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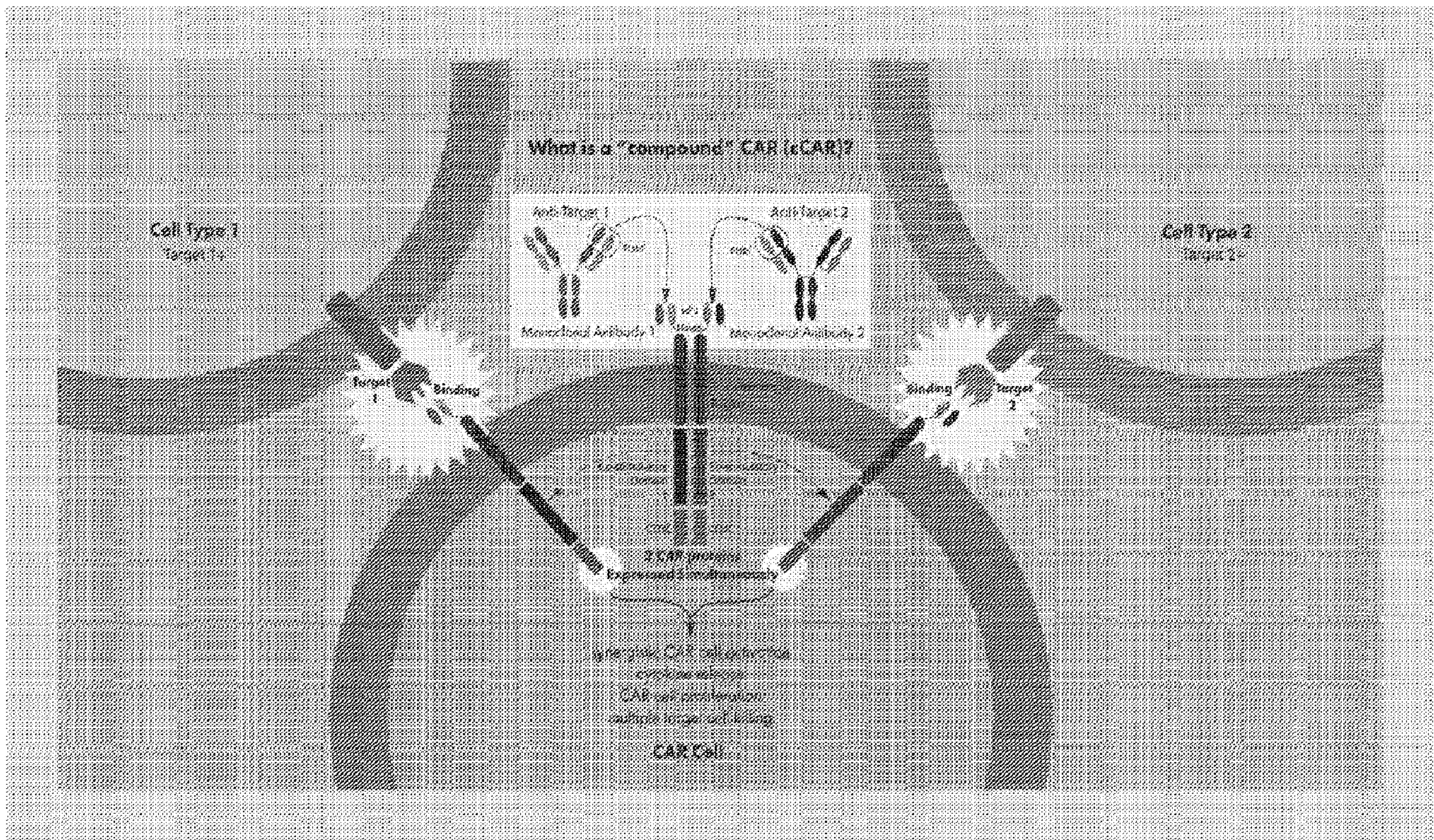
(71) Demandeur/Applicant:
ICELL GENE THERAPEUTICS LLC, US

(72) Inventeurs/Inventors:
MA, YUPO, US;
PINZ, KEVIN, US;
JIANG, XUN, US;
WADA, MASAYUKI, US;
CHEN, KEVIN, US...

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : RECEPTEURS D'ANTIGENES CHIMERIQUES, COMPOSITIONS ET METHODES ASSOCIEES
(54) Title: CHIMERIC ANTIGEN RECEPTORS (CARs), COMPOSITIONS AND METHODS THEREOF

FIG. 1



(57) **Abrégé/Abstract:**

The present invention relates to compositions and methods relating to chimeric antigen receptor (CAR) polypeptides and methods relating thereto. In one embodiment, the present invention relates to engineered cells having chimeric antigen receptor

(57) **Abrégé(suite)/Abstract(continued):**

polypeptides directed to at least two targets. In another embodiment, the present invention relates to engineered cells having chimeric antigen receptor polypeptides and an enhancer moiety.

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(71) Applicant: ICELL GENE THERAPEUTICS LLC
[US/US]; 25 Health Science Drive, Stony Brook, NY
11794 (US).

(72) Inventors; and

(71) Applicants : MA, Yupo [US/US]; Icell Gene Therapeutics LLC, 25 Health Science Drive, Stony Brook, NY 11794 (US). PINZ, Kevin [US/US]; Icell Gene Therapeutics LLC, 25 Health Science Drive, Stony Brook, NY 11794 (US). JIANG, Xun [US/US]; Icell Gene Therapeutics LLC, 25 Health Science Drive, Stony Brook, NY 11794 (US). WADA, Masayuki [US/US]; Icell Gene Therapeutics LLC, 25 Health Science Drive, Stony Brook, NY 11794 (US). CHEN, Kevin [US/US]; Icell Gene Therapeutics LLC, 25 Health Science Drive, Stony Brook, NY 11794 (US).

(74) Agents: HARRINGTON, James .F et al.; Hoffmann & Baron, LLP, 6900 Jericho Turnpike, Syosset, NY 11791 (US).

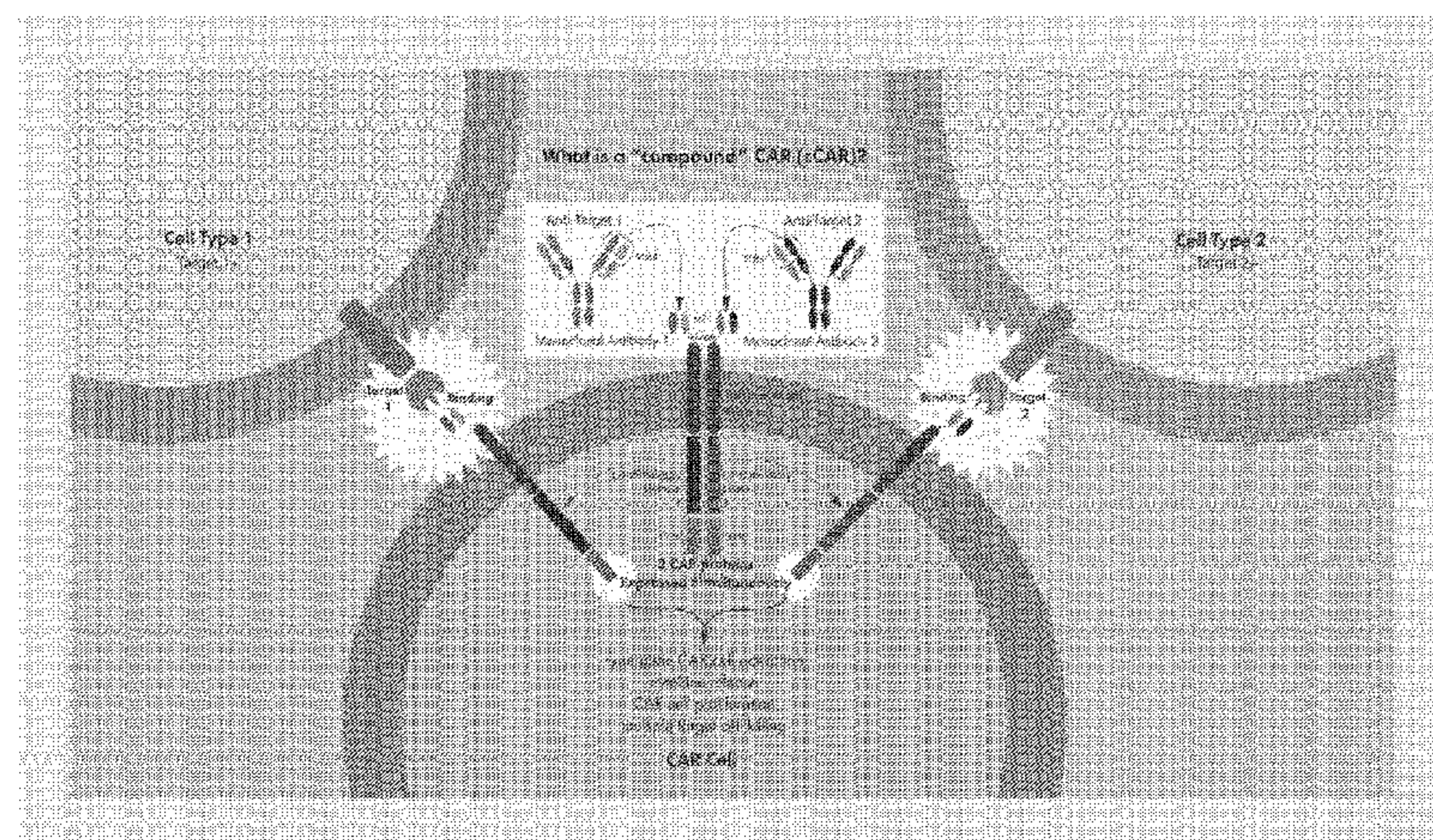
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(54) Title: CHIMERIC ANTIGEN RECEPTORS (CARs), COMPOSITIONS AND METHODS OF USE THEREOF

FIG. 1



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

5 This application is an International PCT Application claiming priority from US Provisional Application Nos. 62/184,321, filed June 25, 2015; 62/235,840, filed on October 1, 2015; and 62/244, 435, filed October 21, 2015 all of which are incorporated herein by reference in its entirety.

BACKGROUND

10 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
15 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”
20 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.

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.

5 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.

10 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
15 cells having multiple chimeric antigen receptor polypeptides, each chimeric antigen receptor polypeptides are 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, BCMA, TACI, LeY, CD5, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD41,
20 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.

5

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-

5 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).

Figure 4. A co-culture assay representing the incubation of CD33CD123-2G CAR-T cells
10 (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
15 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. A co-culture assay representing incubation of cCAR-T cells with AML patient
20 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. 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 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 13. 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.

Figures 14 A-C. BCMA-CS1 cCAR construct scheme (BC1cCAR). (A) 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 CS1. Two unit CARs use same co-stimulatory domain, 4-1BB. (B) Flow cytometry analysis of BC1cCAR expression on T cell surface for vector (left) and BC1cCAR (right, highlighted by a square) showing 15.3% positive for F(Ab)2. Gating done against isotype controls. (C) Preliminary functional validation of BC1cCAR-T cells by co-culturing K562 cells transduced with BCMA cDNA (BCMA-K562) (obtained from Kochenderfer, NIH). Bar graph shows lysis of the BCMA-K562 cell line vs. control T-cells as well as lysis of wild-type K562 (wt-K562) vs. control.

Figure 14D. BCMA-CS1-2G construct using two different co-stimulatory domains either 4-1BB or CD28 for each unit. The construct includes 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 targets expressing BCMA and /or CS1. Two unit CARs use a different co-stimulatory domain, either 4-1BB or CD28. Flow cytometry analysis of BC1cCAR expression on T cell surface for vector (left) and BC1cCAR (right, highlighted by a square) showing rare positive cells for F(Ab)2. Gating done against isotype controls.

Figure 14E. 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), CD269-CS1-2G (lane 3) 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.

Figures 15A-B. MM1S cell line co-culture. Co-cultures were carried out under 24 hours and collected and analyzed via flow cytometry. Target MM1S cells (myeloma cells) were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. Figure 15A: Flow cytometry depictions of co-cultures. Figure 15B: right: graphical summary of lysis vs. E:T ratio.

Figures 16A-B. RPMI-8226 cell line co-culture. Co-cultures were carried out under 24 hours and collected and analyzed via flow cytometry. Target RPMI-8226 cells were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. Figure 16A: flow cytometry depictions of co-cultures. Figure 16B: graphical summary of lysis vs. E:T ratio.

Figures 17 A-B. U266 cell line co-culture. Co-cultures were carried out under 24 hours and collected, and analyzed via flow cytometry. Target U266 cells were labeled with Cytotracker

(CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. (A) flow cytometry depictions of co-cultures. (B) graphical summary of lysis vs. E:T ratio.

Figures 18A-B. MM10-G primary patient sample co-culture and specific lysis. Co-cultures were carried out under 24 hours and collected and analyzed via flow cytometry. Target MM10-G cells were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. Notably, gating shows MM10-G presenting with distinct BCMA⁺ and CS1⁺ populations. Figure 18A: flow cytometry depictions of co-cultures. Figure 18B: graphical summary of lysis vs. E:T ratio.

Figures 19A-B. MM7-G primary patient sample co-culture and specific lysis. Co-cultures were carried out under 24 hours and collected and analyzed via flow cytometry. Target MM7-G cells were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. Figure 19A: flow cytometry depictions of co-cultures. Figure 19B: graphical summary of lysis vs. E:T ratio.

Figures 20A-B. MM11-G primary patient sample co-culture and specific lysis. Co-cultures were carried out under 24 hours and collected and analyzed via flow cytometry. Target MM11-G cells were labeled with Cytotracker (CMTMR) dye to distinguish it from effector T-cells. Populations were gated by anti-BCMA (CD269) and anti-CS1 (CD319) antibodies. Figure 20A: flow cytometry depictions of co-cultures. Figure 20B: graphical summary of lysis vs. E:T ratio.

Figure 21. CD269-CS1-BBCAR NK cells demonstrate anti-leukemic effects *in vivo*.

NSG mice were sublethally irradiated and intravenously injected the following day with luciferase-expressing MM.1S multiple myeloma cells to induce measurable tumor formation.

After 3 days, the mice were intravenously injected with 8×10^6 CD269-CS1-BBCAR NK cells

5 or vector control NK control cells. On days 3, 6, and 8, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Average light intensity measured for the CD269-CS1-BBCAR NK injected mice was compared to that of vector control NK injected mice.

Figure 22. Percent survival of mice was measured and compared between the two groups

10 based on the studies from Figure 21.

Figure 23. 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 24. A schematic providing an example of the steps for generation of CAR T or

15 NK cell targeting hematologic malignancies.

Figure 25. 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 transduction of sgCD45B CRISPR into NK-92 cells, transduced cells were cultured in medium containing puromycin for a few weeks. CD45 negative

20 NK-92 cells were determined using CD45 antibody and were sorted. The purity of stable NK⁴⁵ⁱ-92 (CD45 knockdown) NK-92 cells were determined by Flow cytometry analysis (right panel). This data showed that NK⁴⁵ⁱ-92 cells were successfully generated and obtained.

Figure 26. Cell growth curve of wild type, GFP transduced NK-92 or NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-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.

Figures 27 A-B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92 or GFP NK⁴⁵ⁱ-92 cells (effector: E), 5:1 (E:T) ratio. 16 hours incubation. (A) 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 NK⁴⁵ⁱ-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. All of incubation time were 16 h and the ratio of effector T-cells: target cell was 5:1. All experiments were performed in duplicate. (B) Bar graph indicates the percent of cell lysis by the GFP transduced NK⁴⁵ⁱ-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. Blue dots are in the upper left quadrant.

Figures 28 A-B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92, CD5CAR NK-92 or CD5CAR NK⁴⁵ⁱ-92 cells (effector: E). 5:1 (E:T) ratio. 16 hours incubation (A) 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 NK⁴⁵ⁱ-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 times were 16 h and the ratio of effector T-cells: target cell is 5:1. All experiments were performed in duplicate. (B) Bar graph indicates the percent of cell lysis by the CD5CAR NK-92 cells or CD5CAR NK⁴⁵ⁱ-92 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 NK⁴⁵ⁱ-92 cells shows near to 100 % cell killing activity against CD5-positive CCRF-CEM compared to control GFP NK-92 cells. These data suggest that CD5CAR NK-cells and CD5CAR NK⁴⁵ⁱ-92 cells can effectively lyse CCRF-CEM cells that express CD5 compared to GFP-control NK-92 cells in vitro co-culture assay, and provide proof that knockdown of CD45 does not affect cell function for killing activity in NK-92 cells. Blue dots are in the upper left quadrant of the first two panels starting from the left.

Figures 29A-B. Organization of the CD45CAR construct and its expression. (A) 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. (B), 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.

Figures 30 A-B. Transduction of CD45CAR into NK⁴⁵ⁱ-92 cells and cell sorting of CD45CAR transduced cells. (A) The expression levels of CD45CAR on NK⁴⁵ⁱ-92 were determined by flow cytometry analysis (circled in blue at middle panel) compared to NK⁴⁵ⁱ-92

cells (left panel) after CD45CAR lentiviruses were transduced into NK⁴⁵ⁱ-92 cells. CD45CAR expressed NK⁴⁵ⁱ-92 cells were sorted and CD45 expression levels on cell surface were determined by Flow cytometry analysis (right panel). (B) About 87% of CD45CAR expression on cell surface was detected by flow cytometry analysis.

5 Figures 31A-B. Co-culture assay with CCRF-CEM (target: T) and GFP NK-92 or CD45CAR NK⁴⁵ⁱ-92 cells (effector: E). 5:1 (E:T) ratio. 16 hours incubation. (A) Flow cytometry analysis of in co-culture with CCRF-CEM and control GFP transduced NK-92 cells (left panel) or CD45CAR NK⁴⁵ⁱ-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.

10 All of incubation times were 16 h and the ratio of effector T-cells: target cell is 5:1. All experiments were performed in duplicate. (B) Bar graph indicates the percent of cell lysis by CD45CAR NK⁴⁵ⁱ-92 cells compared to the control GFP NK92 cells in co-culture assay with CCRF-CEM. Data are mean $\pm$ S.D. CD45CAR NK⁴⁵ⁱ-92 cells shows about 70% cell lysis against CCRF-CEM cells compared to control GFP NK-92 cells. These data suggest that CD45CAR

15 NK⁴⁵ⁱ-92 cells effectively lyse CCRF-CEM cells that express CD45 compared to GFP-control NK-92 cells in vitro co-culture assay.

 Figures 32 A-C. Co-culture assay with Jurkat cells (target: T) and GFP-control or CD45CAR NK⁴⁵ⁱ-92 cells (effector: E). 5:1 or 2:1 (E:T) ratio. 6 hours incubation. (A) Flow cytometry analysis was carried out after Jurkat cells were stained by CMTMR cell tracker dye.

20 These data shows that Jurkat cells are CD45 positive (left panels) and mostly CD56 negative cells (right panel). (B) Flow cytometry analysis of co-culture assay with Jurkat cells (target: T) and control or CD45CAR NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-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. (C) Bar graph shows percent cell lysis by CD45CAR NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-92 cells effectively lyse Jurkat cells that express CD45 on cell surface compared to GFP-control NK-92 cells in vitro co-culture assay.

Figures 33A-C. Co-culture assay with GFP-NK-92 cells (target: T) and non-transduced NK-92 cells or CD45CAR NK⁴⁵ⁱ-92 cells (effector: E). 5:1 or 2:1 (E:T) ratio. 6 hours incubation (A) 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). (B) Flow cytometry analysis of co-culture assay with GFP control NK-92 cells (target: T) and non-transduced or CD45CAR NK⁴⁵ⁱ-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 non-transduced or CD45CAR NK⁴⁵ⁱ-92 cells in 5:1 (E:T) ratio and right panels indicated that in co-culture with non-transduced or CD45CAR NK⁴⁵ⁱ-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. The incubation time was 6 h. All experiments were performed in duplicate. (C) Bar graph shows percent cell lysis of GFP NK-92 cells by CD45CAR NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-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. Green dots are in the upper right quadrant of each panel.

Figures 33D-E. Transduction of CD45b-BB or CD45b-28 into NK⁴⁵ⁱ-92 cells and cell sorting of CD45b-BB or CD45b-28 transduced NK⁴⁵ⁱ-92 cells. (D) The surface expression levels of CD45b-BB CAR or CD45b-28 CAR on NK⁴⁵ⁱ-92 were determined by flow cytometry analysis (circled in blue at middle panel) compared to NK⁴⁵ⁱ-92 cells (left panel) after CD45b-BB or CD45b-28 lentiviruses transduced into NK⁴⁵ⁱ-92 cells. (E) NK⁴⁵ⁱ-92 cells expressing the CD45b-BB or CD45b-28 CAR were sorted by Flow cytometry analysis. About 74% of CD45b-BB CAR or 82% of CD45b-28 CAR expression on cell surface was detected by flow cytometry analysis.

Figures 33 F-G. Co-culture assay with REH cells (target: T) and GFP NK-92 cells or CD45CAR NK⁴⁵ⁱ-92 cells or CD45b-BB NK⁴⁵ⁱ-92 cells or CD45b-28 NK⁴⁵ⁱ-92 cells (effector: E). 5:1 (E:T) ratio. 20 hours incubation. (F) Flow cytometry analysis of REH cells only (left panel), in co-culture with REH cells and control GFP transduced NK-92 cells (2nd left panel), CD45CAR NK⁴⁵ⁱ-92 cells (middle panel), CD45b-BB NK⁴⁵ⁱ-92 cells (4th from left panel) or CD45b-28 NK⁴⁵ⁱ-92 cells (right panel). Blue dots in all of panels indicate the leftover target REH cells and red dots shows effector GFP or CARs-NK-92 cells by co-culture assay. REH is a B acute lymphoblastic cell line. All of incubation times were 20h and the ratio of effector NK-cells: target cell is 5:1. All experiments were performed in duplicate. (G) Bar graph indicates the percent of cell lysis by CD45CAR NK⁴⁵ⁱ-92 cells, CD45b-BB NK⁴⁵ⁱ-92 cells or CD45b-28 NK⁴⁵ⁱ-92 cells compared to the control GFP NK92 cells in co-culture assay with REH cells. Data are mean + S.D. CD45CAR NK⁴⁵ⁱ-92 cells shows about 76% cell lysis, CD45b-BB NK⁴⁵ⁱ-

92 cells shows about 79% cell lysis and CD45b-28 NK⁴⁵ⁱ-92 shows 100% cell lysis against REH cells compared to control GFP NK-92 cells. These data suggest that all three CD45CARs effectively lyse REH cells.

Figures 34 A-B. Schematic diagram to elucidate the construct and its expression in T or NK cells. (A) a combination of CAR, (third generation), sushi/IL-15 is assembled on an expression vector and their expression is driven by the SFFV promoter. CAR with sushi/IL-15 is linked with the P2A cleaving sequence. The sushi/IL-15 portion is composed of IL-2 signal peptide fused to sushi domain and linked to IL-5 via a 26-amino acid poly-proline linker. (B) CAR and sushi/IL15 are present on the T or NK cells.

Figures 35 A-B. CD4IL15RA-CAR expression. (A) HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1) and CD4IL15RA CAR (lane 2), and positive control, CD4CAR (lane 3). 48 hours after transfection, supernatant was removed, and cells were also removed for a Western blot with mouse anti-human CD3z antibody. (B) HEK-293 cells were transduced with either GFP (left) or CD4IL15RA-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 36. Transduction of NK cells with CD4IL15RACAR. NK-92 cells were transduced with either GFP (left) or 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')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 37. Transduction of T cells with CD4IL15RACAR. Left is the Western blot. HEK-293FT cells were transfected with lentiviral plasmids for GFP (lane 1) and CD4IL15RACAR (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 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')₂ or isotype control for 30 minutes. Transduced with either GFP (left) or CD4IL15RACAR (right). Cells were washed and stained with streptavidin-PE conjugate at 1:250, washed, suspended in 2% formalin, and analyzed by flow cytometry

Figures 38 A-B. CD4CAR NK-92 cells and CD4IL15RA CAR NK-92 cells eliminate KARPAS 299 T leukemic cells in co-culture. (A) NK-92 cells transduced with either GFP control (upper right), CD4CAR (lower left), or CD4IL15RA (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. (B) The percentage of target cells lysed is shown in the graph.

Figure 39. CD4CAR NK-92 cells and CD4IL15RA CAR NK-92 cells eliminate MOLT4 T leukemic cells expressing CD4 in co-culture. NK-92 cells transduced with either GFP control (left), CD4CAR (center), or CD4IL15RA (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.

5 Figure 40. CD4IL15RACAR T cells demonstrate more potent anti-leukemic effects *in vivo* than CD4CAR. NSG mice were sublethally irradiated and intravenously (tail vein) injected the following day with luciferase-expressing MOLM13 cells to induce measurable tumor formation. After 3 days, the mice were intravenously injected with one course of 8×10^6 CD4CAR, or CD4IL15RACAR T cells, or vector control T control cells. On days 3, 6, 9 and 11,
10 mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging.

Figure 41. Percent tumor reduction in mice was measured and compared between the three groups based on the studies from Figure 40. Average light intensity measured for the CD4CAR and CD4IL15RACAR T injected mice was compared to that of vector control T injected mice, and correlated with remaining tumor burden. In each set of two, CD4CAR T is on
15 the left and CD4IL15RA CAR T is on the right.

Figure 42. 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.

20 Figure 43. 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 44 A-B. 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
5 channel to determine the percentage of GFP+ cells, 7, 14, 21 and 28 days after transduction.

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

(B) 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 μ L of SFFV-GFP and 1 mL of EF1-GFP supernatant was used). (N=2).

10 Figure 45. Ligand receptor interactions in malignant plasma cells. The APRIL ligand binds TAC1 or BCMA. The BAFF ligand binds TAC1, BCMA, or BAFF-R.

DETAILED DESCRIPTION

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

15 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
20 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, without limiting, 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 include 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, 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.

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
5 protein of type I, II, III, or IV. In an embodiment, the signal peptide includes an immunoglobulin heavy chain signal peptide.

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
10 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
15 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
20 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.

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 an invention disclosed herein, 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 an invention disclosed herein, 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
 5 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, CD5, 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,
 10 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, immunoglobulin 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, CD5, CD13,
 15 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, immunoglobulin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

20 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, CD5, 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, immunoglobulin kappa and lambda, CD38, CD52, CD3, CD4, CD8, CD5, CD7, CD2, and CD138 polypeptides.

5 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.

10 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.

15 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

20 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, CD3 ϵ , CD45, CD4, CD5, CD8, CD8a, CD9, CD16, CD22, CD33, CD37, CD64, 5 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 is not limited to, immunoglobulin (e.g. IgG1, IgG2, IgG3, IgG4, and IgD).

10 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 15 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 20 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, CD3 ϵ , 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 R1a, 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 (CD11a/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 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 GenBank 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).

5 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.

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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.

20

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.

5 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
10 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 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
15 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.
20 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-1 α promoter has been commonly selected for the CAR expression.

The present invention relates to 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 α (EF- 1 α). 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 1 α (EF) promoter, CAG (chicken beta-actin promoter with CMV enhancer) promoter human elongation factor 1 α (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 cosmid, 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 vectors. 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
5 (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
10 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
15 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).

20 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.

The disclosure includes a method of generating a cCAR. In some embodiments, the cCAR is generated using T-cells. In other embodiments, cCAR is using primary NK cells isolated from the peripheral blood or cord blood and NK-92 cells, such that they are administered “off-the-shelf” to any mammal with a disease or cancer.

According to one aspect of the present invention, NK cells can be expanded and transfected with CAR polynucleotides in accordance to the present invention. NK cells can be derived from cord blood, peripheral blood, iPS cells and embryonic stem cells. According to one aspect of the present invention, 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.

5 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.

10 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
15 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 CD19 or truncated epidermal growth factor receptor in T cells. All possible safety switches are have been contemplated and are embodied in the present invention.

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

20 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.

Multiple CAR units

5 The present disclosure provides an engineered cell having at least two distinct CAR polypeptides.

As used herein, compound CAR (cCAR) or multiple CAR refers to an engineered cell having at least two distinct chimeric antigen receptor polypeptides. As used herein, a “distinct chimeric antigen receptor polypeptide” has a unique antigen recognition domain, a signal
10 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
15 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
20 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 a 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 units 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 invention, 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 a preferred embodiment, the engineered cell having at least two distinct chimeric antigen receptor polypeptides is a primary NK cells isolated from the peripheral blood or cord blood and NK-92 cells, such that they are administered “off-the-shelf” to any mammal with a disease or cancer.

5 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
10 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
15 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,
20 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.

5 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.

10 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.

15 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, 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.

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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 CS1 antigen recognition domain.

5 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.

10 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
15 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
20 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
5 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 invention may target same or different tumor populations in T or NK cells. The first CAR, for example, may target the bulky tumor
10 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 invention 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
15 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

20 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-21, PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, and TGFR beta, receptors for the same, and functional
5 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
10 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 peptide and secreted into the extracellular space.

15 In one embodiment, the enhancer is IL-15. In this instance, the additional factor 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. An example of a suitable sushi domain includes SEQ ID NO. 35. In accordance with the present disclosure, any chimeric antigen receptor polypeptide disclosed herein includes the Human Interleukin 15 with human interleukin
20 2 signal peptide SEQ ID NO. 36.

Interleukin (IL)-15 and its specific receptor chain, IL-15R α (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 could overcome 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
5 enhances its survival and proliferation in-vitro and in-vivo. In some embodiments, CD4CAR or any CAR can include expressing any one or more of moieties, 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 relates to an engineered cell having a CAR as described herein and
10 any one or more of moieties 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 persistent or proliferation of CAR T or NK for treating cancer in a patient.

In one embodiment, the engineered cell includes a CD4 chimeric antigen receptor polypeptide and IL-15RA (SEQ ID NO. 1), and corresponding polynucleotide (SEQ ID NO. 2).

15 **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.

20 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 invention 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 invention, any of the engineered cells disclosed herein may be constructed in a transposon system (also called a “Sleeping Beauty”), which
5 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
10 bicistronic or multicistronic expression vectors. There are several strategies 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
15 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.

20 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
 5 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,
 10 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

15 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 can be employed to construct bicistronic or multicistronic vectors including, but not limited to, (1)
 20 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, 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 invention provides a method of targeting CD45 for conditioning prior to allogeneic 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.

5 It was surprisingly found that multiple CARs (Compound CARs, cCAR) of the present invention 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
10 combination of two or more antigens to rapidly eliminate the tumor.

The invention 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 invention includes compound (multiple or compound) cCAR in a T or NK
15 cell targeting different or same surface antigens present in tumor cells. The compound chimeric antigen receptors of present invention comprise at least multiple chimeric receptor constructs linked by a linker and target 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
20 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 embodiment, the compound CARs of the present invention target Myelodysplastic Syndrome and acute myeloid leukemia (AML) population. 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 invention 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 invention 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 invention 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 invention 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 plasmacytoid dendritic neoplasms (BPDCN), Hodgkin's lymphoma, mastocytosis, and hairy cell leukemia cells.

In another embodiment, the present disclosure provides a method of providing myeloblastic 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.

5 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
10 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, immunoglobulin kappa and
15 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 targets cells expressing CD19 or CD123 antigen or both. The targeted cells are cancer cells, such as, without limiting, B-cell lymphomas
20 or leukemias.

In further embodiments, the compound CAR targets cells expressing CS1 and/or B-cell maturation antigens (BCMA) or both. In another embodiment, the targeting 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 , CS1, BCMA, CD267, BAFF-R, CD38, CD138, CD52, CD19, CD20, interleukin 6 receptor and NY-ESO-1 antigens. In another embodiment, the targeting cells are malignant plasma cells such as, without limiting, multiple myeloma.

5 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, 10 ovarian and extragonadal yolk sac tumors, ovarian choriocarcinoma, teratomas, ovarian clear cell carcinoma, and placental site trophoblastic tumor.

In accordance with the present invention, the T or NK cell comprising compound CARs targeting different or same antigens offset tumor escape and enables simultaneous targeting of tumor cells.

15 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, vectors of the compound CAR is considered to be part of the present disclosure and is embodied herein.

In some embodiments, the compound CAR is administrated in combination with any chemotherapy agents currently being developed or available in the market. In some 20 embodiments, the compound CAR 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 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.

5 In one embodiment, the cells expressing a compound CAR 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.

10 In one embodiment, cells expressing a compound CAR 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
15 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 invention 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
20 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 invention 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 invention may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth.

In some embodiments, the invention discloses a method of depletion 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 with CAR or compound CAR T cells or NK cells. CAR targeted cells are B or plasma cells expressing one or two or all of 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, 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 invention, 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
5 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,
10 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 into 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.
15 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
20 (“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 invention target both of these populations and is embodied herein.

In accordance to the present invention, 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 invention, 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 invention 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 invention, NK cells can be expanded and transfected with cCAR in accordance to the present invention. NK cells can be derived from cord blood, peripheral blood, iPS cells and embryonic stem cells. According to one aspect of the present invention, 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 invention, 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
5 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 invention, CD 33 antigen is one of the targets for cCAR therapy. CD33 is a transmembrane receptor expressed on 90% of malignant cells in acute
10 myeloid leukemia. Thus, according to the present invention, CD123 and CD33 target antigens are particularly attractive from a safety standpoint.

In accordance with the present invention, 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
15 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
20 CAR disclosed in the present invention. CD123 expression is high in the CD34+CD38- population. In accordance with the present invention, the compound CD33CD123 CARs is highly effective for therapeutic treatment of this population.

In one embodiment of the present invention, 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 (Griffin, Linch et al. 1984). CD33 is widely expressed in leukemic cells
5 in CML, myeloproliferative neoplasms, and MDS.

As a significant number of patient 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
10 CD33, giving broad clinical applicability to the compound CD33CD123 CAR disclosed herein.

Thus, the present invention discloses a novel multiple cCAR T/NK cell construct comprising multiple CAR 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 and thereby providing a more potent, safe and effective therapy.

15 The present invention further discloses 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 potent killing capability in presence of the specific target.

In pre-clinical studies on dual specificity, trans-signaling CARs targeting solid tumors
20 including breast cancer and epithelial ovarian cancer, a CD3 ζ 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 invention, 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 invention.

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 (Kumar, Rajkumar et al. 2008). Anti-Myeloma CARs in Pre-clinical Development have been developed and CAR targets include CD38, CS1, B cell maturation Antigen (BCMA) and CD38. However, heterogeneity of surface antigen expression commonly occurs in malignant plasma cells (Ruiz-Arguelles and San Miguel 1994), 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).

5 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.

10 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 certainly 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 invention, BCMA and CS1 are the targets for BCMACS1 CAR therapy.

20 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 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
5 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
10 factors for malignant plasma cells.

Ligand-receptor interactions in the malignant plasma cells are described in Figure 45.

In some embodiments, a compound CAR targets cells expressing TACI or CS1 antigens or both. In another embodiment, a compound CAR targets cells expressing TACI or CS1 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas,
15 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,
20 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, a compound CAR targets cells expressing BAFF-R or CS1 antigens or both. In another embodiment, a compound CAR targets cells expressing BAFF-R or
5 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 macroglobulinemia, heavy chain diseases, solitary bone
10 plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

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
15 BAFF-R, BCMA, TACI and 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) BCMA or TACI or BAFF-R binding domain, or APRIL binding domain; 2) a hinge region; 3) co-stimulatory domain (s) and intracellular signaling domain.

20 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 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, 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-
5 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, immunoglobulin 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.

10 In some embodiments, a cCAR targets a cell expressing either CD19 or CD20 antigens or both of them. In another embodiment, a cCAR targets a cell expressing either CD19 or CD22 antigens or both of them. The targeting cells are cancer cells such as B-cell lymphomas or leukemias.

Acute graft-versus-host disease (GVHD) remains the most important cause of morbidity
15 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
20 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-deficient mice have normal cytotoxic activity against the prototypic tumor cell line, Yac-1. In

addition, CD45-deficient NK cells proliferate normally and respond to IL15 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

5 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.

10 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
15 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
20 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.

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 inventor proposes to inactivate
5 CD45 gene 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 some embodiments, malignant cells expressing CD45 are
10 present in patients with acute leukemia,, chronic leukemia, B and 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-
15 transplant lymphoproliferative disorders, etc.

In some embodiments, CD45CAR cells can be 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 a further embodiment, CD45CAR cells may be used for pre-treatment of patients before their
20 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 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 may include 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, 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, safety switch can include the amino acid sequence of TNF α bound by monoclonal anti-TNF α drugs.

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
5 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
10 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.

15 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
20 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.

5 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.

10 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
15 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
20 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, 5 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. Therefore, XXXX is the target. 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 10 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 15 exemplary and are not intended to limit the disclosure.

EXAMPLES

Generation of compound CAR (cCAR)

The construction of the CD33CD123 cCAR follows the schematic in Figure 1A. It includes SFFV (spleen focus-forming virus) promoter that drives the expression of the functional 20 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 μ M 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.

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
5 onto retronectin-coated multiwell plates. Activated T cells were transduced in multiple wells with lentiviral supernatant at a low concentration of about 0.3×10^6 cells/mL to increase transduction efficiency (Figure 2).

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.

10 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
15 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
20 antibody showed bands of predicted size for the compoundCAR CD3ζ fusion protein (Figure 1B). 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 1C) and primary T cells (Figure 1C).

The compound CD33CD23CAR lentivirus was tested for transduction efficiency in the HEK293 cell line and analyzed by flow cytometry (Beckman Coulter) (Figure 1C). Flow
5 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 1C).

10 RESULTS

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
15 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
20 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. About 100% of its cell population is CD33+ with a small subset (<10%) of it being dim CD123+. In culture, this cell
25 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 3). 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 4), 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. 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.

5 **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
10 (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).

15 Leukemic cells that were CD123 positive and CD33 negative were also tested. For this purpose, human B cell acute lymphoblastic 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
20 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
25 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
5 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
10 analyzed by flow cytometry. NK-92 cells expressing CD33CD123 cCAR were then labeled as above and sorted on FACS Aria, 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

15 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.

20 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).

Generation of CD19CD20, CD19CD22, CD19CD138 cCARs

The three cCARs have been generated (Figure 13) using the similar strategy to that of the CD33CD123 cCAR described above.

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

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(Ruiz-Arguelles and San Miguel 1994), it is unlikely that a single target is sufficient to eliminate this disease. BCMA CS1 cCAR, BCMA CD19 cCAR, BCMA CD38

5 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

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

BC1cCAR's modular design consists of an anti-CD269 (BCMA, B-cell maturation antigen) 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
15 tandem 4-1BB co-activation domains linked to the CD3 ζ signaling domain (Figure 14A). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BC1cCAR)CAR molecule on the T-cell surface. Two unit CARs use same co-stimulatory domain, 4-1BB. Transfected BC1cCAR HEK293T cells were subjected to Western blot analysis in order to confirm the compound construct. Immunoblot with an anti-
20 CD3 ζ monoclonal antibody showed bands of predicted size for the compound CAR CD3 ζ fusion protein (Figure 14E). 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 CD3 ζ expression was seen for the GFP control vector as expected.

Generation of BC1cCAR (cCAR) T-cells

T-cells isolated from umbilical cord blood (UCB) buffy coats were transduced with BC1cCAR lentivirus after 2 days of activation. Two unit CARs used the same co-stimulatory domain, 4-1BB. BC1cCAR's transduction efficiency was determined to be about 15% as
 5 determined by flow cytometry (Figure 14B). BC1cCAR T-cells were first tested on a CML (chronic myeloid leukemia) cell line negative for the myeloma markers, BCMA and CS1. As expected, there was no lysis from either control T-cells or BC1cCAR T-cells against wild-type K562 (Figure 14C). BCMA-K562 (Kochenderfer, NIH) were K562 cells transduced with BCMA expressing cDNA to express BCMA at >80% of the cell population. BC1cCAR T-cells were co-
 10 cultured with this cell line at E:T ratios of 2:1 and 5:1 and show over 30% lysis as compared to control (undetectable)(Figure 14C). These results are compatible with other cultures performed on antigen-transduced cell lines for other CARs, such as CS1CAR T-cells.

However, when BCMA-CS1-2G (a cCAR) used a different co-stimulatory domain, either 4-BB or CD28 for each unit, rare surface CAR expression was detected, which indicate
 15 that an appropriate selection of a co-stimulatory domain may be important for ensuring the surface CAR expression on T cells (Figure 14D). Although protein was detected in HEK cells by Western blotting (Fig. 14E), we were unable to detect surface expression in activated T cells transduced with CD269-CS1-2G lentiviral supernatant. This may be due to an inability to export the expressed protein to the cell membrane. In future, we may need to optimize the sequence of
 20 this construct to allow for greater cell surface expression.

BC1cCAR T-cells specifically lyse BCMA⁺ and CS1⁺ cell lines

To assess the cytotoxicity ability of BC1cCAR T-cells, we conducted co-culture assays with myeloma cell lines: MM1S (BCMA⁺ 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 (Figure 15). 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 16). 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 17).

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

Flow cytometry analysis of the MM10-G patient sample reveals distinct and consistent BCMA⁺ and CS1⁺ population subsets (Figure 18). 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. After 24 hours, BC1cCAR T-cells show robust 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 19). Against the MM11-G (Figure 20), BC1cCAR T-cells were able to lyse over 45% of BCMA⁺ CS1⁺ population at an E:T of 10:1.

BC1cCAR show targeted and specific lysis ability, by significantly ablating both the BCMA⁺ CS1⁺ and the BCMA⁻ CS1⁺ population subsets in MM10-G co-cultures over 24 hours. 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. At an E:T ratio of 5:1, the ablation of CS1⁺ only population increases to 80% (Figure 18).

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 MM.1S cells, a multiple myeloma cell line, to induce measurable leukemic formation. Three days following tumor cell injection, mice were intravenously injected with 8 x 10⁶ BC1cCAR T cells or vector control cells in a single dose. On days 3, 6, and 8, mice were injected subcutaneously with RediJect D-Luciferin (Perkin Elmer) and subjected to IVIS imaging to measure tumor burden (Figure 21). Average light intensity measured for the BC1cCAR T cells injected mice was compared to that of vector control injected mice in order to determine the percentage of tumor cells in treated versus control mice (Figure 21 and 22). Unpaired T test analysis revealed an extremely significant difference (P=0.0001) between the two groups by day 8 with less light intensity and thus less tumor burden in the BC1cCAR T cells injected group compared to control (p <0.0001). On day 1, and every other day afterwards, tumor size area was measured and the average tumor size between the two groups was compared (Figure 21). In summary, these *in vivo* data indicate that CD269-CS1-BBCAR T cells significantly reduce tumor burden in MM.1S-injected NSG mice when compared to vector control NK control cells.

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 23).

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

In a non-limiting embodiment of the invention, 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 24 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 25). 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 (NK⁴⁵ⁱ-92) after CRISPR/Cas nucleases target

We demonstrated that, following CRISPR/Cas nuclease inactivation of CD45, the growth of NK⁴⁵ⁱ-92 cells was similar to that of the wild NK-92 cells (Figure 26). Inactivation of CD45 did not significantly affect the cell proliferation of NK-92. In addition, we showed that the lysis ability of NK⁴⁵ⁱ-92 cells was compatible to that of wild type, NK-92 when cells were co-cultured with leukemic cells, CCRF (Fig 27).

To demonstrate that CD45⁻-inactivated NK-92 was compatible with CAR lysis, NK⁴⁵ⁱ-92 cells and their wild type, NK-92 were transduced with lentiviruses expressing CD5CAR or GFP. The resulting CD5CAR NK⁴⁵ⁱ-92 cells and GFP NK⁴⁵ⁱ-92 were sorted by FACS, and used to compare their ability of killing targeted cells. CD5CAR NK⁴⁵ⁱ-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 NK⁴⁵ⁱ-92 and CD5 CAR NK-92 cells (Figure 28). This suggests that the loss of CD45 expression does not diminish the anti-tumor activity of CAR NK-cells.

10 **Generation of CD45CAR construct**

We next investigate that CD45CAR in NK⁴⁵ⁱ-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 CD3 ζ signaling domain (Figure 29A). 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 29B).

20 **CD45CAR NK⁴⁵ⁱ-92 NK cells**

Following fluorescence-activated cell sorting (FACS) to enrich for NK⁴⁵ⁱ-92 cells, CD45CAR NK-92 transduction efficiency was determined to be 87%, as determined by flow cytometry (Figure 30) after sorting. After FACS collection of NK⁴⁵ⁱ-92 cells, CD45CAR expression levels remained consistently stable for at least 10 passages.

CD45CAR NK⁴⁵ⁱ-92 cells specifically lyse CD45+ leukemic cells.

To assess CD45CAR NK⁴⁵ⁱ-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 31, 32 and 33). We demonstrated that CD45CAR NK⁴⁵ⁱ-92 cells

5 consistently displayed robust lysis of leukemic cells. Following 6-hour incubation at a low effective to target cell (E:T ratio 5:1), CD45CAR NK⁴⁵ⁱ-92 cells effectively lysed more than 60% of CCRF-CEM cells (Figure 31). After 6-hour co-culture, CD45CAR NK⁴⁵ⁱ-92 cells were also able to eliminate about 60% of Jurkat cells at a ratio of E:T, 2:1 or 5:1 (Figure 32). After 6 hours of co-culture, CD45CAR NK⁴⁵ⁱ-92 cells efficiently lysed 20% CD45 positive NK-92 cells

10 at an E:T ratio of 2:1, with close to 60% lysis at an E:T of 5:1 (Figure 33A-33C).

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

15 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 CD3 ζ 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

20 compared to control GFP NK-92 cells (Fig. 33D-G). CD45b-28 CAR NK⁴⁵ⁱ-92 cells exhibited the highest ability of lysis of REH cells.

IL15 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
5 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
10 IL-15 receptor alpha chain (also called IL15RA or RA) contributing to IL-15-mediated effects such as T-cell survival, proliferation and memory T cell generation.

IL-15RA binds the $\beta\gamma$ complex in the surface of T cells and IL15 signals by binding with this IL-15RA/ $\beta\gamma$ complex on the cell surface of T cells and other types of cells.

Recent data have shown that while transfection of IL-15 alone does not significantly
15 influence T-cell function, transfection of IL-15/IL-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
20 with IL-15 alone results in robust proliferation and maintenance of memory CD8 T cells and NK cells. Recent studies have shown that a portion of the extracellular region of IL-15RA called sushi domain is required for its binding of IL15 (WEI et al., J. Immunol., vol.167(1), p:277-282, 2001). The IL-15/RA fusion protein or IL-15/sushi fusion protein containing the linker is more

potent than IL-15 and soluble IL-15RA alone. The combination of IL-15/RA or IL-15/sushi can maximize IL-15 activity. However, it is unclear if a design incorporating both CAR and IL-15/RA or IL15/sushi in the same construct maintains its desired biological properties in T or NK cells as insert sequence length is able to affect transfection efficiency and gene expression levels.

5 The present disclosure provides an engineered cell having both CAR and IL15/RA or IL15/sushi 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 IL15/RA or IL-15/sushi.

10 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) IL15RA (RA) or sushi. In further embodiments, CAR comprises chimeric antigen receptor, one or more of co-stimulatory endodomains, such as CD28, CD2, 4-1BB 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.

Characterization of CD4IL15RA-CAR

20 The CD4IL15RA-CAR has been generated and it contains the third generation of CD4CAR linked to IL15RA (Figure 34). 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 34). CAR with sushi/IL-15 is linked with the P2A cleaving sequence. The sushi/IL-15 portion is

composed of IL-2 signal peptide fused to sushi domain and linked to IL-5 via a 26-amino acid poly-proline linker (Figure 34).

To verify the CD4IL15RA construct, HEK293FT cells were transfected with lentiviral plasmids for either GFP (control) or CD4IL15RA. 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 CD4IL15RA-CAR was successfully expressed in HEK 293 cells (Lane 2, Figure 35a, as shown next to recombinant IL-15 protein in Lane 3 (arrow). The CD4IL15RA-CAR lentiviral supernatant was further examined by the transduction of fresh HEK-293 cells (Figure 35a). HEK-293 cells were transduced with either GFP or CD4IL15RA-CAR viral supernatant from transfected HEK-293FT cells. Polybrene was added to 4 uL/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 34b shows that HEK-293 cells that were transduced with the CD4IL15RA-CAR lentivirus were 80% positive for F(Ab)₂-PE (circled, Figure 35b), while transduction with GFP control lentivirus was minimal for F(Ab)₂-PE (Figure 35b,left).

Production of CD4IL15RA-CAR NK cells

NK-92 cells were transduced with CD4IL15RA-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 CD4IL15RA-CAR (circled, Figure 36). Further experimental tests for CD4IL15RA-CAR will include 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 CD19IL15RA-CAR.

Production of CD4IL15RA-CAR T cells

Human umbilical cord buffy coat cells were transduced with CD4IL15RA-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 CD4IL15RA-CAR (circled, Figure 37). Further experimental tests for CD4IL15RA-CAR will include leukemia/lymphoma killing assays in vitro and vivo, and comparison of target killing and proliferation rates with cells transduced with CD4CAR.

CD4IL15RACAR 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, CD4IL15RA 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

38). Similarly, when co-cultured 1:1 with MOLT4 cells, CD4IL15RA CAR NK cells lysed target cells at a higher rate (84% to 65%) than CD4CAR NK cells in an overnight assay (Figure 39).

These results show that CD4IL15 CAR NK cells can ablate tumor cells at least as well as CD4CAR NK cells.

5 **CD4CAR and CD4IL15RA CAR T cells exhibit more potent anti-tumor activity *in vivo* than CD4CAR**

In order to evaluate the *in vivo* anti-tumor activity of CD4CAR and CD4IL15RACAR T cells, and to determine the possible increase in persistence of the CD4IL15RA CAR T cells
 10 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 (M5) that is 100% CD4, to induce measurable tumor formation. Three days following tumor cell injection, 6 mice each were intravenously injected with a course of 8×10^6 CD4CAR, CD4IL15RACAR T cells or vector control T cells. On days 3, 6, 9 and 11,
 15 mice were injected subcutaneously with RediJect D-Luciferin (Perkin Elmer) and subjected to IVIS imaging to measure tumor burden (Figure 40). CD4CAR T cell-treated mice had a 52% lower tumor burden relative to control on Day 6, whereas CD4IL15RA CAR T cell-treated mice had a 74% lower tumor burden (Figure 41). On Day 11, nearly all tumor cells had been lysed in both of these groups. Unpaired T test analysis revealed an very significant difference ($P=0.0045$)
 20 between control and the two groups by day 9 with less light intensity and thus less tumor burden in the CD4CAR and CD4IL15RACAR T cells treated group compared to control.

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
 25 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 uL/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; Figure 23).

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.

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,

CD138, and GPC3, or a part or a combination of a hinge region and T- cell activation domains is provided. All references cited and/or disclosed herein are hereby incorporated by reference in their entirety.

The provided methods also include: 1) generating of the CAR T or NK cells targeting
5 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 invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims. Various
10 publications, including patents, published applications, technical articles and scholarly articles are cited throughout the specification. Each cited publication is incorporated by reference herein, in its entirety and for all purposes. Various terms relating to aspects of the invention 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
15 manner consistent with the definition provided herein.

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
20 293 cells were transduced with either EF1-GFP or SFFV-GFP viral supernatant, with either 30 μ L (low), 125 μ L (medium), or 500 μ L (high) per well of a 12 well tissue-culture treated plate. Culture media was changed the following morning to DMEM plus 10% FBS.

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 43). 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 42). 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 μ L 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 $1.0-4.0 \times 10^6$ /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 44A), 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 μ L, or 20-fold less) of SFFV-GFP, between Day 7 and Day 28, from over 60% to under 40% (Figure 44B). This suggests that transduction using the SFFV promoter led to greater persistence of transduced cells.

BCMA or TACI or BAFF-R CAR NK cells or T-cells targeting cells expressing at least one of BCMA or TACI or BAFF-R CAR antigen

To assess the cytotoxicity ability of CAR targeting at least one of BCMA or TACI or BAFF-R NK cells or T cells, co-culture assays are conducted with cell lines or primary human cells expressing at least one of BCMA or TACI or BAFF-R. The ability of the aforementioned CAR NK cells or T cells to lyse the target cells was quantified by flow cytometry analysis, and target cells were stained with Cytotracker dye (CMTMR). Lysis is observed at 24 hour long cultures.

BAFF or APRIL CAR NK or T cells targeting cells expressing at least one of BCMA or TACI or BAFF-R antigen.

The chimeric antigen receptor in the CAR is the ligand for BCMA or TACI or BAFF-R.

To assess the cytotoxicity ability of CAR targeting at least one of BCMA or TACI or BAFF-R NK or T cells, co-culture assays are conducted with cell lines or human primary cells expressing at least one of of BCMA or TACI or BAFF-R. The ability of the aforementioned CAR NK or T cells to lyse the target cells was quantified by flow cytometry analysis, and target cells were stained with Cytotracker dye (CMTMR). Lysis is observed at 24 hour-long cultures.

References

- Arai, S., R. Meagher, M. Swearingen, H. Myint, E. Rich, J. Martinson and H. Klingemann (2008). "Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial." Cytotherapy **10**(6): 625-632.
- Boissel, L., M. Betancur-Boissel, W. Lu, D. S. Krause, R. A. Van Etten, W. S. Wels and H. Klingemann (2013). "Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity." Oncoimmunology **2**(10): e26527.
- Burnett, A. K. (2012). "Treatment of acute myeloid leukemia: are we making progress?" Hematology-American Society Hematology Education Program: 1-6.
- Chu, J., Y. Deng, D. M. Benson, S. He, T. Hughes, J. Zhang, Y. Peng, H. Mao, L. Yi, K. Ghoshal, X. He, S. M. Devine, X. Zhang, M. A. Caligiuri, C. C. Hofmeister and J. Yu (2014). "CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma." Leukemia **28**(4): 917-927.
- Corbin, A. S., A. Agarwal, M. Loriaux, J. Cortes, M. W. Deininger and B. J. Druker (2011). "Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity." J Clin Invest **121**(1): 396-409.
- Dinndorf, P. A., R. G. Andrews, D. Benjamin, D. Ridgway, L. Wolff and I. D. Bernstein (1986). "Expression of normal myeloid-associated antigens by acute leukemia cells." Blood **67**(4): 1048-1053.
- Djokic, M., E. Bjorklund, E. Blennow, J. Mazur, S. Soderhall and A. Porwit (2009). "Overexpression of CD123 correlates with the hyperdiploid genotype in acute lymphoblastic leukemia." Haematologica **94**(7): 1016-1019.
- Ehninger, A., M. Kramer, C. Rollig, C. Thiede, M. Bornhauser, M. von Bonin, M. Wermke, A. Feldmann, M. Bachmann, G. Ehninger and U. Oelschlagel (2014). "Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia." Blood Cancer J **4**: e218.
- Firor, A. E., A. Jares and Y. Ma (2015). "From humble beginnings to success in the clinic: Chimeric antigen receptor-modified T-cells and implications for immunotherapy." Exp Biol Med (Maywood).
- Garfall, A. L., M. V. Maus, W. T. Hwang, S. F. Lacey, Y. D. Mahnke, J. J. Melenhorst, Z. Zheng, D. T. Vogl, A. D. Cohen, B. M. Weiss, K. Dengel, N. D. Kerr, A. Bagg, B. L. Levine, C. H. June and E. A. Stadtmauer (2015). "Chimeric Antigen Receptor T Cells against CD19 for Multiple Myeloma." N Engl J Med **373**(11): 1040-1047.
- Ghosh, N. and W. Matsui (2009). "Cancer stem cells in multiple myeloma." Cancer Lett **277**(1): 1-7.
- Griffin, J. D., D. Linch, K. Sabbath, P. Larcom and S. F. Schlossman (1984). "A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells." Leuk Res **8**(4): 521-534.
- Jilani, I., E. Estey, Y. Huh, Y. Joe, T. Manshouri, M. Yared, F. Giles, H. Kantarjian, J. Cortes, D. Thomas, M. Keating, E. Freireich and M. Albitar (2002). "Differences in CD33 intensity between various myeloid neoplasms." Am J Clin Pathol **118**(4): 560-566.
- Jordan, C. T., D. Upchurch, S. J. Szilvassy, M. L. Guzman, D. S. Howard, A. L. Pettigrew, T. Meyerrose, R. Rossi, B. Grimes, D. A. Rizzieri, S. M. Luger and G. L. Phillips (2000). "The

- interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells." Leukemia **14**(10): 1777-1784.
- Klingemann, H. (2014). "Are natural killer cells superior CAR drivers?" Oncoimmunology **3**: e28147.
- 5 Kumar, S. K., S. V. Rajkumar, A. Dispenzieri, M. Q. Lacy, S. R. Hayman, F. K. Buadi, S. R. Zeldenrust, D. Dingli, S. J. Russell, J. A. Lust, P. R. Greipp, R. A. Kyle and M. A. Gertz (2008). "Improved survival in multiple myeloma and the impact of novel therapies." Blood **111**(5): 2516-2520.
- Lang, S., N. L. Vujanovic, B. Wollenberg and T. L. Whiteside (1998). "Absence of B7.1-
10 CD28/CTLA-4-mediated co-stimulation in human NK cells." Eur J Immunol **28**(3): 780-786.
- Lanitis, E., M. Poussin, A. W. Klattenhoff, D. Song, R. Sandaltzopoulos, C. H. June and D. J. Powell, Jr. (2013). "Chimeric antigen receptor T Cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity in vivo." Cancer Immunol Res **1**(1): 43-53.
- 15 Loke, J., J. N. Khan, J. S. Wilson, C. Craddock and K. Wheatley (2015). "Mylotarg has potent anti-leukaemic effect: a systematic review and meta-analysis of anti-CD33 antibody treatment in acute myeloid leukaemia." Annals of Hematology **94**(3): 361-373.
- Maus, M. V., J. A. Fraietta, B. L. Levine, M. Kalos, Y. Zhao and C. H. June (2014). "Adoptive immunotherapy for cancer or viruses." Annu Rev Immunol **32**: 189-225.
- 20 Olson, J. A., D. B. Leveson-Gower, S. Gill, J. Baker, A. Beilhack and R. S. Negrin (2010). "NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects." Blood **115**(21): 4293-4301.
- Ruiz-Arguelles, G. J. and J. F. San Miguel (1994). "Cell surface markers in multiple myeloma." Mayo Clin Proc **69**(7): 684-690.
- 25 Testa, U., E. Pelosi and A. Frankel (2014). "CD 123 is a membrane biomarker and a therapeutic target in hematologic malignancies." Biomark Res **2**(1): 4.
- Vergez, F., A. S. Green, J. Tamburini, J. E. Sarry, B. Gaillard, P. Cornillet-Lefebvre, M. Pannetier, A. Neyret, N. Chapuis, N. Ifrah, F. Dreyfus, S. Manenti, C. Demur, E. Delabesse, C. Lacombe, P. Mayeux, D. Bouscary, C. Recher and V. Bardet (2011). "High levels of
30 CD34+CD38low/-CD123+ blasts are predictive of an adverse outcome in acute myeloid leukemia: a Groupe Ouest-Est des Leucemies Aigues et Maladies du Sang (GOELAMS) study." Haematologica **96**(12): 1792-1798.
- Wilkie, S., M. C. van Schalkwyk, S. Hobbs, D. M. Davies, S. J. van der Stegen, A. C. Pereira, S. E. Burbidge, C. Box, S. A. Eccles and J. Maher (2012). "Dual targeting of ErbB2 and MUC1 in
35 breast cancer using chimeric antigen receptors engineered to provide complementary signaling." J Clin Immunol **32**(5): 1059-1070.

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 June 24, 2016. The sequence-listing.txt file is 140KB in size.

CLAIMS

1. 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
5 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
10 different.

2. The engineered cell according to claim 1, 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
15 receptor polypeptide.

3. The engineered cell according to claim 2, wherein the high efficiency cleavage site is
selected from the group consisting of P2A, T2A, E2A, and F2A.

4. The engineered cell according to any one of claims claim 1-3, wherein the first co-stimulatory
domain and the second co-stimulatory domain are different.

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

6. The engineered cell according to any one of claims 1-5, 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.
7. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD267 or CD269; and the target of the second antigen recognition domain is selected from the group consisting of CD19, CD38, CD138, CD138, and CS1.
8. The engineered cell according to any one of claims 1-6, 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, CD267, CD269, CD38, and CS1.
9. The engineered cell according to any one of claims 1-6, 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.
10. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition domain comprises LeY or CD123.

11. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises BCMA; and the target of the second antigen recognition domain comprises CS1, CD19, CD38, CD138, or CS1.
12. The engineered cell according to any one of claims 1-11, wherein the engineered cell is a
5 T-cell or Natural Killer cell.
13. The engineered cell according to any one of claims 12, wherein the T-cell is a CD4 T-cell or CD8 T-cell.
14. The engineered cell according to any one of claims 12, wherein the Natural Killer cell is a NKT cell or NK-92cell.
- 10 15. An engineered polypeptide comprising a chimeric antigen receptor polypeptide and an enhancer.
16. The engineered polypeptide according to claim 15, 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.
- 15 17. The engineered polypeptide according to claim 15, wherein the chimeric antigen receptor polypeptide comprises an antigen recognition domain selective for a target selected from the group consisting of: CD19, CD20, CD22, CD33, CD38, CD123, CD138, CD267, CD269, CD38, and CS1.
18. The engineered polypeptide according to claim 15, wherein the chimeric antigen receptor
20 polypeptide comprises the CD45 antigen recognition domain.

19. The engineered polypeptide according to any one of claims 15-18, wherein said enhancer is selected from the group consisting of PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, TGFR beta, IL-2, IL-7, IL-12, IL-15, IL-21, functional fragments thereof, and combinations thereof.
20. The engineered polypeptide according to any one of claims 15-18, wherein the
5 engineered polypeptide further comprises an enhancer receptor or functional fragment thereof.
21. The engineered polypeptide of 20, wherein the enhancer receptor comprises IL-15RA or a functional fragment thereof.
22. The engineered polypeptide of 21, wherein the functional fragment comprises the sushi domain.
- 10 23. The engineered polypeptide according to claim 15-22, wherein chimeric antigen receptor polypeptide and enhancer are on a single polypeptide molecule.
24. The engineered polypeptide of claim 23, wherein a high efficiency cleavage site is disposed between the chimeric antigen receptor and enhancer.
25. The engineered polypeptide according to claim 24, wherein the high efficiency cleavage
15 site is selected from the group consisting of P2A, T2A, E2A, and F2A.
26. The engineered polypeptide according to any one of claims 15-25, 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 or IL-15RA.
- 20 27. An engineered polynucleotide, the polynucleotide encodes for any one of the polypeptides according to claims 15-26.

28. The engineered polynucleotide according to claim 27, wherein the polynucleotide is in a vector.
29. An engineered cell comprising the polypeptide according to any one of claims 15-26.
30. An engineered cell comprising the polynucleotide according to any one of claims 24-25.
- 5 31. The engineered cell according to any one of claims 29-30, wherein the engineered cell comprises T-cells or Natural Killer cells.
32. The engineered cell according to any one of claims 31, wherein the cell is a T-cell or Natural Killer cell.
33. The engineered cell according to any one of claims 32, wherein the T-cell is a CD4 T-cell
10 or CD8 T-cell.
34. The engineered cell according to any one of claims 31, wherein the Natural Killer cell is a NKT cell or NK-92 cell.
35. A method of treating B-cell lymphoma comprising administering to a patient in need thereof an engineered cell according to any one of claims 1-15 and 29-34.
- 15 36. A method of treating T-cell lymphoma comprising administering to a patient in need thereof an engineered cell according to any one of claims 1-15 and 29-34.
37. A method of treating multiple myeloma comprising administering to a patient in need thereof an engineered cell according to any one of claims 1-15 and 29-34.
38. A method of treating chronic myeloid leukemia comprising administering to a patient in
20 need thereof an engineered cell according to any one of claims 1-15 and 29-34, wherein the

target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition domain comprises CD123.

39. 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 1-15, wherein the
5 target of the first antigen recognition domain comprises CD19; and the target of the second antigen recognition domain comprises CD123.

40. A method of treating multiple myeloma comprising administering to a patient in need thereof an engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain is selected from the group consisting of CD38, CS1, BCMA, and
10 CD38; and the target of the second antigen recognition domain is selected from the group consisting of CD38, CS1, BCMA, and CD38.

41. 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 1-15, wherein the target of the first antigen recognition domain is selected from the group consisting of BCMA, TAC1, CS1,
15 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.

42. The method of treating a cell proliferative disease according to claim 41, wherein the cell proliferative disease is selected from the group consisting of lymphomas, leukemias, and plasma cell neoplasms.

20 43. The method of treating a cell proliferative disease according to claim 42, 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.

44. 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 30-34.

5 45. 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,
10 CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, and CS1; and CAR enhancing agent.

46. A method of treating a cell proliferative disease according to claim 45, 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-
15 12, IL-15, CSF1R, CTAL-4, TIM-3, and TGFR beta.

47. 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 13-23.

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

49. The engineered chimeric antigen receptor polypeptide according to claim 48, wherein said CD45 antigen recognition domain comprises the binding portion or variable region of a monoclonal antibody selective for CD45.

50. The engineered chimeric antigen receptor polypeptide of claim any one of claims 48-49,
5 wherein said CD45 antigen recognition domain comprises the CD45 scFv.

51. The engineered chimeric antigen receptor polypeptide of any one of claims 48-50, 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 52. The engineered chimeric antigen receptor polypeptide of any one of claims 48-51, 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.

53. The engineered chimeric antigen receptor polypeptide of any one of claims 48-52,
15 wherein said signaling domain comprises the signaling domain selected from the group consisting of CD3 zeta, common FcR gamma (FCER1G), Fc gamma R1a, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DAP10, DAP12, active fragments thereof, and combinations thereof.

54. The engineered chimeric antigen receptor polypeptide of any one of claims 48-53,
20 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 (CD11a/CD18), ICOS, and 4-1BB (CD137), active fragments thereof, and combinations thereof.

55. An engineered polynucleotide that encodes a polypeptide according to any one of claims 48-54.

5 56. The engineered polynucleotide according to claim 55, wherein the polynucleotide is in a vector.

57. An engineered cell comprising the polynucleotide according to any one of claims 55-56.

58. An engineered cell comprising the polypeptide according to any one of claims 48-54.

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

60. The engineered cell according to claim 59, wherein the T-cell is a CD4 T-cell or CD8 T-cell.

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

15 62. 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 1-14, 29-34, and 57-61; 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

20 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, CD267, CD269, CD38, Flt3 receptor, and CS1.

63. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises BCMA; and the second antigen recognition domain
5 comprises CS1.

64. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises BCMA.

65. The engineered cell according to any one of claims 1-6, wherein the target of the first
10 antigen recognition domain comprises CD19; and the target of the second antigen recognition domain comprises CD22.

66. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises CD20.

15 67. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD19; and the second antigen recognition domain comprises CD123.

68. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD33; and the target of the second antigen recognition
20 domain comprises CD123.

69. The engineered cell according to any one of claims 1-6, wherein the target of the first antigen recognition domain comprises CD269; and the target of the second antigen recognition domain comprises CS1.

70. The engineered cell according to any one of claims 29-34, wherein the target of the antigen recognition domain comprises CD4 and the enhancer comprises IL-15RA.

71. The engineered cell according to any one of claims 1-6, wherein TAC1 antigen recognition domain comprises the APRIL ligand or the BAFF ligand or a portion thereof.

72. The engineered cell according to any one of claims 1-6, wherein the BCMA antigen recognition domain comprises APRIL ligand or BAFF ligand or a portion thereof.

73. The engineered cell according to any one of claims 1-6, wherein the BAFF-R antigen recognition domain comprises the BAFF ligand or a portion thereof.

74. The engineered cell according to any one of claims 1-6, wherein the first co-stimulatory domain and the second co-stimulatory domain are same.

75. The engineered cell according to any one of claims 1-6 and 11, wherein the first co-stimulatory domain and the second co-stimulatory domain comprise 4-1BB co-stimulatory domain.

FIG. 1

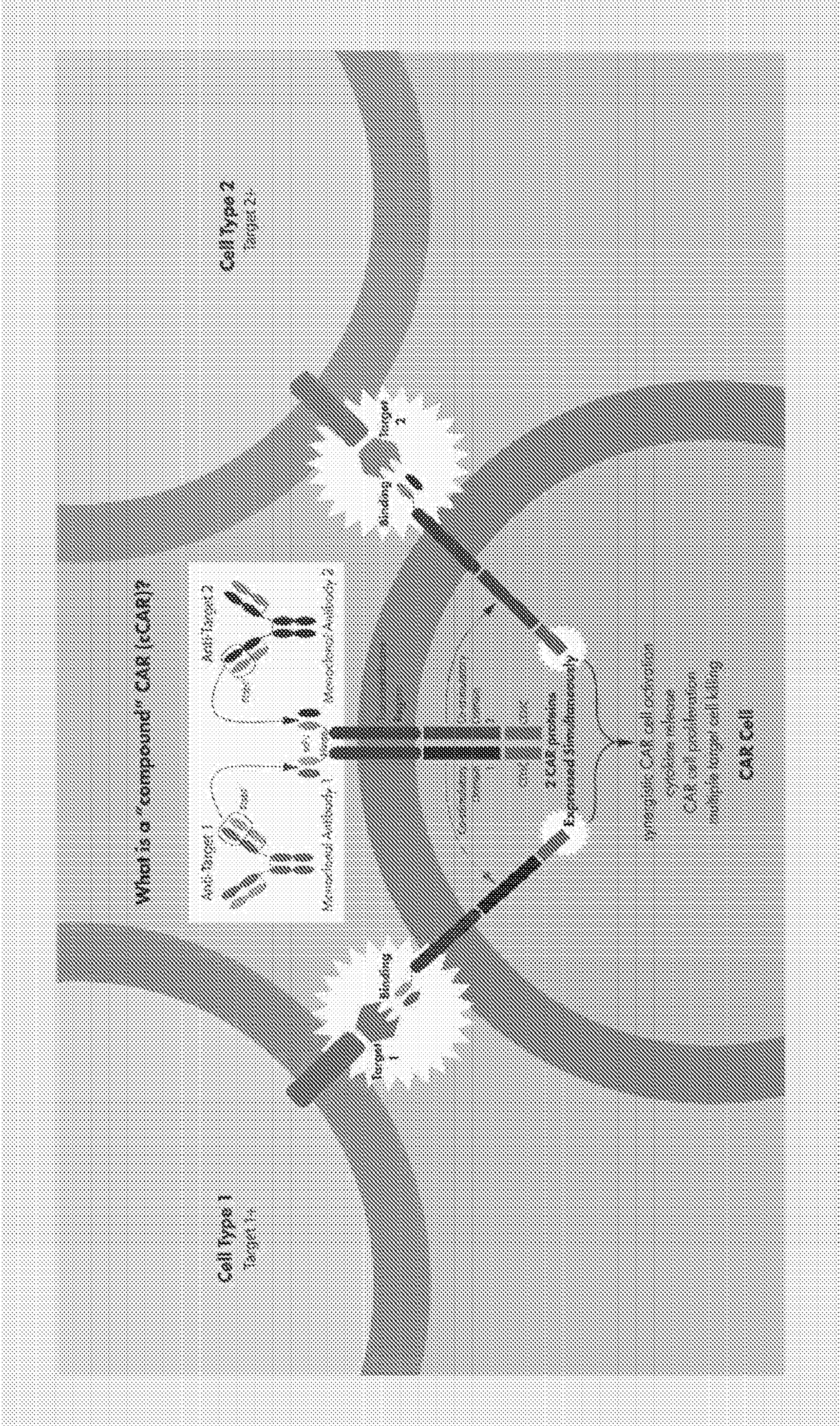


FIG. 2A

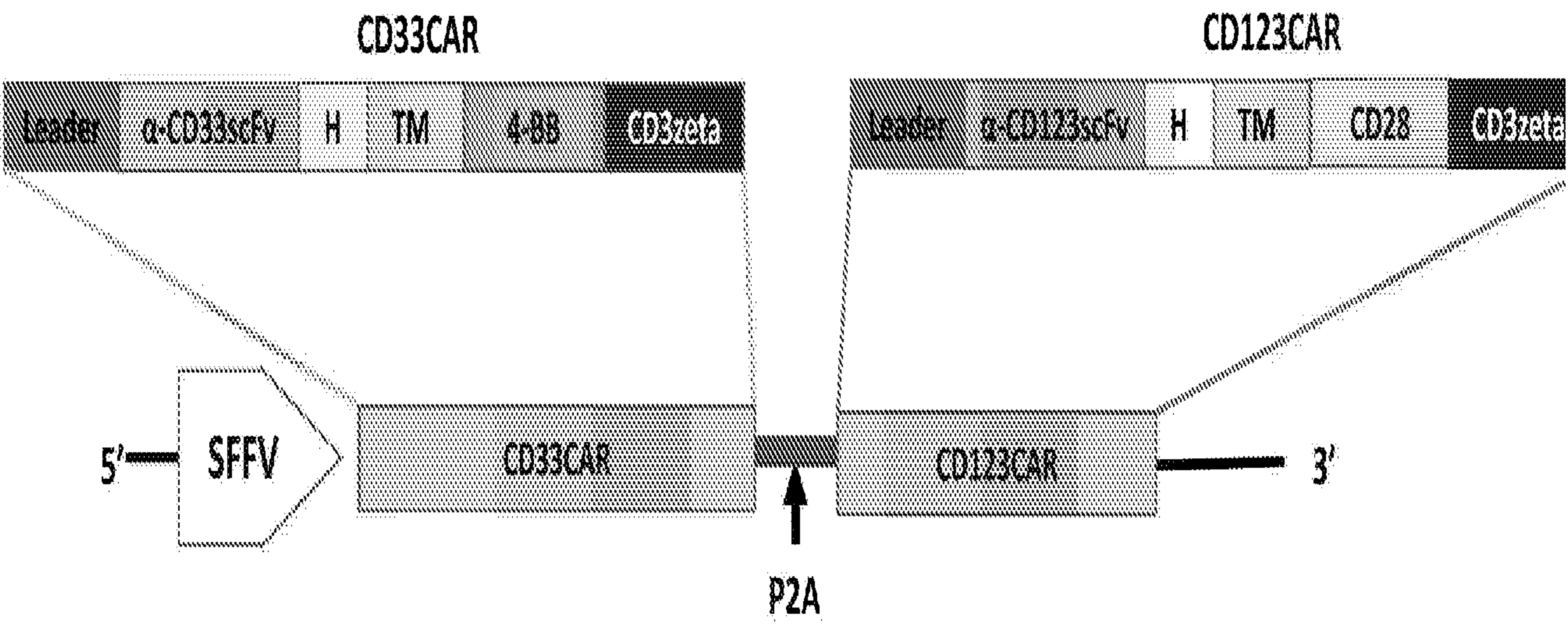


FIG. 2B

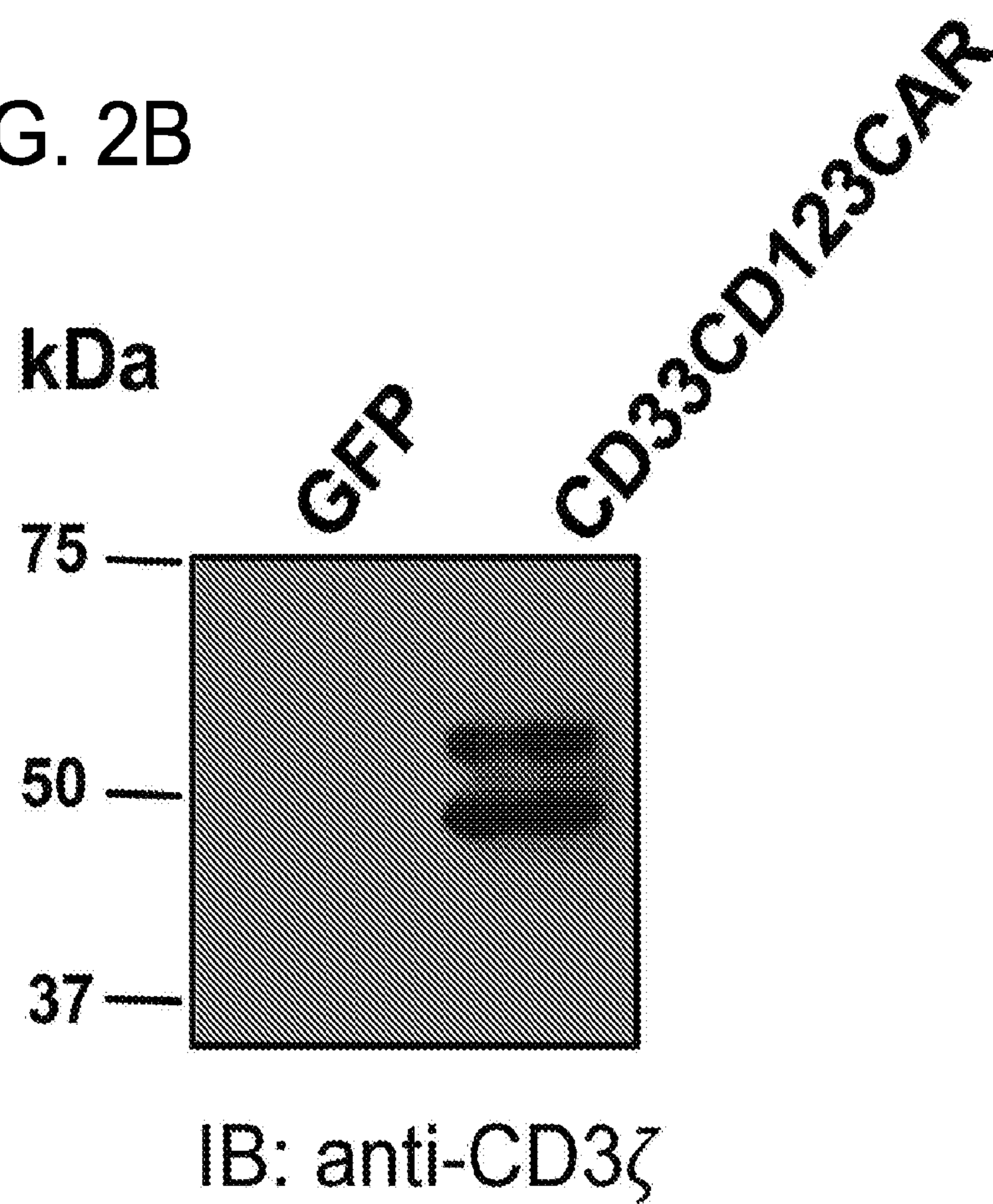


FIG. 2C

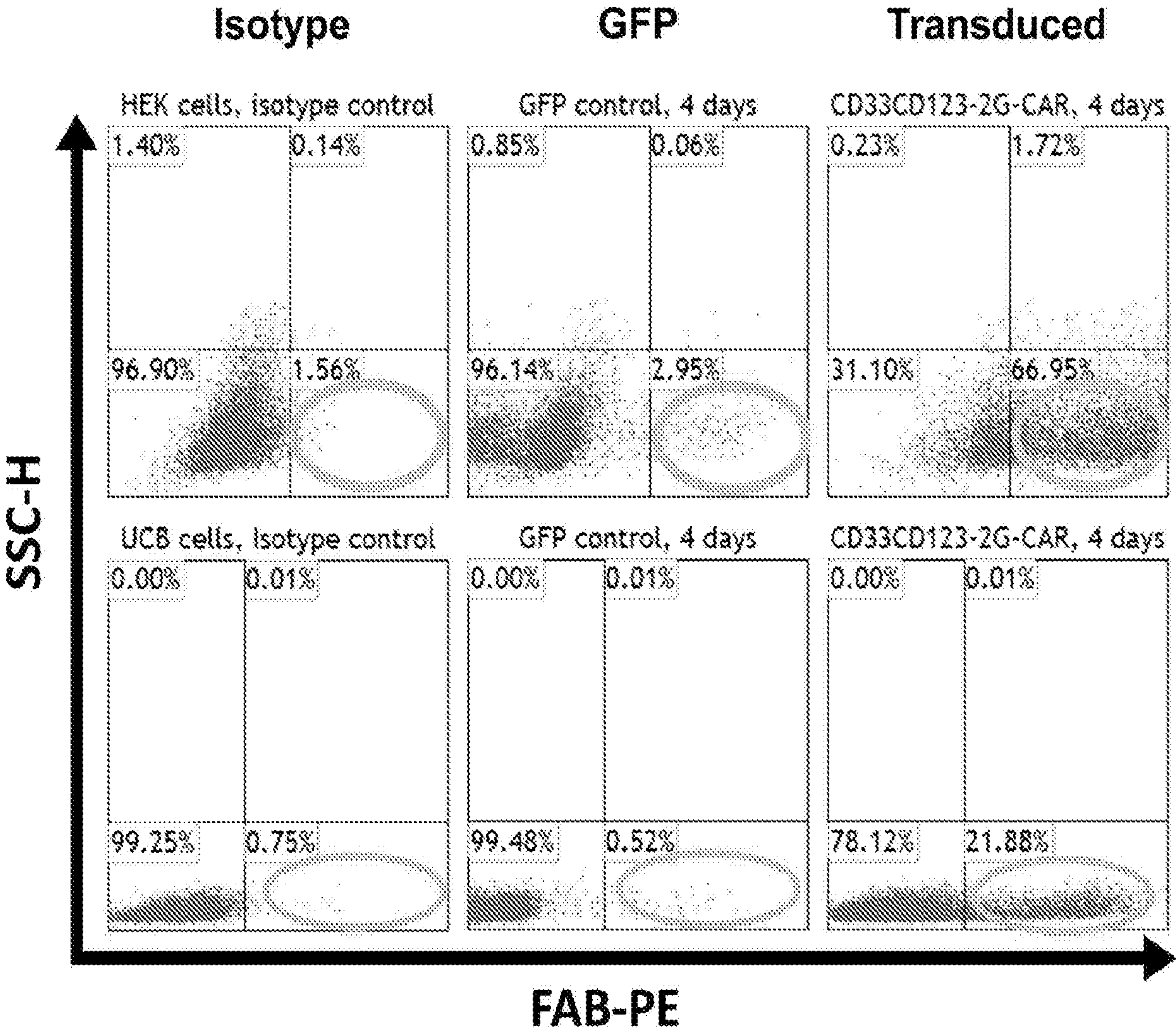


FIG. 3

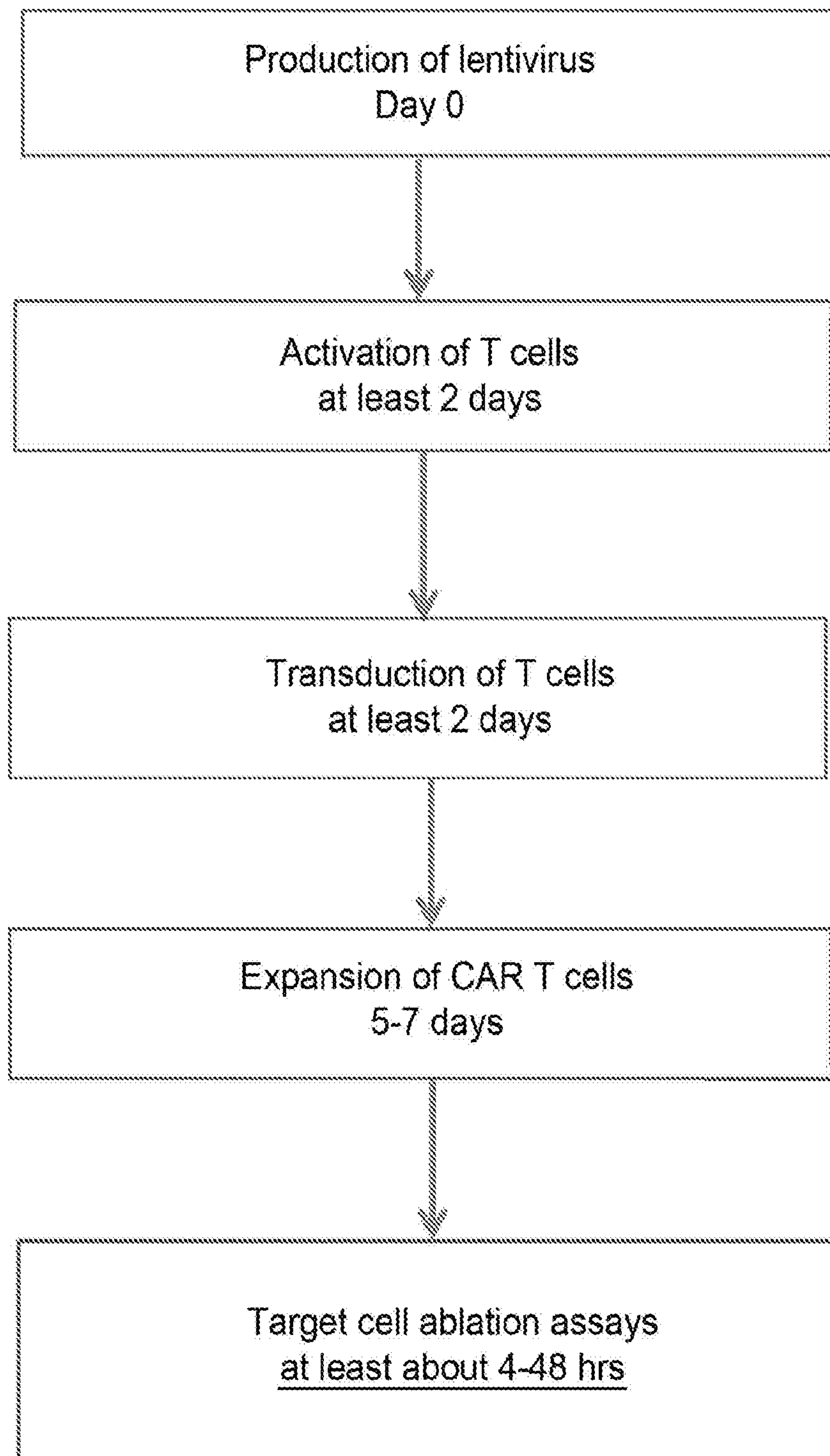


FIG. 4

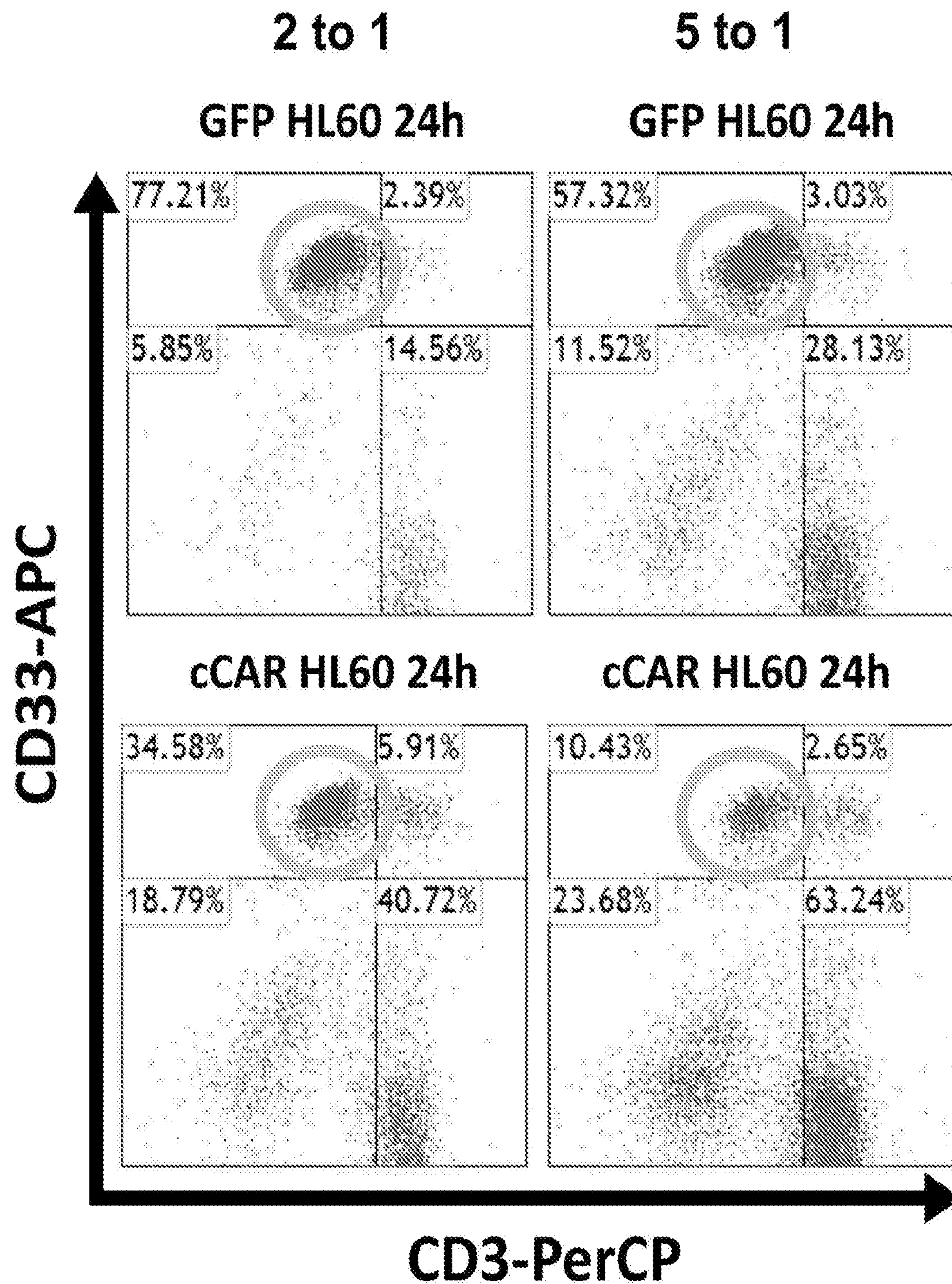


FIG. 5

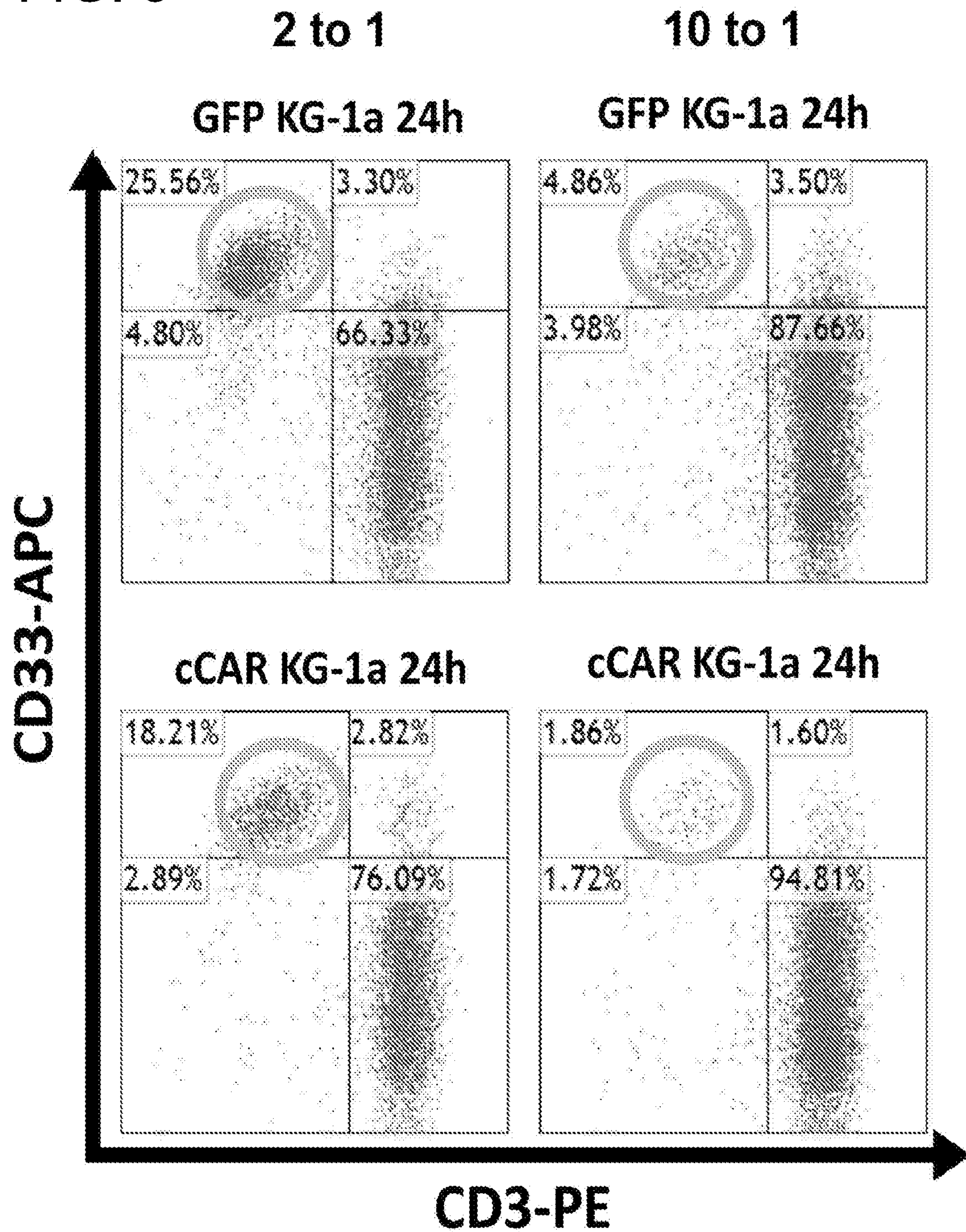


FIG. 6

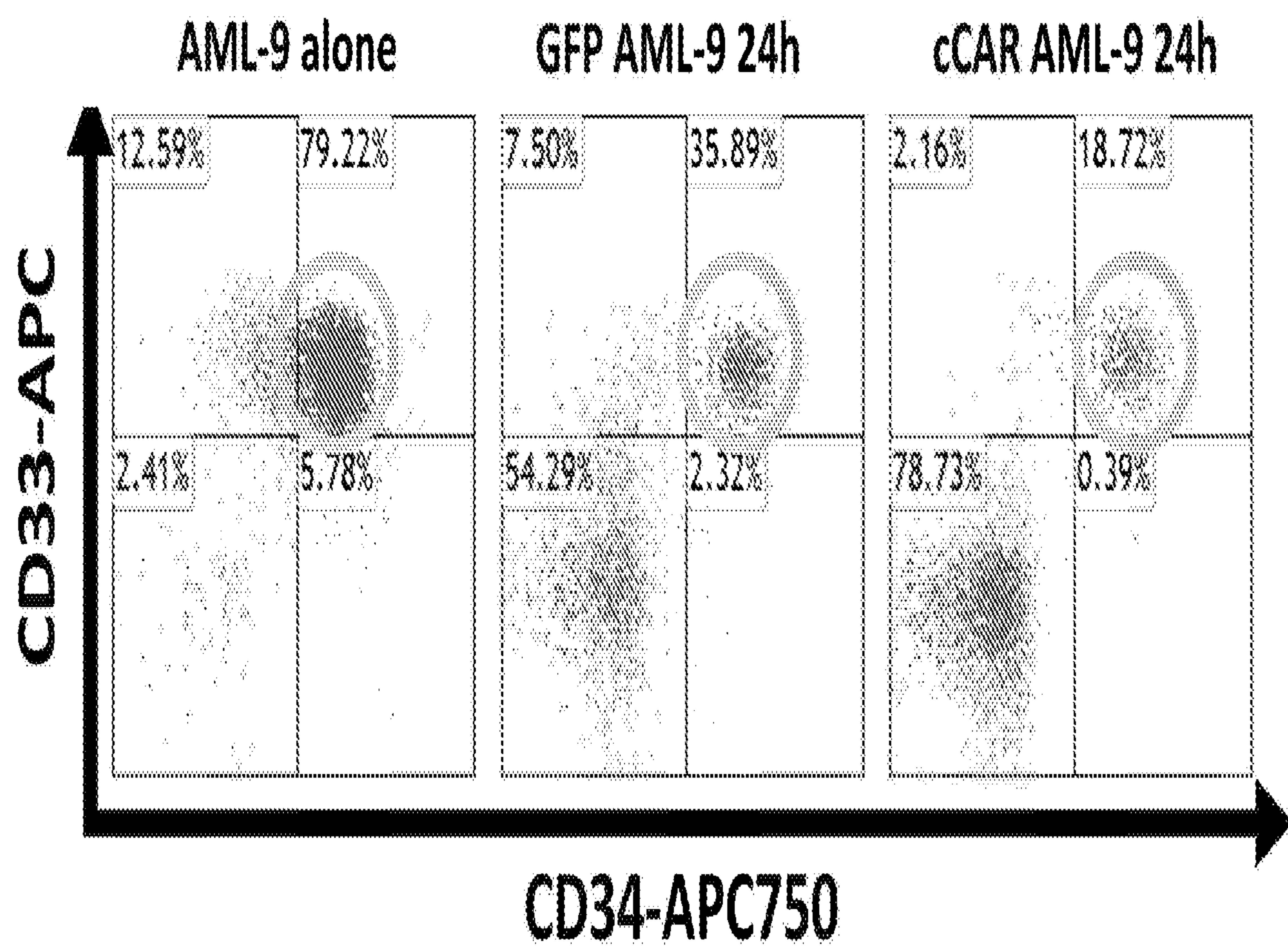


FIG. 7

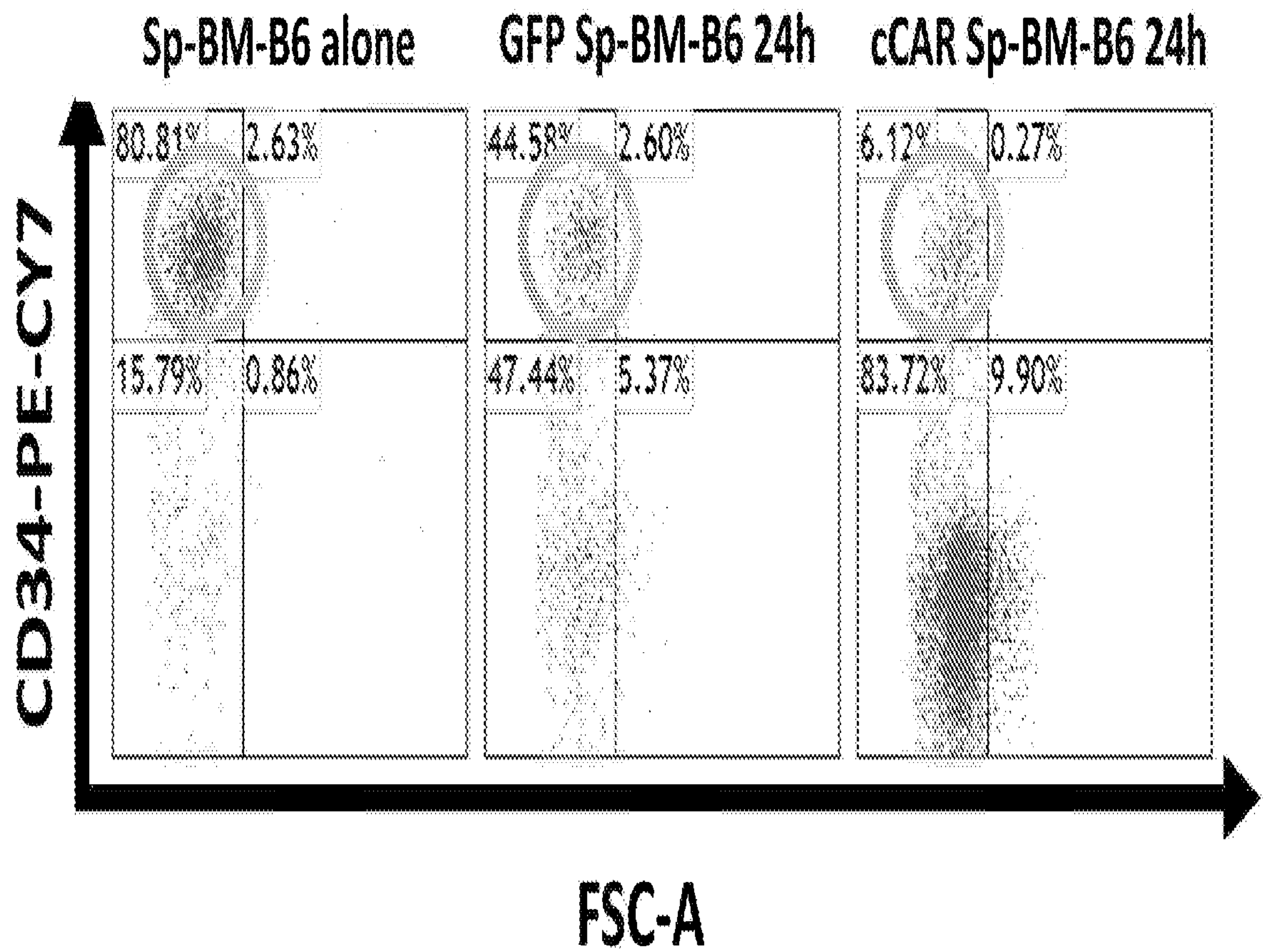


FIG. 8

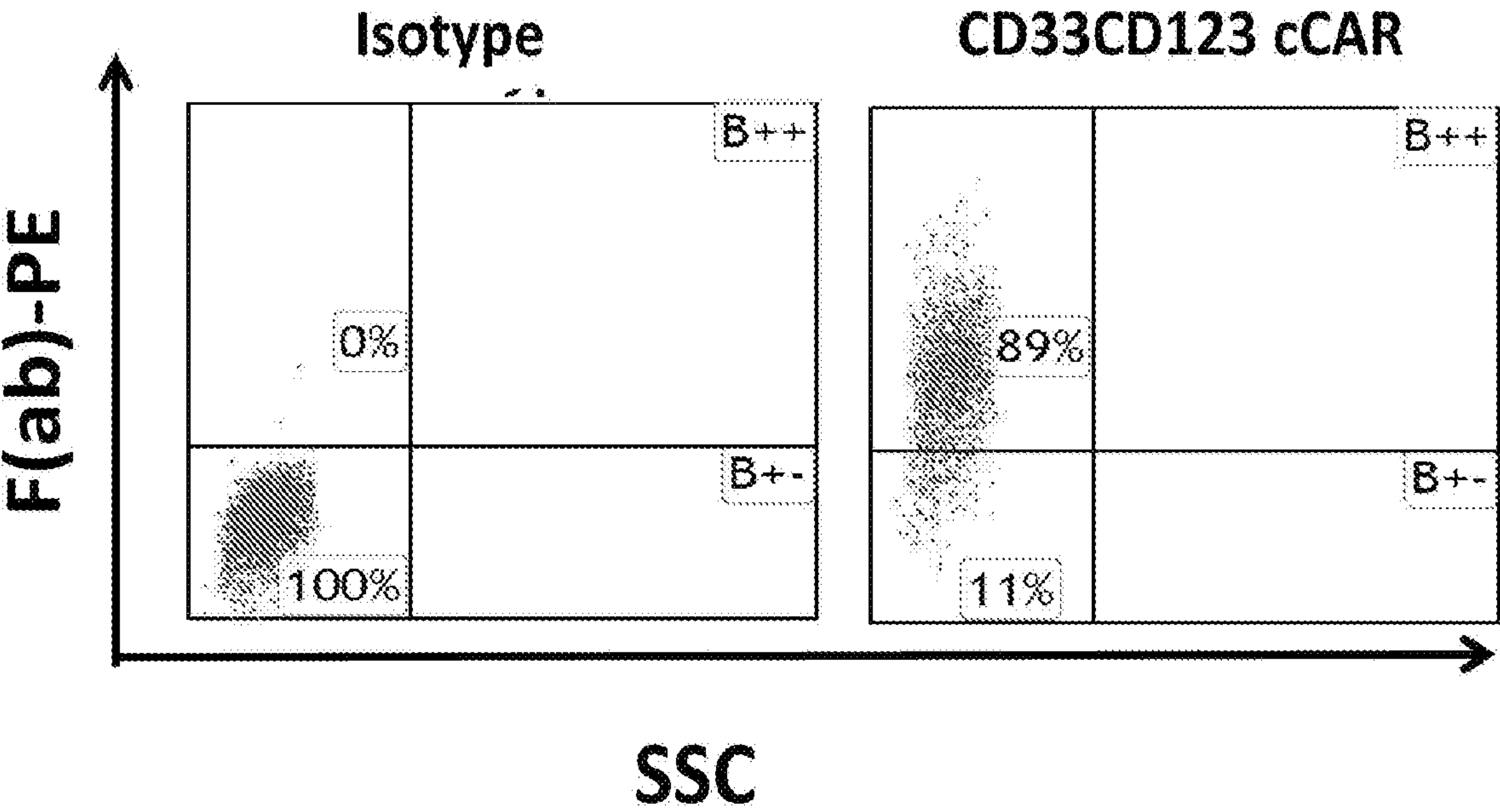


FIG. 9

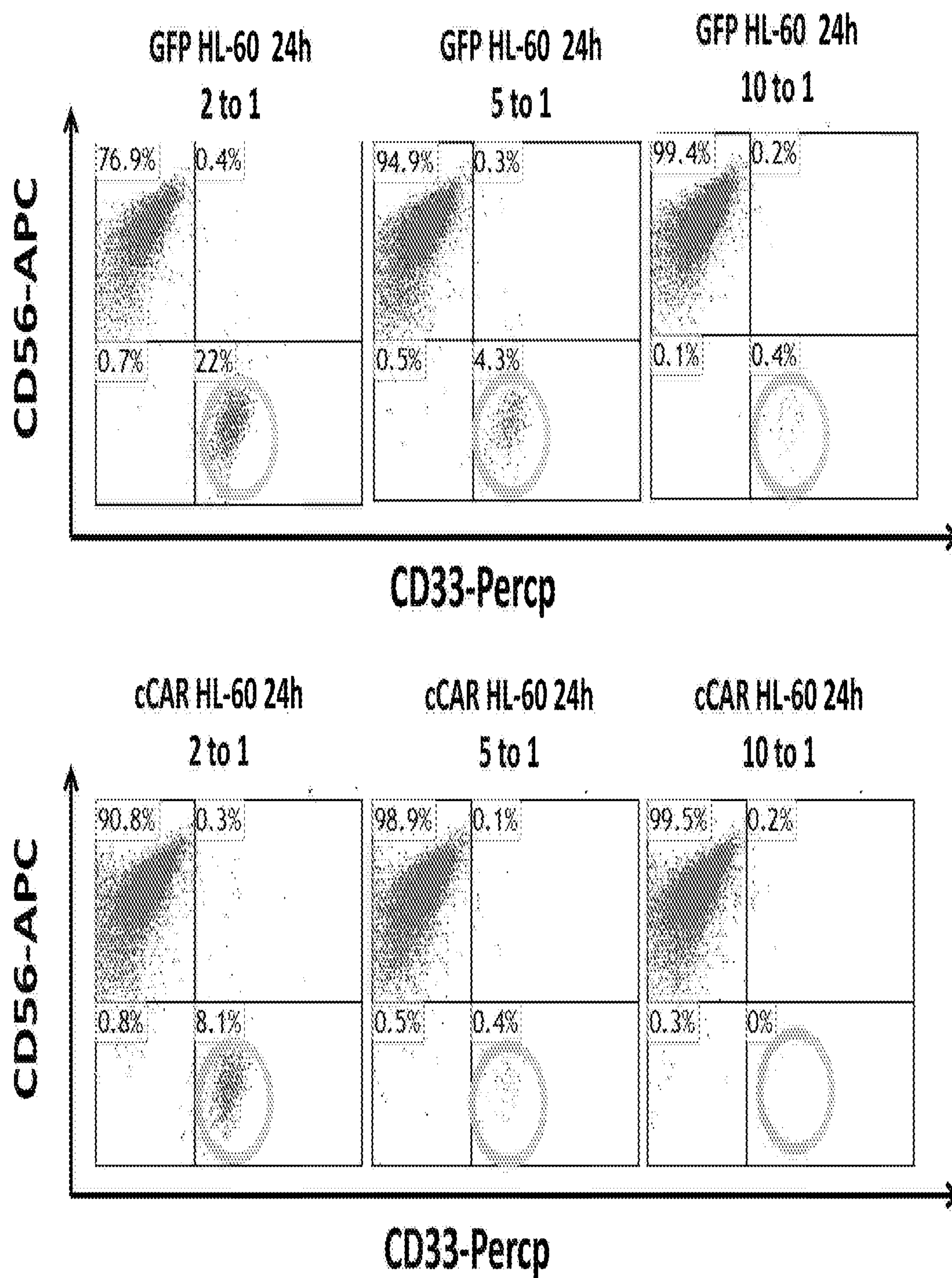


FIG. 10

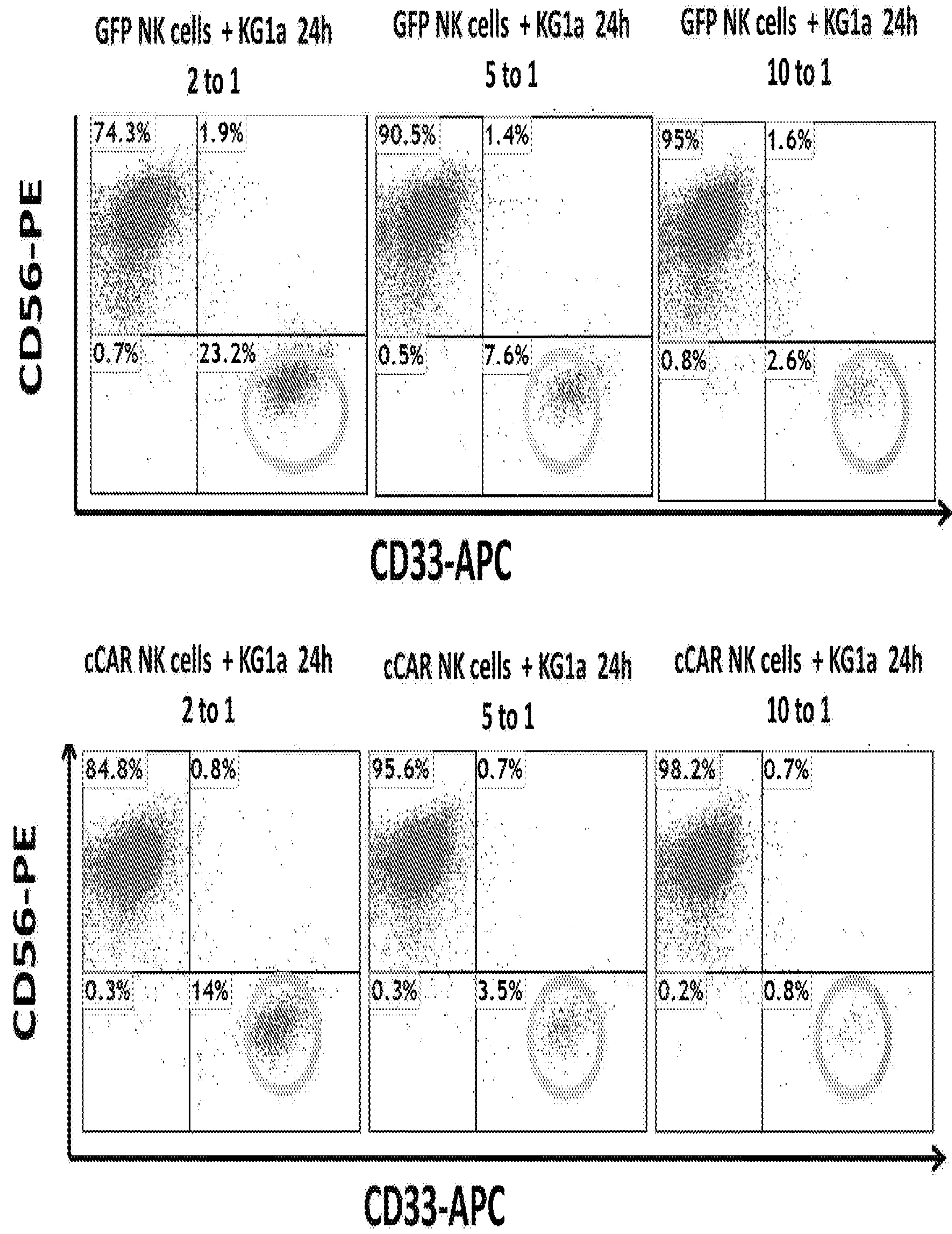


FIG. 11

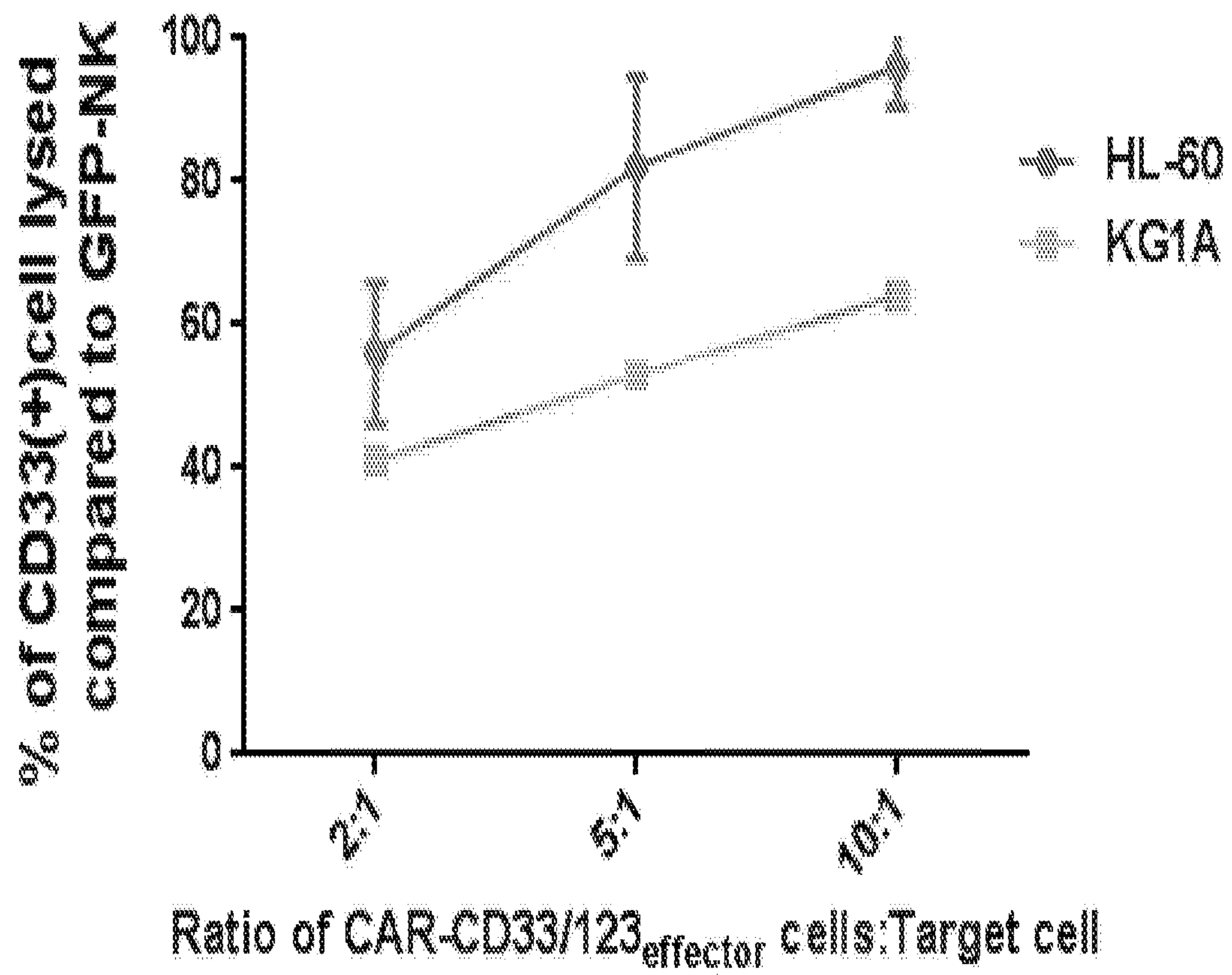


FIG. 12

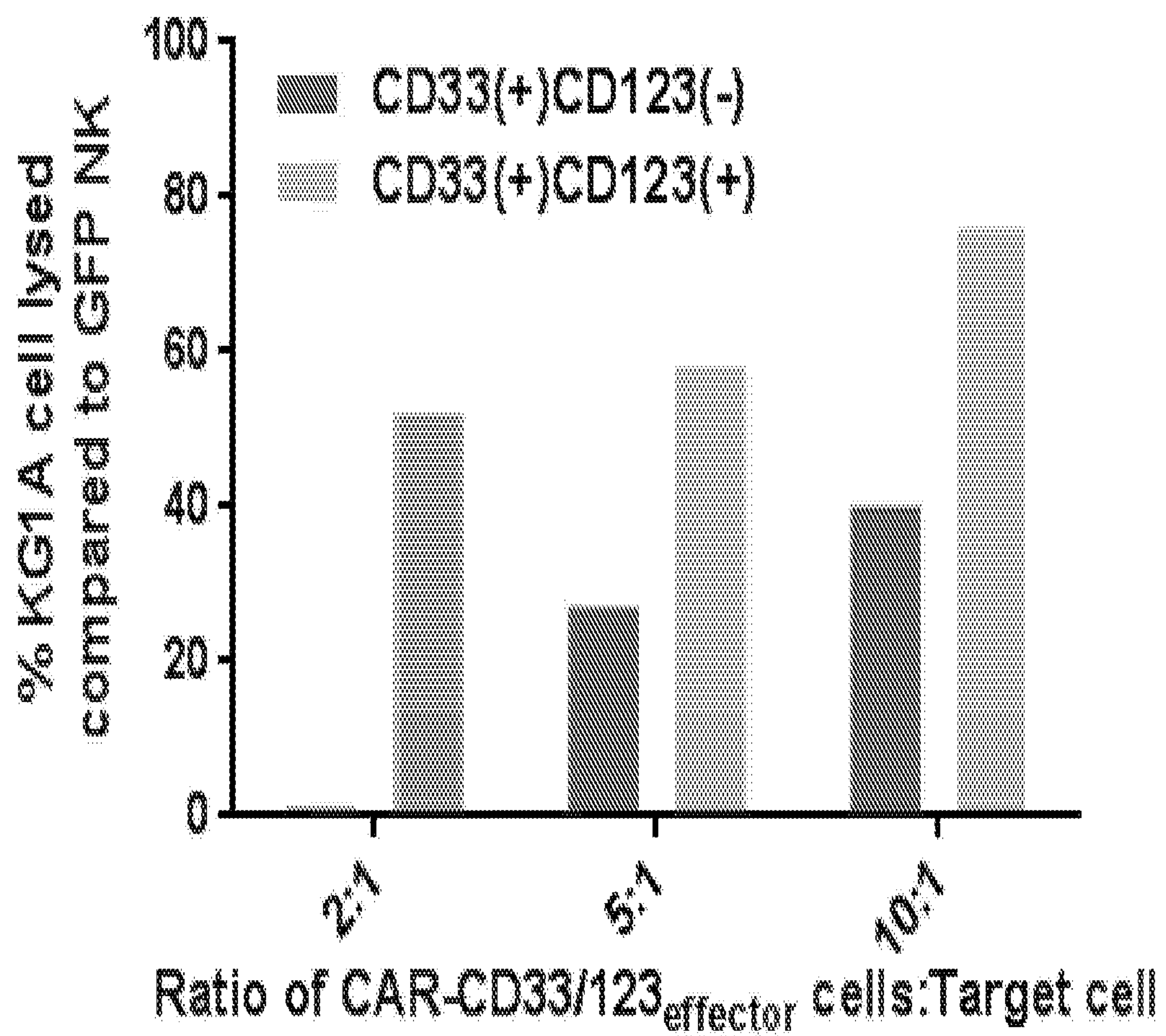


FIG. 13

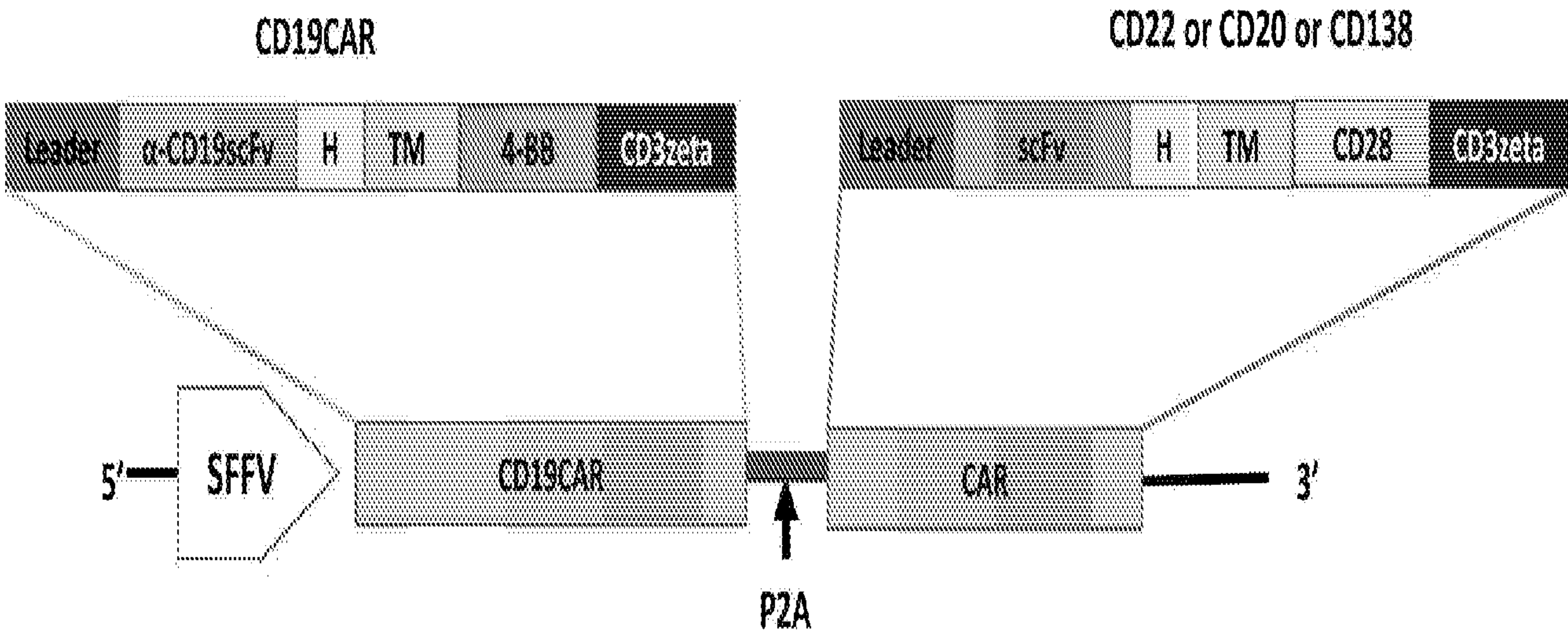


FIG. 14A

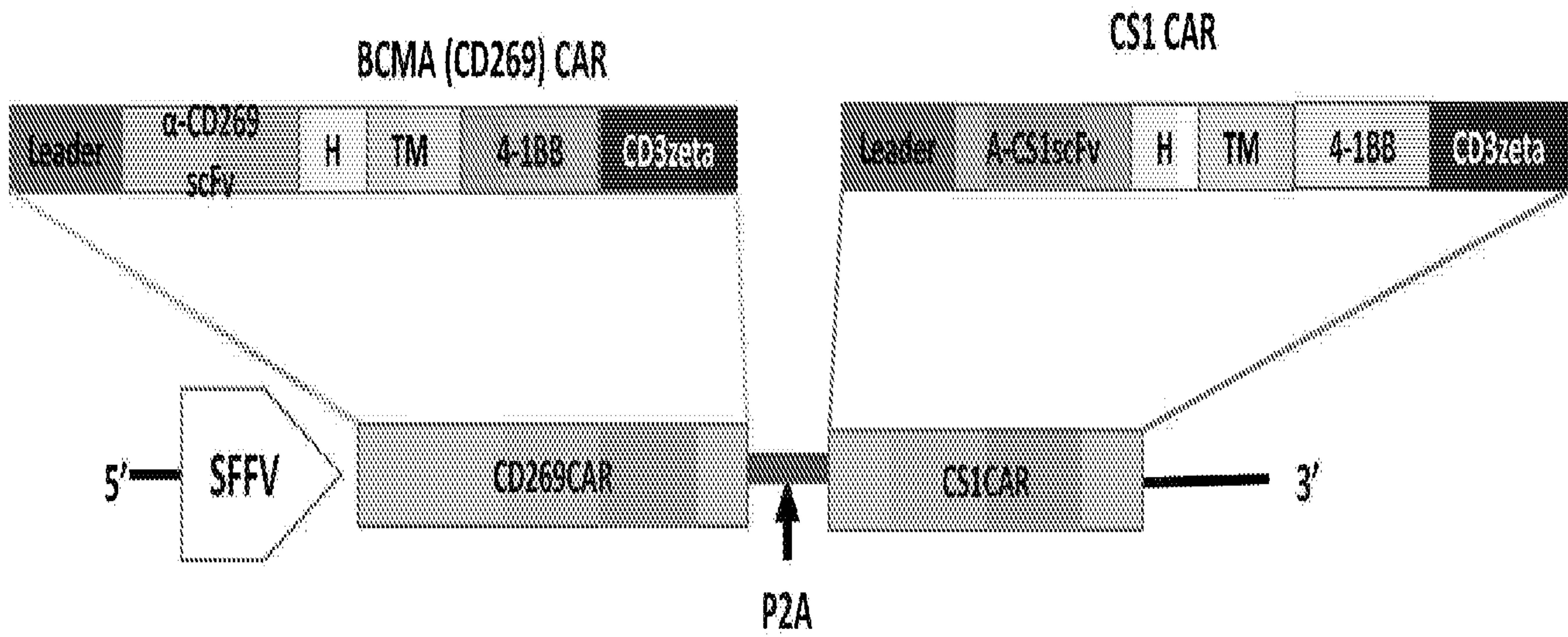


FIG. 14B

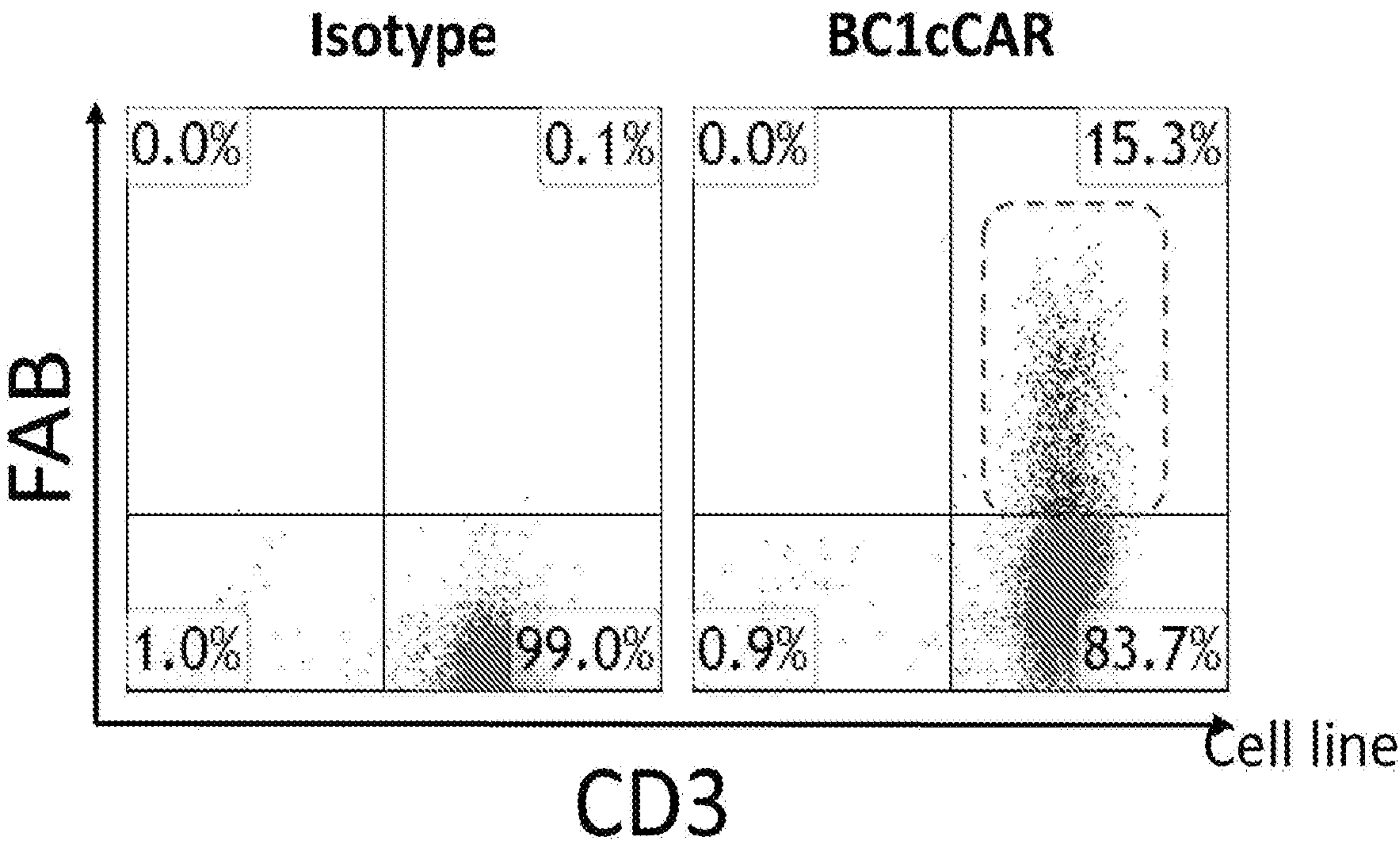


FIG. 14C

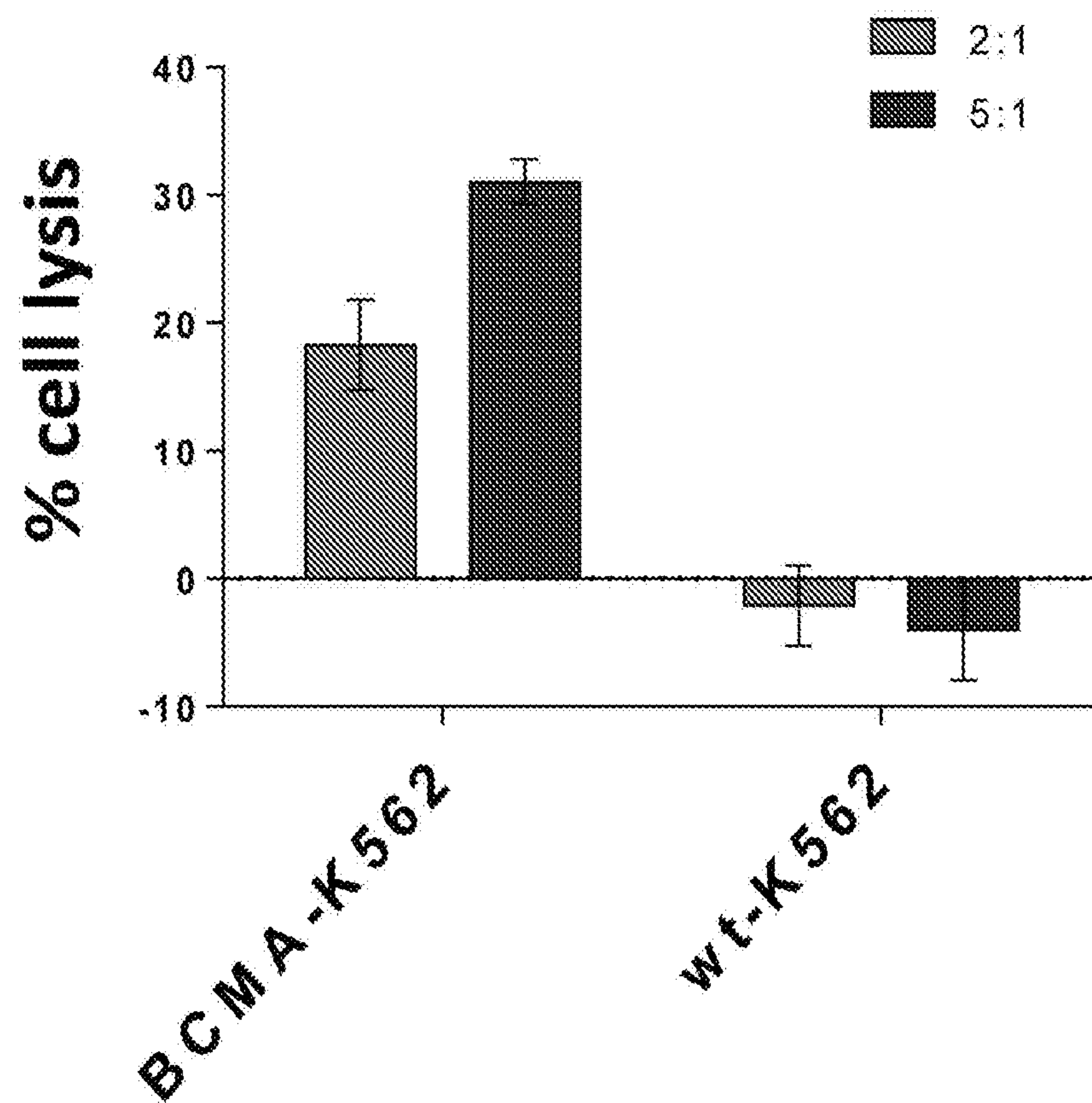


FIG. 14D

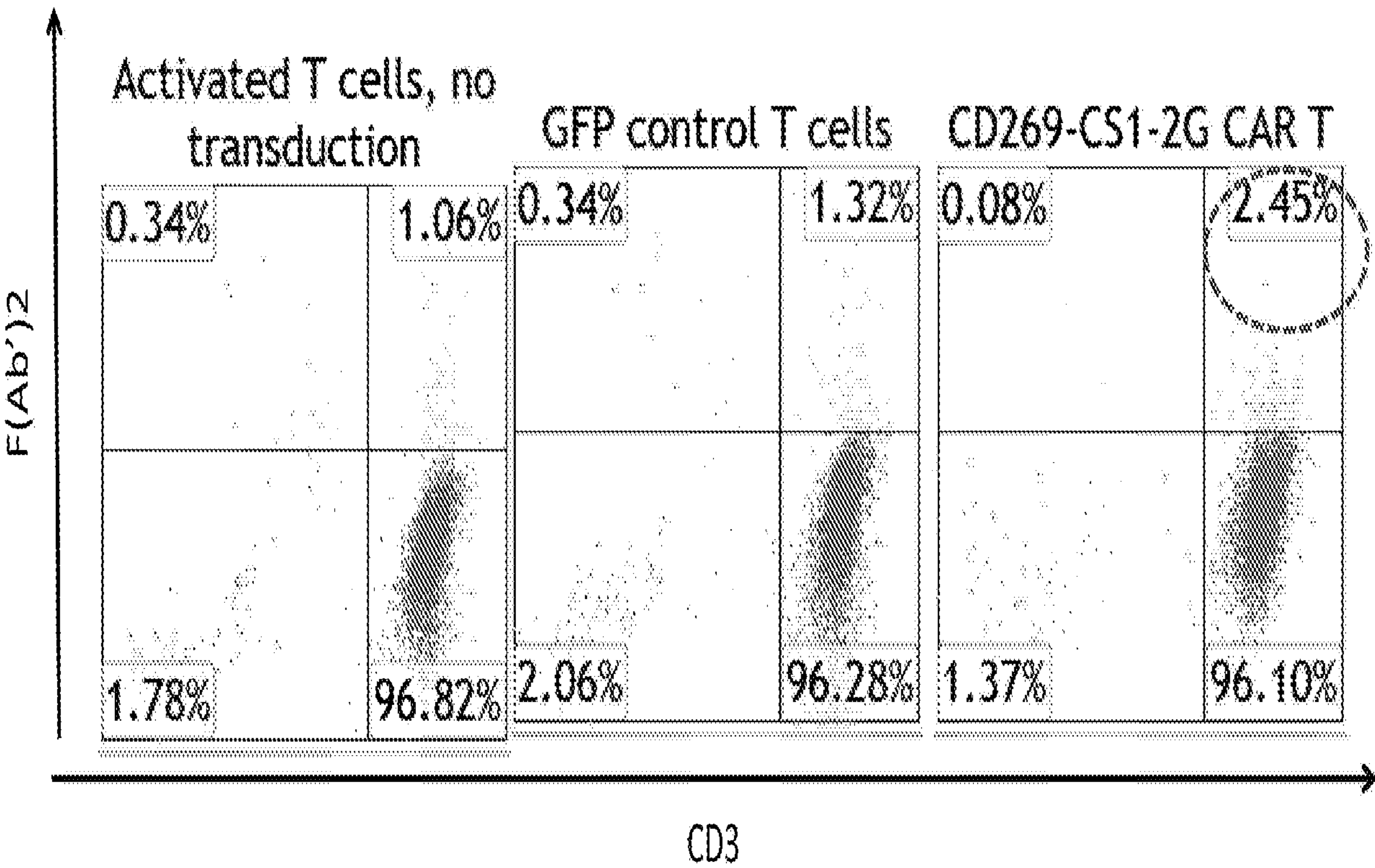


FIG. 14E

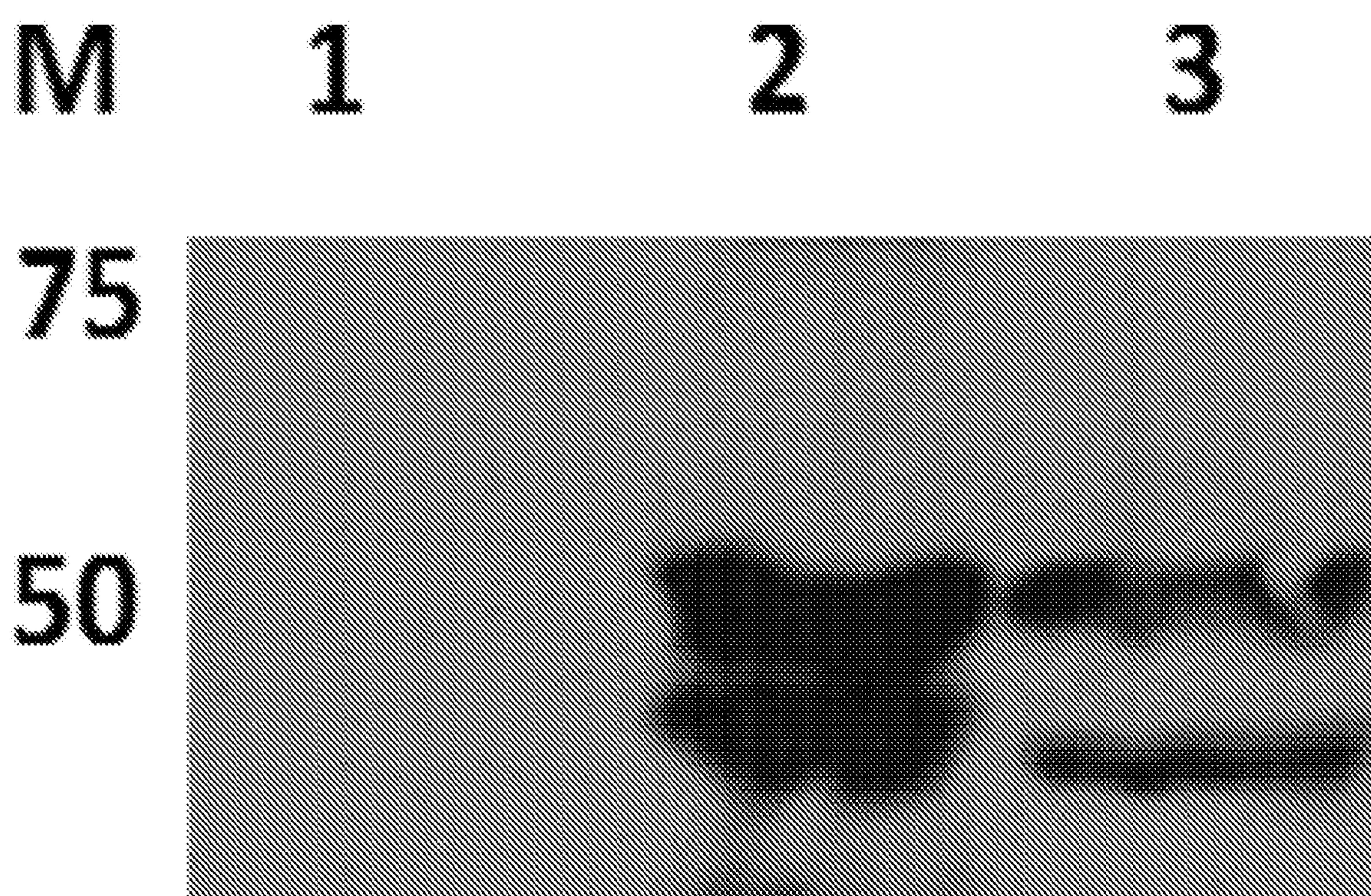


FIG. 15A

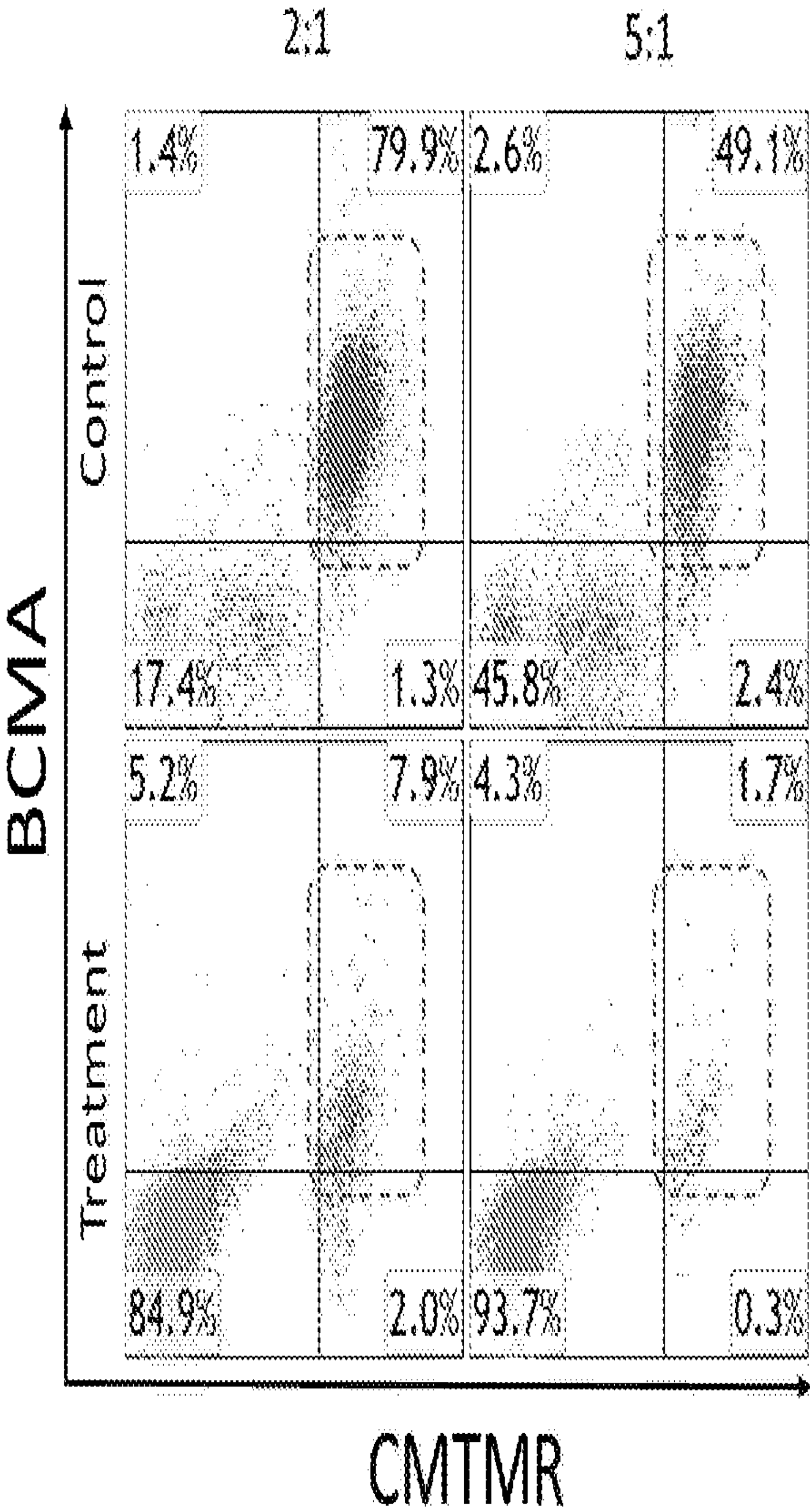


FIG. 15B

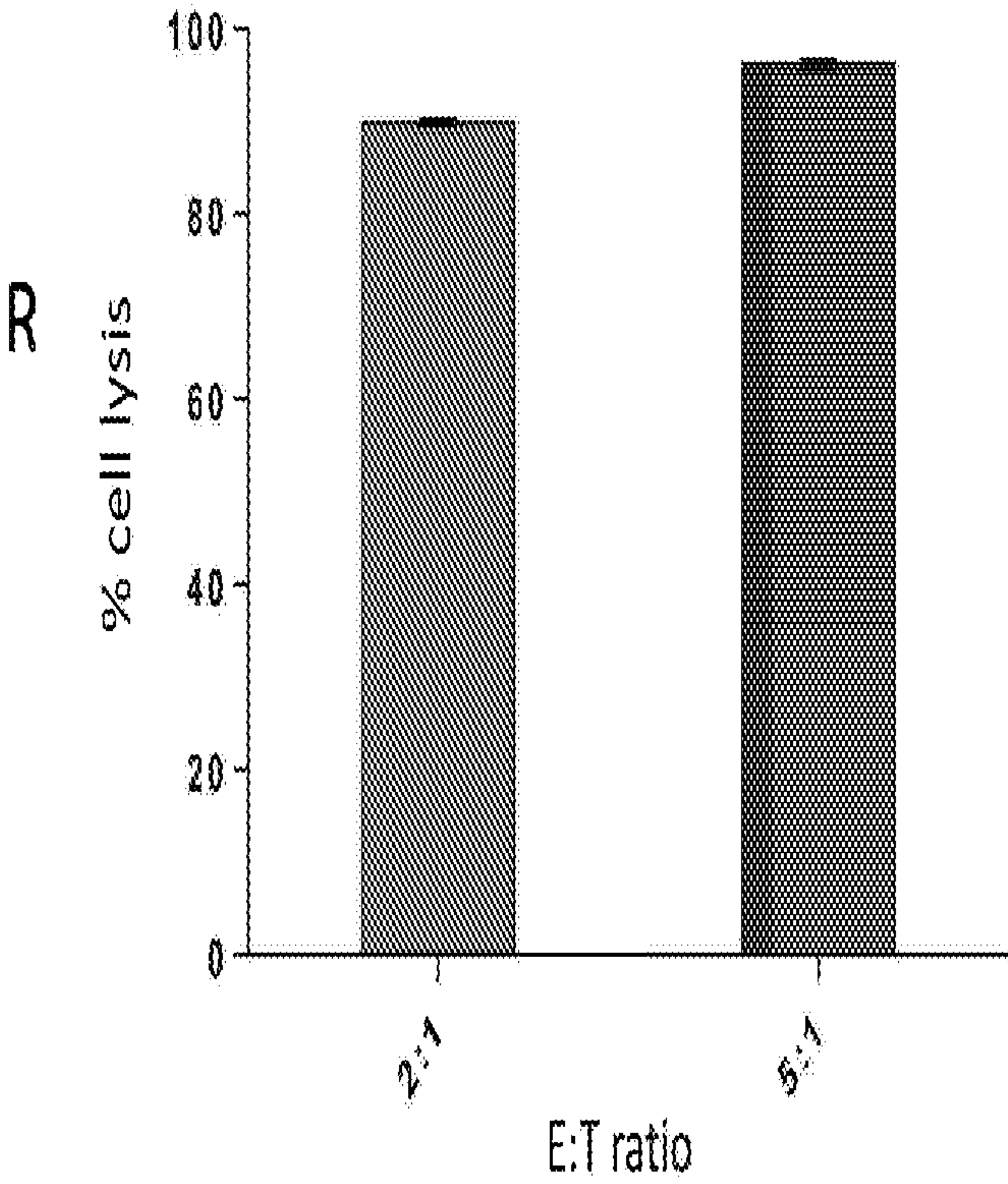


FIG. 16A

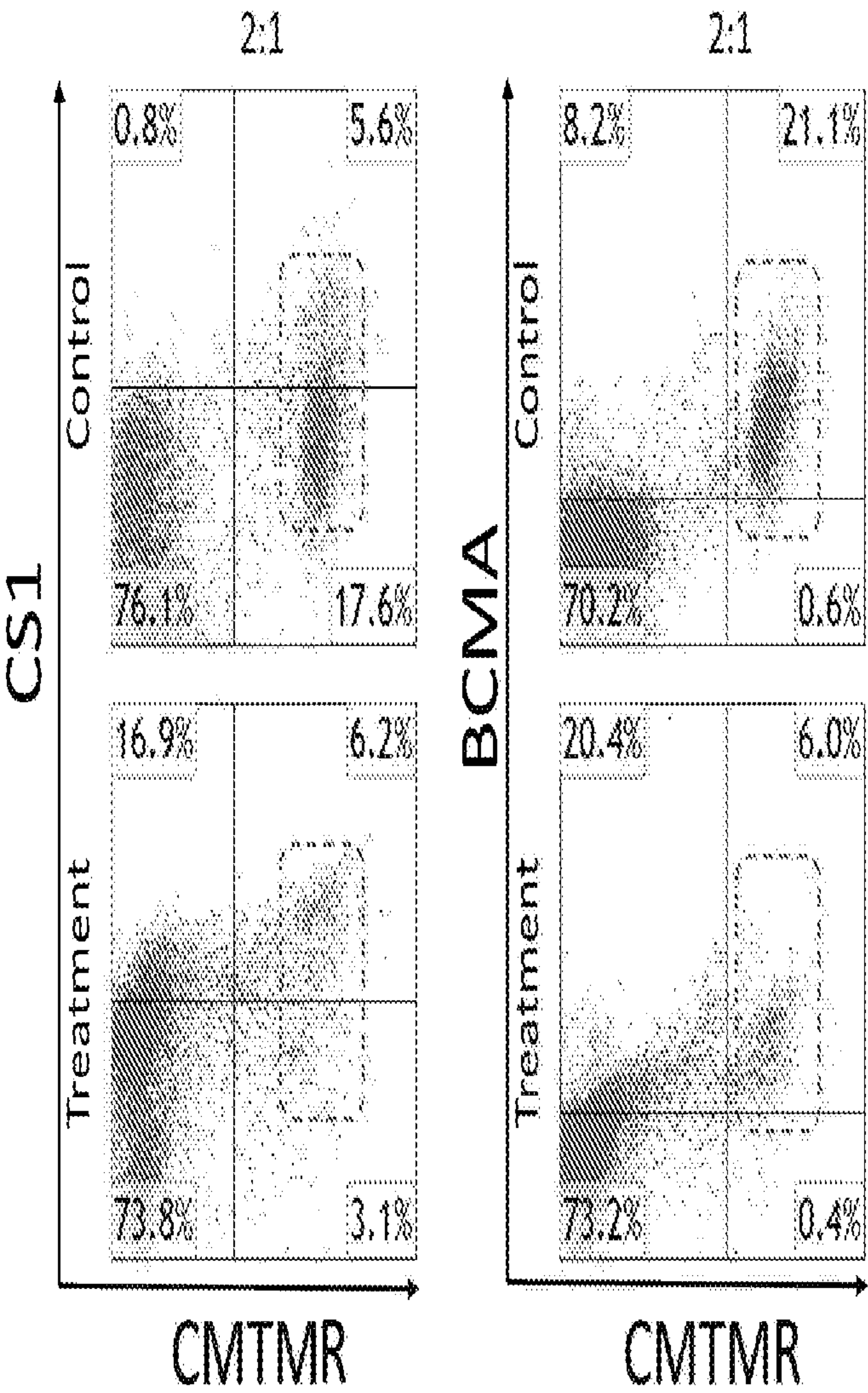


FIG. 16B

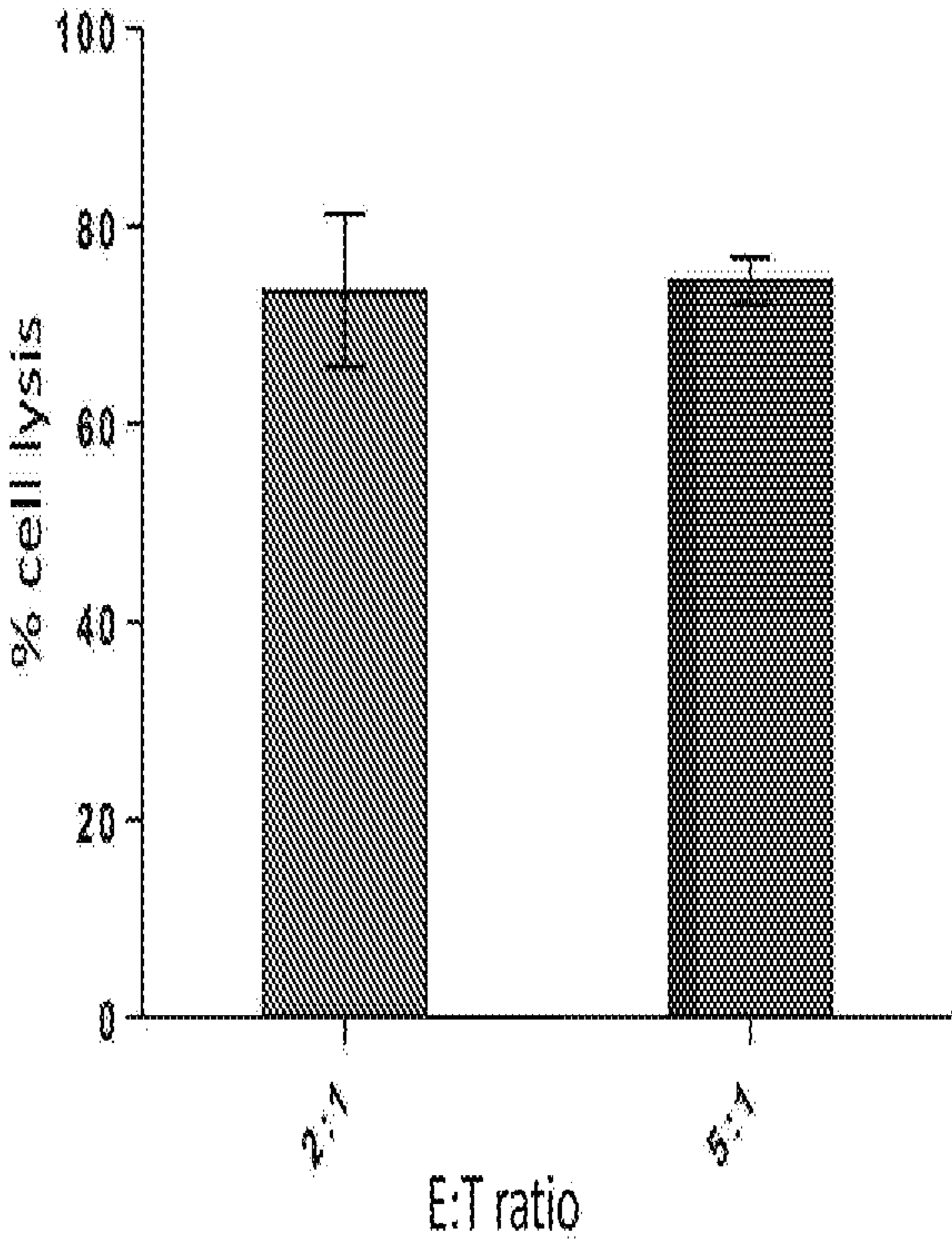


FIG. 17A

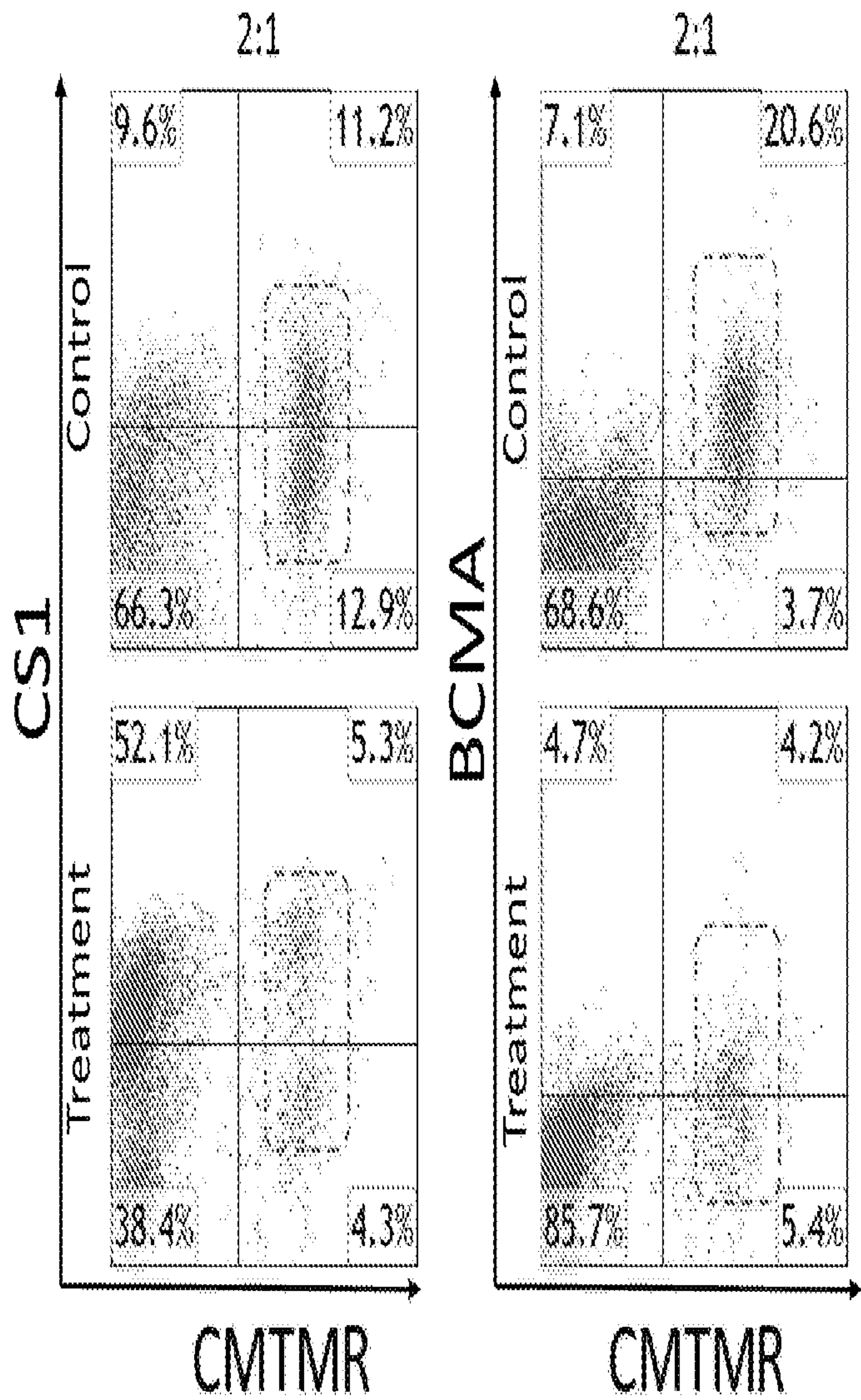


FIG. 17B

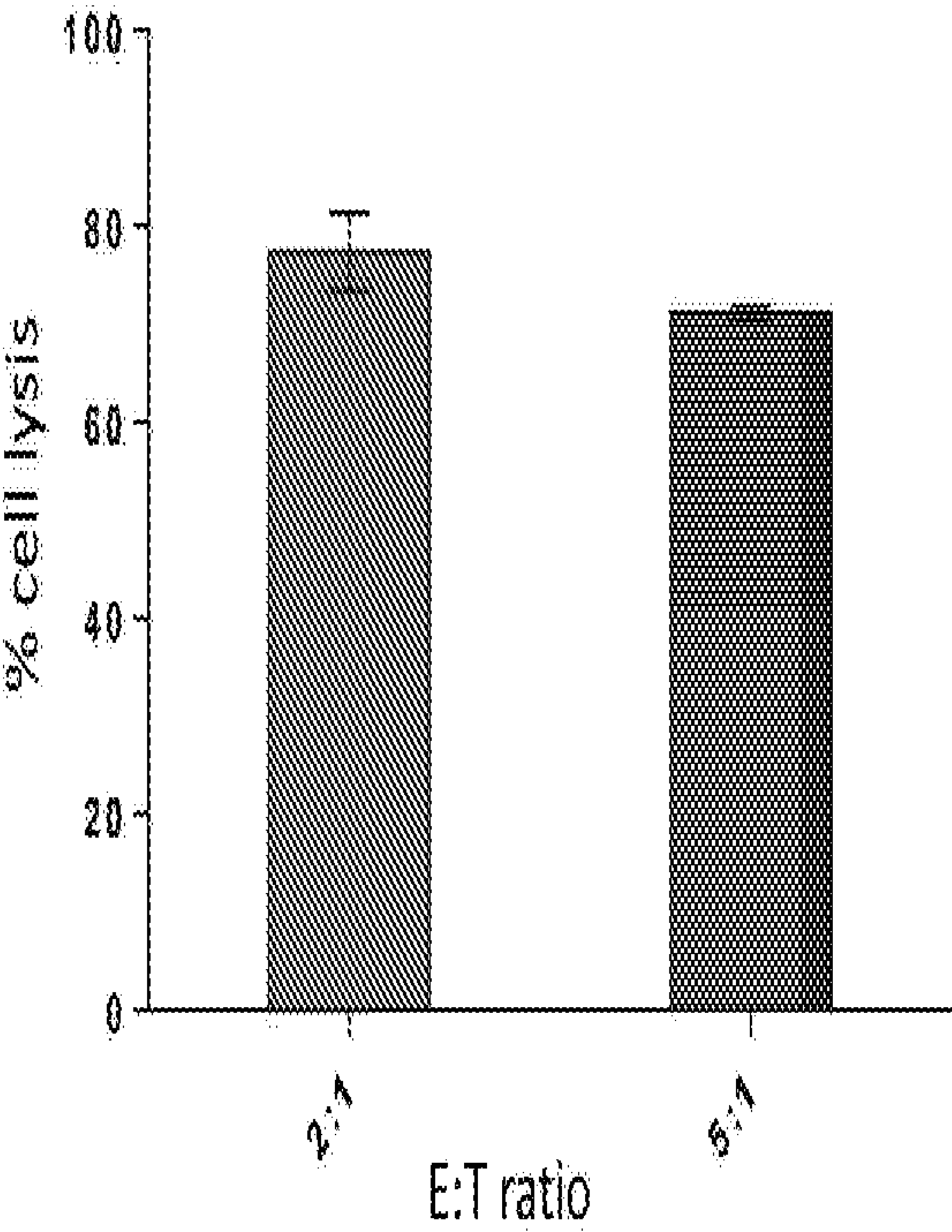


FIG. 18A

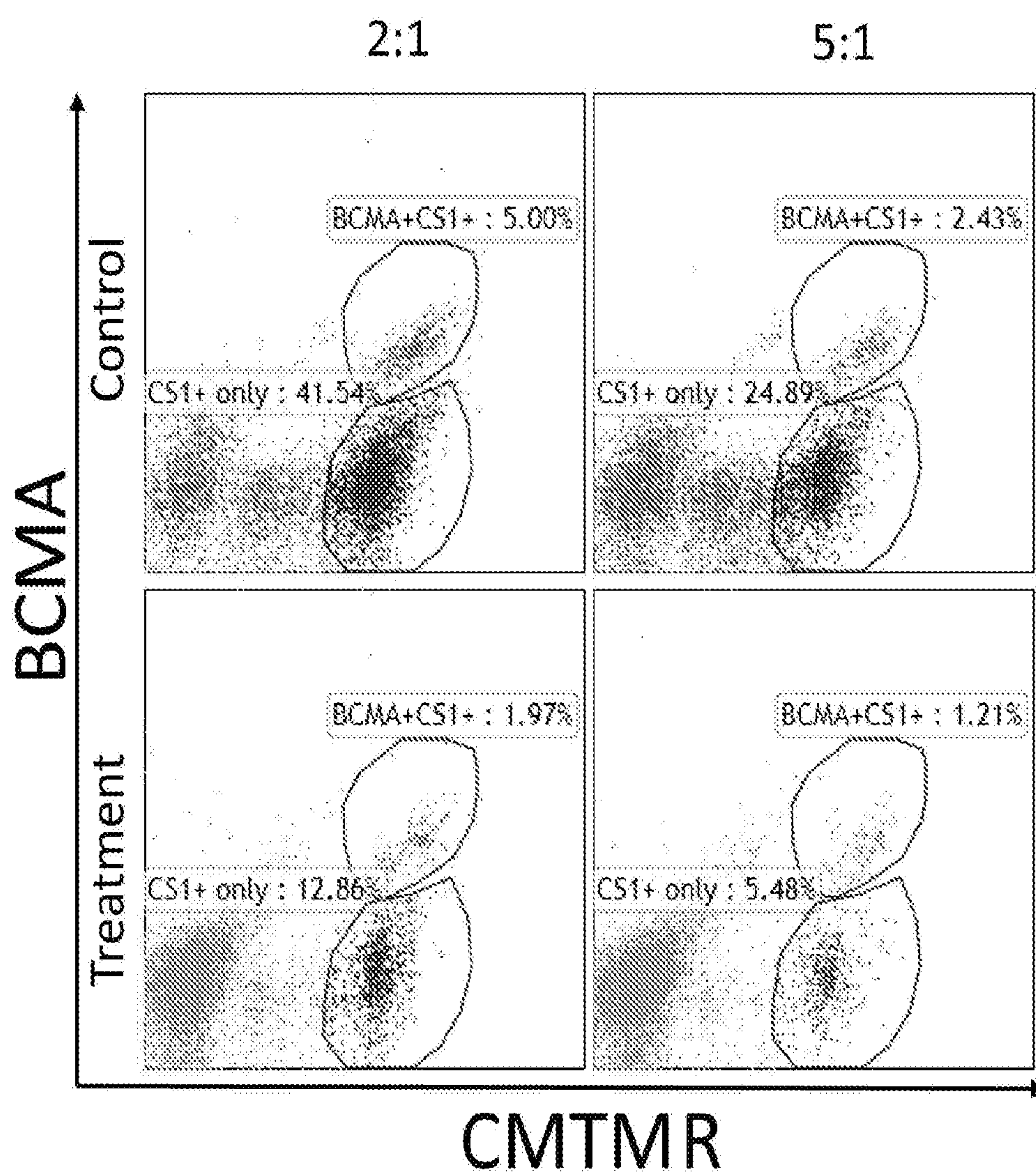


FIG. 18B

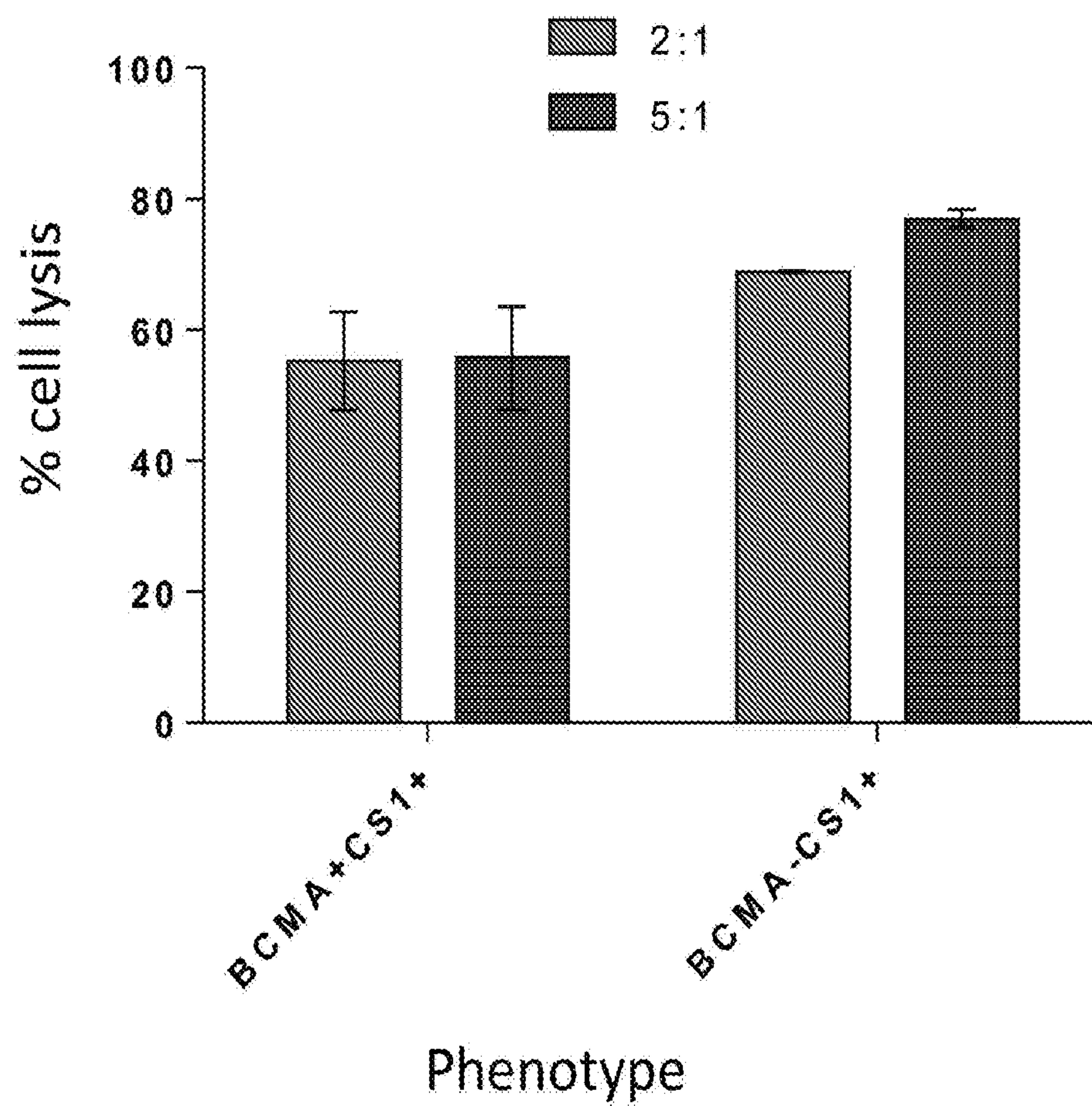


FIG. 19A

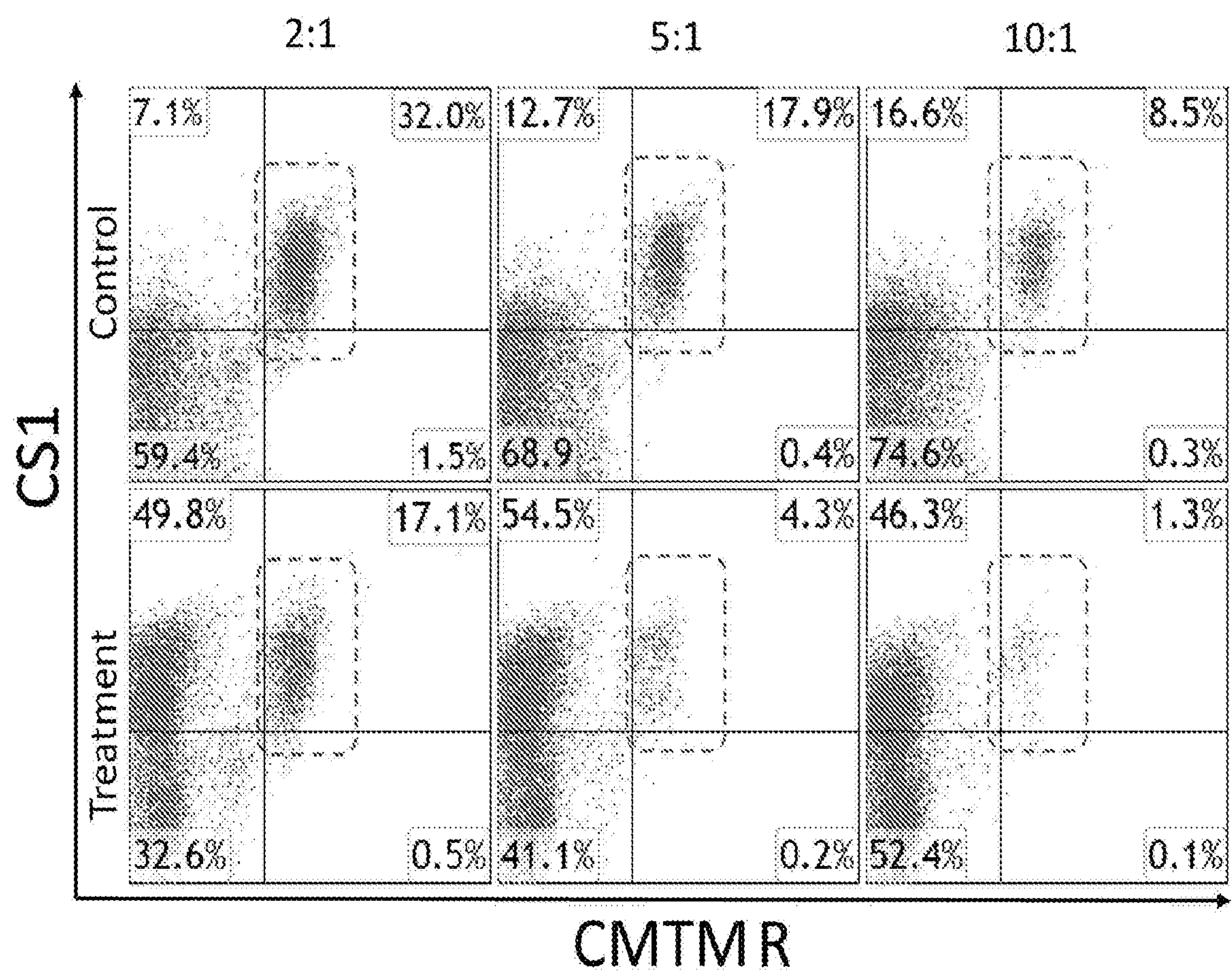


FIG. 19B

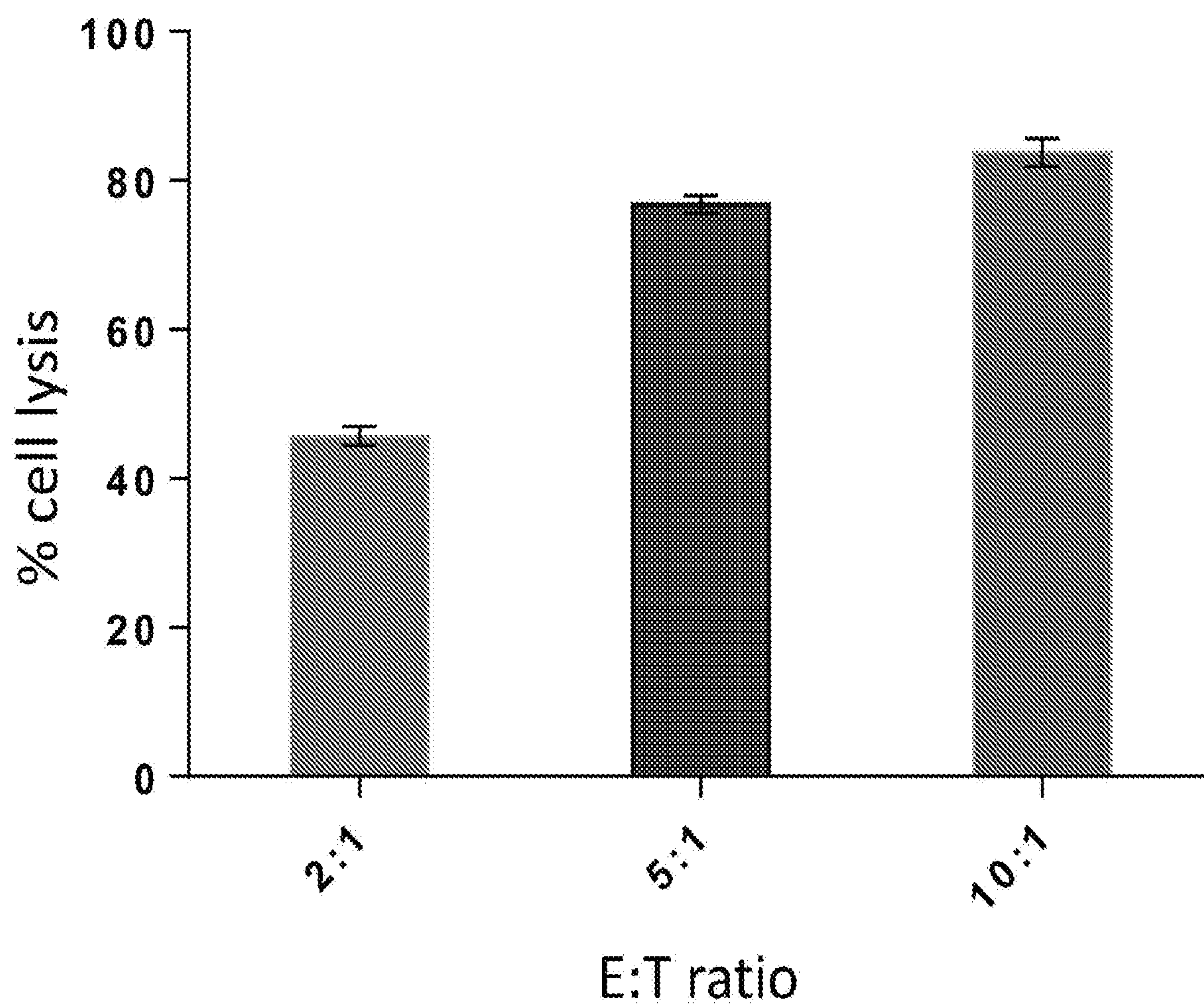


FIG. 20A

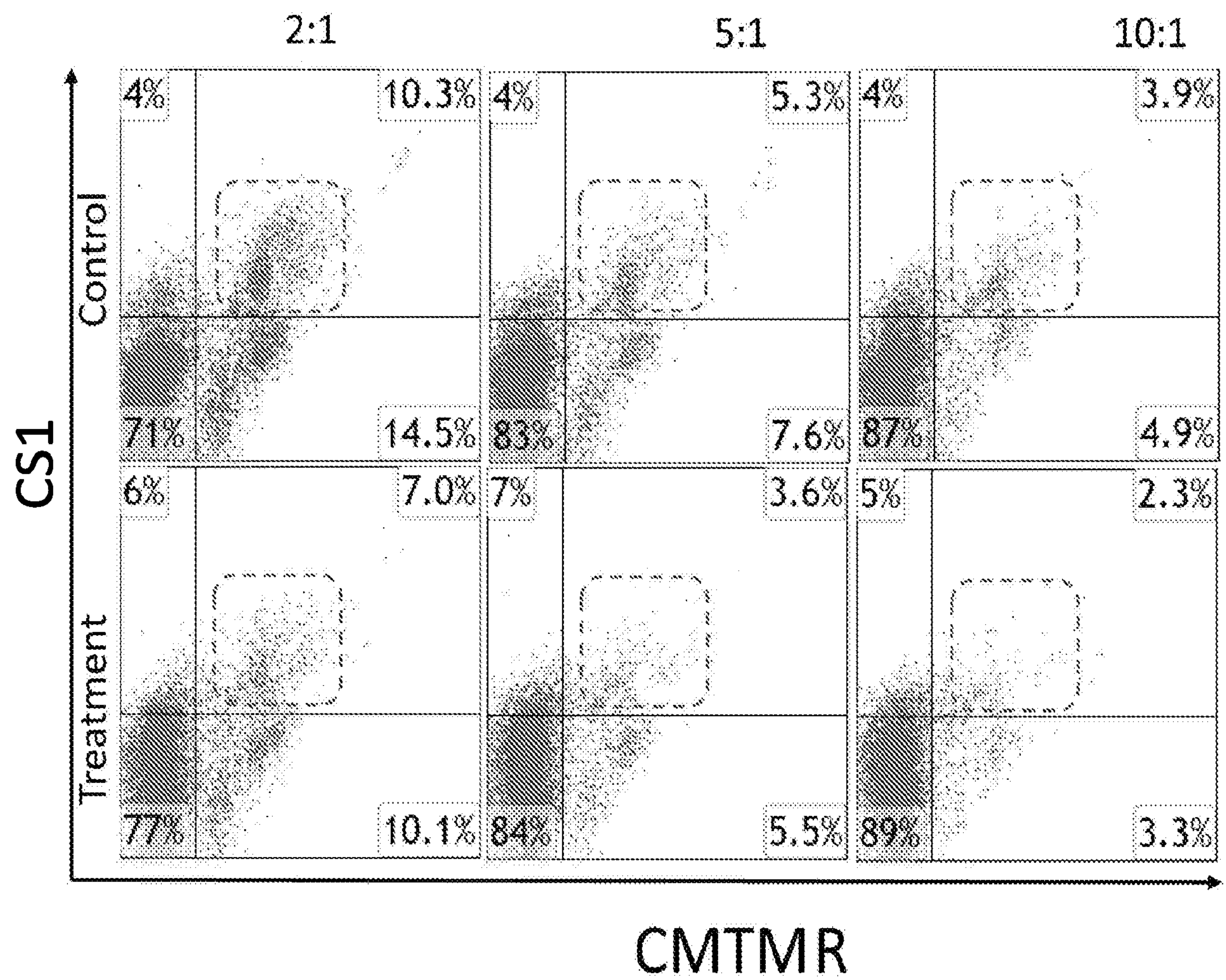


FIG. 20B

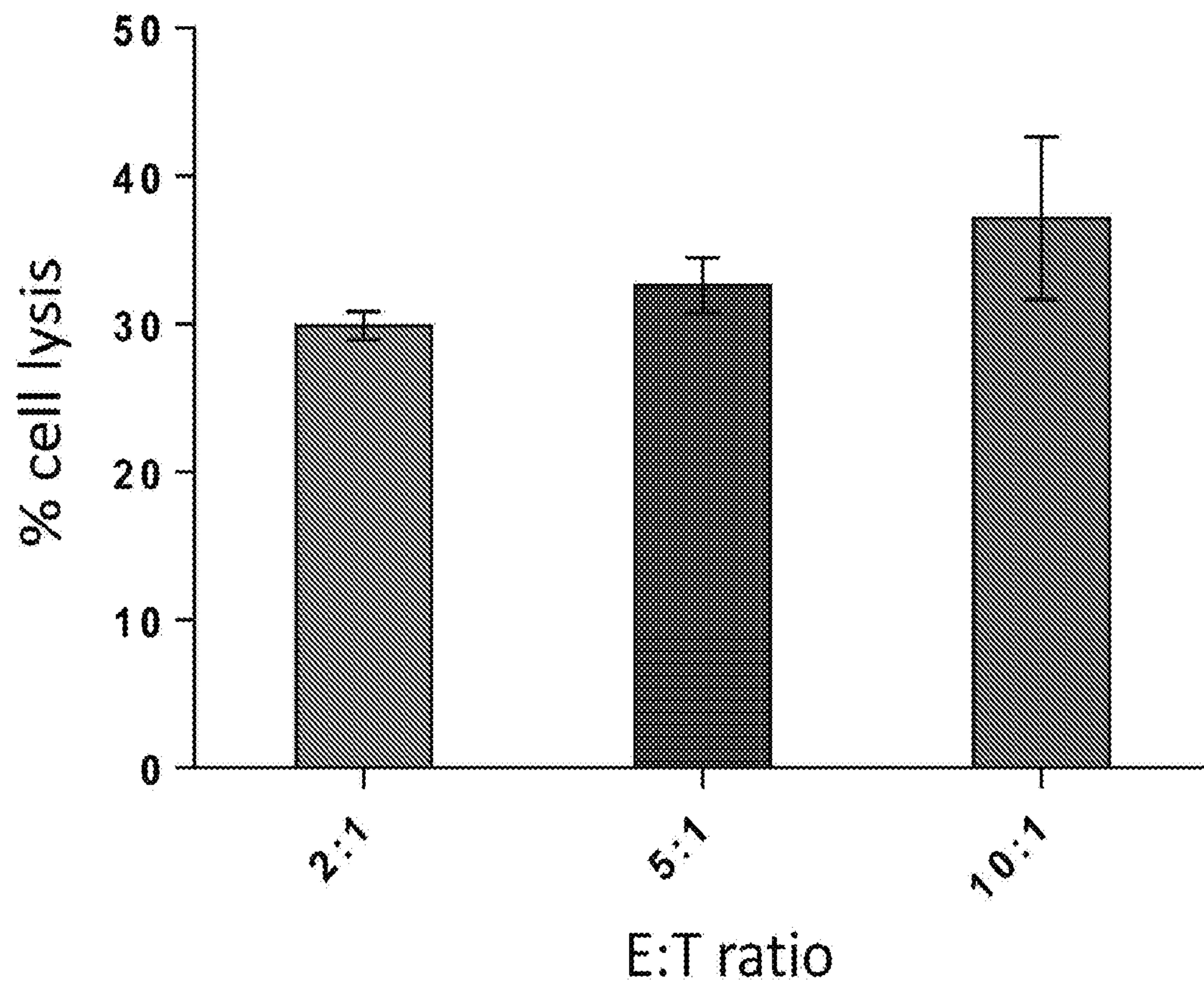


FIG. 21

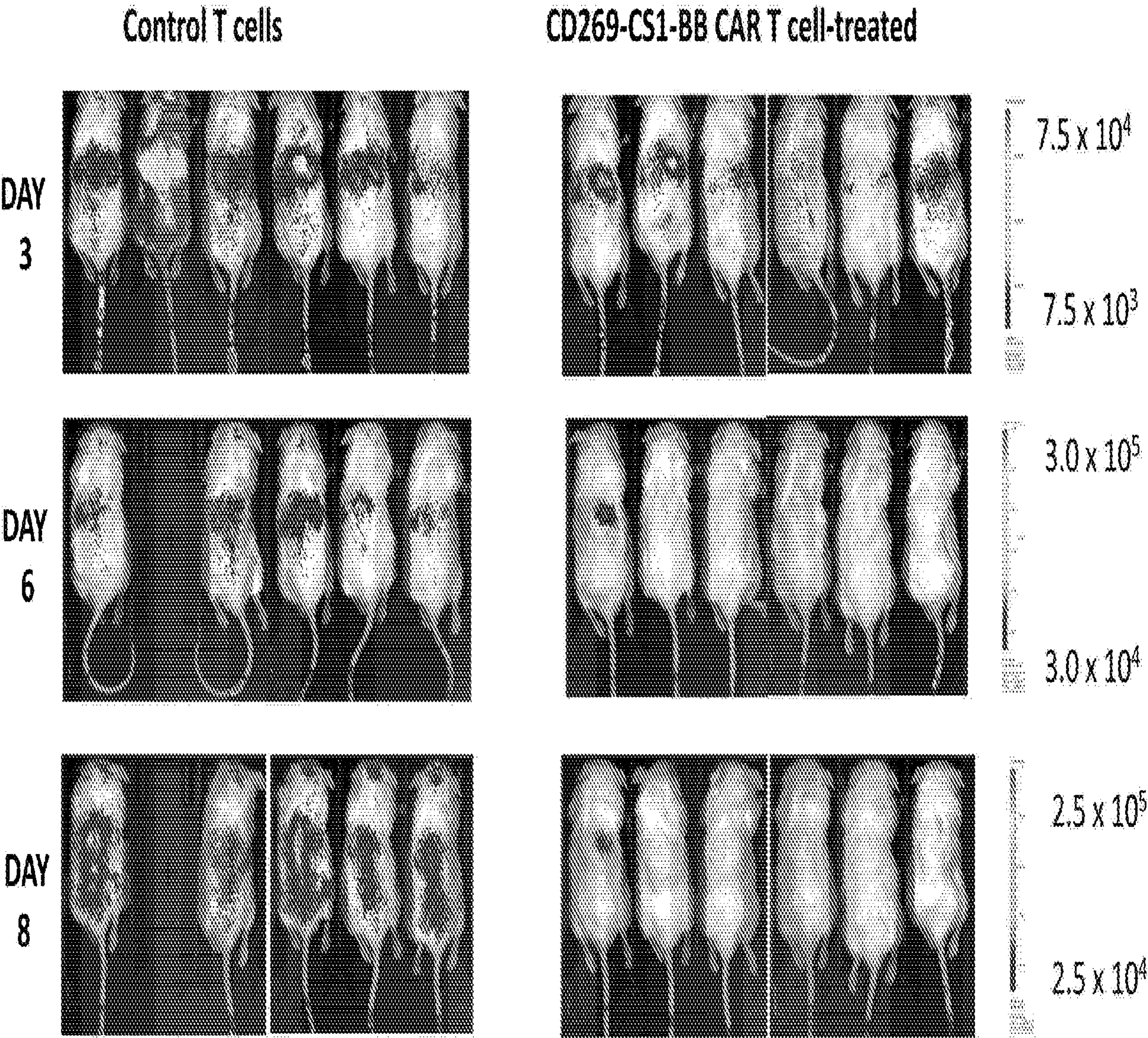


FIG. 22

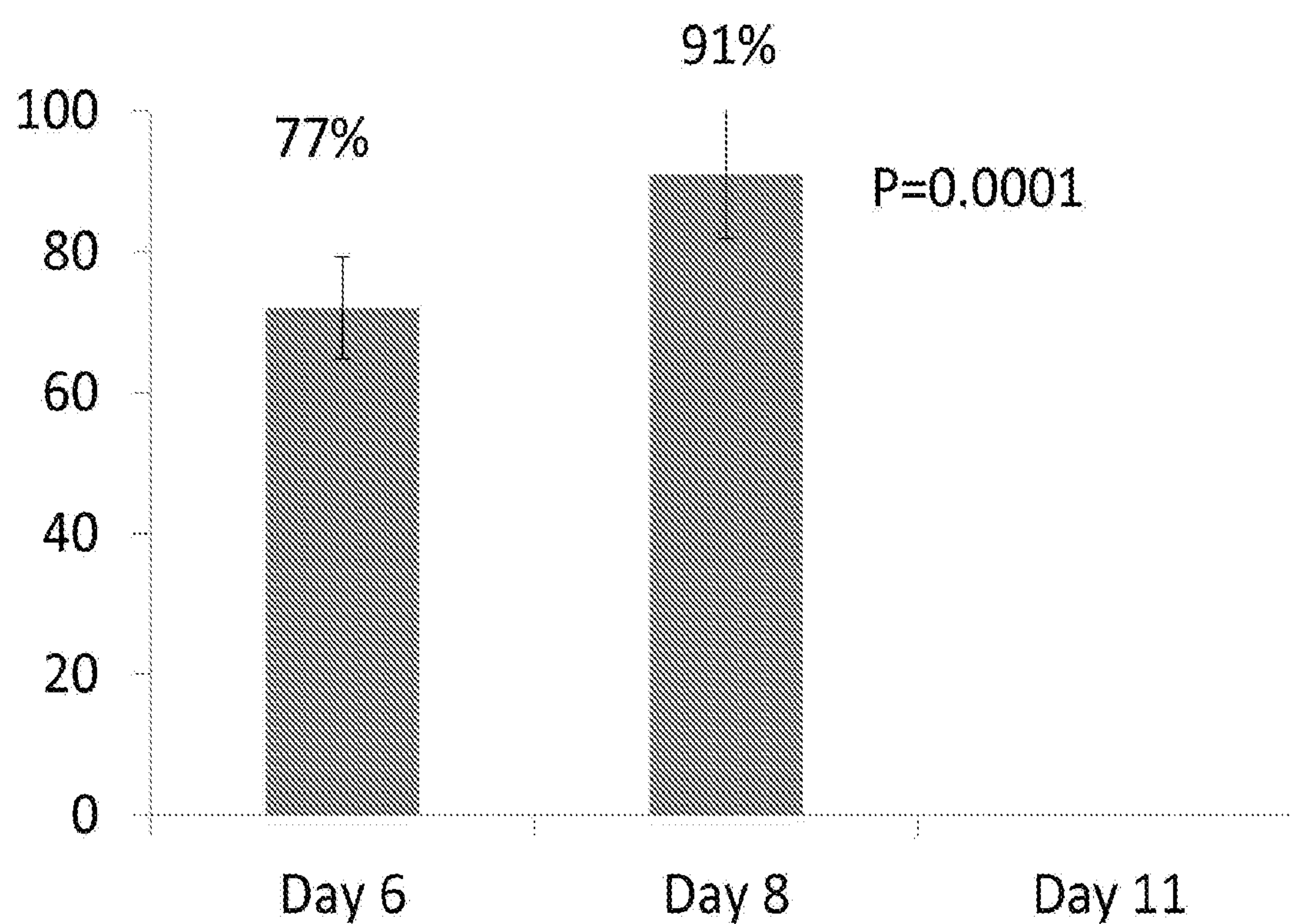


FIG. 23

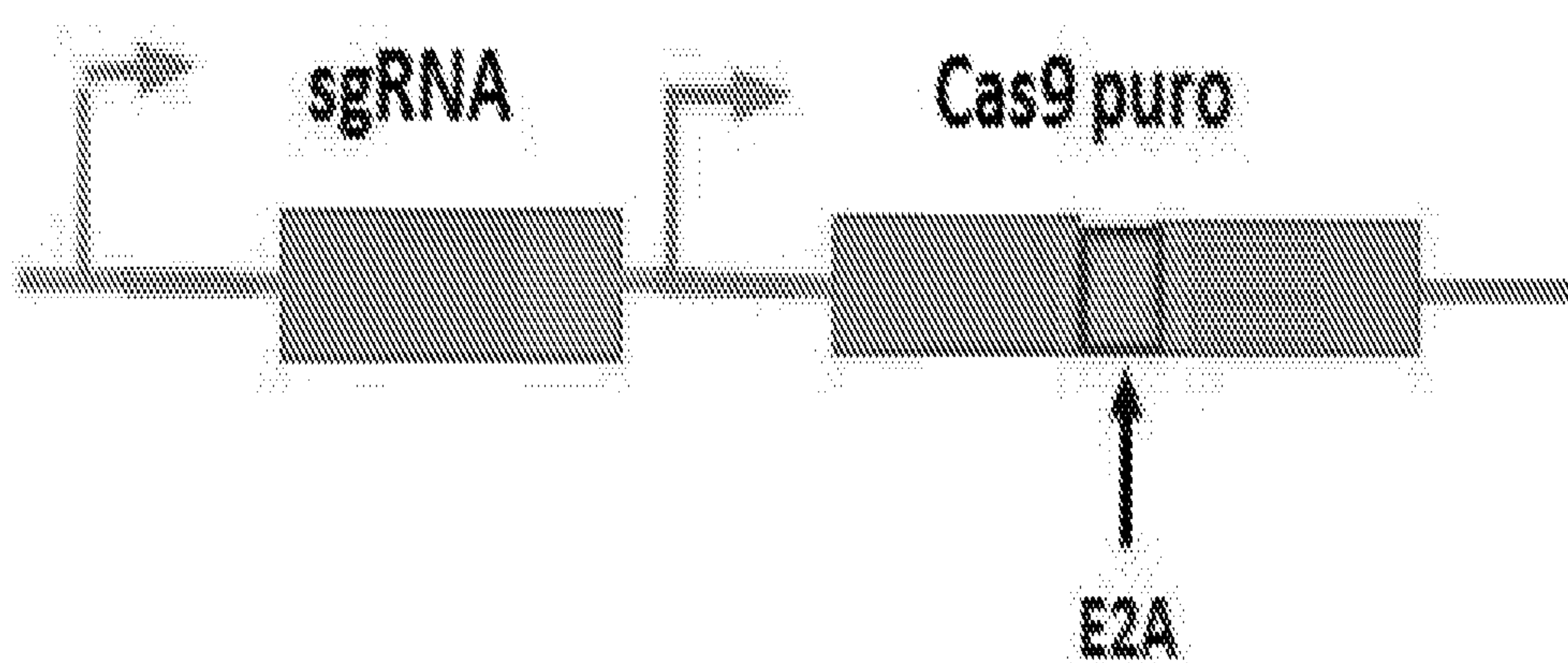
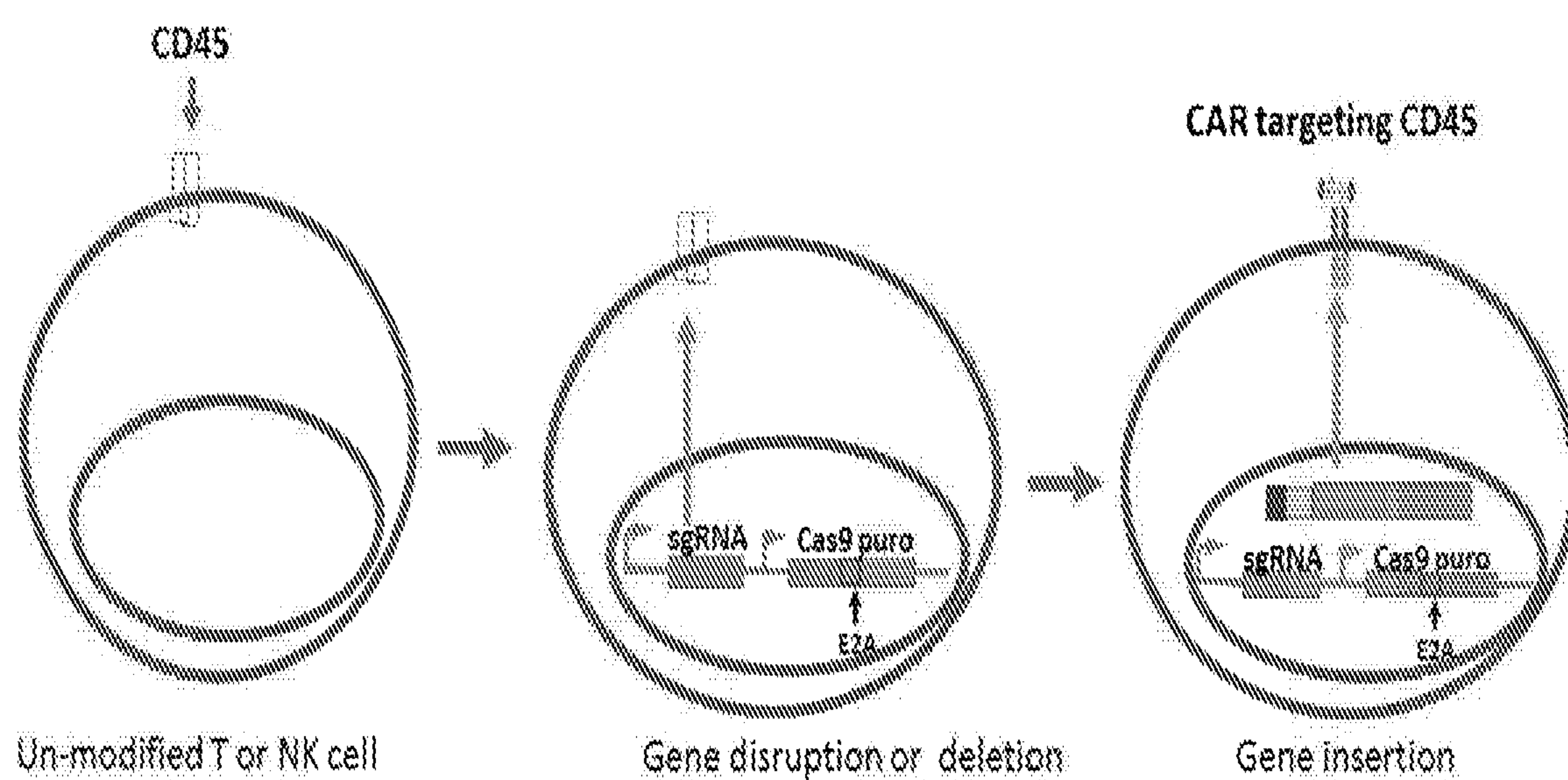


FIG. 24



- 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

FIG. 25

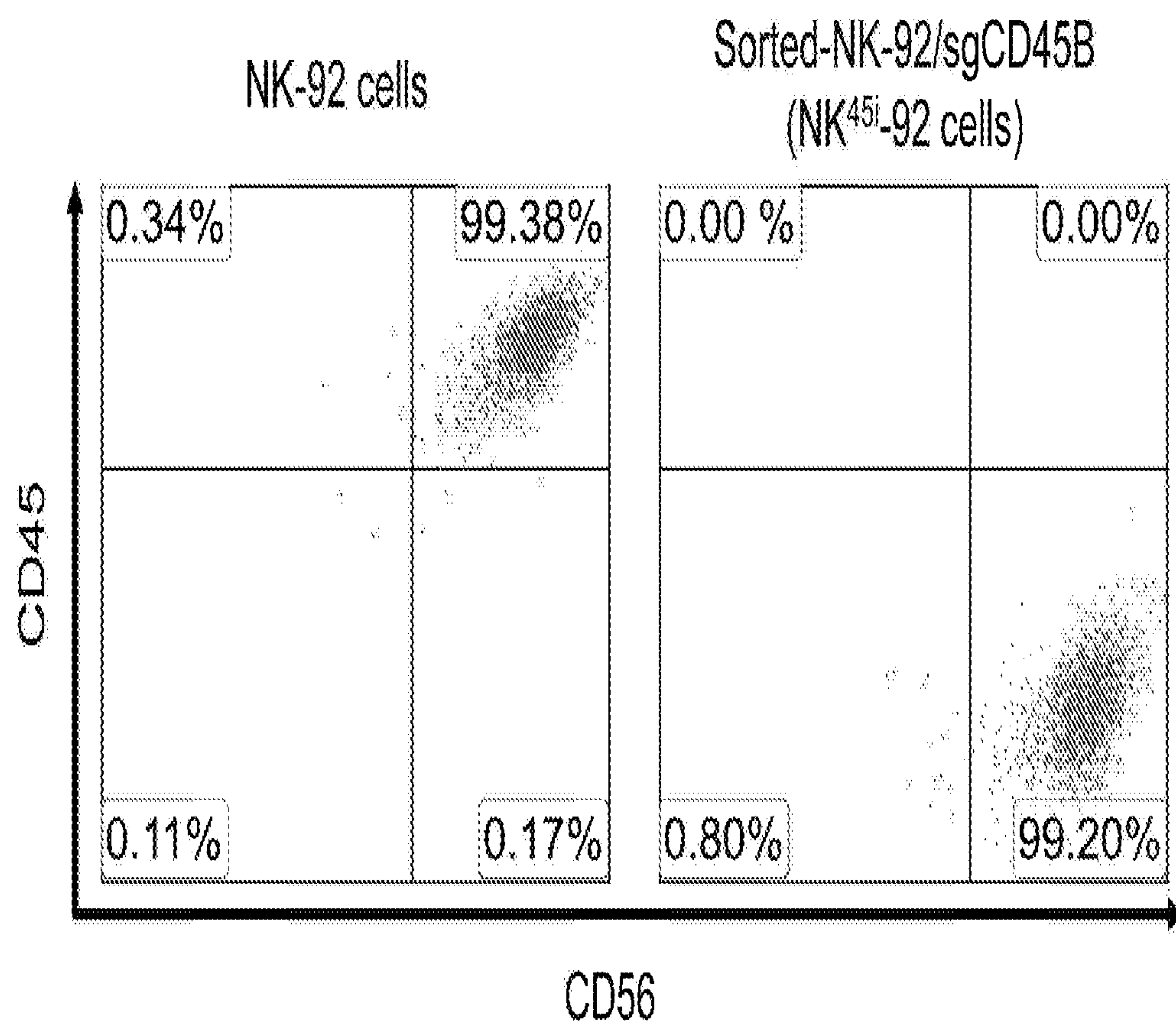
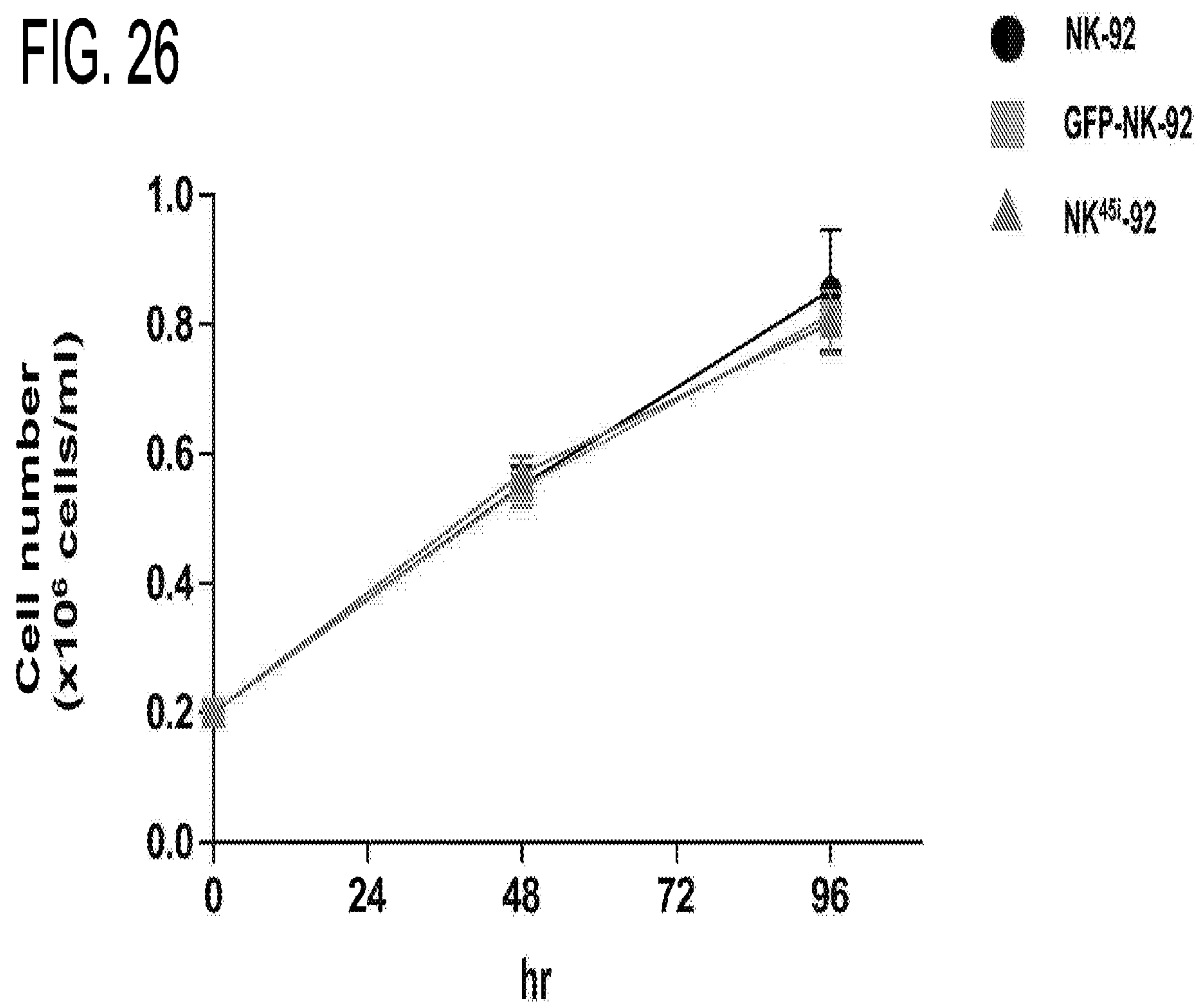


FIG. 26



24 well, duplicate, n=3

IL-2 was added at 48hr time point

FIG. 27A

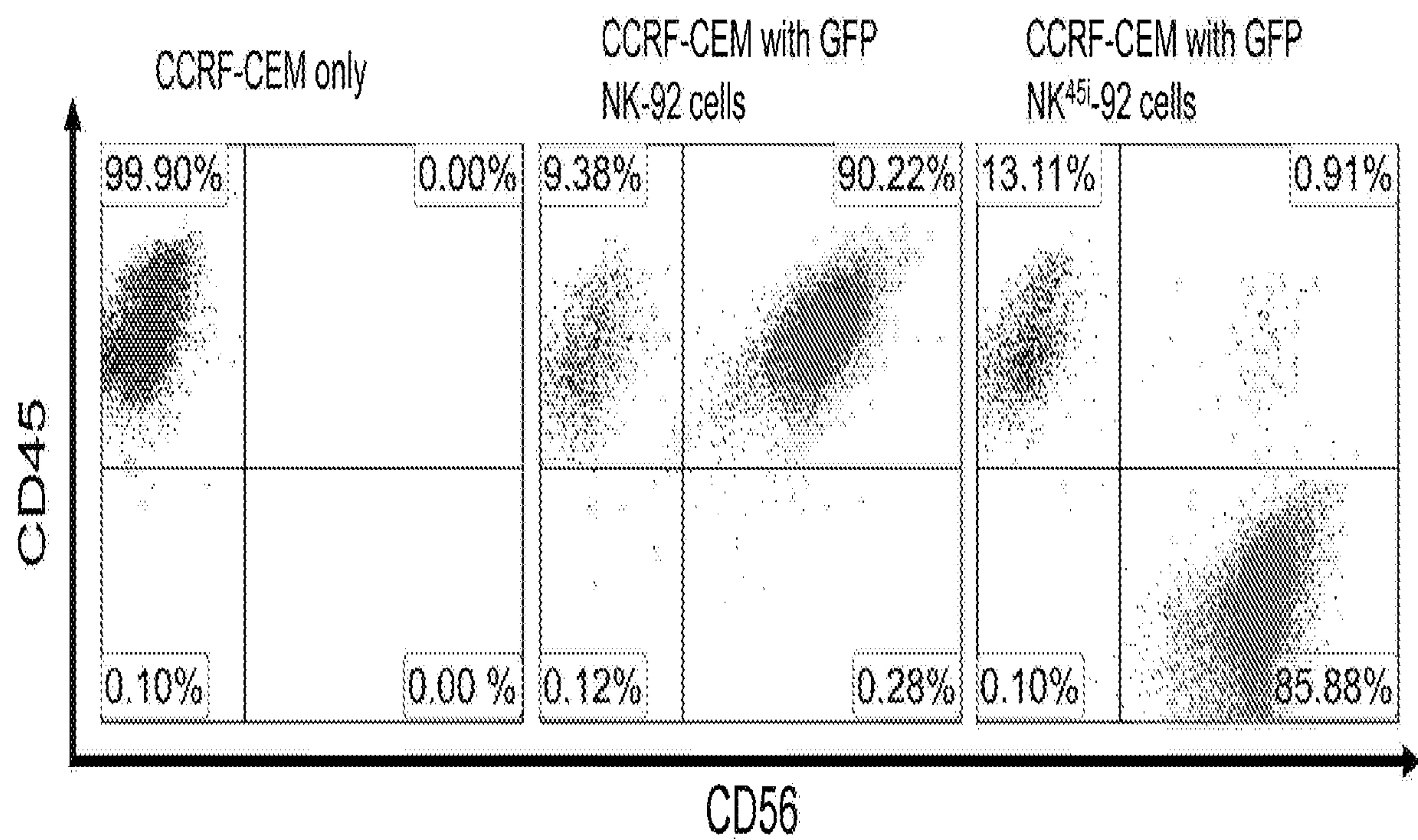


FIG. 27B

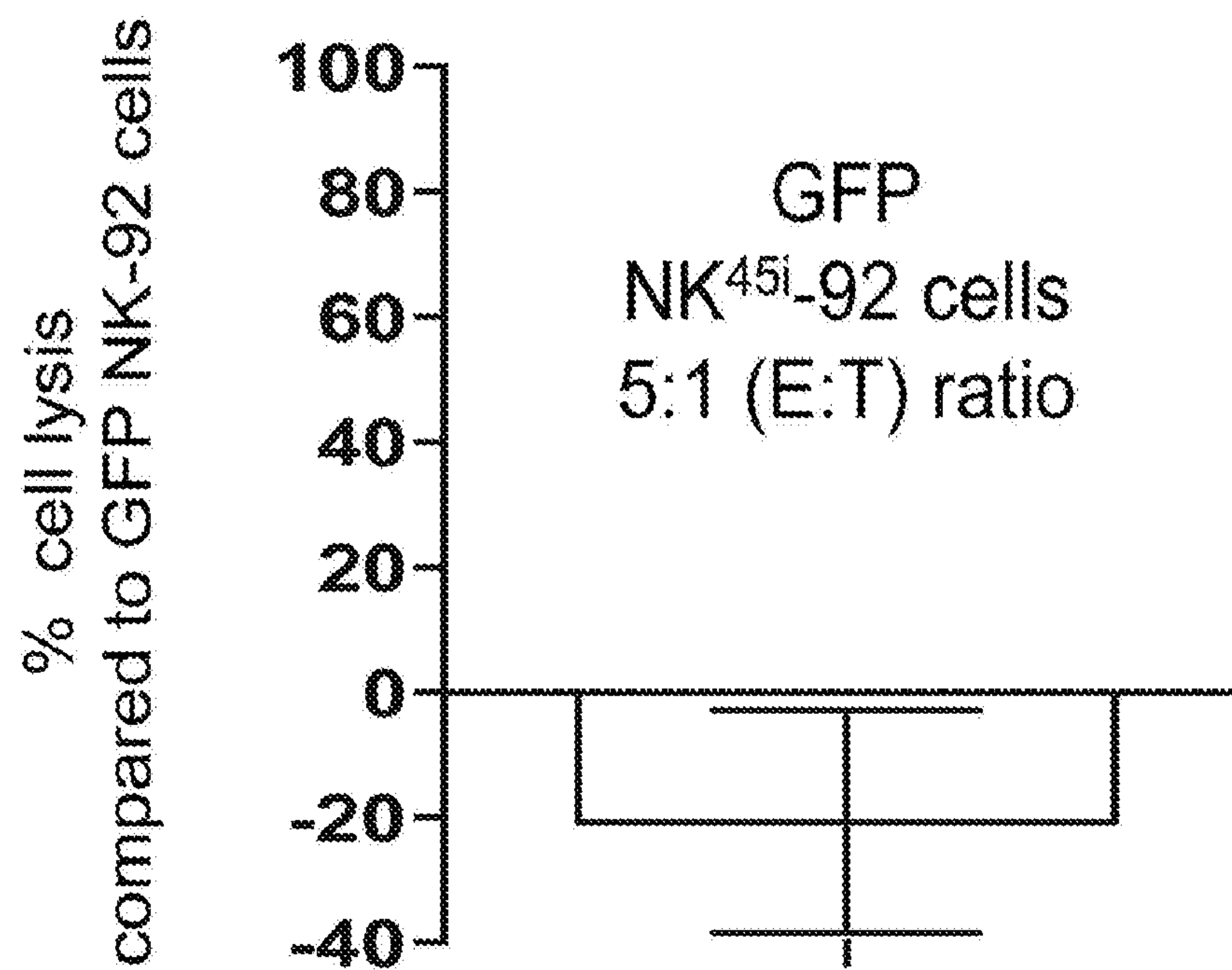


FIG. 28A

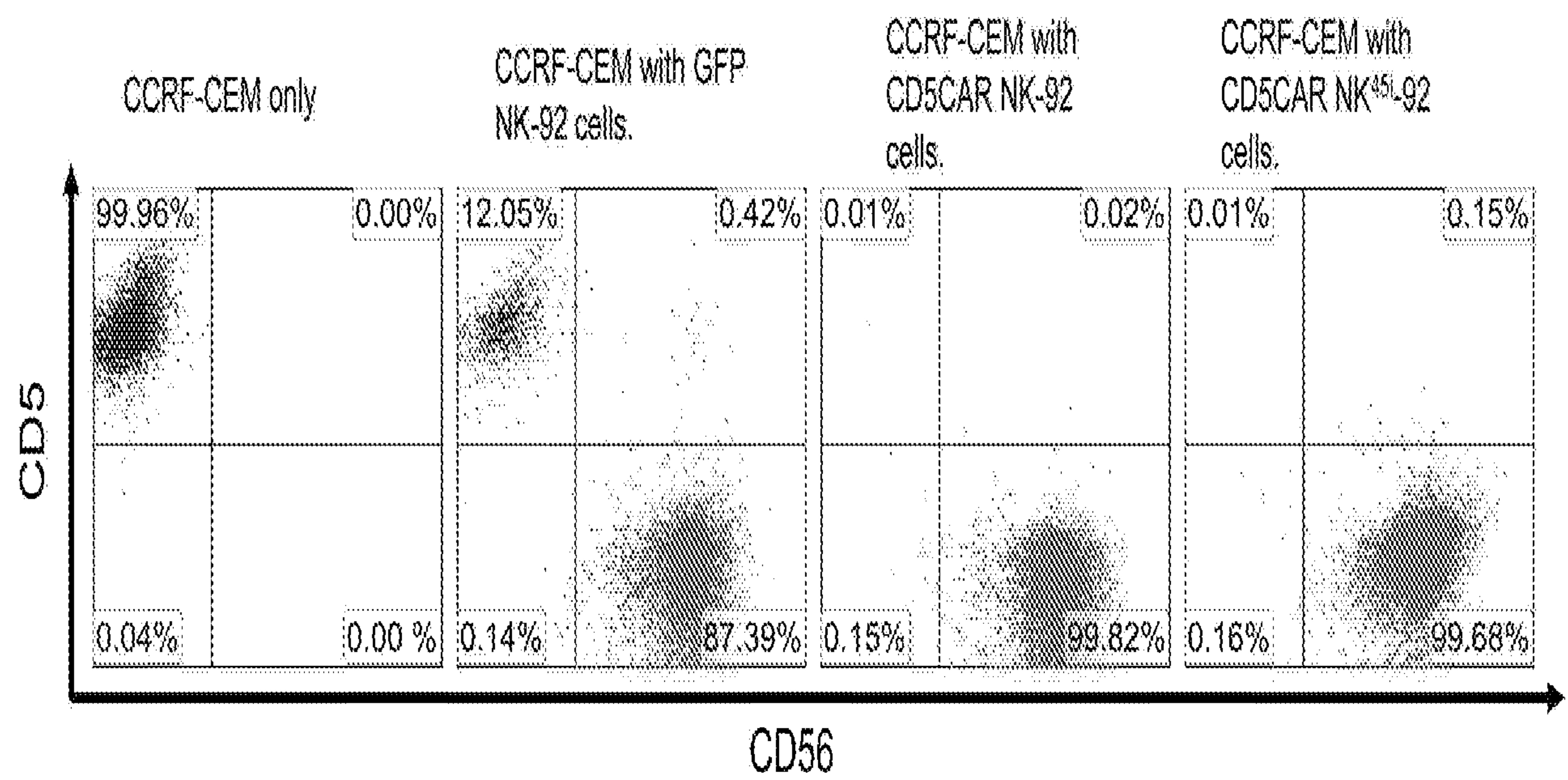


FIG. 28B

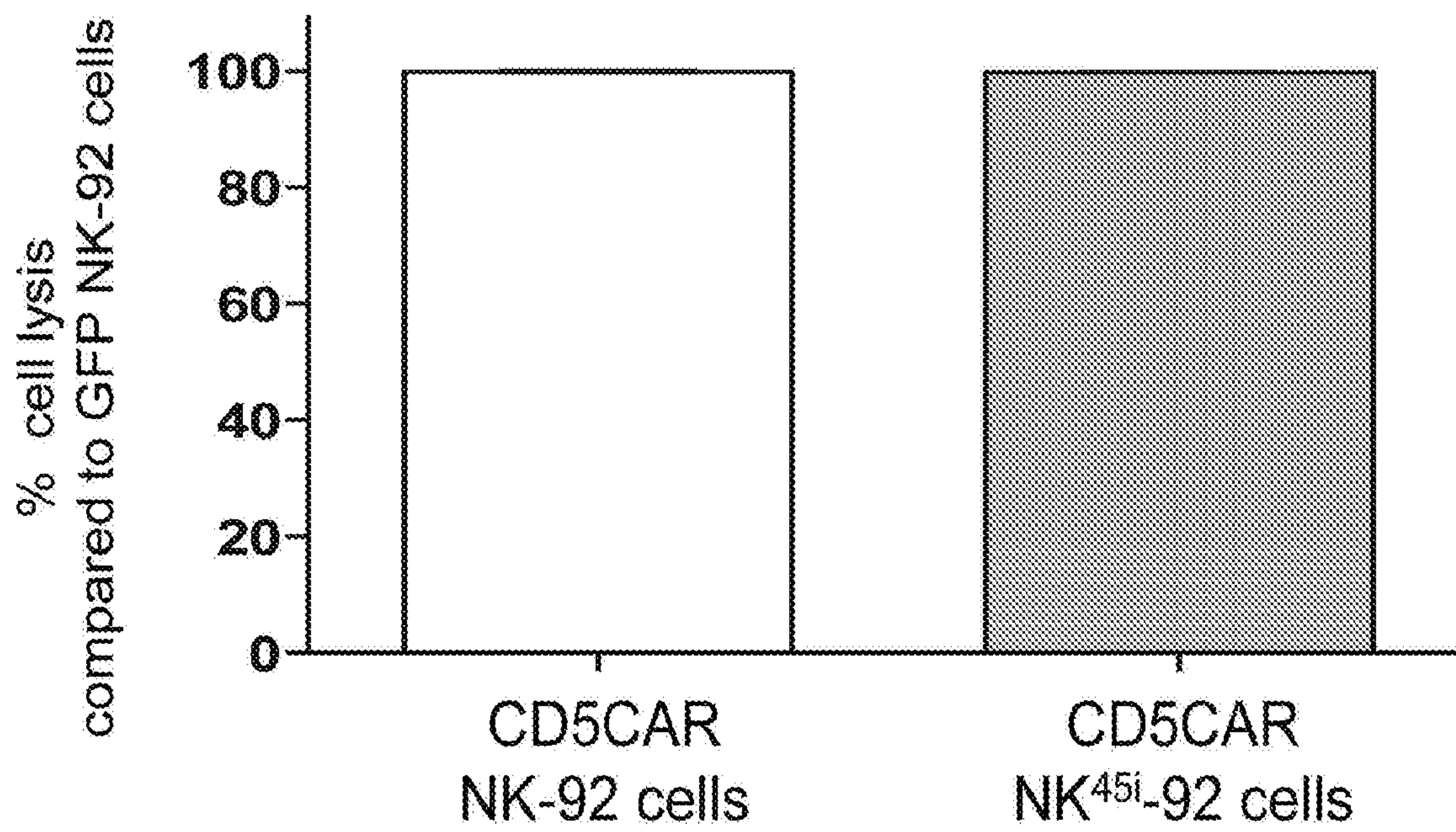


FIG. 29A

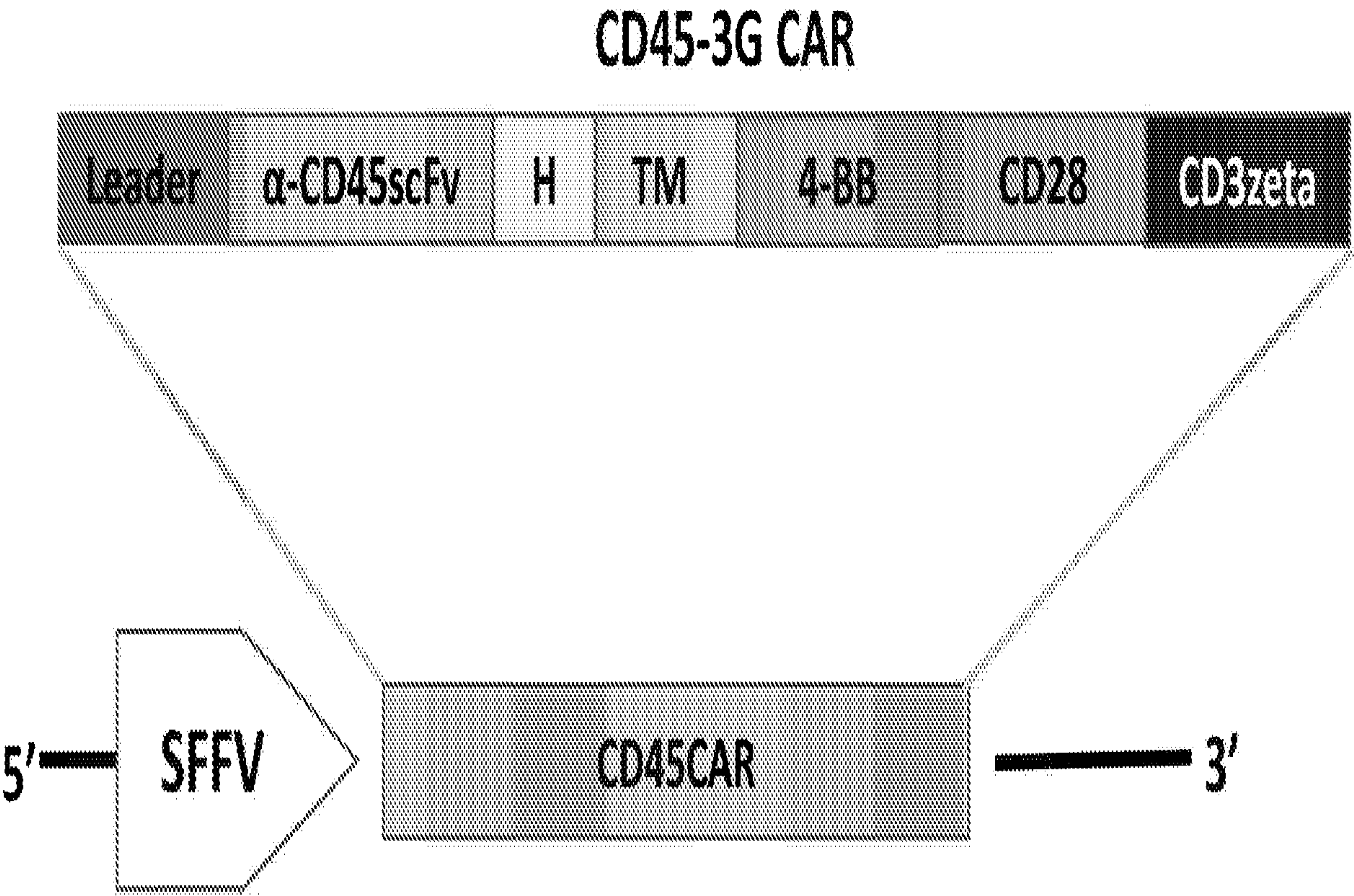


FIG. 29B

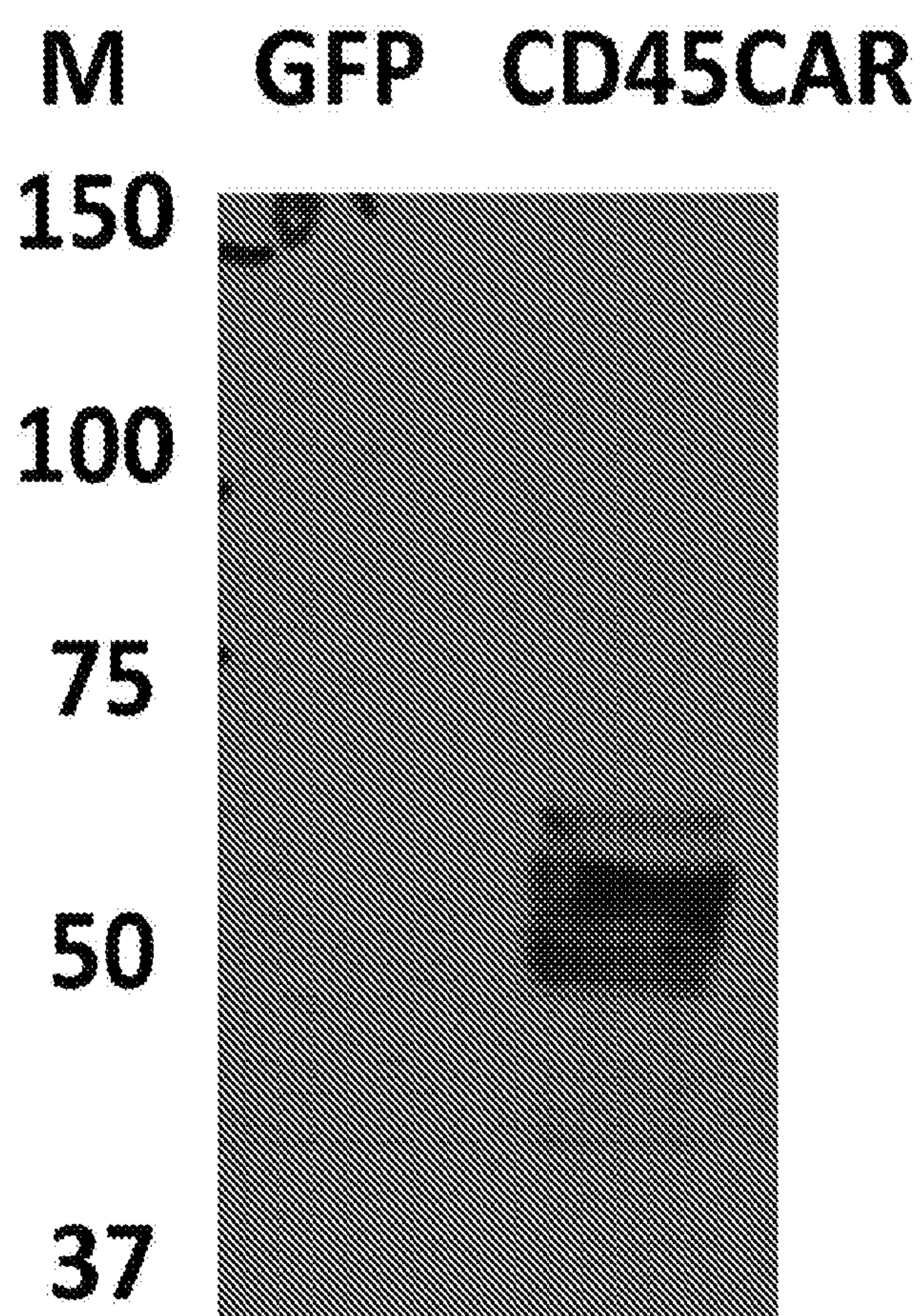


FIG. 30A

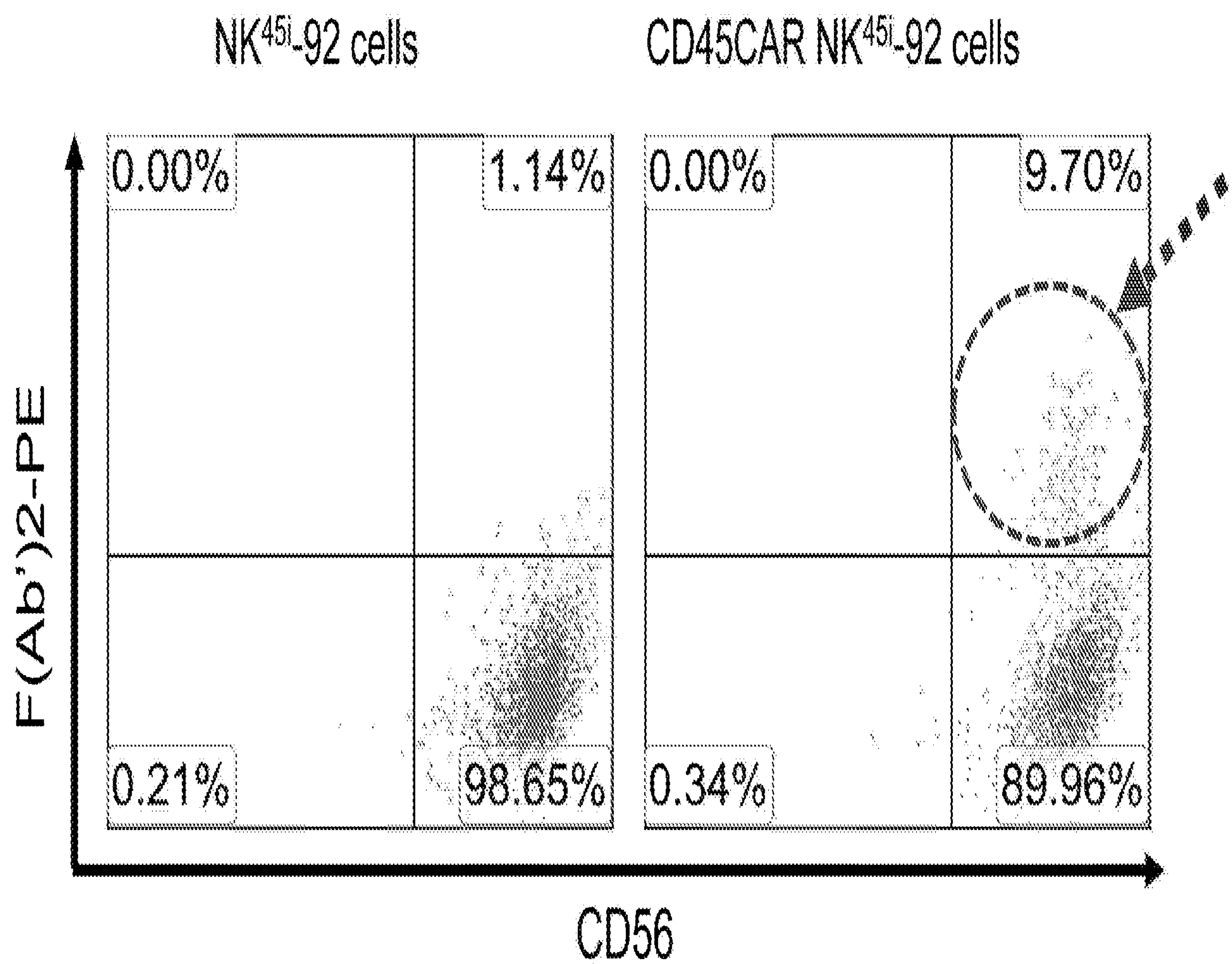


FIG. 30B

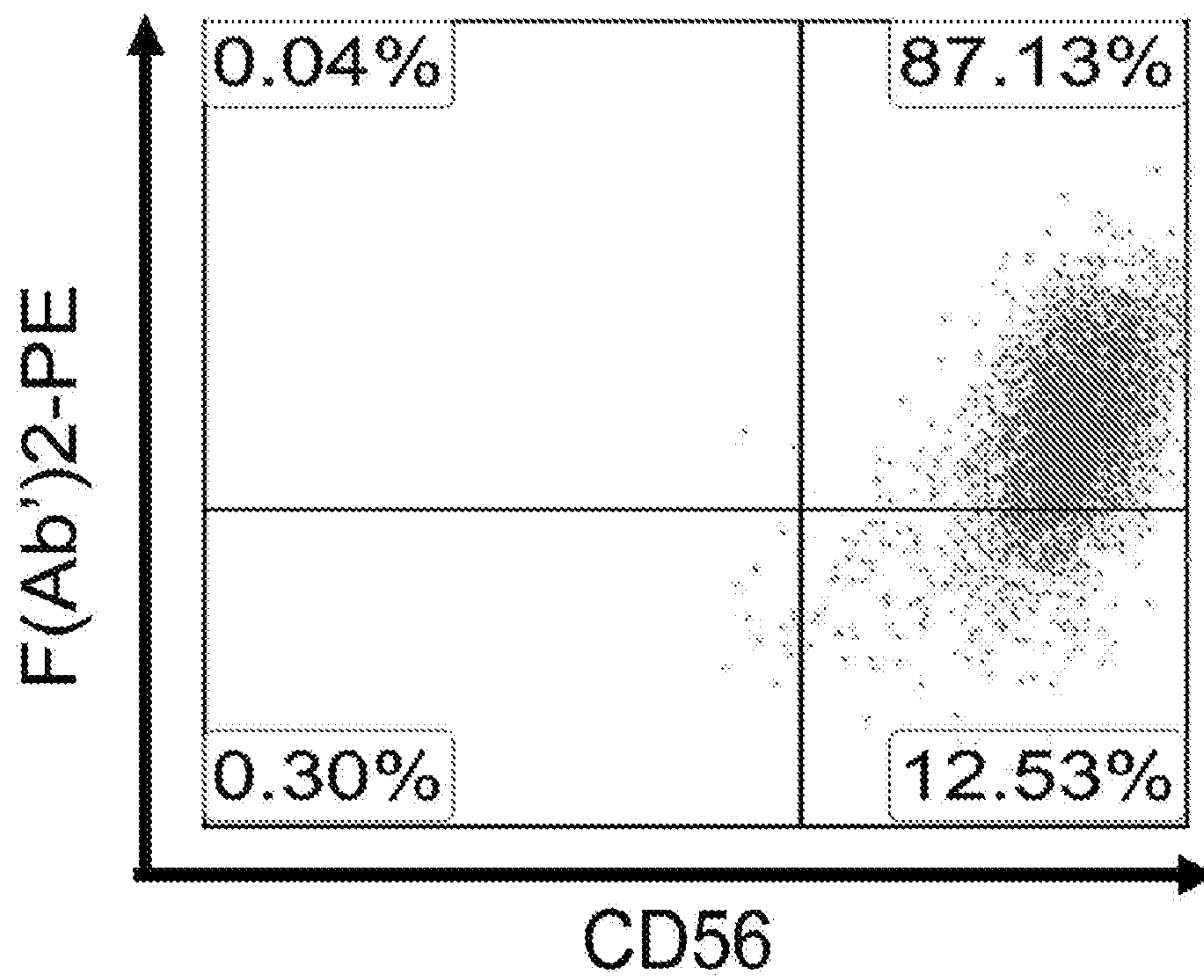


FIG. 31A

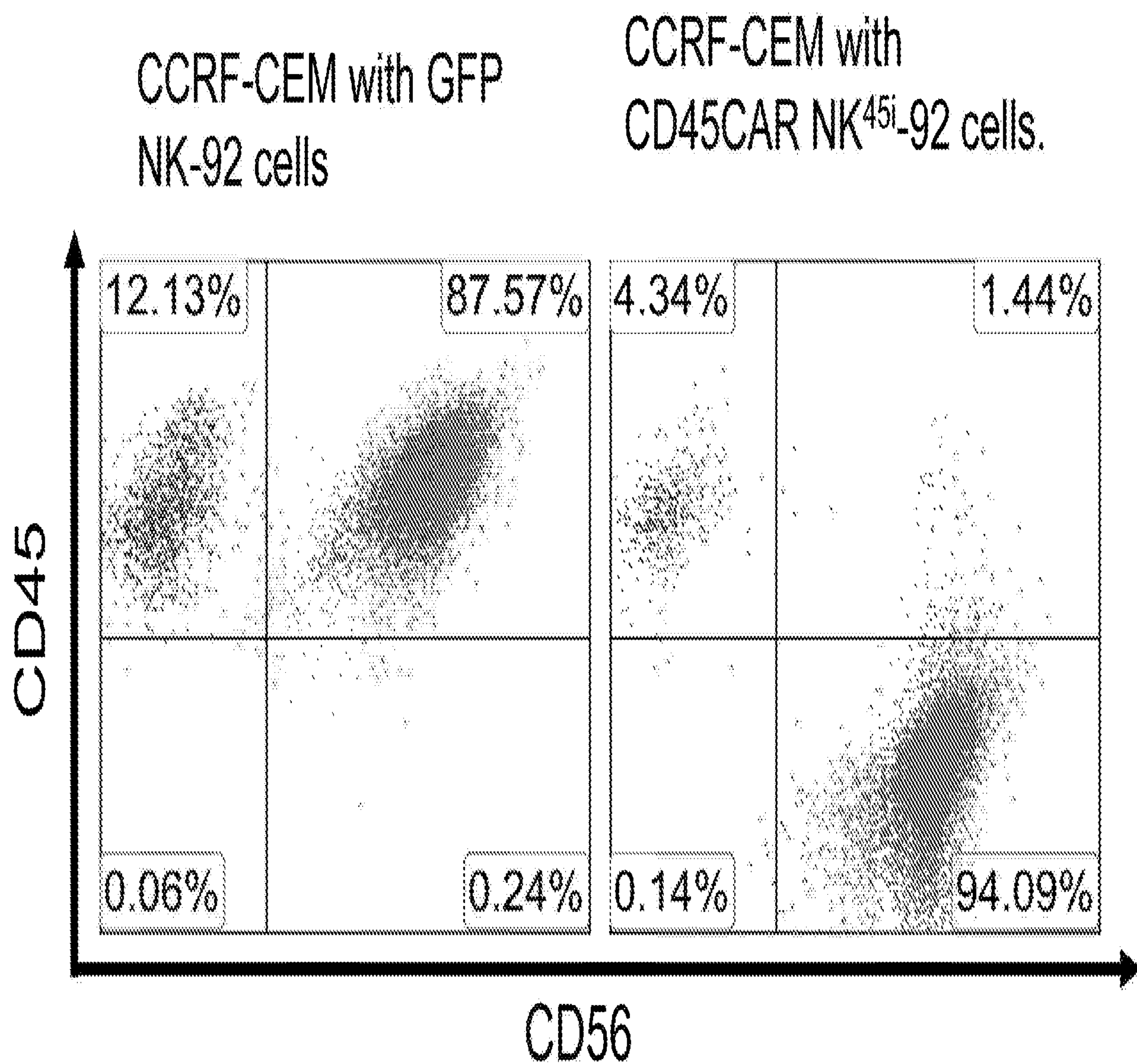


FIG. 31B

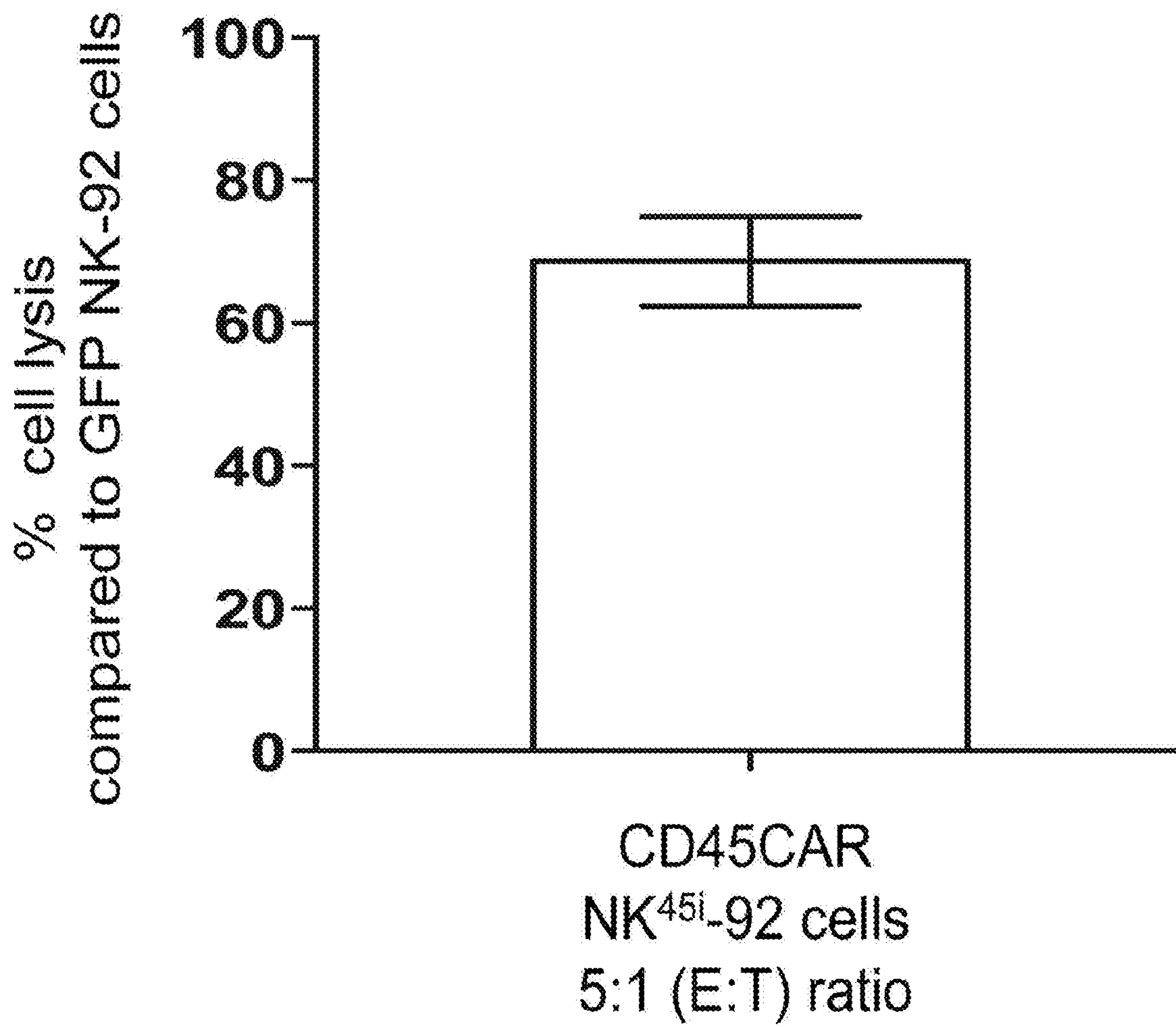


FIG. 32A

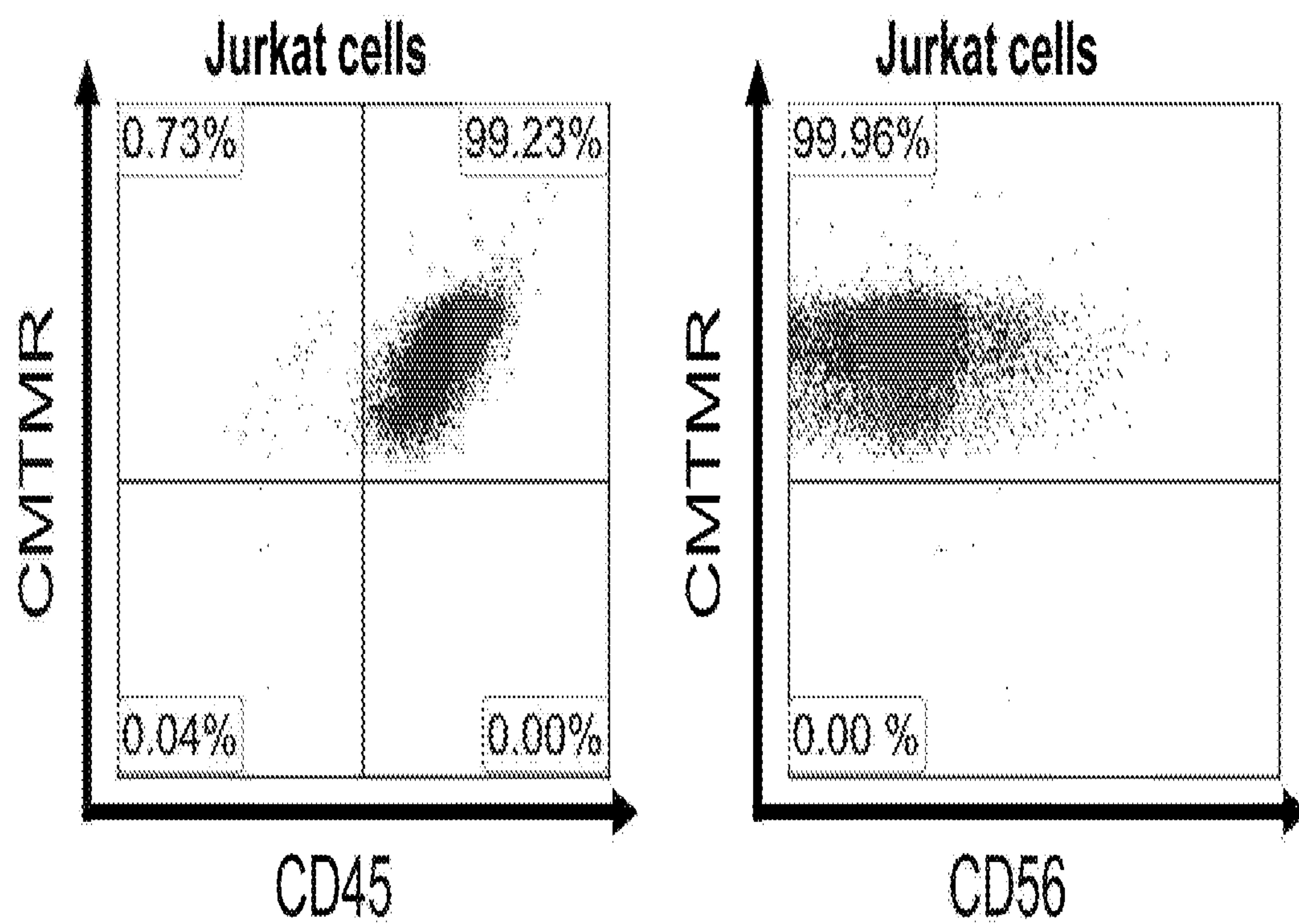


FIG. 32B

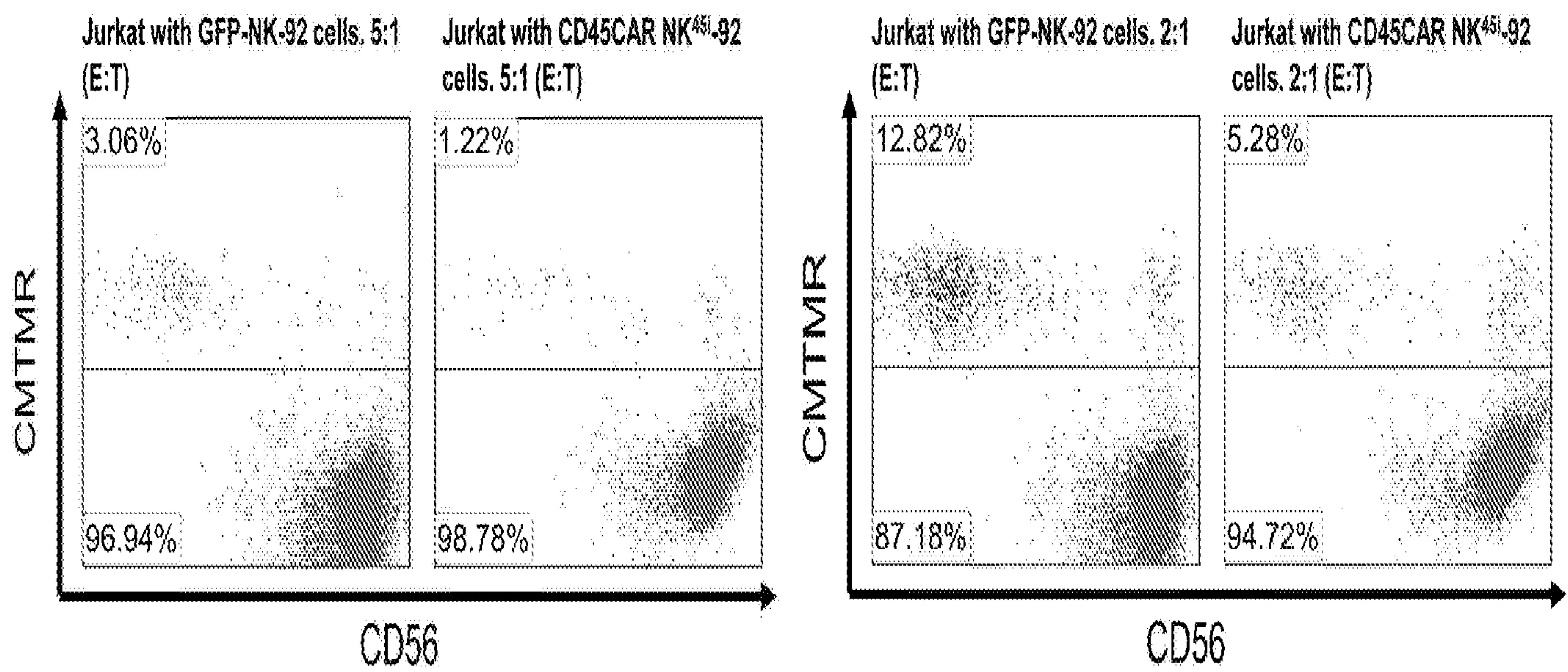


FIG. 32C

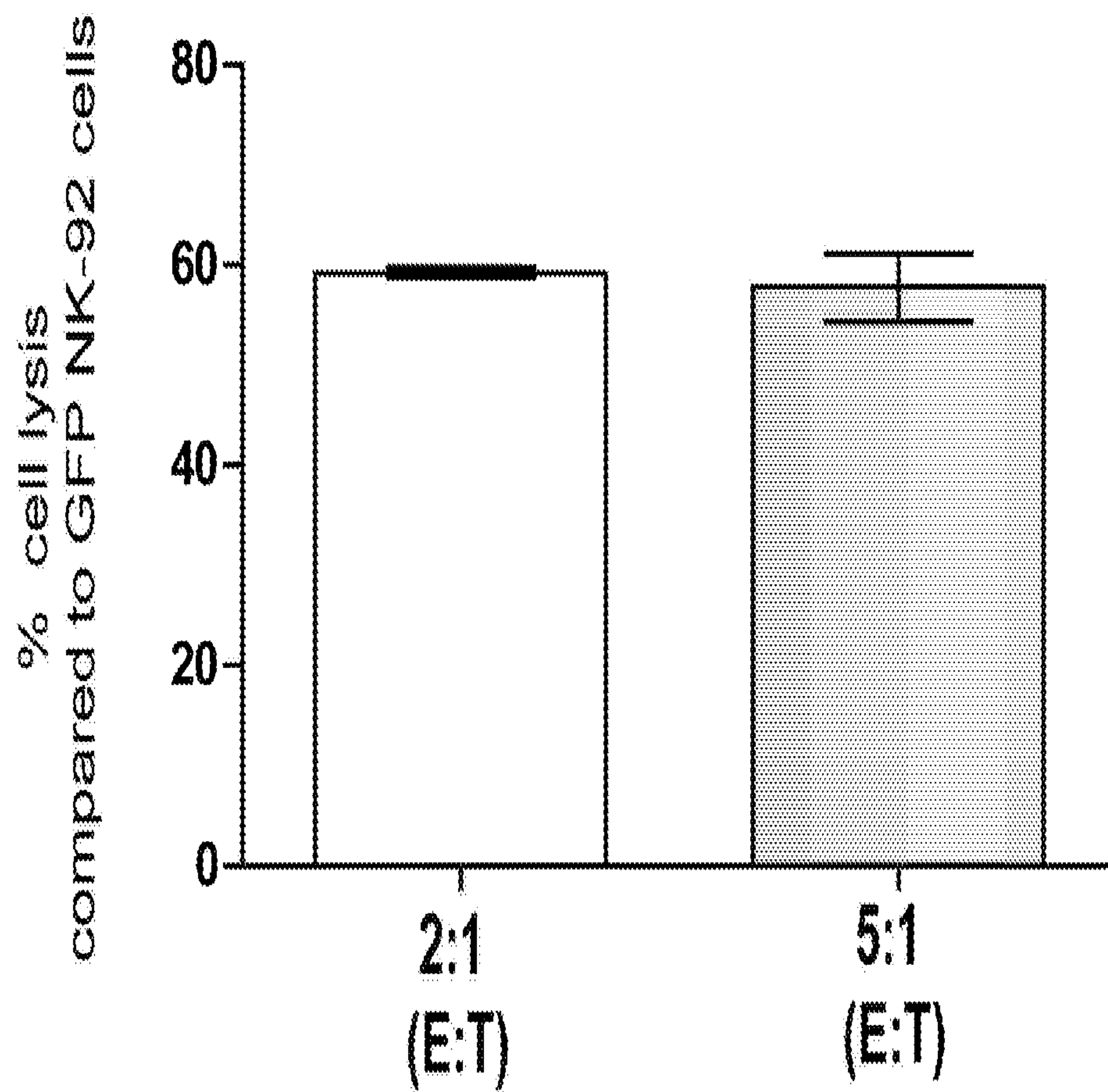


FIG. 33A

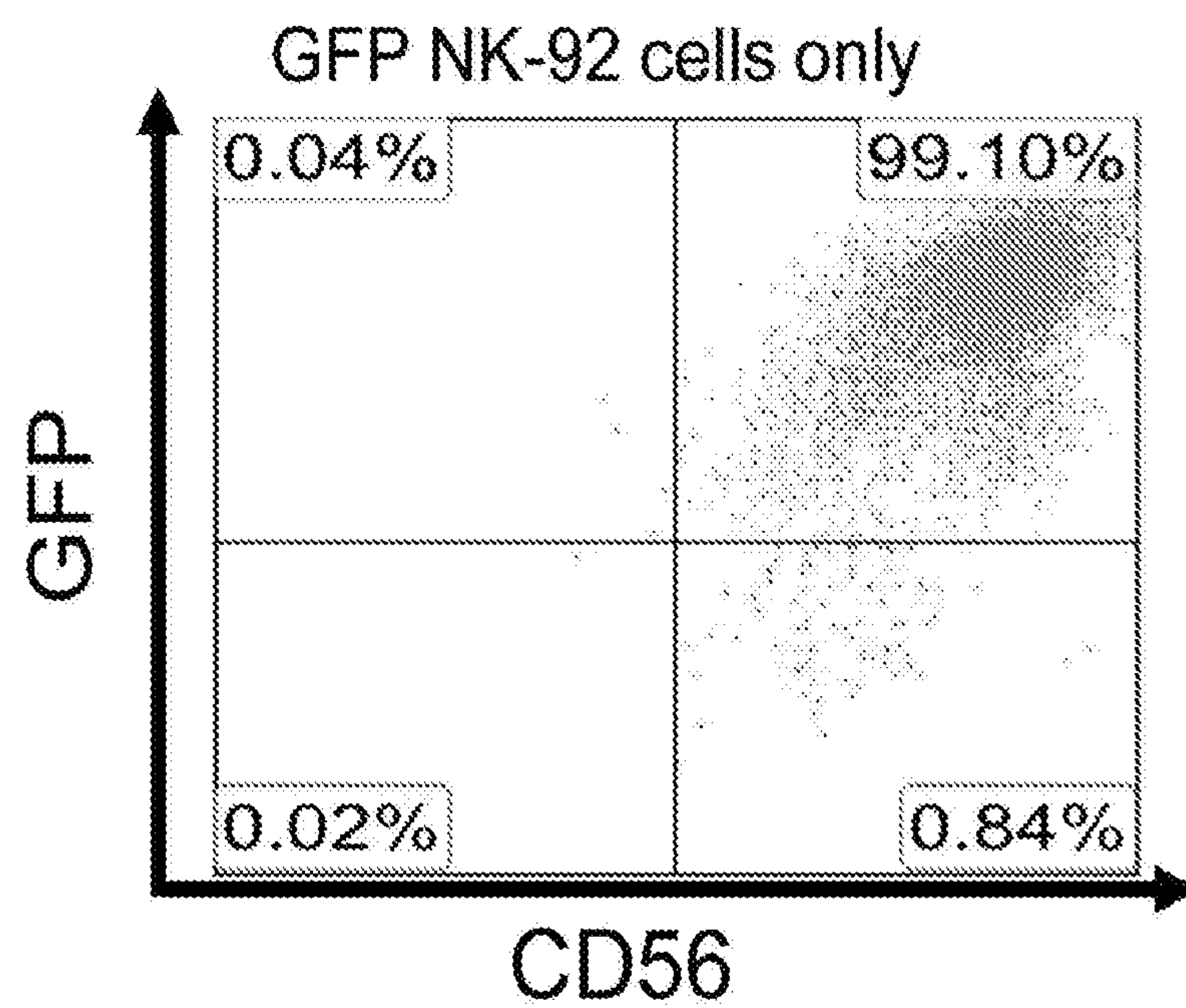


FIG. 33B

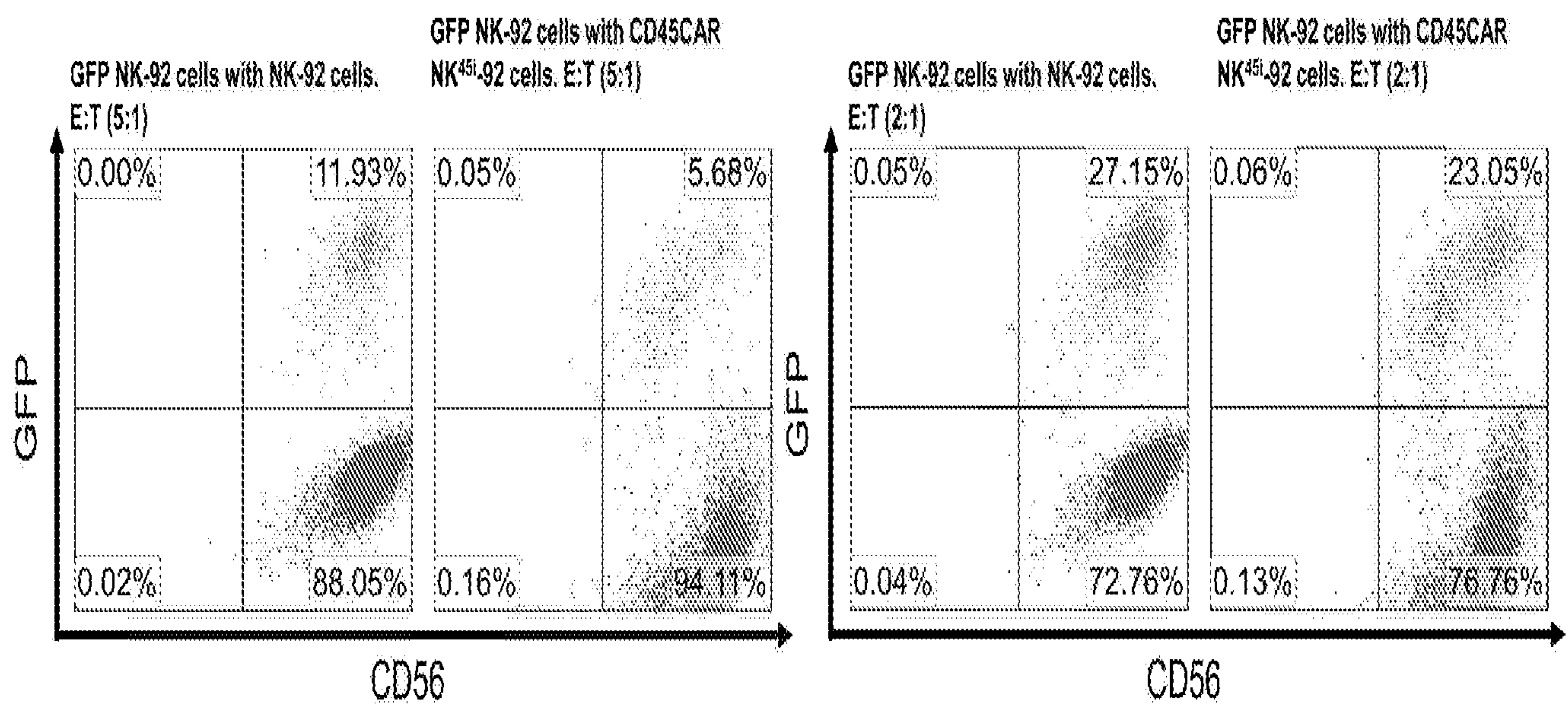


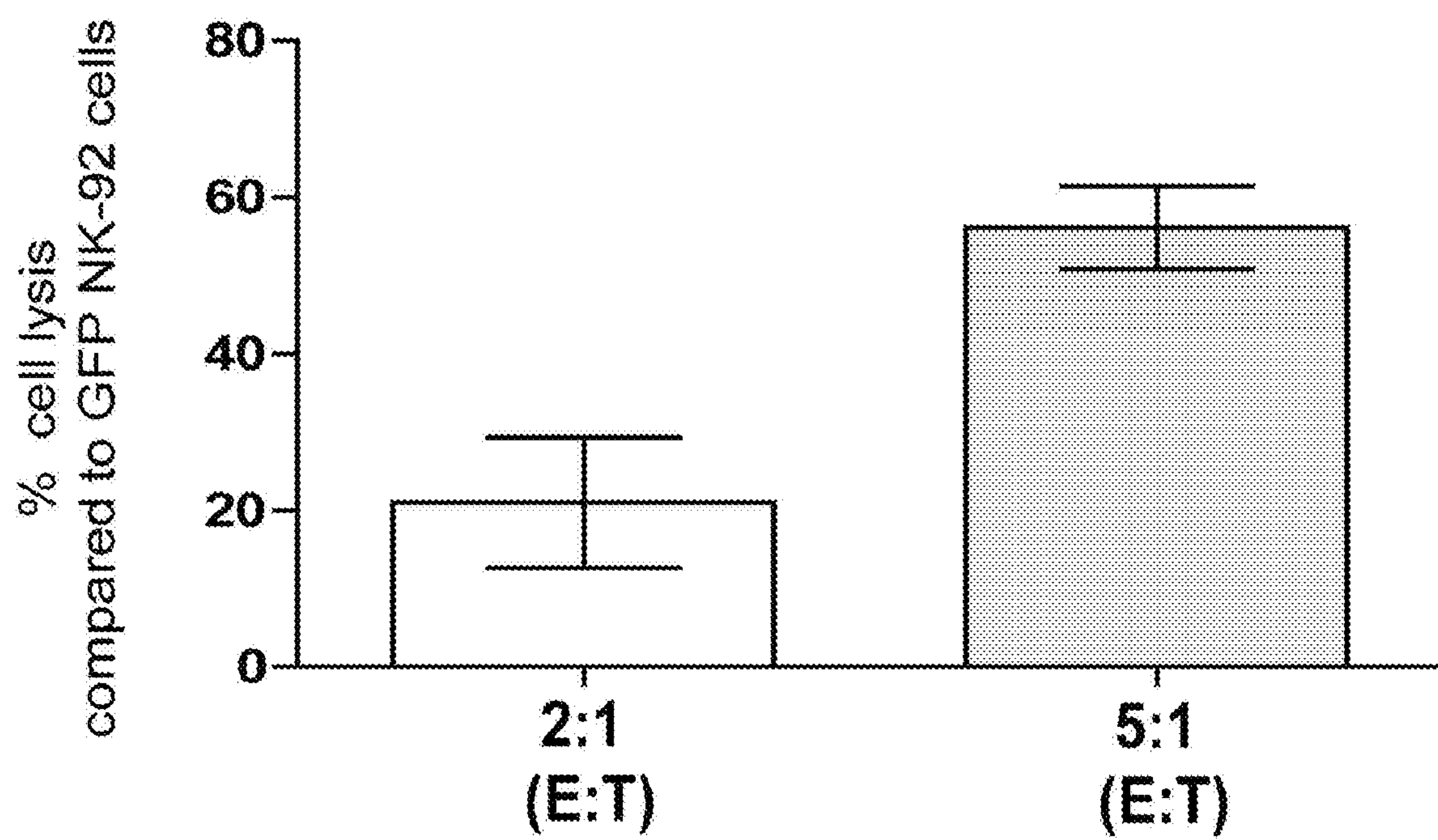
FIG. 33C

FIG. 33D

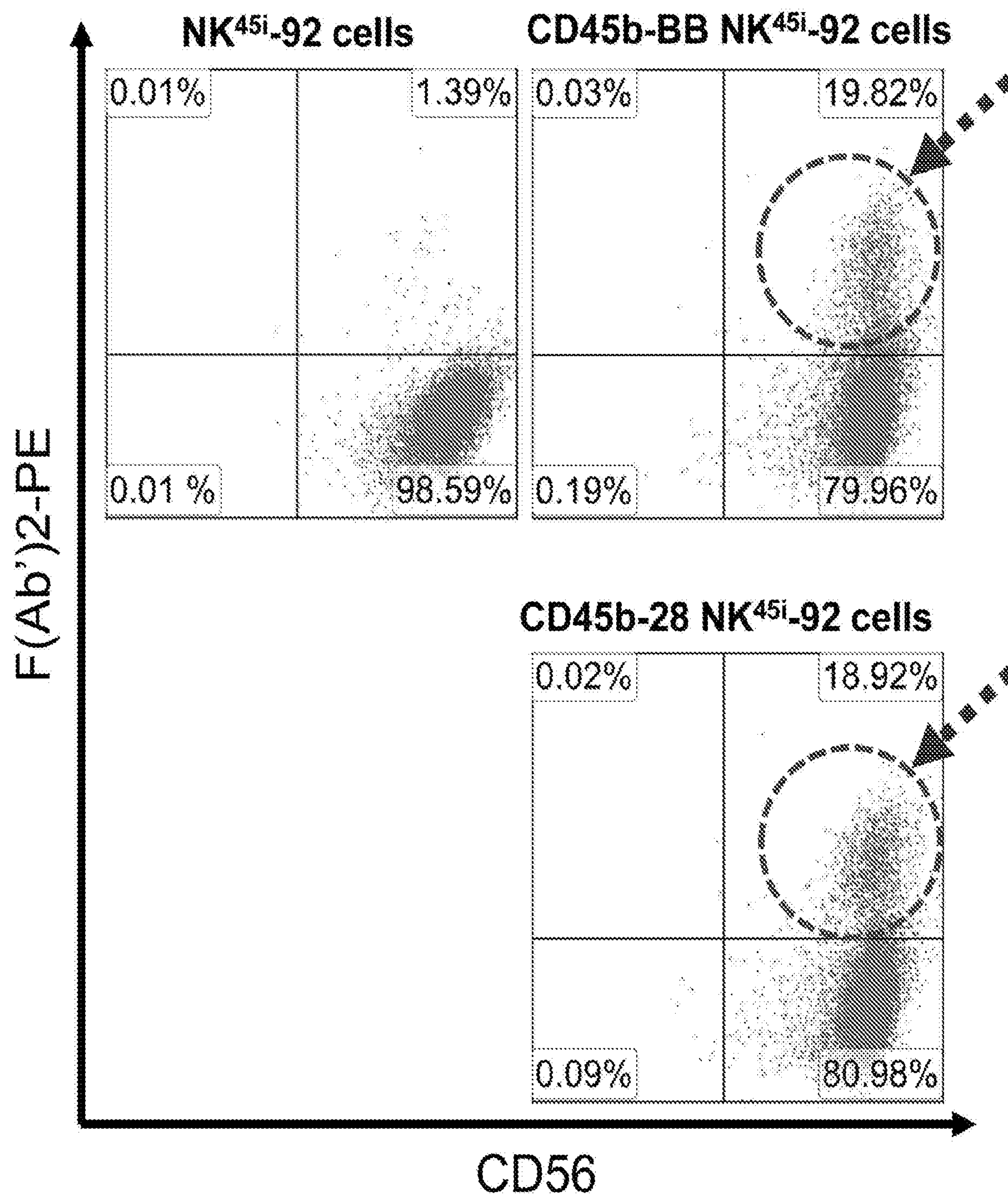


FIG. 33E

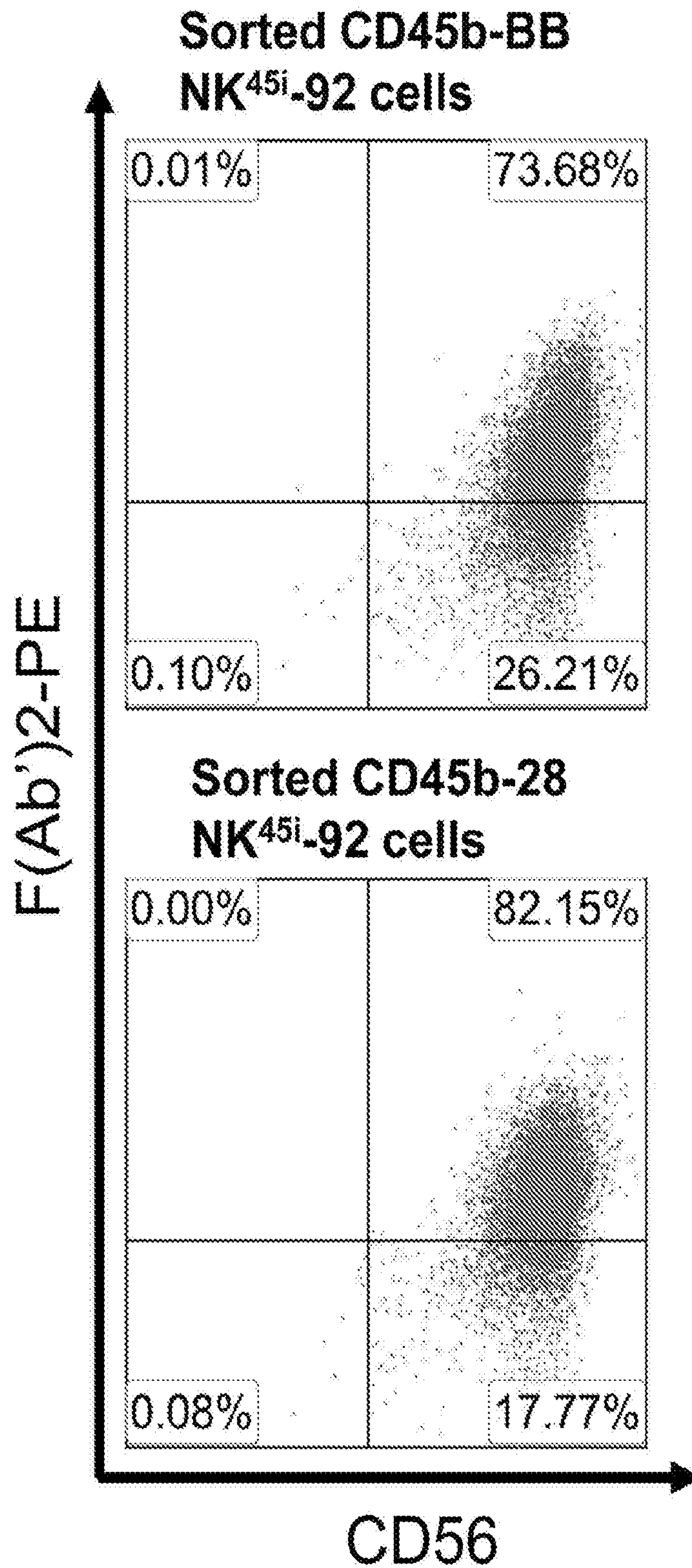


FIG. 33F

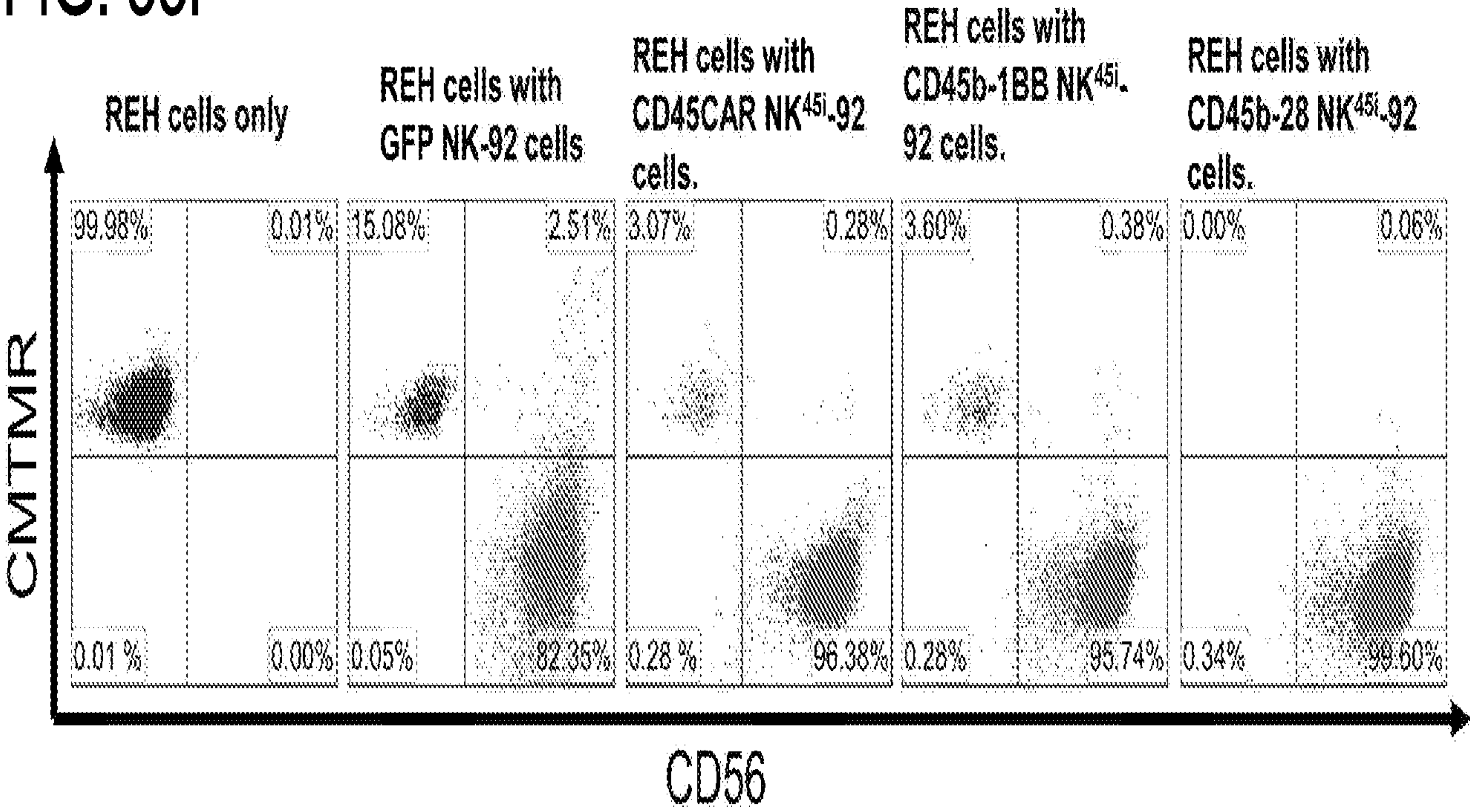


FIG. 33G

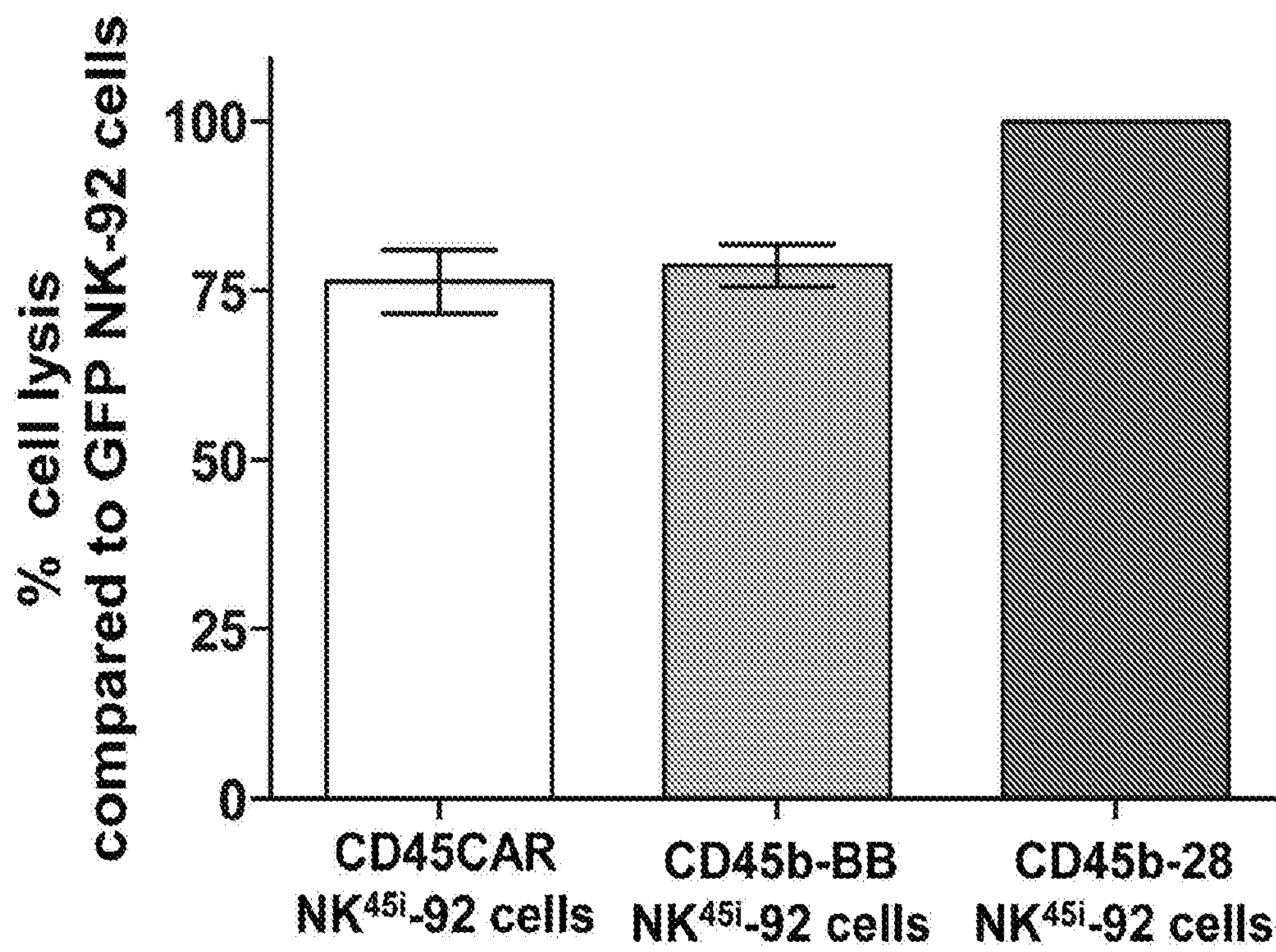


FIG. 34A

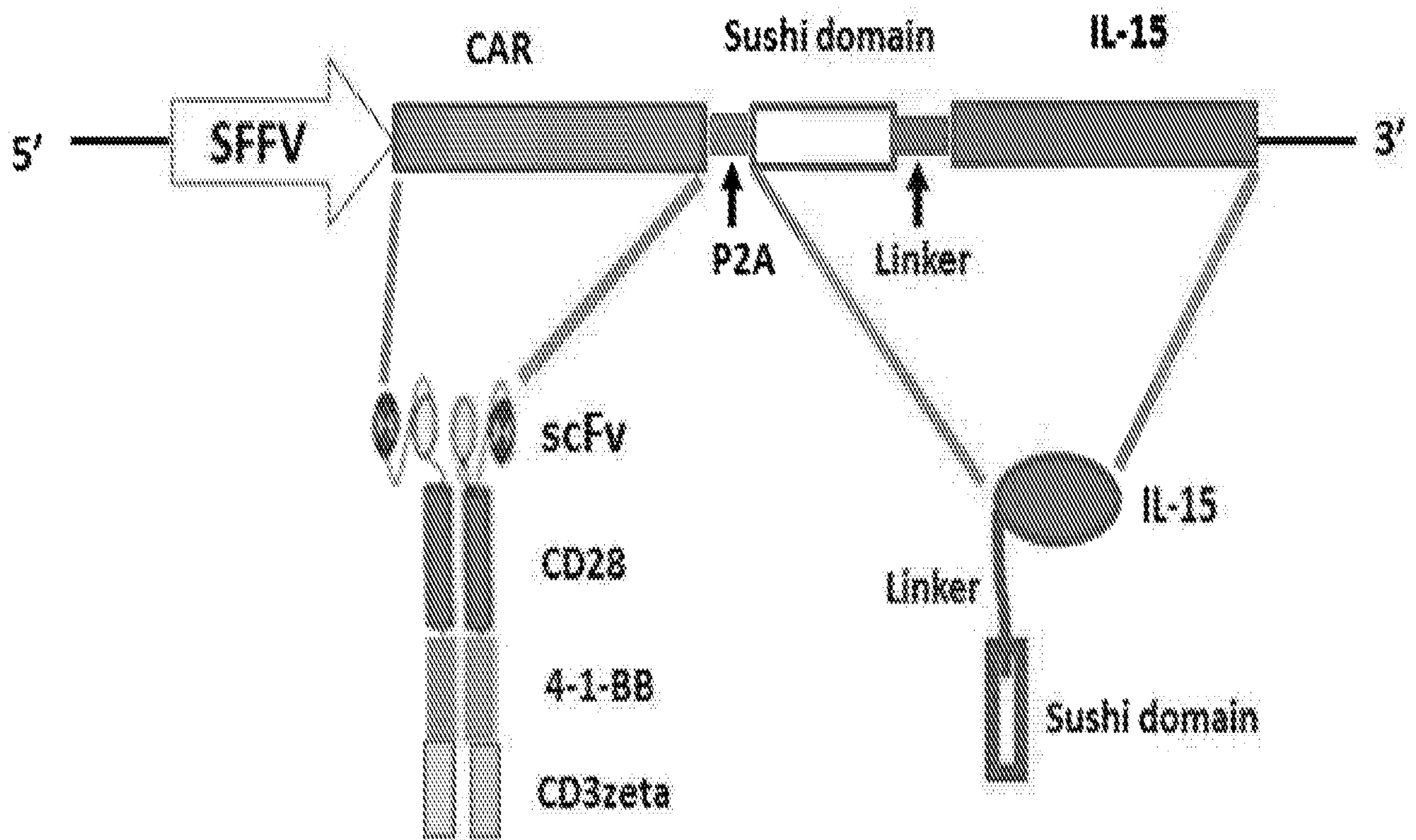


FIG. 34B

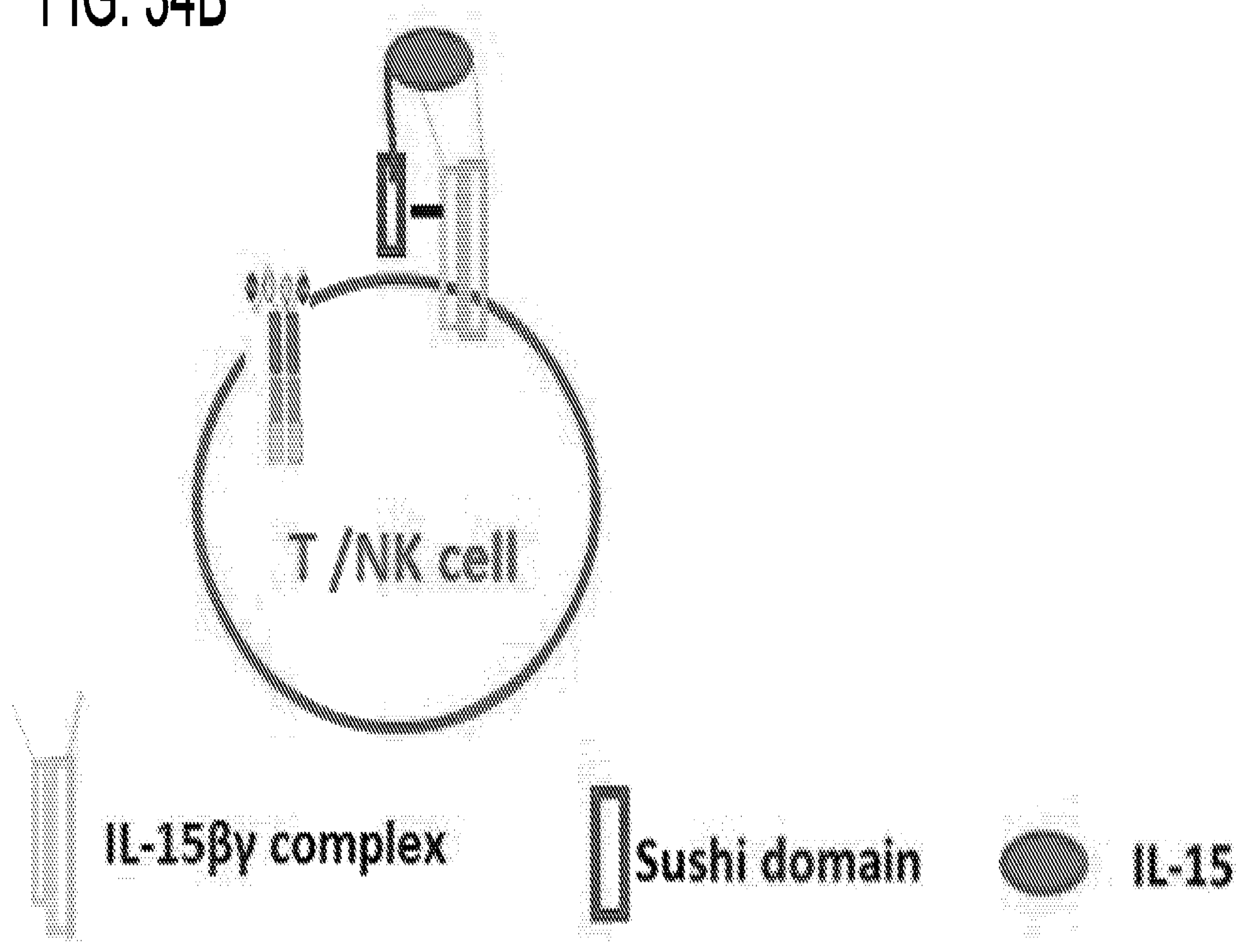


FIG. 35A

HEK CD4IL15RA

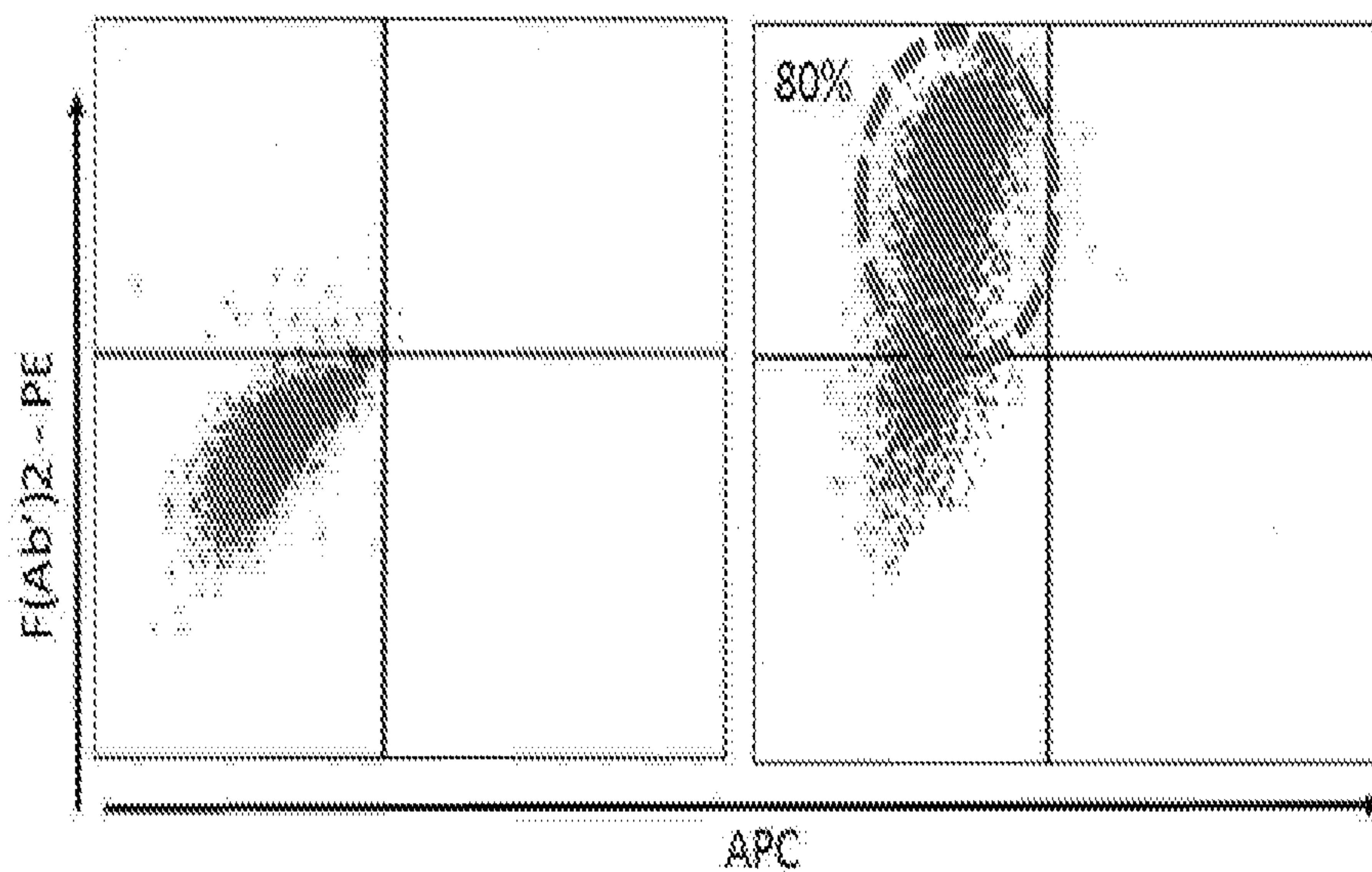
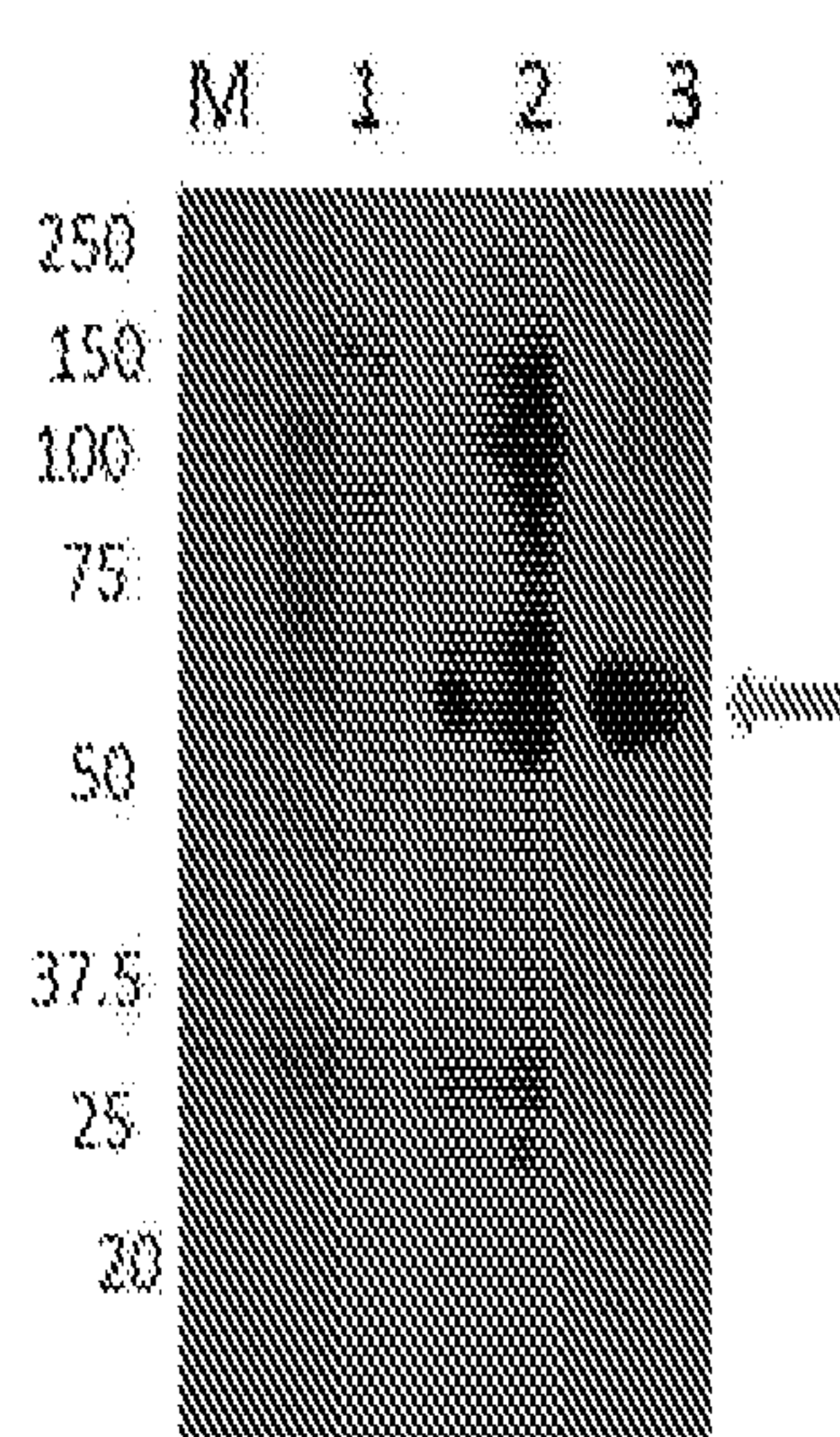


FIG. 35B

FIG. 36

CD4IL15RA NK cells

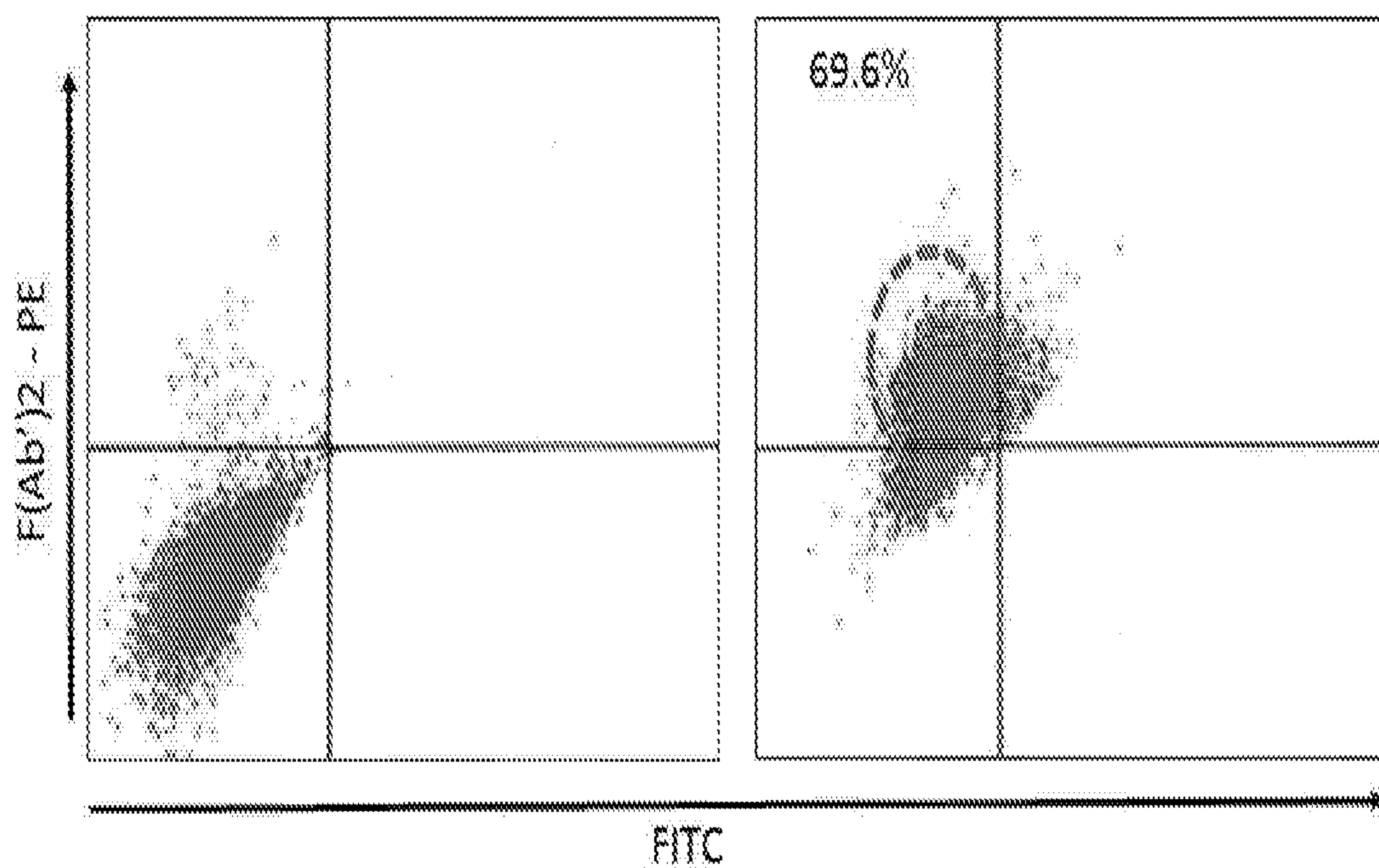


FIG. 37
CD4⁺IL15RA⁺ T cells

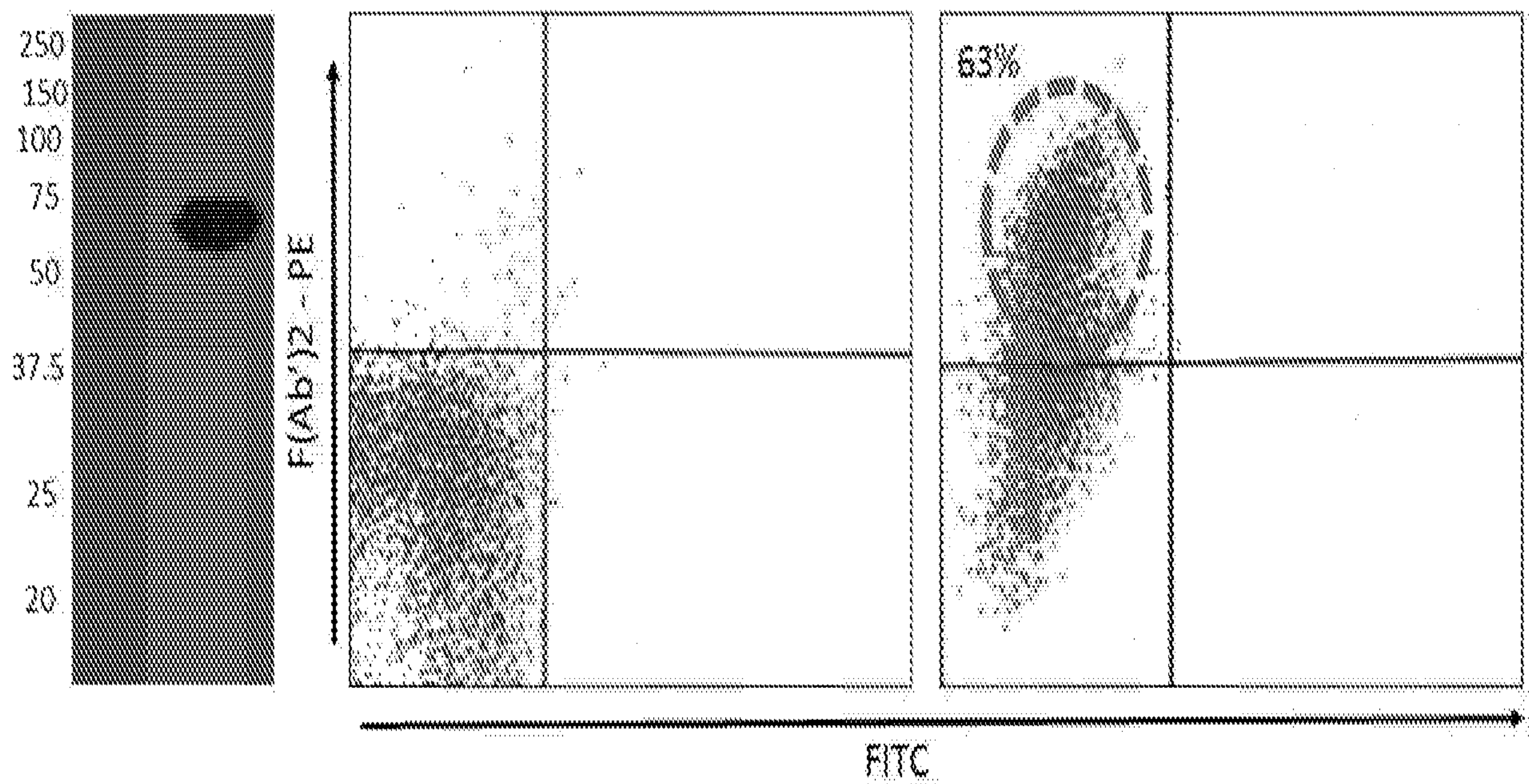


FIG. 38A

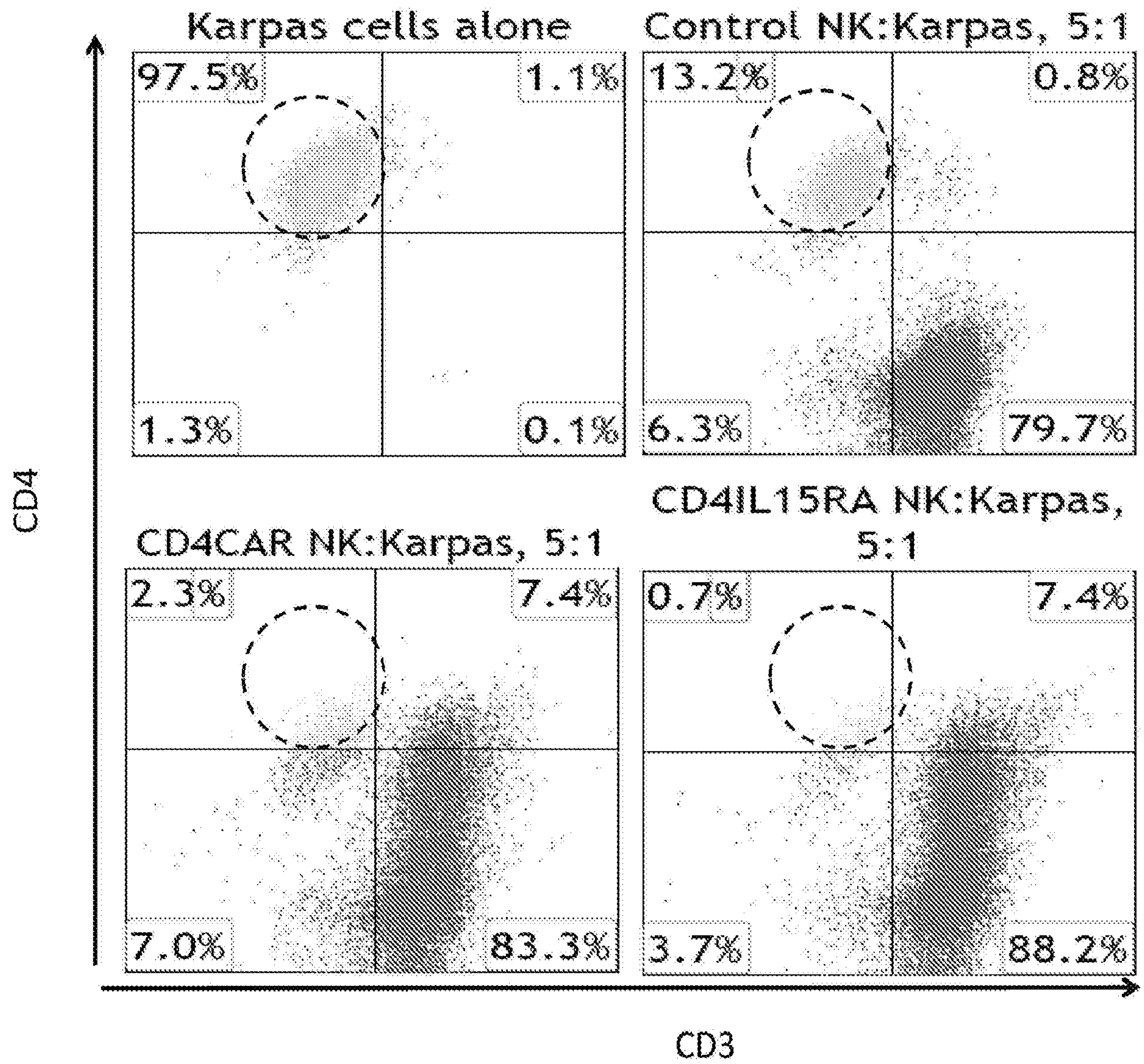


FIG. 38B

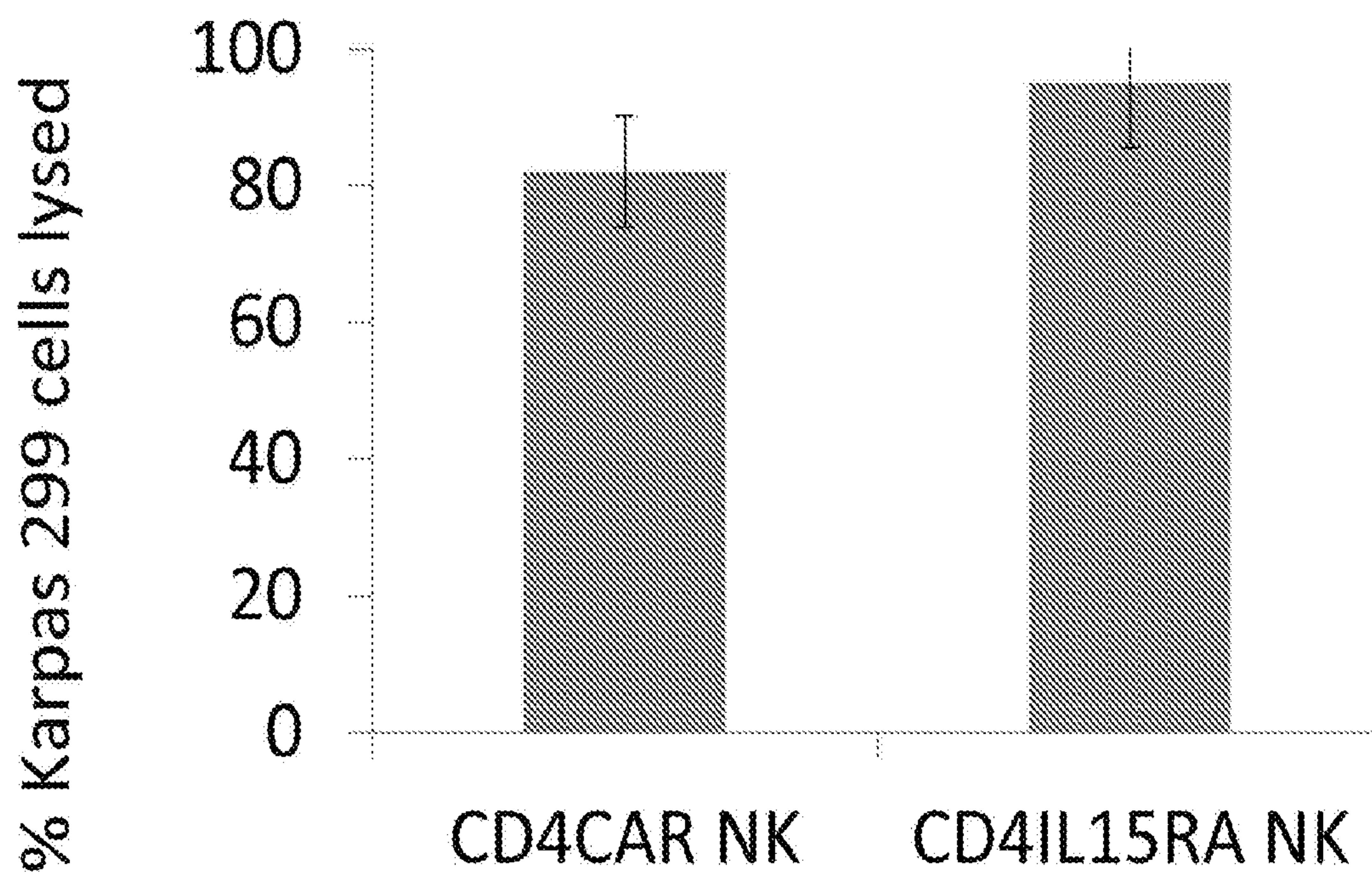


FIG. 39

CD4 and CD4IL15RA NK CAR cells lyse MOLT4 tumor cells, 1:1

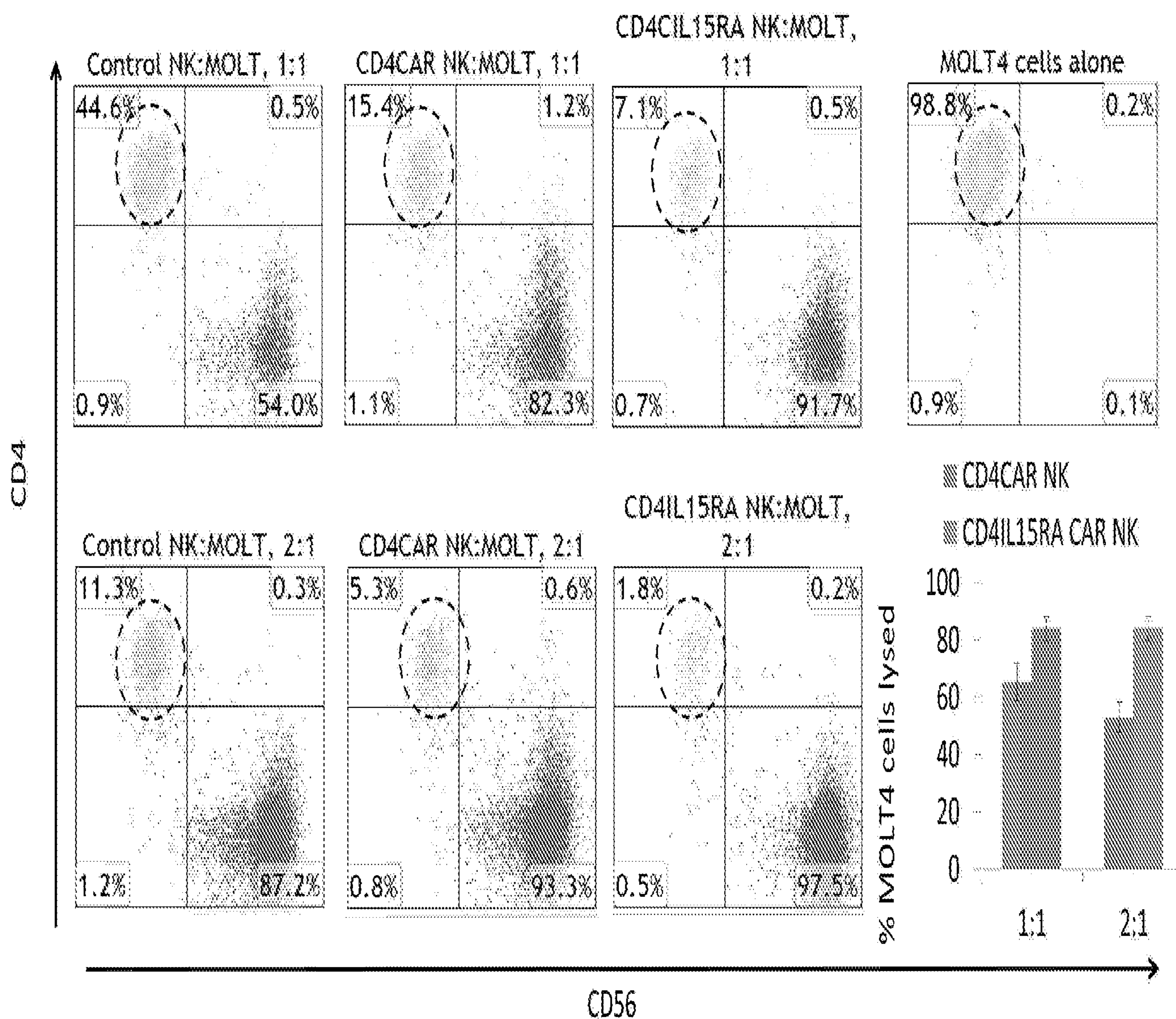


FIG. 40

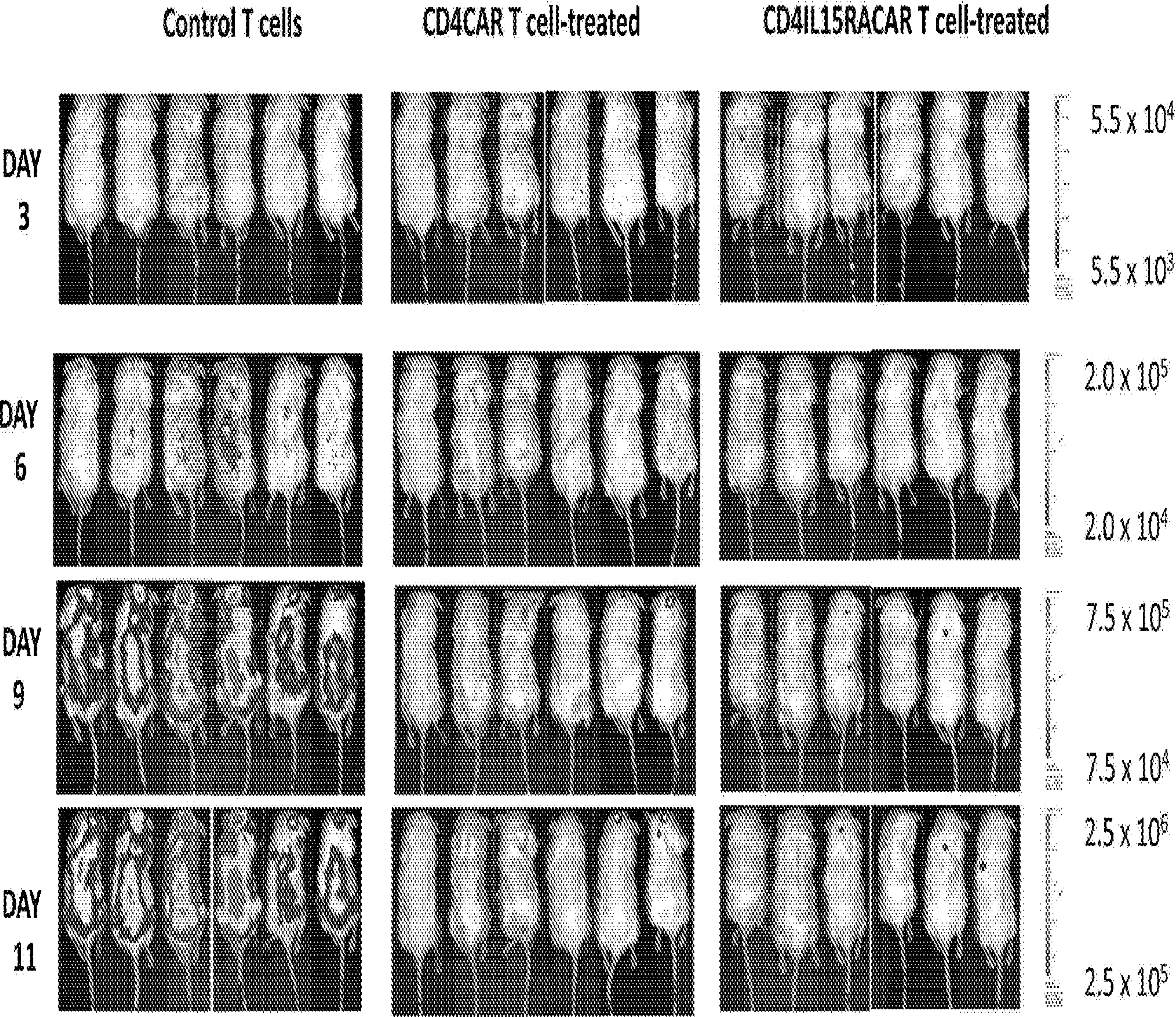


FIG. 41

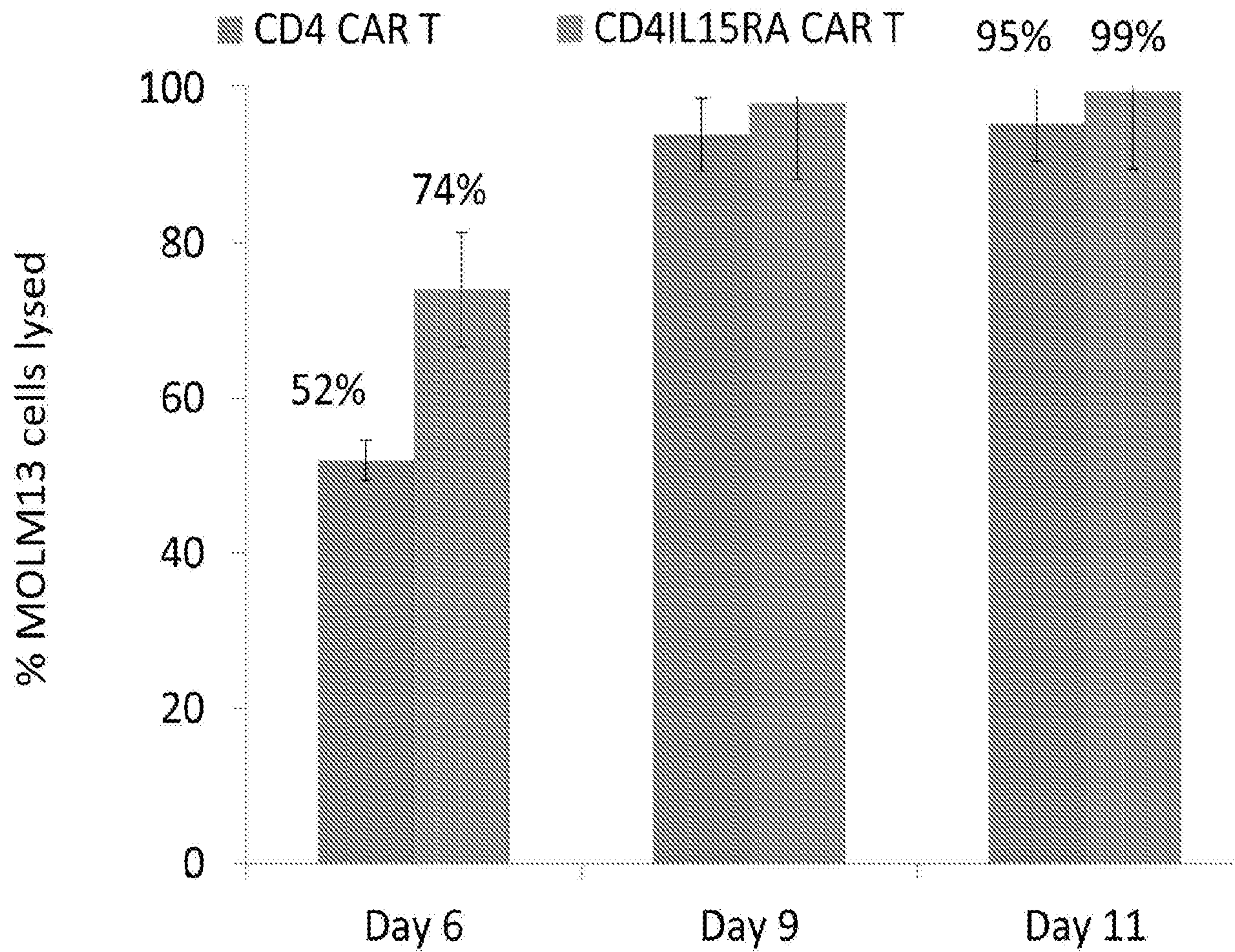


FIG. 42

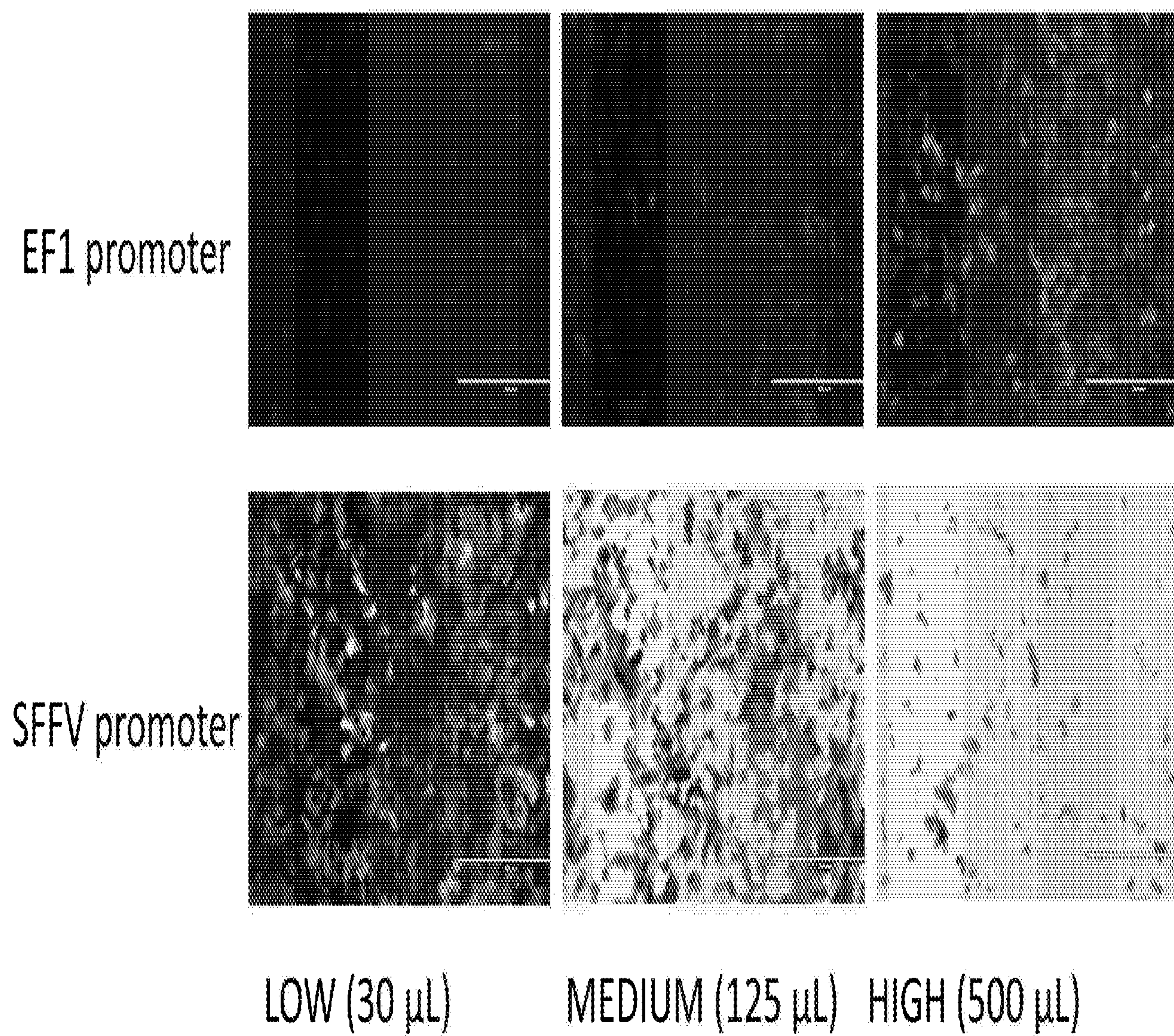


FIG. 43

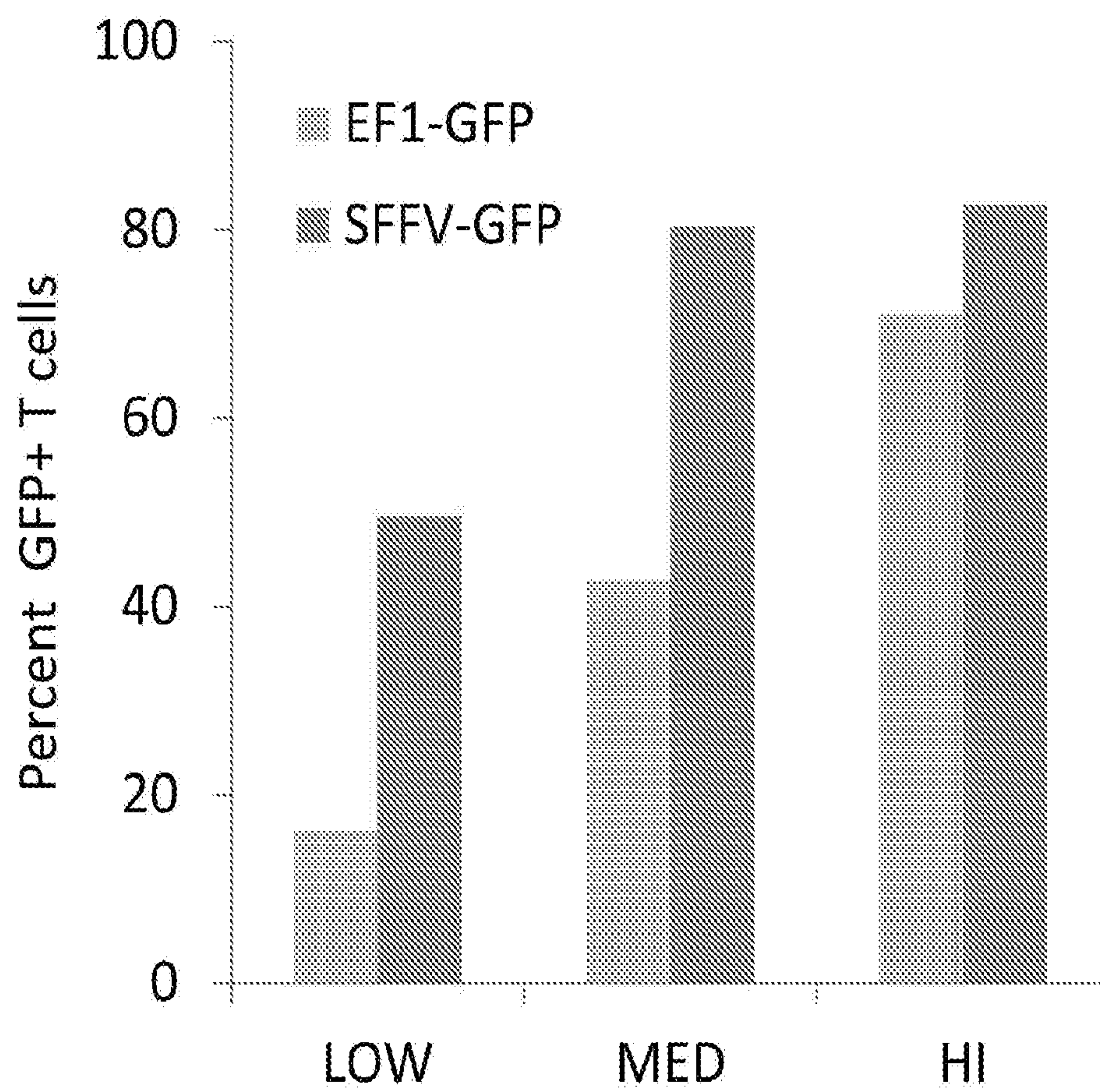


FIG. 44A

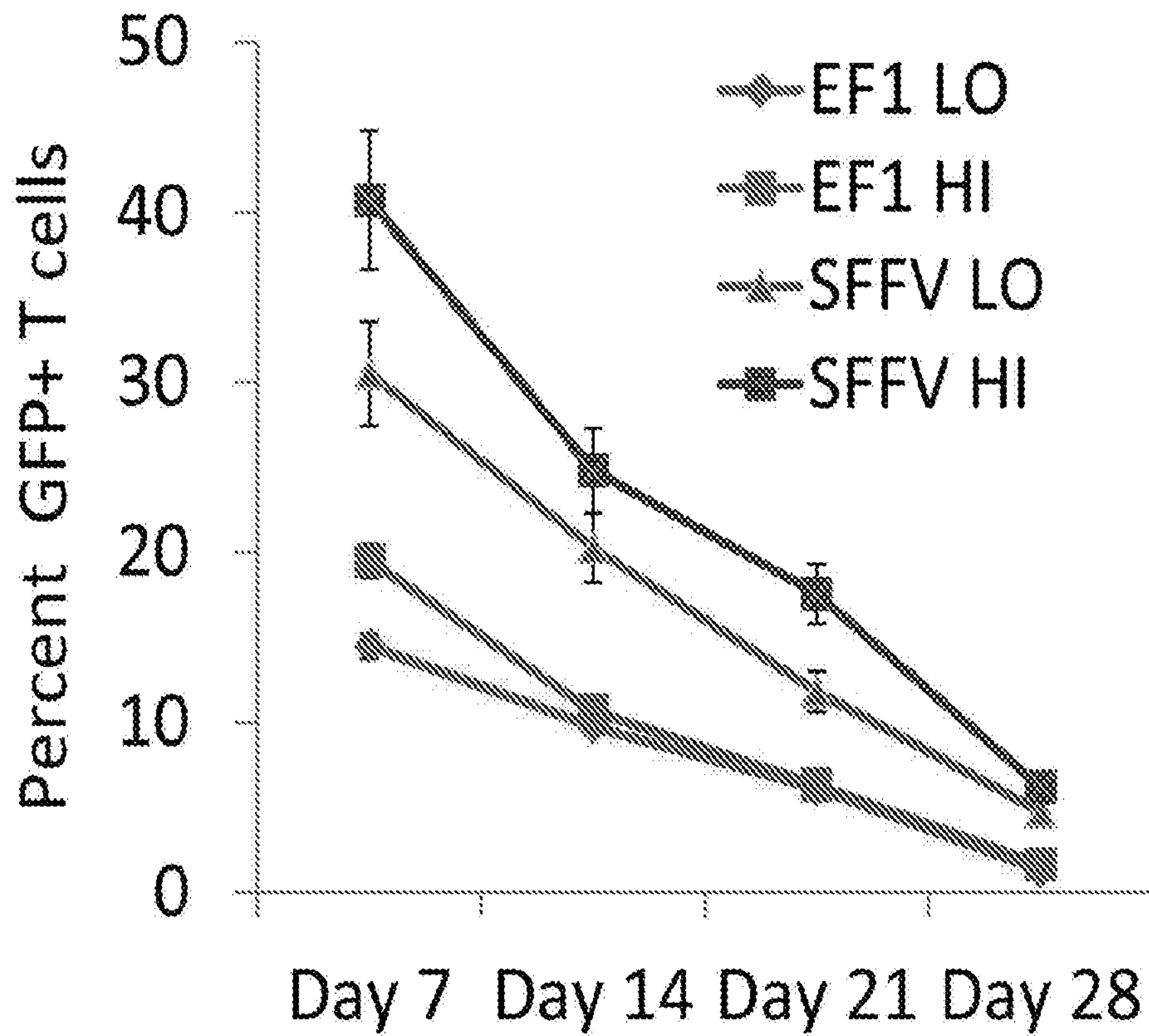


FIG. 44B

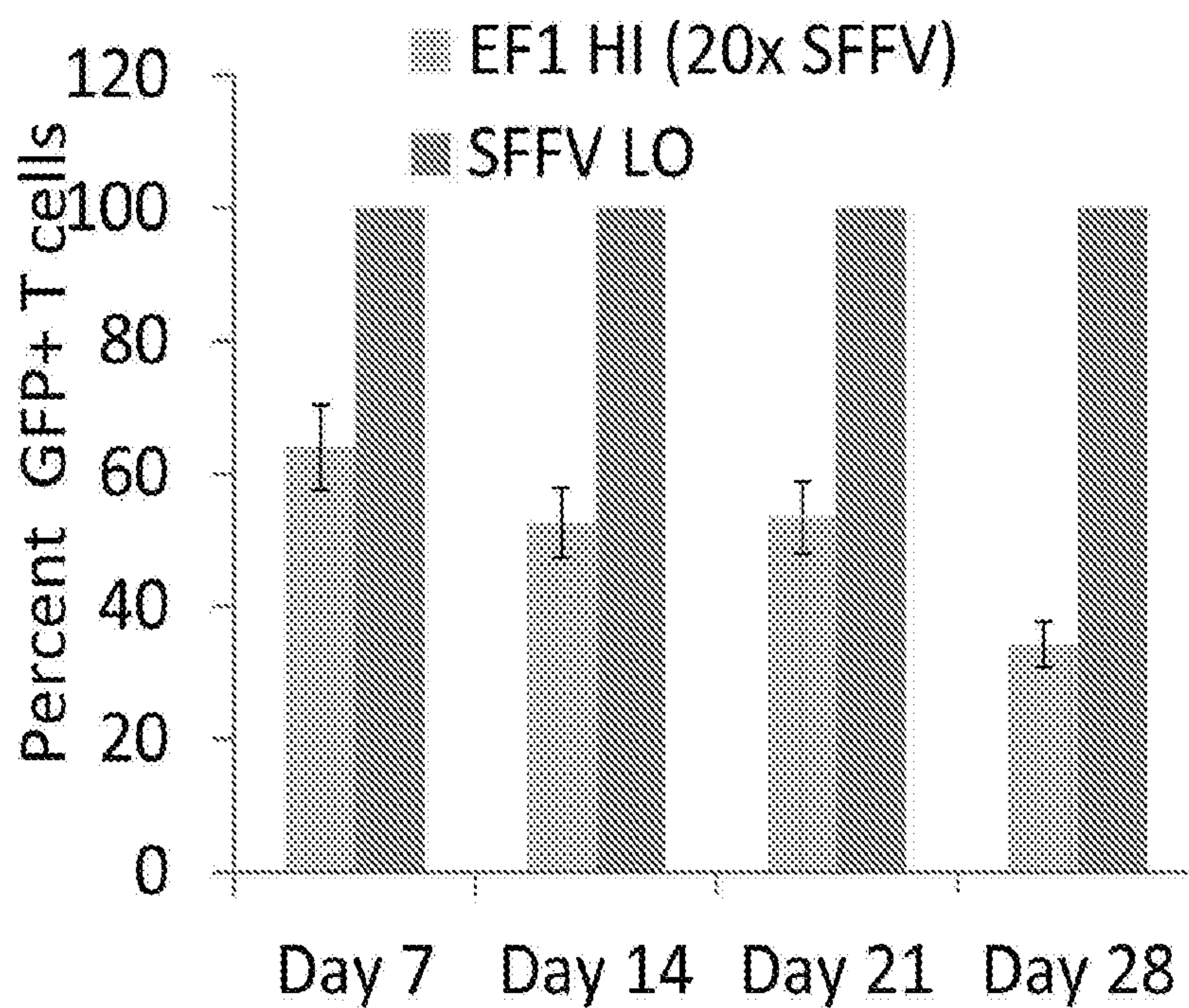
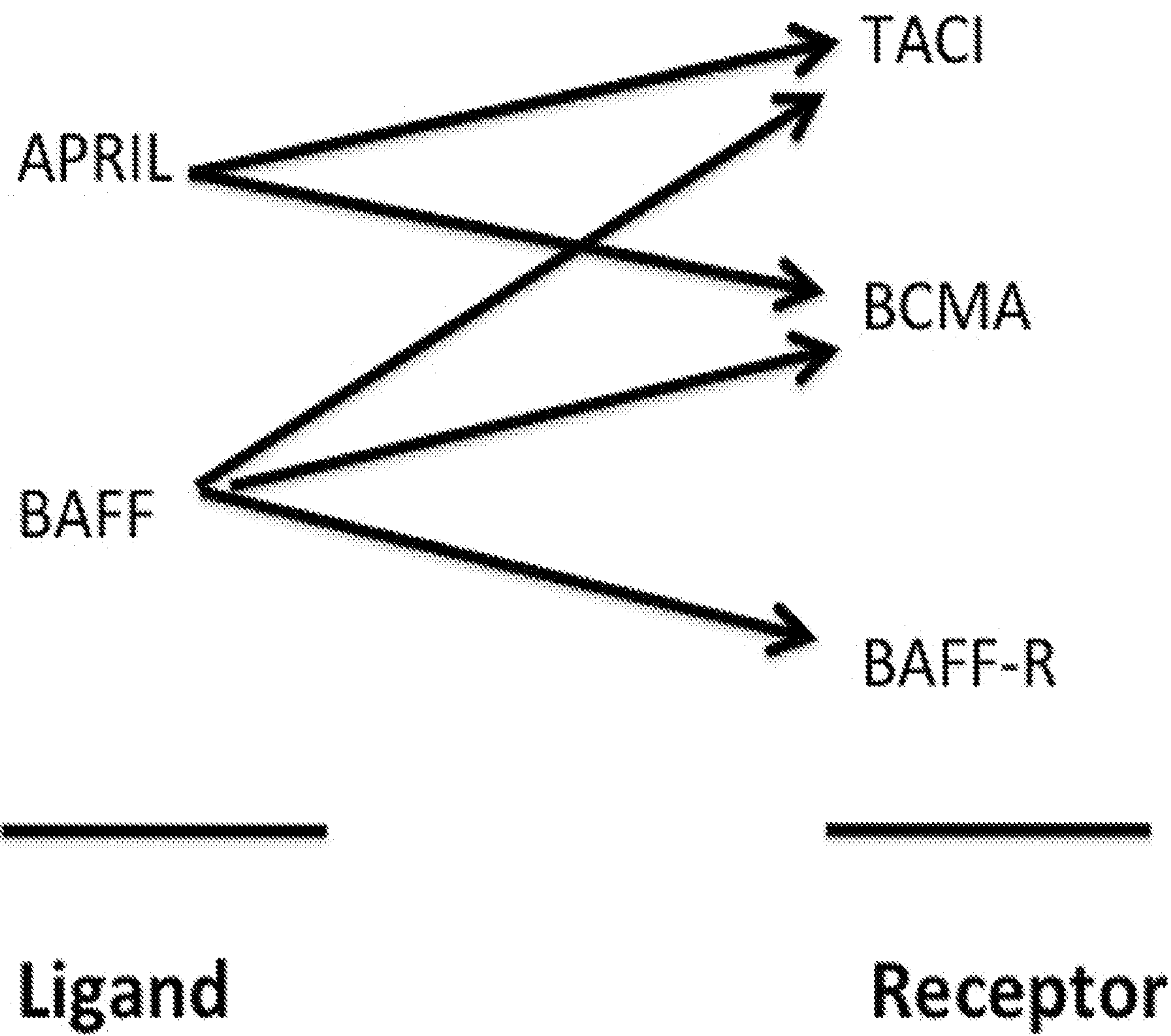


FIG. 45



What is a "compound" CAR (cCAR)?

The diagram illustrates the structure and function of a compound CAR (cCAR). The cCAR protein is composed of an extracellular domain (containing two antibody arms: Anti-Target 1 and Anti-Target 2), a transmembrane domain, and an intracellular domain (containing a signaling domain). The cCAR proteins are shown binding to Target 1 and Target 2 on adjacent cells. The intracellular domains of the cCAR proteins are shown interacting with a signaling pathway, leading to the activation of the CAR cell.

Cell Type 1
Target 1

Cell Type 2
Target 2

Anti-Target 1
Anti-Target 2

Membrane Antibody 1
Membrane Antibody 2

Target 1
Target 2

Binding

Transmembrane Domain

Signaling Domain

cCAR protein
Expressed Simultaneously

upregulate CAR cell activation
activate CAR
CAR cell activation
induce target cell killing

CAR Cell