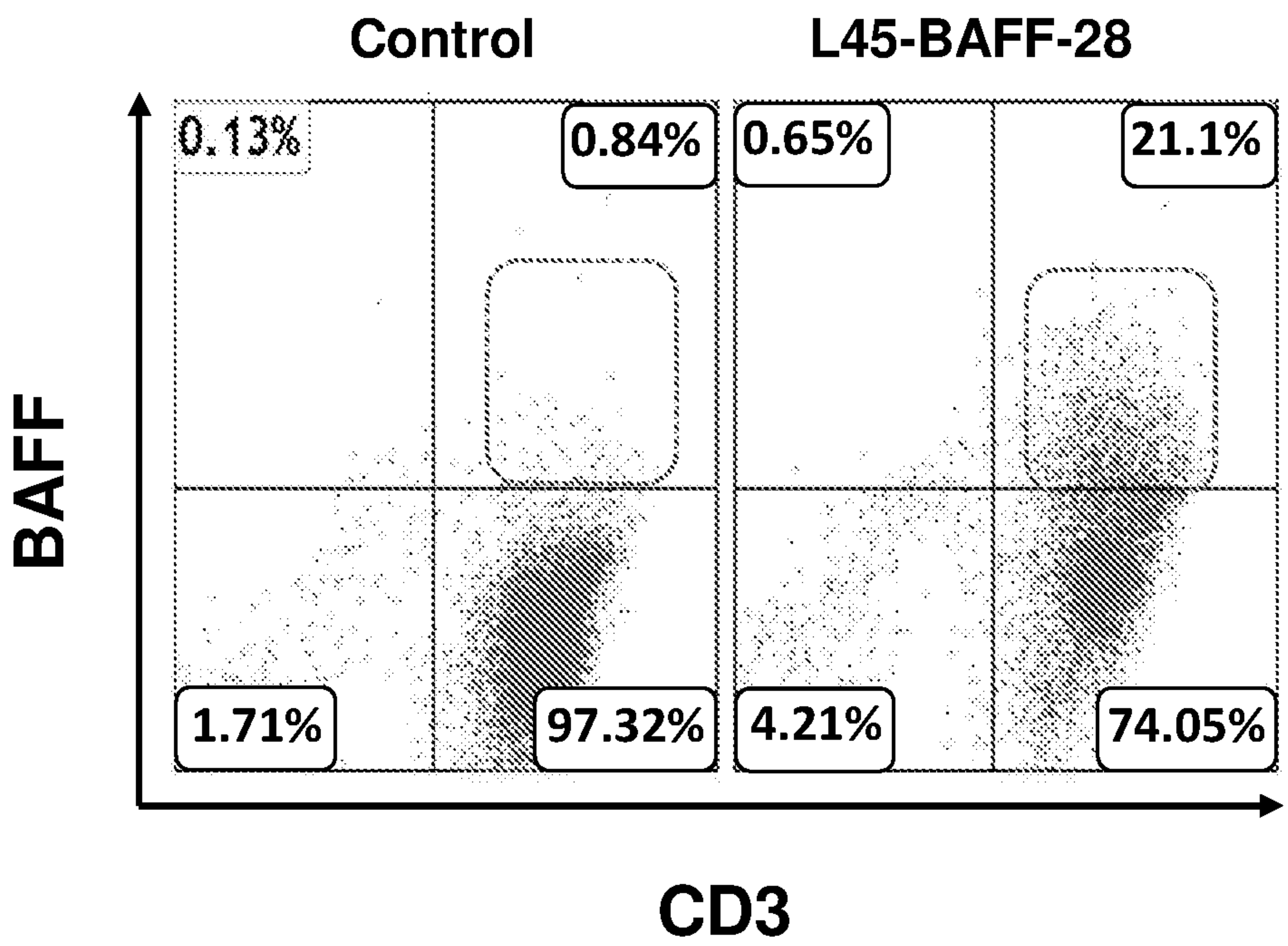
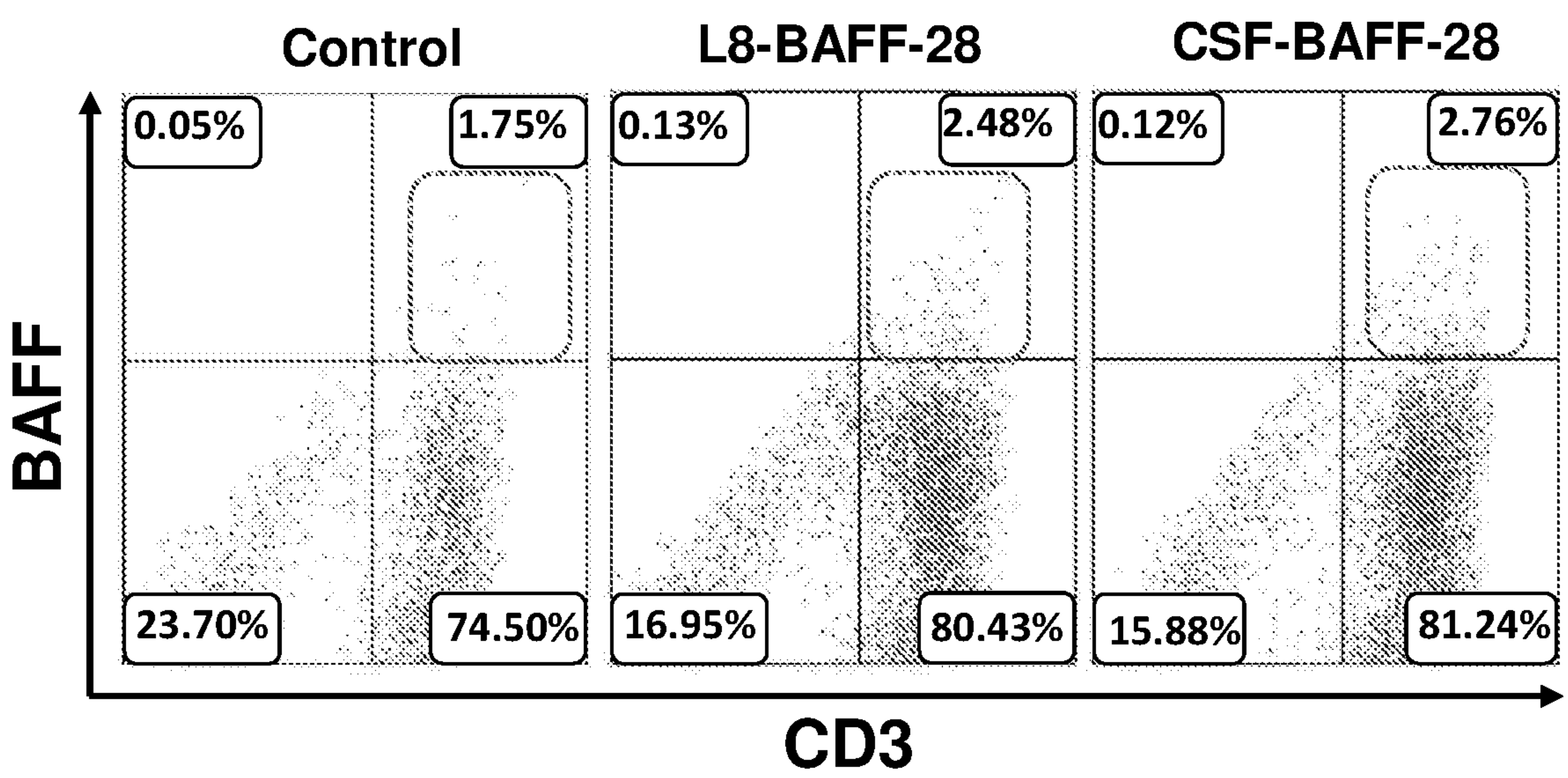


FIGURE 32B



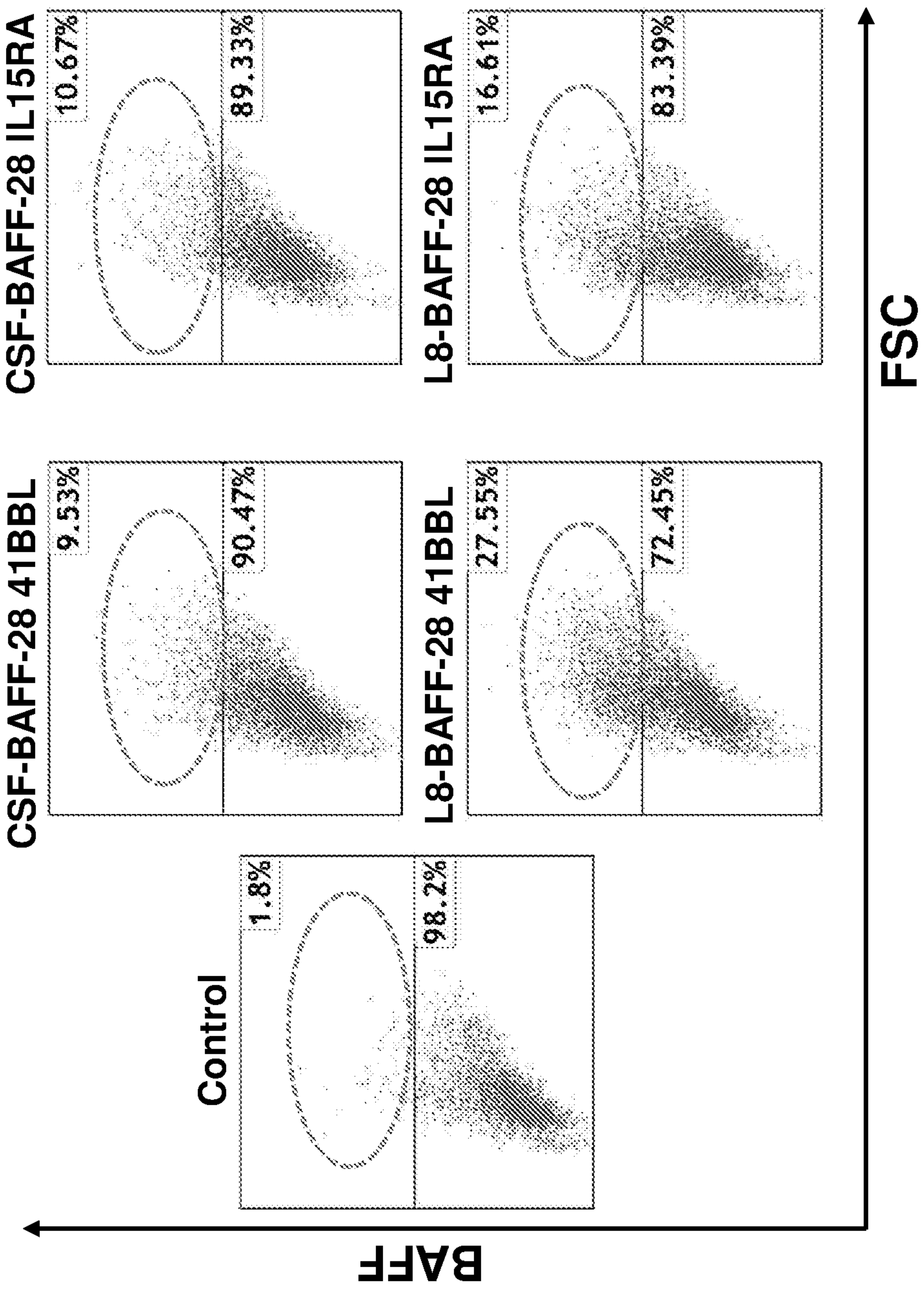

Control
1.8%
98.2%
CSF-BAFF-28 41BBL
9.53%
90.47%
CSF-BAFF-28 IL15RA
10.67%
89.33%
L8-BAFF-28 41BBL
27.55%
72.45%
L8-BAFF-28 IL15RA
16.61%
83.39%
FSC
BAFF

FIGURE 32C

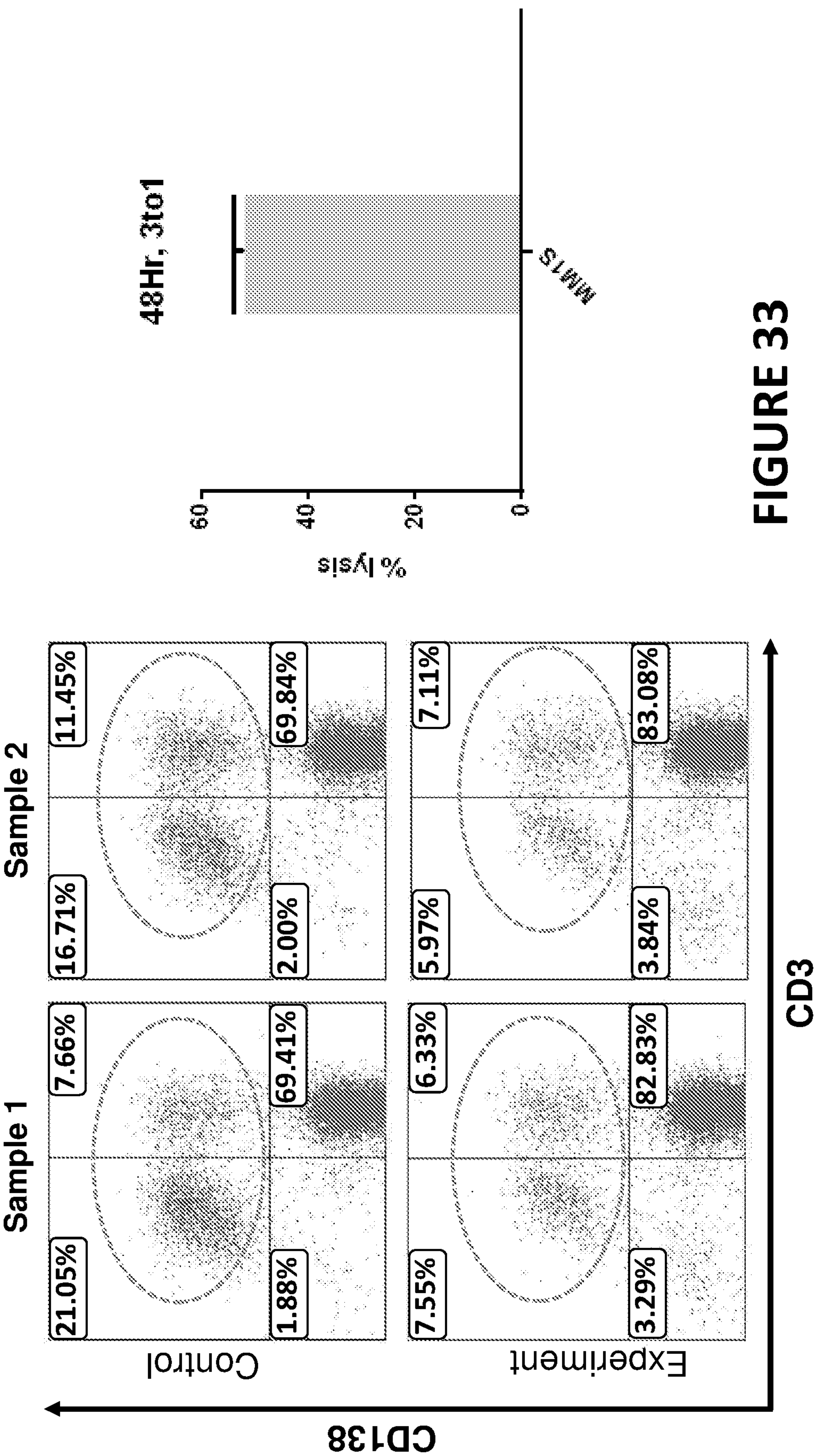

48Hr, 3to1
% lysis
60
40
20
0
MM1S
Sample 1
Sample 2
Control
Experiment
21.05%
7.66%
1.88%
69.41%
16.71%
11.45%
2.00%
69.84%
7.55%
6.33%
3.29%
82.83%
5.97%
7.11%
3.84%
83.08%
CD138
CD3

FIGURE 33

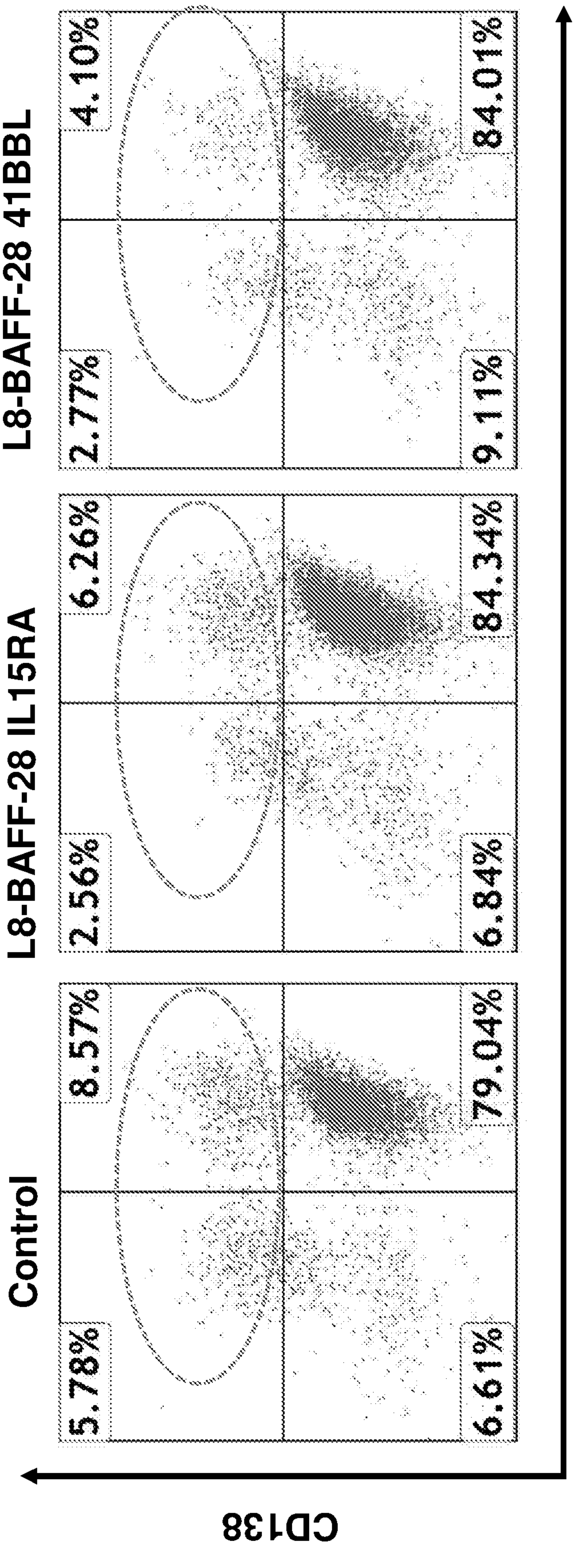
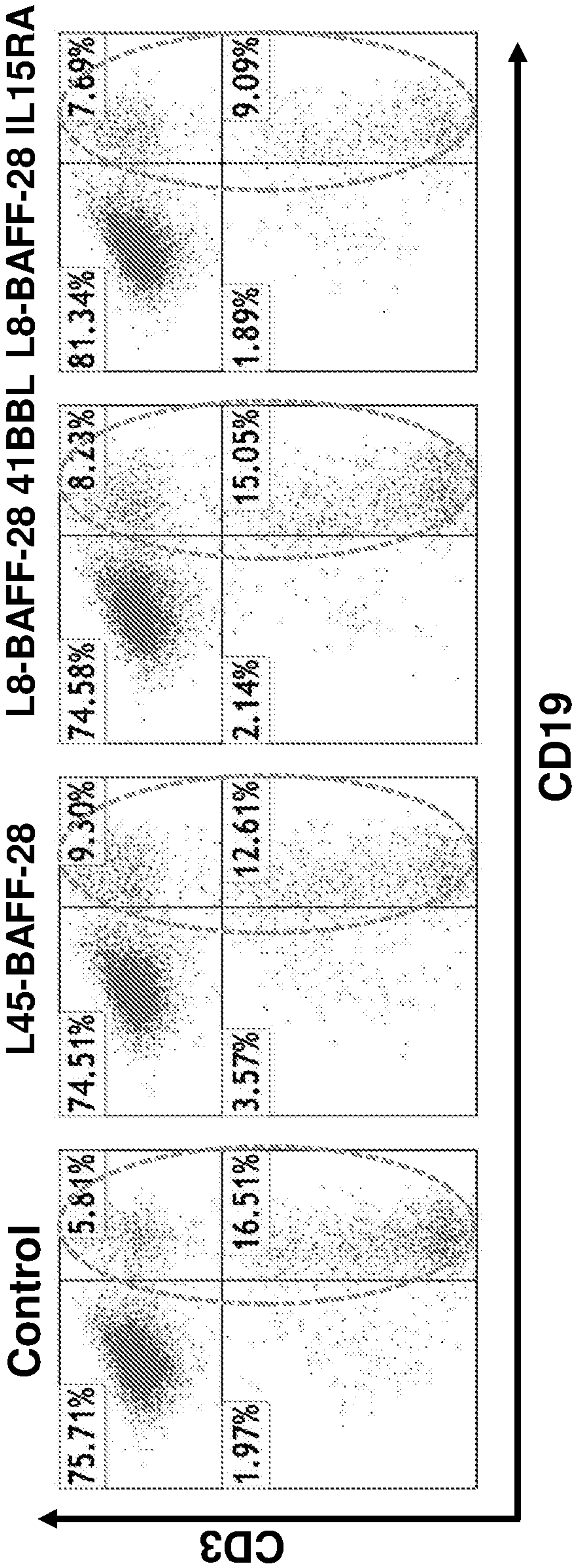

Control
L8-BAFF-28 IL15RA
L8-BAFF-28 41BBL
5.78%
8.57%
6.61%
79.04%
2.56%
6.26%
6.84%
84.34%
2.77%
4.10%
9.11%
84.01%
CD3
CD138

FIGURE 34A

FIGURE 34B


Control
75.71%
5.81%
1.97%
16.51%
L45-BAFF-28
74.51%
9.30%
3.57%
12.61%
L8-BAFF-28 41BBL
74.58%
8.23%
2.14%
15.05%
L8-BAFF-28 IL15RA
81.34%
7.69%
1.89%
9.09%
CD3
CD19

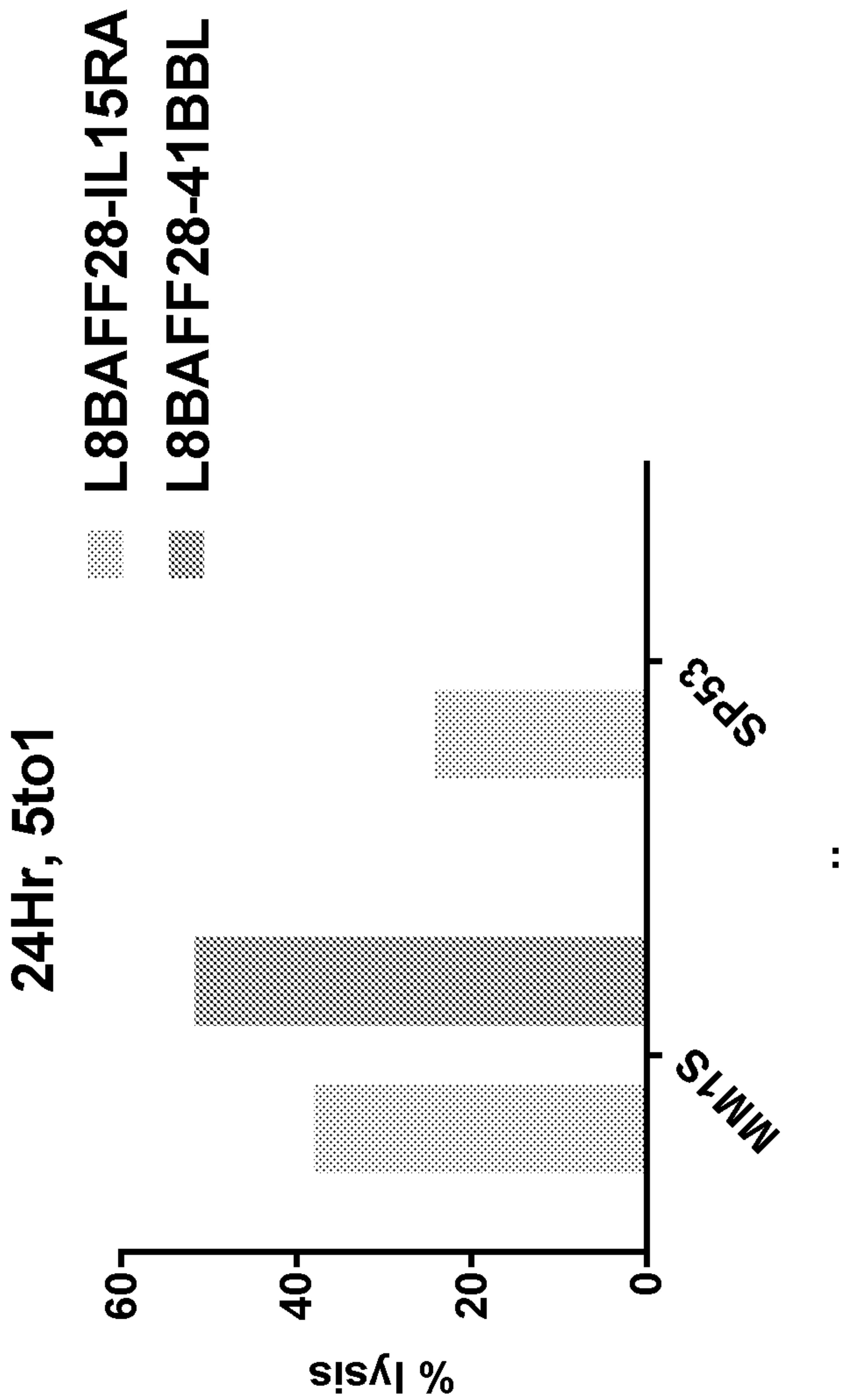
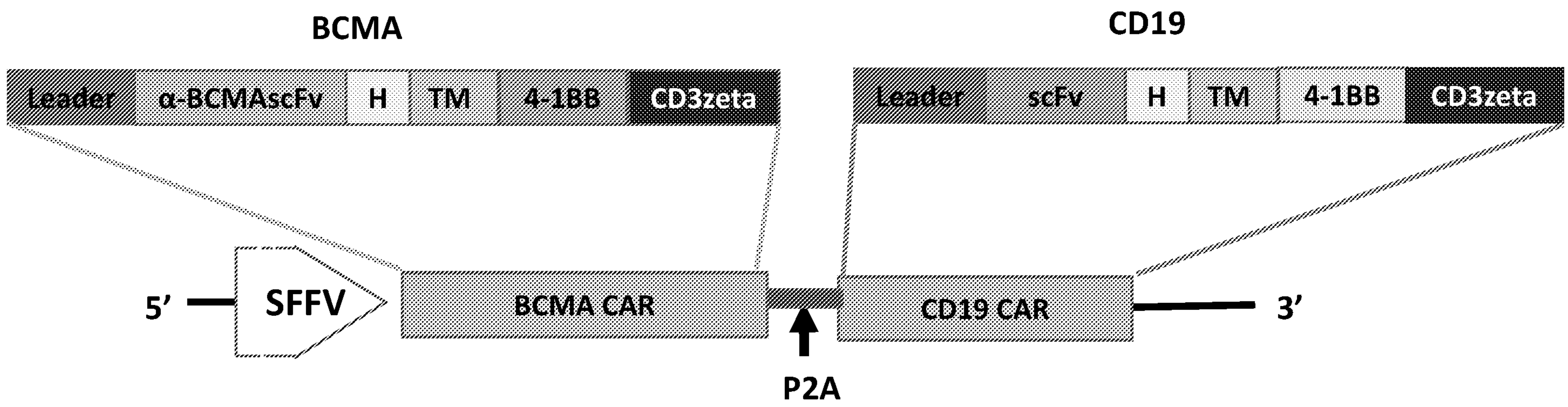

24Hr, 5to1
L8BAFF28-IL15RA
L8BAFF28-41BBL
% lysis
60
40
20
0
MM1S
SP53

FIGURE 34C

FIGURE 35


BCMA
Leader
α-BCMAscFv
H
TM
4-1BB
CD3zeta
CD19
Leader
scFv
H
TM
4-1BB
CD3zeta
5'
SFFV
BCMA CAR
CD19 CAR
3'
P2A

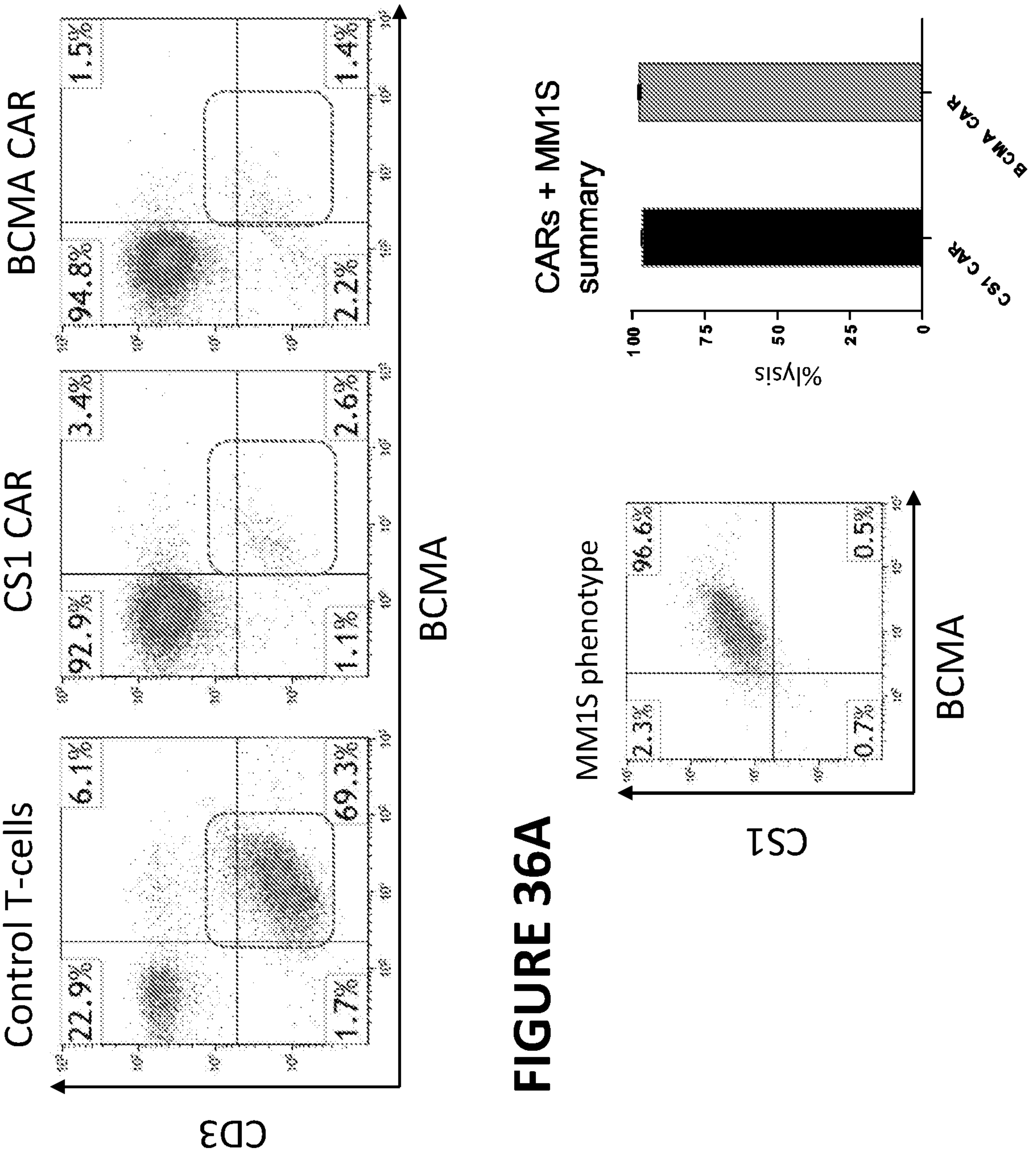

Control T-cells
CS1 CAR
BCMA CAR
CD3
BCMA
22.9%
6.1%
1.7%
69.3%
92.9%
3.4%
1.1%
2.6%
94.8%
1.5%
2.2%
1.4%
MM1S phenotype
CS1
2.3%
96.6%
0.7%
0.5%
CARs + MM1S
summary
%lysis
100
75
50
25
0
CS1 CAR
BCMA CAR

FIGURE 36A

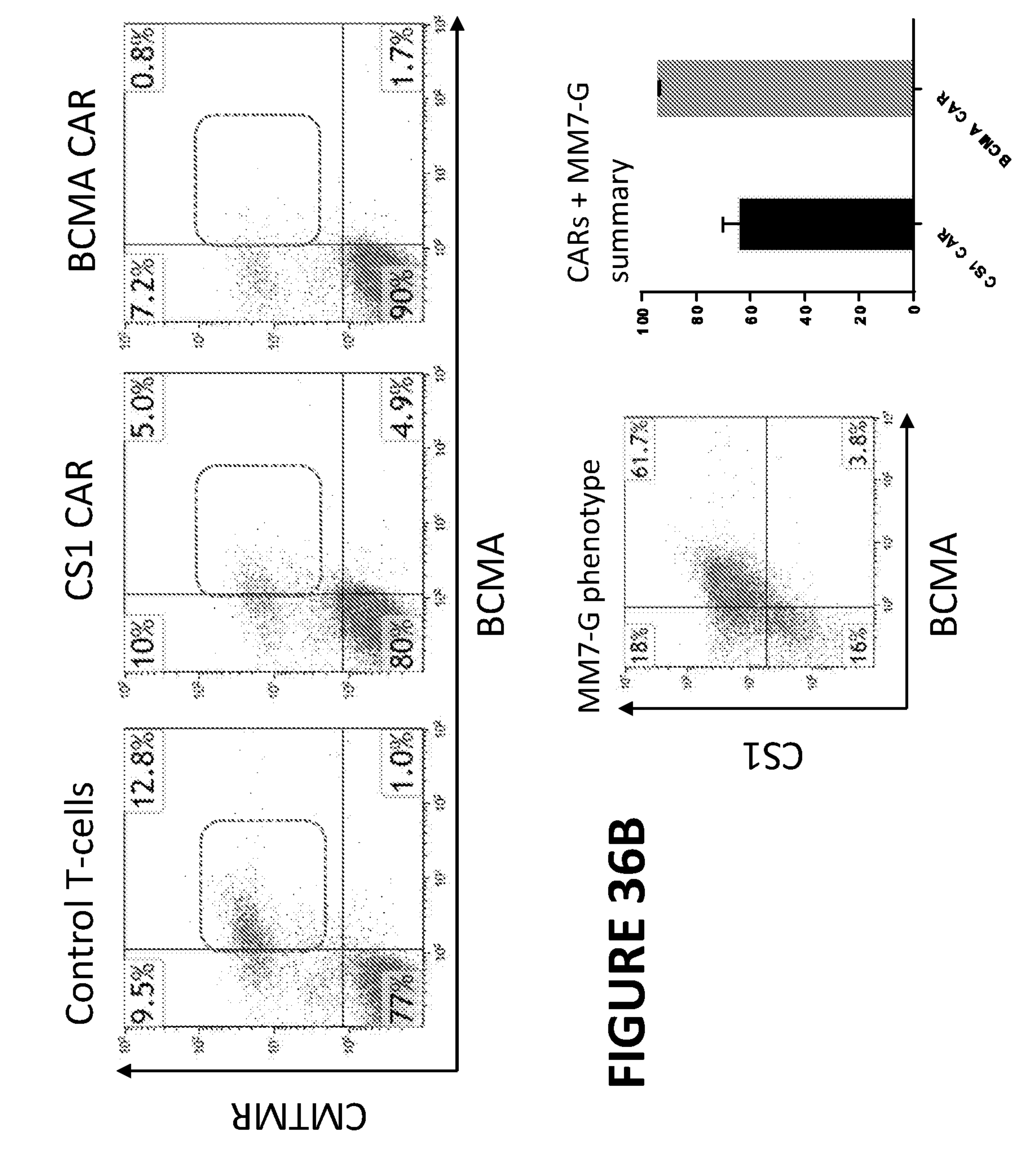

Control T-cells
9.5%
12.8%
77%
1.0%
CS1 CAR
10%
5.0%
80%
4.9%
BCMA CAR
7.2%
0.8%
90%
1.7%
CMTMR
BCMA
MM7-G phenotype
18%
61.7%
16%
3.8%
CS1
BCMA
CARs + MM7-G
summary
100
80
60
40
20
0
CS1 CAR
BCMA CAR

FIGURE 36B

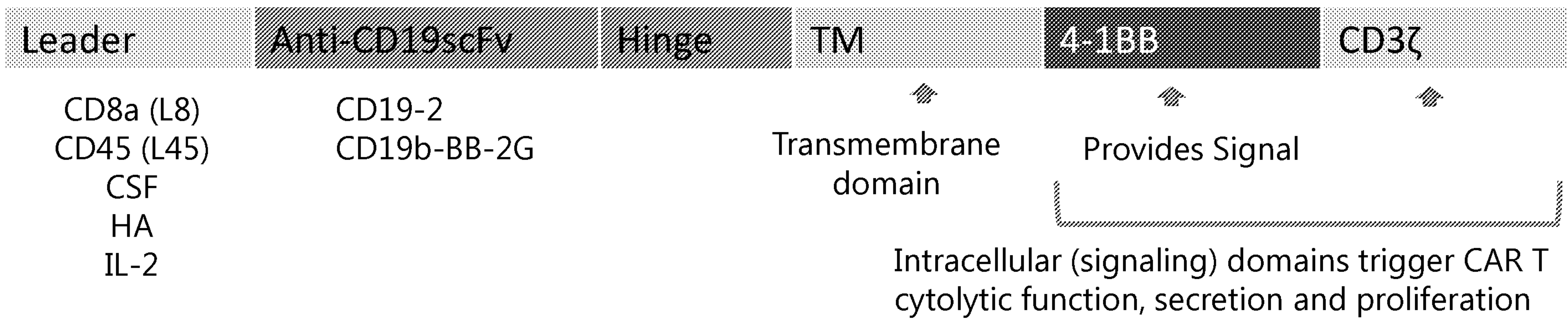

Leader
Anti-CD19scFv
Hinge
TM
4-1BB
CD3ζ
CD8a (L8)
CD45 (L45)
CSF
HA
IL-2
CD19-2
CD19b-BB-2G
Transmembrane domain
Provides Signal
Intracellular (signaling) domains trigger CAR T cytolytic function, secretion and proliferation

FIGURE 37A

FIGURE 37B

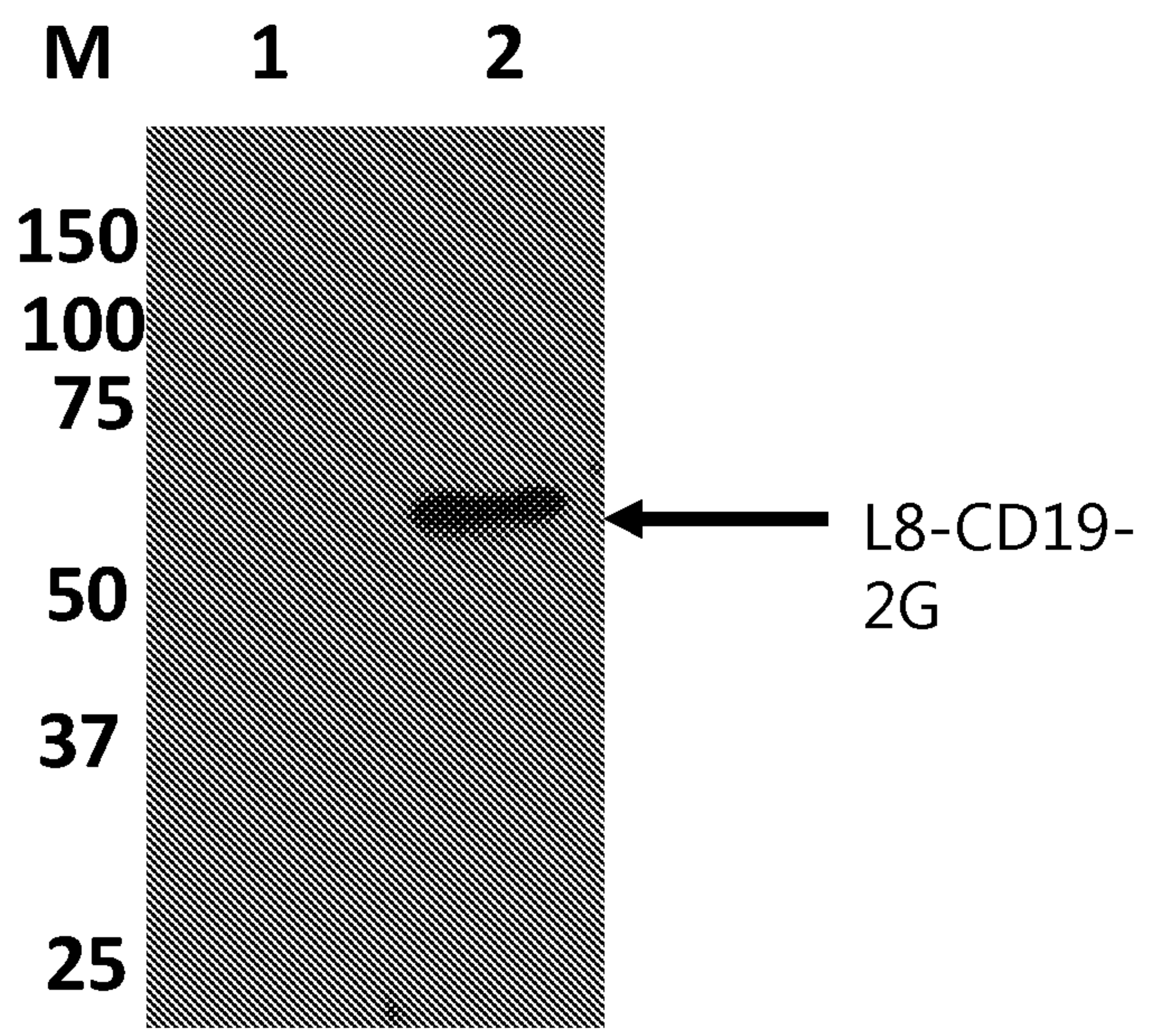
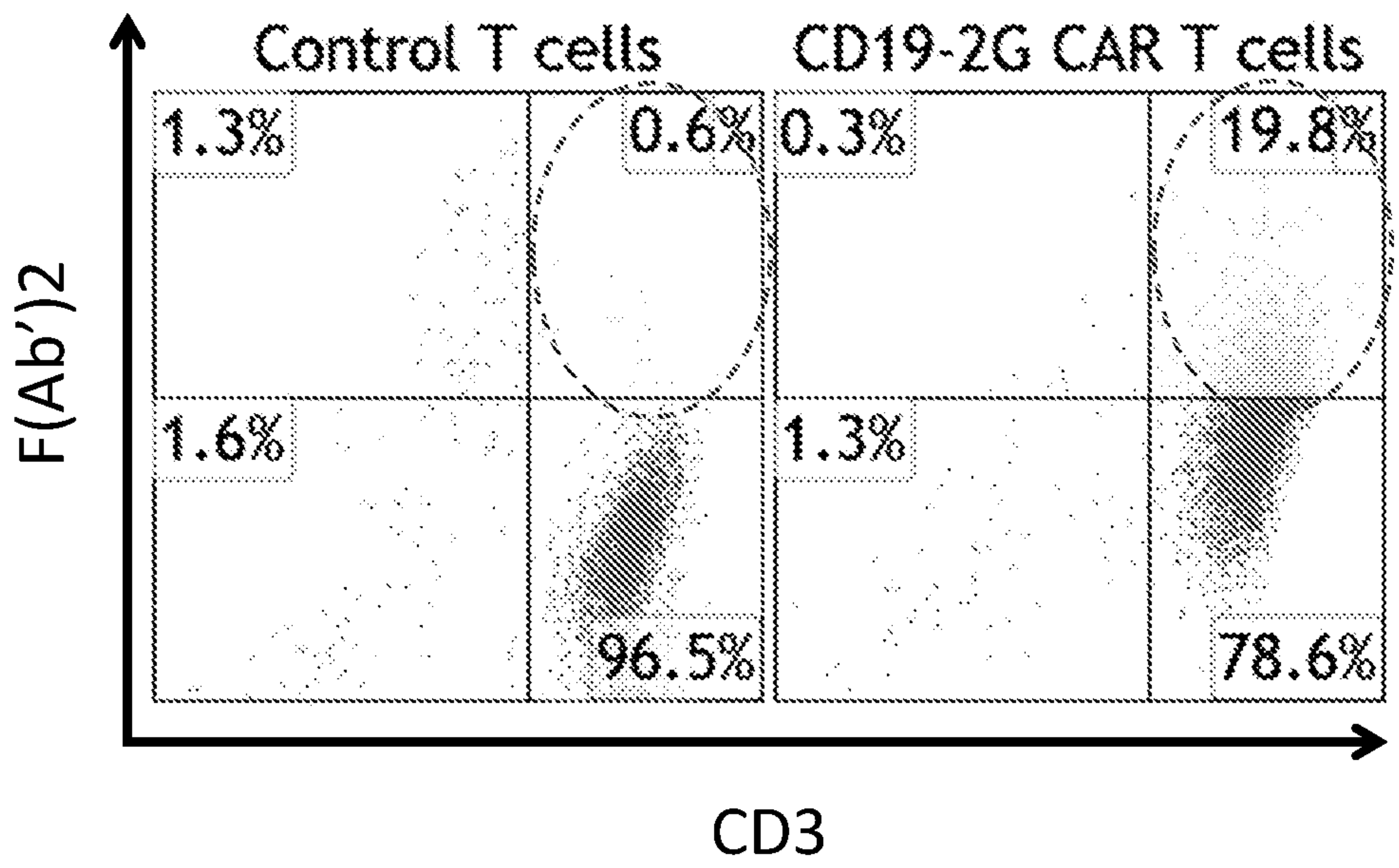


FIGURE 37C



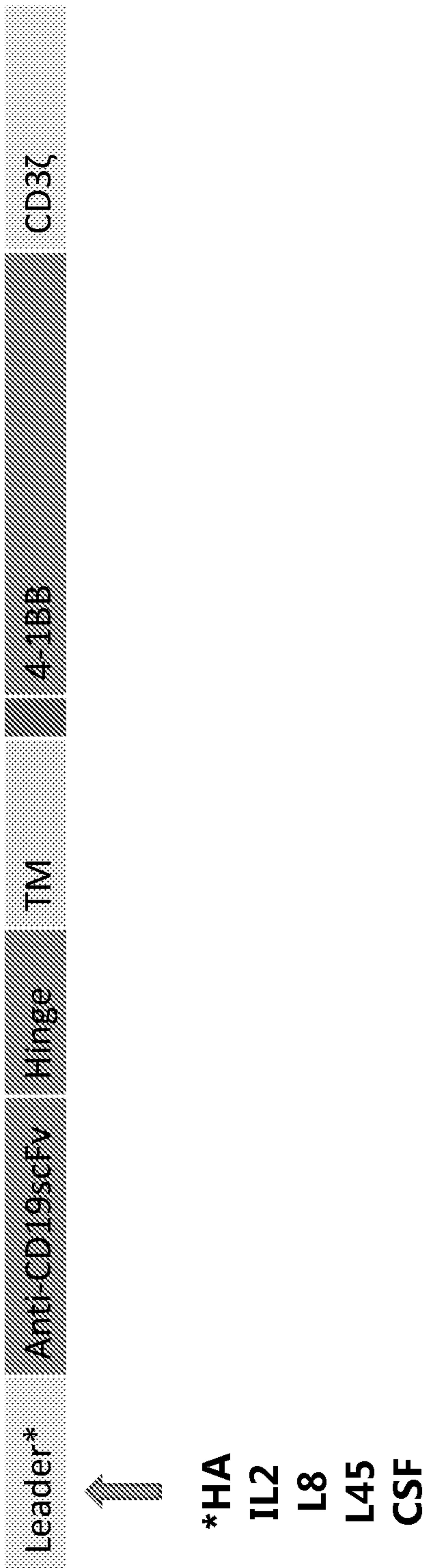


FIGURE 38A

FIGURE 38B

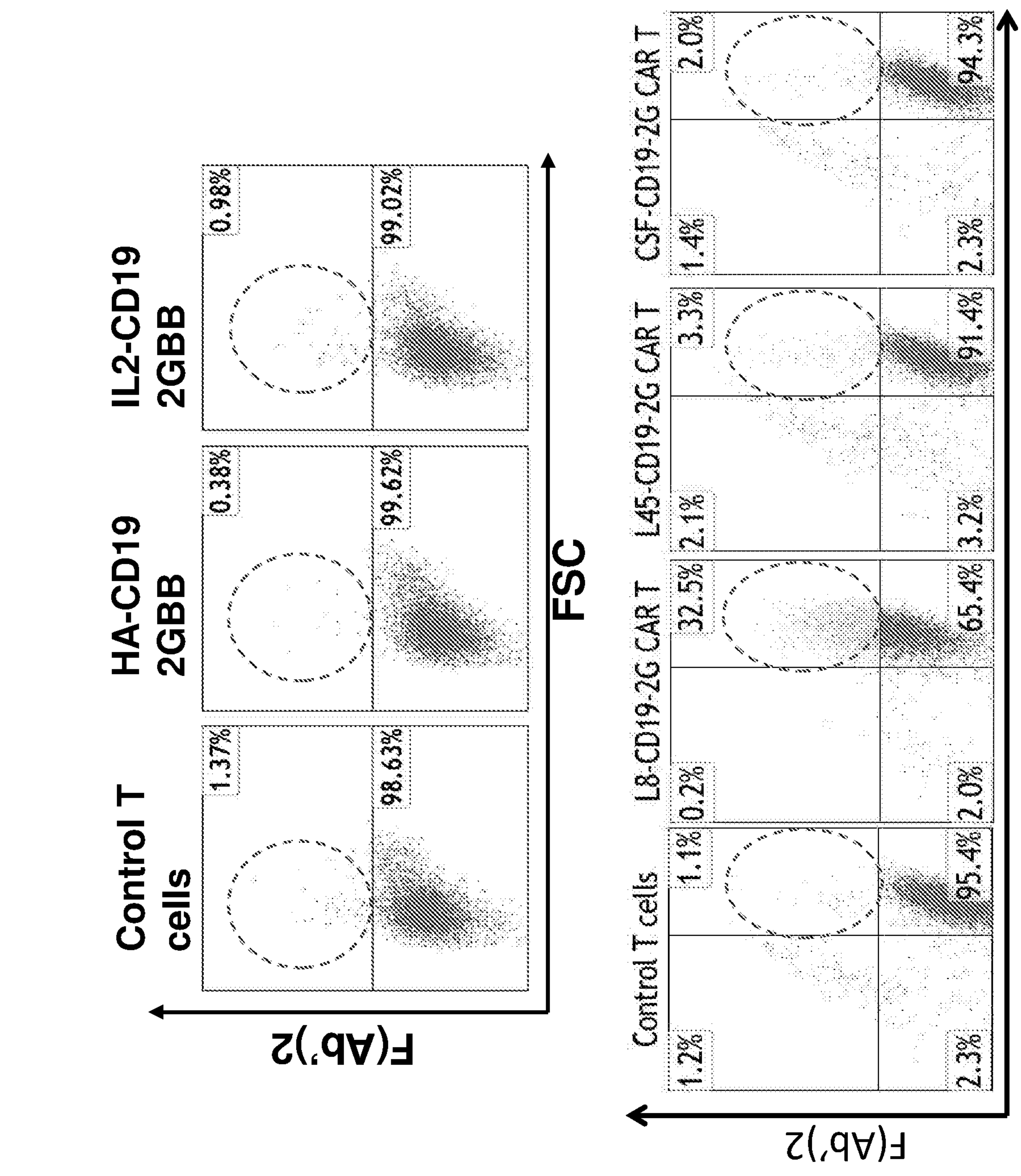

Control T cells
1.37%
98.63%
HA-CD19 2GBB
0.38%
99.62%
IL2-CD19 2GBB
0.98%
99.02%
FSC
F(Ab')2
Control T cells
1.2%
1.1%
2.3%
95.4%
L8-CD19-2G CAR T
0.2%
32.5%
2.0%
65.4%
L45-CD19-2G CAR T
2.1%
3.3%
3.2%
91.4%
CSF-CD19-2G CAR T
1.4%
2.0%
2.3%
94.3%
CD3
F(Ab')2

FIGURE 39A

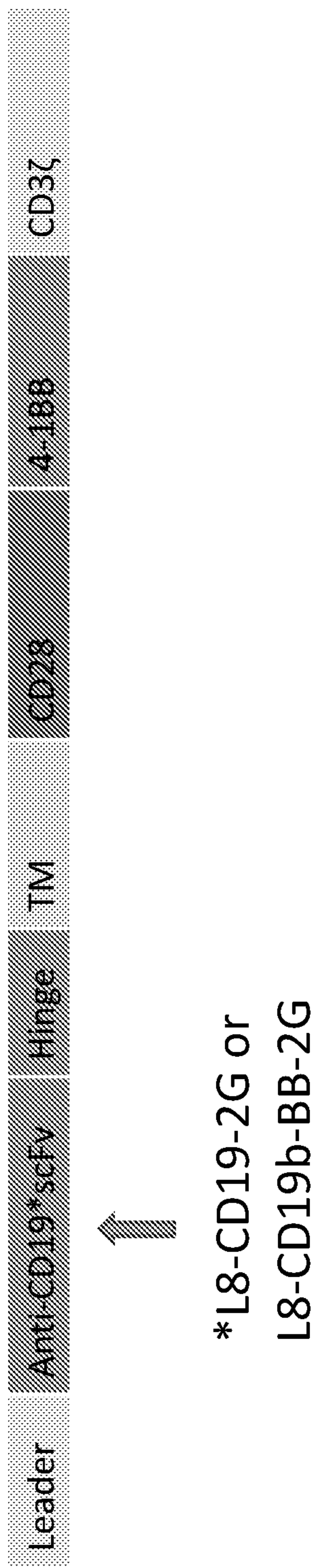


FIGURE 39B

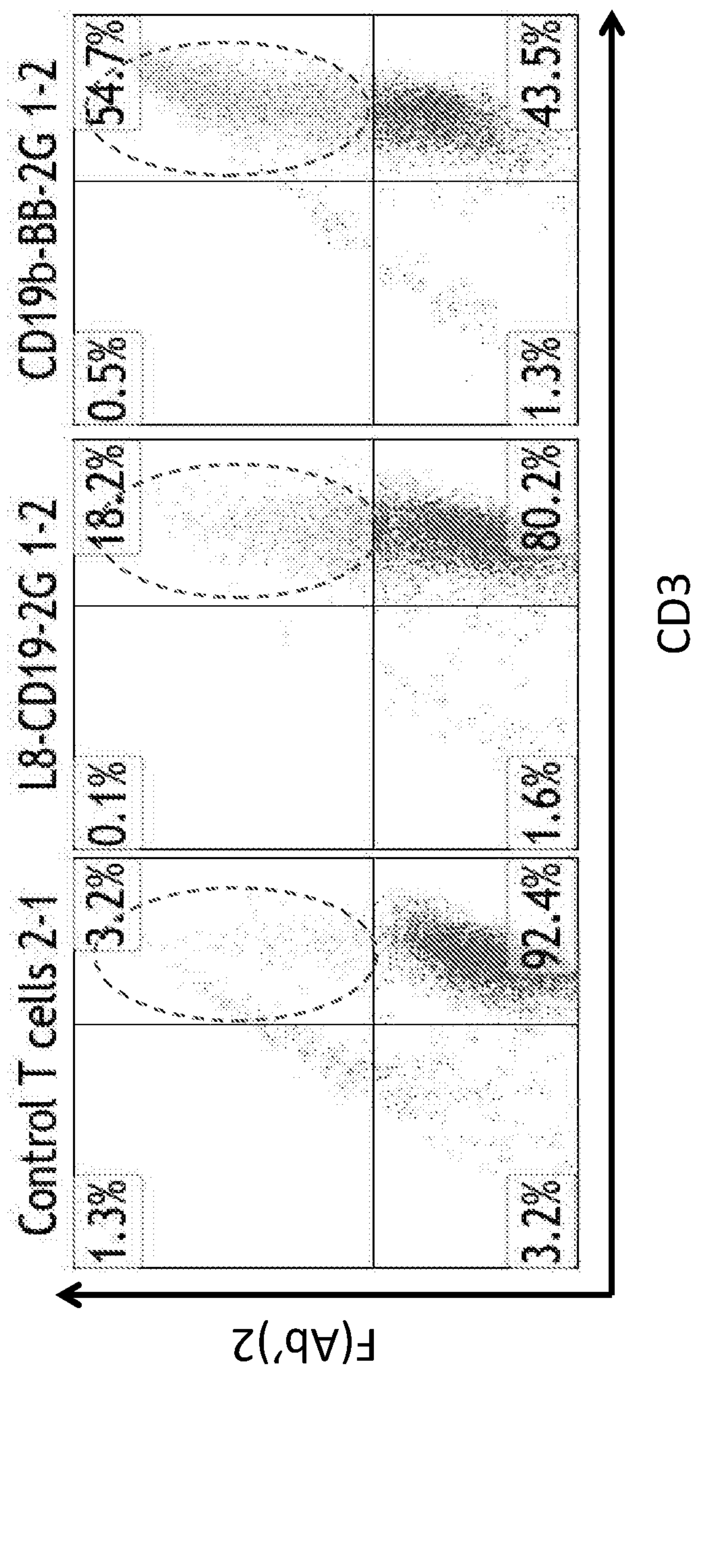


FIGURE 40

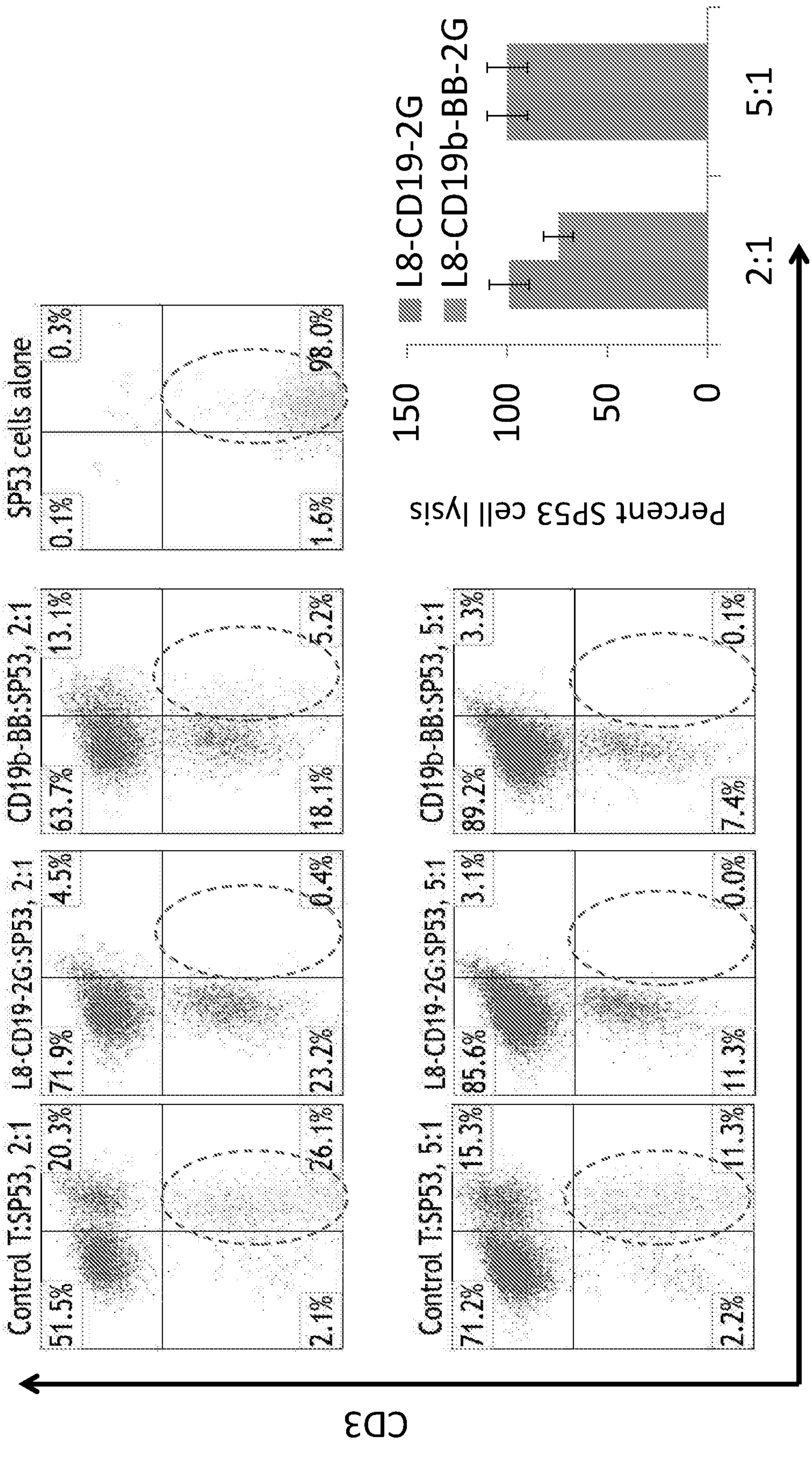

Control T:SP53, 2:1
51.5%
20.3%
2.1%
26.1%
L8-CD19-2G:SP53, 2:1
71.9%
4.5%
23.2%
0.4%
CD19b-BB:SP53, 2:1
63.7%
13.1%
18.1%
5.2%
SP53 cells alone
0.1%
0.3%
1.6%
98.0%
Control T:SP53, 5:1
71.2%
15.3%
2.2%
11.3%
L8-CD19-2G:SP53, 5:1
85.6%
3.1%
11.3%
0.0%
CD19b-BB:SP53, 5:1
89.2%
3.3%
7.4%
0.1%
Percent SP53 cell lysis
150
100
50
0
L8-CD19-2G
L8-CD19b-BB-2G
2:1
5:1
CD3
CD19

FIGURE 41

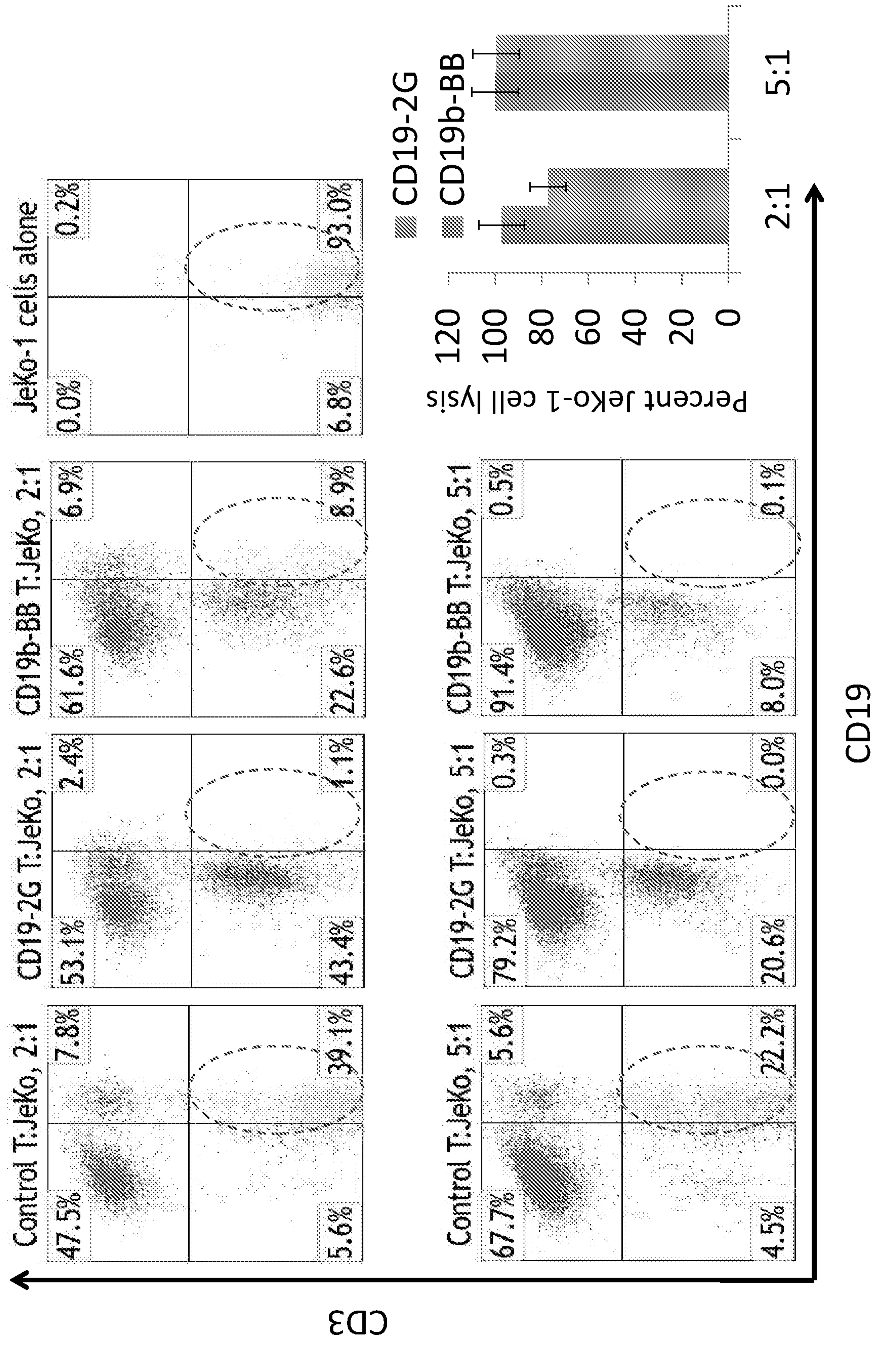
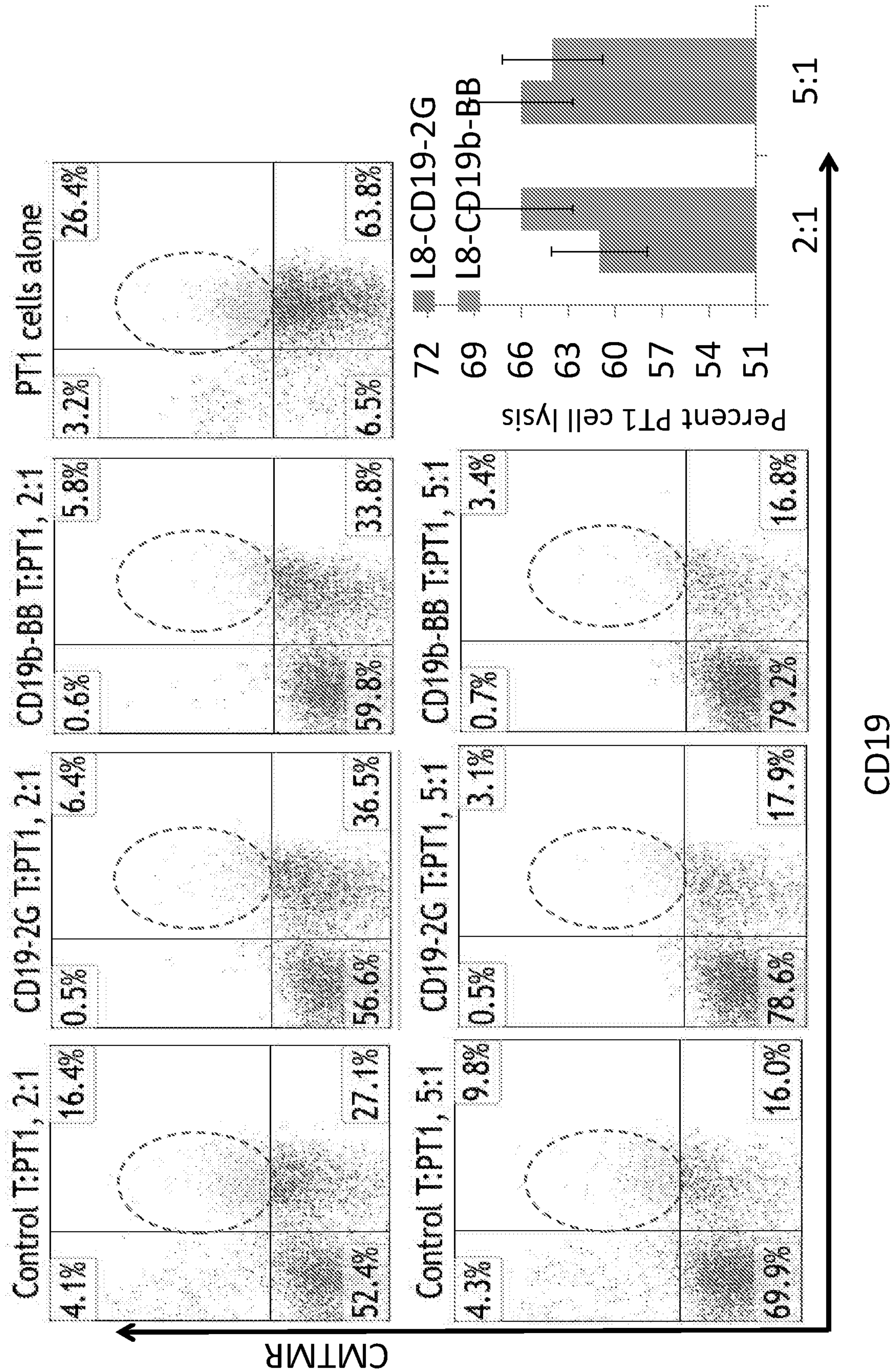

Control T:JeKo, 2:1
47.5%
7.8%
5.6%
39.1%
CD19-2G T:JeKo, 2:1
53.1%
2.4%
43.4%
1.1%
CD19b-BB T:JeKo, 2:1
61.6%
6.9%
22.6%
8.9%
JeKo-1 cells alone
0.0%
0.2%
6.8%
93.0%
Control T:JeKo, 5:1
67.7%
5.6%
4.5%
22.2%
CD19-2G T:JeKo, 5:1
79.2%
0.3%
20.6%
0.0%
CD19b-BB T:JeKo, 5:1
91.4%
0.5%
8.0%
0.1%
CD19-2G
CD19b-BB
Percent JeKo-1 cell lysis
120
100
80
60
40
20
0
2:1
5:1
CD19
CD3

FIGURE 42


Control T:PT1, 2:1
4.1%
16.4%
52.4%
27.1%
CD19-2G T:PT1, 2:1
0.5%
6.4%
56.6%
36.5%
CD19b-BB T:PT1, 2:1
0.6%
5.8%
59.8%
33.8%
PT1 cells alone
3.2%
26.4%
6.5%
63.8%
Control T:PT1, 5:1
4.3%
9.8%
69.9%
16.0%
CD19-2G T:PT1, 5:1
0.5%
3.1%
78.6%
17.9%
CD19b-BB T:PT1, 5:1
0.7%
3.4%
79.2%
16.8%
Percent PT1 cell lysis
72
69
66
63
60
57
54
51
L8-CD19-2G
L8-CD19b-BB
2:1
5:1
CMTMR
CD19

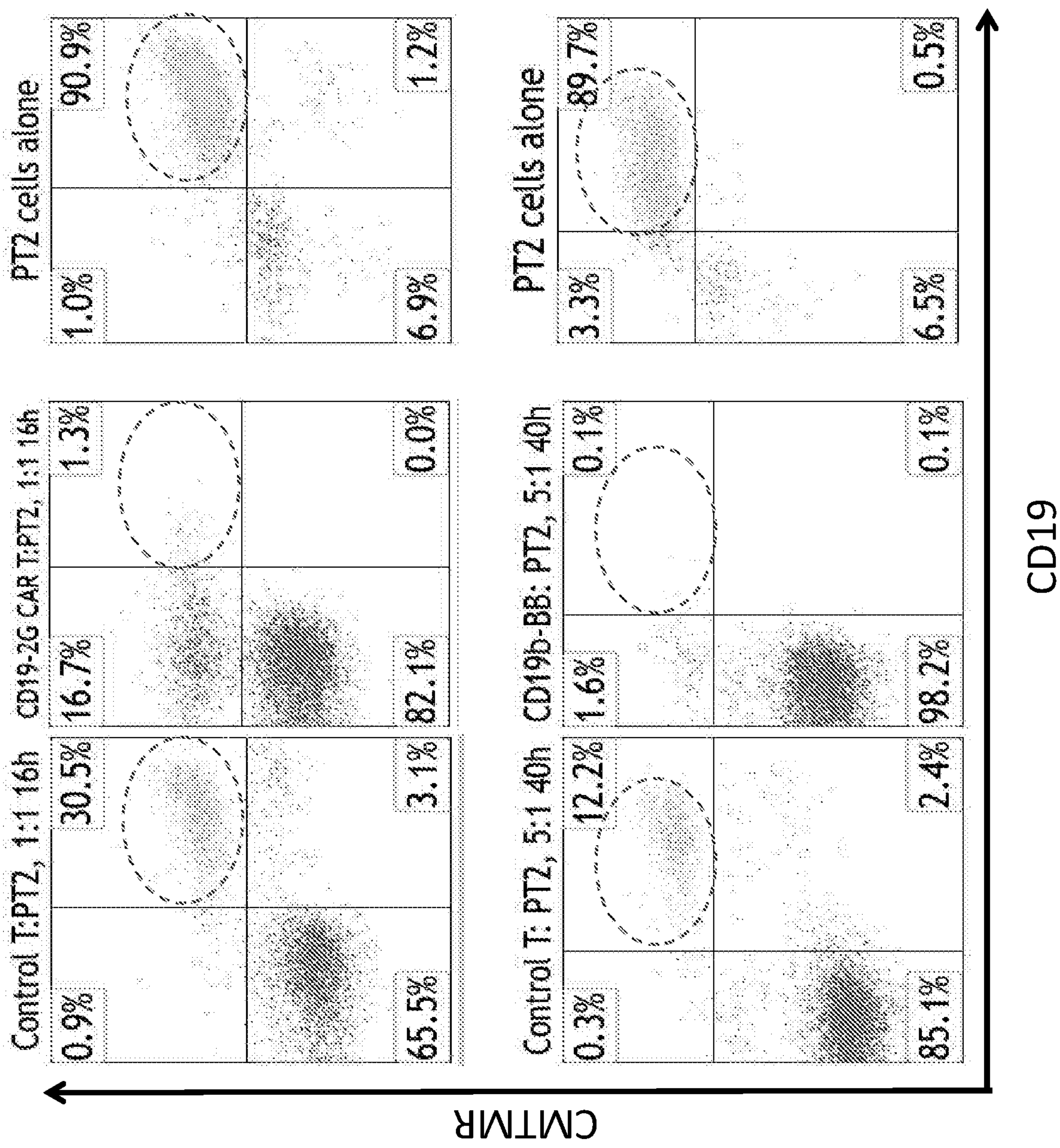

Control T:PT2, 1:1 16h
0.9%
30.5%
65.5%
3.1%
CD19-2G CAR T:PT2, 1:1 16h
16.7%
1.3%
82.1%
0.0%
PT2 cells alone
1.0%
90.9%
6.9%
1.2%
Control T: PT2, 5:1 40h
0.3%
12.2%
85.1%
2.4%
CD19b-BB: PT2, 5:1 40h
1.6%
0.1%
98.2%
0.1%
PT2 cells alone
3.3%
89.7%
6.5%
0.5%
CMTMR
CD19

FIGURE 43

FIGURE 44

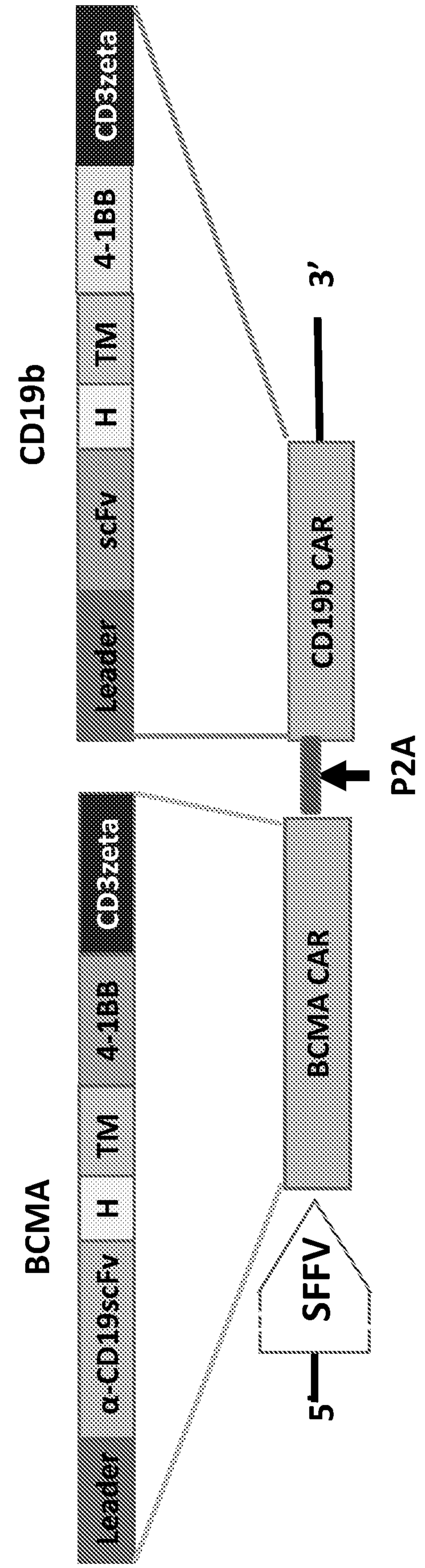

BCMA
Leader
α-CD19scFv
H
TM
4-1BB
CD3zeta
CD19b
Leader
scFv
H
TM
4-1BB
CD3zeta
5'
SFFV
BCMA CAR
P2A
CD19b CAR
3'

FIGURE 45A

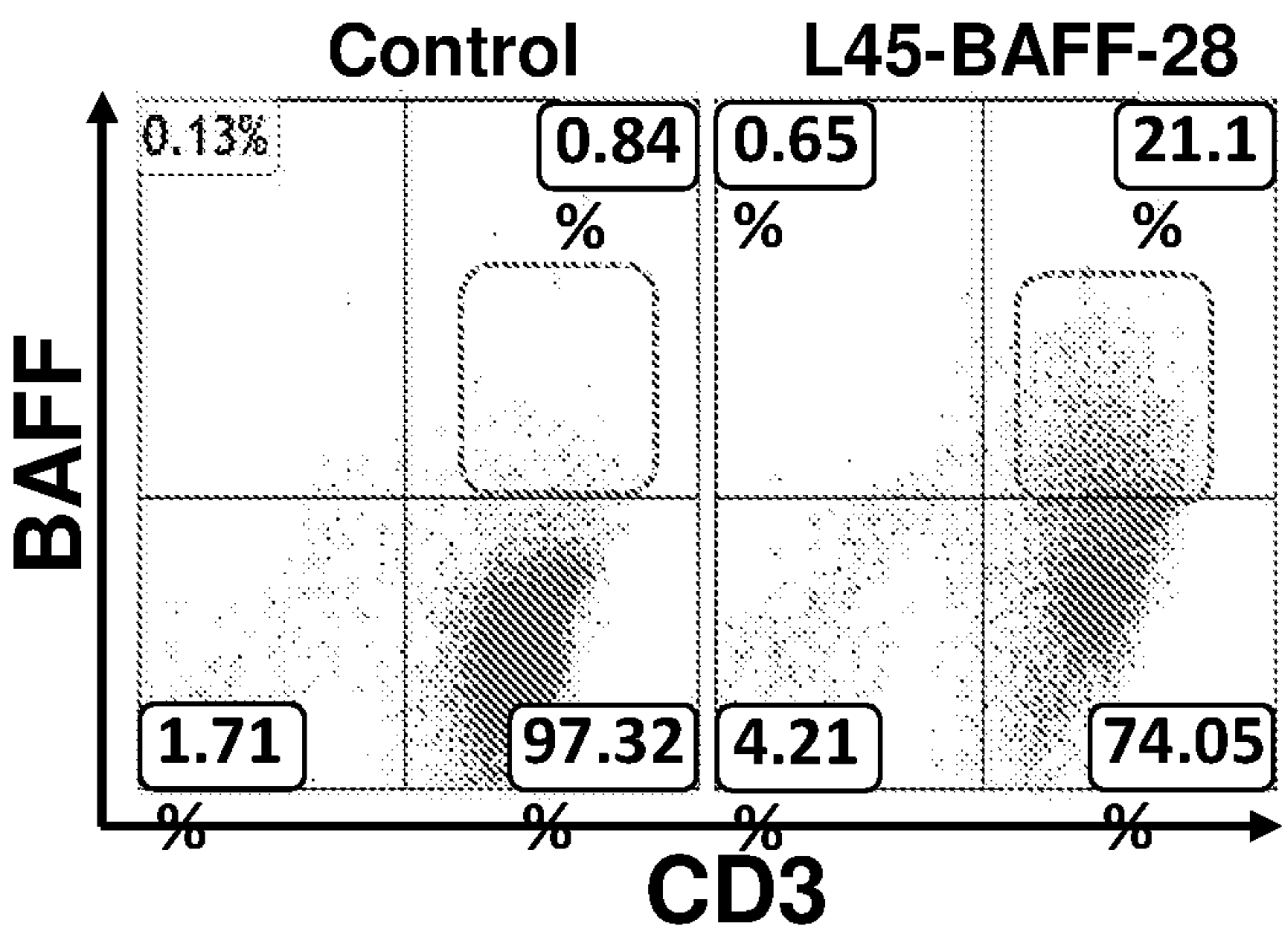


FIGURE 45B

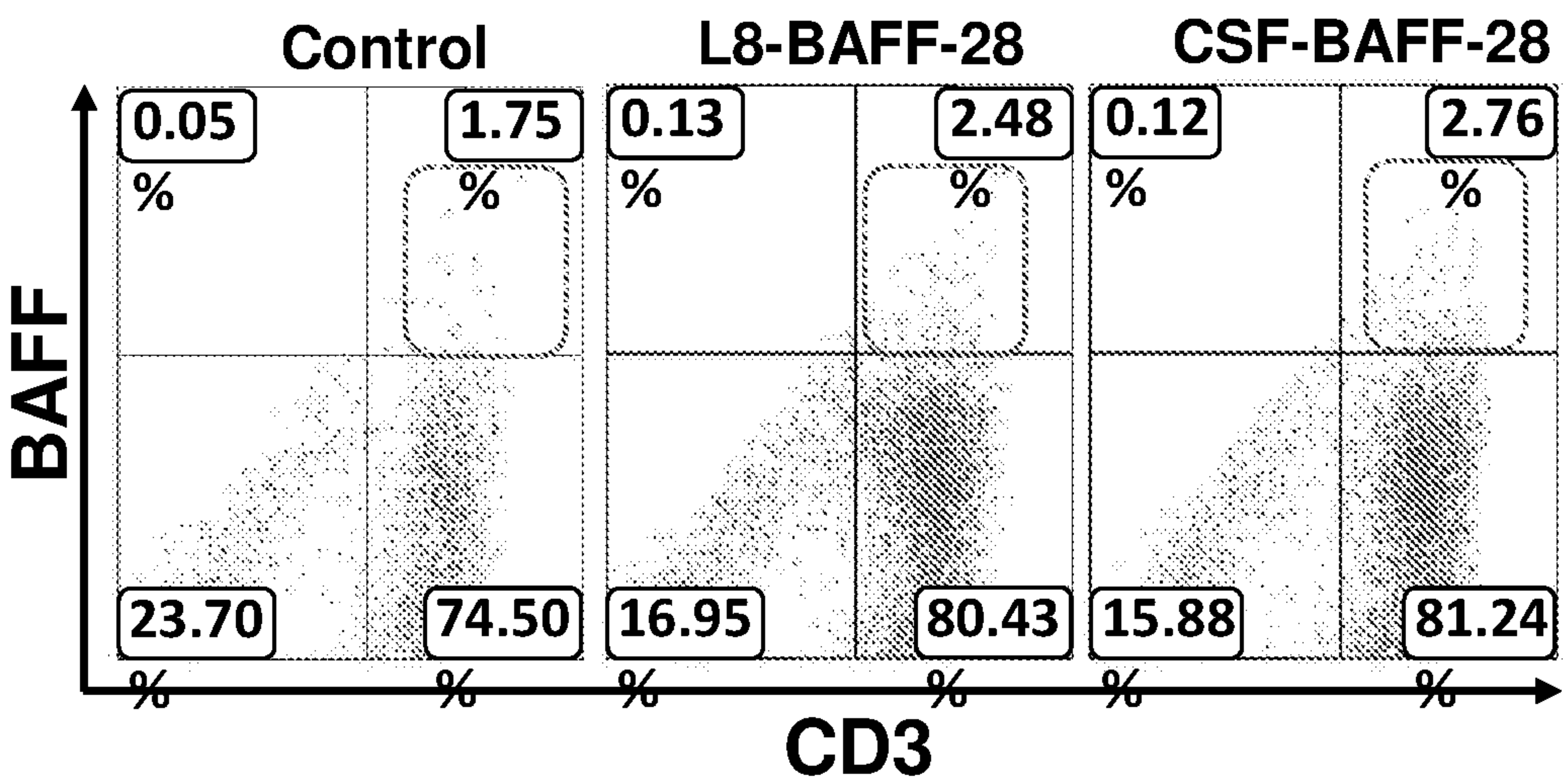


FIGURE 45C

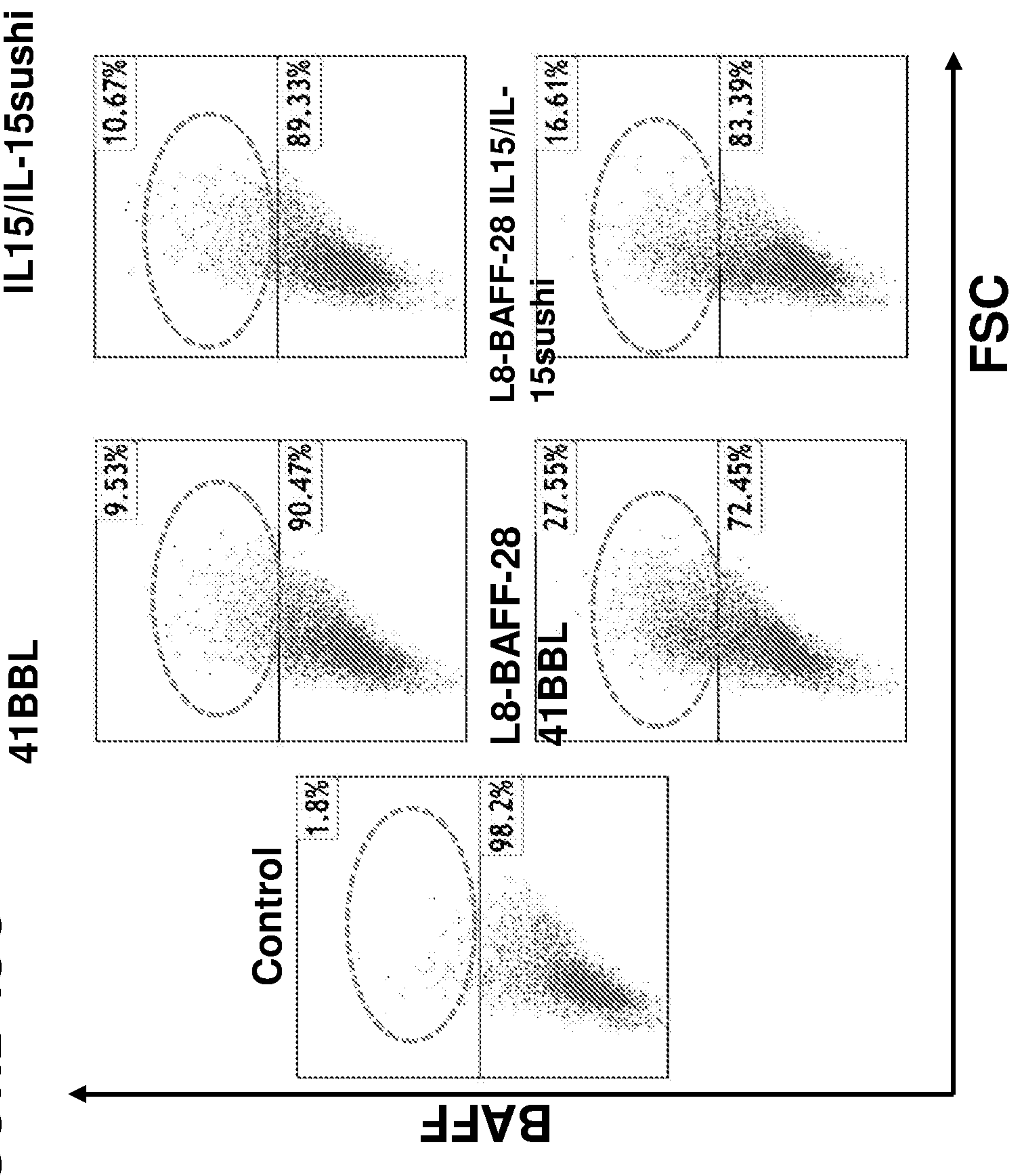

CSF-BAFF-28
IL15/IL-15sushi
10.67%
89.33%
L8-BAFF-28 IL15/IL-
15sushi
16.61%
83.39%
CSF-BAFF-28
41BBL
9.53%
90.47%
L8-BAFF-28
41BBL
27.55%
72.45%
Control
1.8%
98.2%
FSC
BAFF

FIGURE 46A

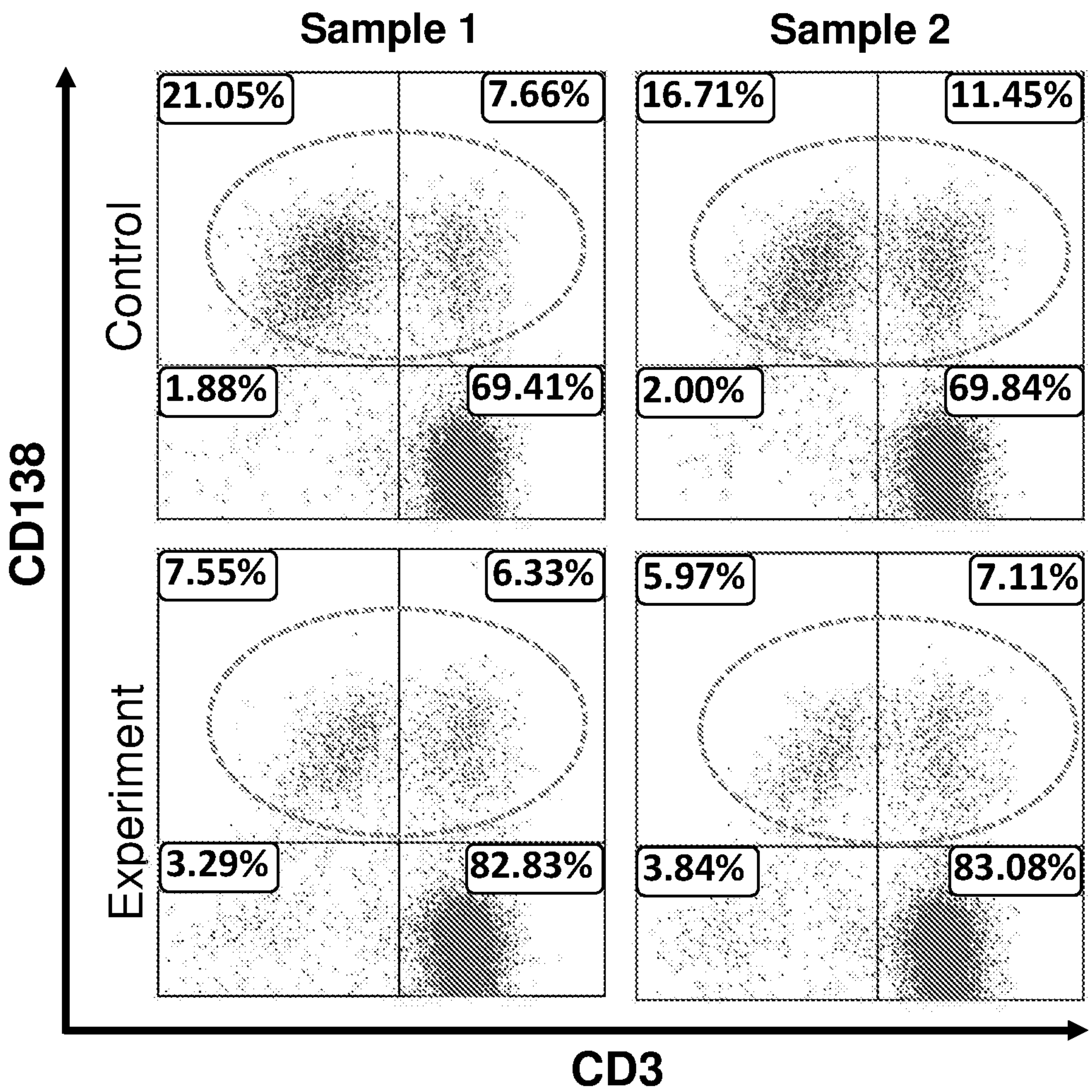


FIGURE 46B

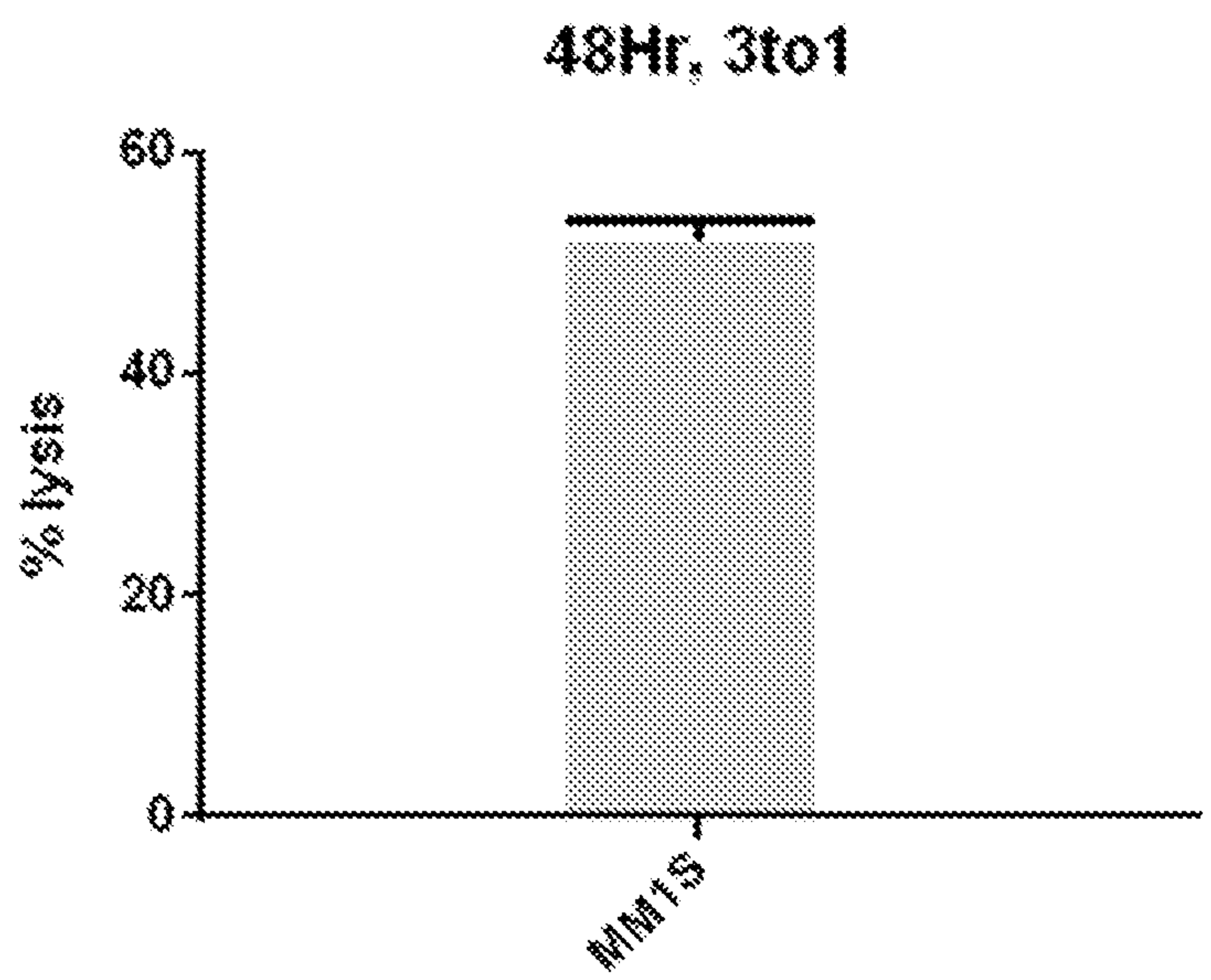


FIGURE 47A

FIGURE 47B

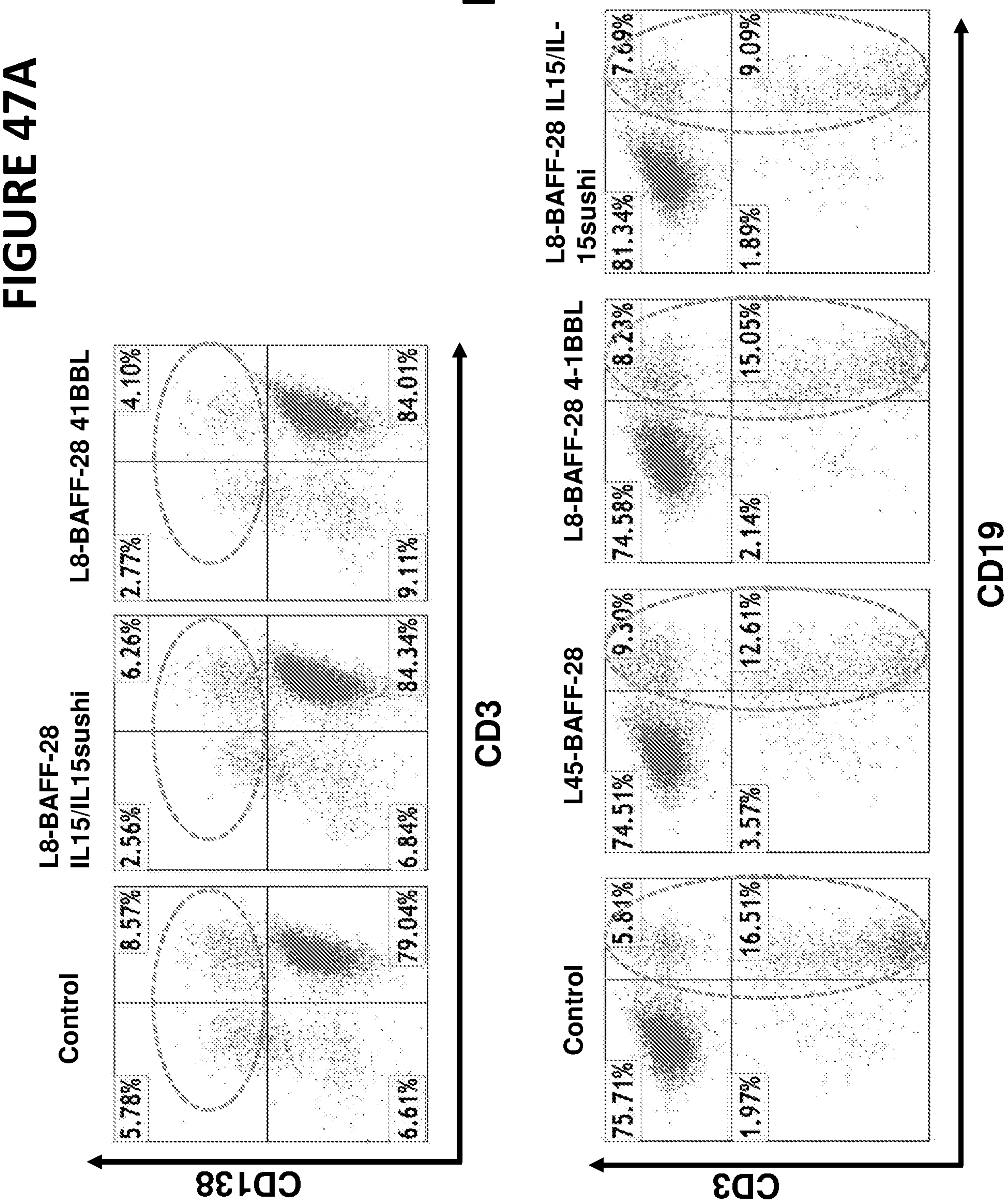


FIGURE 48

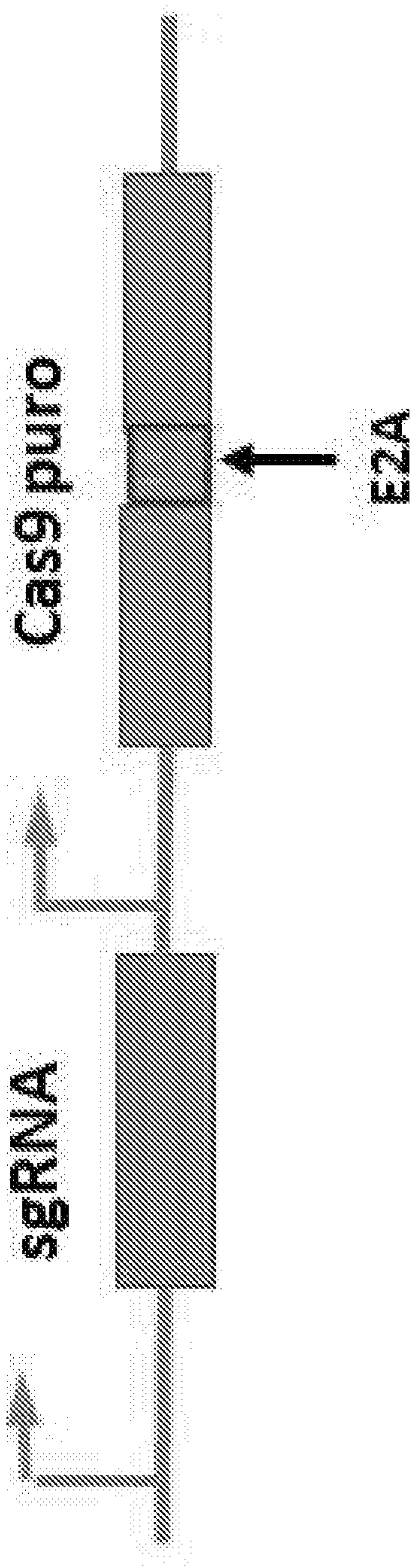
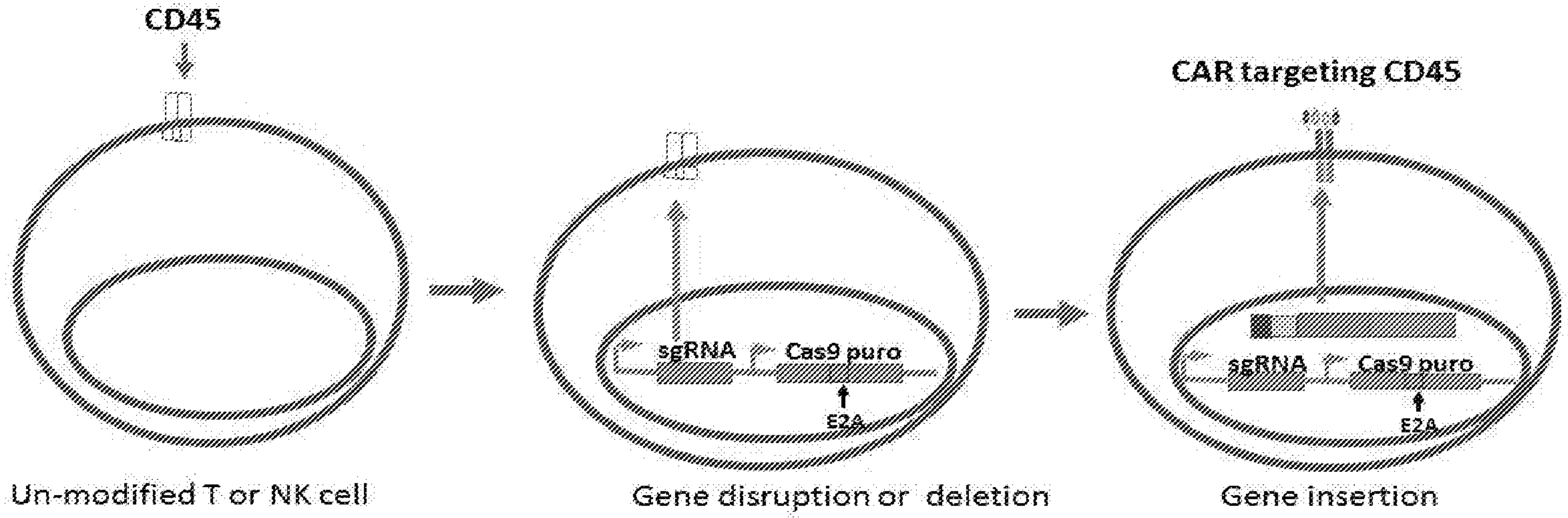


FIGURE 49A



Steps: T or NK cells armed with CARs targeting hematologic malignancies

1. Donor T cells or NK cells
2. Gene disruption or deletion of CD45
3. Insert of scFv CAR targeting CD45

FIGURE 49B

B.

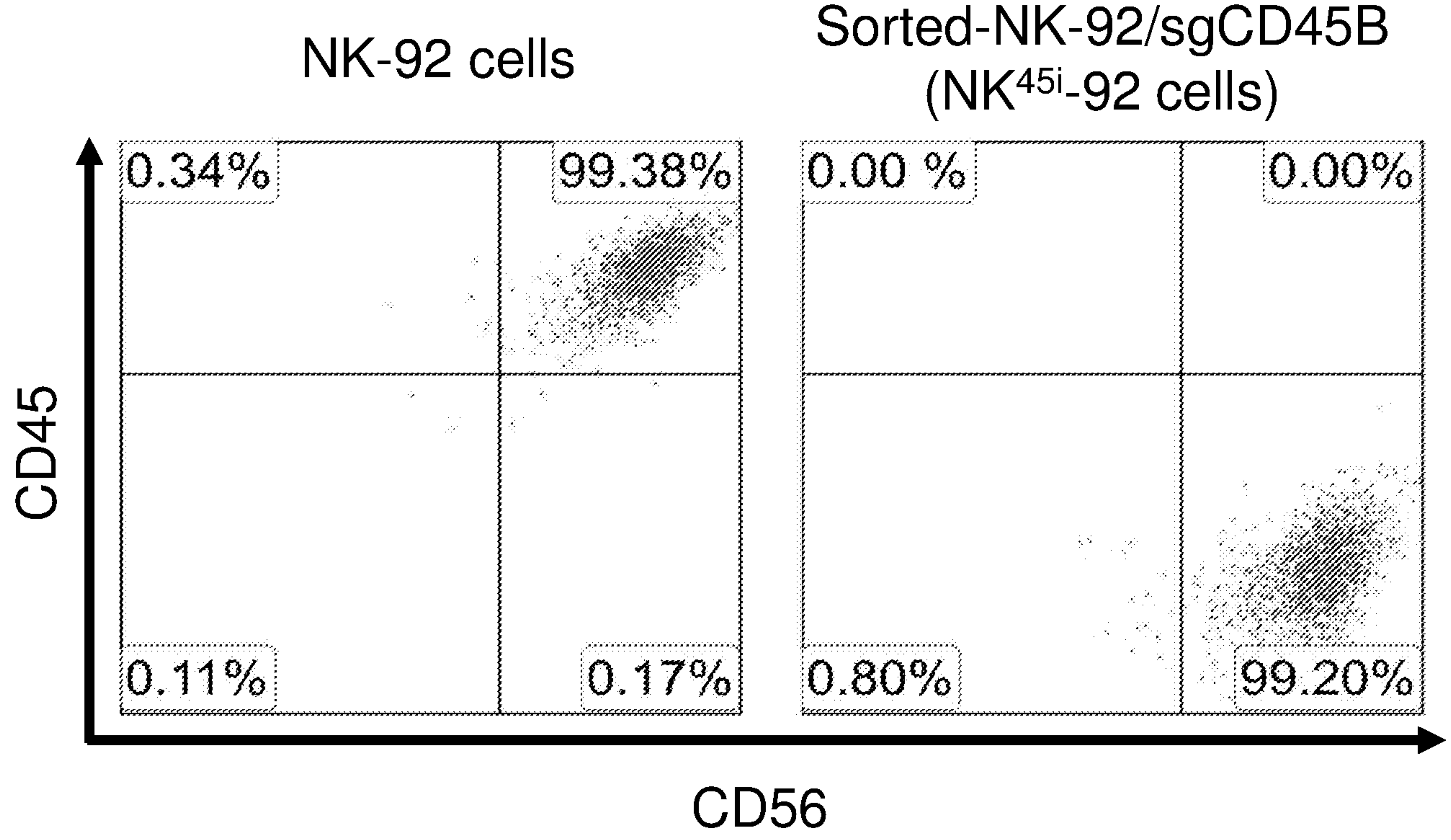

NK-92 cells
Sorted-NK-92/sgCD45B
(NK45i-92 cells)
0.34%
99.38%
0.11%
0.17%
0.00 %
0.00%
0.80%
99.20%
CD45
CD56

FIGURE 50

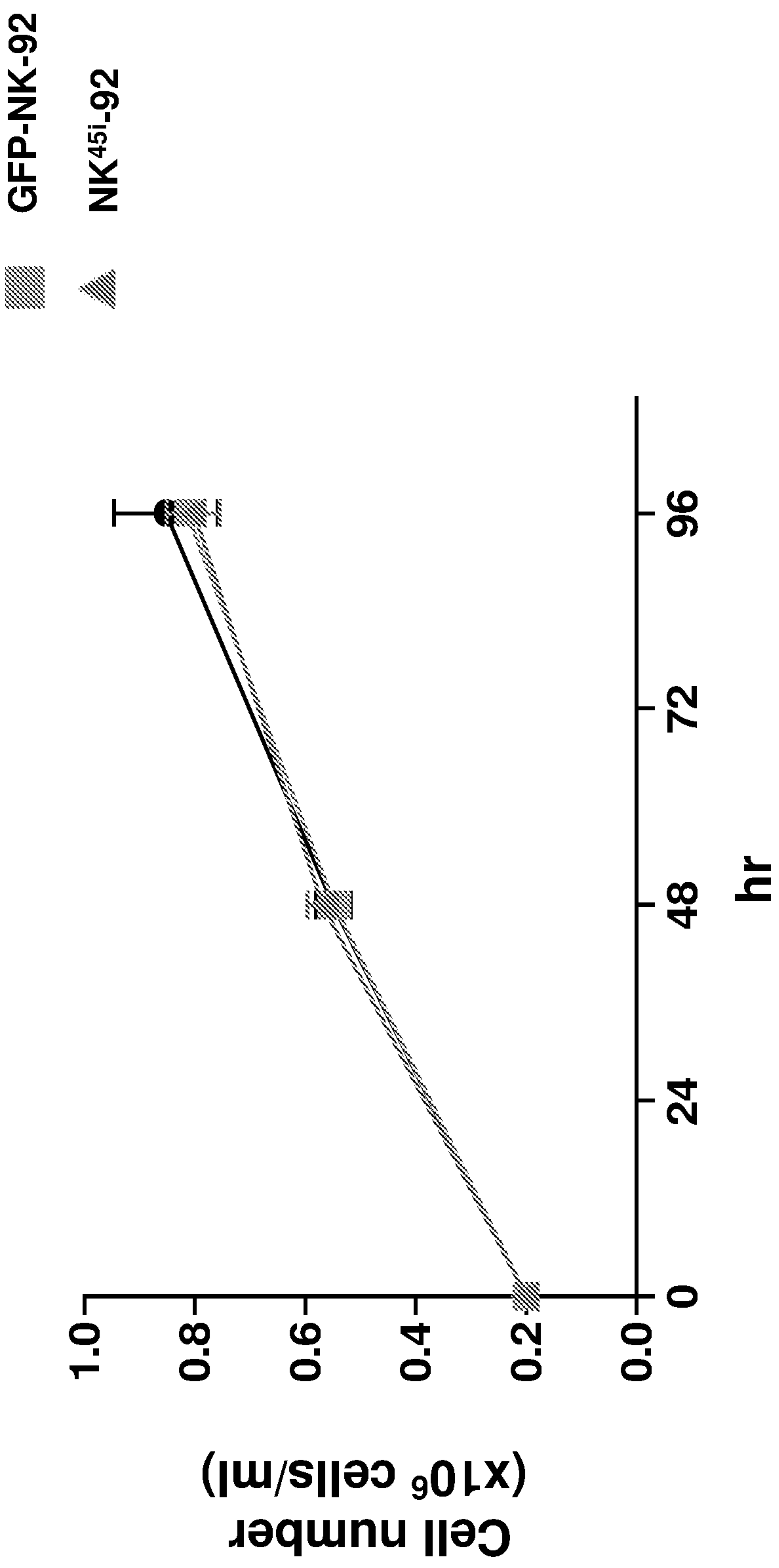

NK-92
GFP-NK-92
NK45i-92
Cell number
(x106 cells/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
hr

FIGURE 51A

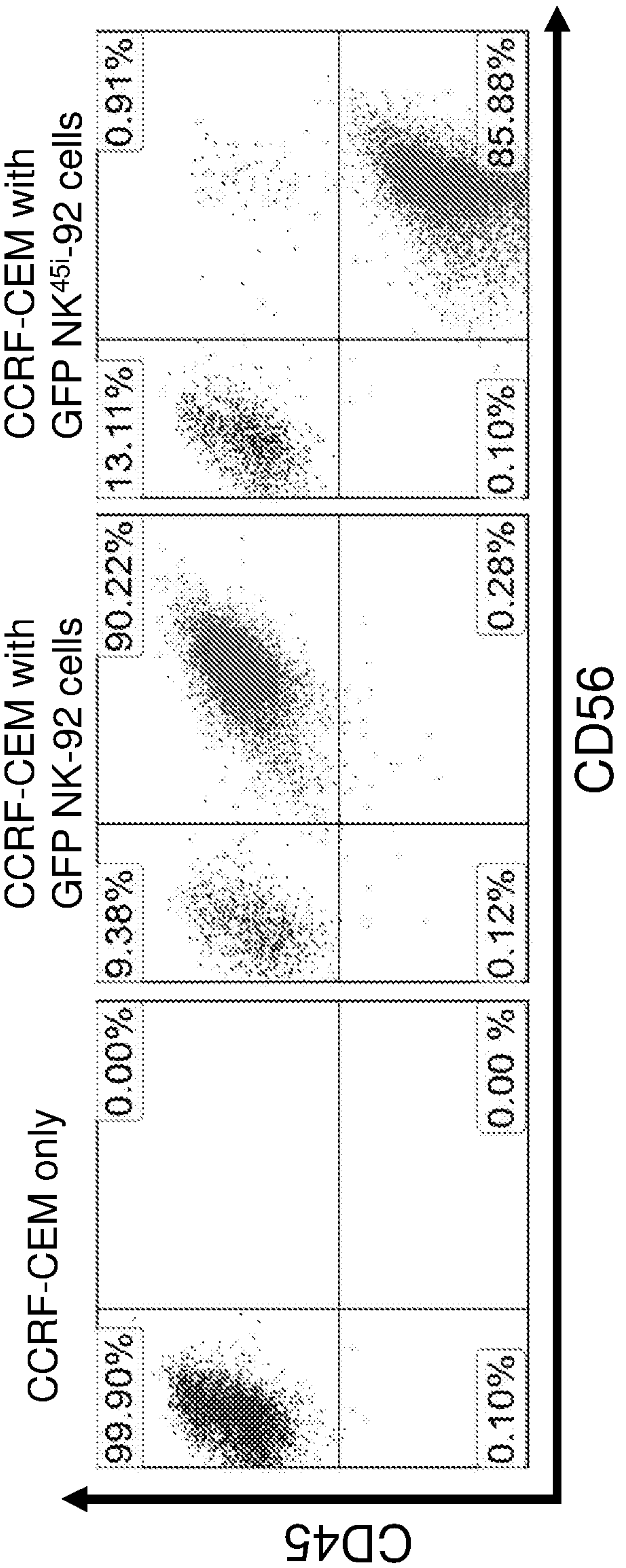


FIGURE 51B

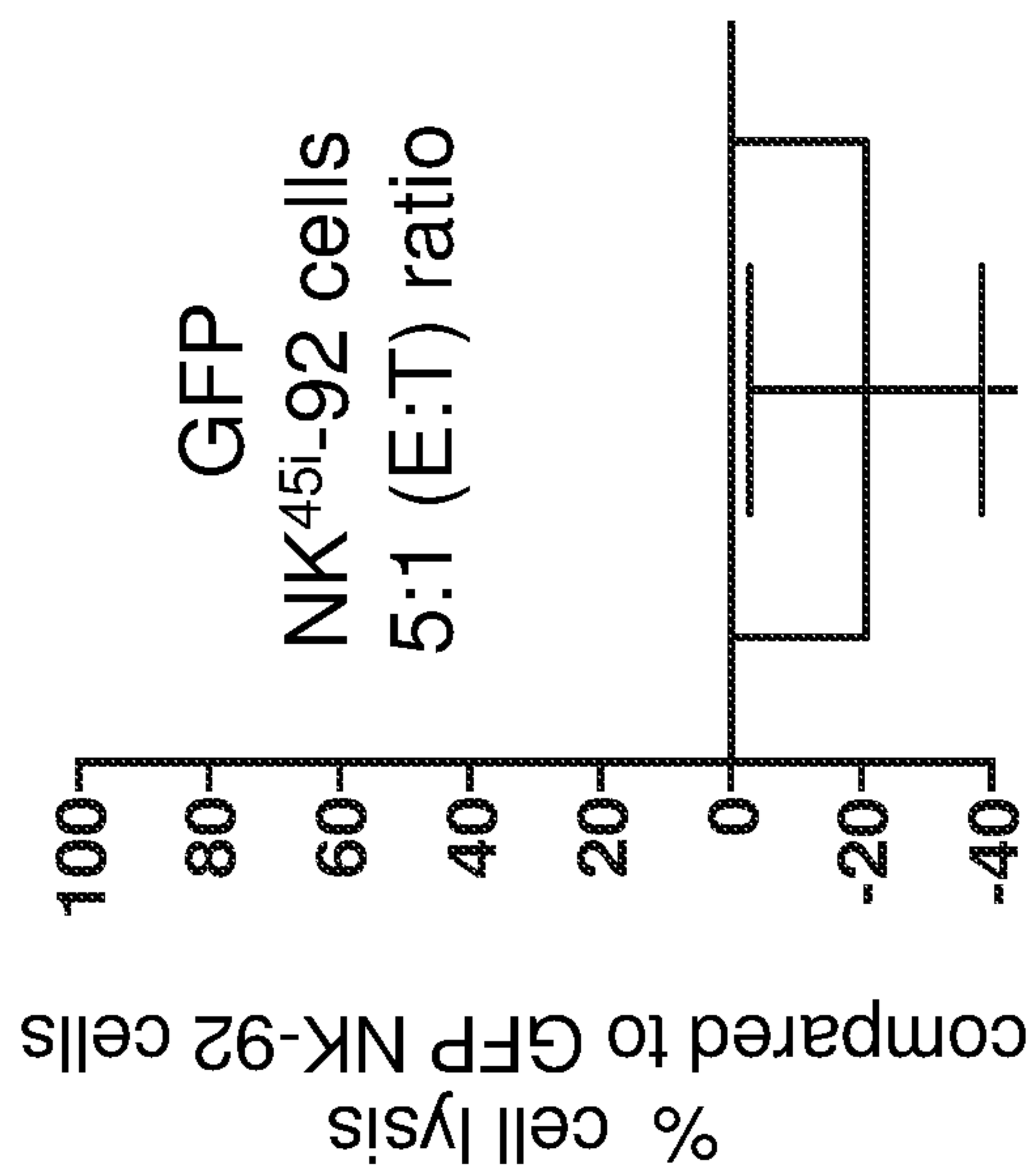


FIGURE 52A

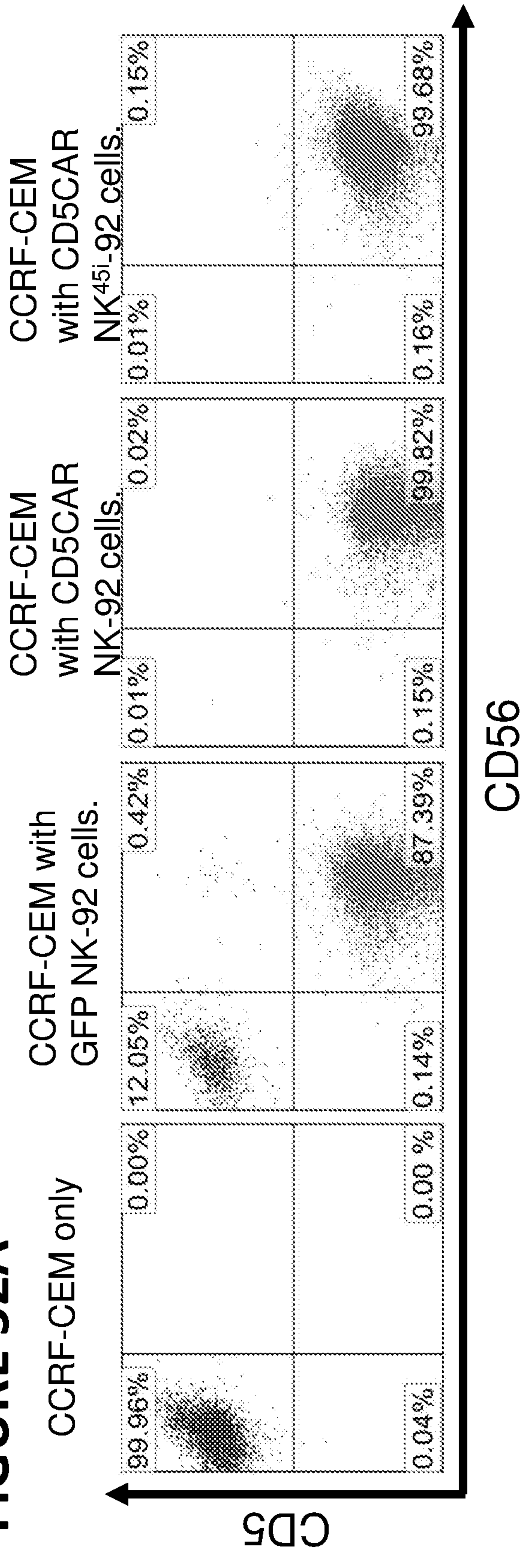


FIGURE 52B

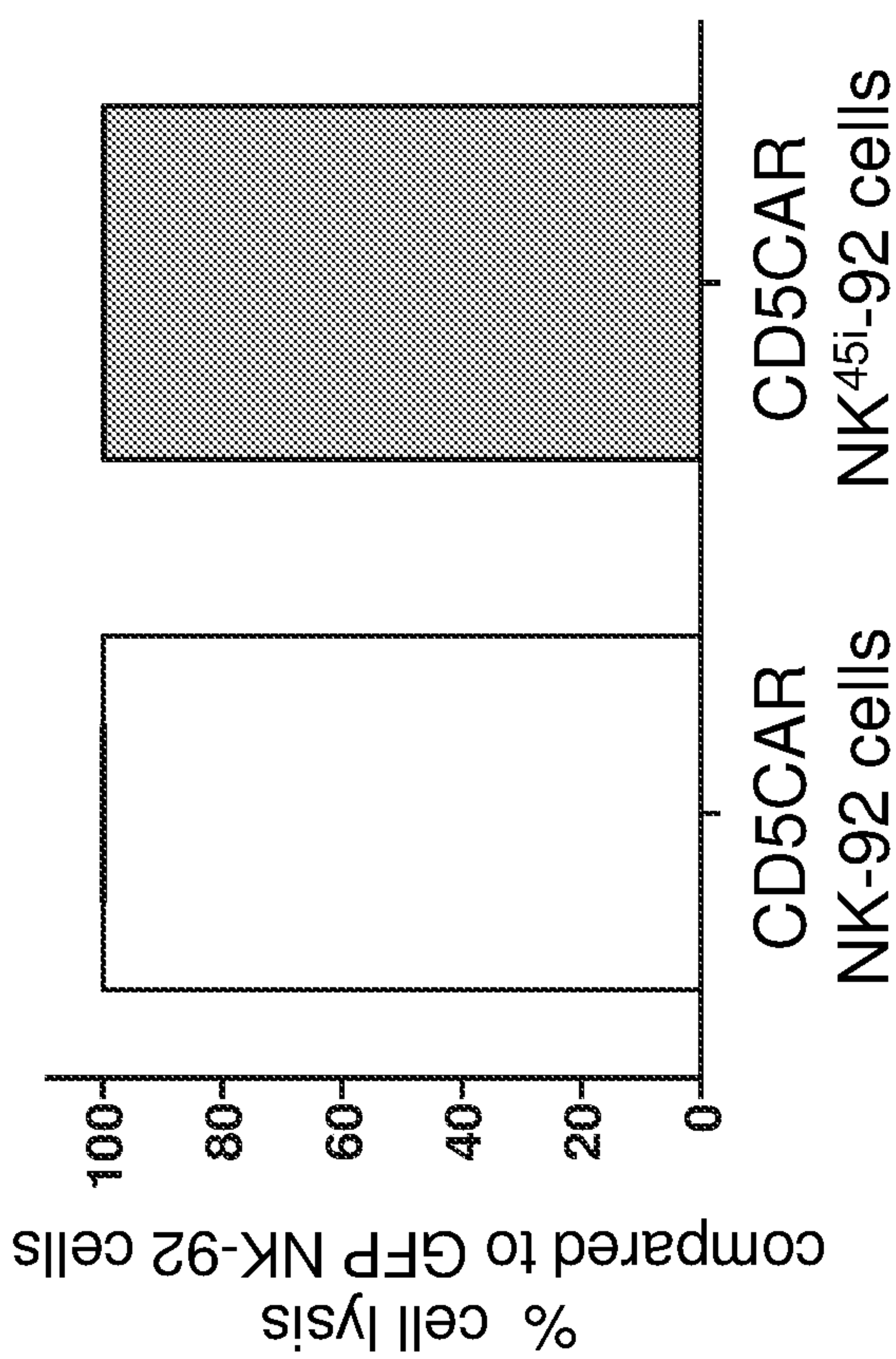


FIGURE 53A

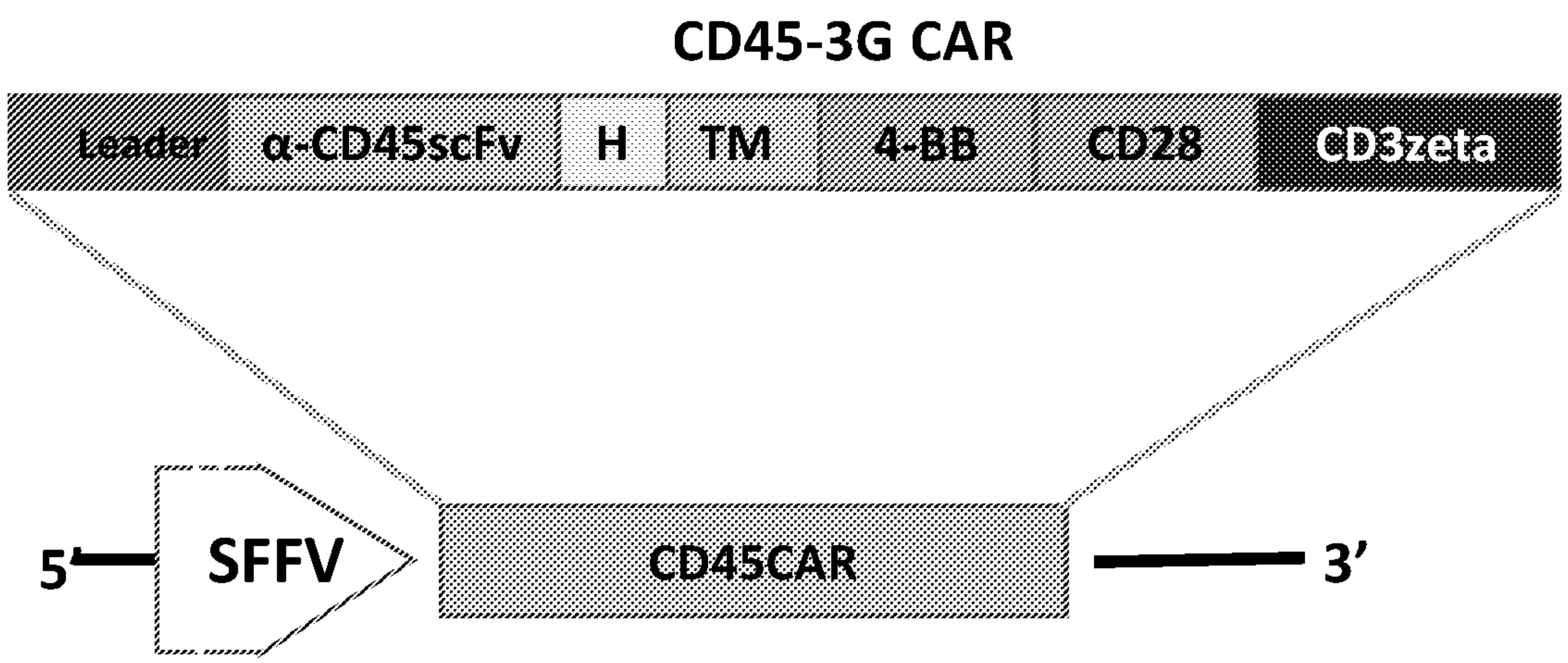


FIGURE 53B

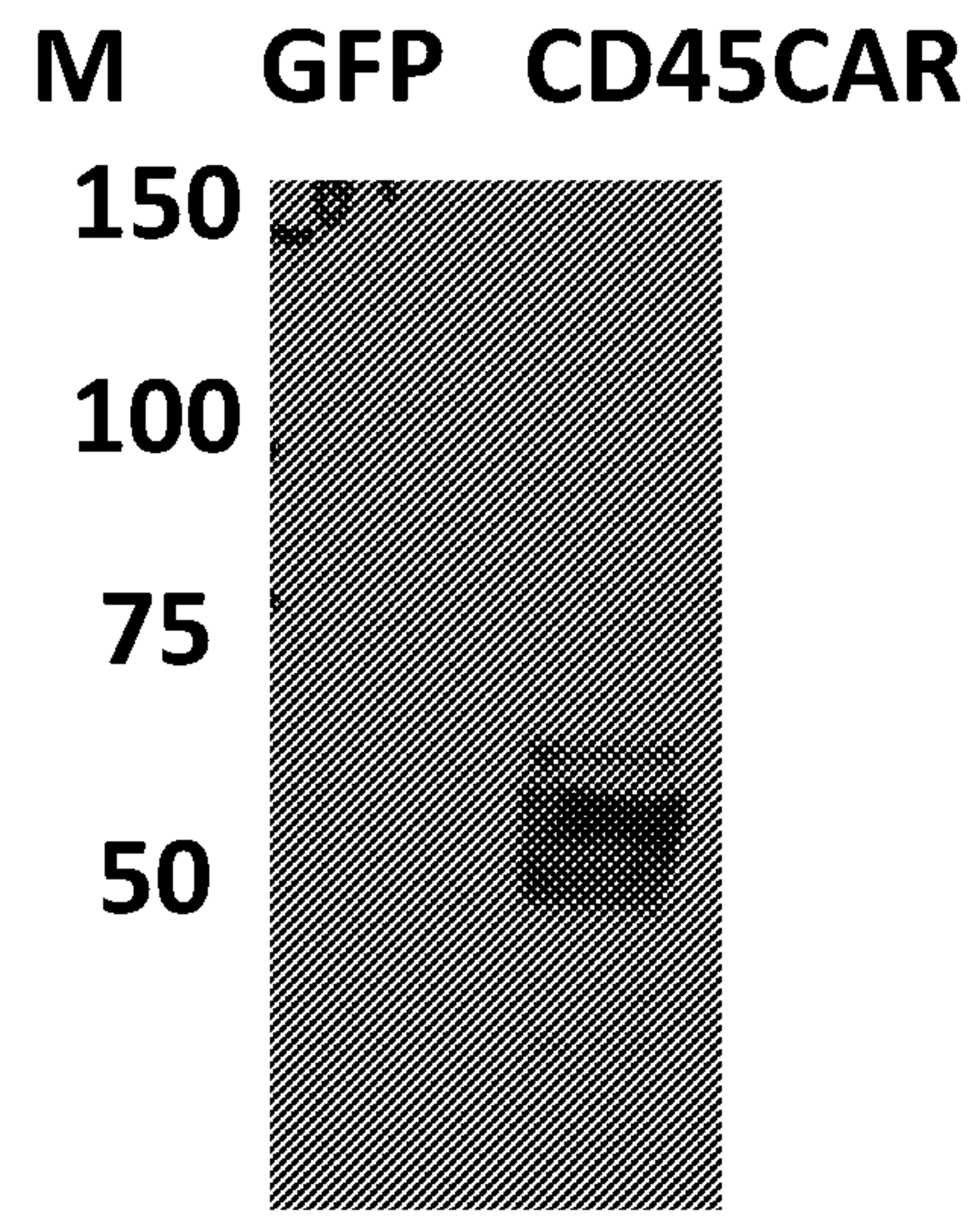


FIGURE 54

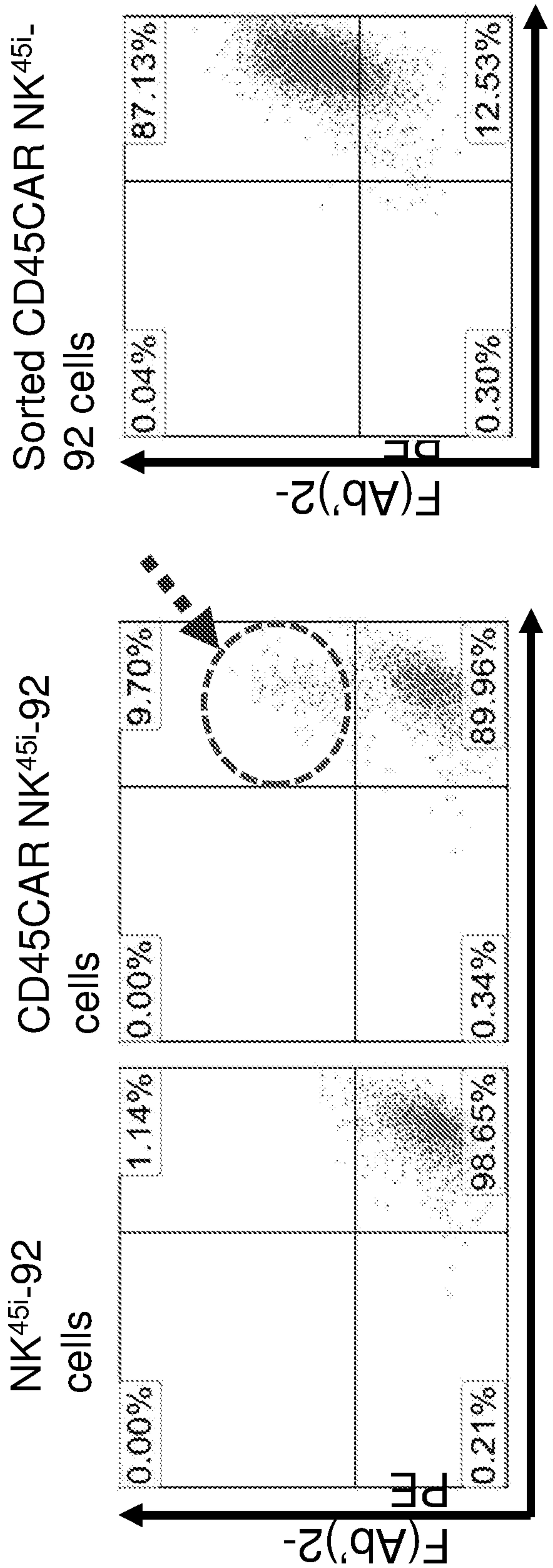

NK45i-92 cells
CD45CAR NK45i-92 cells
Sorted CD45CAR NK45i-92 cells
0.00%
1.14%
0.21%
98.65%
0.00%
9.70%
0.34%
89.96%
0.04%
87.13%
0.30%
12.53%
F(Ab')2-PE
CD56
F(Ab')2-PE
CD56

FIGURE 55A

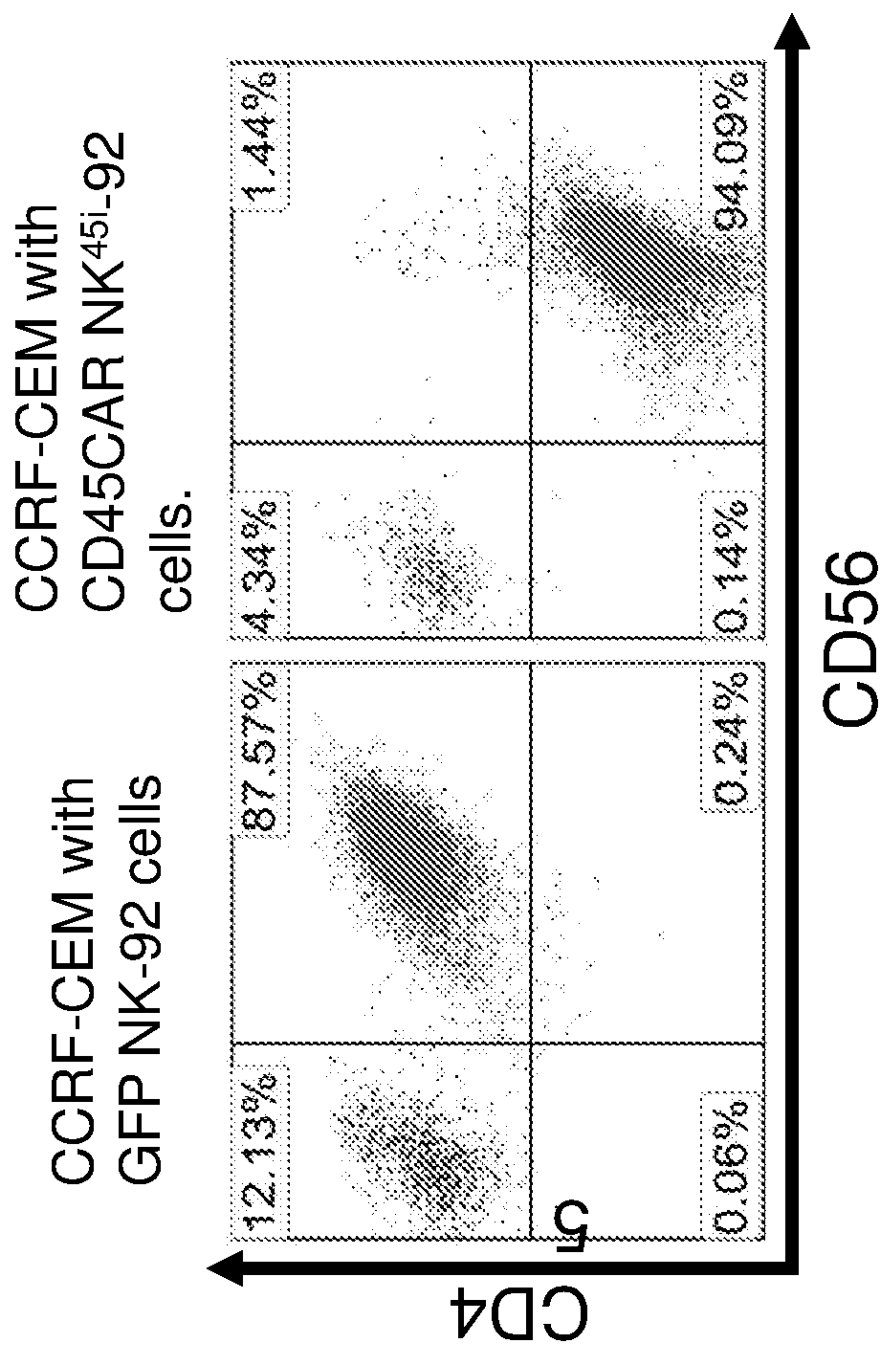
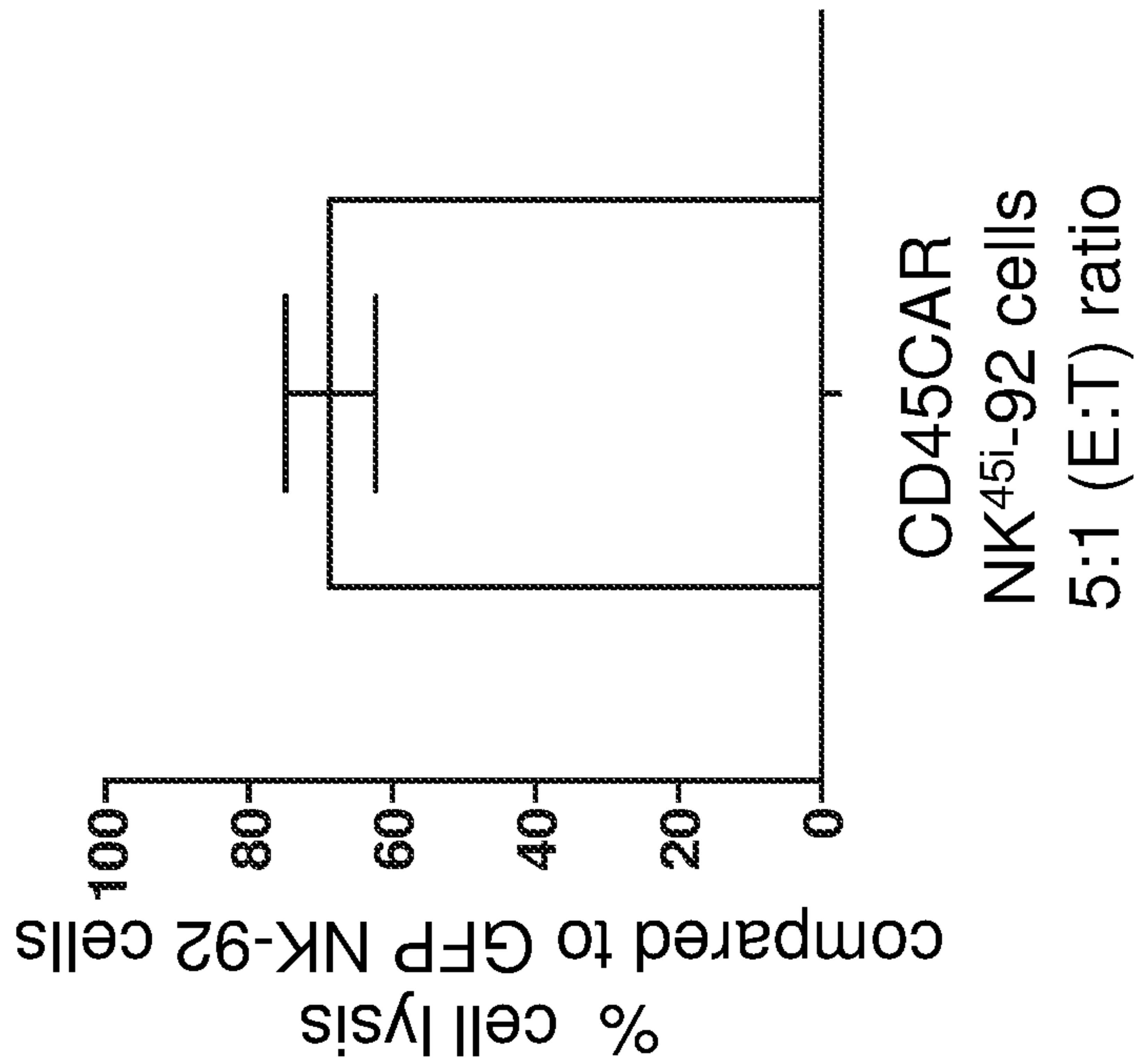


FIGURE 55B



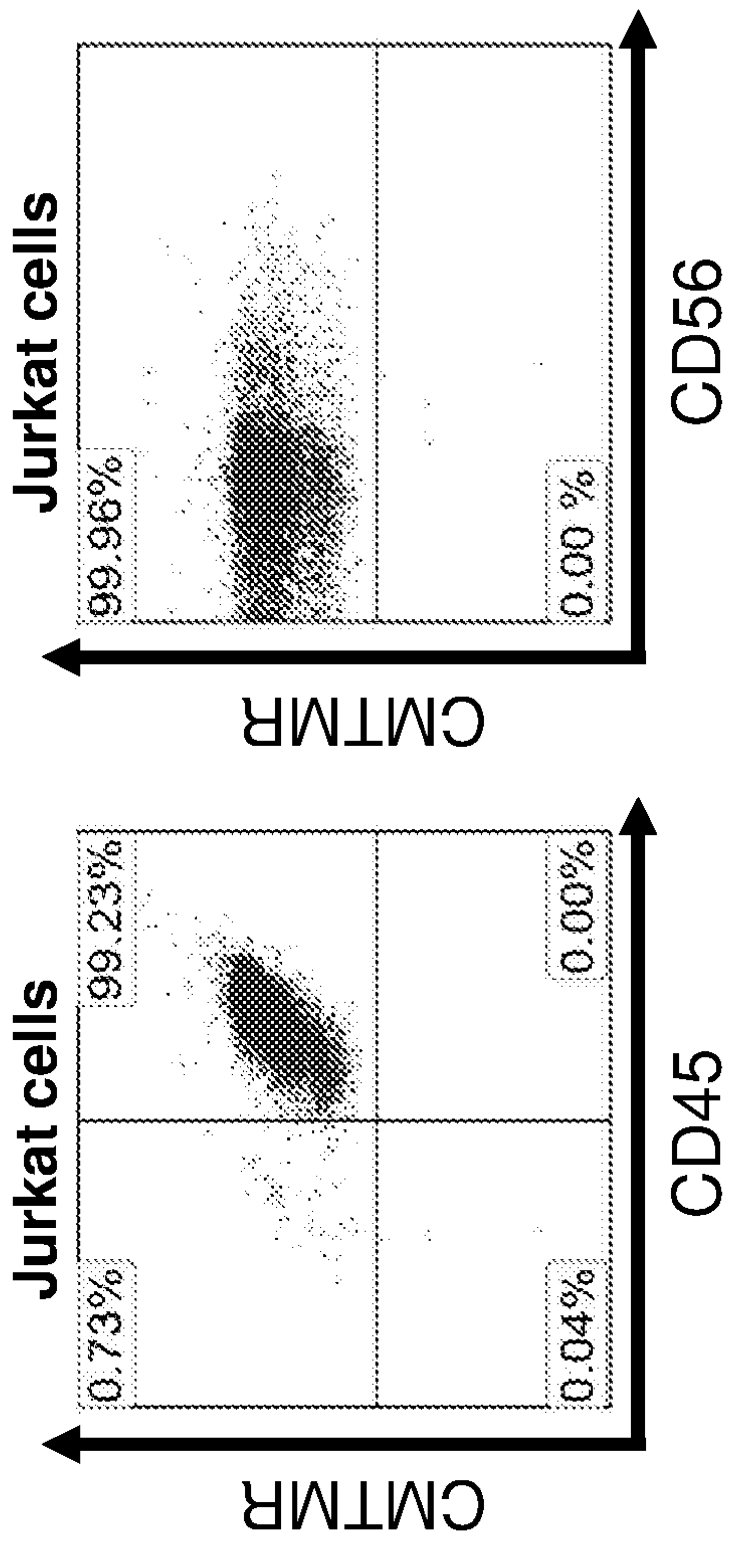


FIGURE 56A

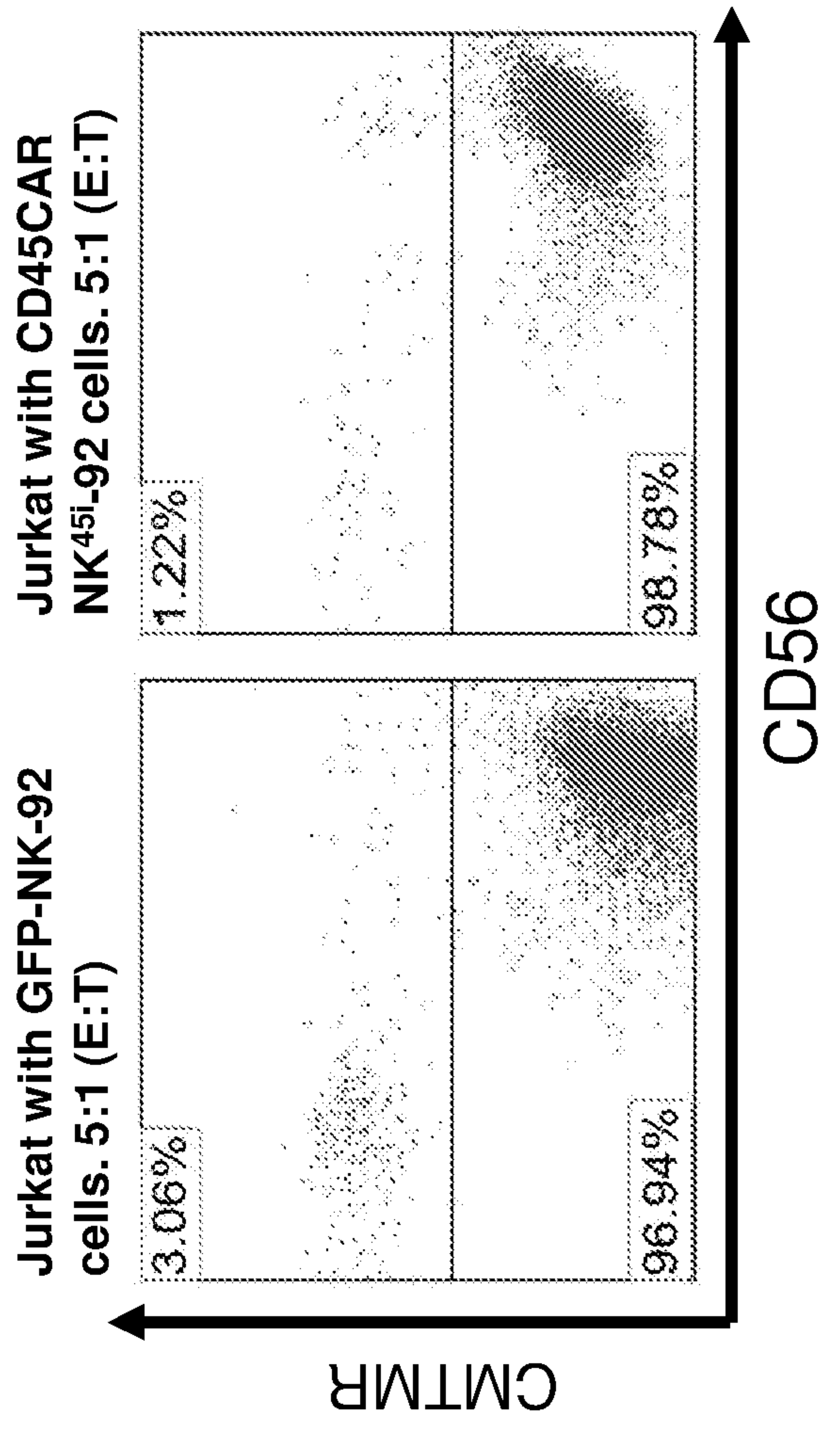


FIGURE 56B

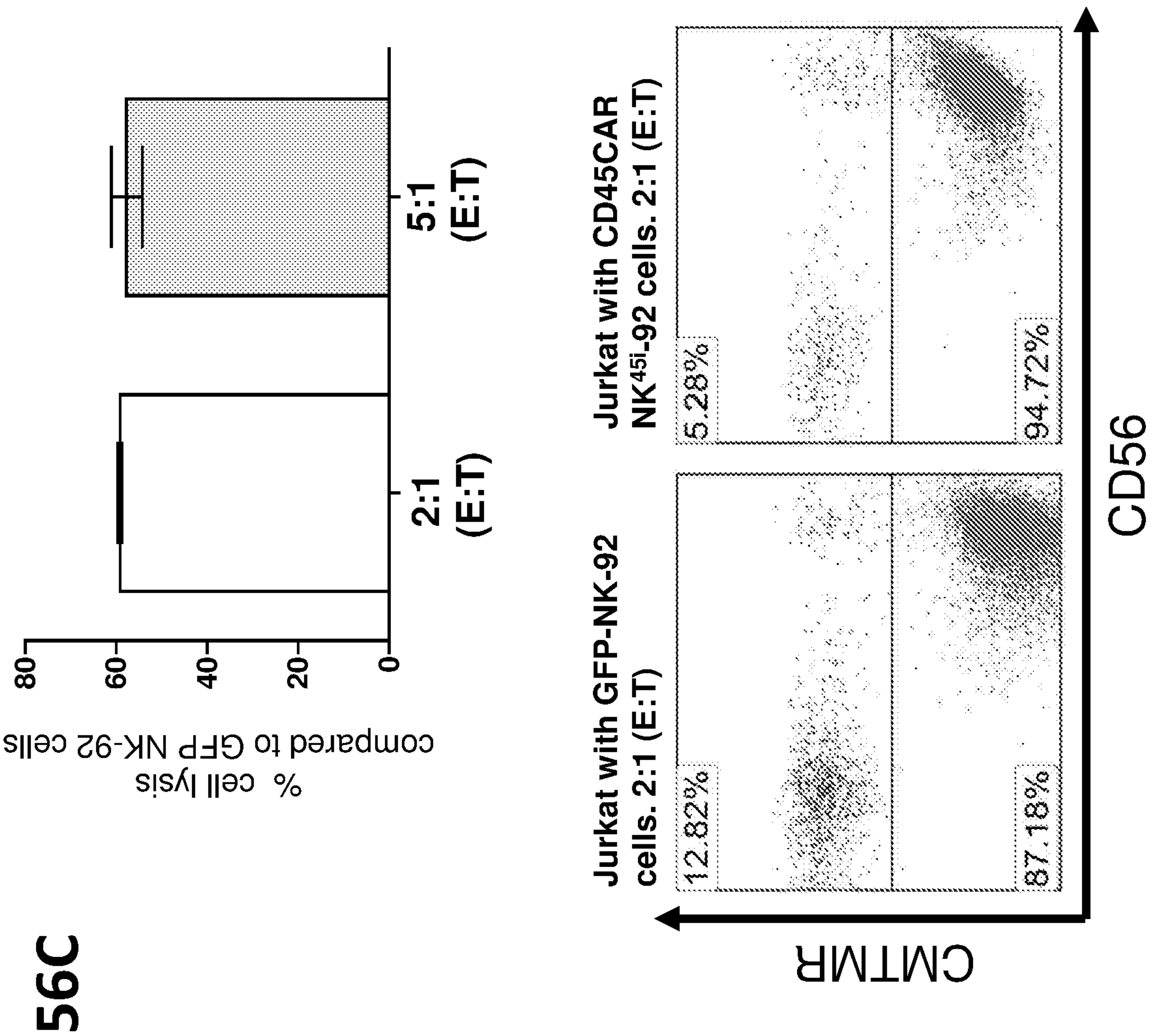

% cell lysis
compared to GFP NK-92 cells
80
60
40
20
0
2:1
(E:T)
5:1
(E:T)
Jurkat with GFP-NK-92
cells. 2:1 (E:T)
12.82%
87.18%
Jurkat with CD45CAR
NK45i-92 cells. 2:1 (E:T)
5.28%
94.72%
CMTMR
CD56

FIGURE 56C

FIGURE 57A

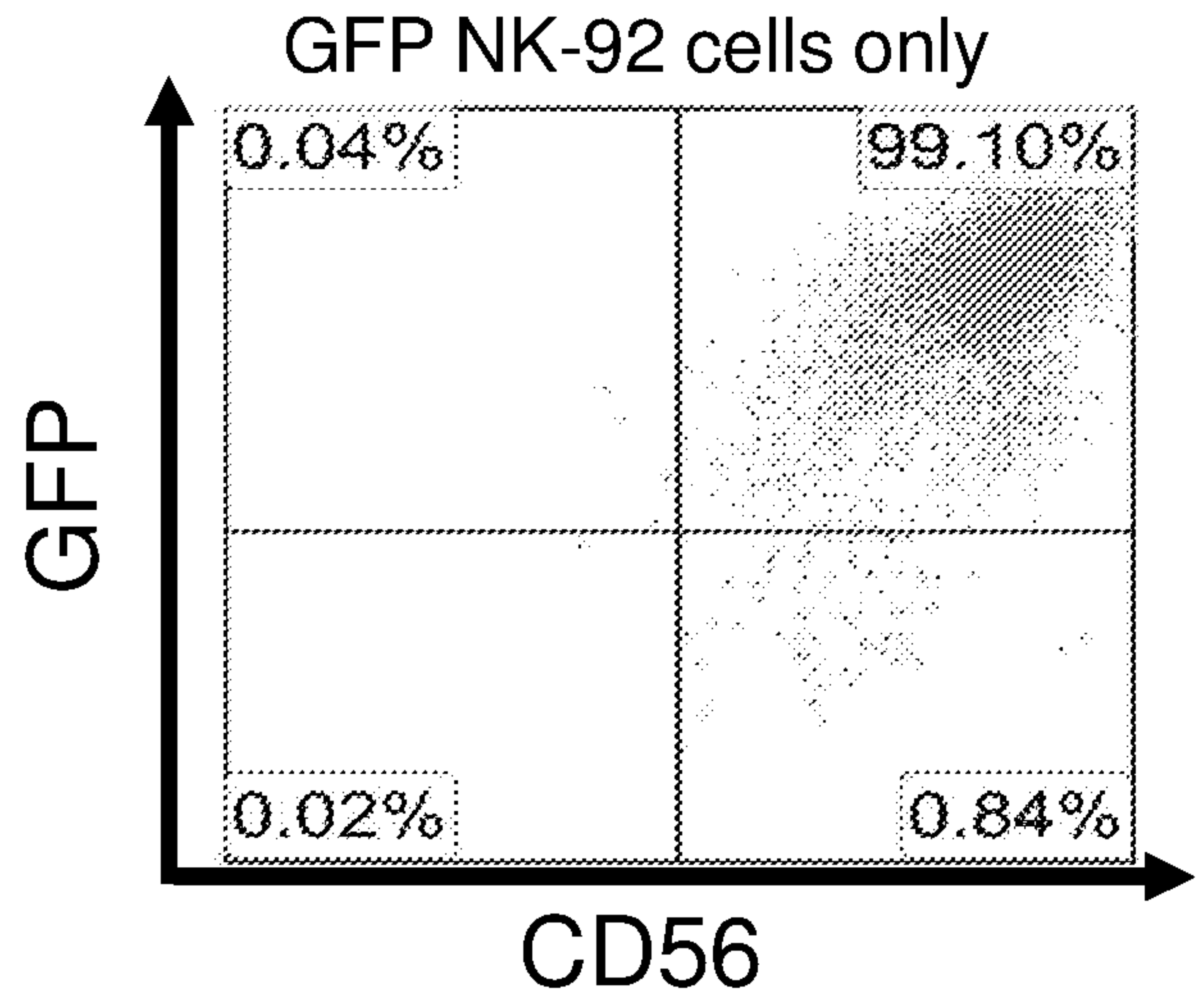


FIGURE 57B

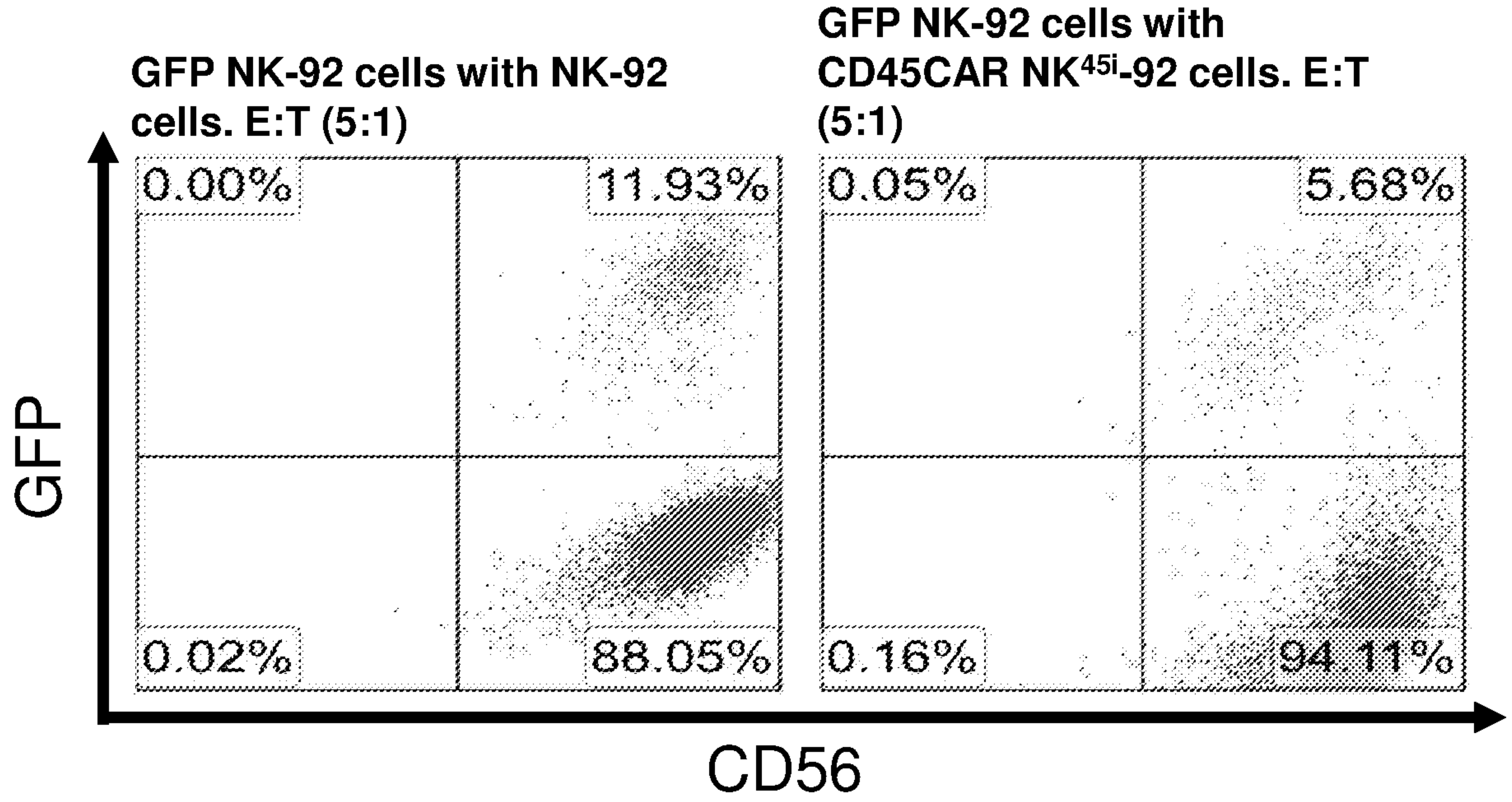


FIGURE 57C

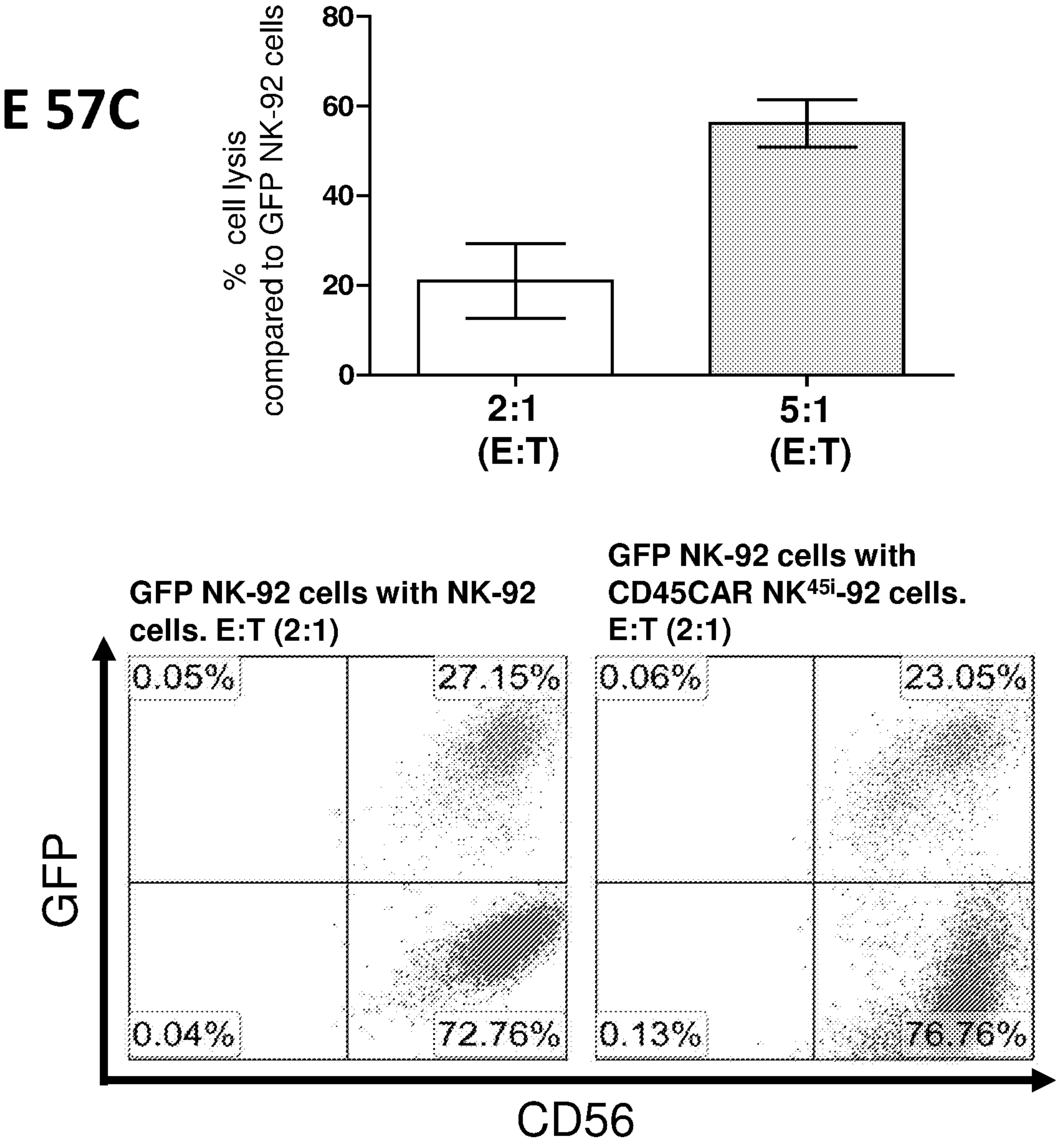

% cell lysis compared to GFP NK-92 cells
80
60
40
20
0
2:1 (E:T)
5:1 (E:T)
GFP NK-92 cells with NK-92 cells. E:T (2:1)
0.05%
27.15%
0.04%
72.76%
GFP NK-92 cells with CD45CAR NK45i-92 cells. E:T (2:1)
0.06%
23.05%
0.13%
76.76%
GFP
CD56

FIGURE 57D

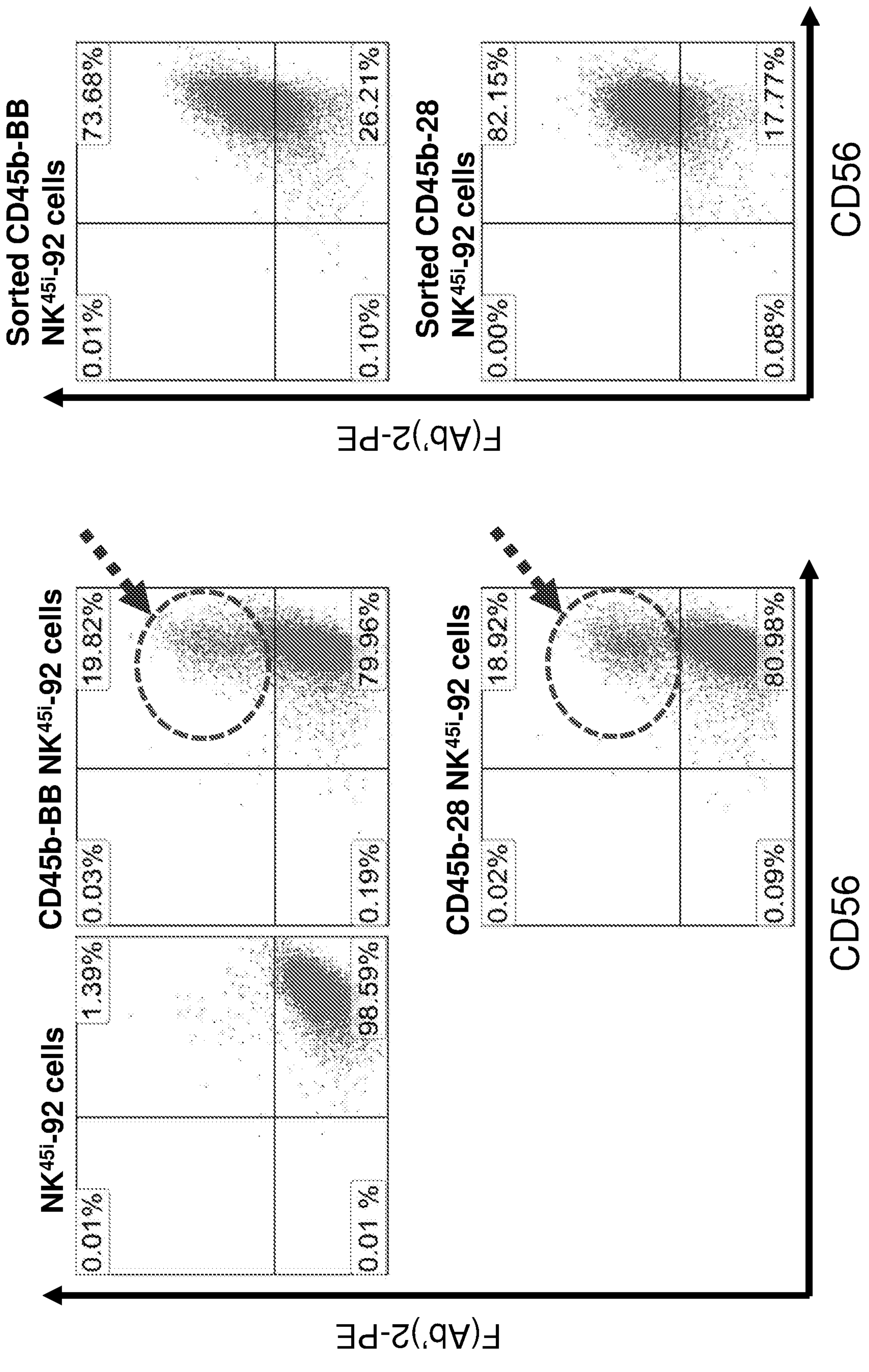
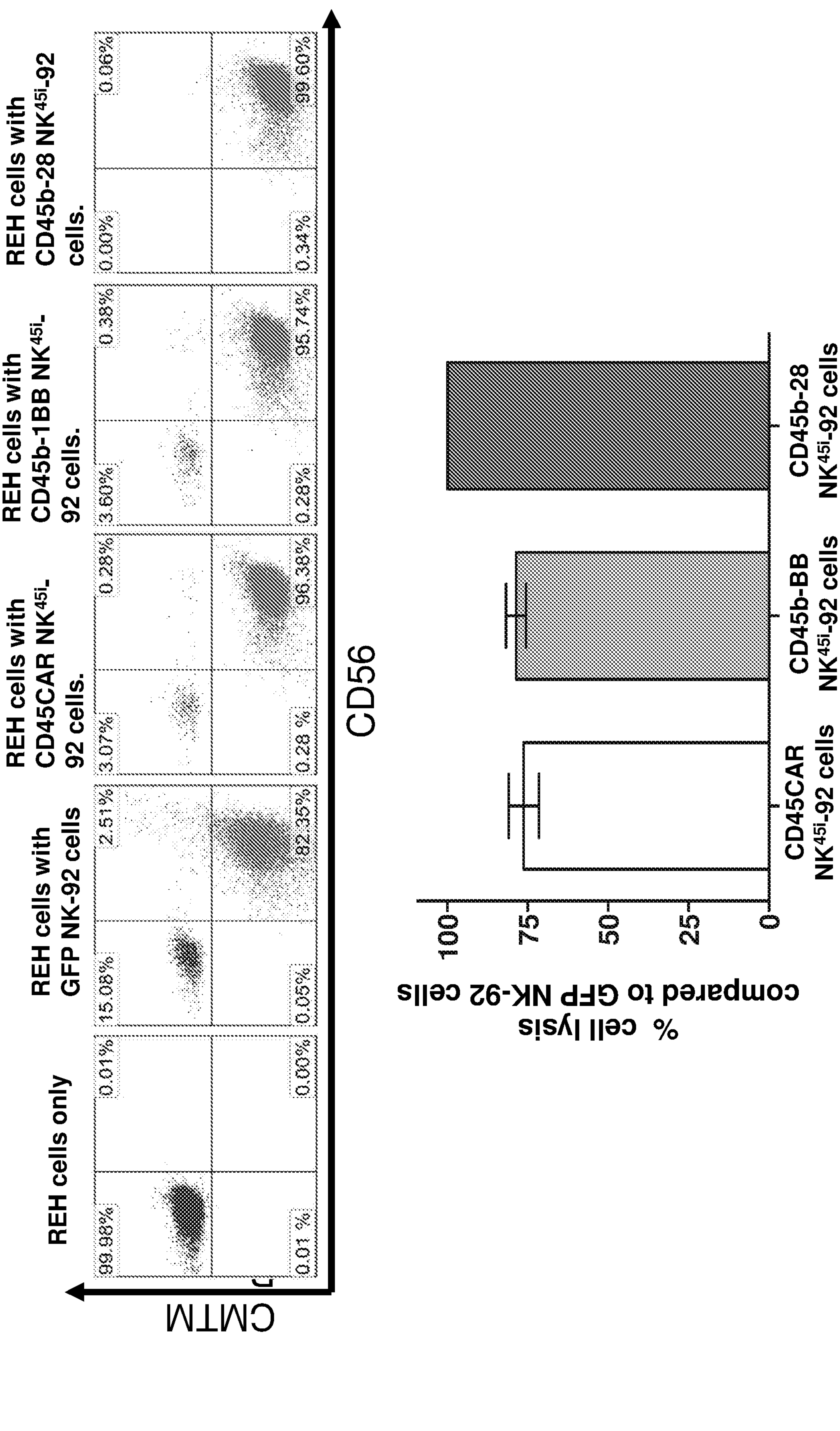

NK45i-92 cells
0.01%
1.39%
0.01 %
98.59%
CD45b-BB NK45i-92 cells
0.03%
19.82%
0.19%
79.96%
CD45b-28 NK45i-92 cells
0.02%
18.92%
0.09%
80.98%
Sorted CD45b-BB NK45i-92 cells
0.01%
73.68%
0.10%
26.21%
Sorted CD45b-28 NK45i-92 cells
0.00%
82.15%
0.08%
17.77%
F(Ab')2-PE
CD56
F(Ab')2-PE
CD56

FIGURE 57E


REH cells only
REH cells with GFP NK-92 cells
REH cells with CD45CAR NK45i-92 cells.
REH cells with CD45b-1BB NK45i-92 cells.
REH cells with CD45b-28 NK45i-92 cells.
99.98%
0.01%
0.01 %
0.00%
15.08%
2.51%
0.05%
82.35%
3.07%
0.28%
0.28 %
96.38%
3.60%
0.38%
0.28%
95.74%
0.00%
0.06%
0.34%
99.60%
CMTM
CD56
% cell lysis compared to GFP NK-92 cells
100
75
50
25
0
CD45CAR NK45i-92 cells
CD45b-BB NK45i-92 cells
CD45b-28 NK45i-92 cells

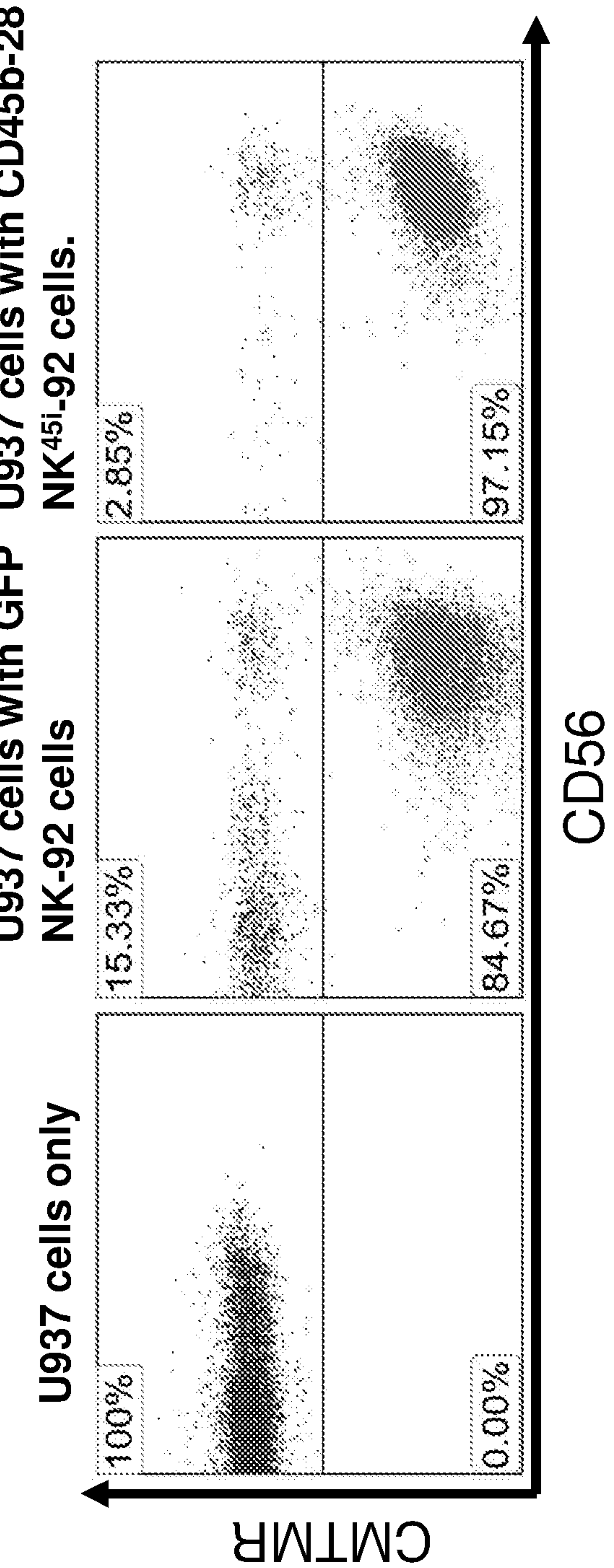


FIGURE 57FA

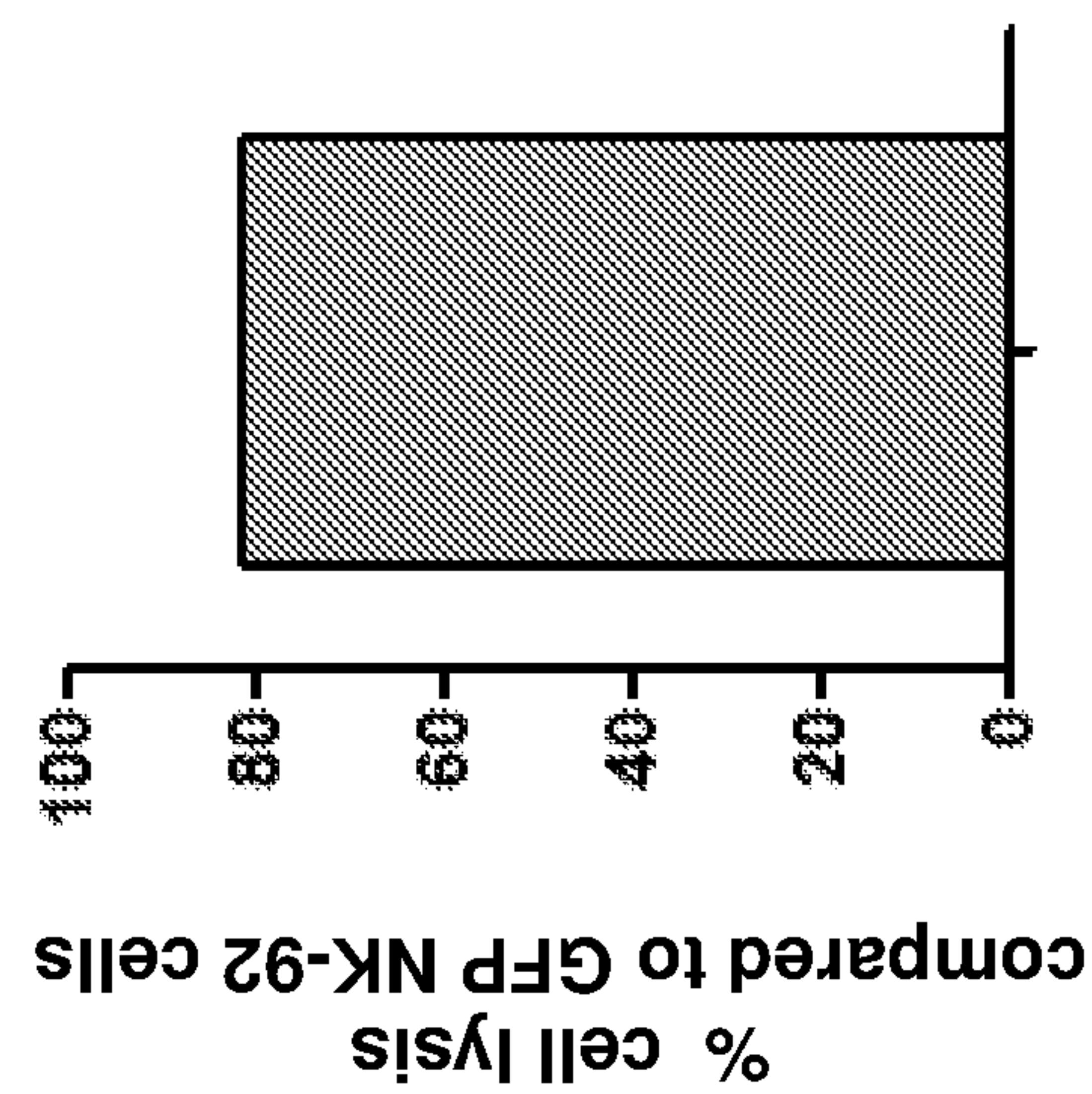


FIGURE 57FB

FIGURE 57GA

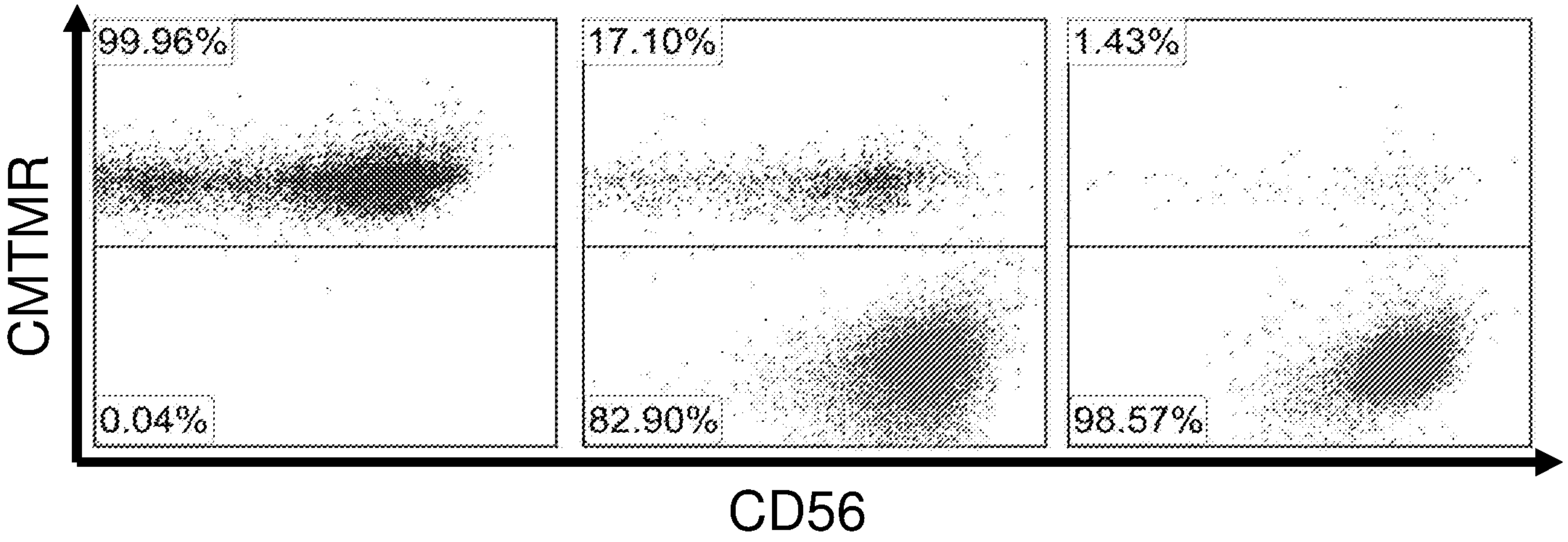


FIGURE 57GB

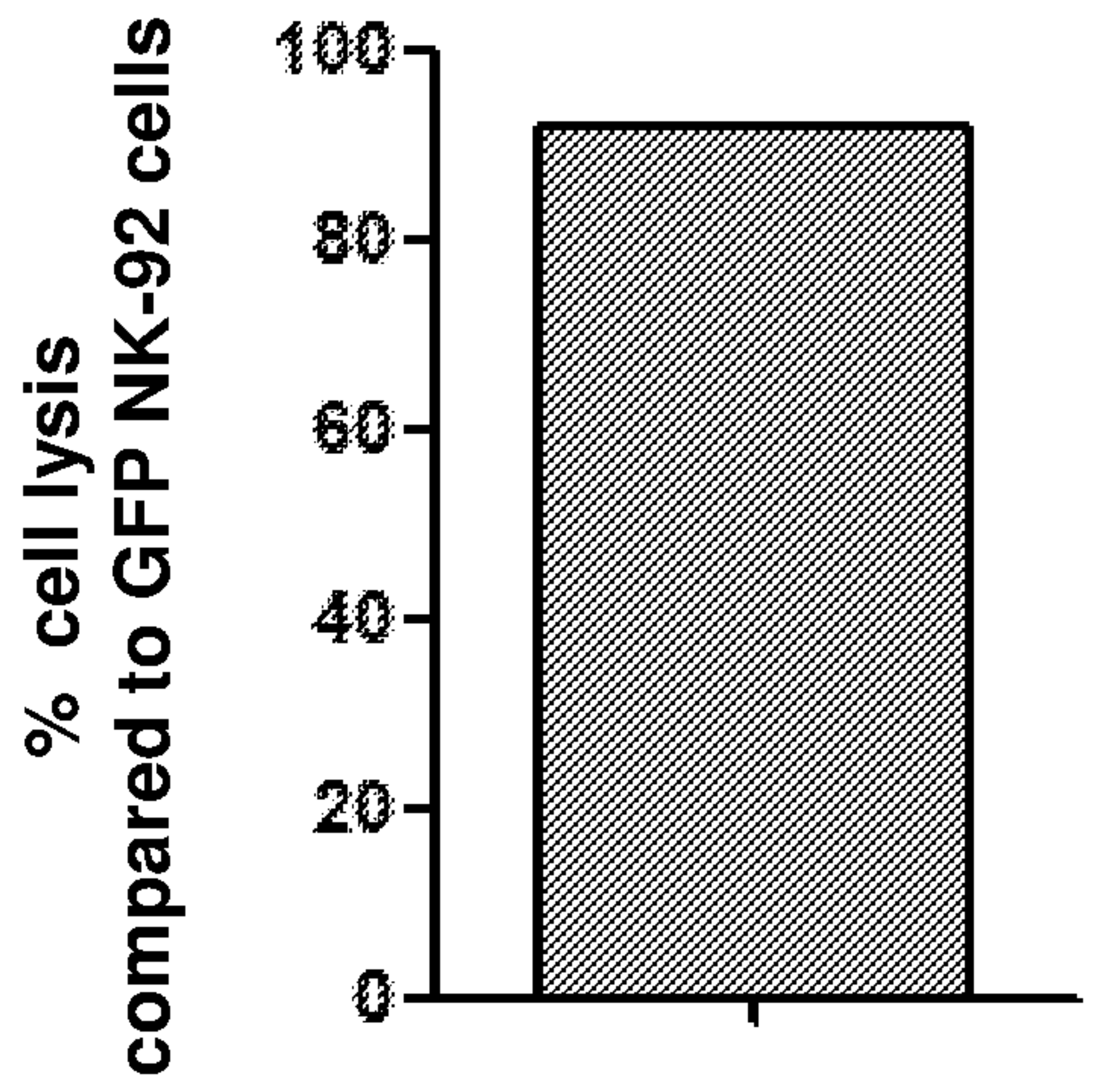
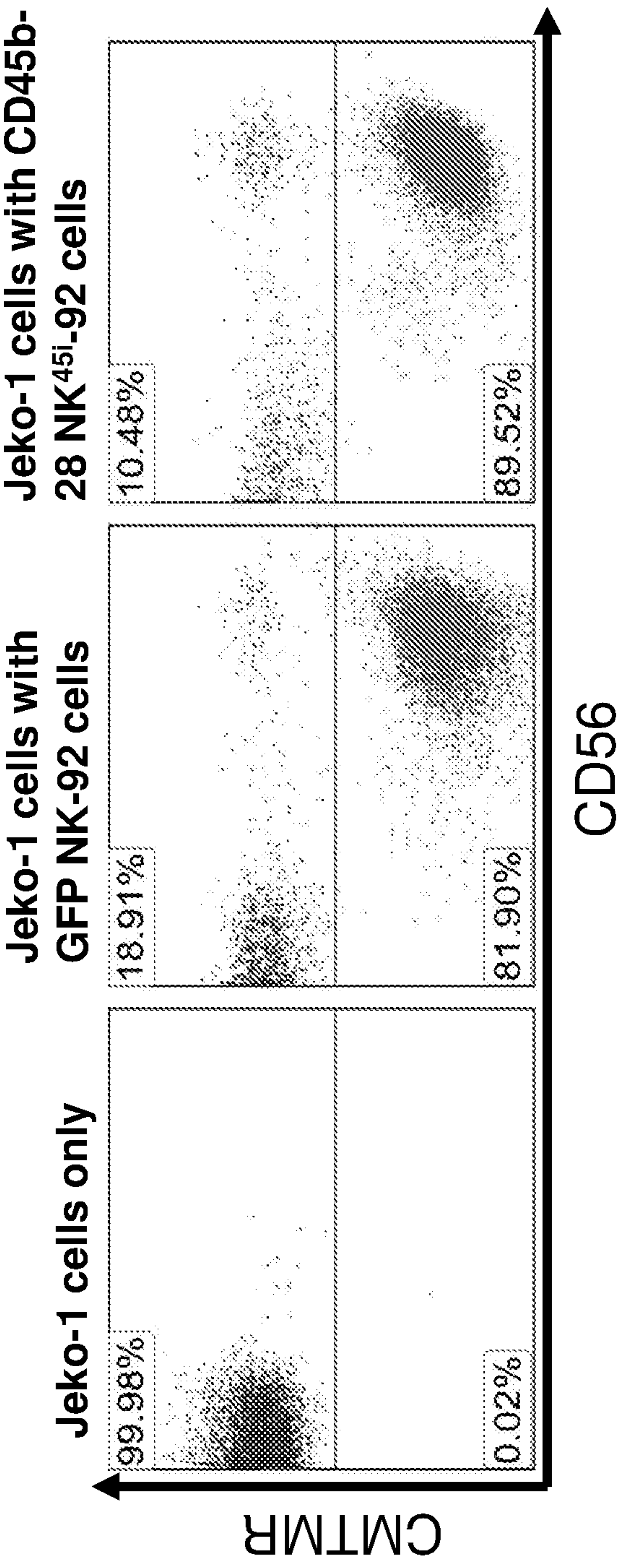


FIGURE 57HA



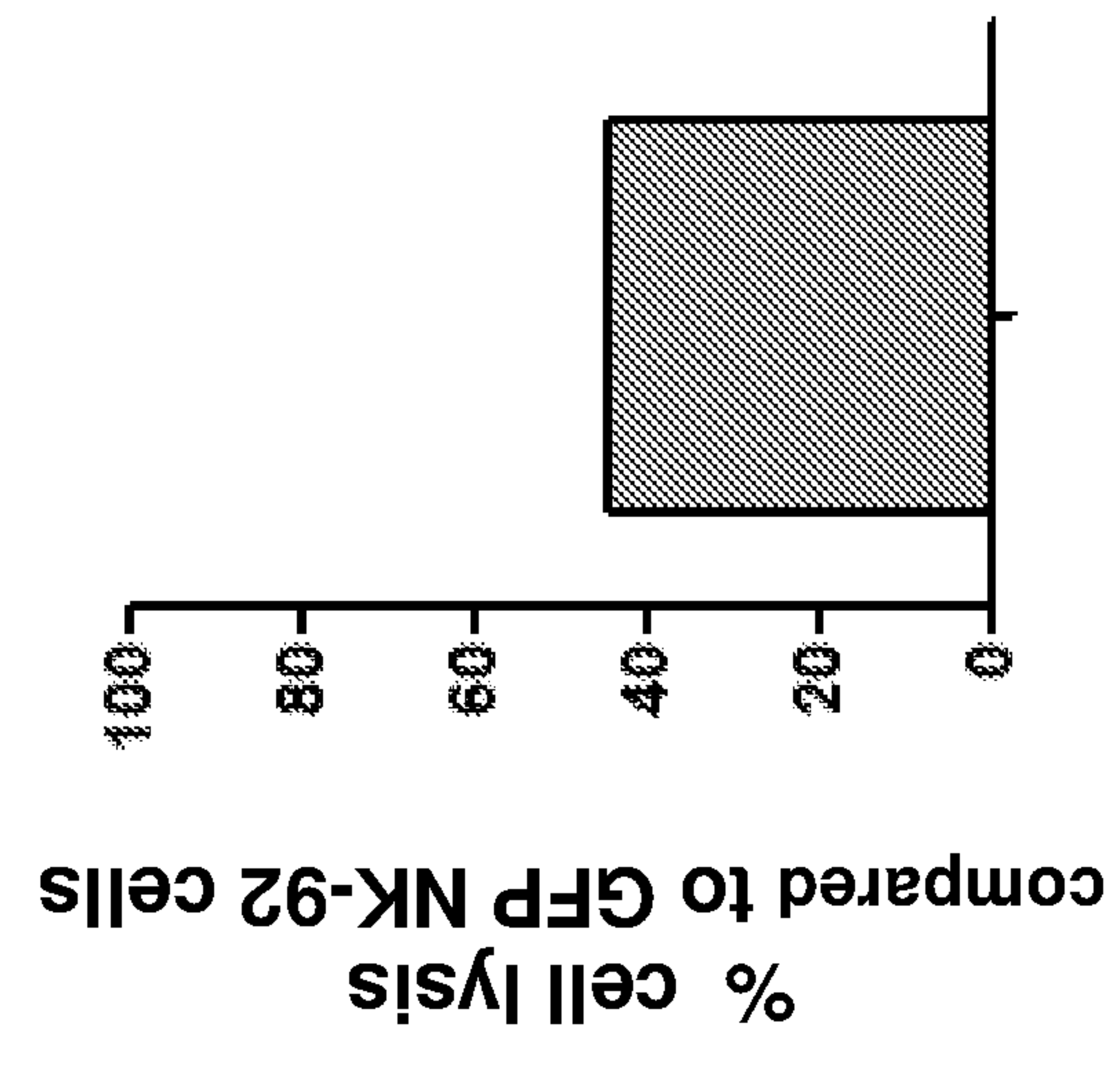
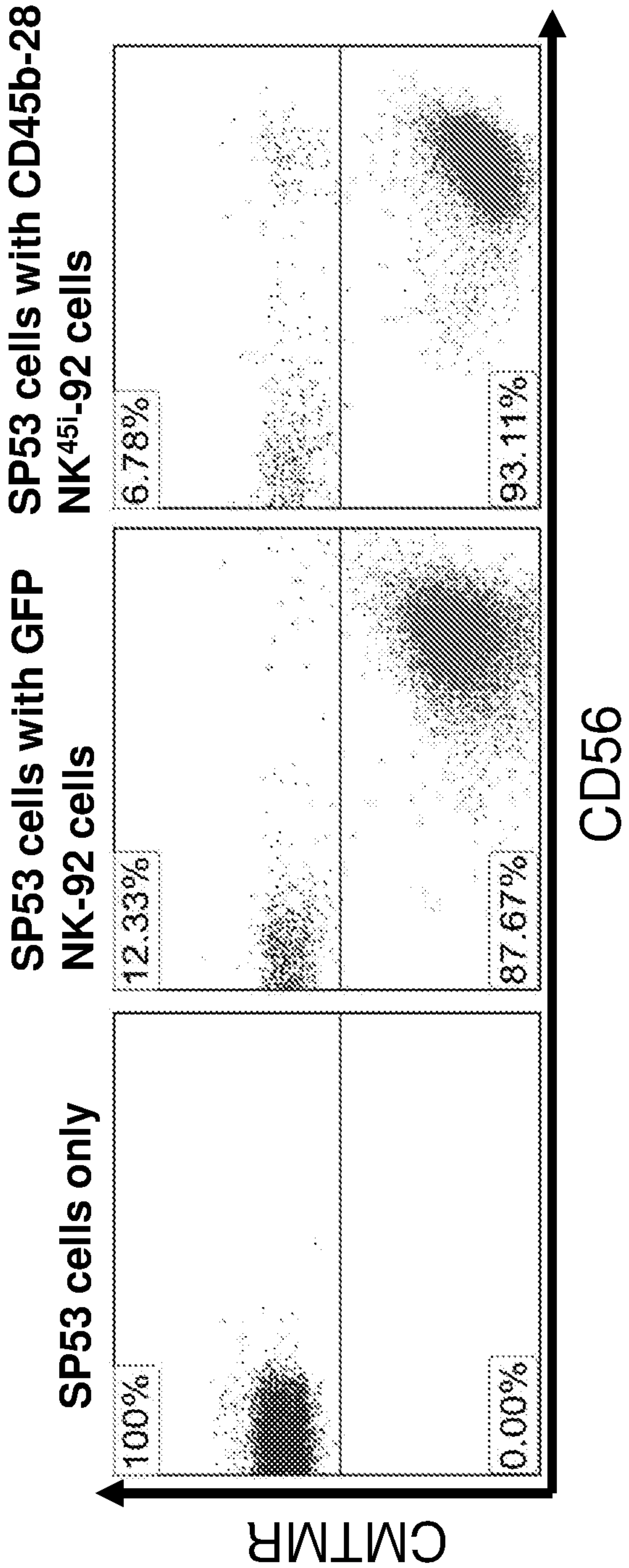


FIGURE 57HB

FIGURE 57IA



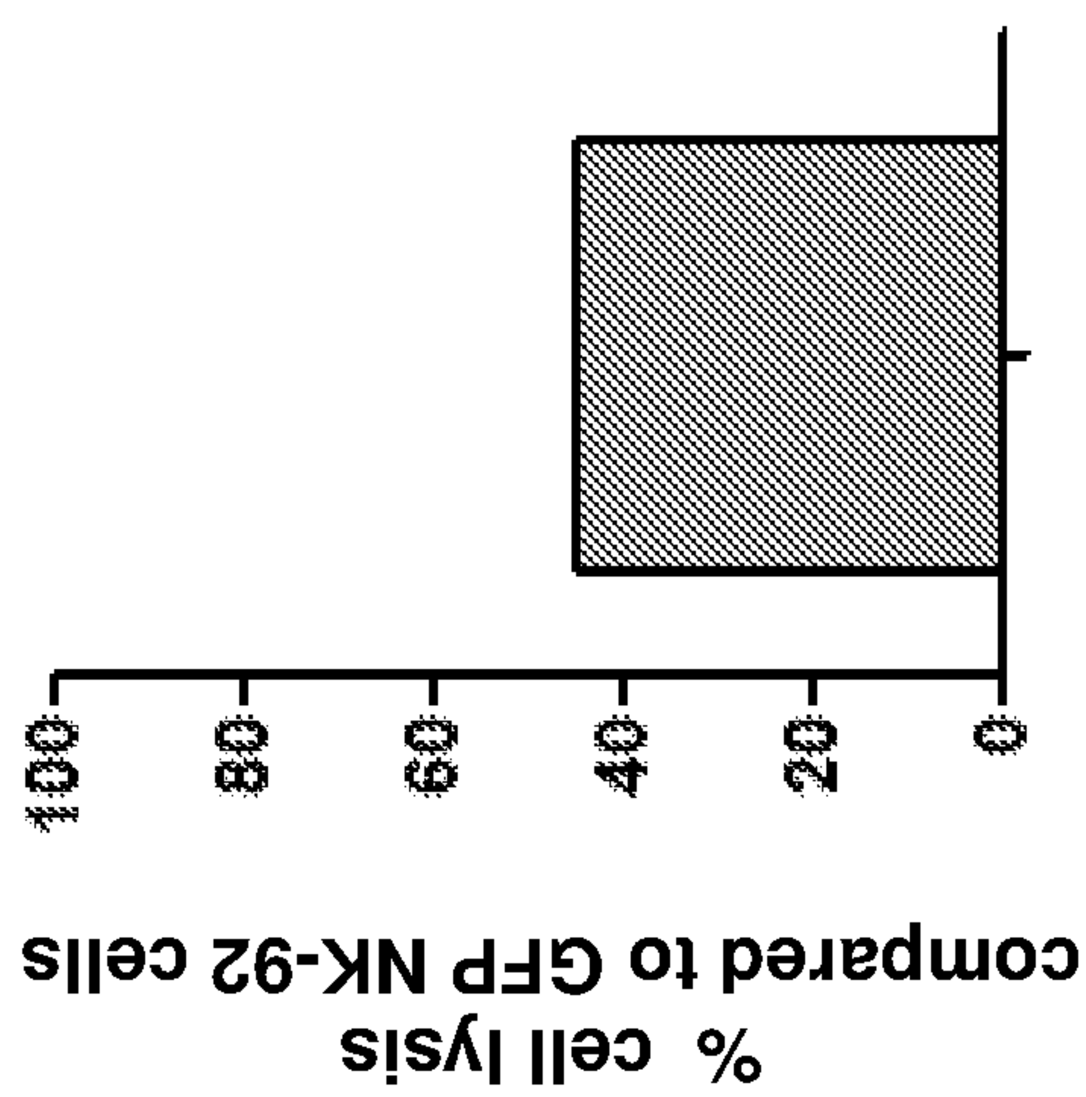


FIGURE 57IB

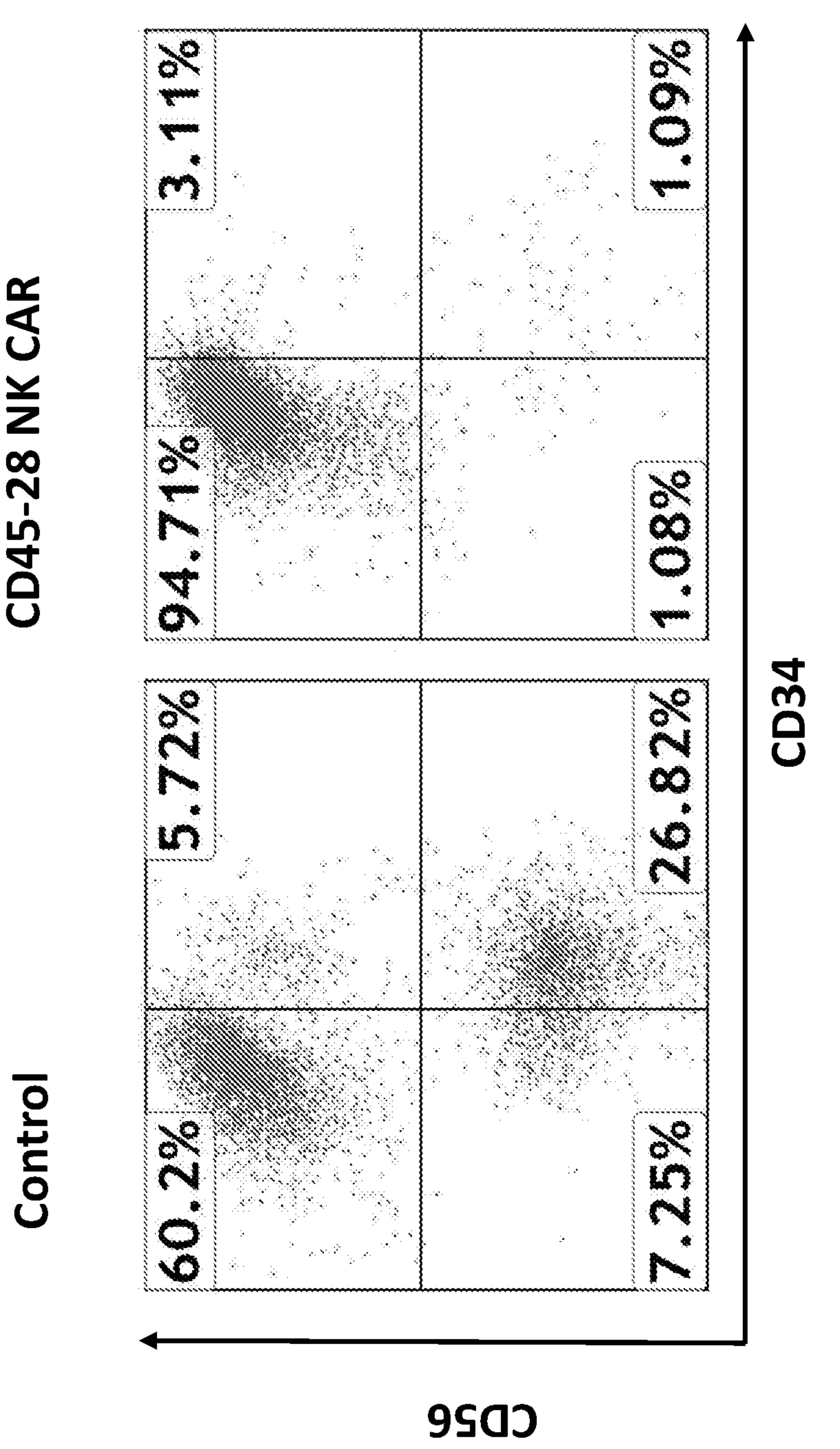

Control
CD45-28 NK CAR
60.2%
5.72%
7.25%
26.82%
94.71%
3.11%
1.08%
1.09%
CD34
CD56

FIGURE 57J

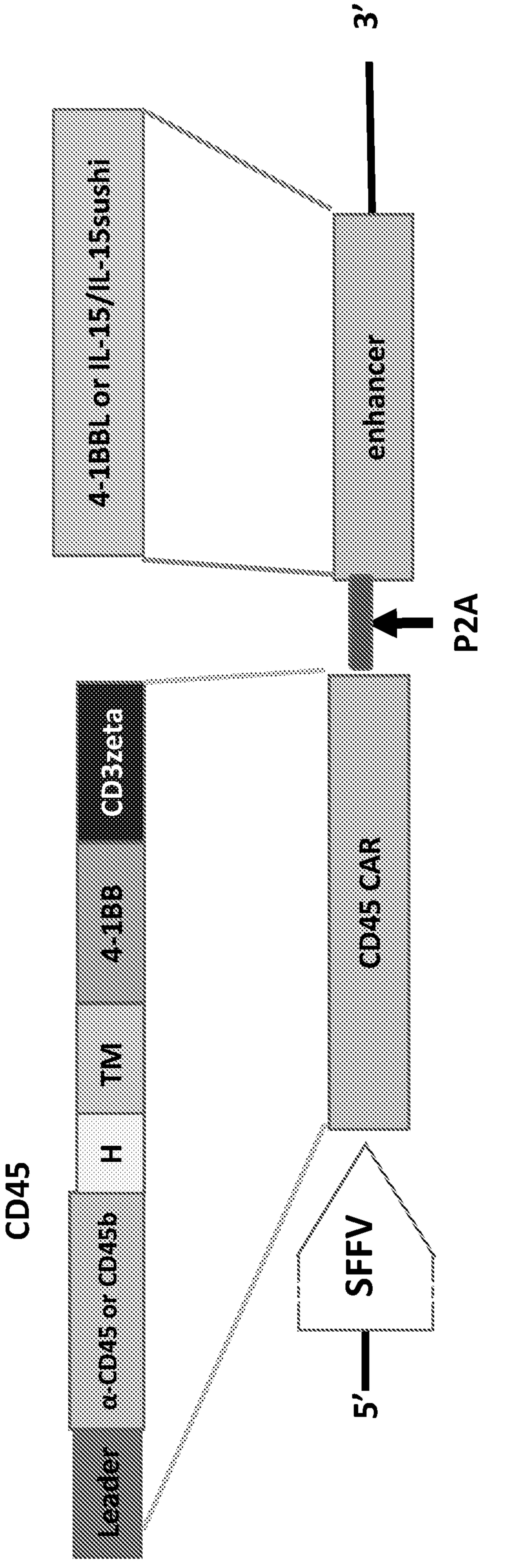

3'
4-1BBL or IL-15/IL-15sushi
enhancer
P2A
CD3zeta
4-1BB
TM
H
α-CD45 or CD45b
Leader
CD45
CD45 CAR
SFFV
5'

FIGURE 58A

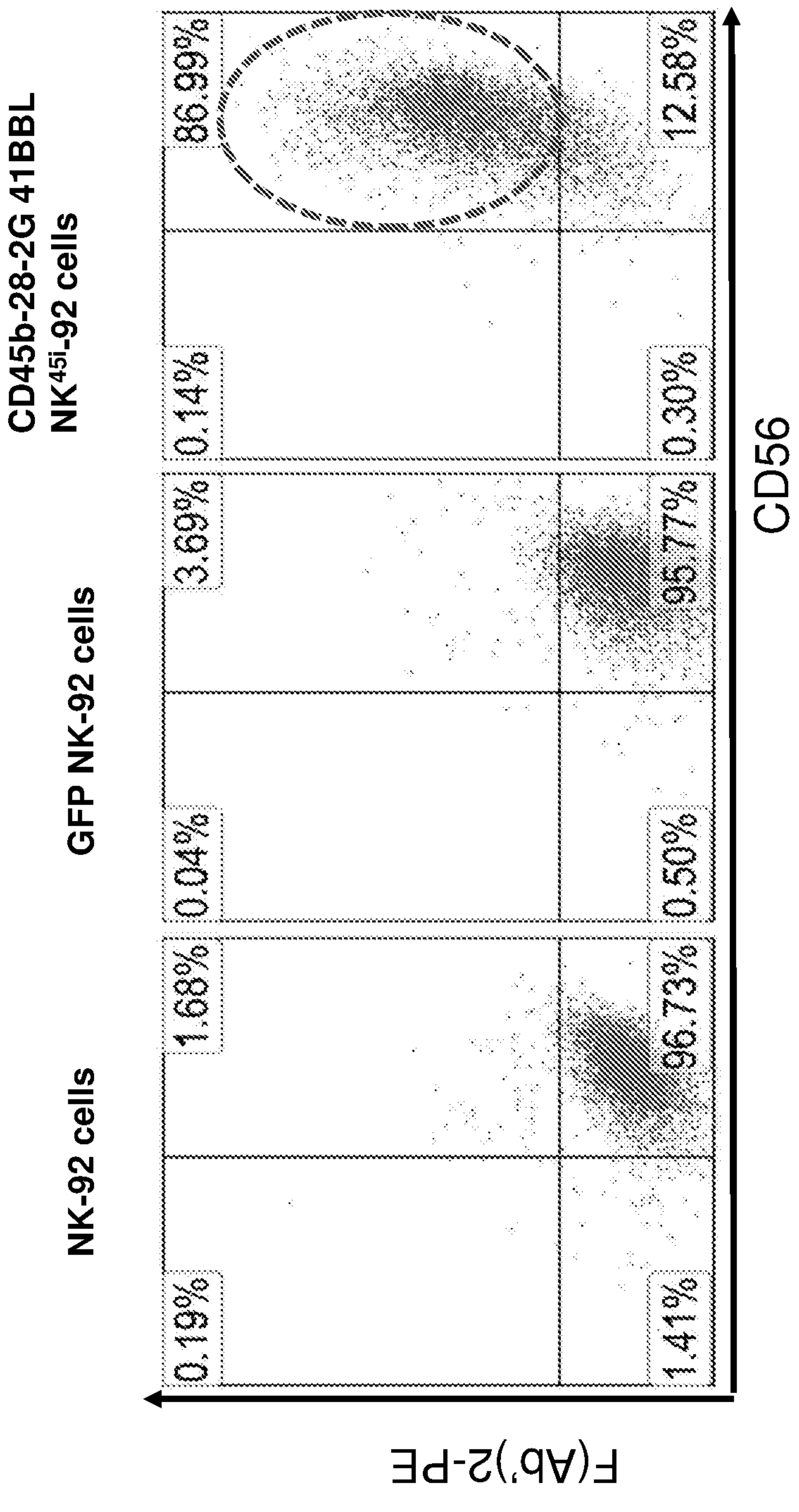

CD45b-28-2G 41BBL
NK45i-92 cells
GFP NK-92 cells
NK-92 cells
86.99%
12.58%
0.14%
0.30%
3.69%
95.77%
0.04%
0.50%
1.68%
96.73%
0.19%
1.41%
CD56
F(Ab')2-PE

FIGURE 58B

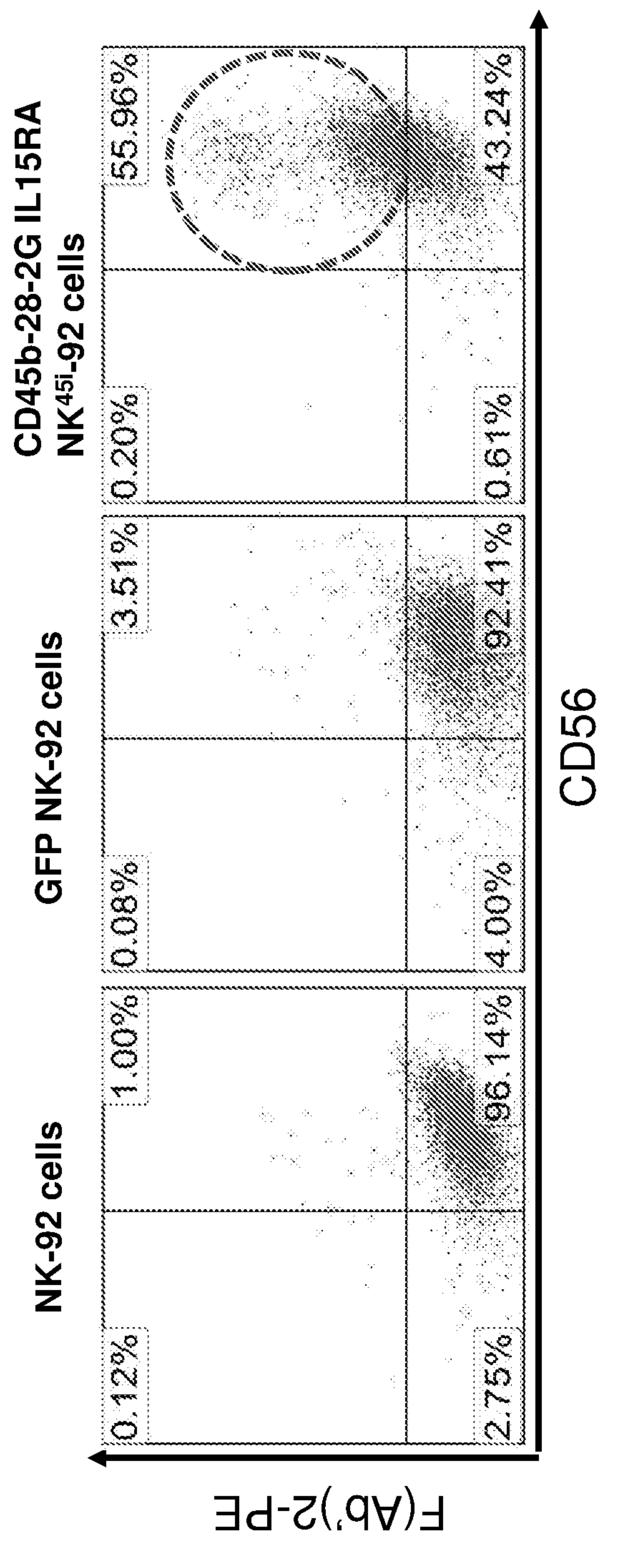

NK-92 cells
0.12%
1.00%
2.75%
96.14%
GFP NK-92 cells
0.08%
3.51%
4.00%
92.41%
CD45b-28-2G IL15RA
NK^{45i}-92 cells
0.20%
55.96%
0.61%
43.24%
CD56
F(Ab')2-PE

FIGURE 58C

FIGURE 59A

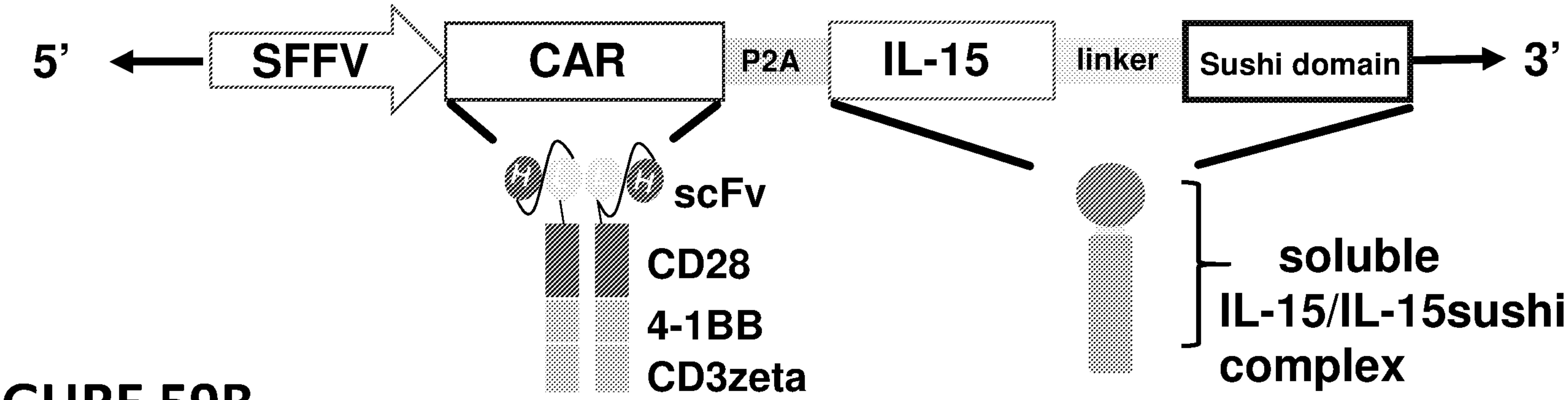


FIGURE 59B

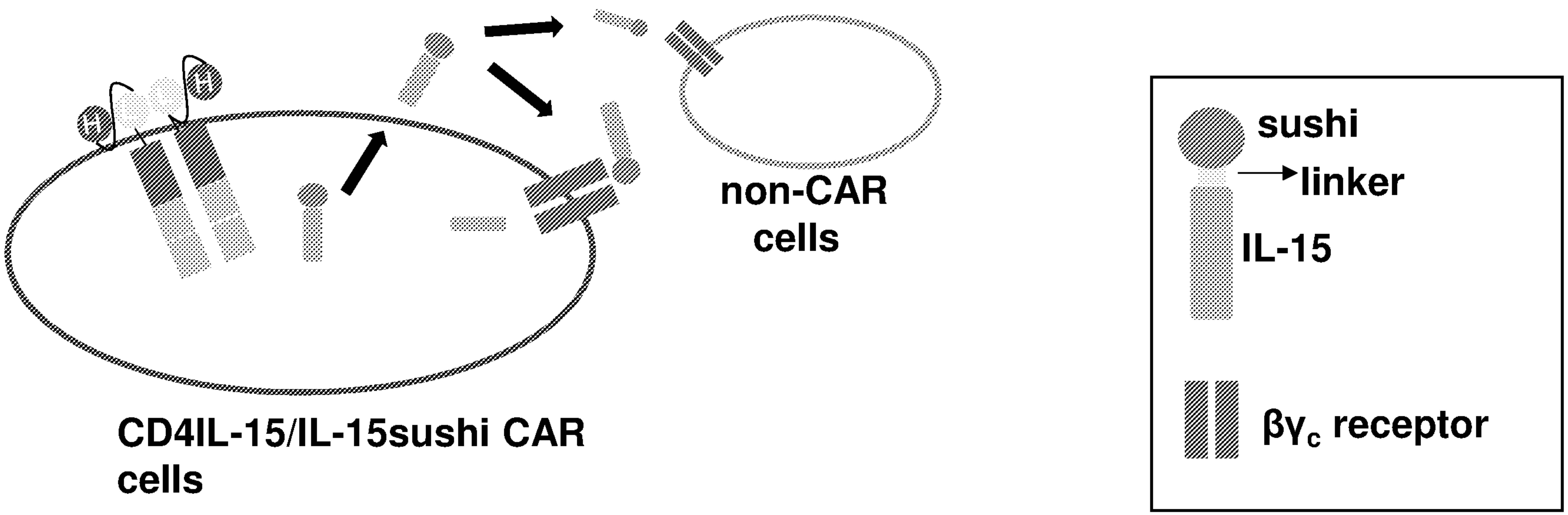


FIGURE 60A

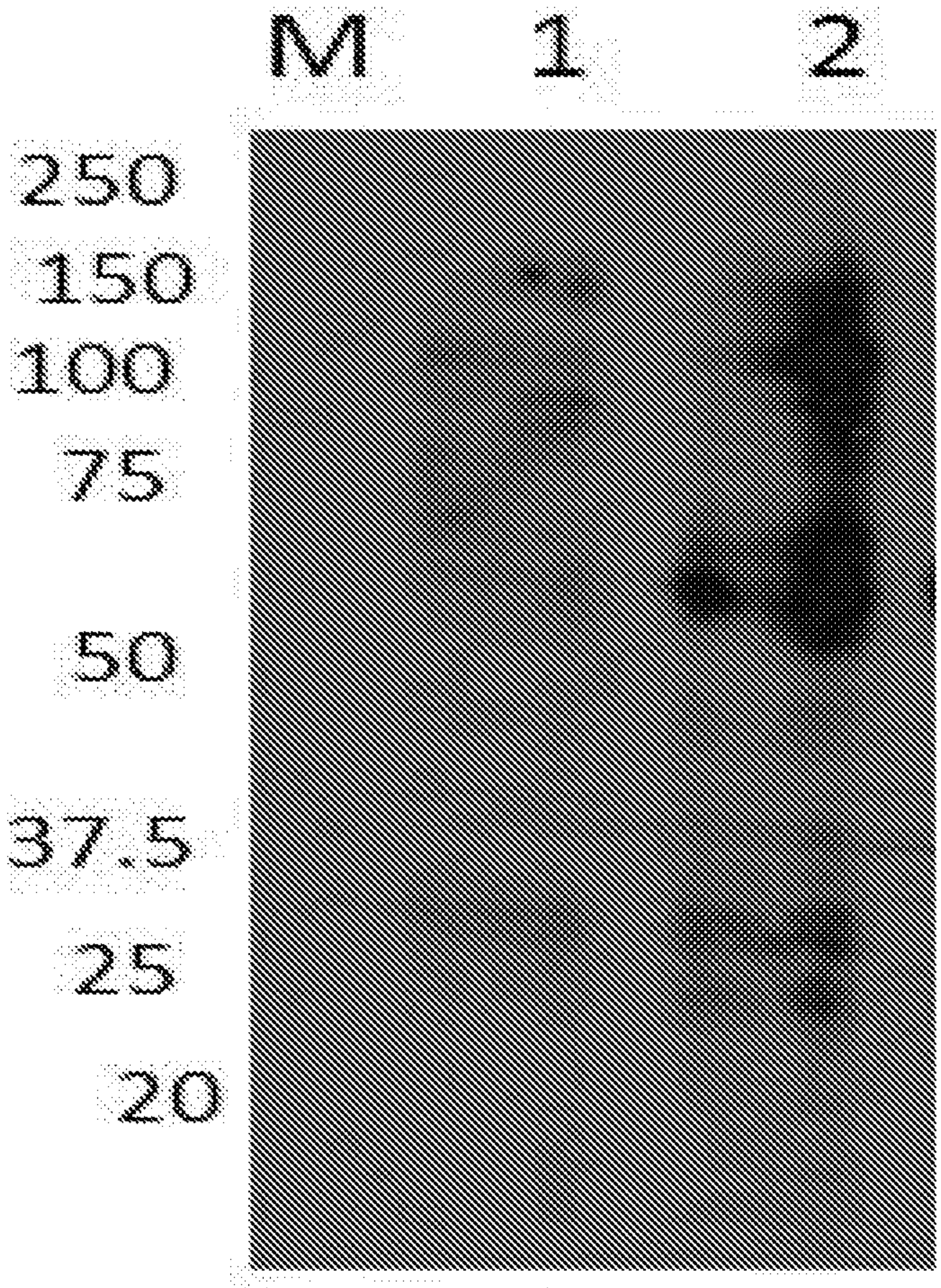


FIGURE 60B

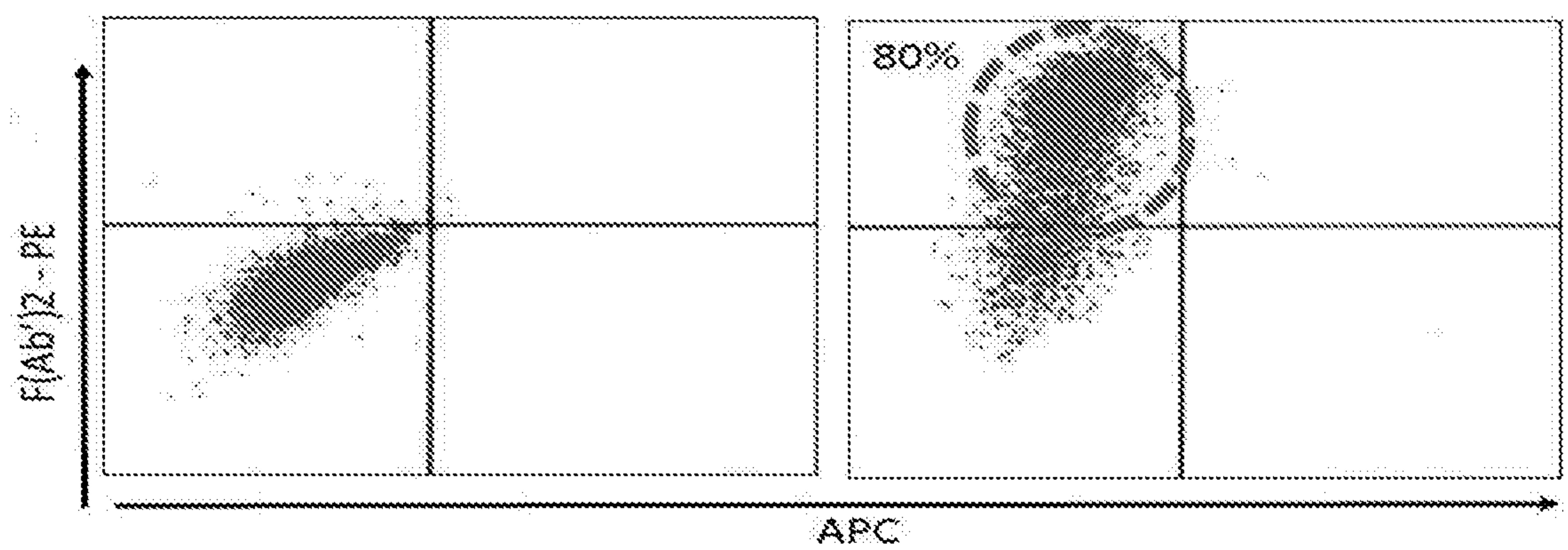


FIGURE 61

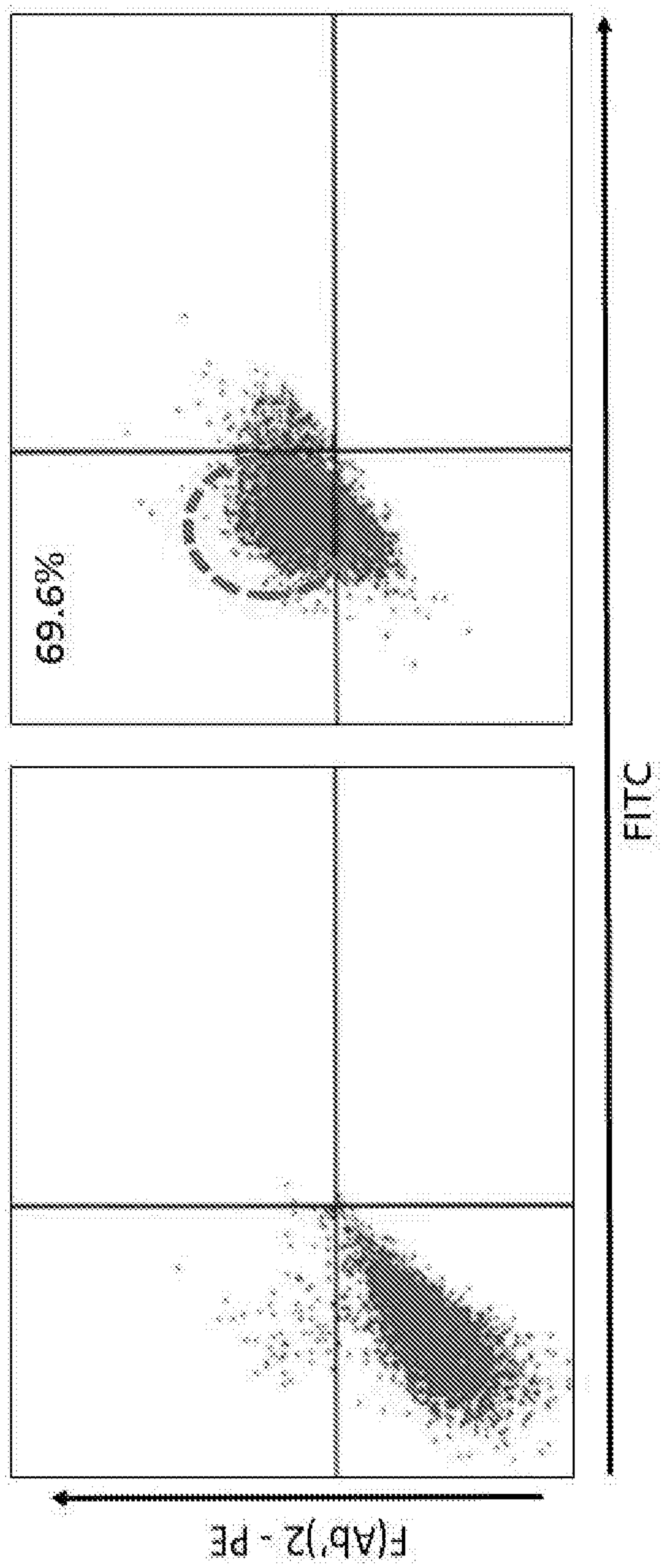


FIGURE 62

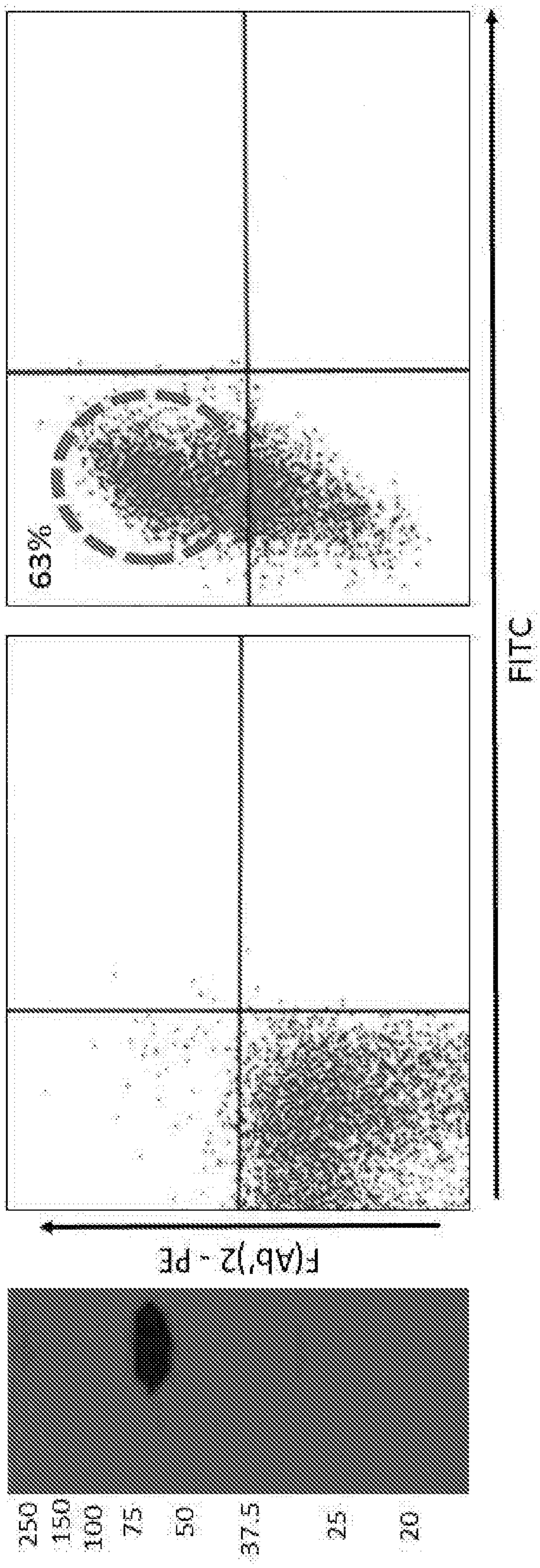
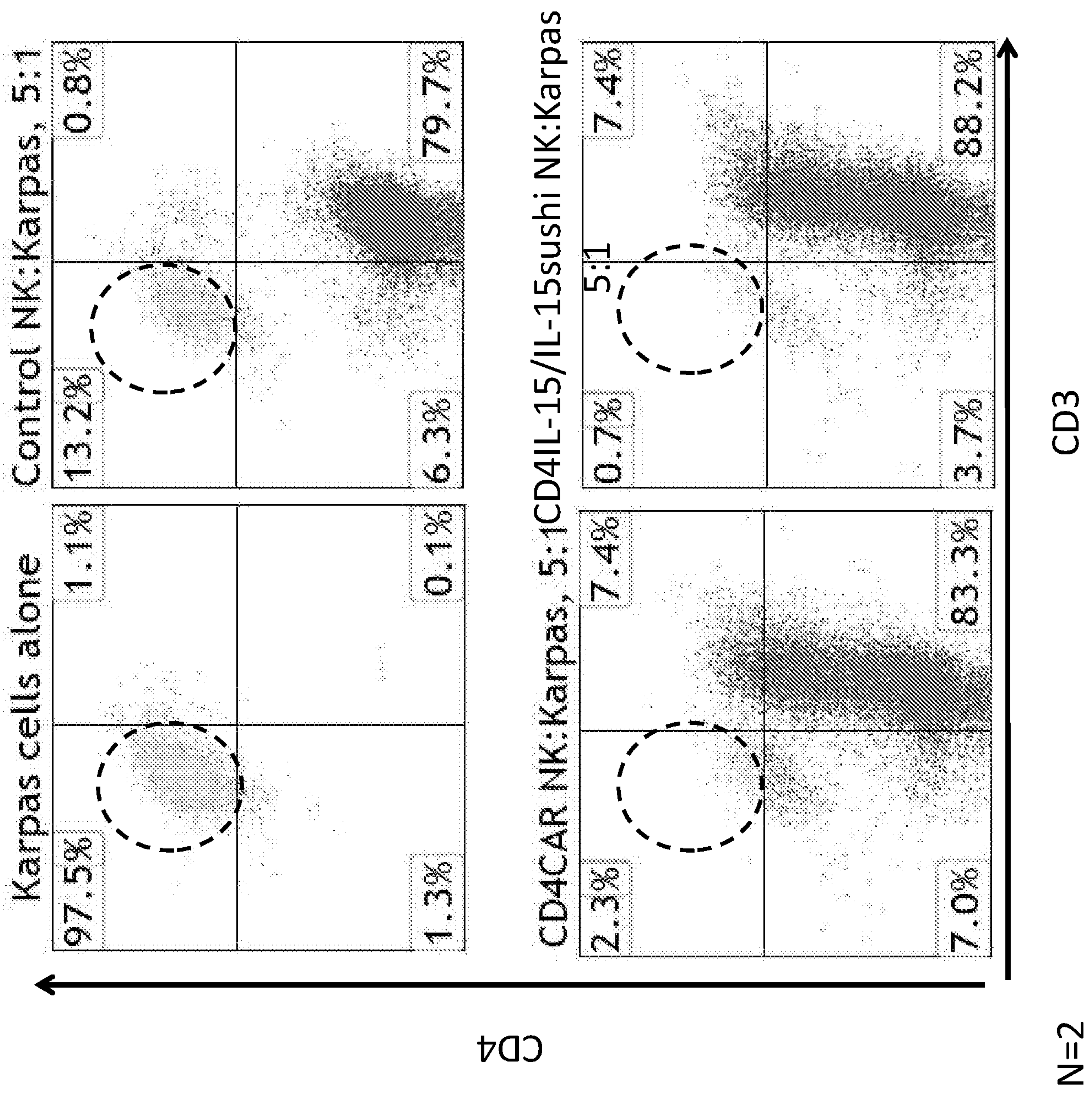


FIGURE 63A


Karpas cells alone
97.5%
1.1%
1.3%
0.1%
Control NK:Karpas, 5:1
13.2%
0.8%
6.3%
79.7%
CD4CAR NK:Karpas, 5:1
2.3%
7.4%
7.0%
83.3%
CD4IL-15/IL-15sushi NK:Karpas 5:1
0.7%
7.4%
3.7%
88.2%
CD4
CD3
N=2

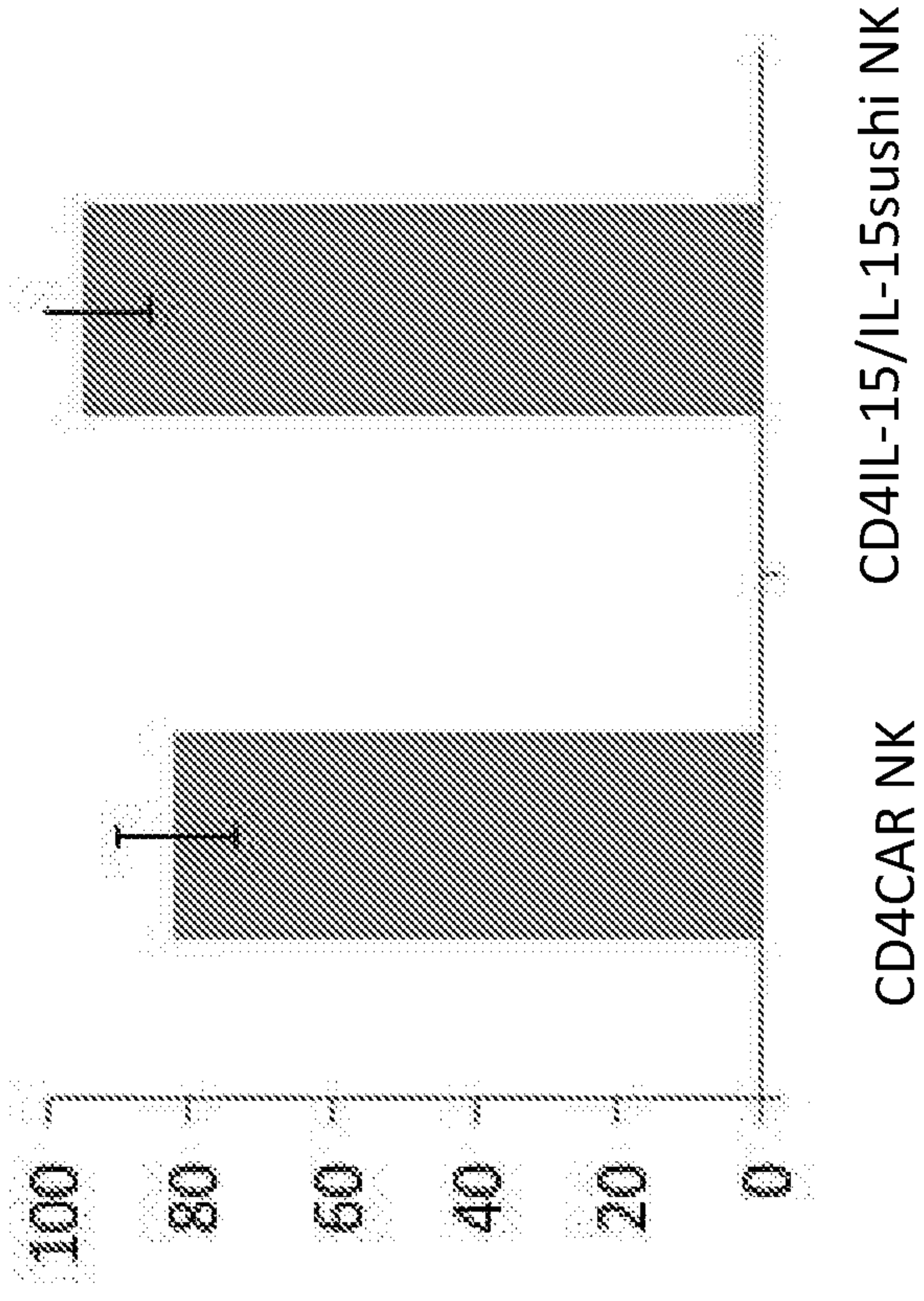

% Karpas 299 cells lysed
100
80
60
40
20
0
CD4CAR NK
CD4IL-15/IL-15sushi NK

FIGURE 63B

FIGURE 64

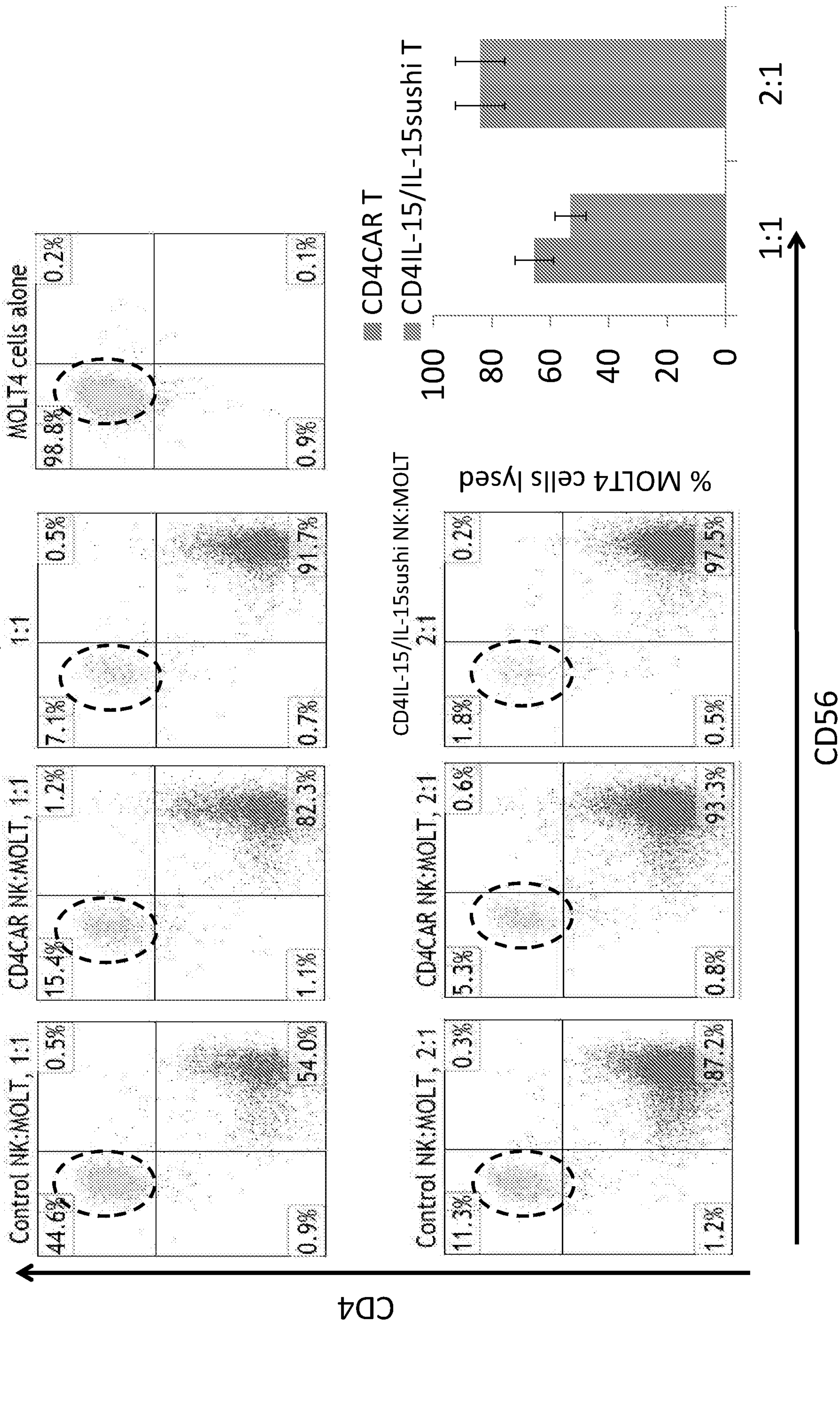
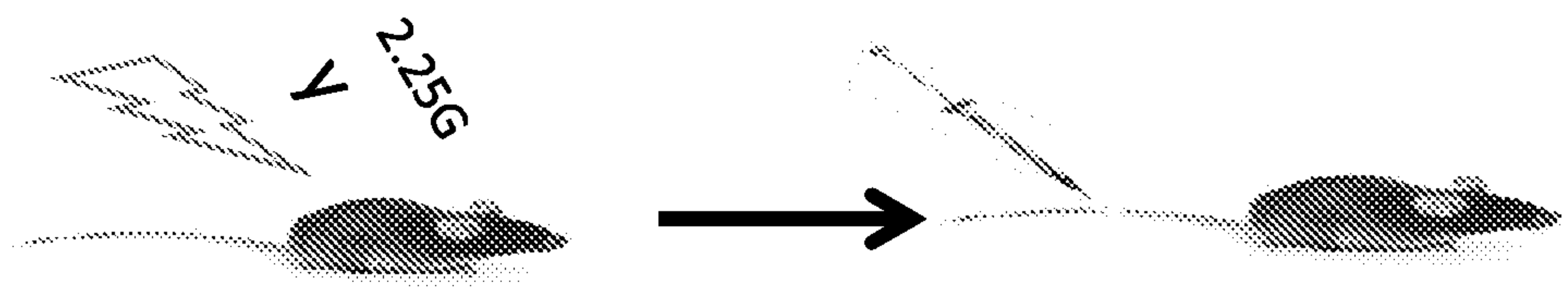

Control NK:MOLT, 1:1
44.6%
0.5%
0.9%
54.0%
CD4CAR NK:MOLT, 1:1
15.4%
1.2%
1.1%
82.3%
CD4IL-15/IL-15sushi NK:MOLT
1:1
7.1%
0.5%
0.7%
91.7%
MOLT4 cells alone
98.8%
0.2%
0.9%
0.1%
Control NK:MOLT, 2:1
11.3%
0.3%
1.2%
87.2%
CD4CAR NK:MOLT, 2:1
5.3%
0.6%
0.8%
93.3%
CD4IL-15/IL-15sushi NK:MOLT
2:1
1.8%
0.2%
0.5%
97.5%
CD4CAR T
CD4IL-15/IL-15sushi T
% MOLT4 cells lysed
100
80
60
40
20
0
1:1
2:1
CD4
CD56

FIGURE 65A



CD4 expression in AML (MOLM13) cells

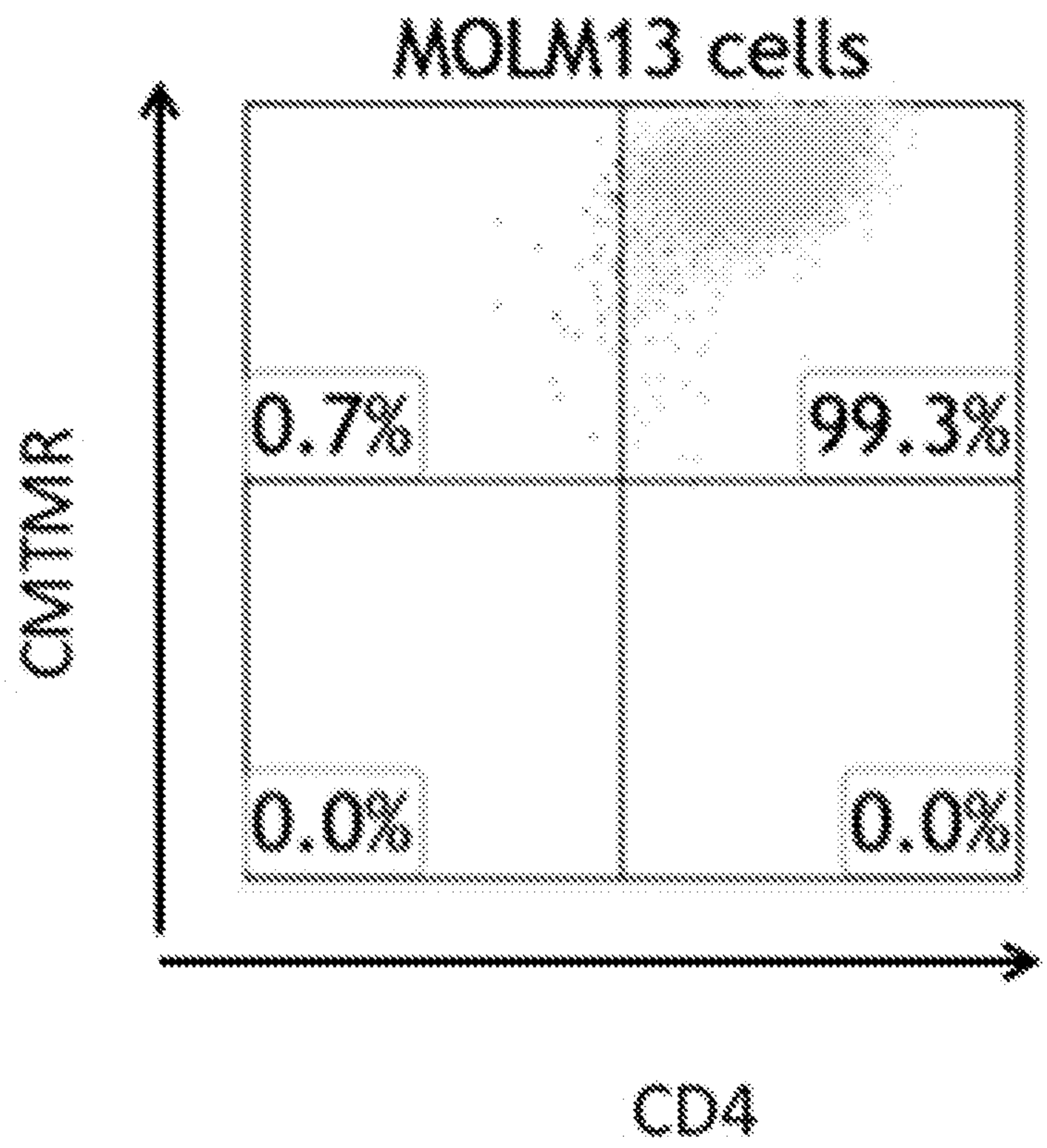


FIGURE 65B

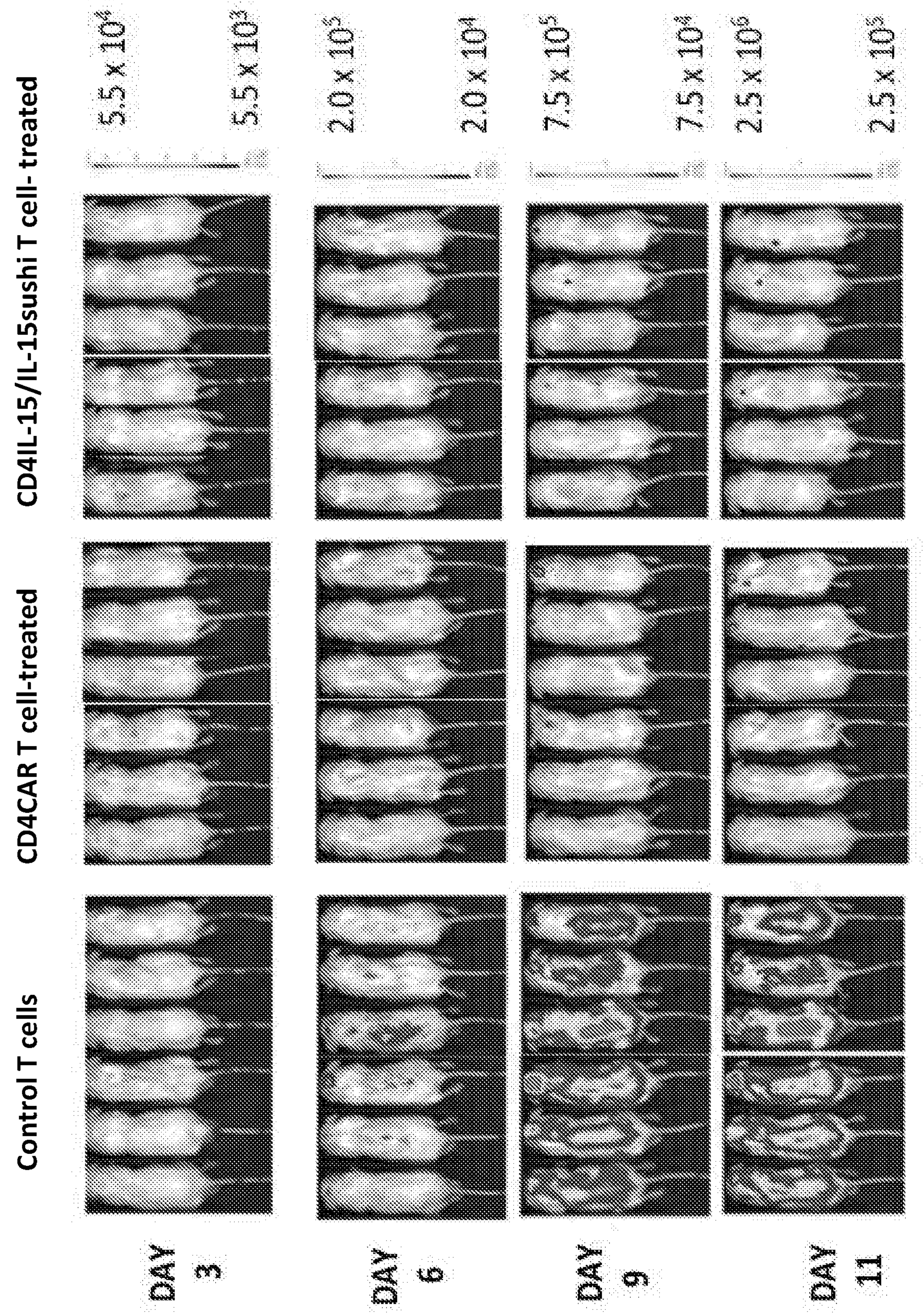

Control T cells
CD4CAR T cell-treated
CD4IL-15/IL-15sushi T cell- treated
DAY 3
DAY 6
DAY 9
DAY 11
5.5×10^4
5.5×10^3
2.0×10^5
2.0×10^4
7.5×10^5
7.5×10^4
2.5×10^6
2.5×10^5

FIGURE 65C

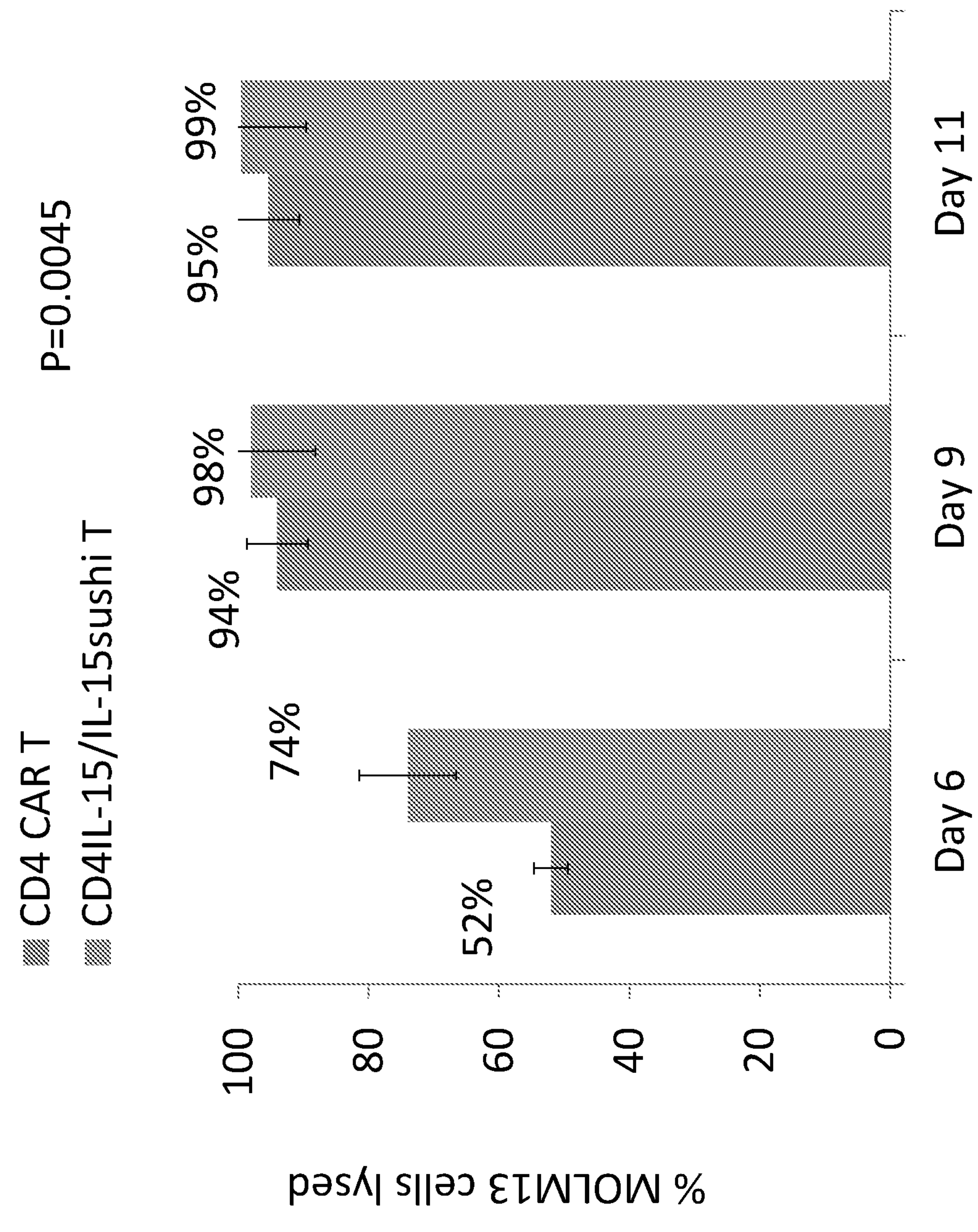

CD4 CAR T
CD4IL-15/IL-15sushi T
P=0.0045
52%
74%
94%
98%
95%
99%
100
80
60
40
20
0
% MOLM13 cells lysed
Day 6
Day 9
Day 11

FIGURE 65D

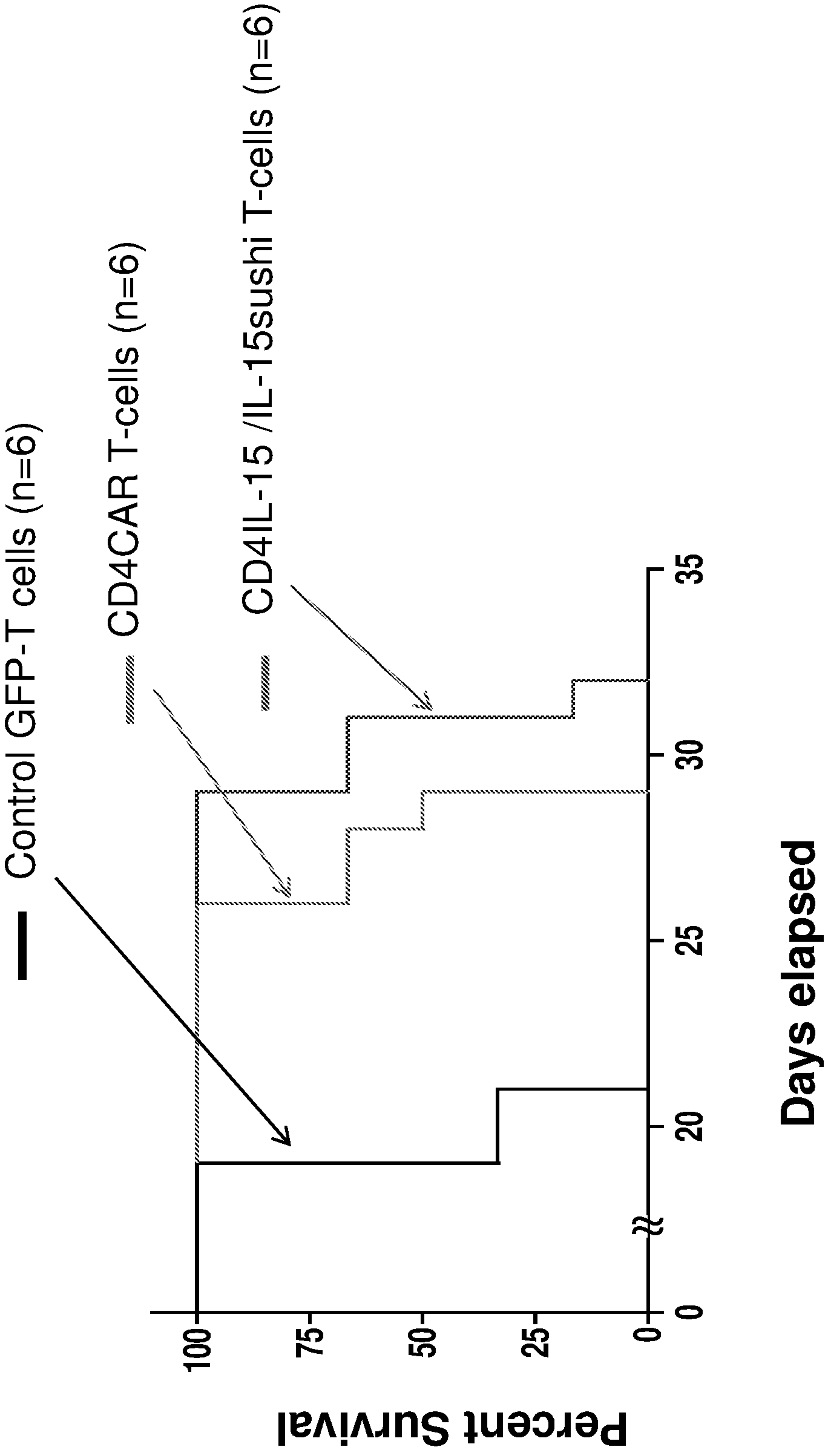
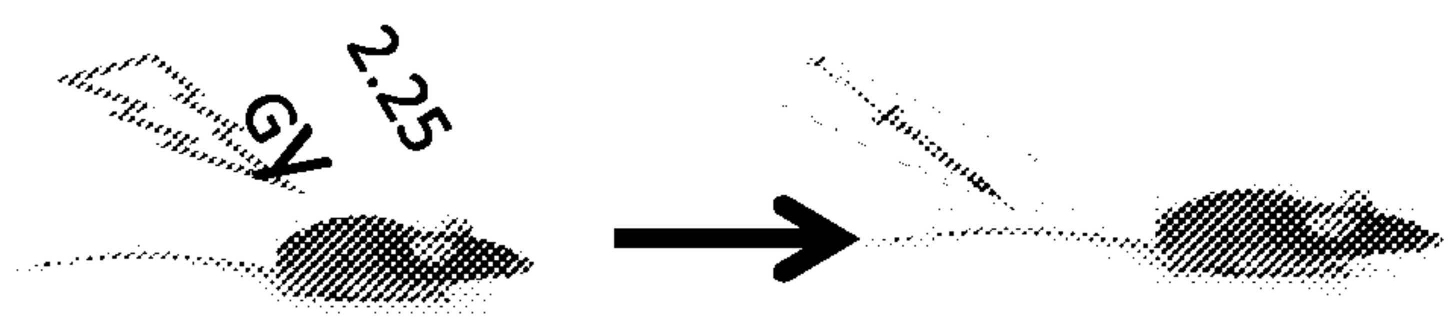

Control GFP-T cells (n=6)
CD4CAR T-cells (n=6)
CD4IL-15 /IL-15sushi T-cells (n=6)
Percent Survival
100
75
50
25
0
0
20
25
30
35
Days elapsed

FIGURE 66A



CD4 expression in MOLM13 and Jurkat cell lines

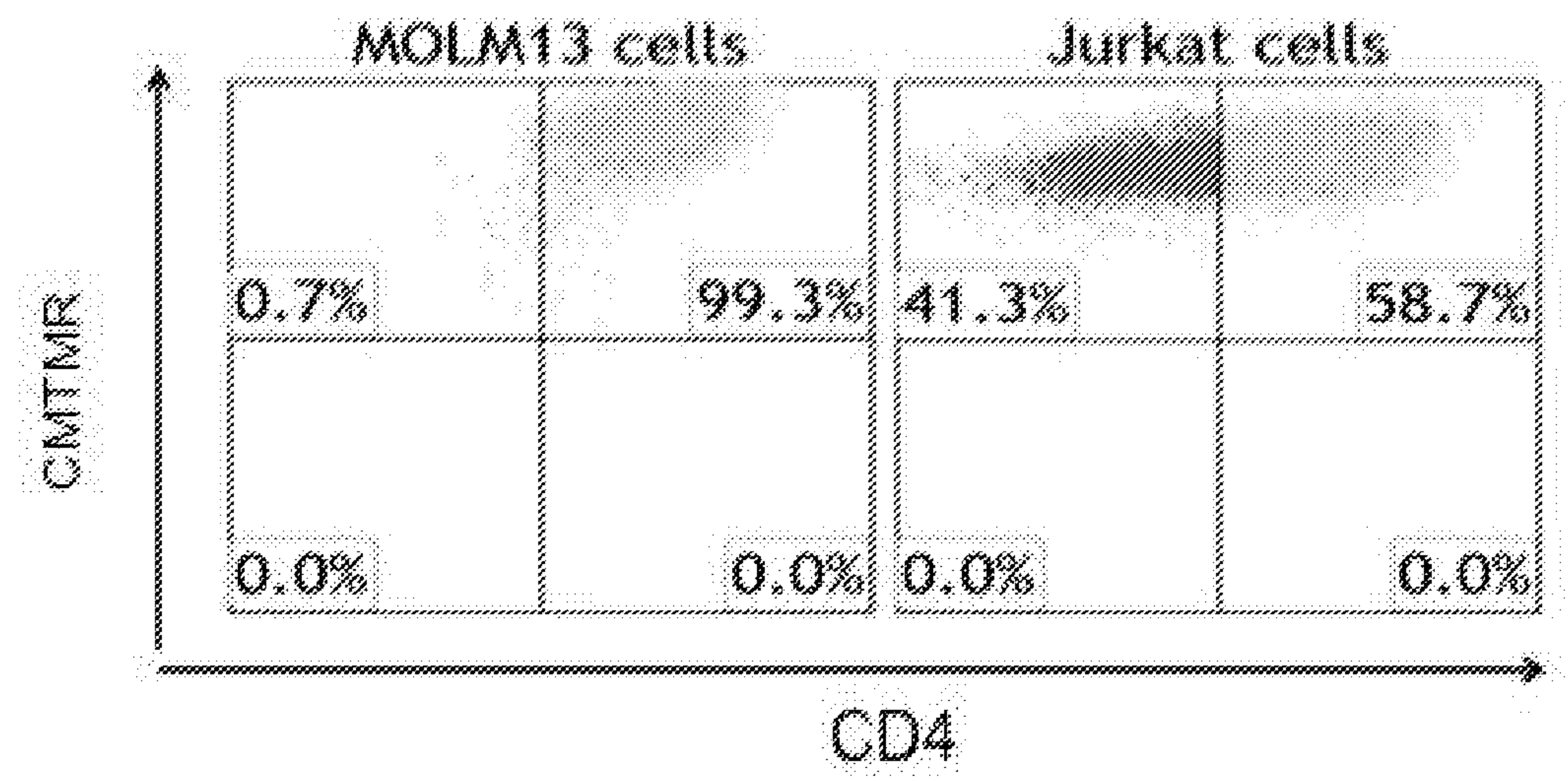


FIGURE 66B

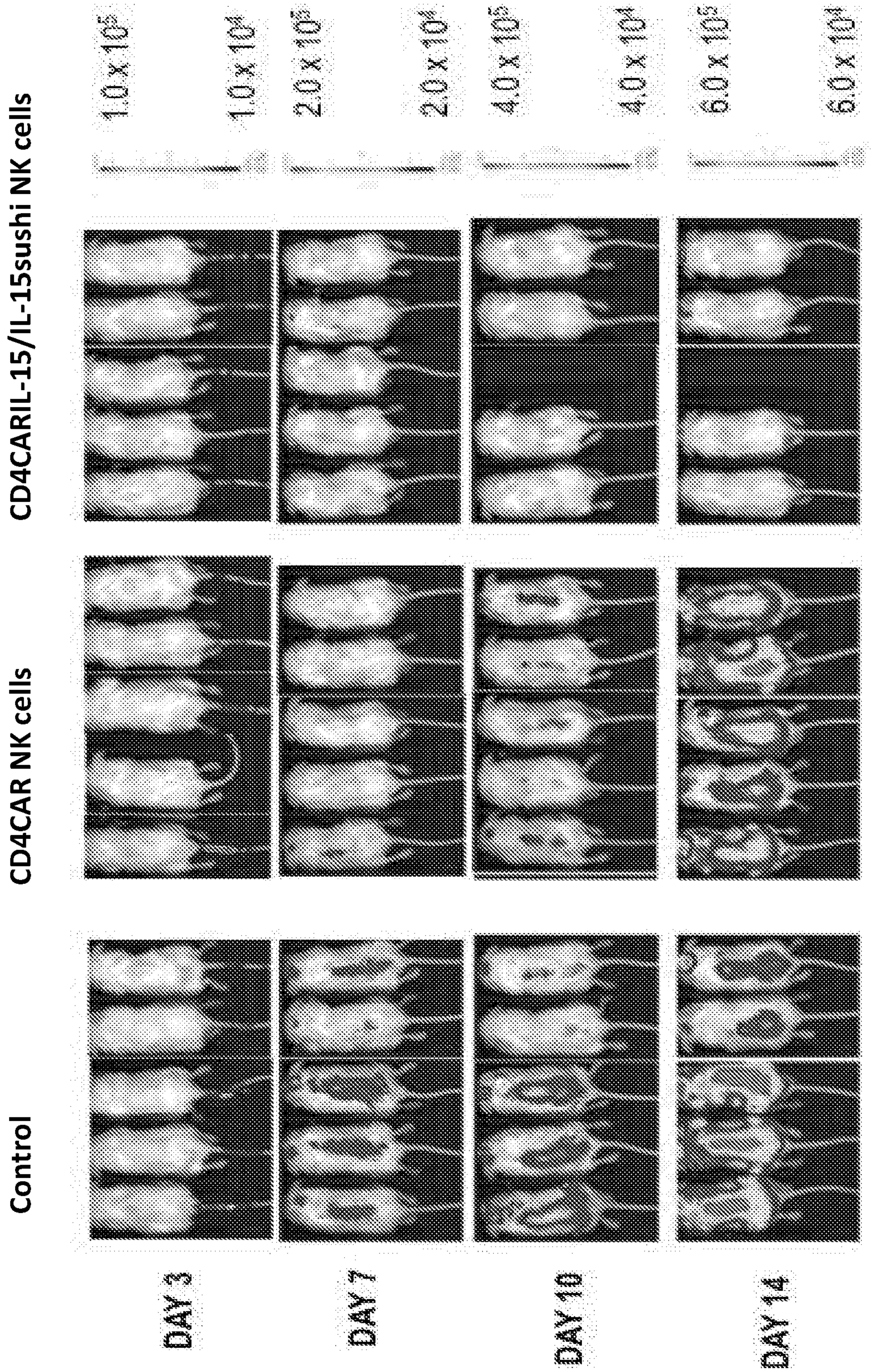

Control
CD4CAR NK cells
CD4CARIL-15/IL-15sushi NK cells
DAY 3
DAY 7
DAY 10
DAY 14
1.0 x 10^5
1.0 x 10^4
2.0 x 10^5
2.0 x 10^4
4.0 x 10^5
4.0 x 10^4
6.0 x 10^5
6.0 x 10^4

FIGURE 66C

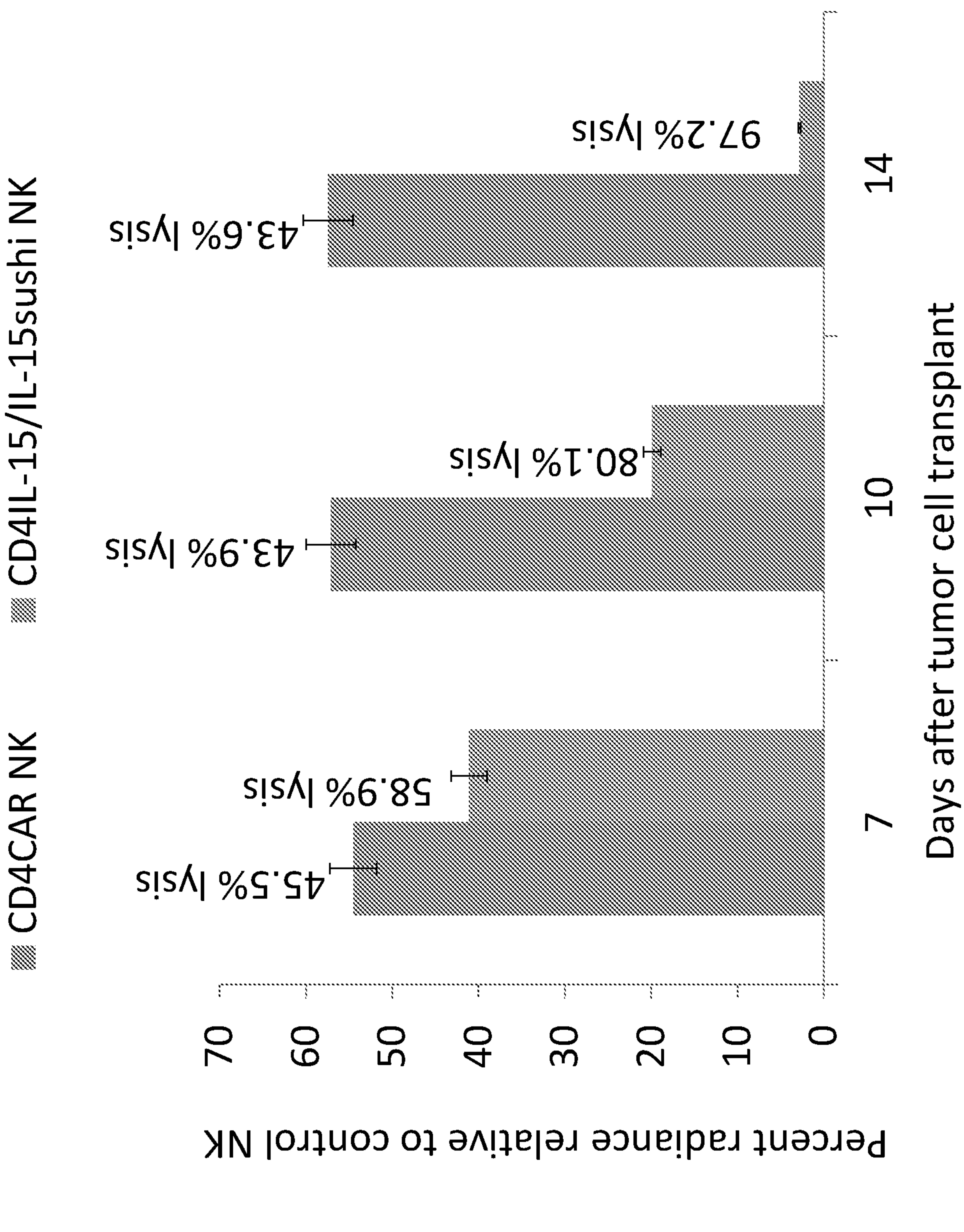

CD4CAR NK
CD4IL-15/IL-15sushi NK
Percent radiance relative to control NK
70
60
50
40
30
20
10
0
45.5% lysis
58.9% lysis
43.9% lysis
80.1% lysis
43.6% lysis
97.2% lysis
7
10
14
Days after tumor cell transplant

FIGURE 66D

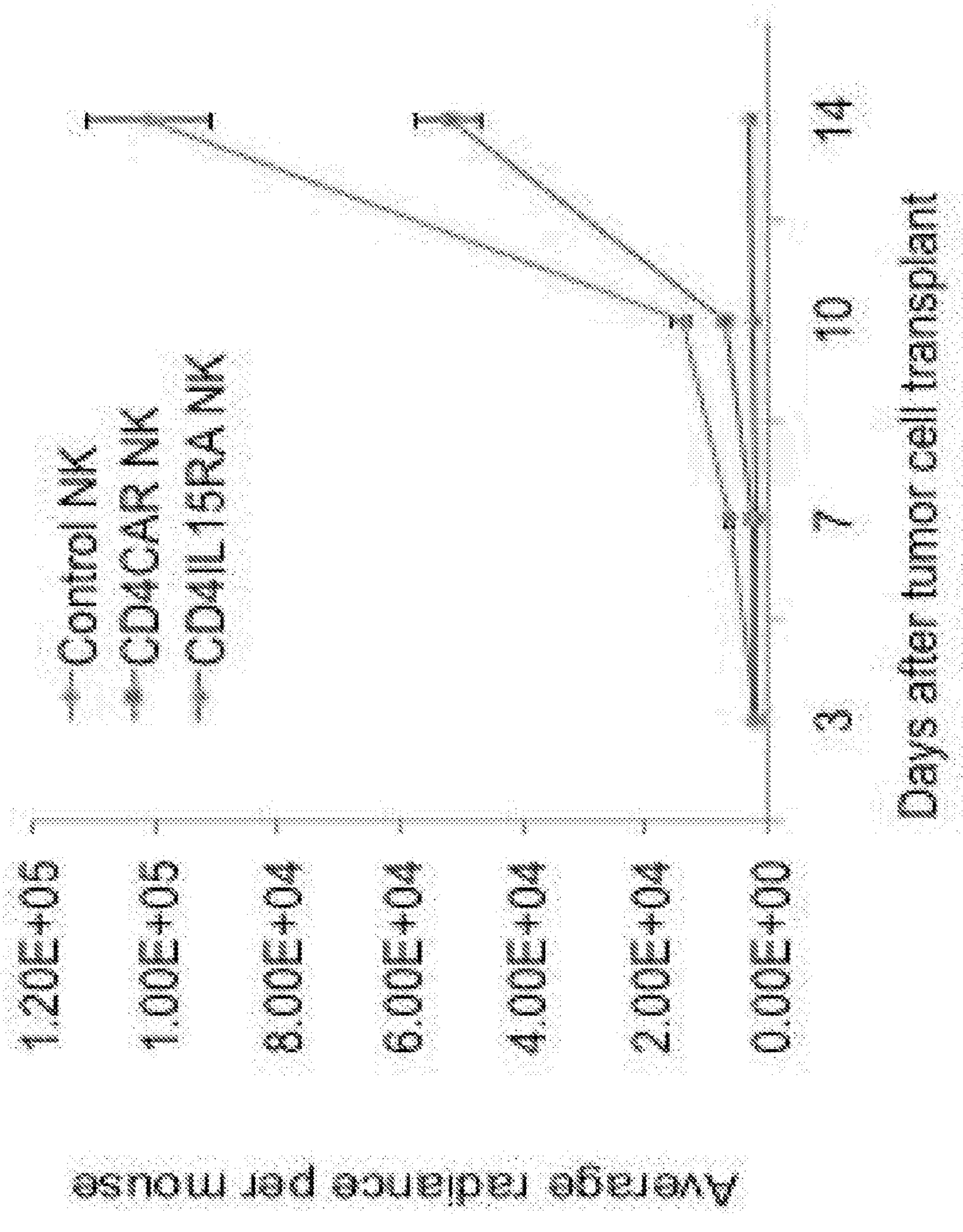
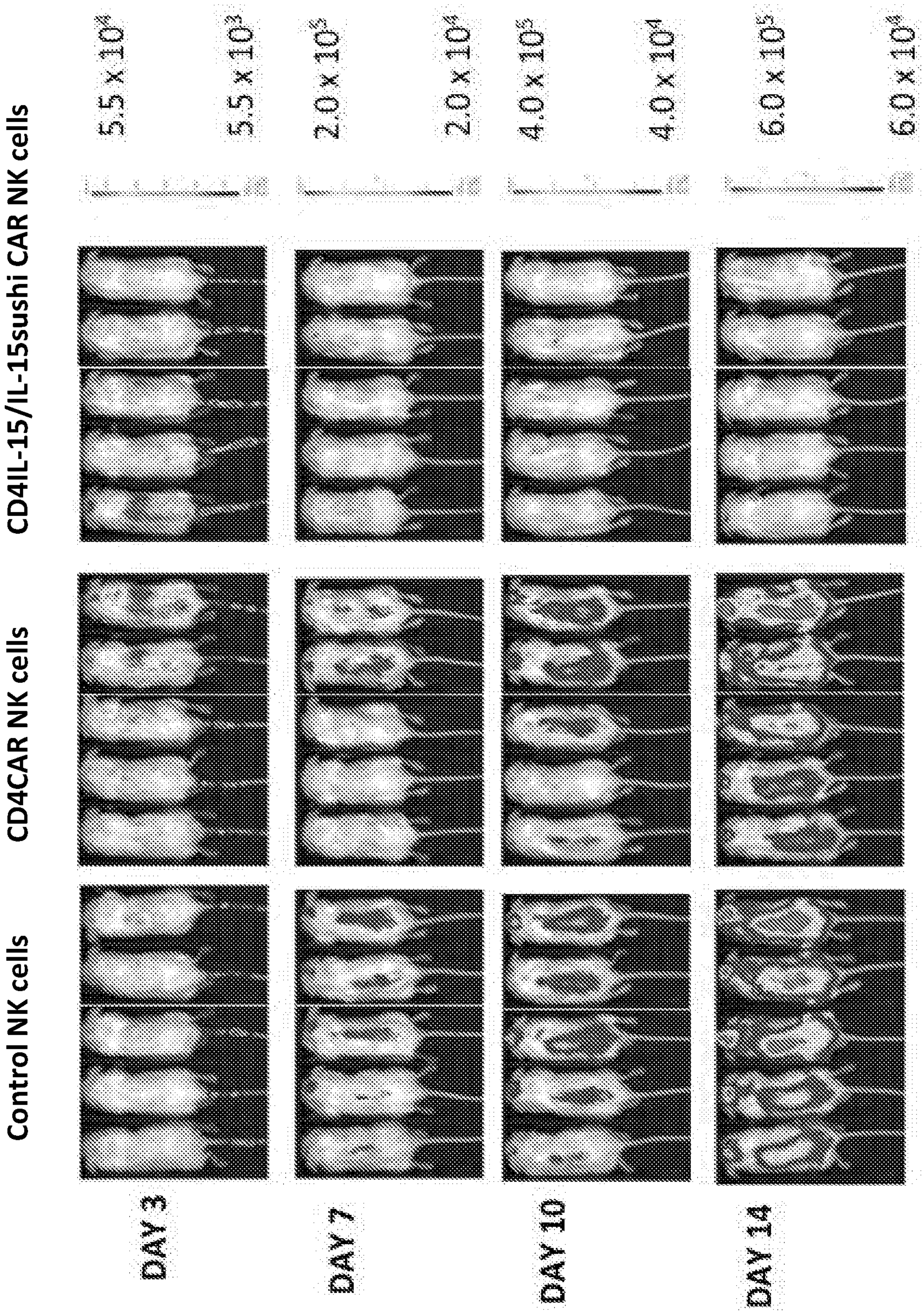

Control NK
CD4CAR NK
CD4IL15RA NK
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
Average radiance per mouse
3
7
10
14
Days after tumor cell transplant

FIGURE 67A


Control NK cells
CD4CAR NK cells
CD4IL-15/IL-15sushi CAR NK cells
DAY 3
DAY 7
DAY 10
DAY 14
5.5×10^4
5.5×10^3
2.0×10^5
2.0×10^4
4.0×10^5
4.0×10^4
6.0×10^5
6.0×10^4

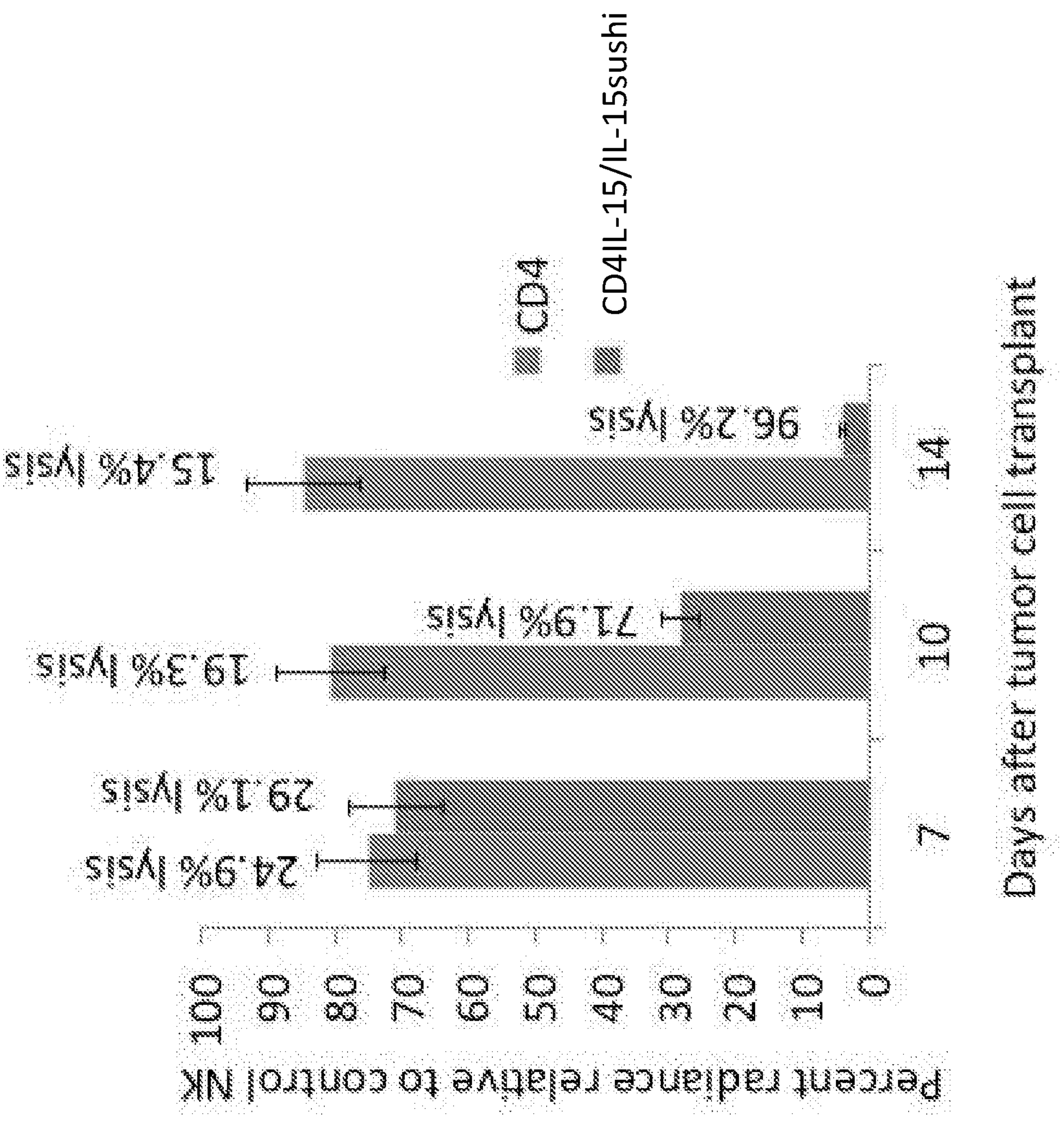

Percent radiance relative to control NK
100
90
80
70
60
50
40
30
20
10
0
24.9% lysis
29.1% lysis
19.3% lysis
71.9% lysis
15.4% lysis
96.2% lysis
7
10
14
Days after tumor cell transplant
CD4
CD4IL-15/IL-15sushi

FIGURE 67B

FIGURE 68A

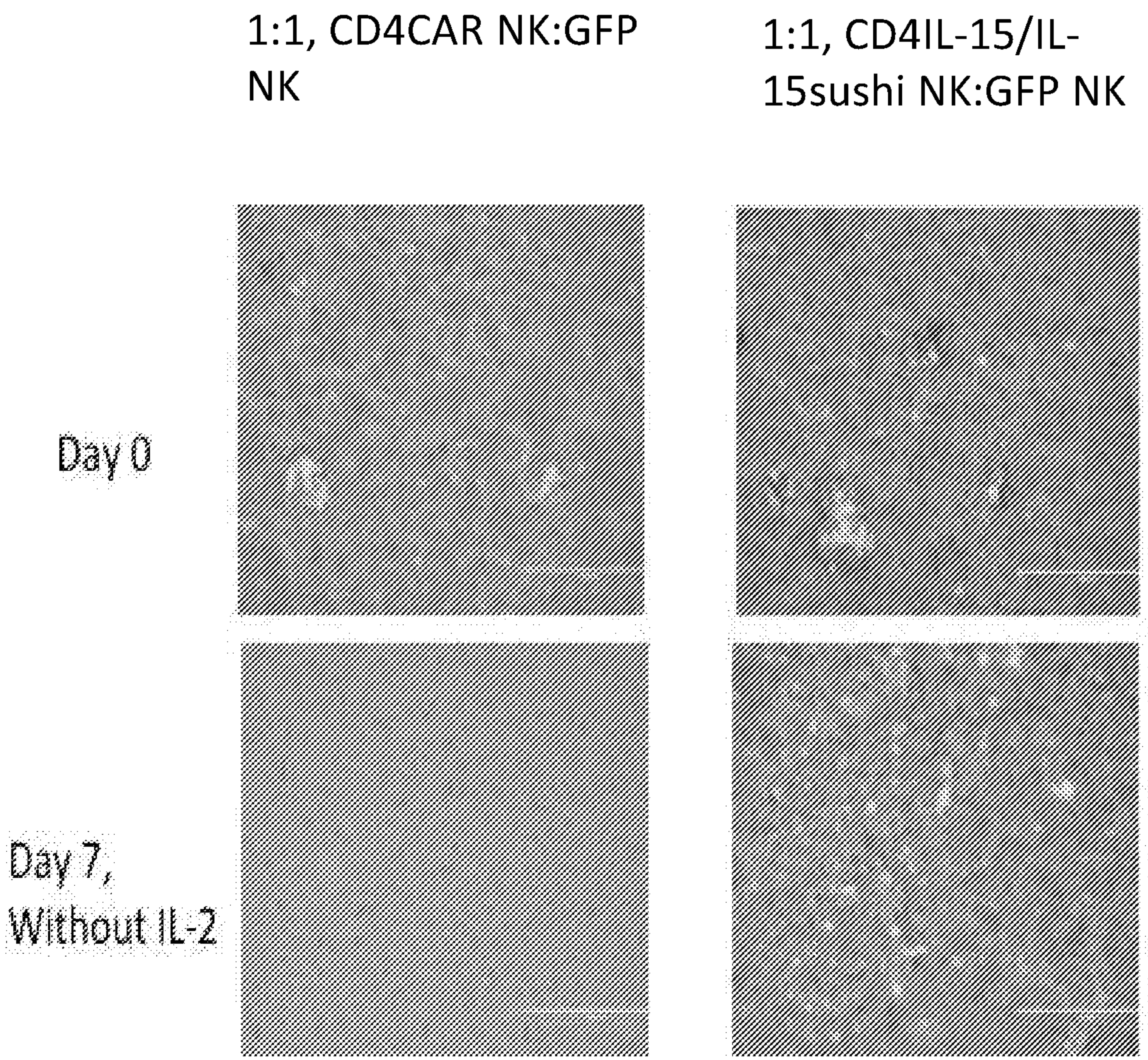

1:1, CD4CAR NK:GFP NK
1:1, CD4IL-15/IL-15sushi NK:GFP NK
Day 0
Day 7, Without IL-2

FIGURE 68B

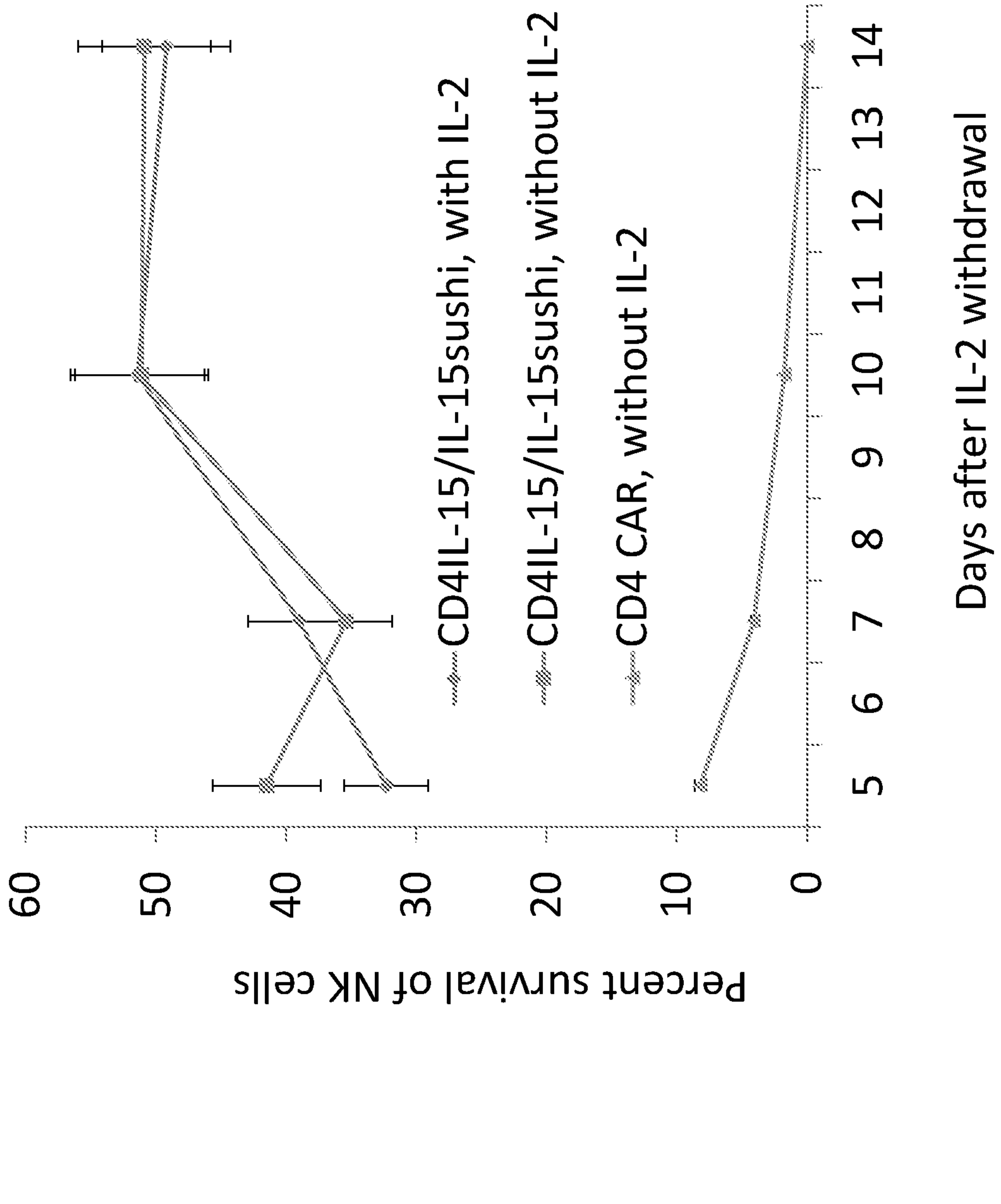

Percent survival of NK cells
60
50
40
30
20
10
0
5
6
7
8
9
10
11
12
13
14
Days after IL-2 withdrawal
CD4IL-15/IL-15sushi, with IL-2
CD4IL-15/IL-15sushi, without IL-2
CD4 CAR, without IL-2

FIGURE 69

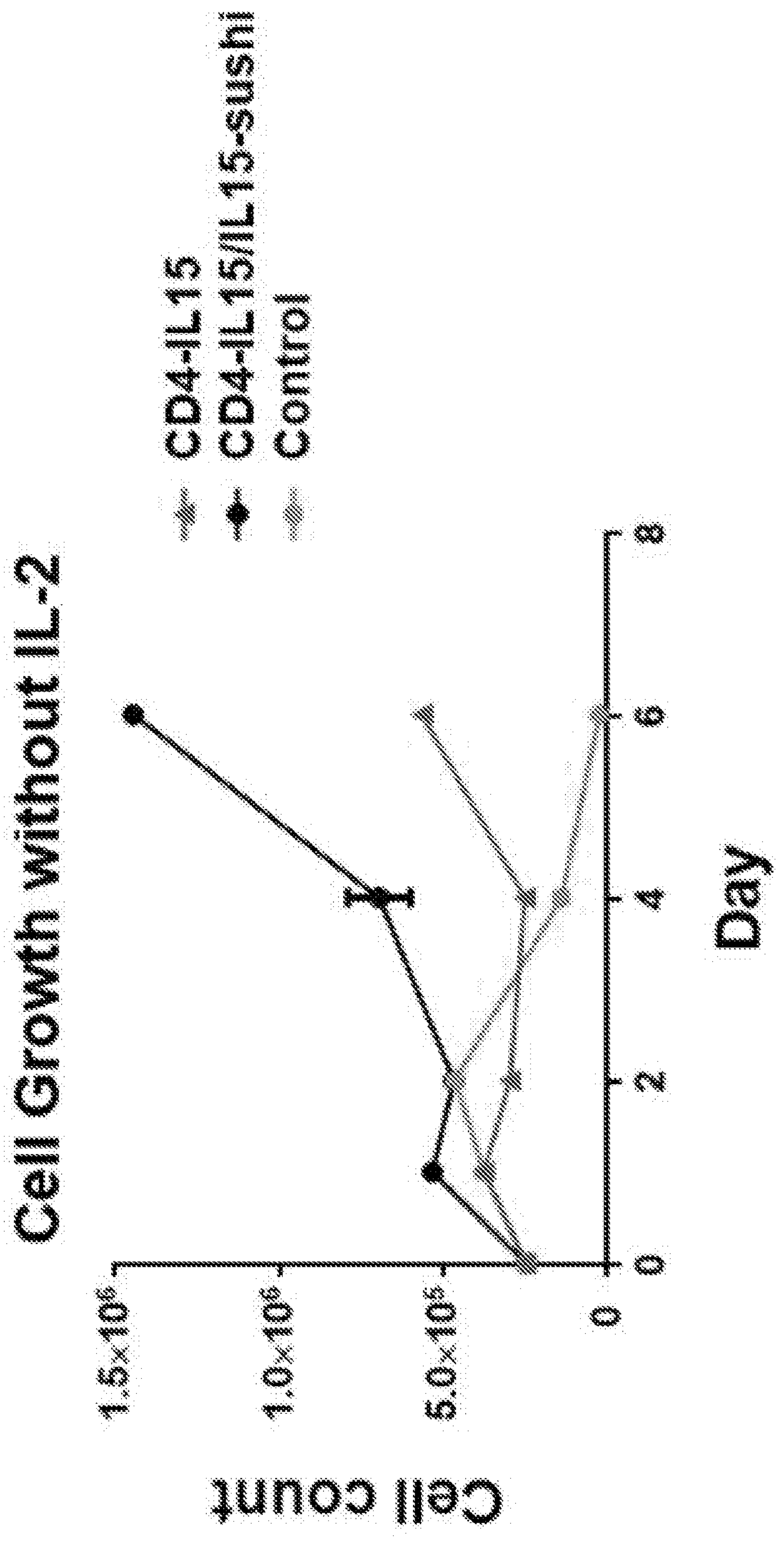

Cell Growth without IL-2
Cell count
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
0
2
4
6
8
Day
CD4-IL15
CD4-IL15/IL15-sushi
Control

FIGURE 70

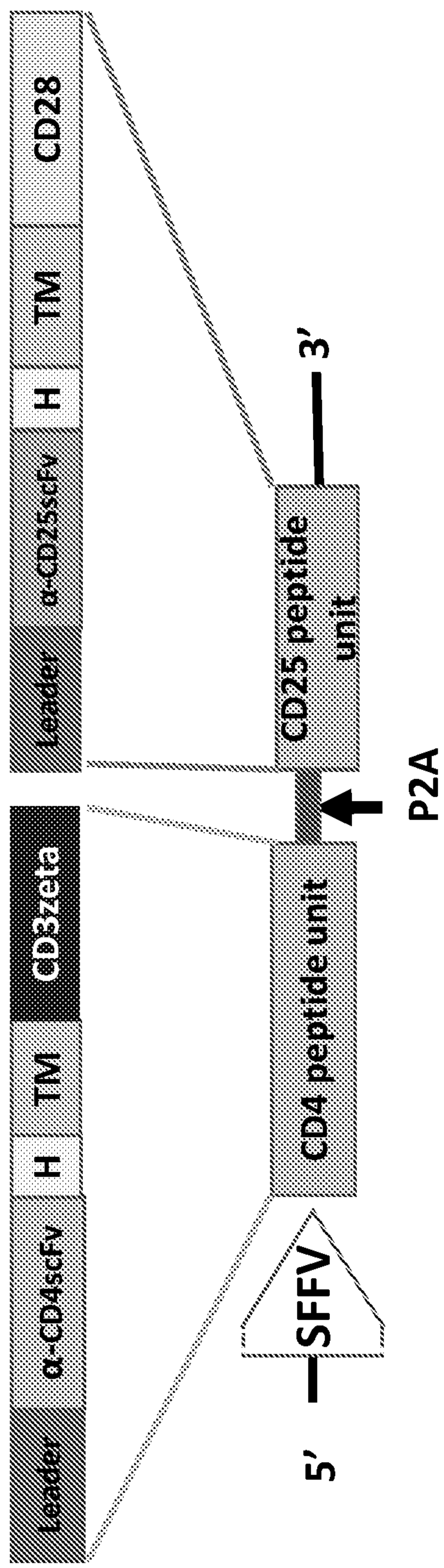

CD4
Leader
α-CD4scFv
H
TM
CD3zeta
CD25
Leader
α-CD25scFv
H
TM
CD28
5'
SFFV
CD4 peptide unit
P2A
CD25 peptide unit
3'

FIGURE 71A

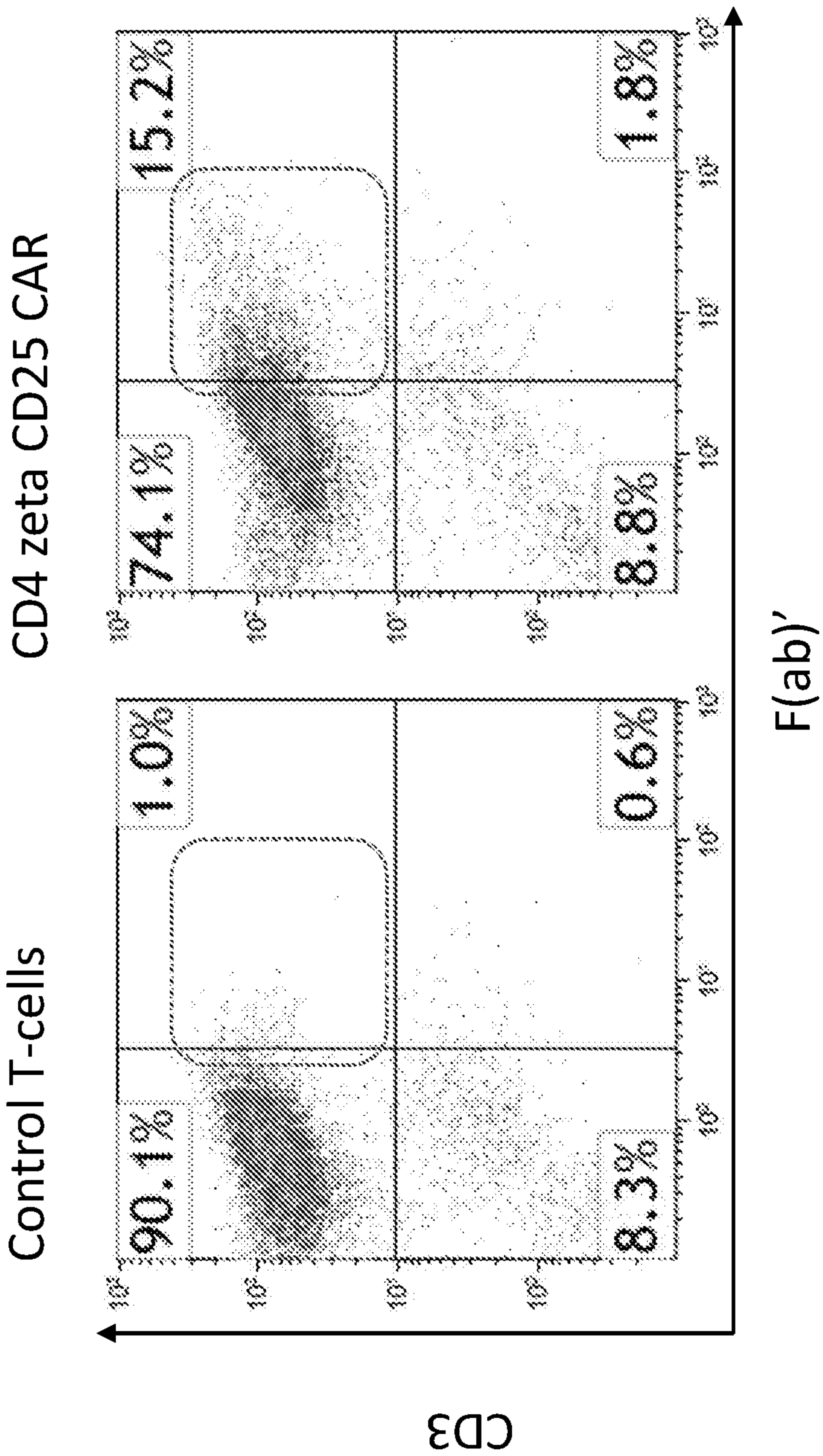
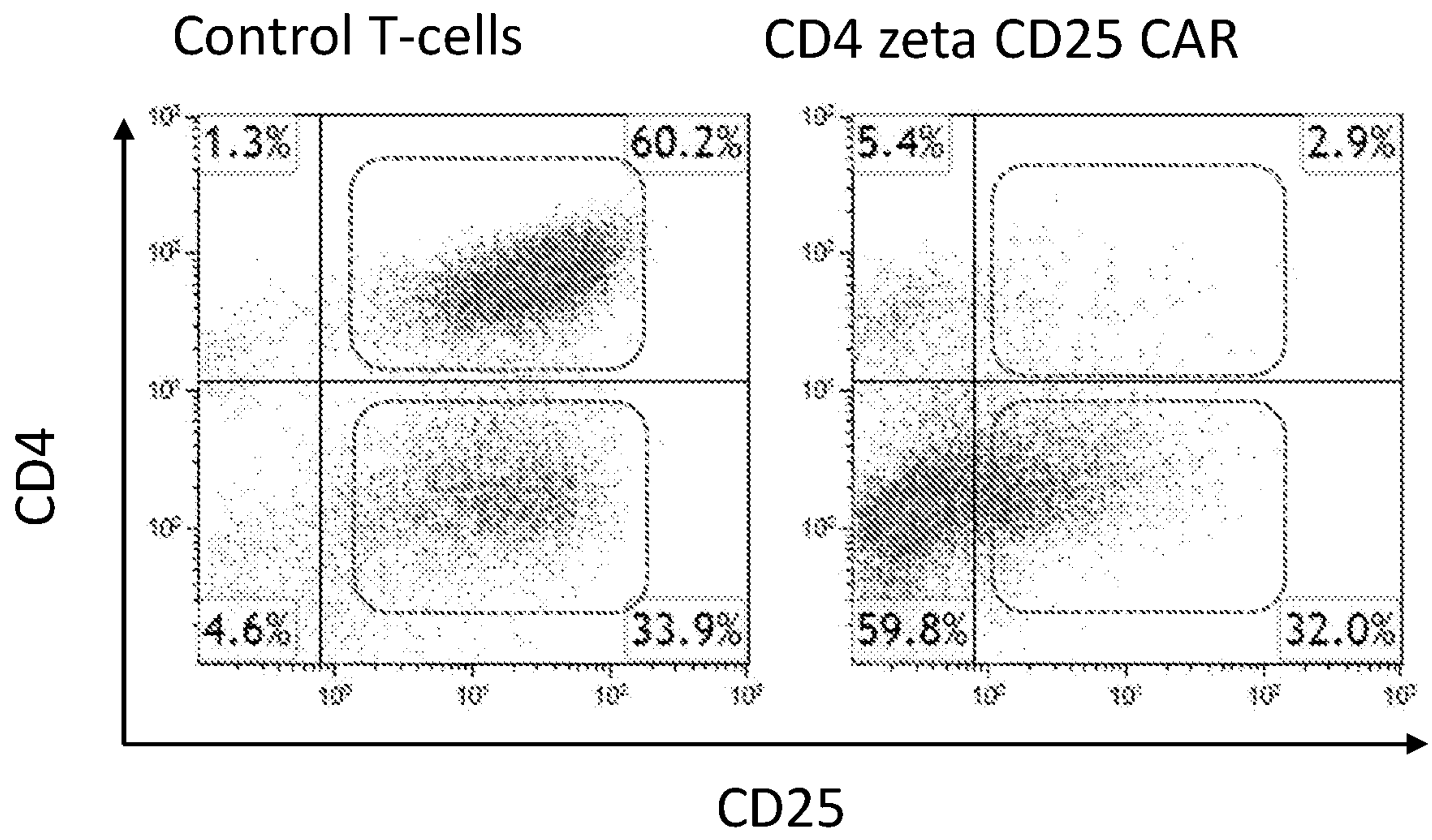
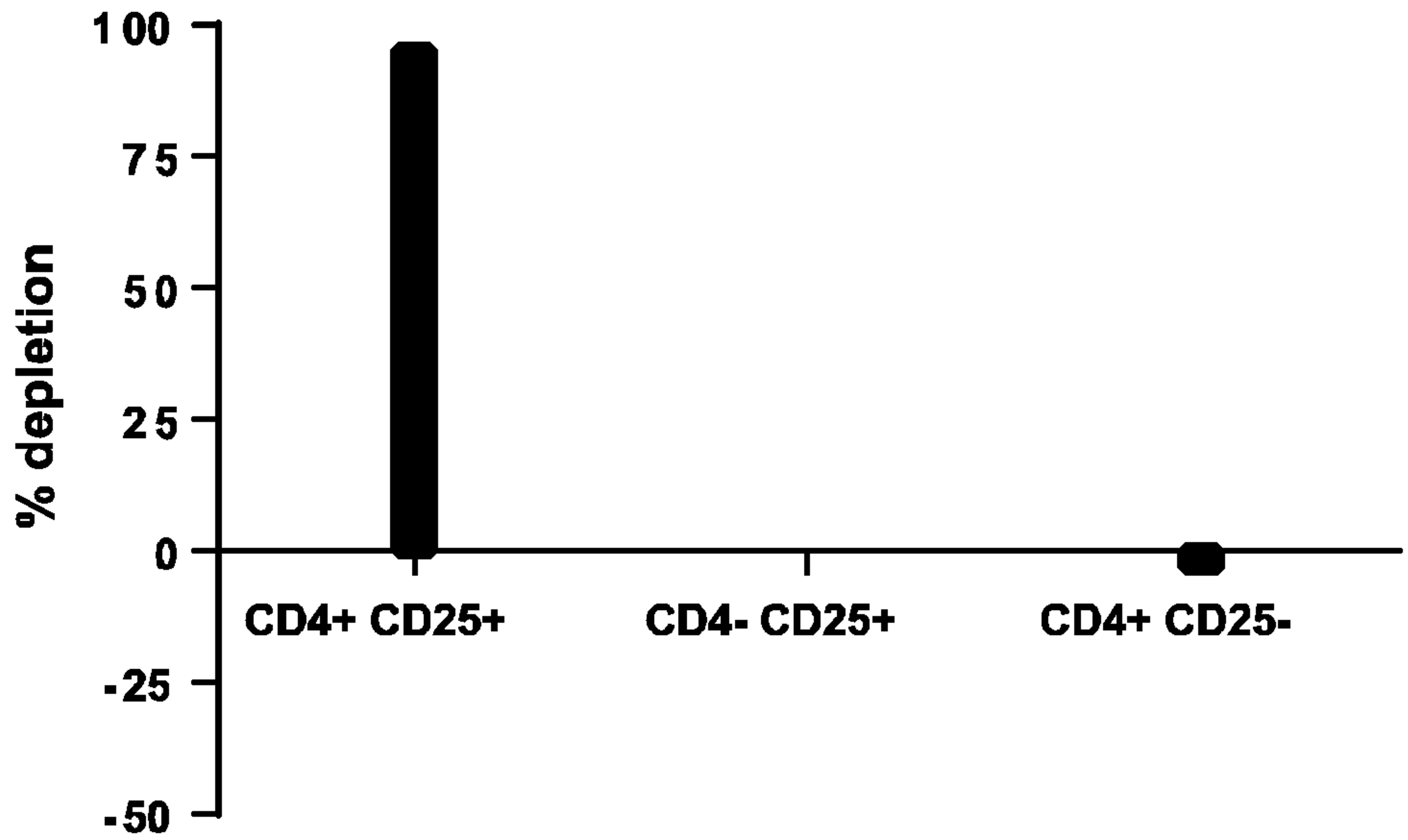

Control T-cells
CD4 zeta CD25 CAR
90.1%
1.0%
8.3%
0.6%
74.1%
15.2%
8.8%
1.8%
F(ab)'
CD3

FIGURE 71B


Control T-cells
CD4 zeta CD25 CAR
1.3%
60.2%
4.6%
33.9%
5.4%
2.9%
59.8%
32.0%
CD4
CD25


100
75
50
25
0
-25
-50
% depletion
CD4+ CD25+
CD4- CD25+
CD4+ CD25-

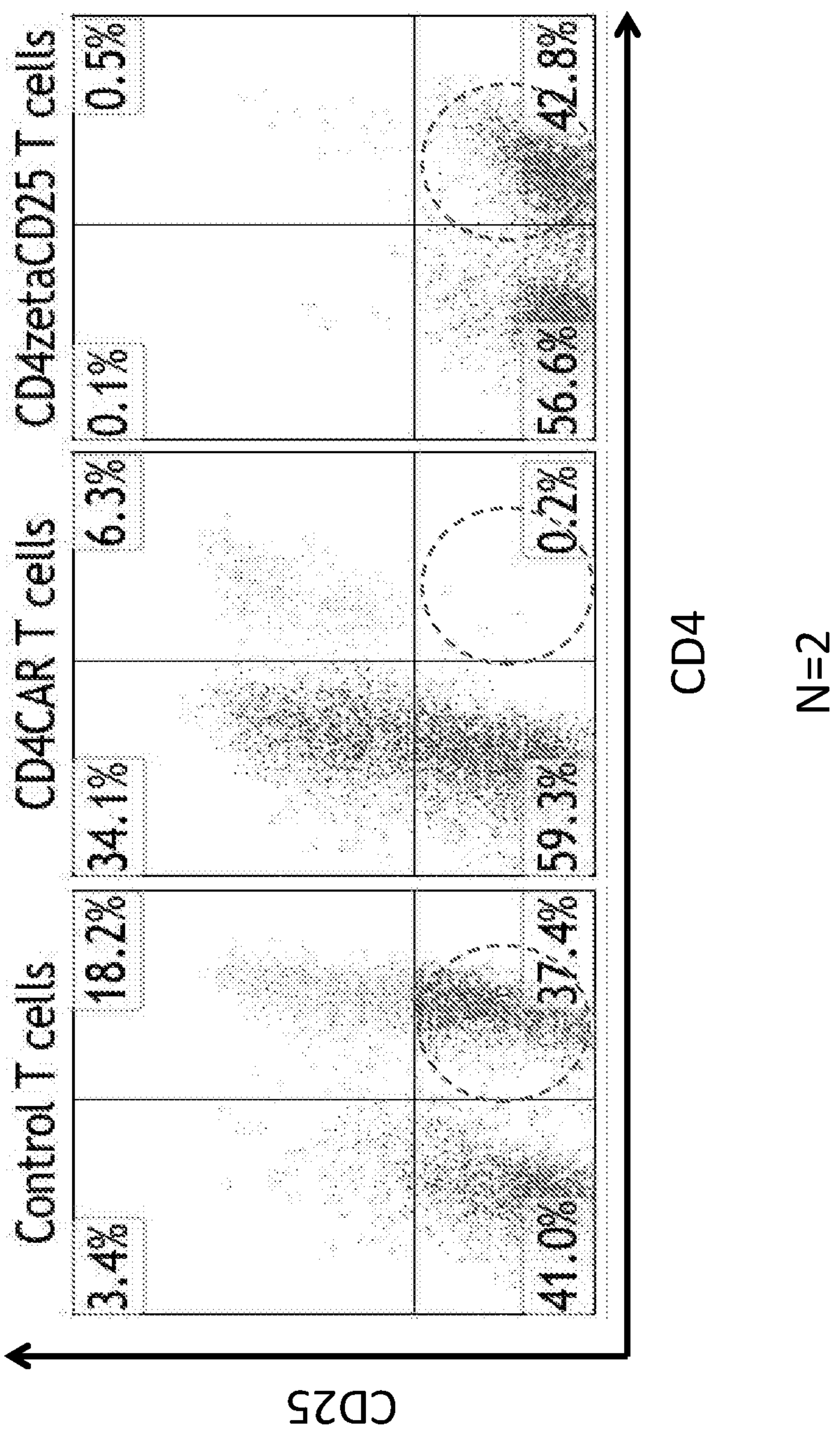

Control T cells
3.4%
18.2%
41.0%
37.4%
CD4CAR T cells
34.1%
6.3%
59.3%
0.2%
CD4zetaCD25 T cells
0.1%
0.5%
56.6%
42.8%
CD25
CD4
N=2

FIGURE 72

FIGURE 73A

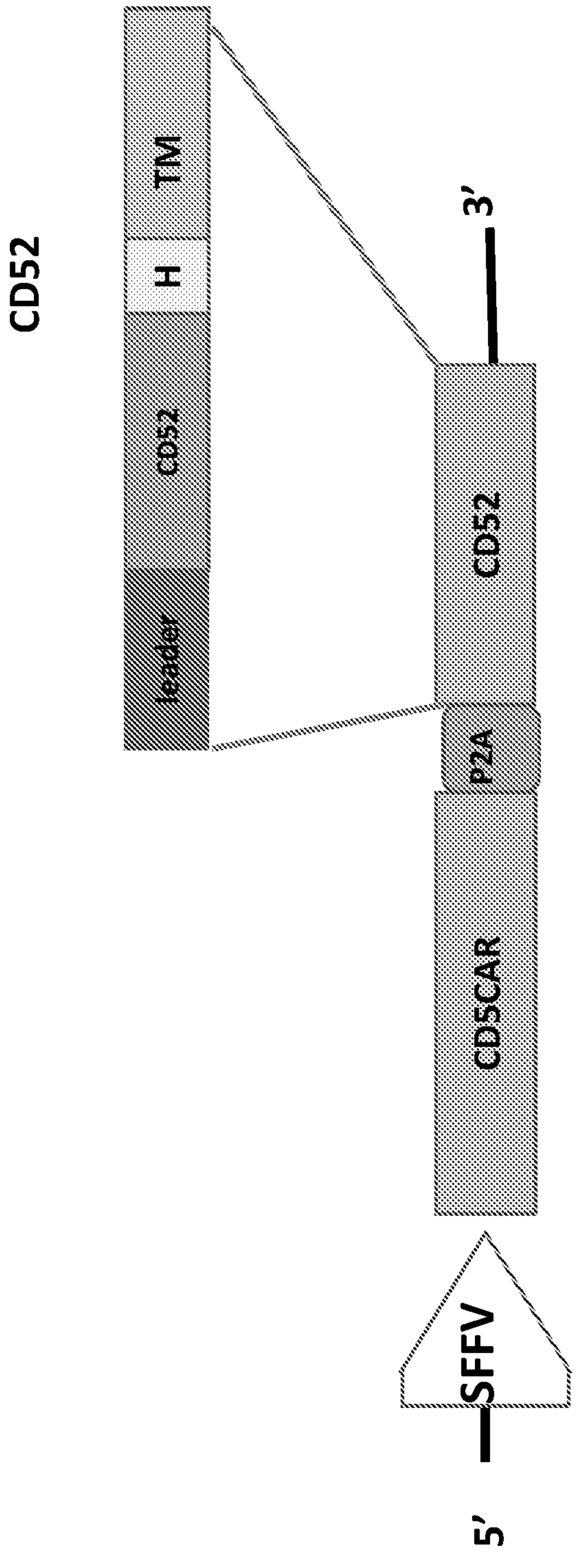


FIGURE 73B

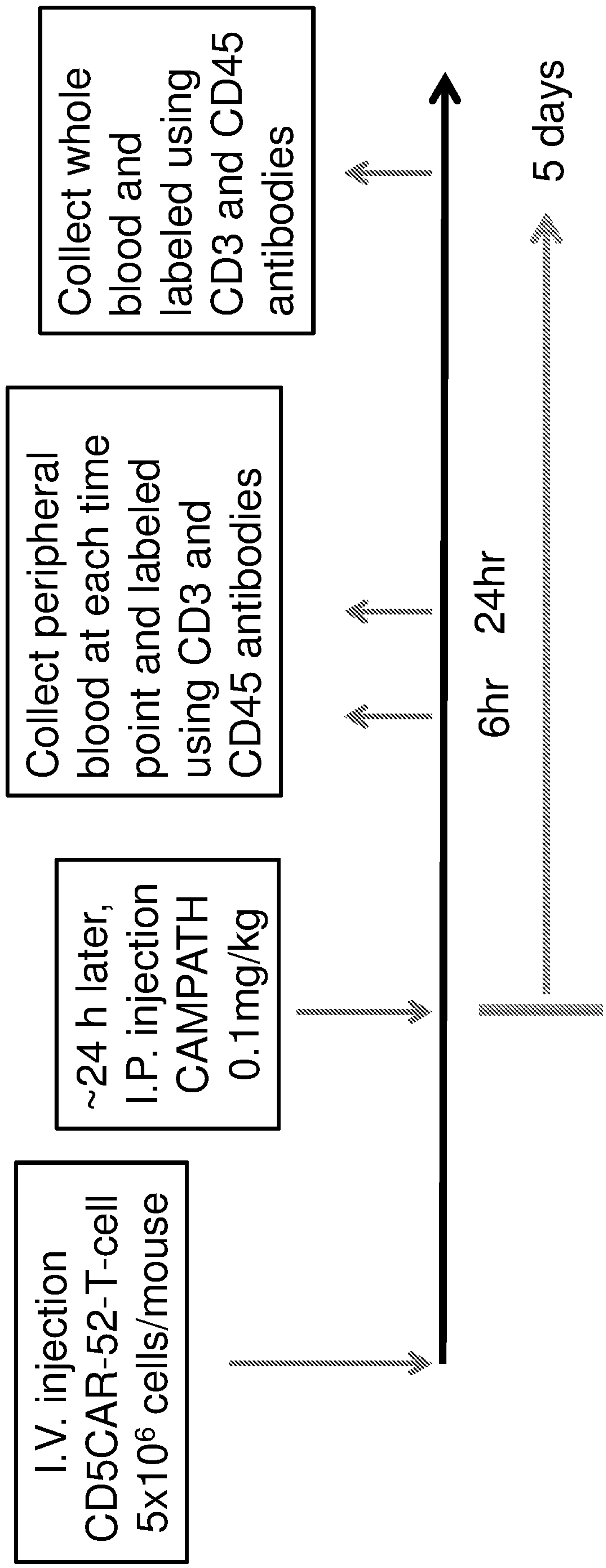

I.V. injection
CD5CAR-52-T-cell
5x10⁶ cells/mouse
~24 h later,
I.P. injection
CAMPATH
0.1mg/kg
Collect peripheral
blood at each time
point and labeled
using CD3 and
CD45 antibodies
Collect whole
blood and
labeled using
CD3 and CD45
antibodies
6hr
24hr
5 days

FIGURE 73C

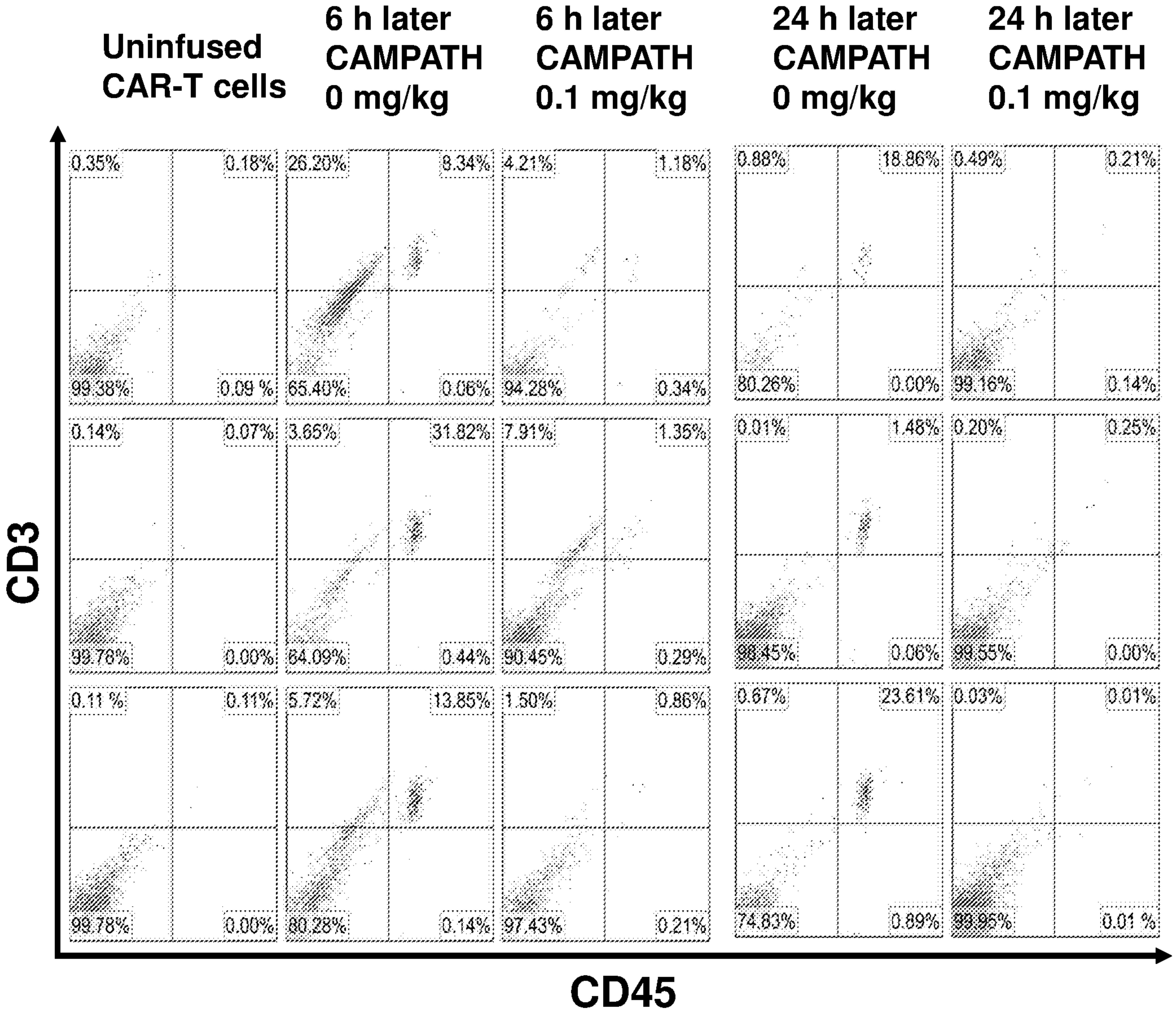

Uninfused
CAR-T cells
6 h later
CAMPATH
0 mg/kg
6 h later
CAMPATH
0.1 mg/kg
24 h later
CAMPATH
0 mg/kg
24 h later
CAMPATH
0.1 mg/kg
0.35%
0.18%
99.38%
0.09 %
26.20%
8.34%
65.40%
0.06%
4.21%
1.18%
94.28%
0.34%
0.88%
18.86%
80.26%
0.00%
0.49%
0.21%
99.16%
0.14%
0.14%
0.07%
99.76%
0.00%
3.65%
31.82%
64.09%
0.44%
7.91%
1.35%
90.45%
0.29%
0.01%
1.48%
98.45%
0.06%
0.20%
0.25%
99.55%
0.00%
0.11 %
0.11%
99.78%
0.00%
5.72%
13.85%
80.28%
0.14%
1.50%
0.86%
97.43%
0.21%
0.67%
23.61%
74.83%
0.89%
0.03%
0.01%
99.95%
0.01 %
CD3
CD45

FIGURE 73D

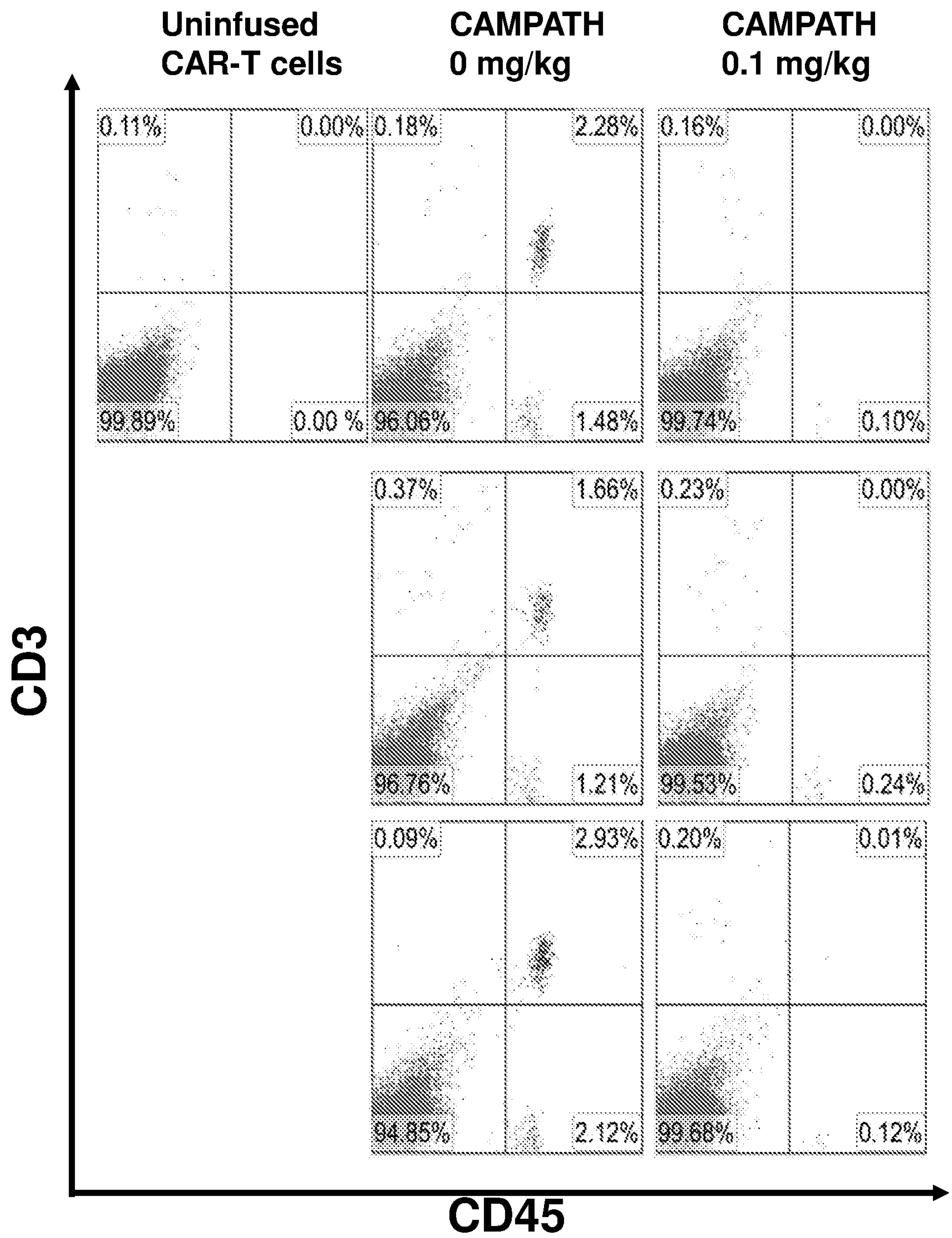

Uninfused
CAR-T cells
CAMPATH
0 mg/kg
CAMPATH
0.1 mg/kg
0.11%
0.00%
99.89%
0.00 %
0.18%
2.28%
96.06%
1.48%
0.16%
0.00%
99.74%
0.10%
0.37%
1.66%
96.76%
1.21%
0.23%
0.00%
99.53%
0.24%
0.09%
2.93%
94.85%
2.12%
0.20%
0.01%
99.68%
0.12%
CD3
CD45

FIGURE 74

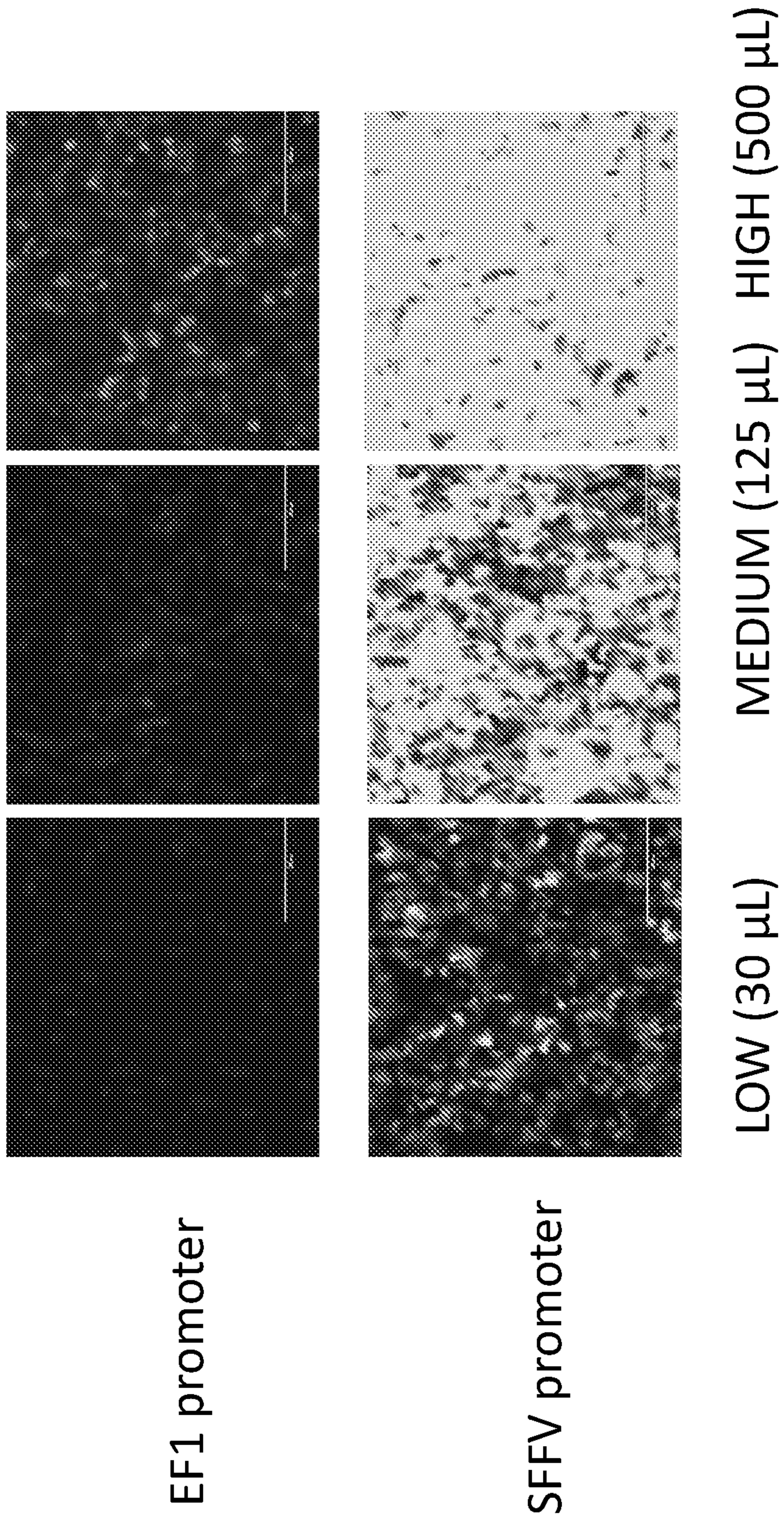

EF1 promoter
SFFV promoter
LOW (30 µL)
MEDIUM (125 µL)
HIGH (500 µL)

FIGURE 75

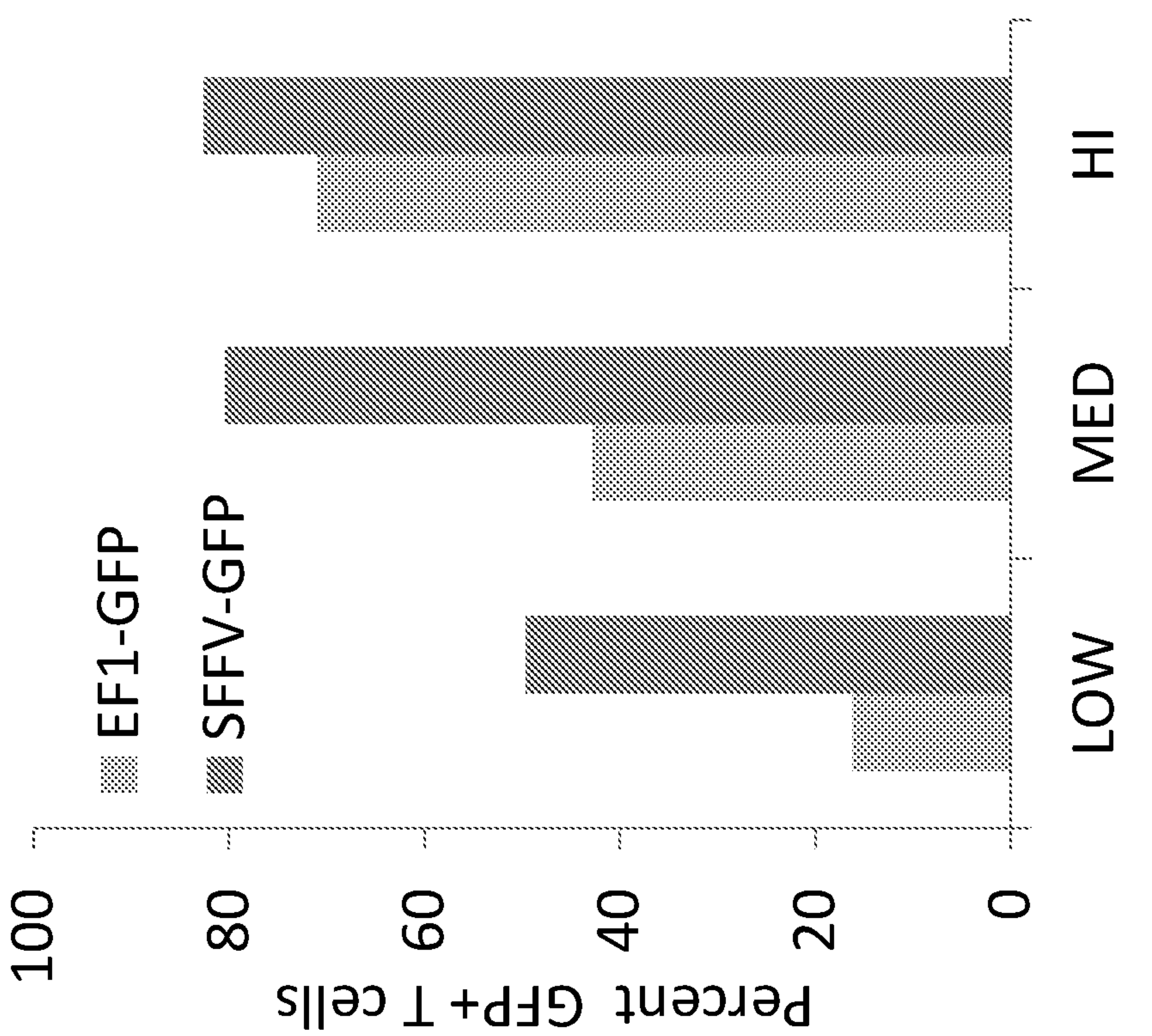


FIGURE 76A

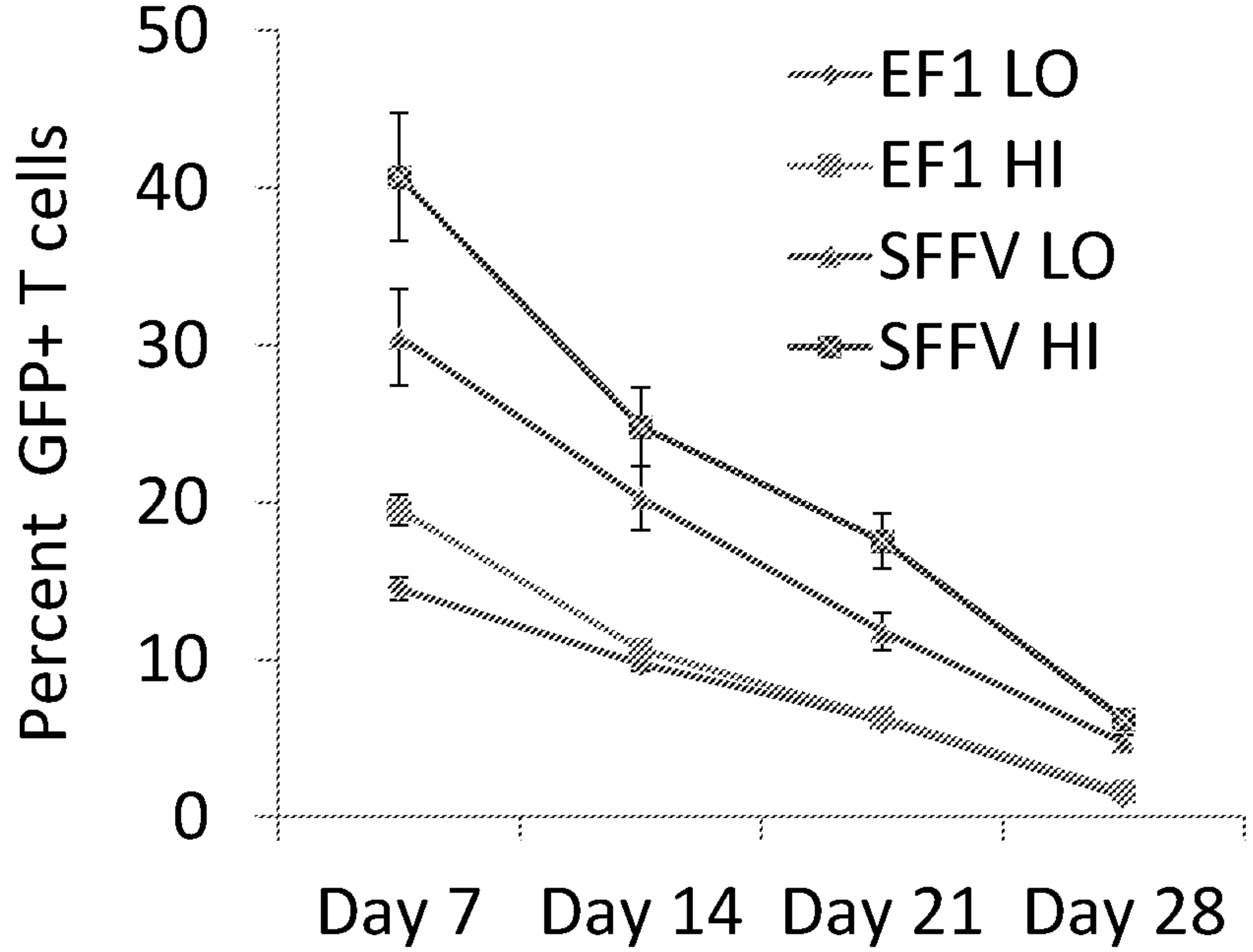


FIGURE 76B

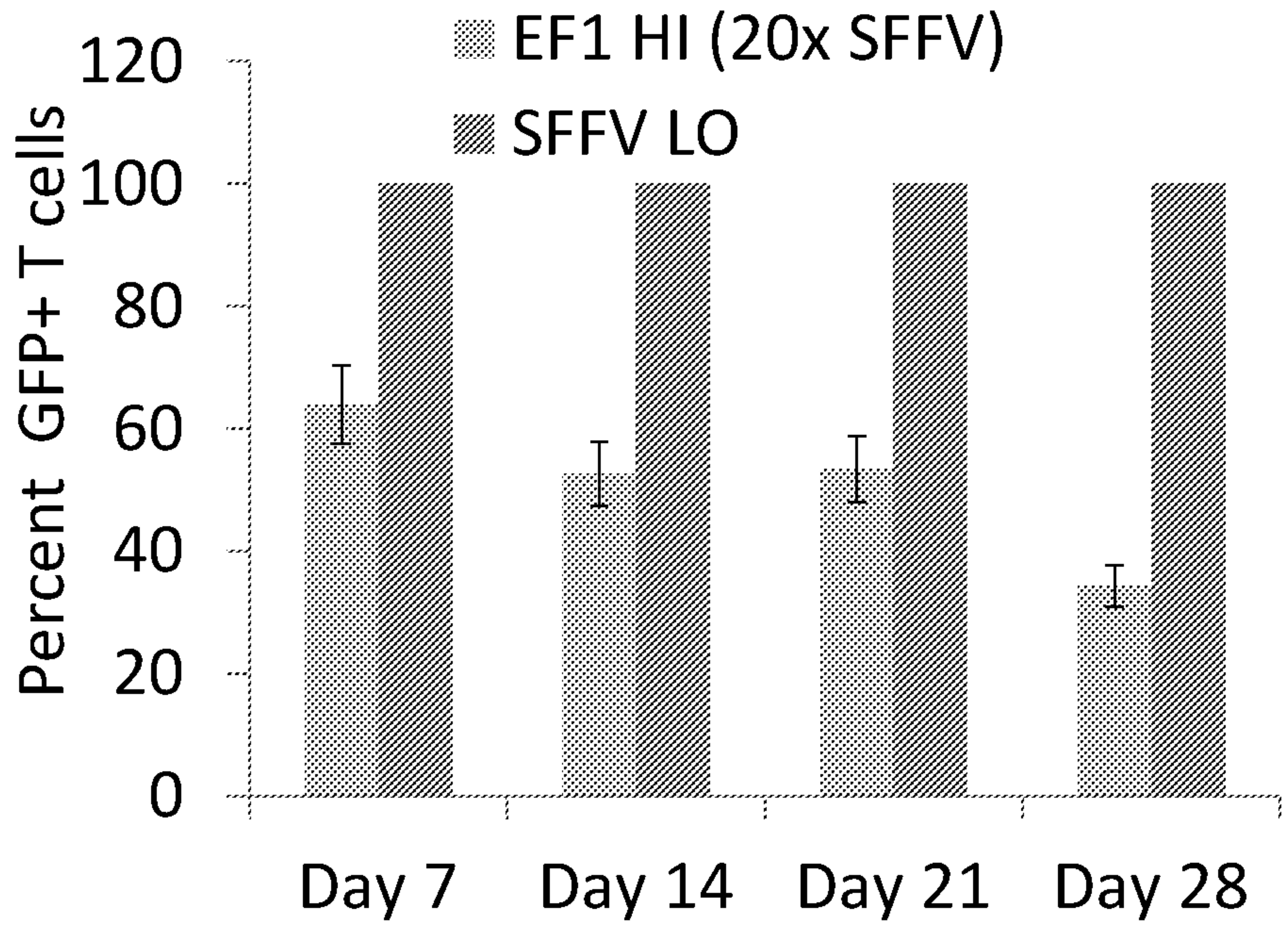
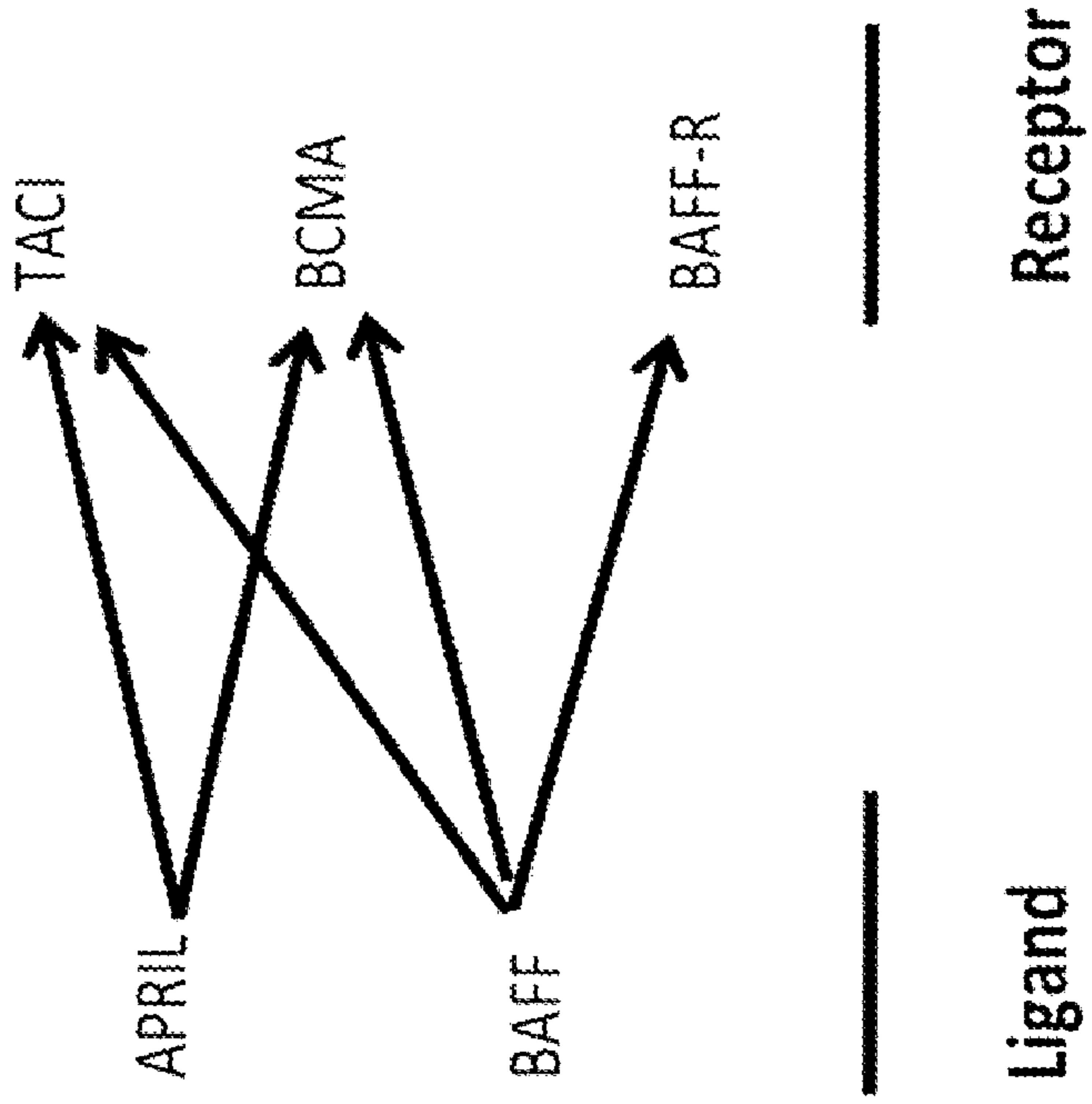


FIGURE 77



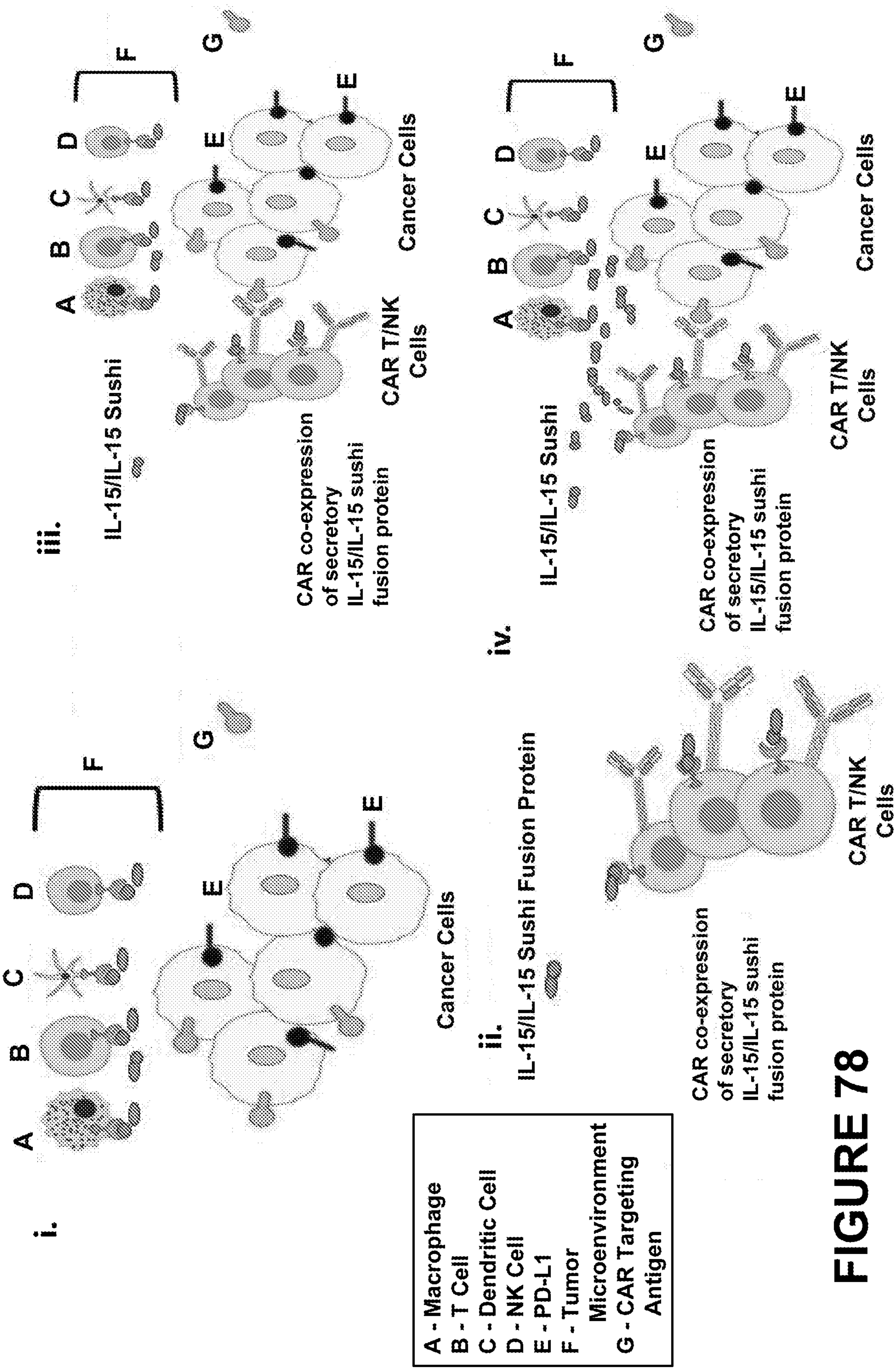

i.
A B C D
F
G
E E
Cancer Cells
A - Macrophage
B - T Cell
C - Dendritic Cell
D - NK Cell
E - PD-L1
F - Tumor Microenvironment
G - CAR Targeting Antigen
ii.
IL-15/IL-15 Sushi Fusion Protein
CAR co-expression of secretory IL-15/IL-15 sushi fusion protein
CAR T/NK Cells
iii.
IL-15/IL-15 Sushi
A B C D
F
G
E E
CAR co-expression of secretory IL-15/IL-15 sushi fusion protein
CAR T/NK Cells
Cancer Cells
iv.
IL-15/IL-15 Sushi
A B C D
F
G
E E
CAR co-expression of secretory IL-15/IL-15 sushi fusion protein
CAR T/NK Cells
Cancer Cells

FIGURE 78

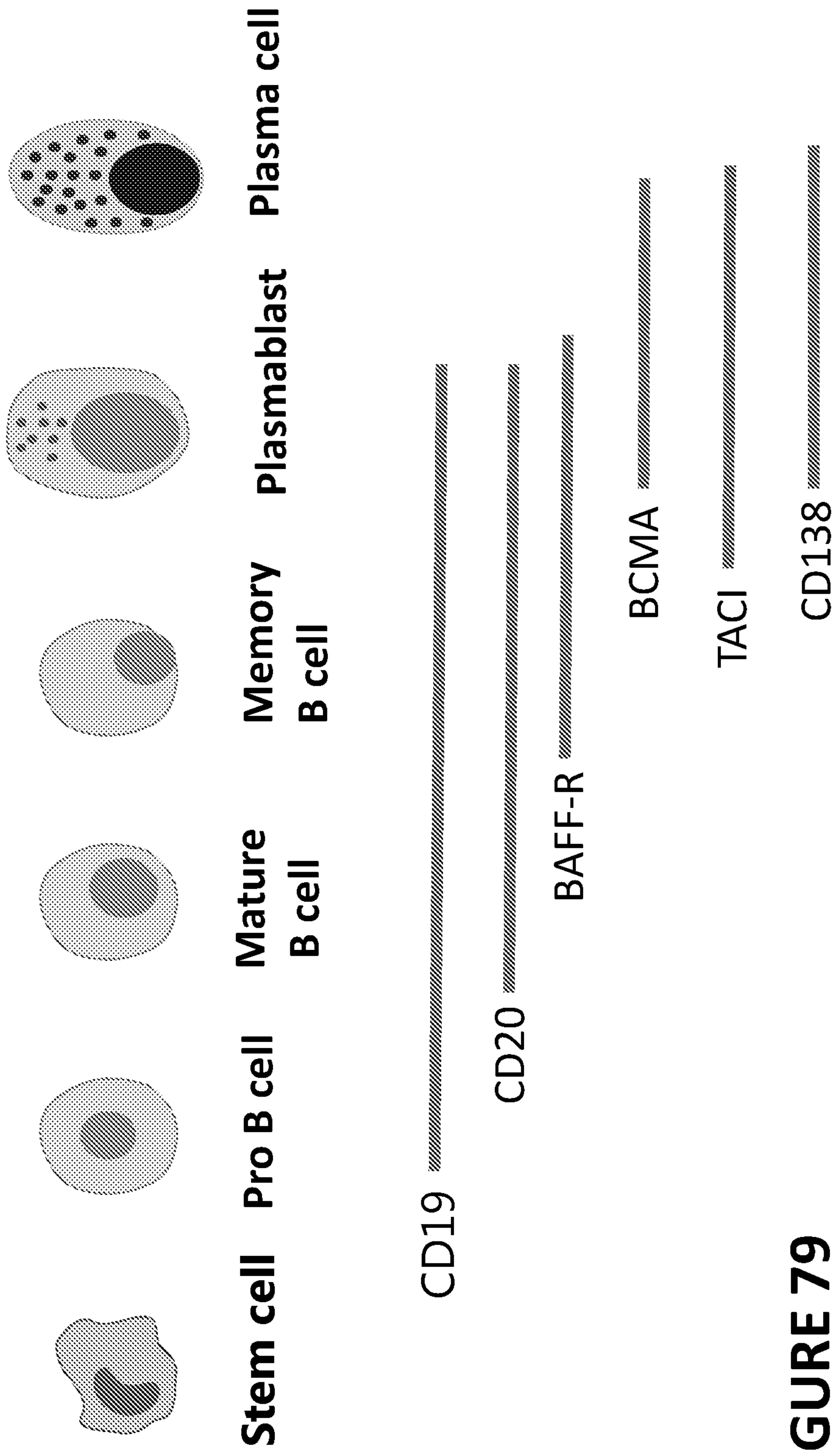

Stem cell
Pro B cell
Mature B cell
Memory B cell
Plasmablast
Plasma cell
CD19
CD20
BAFF-R
BCMA
TACI
CD138

FIGURE 79

H. sapiens (human)	MYRMQLLSCIALSLALVTNS
M. mulatta (rhesus)	MYRMQLLSCIALSLALVTNS
B. taurus (bovine)	MYKIQLLSCIALTLALVANG
O. cuniculus (rabbit)	MYKVQLLSCIALTLALLTSS
M. musculus (mouse)	MYSMQLASCVTLTLVLLVNS

FIGURE 80

```
H. sapiens (human)      1  QVAALQGDLASLRAELQGHHAEKLP----AGAGAPKAGLEEAPAVTAGLK--IFEPPAPG  54
M. mulatta (rhesus)     1  QVAALQGDLASLRAELQSHHAEKLP----ARARAPKAGLGEAPAVTAGLK--IFEPPAPG  54
B. taurus (bovine)      1  -VASLQAELGSLRAELRGRPG-----APQAGAAAPRE----APGVTFALKQRIFAPPAPG  50
O. cuniculus (rabbit)   1  RVAALQADLLSLRAAVQGHQAEQLPELPGSGAAAPKATLGEAPAVTAGLKG-IFAPAAPG  59
M. musculus (mouse)     1  QLAALQADLMNLRMELQSYRGSATP----AAAG--------APELTAGVK--LLTPAAPR  46

H. sapiens (human)     55  EGNSSQNSRNKRAVQGPEETVIQDCLQLIADSETPTIQKGSYTFVPWLLSFKRGSALEEK  114
M. mulatta (rhesus)    55  EGNSSQSSRNKRAIQGAEETVIQDCLQLIADSETPTIQKGSYTFVPWLLSFKRGSALEEK  114
B. taurus (bovine)     51  ESNSSQSGRSKRALQEAEETVTQDCLQLIADSDTPTIRKGAYTFVPWLLSFKRGRALEEK  110
O. cuniculus (rabbit)  60  ESNSSWSSRKKRAVEGVEETVIQDCLQLIADSDTPIIRKGSYTFVPWLLSFKRGRALEEK  119
M. musculus (mouse)    47  PHNSSRGHRNRRAFQGPEETVIQDCLQLIADSDTPTIRKGTYTFVPWLLSFKRGNALEEK  106

H. sapiens (human)    115  ENKILVKETGYFFIYGQVLYTDKTYAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPETLP  174
M. mulatta (rhesus)   115  ENKILVKETGYFFIYGQVLYTDKTYAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPETLP  174
B. taurus (bovine)    111  ENKILVKETGYFFIYGQVLYTDNTFAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPETLP  170
O. cuniculus (rabbit) 120  ENKIVVKETGYFFIYGQVLYTDSTFAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPETLP  179
M. musculus (mouse)   107  ENKIVVRQTGYFFIYSQVLYTDPIFAMGHVIQRKKVHVFGDELSLVTLFRCIQNMPKTLP  166

H. sapiens (human)    175  NNSCYSAGIAKLEEGDELQLAIPRENAQISLDGDVTFFGALKLL  218
M. mulatta (rhesus)   175  NNSCYSAGIAKLEEGDELQLAIPRENAQISLDGDVTFFGALKLL  218
B. taurus (bovine)    171  NNSCYSAGIAKLEEGDELQLAIPREDAKISRDGDGTFFGALKLL  214
O. cuniculus (rabbit) 180  NNSCYSAGIAKLEEGDELQLAIPRENAQISRDGDGTFFGALKLL  223
M. musculus (mouse)   167  NNSCYSAGIARLEEGDETQLAIPRENAQISRNGDDTFFGALKLL  210
```

FIGURE 81


What is a "compound" CAR (cCAR)?
Cell Type 1
Target 1+
Cell Type 2
Target 2+
A
B
C
D
E
F
G
H
J
I
I
K
L
M
N
O
O
P
synergistic CAR cell activation
cytokine release
CAR cell proliferation
multiple target cell killing
CAR Cell
A - Anti-Target 1
B - Anti-Target 2
C - F(ab)'
D - F(ab)"
E - Monoclonal Antibody 1
F - Monoclonal Antibody 2
G - scFv
H - Hinge
I - Binding
J - Target 1
K - Target 2
L - Transmembrane Region
M - Co-stimulatory Domain 1
N - Co-stimulatory Domain 2
O - CD3ζ
P - 2 CAR Proteins Expressed Simultaneously

FIGURE 1