(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
04 July 2024 (04.07.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/141977 A1

(51) International Patent Classification:
C12N 5/0783 (2010.01) *A61K 39/00* (2006.01)
A61K 35/17 (2015.01)

(21) International Application Number:
PCT/IB2023/063330

(22) International Filing Date:
28 December 2023 (28.12.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/477,785 29 December 2022 (29.12.2022) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) **Title:** ENGINEERED NATURAL KILLER CELLS AND RELATED METHODS

(57) **Abstract:** The disclosure provides a population of engineered natural killer cells, wherein the NK cells express at least one polypeptide selected from the group consisting of human leukocyte antigen E, human leukocyte antigen F, and human leukocyte antigen G. The NK cells comprise a genetically engineered disruption of one or more copies of endogenous β-2 microglobulin and a genetically engineered disruption of one or more copies of a human leukocyte antigen class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein, regulatory factor 5, regulatory factor X associated protein, and class II transactivator. A method of producing human pluripotent stem cell-derived engineered natural killer cells under feeder free conditions also is provided, as is a method of treating a disease or disorder in a subject by administering the NK cells to the subject.

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ENGINEERED NATURAL KILLER CELLS AND RELATED METHODS

FIELD OF THE INVENTION

[0001] The invention relates to engineered natural killer (NK) cells and methods of preparing populations of engineered NK cells.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of U.S. Provisional Patent Application No. 63/477,785, filed December 29, 2022, which is hereby incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: 58,824 byte file named "57819P_Seqlisting.XML"; created on December 18, 2022.

BACKGROUND

[0004] The engineering of patient-derived immune cells to express chimeric antigen receptors (CARs) has altered the landscape of adoptive cell therapies, providing scientists and clinicians the ability to harness the powerful cytolytic capabilities of immune cells and direct them to specific antigen-expressing targets in an MHC-independent manner. Natural killer (NK) cells, which arise through the lymphoid lineage, are part of the innate immune system and attractive for adoptive cell therapy as they have been found to detect and kill certain types of tumor cells. Typical sources of NK cells include peripheral blood NK cells (PB-NK), umbilical cord blood NK cells and NK cell lines, such as NK-92. While promising, these sources suffer from genetic instability, inconsistent cytotoxicity, limited expansion, product heterogeneity, and/or host immunogenicity. There is a need in the art for improved NK cell-based therapies to address limitations of current therapy options.

SUMMARY

[0005] The disclosure provides a population of natural killer (NK) cells derived from pluripotent stem cells. The disclosure further provides a population of engineered NK cells, wherein the NK cells express at least one polypeptide selected from the group consisting of human leukocyte antigen E (HLA-E), human leukocyte antigen F (HLA-F), and human leukocyte antigen G (HLA-G). The NK cells also comprise a genetically engineered disruption of one or more copies (e.g., all copies) of endogenous β-2 microglobulin (B2M) and/or a genetically engineered disruption of one or more copies (e.g., all copies) of a human leukocyte antigen (HLA) class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein (RFXANK), regulatory factor 5 (RFX5), regulatory factor X associated protein (RFXAP), and class II transactivator (CIITA). For example, the NK cells comprise a genetically engineered disruption of endogenous B2M and RFXANK, and express HLA-E. In another example, the NK cells comprise a genetically engineered disruption of all copies of endogenous B2M and all copies of RFXANK, and express HLA-E. In various aspects, the NK cells further

comprise a nucleic acid molecule encoding a suicide gene product (e.g., a herpes simplex virus thymidine kinase (TK) suicide gene). The NK cells are optionally derived from pluripotent stem cells (e.g., embryonic stem cells or induced pluripotent stem cells), and optionally express one or more cell surface markers selected from the group consisting of CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and NKp44. Optionally, the NK cells further express a chimeric antigen receptor (CAR); an Fc receptor (FcR); or a non-natural NKG2D receptor (see, for example, U.S. Patent No. 10,259,858, and U.S. Patent Publication No. 2020/0138866), which, in some aspects, is capable of binding an antibody-based bispecific molecule (e.g., a MicAbody); or other type of receptor.

[0006] The disclosure further provides a method of producing pluripotent stem cell-derived NK cells under feeder free, optionally serum-free, conditions. The method comprises (a) culturing pluripotent stem cells (PSCs), optionally genetically engineered to disrupt one or more copies (e.g., all copies) of B2M and/or one or more copies (e.g., all copies) of a HLA class II-related gene selected from the group consisting of RFXANK, RFX5, RFXAP, and CIITA, and optionally express a HLA class I protein, e.g., HLA-E; (b) differentiating the pluripotent stem cells to precursor cells (e.g., using embryoid bodies) capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives; and (c) differentiating the precursor cells to NK cells under feeder free, optionally serum-free, conditions in a culture media comprising one or more of SCF, IL-7, IL-15, and Flt3L. Optionally, the culture media of step (c) further comprises a pyrimido [4,5-b] indole derivative such as (1r,4r)-N1-(2-benzyl-7-(2-methyl-2H-tetrazol-5-yl)-9H-pyrimidol [4,5-b] indol-4-yl)cyclohexane-1,4-diamine dihydrobromide dihydrate ("UM171"). In various aspects, the PSC is a human PSC. In other various aspects, the method further comprises (d) culturing the NK cells in a culture media comprising IL-15 and IL-18 (and, optionally, IL-21), and in the presence of an antibody or ligand that binds a receptor on NK cells to promote activation and expansion of the NK cells. In one aspect, the antibody or ligand binds to a receptor selected from the group consisting of DNAM-1, OX40, NKG2D, 2B4, NKp30, and NKp46. A combination of antibodies or ligands which bind different targets selected from DNAM-1, OX40, NKG2D, 2B4, NKp30, and NKp46 may be included in step (d). For example, step (d) may comprise culturing the NK cells in the presence of an antibody that binds NKp30 and/or an antibody that binds DNAM-1.

[0007] The disclosure also provides a method of treating a disease in a subject in need thereof, the method comprising administering to the subject an effective amount of the population of NK cells, thereby treating the disease in the subject. In some aspects of the disclosure, the disease is cancer. Optionally, one or more cytokines, such as IL-15 and/or IL-2, are administered to the subject prior to, during, or after administering the population of NK cells. Also optionally, one or more antibody constructs are administered to the subject prior to, during, or after administering the population of NK cells.

[0008] While various embodiments in the specification are presented using "comprising" language, under various circumstances, a related embodiment may also be described using "consisting of" or "consisting essentially of" language. The disclosure contemplates embodiments described as "comprising" a feature to include embodiments which "consist of" or "consist essentially of" the feature. The term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

The term "or" should be understood to encompass items in the alternative or together unless context unambiguously requires otherwise.

[0009] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein. However, the description also contemplates the same ranges in which the lower and/or the higher endpoint is excluded. When the term "about" is used in relation to a numerical number or general value, it means the recited number or value plus or minus 1%, 2%, 3%, 4%, 5%, 10%, or 15%, including all the values within this range, of that recited number or value. The actual variation intended is determinable from the context.

[0010] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the disclosure and does not pose a limitation on the scope of the disclosure unless otherwise claimed. Only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

[0011] Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including the figures and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specified as an aspect or embodiment of the invention. The entire document is intended to be related as a unified disclosure, and it should be understood that all combinations of features described herein (even if described in separate sections) are contemplated, even if the combination of features is not found together in the same sentence, or paragraph, or section of this document.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 depicts a representative scheme for an NK differentiation process described herein: Step 0 – PSC Culture; Step 1 – Hematopoietic Differentiation; Step 2 – NK Differentiation; Step 3A – NK Activation; and Step 3B – NK Expansion. Steps 0-3B are referenced in the figure descriptions below.

[0013] Figures 2A-2C are bar graphs illustrating the cell yield (Figure 2A), percentage of $CD56^{+}CD45^{+}$ cells (Figure 2B), and cell viability (Figure 2C) resulting from the method of producing human PSC-derived engineered NK cells (FcR-UDC I-NK cells) described herein. The bar on the left of each graph represents conditions which did not include UM171, and the bar on the right in each graph represents conditions wherein 35 nM UM171 was present.

[0014] Figures 3A-D are bar graphs illustrating the Step 2 (NK Differentiation) yield and expansion of NK cells across multiple gene edited clones (Figures 3A-3B) and PSC cell lines (Figures 3C-D). In Figures 3A-3B, NK

cells were derived from NIH3 PSC cell line (wild-type) or genetically engineered NIH3 PSC cell lines: UDC I-PSC, FcR-UDC I-PSC, and UDC I/II-PSC. In Figures 3C-3D, NK cells were derived from NIH3 and W1C1 PSC cell lines that were genetically engineered (UDC I-PSCs and UDC I/II-PSC) or parental NIH3 cell line (wild-type).

[0015] Figure 4 is a graph illustrating the effects of various combinations of NK receptor agonists on the activation of CD56+ cells, as measured by expression of CD215/IL-15Rα (left panel) or CD25/IL-2Rα (right panel).

[0016] Figures 5A-5B are line graphs illustrating (Figure 5A) cumulative fold-expansion and (Figure 5B) cell viability after Step 3A and Step 3B of the NK differentiation process (illustrated in Figure 1) testing different cell densities plated at the beginning of Step 3A. NK cells were generated from NIH3 UDC I-PSC using the method described herein.

[0017] Figure 6 is a graph comparing cell sizes of NK cells derived from PSCs using the method described herein ("iPSC") and NK cells from peripheral blood ("PBNK") as determined by flow cytometry. NK cells derived from PSCs using the method described herein were on average about 18% larger in size (by volume) compared to NK cells isolated from peripheral blood.

[0018] Figures 7A-7E are bar graphs of the expected phenotype of NK cells derived from different gene edited clones: NIH3 wild-type and engineered NIH3 UDC I-PSC, FcR-UDC I-PSC, and UDC I/II-PSC. Asterisk indicates no data collected.

[0019] Figures 8A-8D are bar graphs of the expected phenotype of NK cells derived from different cells lines: NIH3 and W1C1 wild-type, and gene edited clones engineered UDC I-PSC and UDC I/II-PSC. Asterisk indicates no data collected.

[0020] Figures 9A-9C are bar graphs illustrating expression of common NK and T cell markers in FcR-UDC I-NK cells (Step 2 (left bar for each marker) and Step 3 (middle bar for each marker)) derived from engineered FcR-UDC I-PSC compared to expanded PB-NK (right bar for each marker). Steps 2 and 3 are illustrated in Figure 1.

[0021] Figures 10A-10C are bar graphs illustrating expression of common activation, exhaustion, and "memory-like" markers in FcR-UDC I-NK cells (Step 2 (left bar for each marker) and Step 3 (middle bar for each marker)) derived from engineered FcR-UDC I-PSC compared to expanded PB-NK (right bar for each marker). Steps 2 and 3 are illustrated in Figure 1.

[0022] Figures 11A-11B are bar graphs illustrating expression of cytokine and chemokine receptors in FcR-UDC I-NK cells (Step 2 (left bar for each receptor) and Step 3 (middle bar for each receptor)) derived from engineered FcR-UDC I-PSC compared to expanded PB-NK (right bar for each receptor). Steps 2 and 3 are illustrated in Figure 1.

[0023] Figures 12A-12C are bar graphs illustrating expression of adhesion/homing, costimulatory, and apoptotic markers in FcR-UDC I-NK cells (Step 2 (left bar for each receptor) and Step 3 (middle bar for each

receptor)) derived from engineered FcR-UDC I-PSC compared to expanded PB-NK (right bar for each receptor). Steps 2 and 3 are illustrated in Figure 1.

[0024] Figure 13 is a line graph illustrating natural cellular cytotoxicity (NCC) of the NK cells derived from engineered NIH3 and W1C1 PSC, UDC I-PSC (2F7 (NIH) and 1A6 (W1C1)), FcR-UDC I-PSC (3C1), and UDC I/II-PSC (10A7 (NIH) and 2A6 (W1C1)), against K562 tumor target cells. Percent specific lysis is provided on the y-axis, and effector to target cell ratio (E/T) is provided on the x-axis. The UDC-NK cells disclosed herein mediated cell lysis across multiple PSC cell lines and gene edited clones.

[0025] Figure 15 is a line graph illustrating NCC of FcR-UDC I-NK cells compared to expanded PB-NK. Percent specific lysis is provided on the y-axis, and effector to target cell ratio (E/T) is provided on the x-axis. The UDC-NK cell population disclosed herein mediated cell lysis to a degree comparable to PB-NK.

[0026] Figure 16 is a line graph illustrating that FcR-UDC I-NK exhibits no fratricide nor cytotoxicity against donor PBMCs, but exhibits NCC against K562 tumor target cells.

[0027] Figure 17 is a line graph demonstrating the efficacy of engineered NK cells (FcR-UDC I-NK cell) to kill serially added K562 tumor cells in a dose-dependent manner.

[0028] Figures 18A-18D are line graphs illustrating antibody-dependent cellular cytotoxicity (ADCC) activity of the engineered FcR-UDC I-NK cells generated using the methods described herein. Percent specific lysis is provided on the y-axis, and effector to target cell ratio (E/T) is provided on the x-axis. In Figures 18A-18C, the FcR-UDC I-NK cell population disclosed herein demonstrated ADCC activity against 3 different $CD20^+$ tumor cells lines: Daudi (Figure 18A), Ramos (Figure 18B), and Raji (Figure 18C) using commercially available Rituximab that binds CD20 as compared to an IgG1 control. In Figure 18D, expanded PB-NK did not show any difference in ADCC activity between Rituximab and IgG1 control. Circles represent no antibody, squares represent human IgG1, and triangles represent Rituximab.

[0029] Figure 19 is a bar graph illustrating NCC and ADCC activity of FcR-UDC I-NK cells across a broad range of B lymphoma cell lines.

[0030] Figure 20A is bar graphs identifying the inducible cytokines after stimulation with Rituximab (R-mab)-coated Raji tumor cells in FcR-UDC I-NK cells (left bar for each condition noted) compared to expanded PB-NK (right bar for each condition noted). The x-axis for each graph recites "no stimulation," "Raji+hIgG1," and "Raji+R-mab." Significant elevation was observed for TNF-alpha, interferon-gamma, GM-CSF, and IP-10. Moderate elevation was observed for granzyme-B, MIP-1alpha, MIP-1beta, RANTES, IL-8, IL-10, and MCP-1. Cytokines not detected included IL-1alpha, IL-1beta, IL-6, and IL-12 as shown in Figures 20B-20E.

[0031] Figures 21A-21D are bar graphs identifying cytokines and/or chemokines that were not elevated following co-culture with allogeneic PBMC versus co-culture with Raji plus Rituximab in FcR-UDC I-NK cells (left bar for each condition) compared to expanded PB-NK (right bar for each condition). The x-axis for each graph recites "no stimulation," "Raji+Rituximab," and "AlloPBMC."

[0032] Figures 22A-22G are bar graphs illustrating *in vivo* persistence of FcR-UDC I-NK cells in the absence of any antigen in non-tumor-bearing mice. The NK cells (4 x 10^6) were injected i.v. in the mice followed by administration of IL-2 (30,000 IU, 3x/wk.) and IL-15 (100 ng, daily for 7 days). The percent and absolute number of FcR-UDC I-NK cells in the blood, spleen, and bone marrow were measured.

[0033] Figures 23A-23E are bar graphs illustrating the *in vivo* tissue distribution of FcR-UDC I-NK cells in non-tumor-bearing mice. The NK cells (5 x 10^6, 7.5 x 10^6, and 10 x 10^6) were injected i.v. followed by administration of IL-2 (30,000 IU, 3x/wk.) and IL-15 (100 ng, daily). The percentage of human $CD56^+$ cells (FcR-UDC I-NK cells) within mouse plus human CD45 was measured in the lung (Figure 23B), liver (Figure 23D), spleen (Figure 23E), blood (Figure 23A), and bone marrow (Figure 23C).

[0034] Figure 24 is a graphic of an efficacy study of FcR-UDC I-NK cells plus Rituximab in a Raji i.p. tumor model. NSG mice were irradiated and injected (i.p.) with Raji tumor cells (3 x 10^5). At day 4, FcR-UDC I-NK cells or PB-NK cells (each 5 x 10^6) with or without Rituximab (300 µg) was administered. At day 7, an additional dose of Rituximab (300 µg) was administered. Imaging/monitoring of the NSG mice occur at indicated time points through day 120.

[0035] Figures 25A-25C illustrate (Figure 25A) a survival curve and (Figures 25B-25C) tumor burden following the efficacy study of Figure 24. In Figure 25A, both FcR-UDC I-NK cells plus Rituximab and PB-NK plus Rituximab had a significant increase in median survival (days) as compared to untreated, Rituximab, FcR-UDC I-NK cells, and PB-NK alone. Rituximab and PB-NK alone increased mean survival (days) compared to untreated. In Figures 25B-25C, FcR-UDC I-NK cells plus Rituximab and PB-NK plus Rituximab provided a durable response in delaying tumor onset through day 60 in 50% of the mice.

[0036] Figure 26 is a graphic of an efficacy study of FcR-UDC I-NK cells plus Rituximab in a Raji i.p. tumor model. NSG mice were irradiated and injected (i.p.) with Raji tumor cells (2 x 10^5). At day 2, FcR-UDC I-NK cells (10 x 10^6) with or without Rituximab (100 µg) was administered. At days 6, 10, and/or 16, additional doses of FcR-UDC I-NK cells or PB-NK cells (each 10 x 10^6) were administered. Imaging /monitoring of the NSG mice occur at indicated time points through day 86.

[0037] Figures 27A-27B illustrate (Figure 27A) tumor burden and (Figure 27B) a survival curve following the efficacy study of Figure 26. In Figure 27A, FcR-UDC I-NK cells plus Rituximab (D2, 10, and 16) had longer durable response in delaying tumor onset compared to FcR-UDC I-NK cells plus Rituximab (D2, 6, and 10). In Figure 27B, both FcR-UDC I-NK cells plus Rituximab (regardless of dosing regimen) had a significant increase in median survival (days) as compared to untreated, Rituximab, and FcR-UDC I-NK cells alone.

[0038] Figures 28A-28B are related to an efficacy study using NOGf mice that have mouse FcgR knocked out. Figure 28A is a graphic of the study wherein Rituximab or FcR-UDC I-NK cells plus Rituximab were administered in a Raji tumor model. NOGf mice were injected (i.v.) with Raji tumor cells (1 x 10^5). Rituximab (100 µg, i.p.) was administered on days 1, 8, and 15 following tumor cell implantation, and FcR-UDC-I-NK (10M) was administered i.v. on days 1, 4, 8, and 15. Figure 28B illustrates tumor burden following the efficacy study of Figure 28A.

Cryopreserved FcR-UDC I-NK cells plus Rituximab demonstrated a greater degree of tumor growth inhibition compared to administration of Rituximab alone.

[0039] Figures 29A-29B are related to an efficacy study using NOGf mice engineered to express human interleukin 15 (IL-15) and that have mouse FcgR knocked out. Figure 29A is a graphic of the study wherein Rituximab (50 or 100 µg, i.p.) or FcR-UDC I-NK cells (3 x 10^6 (3M) or 15 x 10^6 (15M), i.v.) plus Rituximab (50 or 100 µg, i.p.) were administered in a Raji tumor model. Figure 29B illustrates tumor burden following the efficacy study of Figure 29A. 15M cryopreserved FcR-UDC I-NK cells plus Rituximab demonstrated a great degree of tumor growth inhibition compared to Rituximab alone.

[0040] Figure 30 is a line graph illustrating the anti-tumor effect of FcR-UDC I-NK cells plus anti-claudin 6 (CLDN6) antibodies (ASP1893 mAb, a humanized anti-CLDN6 antibody and ASP1650, a human/mouse chimeric anti-CLDN6 antibody) against CLDN6-positive tumor cells (the ovarian cancer cell line PA-1). The specific lysis (%) is presented on the y-axis and the amount of antibody ASP1893 mAb or ASP1650 (ng/mL) is presented on the x-axis. Anti-CLDN6 antibodies ASP1893 mAb and ASP1650 induced comparable ADCC with FcR-UDC I-NK cells against CLDN6+ tumor cells.

[0041] Figures 31A-31M are line graphs illustrating the anti-tumor effect of FcR-UDC I-NK cells plus anti-CLDN6 antibodies against tumor cells expressing various levels of CLDN6. Assays were conducted with FcR-UDC I-NK cells with antibody ASP1893 mAb with control IgG1 antibody, or with no antibody. Figures 31A-31D represent assays using tumor cells which highly express CLDN6 (SKOV3-CLDN6, ES-2-CLDN6, PA-1, and NEC14 cells). Figures 31E-31G represent assays using tumor cells which express moderate levels of CLDN6 (ABC-1, OV90, and OVCAR3 cells). Figures 31H-31I represent assays using tumor cells which express low levels of CLDN6 (COV362 and NEC8-Luc cells). Figures 31J-31M represent assays using tumor cells which express no CLDN6 (COV318, ES-2, SKOV3, and NCI-H1373 cells). The specific lysis (%) is presented on the y-axis and the E/T ratio is presented on the x-axis. FcR-UDC I-NK cells mediated ADCC with ASP1893 mAb against tumor cells in CLDN6 expression dependent manner.

[0042] Figures 32A-32B illustrate cytokine secretion of FcR-UDC I-NK cells. The figures are bar graphs correlating interferon-gamma (IFN-g; pg/mL) or tumor necrosis factor-alpha (TNF-a) produced by FcR-UDC I-NK cells in the presence of tumor cells (left bar for each cell line tested), tumor cells with human IgG1 (middle bar for each cell line tested), and tumor cells with anti-CLDN6 antibody (ASP1893 mAb) (right bar for each cell line tested). SKOV3-CLDN6, ES-2-CLDN6, PA-1, and NEC14 cells display high levels of CLDN6. ABC-1 and OV90, cells display moderate levels of CLDN6. COV362 and NEC8-Luc cells express low levels of CLDN6. COV318, ES-2, and SKOV3 cells are CLDN6-negative. FcR-UDC I-NK cells exhibited IFN-g and TNF-a production upon stimulation with tumor cells and ASP1893 mAb in a CLDN6 expression-dependent manner.

[0043] Figure 33 is a schematic of an *in vivo* assay examining the anti-tumor efficacy of UDC-NK cells in conjunction with anti-CLDN6 antibody in a PA-1 xenograft solid tumor mouse model. The NOGf IL-15 mice expressed human IL-15 and lacked mouse FcgR.

[0044] Figures 34A-34B illustrate tumor volume (Figure 34A) and survival curve (Figure 34B) following the efficacy study of Figure 33. The combination treatment of FcR-UDC-NK cells and anti-CLDN6 antibody resulted in tumor growth inhibition (Figure 34A) and prolonged the survival of mice in PA-1 SC tumor model (Figure 34B).

[0045] Figures 35A-35C are related to a study examining the anti-tumor efficacy of UDC-NK cells in conjunction with anti-CLDN6 antibody in a PA-1 xenograft solid tumor mouse model using NOGf mice engineered to express human interleukin 15 (IL-15) and that have mouse FcgR knocked out. Figure 35A is a schematic of the *in vivo* assay wherein 7.5M FcR-UDC-NK cells were administered i.v. on days 16, 20, 23, and 27 and anti-CLDN6 antibody (300 µg, i.p.) was administered on days 16 and 23. Figures 35B-35C illustrate tumor volume (Figure 35B) and survival curve (Figure 35C) following the efficacy study of Figure 35A. The combination treatment of FcR-UDC-NK cells and anti-CLDN6 antibody resulted in tumor growth inhibition (Figure 35B) and prolonged the survival of mice in PA-1 SC tumor model (Figure 35C).

[0046] Figures 36A-36B illustrate ADCC activity of FcR-UDC-NK cells. Figure 36A is a line graph illustrating the anti-tumor effect of FcR-UDC I-NK cells plus anti-claudin 18.2 antibody (Zolbetuximab) against BxPC3-CLDN18.2-Luc tumor cells. The specific lysis (%) is presented on the y-axis and the E:T ratio is presented on the x-axis. Figure 36B is a line graph illustrating the anti-tumor effect of FcR-UDC I/II-NK cells plus anti-claudin 18.2 antibody (Zolbetuximab) against BxPC3-CLDN18.2-Luciferase tumor target cells. The fluorescent intensity of the remaining target cells, normalized to the start of the experiment, is presented on the y-axis and the time of measurement is presented on the x-axis.

[0047] Figures 37A-37B are bar graphs illustrating interferon-gamma (Figure 37A) or tumor necrosis factor-alpha (Figure 37B) secretion by FcR-UDC I-NK in samples comprising BxPC3-CLDN18.2-Luc tumor cells alone, BxPC3-CLDN18.2-Luc tumor cells with human IgG, or BxPC3-CLDN18.2-Luc tumor cells with Zolbetuximab.

DETAILED DESCRIPTION

[0048] The instant disclosure provides a population of natural killers (NK) cells with advantageous attributes for immune cell therapy. The materials and methods described herein enable production of, e.g., a substantially homogenous population of NK cells with reduced occurrence of undesired cells, improved safety profiles, strong persistence *in vivo*, enhanced ability to engraft in a subject, and/or enhanced ability to reduce tumor burden.

[0049] The disclosure provides a population of NK cells derived from pluripotent stem cells, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). The disclosure further provides a population of engineered NK cells, wherein the NK cells express at least one exogenous polypeptide selected from the group consisting of human leukocyte antigen E (HLA-E), human leukocyte antigen F (HLA-F), and human leukocyte antigen G (HLA-G). The NK cells are genetically engineered to disrupt of one or more copies (e.g., all copies) of the cells' endogenous β-2 microglobulin (B2M) and/or one or more copies (e.g., all copies) of a human leukocyte antigen (HLA) class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein (RFXANK), regulatory factor 5 (RFX5), regulatory factor X associated protein (RFXAP), and class II transactivator (CIITA). The disruptions allow the NK cells to, e.g., reduce or evade

immune detection, and allow production of an NK cell population which does not require HLA matching for therapeutic applications. Optionally, the NK cells also express a chimeric antigen receptor (CAR) (e.g., a universal CAR), an Fc receptor (FcR or CD16), a non-natural NKG2D receptor, or other type of receptor.

[0050] NK cells are cytotoxic lymphocytes of the innate immune system capable of immune surveillance. NK cells are attractive therapeutics for their natural cytotoxicity to foreign, transformed, or virally infected cells, their antibody-dependent cellular cytotoxicity, and cytokine release profile which can amplify an immune response. NK cells are typically defined by the expression of CD56 or CD16 and the absence of CD3. In various aspects of the disclosure, the NK cells express one or more (e.g., two or more, three or more, four or more, etc.) cell surface markers selected from CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and/or NKp44. Methods of detecting cell surface markers are known in the art and include, for instance, flow cytometry-based methods.

[0051] The NK cells (or a PSC from which the NK cell is derived or an intermediate thereof) are genetically engineered to disrupt of one or more copies (e.g., all copies) of the cells' endogenous B2M gene. B2M is a critical component of the HLA class I (HLA-I) complex. HLA is a cell surface complex that mediates leukocyte-leukocyte interactions or interactions of leukocytes with other cells. HLA-I molecules are cell surface complexes which present antigens to CD8+ cytotoxic T cells, thereby mediating cellular immunity. HLA-I molecules comprise an HLA-I heavy chain and B2M. Genetic disruption of one or more (e.g., all) copies of the B2M gene reduces or abrogates production of the B2M protein (SEQ ID NO: 5), thereby reducing or abrogating display of HLA-I complexes on the NK cell. A representative nucleic acid sequence encoding B2M is provided in SEQ ID NO: 1.

[0052] Also contemplated are NK cells (or a PSC from which the NK cell is derived or an intermediate thereof) genetically engineered to disrupt one or more copies (e.g., all copies) of a subset of HLA-I. Six HLA class I alpha (α) chains have been identified, including three classical (HLA-A, HLA-B and HLA-C) and three non-classical (HLA-E, HLA-F, and HLA-G), which are responsible for specificity of peptide binding on the HLA-I binding cleft. In this regard, the disclosure contemplates NK cells genetically engineered to disrupt one or more copies (e.g., all copies) of an HLA-I gene selected from HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G, as well as combinations thereof. For example, the NK cells (or a PSC from which the NK cell is derived or an intermediate thereof) may be genetically engineered to disrupt one or more copies (e.g., all copies) of HLA-A, HLA-B, and/or HLA-C. HLA molecules are further described in, e.g., Choo, Yonsei Med J., 48(1): 11–23 (2007); www.ebi.ac.uk/ipd/imgt/hla/; and GenBank Accession Nos. NG_029217 (HLA-A), XP_041536701 (HLA-A), NG_023187 (HLA-B), NP_005505 (HLA-B), NG_029422 (HLA-C), NP_001229971 (HLA-C), NM_005516 (HLA-E), NP_005507 (HLA-E), NG_012009 (HLA-F), NP_001091949 (HLA-F), NG_029039 (HLA-G), and NP_001371219 (HLA-G).

[0053] The NK cells of the disclosure (or a PSC from which the NK cell is derived or an intermediate thereof) are genetically engineered to disrupt one or more (e.g., all) copies of an HLA class II (HLA-II)-related gene selected from RFXANK, RFX5, RFXAP, and CIITA. HLA-II molecules are transmembrane proteins found on antigen-presenting cells (APCs) and, at times, solid organs. HLA-II molecules comprise two homologous

subunits, the alpha (α) subunit and the beta (β) subunit. HLA-II related genes encode HLA-II regulatory proteins that regulate the expression of HLA class II molecules. Genetic disruption of one or more copies (e.g., all copies) of one or more of the HLA-II related genes RFXANK, RFX5, RFXAP, and/or CIITA reduces or abrogates production of the encoded proteins, thereby reducing or abrogating display of HLA-II complexes on the NK cell. The NK cell (or a PSC from which the NK cell is derived or an intermediate thereof) may comprise disruptions of one or more copies (e.g., all copies) of the genes encoding one of RFXANK, RFX5, RFXAP, and/or CIITA (e.g., the cell is engineered to disrupt one or more copies (e.g., all copies) of the RFXANK gene); one or more copies (e.g., all copies) of the genes encoding any combination of two of RFXANK, RFX5, RFXAP, and/or CIITA; one or more copies (e.g., all copies) of the genes encoding any combination of three of RFXANK, RFX5, RFXAP, and/or CIITA; or one or more copies (e.g., all copies) of the genes encoding all of RFXANK, RFX5, RFXAP, and CIITA. RFXANK is encoded by, e.g., the sequence of SEQ ID NOs: 3 and 5; RFX5 is encoded by, e.g., the sequence of SEQ ID NOs: 7 and 9; RFXAP is encoded by, e.g., SEQ ID NO: 11; and CIITA is encoded by, e.g., SEQ ID NO: 13. In various aspects of the disclosure, the NK cells are engineered to disrupt all copies of B2M and RFXANK.

[0054] Any suitable technique for introducing a disruption in a target gene (e.g., an HLA-II related gene, a B2M gene, or any other gene of interest) may be used. Many techniques for disrupting endogenous coding sequences are known in the art, including use of gene editing systems such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) systems, transcription activator-like effector nucleases (TALENs), and zinc finger nucleases, as well as targeting vectors that cleave and/or insert nucleic acid sequences into a target site in the cellular genome. An exemplary technique employs adeno-associated virus (AAV) vector-based editing, which optionally involves targeted insertion of an edited sequence (potentially a transgene) into the cellular genome so as to interrupt an endogenous coding sequence, thereby resulting in reduction or avoidance of production of the endogenous protein. In various aspects, serotype 3B AAV vectors are employed. Use of AAV vectors to engineer stem cells is further described in, e.g., Riolobos et al., Molecular Therapy, 21(6), 1232-1241 (2013), hereby incorporated by reference in its entirety and particularly with respect to the disclosure of use of AAV vectors and disruption of HLA complex production. Methods of confirming disruption of a target gene are known in the art and include, but are not limited to, PCR detection methods (e.g., quantitative real time polymerase chain reaction (qRT-PCR)), RNA sequencing, next generation sequencing methods, flow cytometry, and immunostaining.

[0055] In various aspects, the engineered NK cells express at least one polypeptide selected from the group consisting of HLA-E, HLA-F, and HLA-G. In the context of the disclosure, the NK cell is engineered to express HLA-E, HLA-F, and/or HLA-G, i.e., a nucleic acid encoding HLA-E, HLA-F, and/or HLA-G is introduced into the NK cell (or a PSC from which the NK cell is derived or an intermediate thereof) and the encoded protein is produced and displayed on the cell surface. Optionally, the engineered NK cells express HLA-E. Optionally, the engineered NK cell expresses a single chain fusion HLA-E comprising at least of portion of B2M linked, either directly or via a linker sequence, to at least of portion of the HLA-I α-chain, e.g., HLA-E. Optionally, the engineered NK cell expresses a single chain fusion HLA-F or HLA-G comprising at least of portion of B2M linked,

either directly or via a linker sequence, to at least of portion of the HLA-I alpha-chain, e.g., HLA-F or HLA-G. The engineered NK cell may overexpress a protein which is naturally produced by the cell, may express a protein encoded by an exogenous nucleic acid following knock-out or disruption of the corresponding endogenous coding sequence for the protein, or may express a protein which is not naturally encoded by the wild-type NK cell genome. Representative DNA and protein sequences for HLA-E are provided in SEQ ID NOs: 15 and 16. Representative DNA and protein sequences for HLA-F are provided in SEQ ID NOs: 17 and 18. Representative DNA and protein sequences for HLA-G are provided in SEQ ID NOs: 19 and 20. It will be appreciated that variants of the sequences provided herein may be used and, as such, the disclosure contemplates use of a nucleic acid comprising at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the nucleic acid sequences disclosed herein. The disclosure further contemplates use of a nucleic acid which encodes a peptide comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to any of the amino acid sequences described herein. Preferably, a variant retains HLA-I functions such as, e.g., forming a functional peptide binding cleft to display peptides and/or engaging inhibitory receptors on NK cells. Cells which express HLA-E, HLA-F, and/or HLA-G minimize the risk of rejection when used as an adoptive cell therapy. In various aspects of the disclosure, the NK cells are engineered to disrupt all copies of B2M and RFXANK and engineered to express HLA-E, e.g., single chain fusion HLA-E.

[0056] The NK cells of the population also may express an exogenous receptor (i.e., a receptor encoded by an exogenous nucleic acid introduced into the NK cells or a PSC from which the NK cell is derived or an intermediate thereof). In various aspects, the NK cells of the population express a chimeric antigen receptor. "Chimeric antigen receptor" or "CAR" refers to an artificial immune cell receptor that is engineered to recognize and bind to an antigen expressed by a target cell, such as a tumor cell. Generally, a CAR is a chimera of a signaling domain of the T cell receptor (TCR) complex and an antigen-recognizing domain (e.g., a single chain fragment (scFv) of an antibody or other antibody fragment). See, e.g., Enblad et al., Human Gene Therapy. 2015; 26(8):498-505. There are various formats of CARs, each of which contains different components. "First generation" CARs join an antigen binding domain to the CD3zeta intracellular signaling domain of the T-cell receptor through hinge and transmembrane domains. A cytoplasmic or intracellular signaling domain produces stimulatory signals for proliferation and effector function when the CAR engages with a target antigen. Many intracellular signaling domains contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM containing cytoplasmic signaling sequences include those derived from CD8, CD3zeta, CD3gamma, CD3epsilon, CD32 (Fc gamma RIIa), DAP10, DAP12, CD79a, CD79b, FcγRIγ, FcγRIIIγ, FcεRIβ (FCERIB), and FcεRIγ (FCERIG). Intracellular signaling domains, including those derived from CD3 zeta (CD3), are known in the art (TCR zeta, e.g., GenBank Accession No. BAG36664.1). Exemplary hinge domains include, but are not limited to, a CD28 hinge, a CD8 alpha hinge, a human IgG4 hinge domain, and a human IgG4 hinge domain combined with a CH3 human IgG4 domain. Hinge domains are further described in, e.g., Hudecek et al. (2013) Clin. Cancer Res., 19:3153, International Patent Publication No WO 2014031687,

and U.S. Patent No. 8,822,647. "Second generation" CARs incorporate an additional domain, e.g., CD28, 4-1BB (41BB), or ICOS, to supply a costimulatory signal. Additional costimulatory domains include, but are not limited to, CD27, CD134, OX40, CD149, DAP10, CD30, IL2-R, IL7r6, IL21-R, NKp30, NKp44, CD40, CD137, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, or DNAM-1 costimulatory domains (or any combination thereof). "Third generation" CARs contain two costimulatory domains fused with the TCR CD3zeta chain. Third generation costimulatory domains may include, e.g., a combination of CD3zeta, CD27, CD28, 4-1BB, ICOS, or OX40. CARs so constructed can trigger, e.g., cell activation upon binding the targeted antigen in a manner similar to an endogenous T cell receptor, but independent of the major histocompatibility complex (MHC).

[0057] Optionally, the NK cells may express a fragment crystallizable (Fc) region receptor (FcR), e.g., CD16 (FcγRIII). In this regard, the NK cell may be engineered to express an FcR, i.e., a nucleic acid encoding an FcR is introduced into the NK cell (or a PSC from which the NK cell is derived or an intermediate thereof) and the encoded protein is produced and displayed on the cell surface. Generally, an FcR comprises an extracellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain generally includes a domain which binds to an immunoglobulin (Ig) Fc region. The intracellular domain comprises a sufficient portion of an Fc receptor to allow the ITAM to initiate cell signaling when an Ig Fc region binds to the extracellular domain. Cells expressing FcR mediate the efficacy of both antibody-based therapeutics and, to a lesser extent, B-cell mediated adaptive immune responses. NK cells are mediators of antibody-dependent cell-mediated cytotoxicity (ADCC) driven by Fc binding to FcRs. In ADCC, an NK cell FcR binds an antibody and forms a lytic synapse at the interface with the target cell, triggering the release of cytokines and lytic granules. These granules contain enzymes like perforin and granzyme B, which induce the destruction of the antibody-coated target cell. Engineered NK cells expressing an Fc region receptor are referenced herein as “FcR-UDC-NK cells” or “FcR-NK cells.”

[0058] The NK cell may express an Fc-gamma (γ) receptor, an Fc-alpha (α) receptor, or an Fc-epsilon (ε) receptor. The Fcγ family of receptors bind IgG and include FcγRI (CD64), FcγRII (CD32, including CD32a, CD32b, and CD32c), and FcγRIII (CD16, including CD16a and CD16b). CD16a has two allelic variants, 158V and 158F, that possess different affinities for IgG; the NK cell of the disclosure may express CD16a, CD16b, or both. Cancer patients that are homozygous for the 158V higher affinity allele of CD16a reportedly respond better to monoclonal antibody (mAb) therapeutics (see, e.g., Cartron et al. Blood. 99, 754-58 (2002)). FcRn also binds IgG. The Fcε family of receptors bind IgE and include FcεRI and FcεRII (CD23). The Fcα family of receptors bind IgA and include FcαRI (CD89) and Fcα/μR. The NK cells may express any one or more of these FcRs. Additionally, the FcR may be a chimeric FcR, wherein part of the FcR structure is derived from one type of FcR and another part is derived from a different FcR (or is synthetic by which functions in a similar fashion to an FcR subunit).

[0059] The NK cell may express a non-natural NKG2D receptor, such as a receptor comprising non-natural NKG2D receptor ectodomain that binds modified NKG2D ligand α1-α2 domains with greater affinity than it does natural α1-α2 domains. Non-natural NKG2D receptors are further described in, for example, U.S. Patent No. 10,259,858, and U.S. Patent Publication No. 2020/0138866, each of which are incorporated by reference in their entireties and particularly with respect to their disclosure of modified NKG2D ectodomains.

[0060] The NK cells of the population also may express an exogenous polypeptide (i.e., a polypeptide encoded by an exogenous nucleic acid introduced into the NK cells or a PSC from which the NK cell is derived or an intermediate thereof). In various aspects, the NK cells of the population express a cytokine, chemokine, or the like, including, but not limited to, IL-15 or IL-2

[0061] Optionally, the NK cells further comprise a nucleic acid molecule encoding a suicide gene product. Combinations of nucleic acid molecules encoding different suicide gene products also are contemplated. Suicide genes are useful in selectively killing cells by inducing apoptosis or converting a nontoxic compound to a toxic compound, resulting in death of unwanted cells. Examples of suicide gene products include, but are not limited to, caspases (e.g., caspase 9), thymidine kinases, cytosine deaminases, cytochrome P450, and DNases. See, e.g., U.S. Patent Publication No. 2022/0025001, hereby incorporated by reference. Optionally, the nucleic acid encoding the suicide gene product is operably linked to an inducible promoter. See, e.g., Straathof et al. Blood, 105(11), 4247-4254 (2005), hereby incorporated by reference. Optionally, the engineered NK cells comprise a herpes simplex virus thymidine kinase (TK) suicide gene. See, e.g., Bonini, et al., Science, 276, 1719-1724 (1997), hereby incorporated by reference. Optionally, the engineered NK cells comprise one or more copies of the TK suicide gene. In various aspects, the NK cell comprises a nucleic acid encoding a hygromycin-thymidine kinase fusion protein. For example, NK cells may comprise a nucleic acid encoding HSV TK and a nucleic acid encoding a hygromycin-thymidine kinase fusion protein. In another embodiment, the NK cells may be eliminated by targeted antibody-mediated depletion using any number of NK cell-specific targets, including, but not limited to, CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and/or NKp44.

[0062] Methods of genetically engineering a host cell to stably produce one or more gene products of interest are known in the art. Exemplary methods involve the use of viral vectors. Viral vectors may include any suitable viral vectors including, for example, retrovirus, adenovirus, parvovirus (for example, adeno-associated viruses), coronavirus, ortho-myxovirus (for example, influenza virus), rhabdovirus (for example, rabies and vesicular stomatitis virus), paramyxovirus (for example, measles and Sendai), picornavirus, alphavirus, herpesvirus (for example, Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (for example, vaccinia, fowlpox, and canarypox). Exogenous nucleic acids also may be introduced into a host cell via gene editing techniques using, e.g., the systems described herein. Preferably, the nucleic acid encoding the CAR or FcR and the nucleic acid encoding HLA-E, HLA-F, or HLA-G are integrated into cellular genome, such as into the B2M or the HLA-II loci. Thus, in various aspects, the B2M loci are disrupted by inserting in the B2M loci a nucleic acid encoding a different gene product to replace the expression of the endogenous wild type B2M protein.

Alternatively or in addition, certain HLA-II loci are optionally disrupted by inserting in the HLA-II loci a nucleic acid encoding a different gene product to replace the expression of the endogenous wild type HLA-II protein.

[0063] In various aspects of the disclosure, the NK cells are derived from pluripotent stem cells. The term "pluripotent stem cells" includes embryonic stem cells, embryo-derived stem cells, and induced pluripotent stem cells, regardless of the method by which the pluripotent stem cells are derived. Pluripotent stem cells are typically defined functionally as stem cells that are: (a) capable of inducing teratomas when transplanted in immunodeficient (SCID) mice; (b) capable of differentiating to cell types of all three germ layers (e.g., can differentiate to ectodermal, mesodermal, and endodermal cell types); and (c) express one or more markers of embryonic stem cells (e.g., express Oct 4, alkaline phosphatase, SSEA-3 surface antigen, SSEA-4 surface antigen, Nanog, TRA-1-60, TRA-1-81, SOX2, REX1, etc.). Pluripotent stem cells can be generated using, for example, methods known in the art. Exemplary pluripotent stem cells include embryonic stem cells. "Embryonic stem cells" (ESC) include, e.g., cells derived from the inner cell mass of human blastocysts or morulae, including those that have been serially passaged as cell lines. Embryonic stem cells, regardless of their source or the particular method used to produce them, can be identified based on, for instance, (i) the ability to differentiate into cells of all three germ layers, (ii) expression of at least Oct-4 and alkaline phosphatase, and (iii) ability to produce teratomas when transplanted into immunodeficient animals.

[0064] Another exemplary pluripotent stem cell is an induced pluripotent stem cell (iPSC). iPSCs are generated by, e.g., reprogramming a somatic cell by expressing a combination of factors ("reprogramming factors") such as, but not limited to, Oct4, Sox2, c-Myc, Nanog, Lin28, and/or Klf4. Induced pluripotent stem cells can be generated using, as a starting point, virtually any somatic cell of any developmental stage. For example, the cell can be from an embryo, fetus, neonate, juvenile, or adult donor. Exemplary somatic cells that can be used include CD34+ cord blood cells, fibroblasts, such as dermal fibroblasts obtained by a skin sample or biopsy, synoviocytes from synovial tissue, foreskin cells, cheek cells, or lung fibroblasts. In certain embodiments, the somatic cell is not a fibroblast.

[0065] The pluripotent stem cells can be from any species. Embryonic stem cells have been successfully derived from, for example, mice, multiple species of non-human primates, and humans. Thus, one of skill in the art can generate embryonic stem cells from any species, including but not limited to, human, non-human primates, rodents (mice and rats), ungulates (cows, sheep, etc.), dogs (domestic and wild dogs), cats (domestic and wild cats such as lions, tigers, and cheetahs), rabbits, hamsters, gerbils, squirrel, guinea pig, goats, elephants, panda (including giant panda), pigs, raccoon, horse, zebra, marine mammals (dolphin, whales, etc.) and the like. Similarly, iPSCs can be from any species. iPSCs have been successfully generated using, for instance, mouse and human cells. Accordingly, one can readily generate an iPSC using a donor cell from any species, such as any species described herein.

[0066] The population of NK cells of the disclosure has a variety of attributes making the population advantageous for therapeutic applications, e.g., superior engraftment, homing, viability, persistence, immune

avoidance, and/or cytotoxicity. For example, in various aspects, the population of engineered NK cells is capable of engrafting into a subject following administration, using, for example, protocols known to one of skill in the art. Put another way, the population of NK cells is capable of homing to a target tissue (e.g., bone marrow) and/or persisting in a subject for a period after administration (e.g., from 24 hours to a few days or weeks or months). In various aspects, at least a portion of the population of engineered NK cells (e.g., at least 1%, at least 2%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40% or the population) is capable of engrafting into a target tissue. For example, in various aspects, at least 50% (e.g., at last 55%, at least 60%, at least 65%, at least 70%, at last 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%) of the population of engineered NK cells is capable of engrafting into a target tissue (e.g., blood or bone marrow) of the subject. Optionally, the population of engineered NK cells, once administered to a subject, is capable of remaining engrafted in the target tissue for at least 1 day (e.g., at least 2 days, at least 5 days, at least 10 days). In this regard, at least a portion of the population of NK cells is capable of remaining engrafted in the target tissue for at least 20 days (e.g., at least 50 days, at least 100 days, at least 200 days, at least 500 days, or at least 1000 days) following administration.

[0067] Alternatively or in addition, the NK cells of the population are optionally capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) and/or natural cellular cytotoxicity (NCC) of a cancer cell. NK cells mediate NCC via adhesion and conjugation to target cells and release of perforin and granzymes, which induce apoptosis in the target cell. ADCC is a mechanism by which immune cells bearing the Fc receptor kill cells coated with antibody upon binding of the Fc receptor to the Fc portion of the antibody. NK cells mediate ADCC through, e.g., CD16, engagement of which results in release of cytolytic granules and inflammatory mediators. Methods of characterizing ADCC activity and NCC activity are known in the art and described herein and in, e.g., U.S. Patent Publication No. 2020/0131475 (which describes an assay involving use of Raji cells (derived from Burkett's lymphoma) preincubated with anti-CD20 antibody as targets for ADCC assays (Tsirigotis et al., J of Steroid Biochem and Mol Bio 108: 267-271 (2008)); U.S. Patent Publication No. 2022/0040230; and Bhatnagar et al., Eur. J. Immunol., 44(11): 3368 (2014). In various aspects, the administration of the population of NK cells to a subject suffering from cancer decreases tumor load or burden in the subject by at least 1%, at least 2%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100%. Tumor load may be characterized using any suitable method including, but not limited to, X-ray, computerized tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound, endoscopy and laparoscopy, tumor marker levels, cytology, histology, biopsy sampling, and/or counting of target cells in circulation.

[0068] Optionally, at least a portion of the NK cells in the population (e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100%) are larger in volume compared to peripheral blood NK cells. Methods of determining cell volume are known in the art, as illustrated in Model, Cytometry, 93(3): 281 (2018), incorporated by reference in its entirety and particularly with respect to descriptions of methods of measuring cell volume. In various aspects of the disclosure, NK cells of the population have at least about a 5% increase in cell volume as compared to peripheral blood NK cells. For

example, NK cells of the population have at least about a 10% increase or at least about a 15% increase in cell volume (e.g., about 5% to about 20%, about 7% to about 18%, about 10% to about 15%, about 10% to about 20%, or about 15% to about 20% increase) as compared to peripheral blood NK cells.

[0069] The disclosure also provides a kit comprising one or more containers comprising the population of NK cells described herein. The kit may further comprise instructions and written information on indications and usage of the NK cells. Sterile sealed containers, e.g., vials, bottle, vessel, and/or packages comprising the population of NK cells, optionally with suitable instructions for use, are also contemplated. In a further aspect, the disclosure provides an article of manufacture, or unit dose form, comprising: (a) the population of NK cells described herein; (b) a container containing said population; and (c) a label affixed to said container, or a package insert included in said container referring to the use of said NK cells in the treatment of a disease or disorder (e.g., cancer).

[0070] Also provided herein are pharmaceutical compositions comprising the population of NK cells and a pharmaceutically acceptable carrier, excipient, or diluent. In exemplary aspects, the composition is a sterile composition. The pharmaceutical composition according to the disclosure may be formulated for delivery via any route of administration. "Route of administration" may refer to any administration pathway known in the art, including but not limited to aerosol, nasal, oral, transmucosal, transdermal, or parenteral. "Parenteral" refers to a route of administration that is generally associated with injection, including intraorbital, infusion, intraarterial, intracapsular, intracardiac, intradermal, intramuscular, intraperitoneal, intrapulmonary, intraspinal, intrasternal, intrathecal, intrauterine, intravenous, subarachnoid, subcapsular, subcutaneous, intratumoral, transmucosal, or transtracheal. The composition may be in the form of a solution or suspension for infusion or for injection. Each component of the carrier is "pharmaceutically acceptable" in that it must be compatible with the other ingredients of the formulation and safe for use *in vivo*.

[0071] The disclosure further provides a method of treating a disease or disorder in a subject in need thereof, the method comprising administering to the subject an effective amount of the population of engineered NK cells or the pharmaceutical composition comprising the population of engineered NK cells, thereby treating the disease or disorder in the subject. Similarly, the disclosure provides use of the population of engineered NK cells in the treatment of a disease or disorder, as well as use of the population of NK cells in the preparation of a medicament for treating a disease or disorder in a subject in need thereof.

[0072] In various aspects, the disease or disorder is cancer. Examples of cancers include, but are not limited to, prostate cancer, lung cancer (e.g., non-small cell lung cancer), colon cancer, endometrial cancer, soft tissue carcinoma, rectum cancer, urinary bladder cancer, melanoma, kidney cancer, renal cancer (e.g., renal cell carcinoma), oral cavity cancer, pharynx cancer, pancreatic cancer, uterine cancer, thyroid cancer, parathyroid cancer, skin cancer, head and neck cancer (e.g., head and neck squamous cell carcinoma (HNSCC), cervical cancer, brain cancer (e.g., glioblastoma), liver cancer (e.g., hepatocellular carcinoma), bone cancer (e.g., osteosarcoma), or ovarian cancer. Hematopoietic cancers are particularly contemplated. Examples of cancers also include, but are not limited to, leukemias and lymphomas, such as acute myeloid leukemia, Hairy Cell

Leukemia, Chronic Lymphocytic Leukemia, and Non-Hodgkin's Lymphoma (e.g., Diffuse Large B-cell Lymphoma, Burkitt Lymphoma, Mantel cell Lymphoma, and follicular lymphoma). Other cancers include, but are not limited to, alveolar rhabdomyosarcoma, breast cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the nose, nasal cavity, or middle ear, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, malignant mesothelioma, multiple myeloma, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, and ureter cancer.

[0073] In various aspects, the disease or disorder is an autoimmune disease or disorder. Autoimmunity is an immune response to self-antigens, which results in the body attacking normal cells and tissues. There are more than 80 types of autoimmune diseases. The body's response to autoimmune conditions mimics the response against infection, involving activation of immune cells, inflammation, and tissue damage. In various aspects the autoimmune condition is one of over 80 diseases or syndromes hallmarked by the loss of B-cell tolerance and the production of autoantibodies. In various aspects, the autoimmune condition is Graft versus Host Disease (GvHD), systemic lupus erythematosus (SLE), multiple sclerosis, Sjogren's syndrome, systemic sclerosis/scleroderma, cutaneous sclerosis, ulcerative colitis, inflammatory bowel disease, bullous pemphigus, insulin dependent diabetes, mellitus or Type 1 diabetes, Crohn's disease, psoriatic arthritis or rheumatoid arthritis.

[0074] The method may comprise administering the population of NK cells prior to, simultaneously with, or following, a different therapy, such as chemotherapy, surgical resection of a tumor, immunotherapy (such as cytokine therapy or administration of an antibody construct), or radiation therapy. For example, the disclosure contemplates a method wherein the population of engineered NK cells described herein is administered to a subject in need thereof as part of a treatment regimen that also comprises administration of one or more cytokines to the subject. In some aspects, the cytokine is an interleukin (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20. IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, or IL-33). For example, in various aspects of the method, the method further comprises administering IL-15 and/or IL-2 to the subject.

[0075] In various aspects, the method may comprise administering the population of engineered NK cells described herein (such as FcR-UDC I-NKs (e.g., differentiated cells having a disruption in the B2M gene resulting in the abrogation of the HLA class I protein and expressing an exogenous single chain fusion HLA-E as well as CD16) or FcR-UDC I/II-NKs (e.g., FcR-UDC I-NKs further engineered with a knockout mutation of a HLA class II related gene, such as RFXANK, resulting in disruption of the HLA class II protein)) as part of a treatment regimen which also comprises administration of one or more antibody constructs (e.g., antibodies) to the subject. The disclosure further contemplates a treatment regimen wherein an antigen binding antibody fragment or an antibody-like protein product (i.e., a protein based on antibody structural features that allow antigen binding) is administered to a subject. "Antibody construct" refers to antibodies, antigen-binding fragments of antibodies, or antibody-like protein products. "Antibody" refers generally to an intact immunoglobulin. Disclosure herein

referencing antibodies also applies to antibody fragments and antibody-like protein products. Suitable antigen-binding antibody fragments include, e.g., a F(ab)2 or a Fab fragments. Antibody-like protein products include, but are not limited to, single chain Fvs (scFv), diabodies, nanobody constructs, antibody mimetics, and the like. In various aspects, the method comprises administering an intact antibody or antibody fragment or antibody-like protein product that comprises an Fc region. In various aspects, the antibody, antibody fragment, or antibody-like protein product binds a cell surface antigen (e.g., a tumor antigen), such as, but not limited to, 5T4, ACE, ADRB3, AKAP-4, ALK, Androgen receptor, AOC3, APP, Axin1, AXL, B7H3, B7-H4, BCL2, BCMA, bcr-abl, BORIS, BST2, C242, C4.4a, CA125, CA6, CA9, CAIX, CCL11, CCR5, CD123, CD133, CD138, CD142, CD15, CD15-3, CD171, CD179a, CD18, CD19, CD19-9, CD2, CD20, CD22, CD23, CD24, CD25, CD27L, CD28, CD3, CD30, CD31, CD300LF, CD33, CD352, CD37, CD38, CD4, CD40, CD41, CD44, CD44v6, CD5, CD51, CD52, CD54, CD56, CD62E, CD62P, CD62L, CD70, CD71, CD72, CD74, CD79a, CD79b, CD80, CD90, CD97, CD125, CD138, CD141, CD147, CD152, CD154, CD326, CEA, CEACAM5, CFTR, CLDN3, CLDN18.2, CLDN18.1, CLDN6, CLEC12A, CLL-1, cll3, c-MET, Crypto 1 growth factor, CS1, CTLA-4, CXCR2, CXORF61, Cyclin B1, CYP1B1, Cadherin-3, Cadherin-6, DLL3, E7, EDNRB, EFNA4, EGFR, EGFRvIII, ELF2M, EMR2, ENPP3, EPCAM, EphA2, Ephrin A4, Ephrin B2, EPHB4, ERBB2 (Her2/neu), ErbB3, ERG (TMPRSS2 ETS fusion gene), ETBR, ETV6-AML, FAP, FCAR, FCRL5, FGFR1, FGFR2, FGFR3, FGFR4, FLT3, Folate receptor alpha, Folate receptor beta, FOLR1, Fos-related antigen 1, Fucosyl GM1, GCC, GD2, GD3, GM3, GPC1, GPC2, GPC3, GPNMB, GPR20, GPRC5D, GUCY2C, HAVCR1, HER2, HER3, HGF, HMI.24, HMWMAA, HPV E6, hTERT, ICAM, ICOS-L, IGF-I receptor, IGLL1, IL-2 receptor, IL-4 receptor, IL-13Ra2, IL-1 1Ra, interferon receptor, integrins (including α_4, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_1\beta_4$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_6\beta_4$, $\alpha_{11b}\beta_3$ integrins), KIT, LAGE-la, LAIR1, LAMP-1, LCK, LFA-1 (CD11a), L-selectin (CD62L), LILRA2, LIV-1, LMP2, LRRC15, LY6E, LY6K, LY75, MAD-CT-1, MAD-CT-2, MAGE A1, MelanA/MART1, Mesothelin, ML-IAP, MSLN, mucin, MUC1, MUC16, MYCN, myostatin, NA17, NCA-90, NCAM, NOTCH1, NOTCH2, NOTCH3, NOTCH4, NY-BR-1, NY-ESO-1, OR51E2, OY-TES1, p53, p53 mutant, PANX3, PAP, PAX3, PAX5, p-CAD, PD-L1, PD-L2, PDGFR, PDGFR-beta, PIK3CA, PLAC1, Polysialic acid, PRSS21, PSCA, PSMA, PTK7, RAGE-1, STEAP1, TAG72, TARP, TCRβ, TEM1/CD248, TEM7R, TGF-1, TGF- β2, TGS5, Tie 2, TIM-1, Tn Ag, TRAC, TRAIL-R1, TRAIL-R2, TROP-2, TRP-2, TRPV1, TSHR, tumor antigen CTAA16.88, UPK2, VEGF, VEGFR1, VEGFR2, vimentin, WT1, XAGE1, or combinations thereof. Examples of antibodies for use in connection with the method disclosed herein include, but are not limited to, Rituximab, Cetuximab, Trastuzumab, Panitumumab, Ofatumumab, Brentuximab, Pertuzumab, Ado-trastuzumab emtansine, Obinutuzumab, Nimotuzumab, Bevacizumab, Alemtuzumab, Gemtuzumab, Ranibizumab, Olaratumab, Ontuximab, Isatuximab, Sacituzumab, Daratumumab, Lintuzumab, Balantamab, Indatuximab, Dinutuximab, Alemtuzumab, Ibritumomab, Tositumomab, Panitumumab, Tremelimumab, Ticilimumab, Catumaxomab, Oregovomab, Zolbetuximab (described in, e.g., International Patent Publication No. WO 2016166124, hereby incorporated by reference in its entirety and particularly with respect to the description of Zolbetuximab), ASP1650 (described in, e.g., International Patent Publication No. WO 2012156018, hereby incorporated by reference in its entirety and particularly with respect to the description of SEQ ID NOs: 35 and 36, CDR sequences within SEQ ID NOs: 35 and 36, and SEQ ID NOs: 27 and 25), ASP1893 mAb (a humanized

version of ASP1650 (i.e., sharing the CDR sequences of ASP1650)), and Veltuzumab. Additional examples of antibodies include, but are not limited to, Adalimumab, Eculizumab, and Natalizumab. In some aspects, the antibody is Rituximab, ASP1893 mAb, ASP1650, or Zolbetuximab.

[0076] For example, the disclosure provides a method of treating a disease or disorder in a subject in need thereof, the method comprising administering to the subject an effective amount of the population of engineered NK cells described herein and further administering to the subject an antibody which binds a cell surface antigen on a target cell, the elimination of which provides a therapeutic benefit to the subject. In various aspects, the disclosure provides a method of treating cancer in a subject in need thereof, wherein the method comprises administering to the subject an effective amount of the population of engineered NK cells and an effective amount of antibody specific for a cancer cell antigen (such as any of the anti-cancer antibodies described herein). In various aspects of the disclosure, an effective amount refers to treating cancer in a patient by administering multiple doses and/or multiple rounds of doses of NK cells and antibodies as a treatment course. As merely examples of aspects of the disclosure, the subject may suffer from breast cancer, and the NK cells are administered with an anti-HER2 antibody (such as Trastuzumab); or the subject may suffer from colon carcinoma, and the NK cells are administered with an anti-EGFR antibody (such as cetuximab or panitumumab); or the subject may suffer from AML, and the NK cells are administered with an anti-CD123 antibody or an anti-FLT3 antibody. The subject may suffer from a CD20-positive cancer. In various aspects, the subject is suffering from a CLDN18.2-positive cancer, such as gastric or gastroesophageal junction (GEJ) adenocarcinoma, and the NK cells are administered with an anti-CLDN18.2 antibody. Alternatively, the subject may be suffering from a CLDN6-positive cancer (e.g., ovarian cancer, in particular ovarian adenocarcinoma or ovarian teratocarcinoma, lung cancer, including small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), in particular squamous cell lung carcinoma and adenocarcinoma, gastric cancer, breast cancer, hepatic cancer, pancreatic cancer, skin cancer, in particular basal cell carcinoma or squamous cell carcinoma, malignant melanoma, head and neck cancer, in particular malignant pleomorphic adenoma, sarcoma, in particular synovial sarcoma or carcinosarcoma, bile duct cancer, cancer of the urinary bladder, in particular transitional cell carcinoma and papillary carcinoma, kidney cancer, in particular renal cell carcinoma including clear cell renal cell carcinoma or papillary renal cell carcinoma, colon cancer, small bowel cancer, including cancer of the ileum, in particular small bowel adenocarcinoma and adenocarcinoma of the ileum, testicular embryonal carcinoma, placental choriocarcinoma, cervical cancer, testicular cancer, in particular testicular seminoma, testicular teratoma and embryonic testicular cancer, uterine cancer, a germ cell tumor such as a teratocarcinoma or an embryonal carcinoma, in particular a germ cell tumor of the testis, and the metastatic forms thereof), and the NK cells are administered with an anti-CLDN6 antibody. Optionally, the antibody is Rituximab, Zolbetuximab, ASP1893 mAb, or ASP1650.

[0077] In various aspects, the disclosure provides a method of treating an autoimmune condition in a subject in need thereof, wherein the method comprises administering to the subject an effective amount of the population

of engineered NK cells and an effective amount of antibody specific for an immune cell antigen. Optionally, the antibody is Rituximab.

[0078] In some aspects of the disclosure, the engineered NK cells are optionally co-administered with an antibody as part of a treatment regime which may optionally include a chemotherapeutic treatment that is not an antibody. Optionally, the chemotherapeutic agent is selected from fluorouracil, oxaliplatin, and/or capecitabine.

[0079] As used herein, the term "treat," as well as words related thereto, do not necessarily imply 100% or complete treatment or remission. Rather, there are varying degrees of treatment of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the methods of treating a disease or disorder can provide any amount or any level of treatment. Furthermore, the treatment provided by the method may include treatment of one or more conditions or symptoms or signs of the disease being treated. For instance, the treatment method of the present disclosure may inhibit one or more symptoms of the disease. Also, the treatment provided by the methods of the present disclosure may encompass slowing the progression of the disease.

[0080] Treatment for cancer may be determined by any of a number of ways. Any improvement in the subject's wellbeing is contemplated (e.g., at least or about a 10% reduction, at least or about a 20% reduction, at least or about a 30% reduction, at least or about a 40% reduction, at least or about a 50% reduction, at least or about a 60% reduction, at least or about a 70% reduction, at least or about an 80% reduction, at least or about a 90% reduction, or at least or about a 95% reduction of any parameter described herein). For example, a therapeutic response would refer to one or more of the following improvements in the disease: (1) a reduction in the number of neoplastic cells; (2) an increase in neoplastic cell death; (3) inhibition of neoplastic cell survival; (5) inhibition (i.e., slowing to some extent, preferably halting) of tumor growth or appearance of new lesions; (6) decrease in tumor size or load (i.e., burden); (7) absence of clinically detectable disease, (8) decrease in levels of cancer markers; (9) an increased patient survival rate or survival time; and/or (10) some relief from one or more symptoms associated with the disease or condition (e.g., pain).

[0081] For example, in various aspects, administration of the engineered NK cells of the disclosure increases survival time of the subject by at least 20 days (e.g., at least 30 days, at least 45 days, at least 60 days, at least 90 days, or more) relative to survival time of the subject in the absence of administering the population of engineered NK cells. Alternatively or in addition, administering the NK cells to the subject decreases tumor load in the subject. In various aspects, the methods of the disclosure further comprise monitoring treatment in the subject. Disease states may be monitored by, e.g., clinical examination, X-ray, computerized tomography (CT, such as spiral CT), magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound, endoscopy and laparoscopy, tumor marker levels in the context of cancer (e.g., carcinoembryonic antigen (CEA)), cytology, histology, biopsy sampling, and/or counting of target cells in circulation. Other methods and criteria known in the field include, e.g., RECIST (Response Evaluation Criteria In Solid Tumors) and irRC (immune response criteria). The term “effective amount” refers to a sufficient quantity of NK cells (or co-therapy,

such as antibody therapy) to achieve a desired biological response in a clinically relevant time period; for example, "therapeutically effective" refers to a sufficient quantity of NK cells which ameliorates one or more causes or symptoms of a condition or disease. The amount of engineered NK cells administered to a subject may comprise, e.g., a dose of 10^4 to 10^{11}, 10^5 to 10^{10}, or 10^6 to 10^9 cells/kg body weight.

[0082] The subject is a mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters; mammals of the order Logomorpha, such as rabbits; mammals from the order Carnivora, including Felines (cats) and Canines (dogs); mammals from the order Artiodactyla, including Bovines (cows) and Swines (pigs); or of the order Perssodactyla, including Equines (horses). In some aspects, the mammal is of the order Primate, Ceboid, or Simoid (monkey) or of the order Anthropoid (humans and apes). In some aspects, the mammal is a human. The terms "subject in need thereof" include subjects already afflicted with a disease or disorder, as well as those in which the disease or disorder is to be prevented.

[0083] The disclosure also provides a method of producing pluripotent stem (PS) cell-derived natural killer (NK) cells under feeder free, optionally serum-free, conditions. The method comprises (a) culturing pluripotent stem cells, optionally the PSCs are genetically engineered to disrupt one or more copies (e.g., all copies) of endogenous B2M and/or one or more copies (e.g., all copies) of a HLA class II-related gene selected from the group consisting of RFXANK, RFX5, RFXAP, and CIITA, and optionally expresses an engineered non-classical HLA class I protein associated with B2M, e.g., HLA-E; (b) differentiating the pluripotent stem cells to precursor cells capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives; and (c) differentiating the precursor cells to NK cells under feeder free, optionally serum-free, conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L (Flt3 ligand). In various aspects, step (b) comprises differentiating the pluripotent stem cells using embryoid bodies to obtain precursor cells capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives. In various aspects, the PSC is a human PSC. In other various aspects, the method further comprises (d) culturing the NK cells in a culture media comprising IL-15 and IL-18 (and, optionally, IL-21). In various aspects, step (d) comprises culturing the NK cells in the presence of an antibody or ligand that binds a receptor on NK cells to promote activation and/or expansion of the NK cells. In various aspects, the antibody or ligand binds to DNAM-1, OX40, NKG2D, 2B4, NKp30, or NKp46. Optionally, the method comprises culturing the NK cells in the presence of an antibody that binds DNAM-1, an antibody that binds OX40, an antibody that binds NKG2D, an antibody that binds 2B4, an antibody that binds NKp30, and/or an antibody that binds NKp46; a combination of any of the foregoing antibodies may be provided during the culturing step. Alternatively, the method may comprise culturing the NK cells in the presence of a ligand that binds DNAX Accessory Molecule 1 (DNAM-1), a ligand that binds OX40 (e.g., OX40L), a ligand that binds Natural Killer Group 2D (NKG2D) (e.g., MHC class I chain-related protein A (MICA) or UL16 binding protein 1 (ULBP1)), a ligand that binds 2B4 (CD244), a ligand that binds Natural Killer Protein 30 (NKp30) (e.g., B7H6), and/or a ligand that binds Natural Killer Protein 46 (NKp46, CD355), to activate and/or expand the NK cell. A combination of any of the foregoing ligands may be provided during the culturing

step. Optionally, the ligand comprises at least the portion of a native ligand that mediates receptor binding and activation fused to an Fc domain.

[0084] In various aspects, the method described herein is particularly well suited for the large-scale production of human PSC-derived NK cells. Previous methods of NK production under feeder-free conditions allowed up to 5- to 500-fold expansion of PBNK cells. See, e.g., Lu et al., J. Hematol. Oncol. 14:7 (2021). Remarkably, the method of the disclosure allows for at least a 1000-fold expansion of cells. Indeed, the method of the disclosure allows, in some embodiments, at least a 2000-fold, at least a 3000-fold, at least a 4000-fold, at least a 5000-fold, at least a 6000-fold, at least a 7000-fold, at least an 8000-fold, at least a 9000-fold, or at least a 10,000-fold expansion (or any range therein having these values as endpoints). The level of expansion achieved under feeder-free conditions using the materials and methods described herein is superior to previous methods.

[0085] The method of producing human PSC-derived engineered NK cells is performed under "feeder free conditions." "Feeders" are cells often co-cultured with cells of interest, e.g., PSC and/or NK cells; feeder cells which release growth factors and nutrients into the cellular milieu which support and promote target cell (e.g., PSC) growth. Cell types used as feeder cells include, e.g., fibroblasts (mouse or human), as well as endothelial cells and other stromal cells (e.g., OP9 cells). Feeder free conditions refers to culture conditions (or media) essentially free of feeder (e.g., stromal) cells and/or which has not been pre-conditioned by the cultivation of feeder cells. The method of the disclosure surprisingly provides a robust population of NK cells without the need for feeder cells or media conditioned by feeder cells. Indeed, even when compared to methods of NK production under feeder condition, the method of this disclosure allows for comparable to NK production. The use of feeder-free condition is cost-effective and minimizes xenogeneic contaminants.

[0086] "Culturing" refers to maintaining a cell in culture medium under conditions suitable for the survival and/or proliferation of the cell. The steps described herein may be performed using any suitable vessel (e.g., microwell plate, flask, bioreactor, etc.) and environmental conditions which permit cell growth and differentiation. Any suitable cell culture media for growth and differentiation of stem cells may be used in the method. Examples of media include, but are not limited to, mTeSR™1, TeSR™2, mTeSR™ Plus, TeSR™E8, APEL™, STEMdiff™ APEL™2, or StemSpan™ from STEMCELL Technologies (Vancouver, Canada), primate ES/iPS cell medium from ReproCELL (Boston, MA), and StemPro®-34 and StemPro® hESC SFM from Invitrogen (Carlsbad, CA), Gibco® PFHM-II media from ThermoFisher Scientific (Waltham, MA), and X-VIVO™ from Lonza (Basel, Switzerland). The media optionally comprises supplements such as, for example, amino acids (e.g., glutamine, arginine, or asparagine), hydrolysate, vitamins, nucleosides, hormones and/or other growth factors (such as insulin, transferrin, insulin-like growth factor, or epidermal growth factor), ions (e.g., sodium, chloride, calcium, or magnesium), buffers, small molecules (e.g., a GSK-3 inhibitor, a MEK inhibitor, or a Rho Kinase (ROCK) inhibitor), and/or sugar(s) (e.g., glucose or galactose). Medium lacking one or more of these supplements also is contemplated. In various aspects, the media is chemically defined media, i.e., cell culture media in which all of the chemical components are known, and which typically does not include serum, serum albumin, and hydrolysate.

[0087] The method comprises culturing human pluripotent stem cells, optionally, genetically engineered to disrupt one or more copies (e.g., all copies) of endogenous B2M and/or one or more copies (e.g., all copies) of RFXANK, RFX5, RFXAP, or CIITA (or a combination thereof), and optionally express an engineered non-classical HLA class I protein associated with B2M, e.g., HLA-E, as described above with respect to the generation of a population of NK cells. The disclosure relating to PSCs and NK cells provided above also apply to the PSC for use in the method. For example, the human PSCs optionally comprise a nucleic acid molecule encoding one or more suicide gene products which may be present in one or more copies, such as HSV thymidine kinase, and are optionally embryonic stem cells or induced PS cells, as described above.

[0088] Any suitable initial seeding density may be employed to establish a culture for differentiation. For example, an initial seeding density of about $0.5x10^5$ to about $10x10^5$ human pluripotent stem cells /mL may be used, (e.g., about $1x10^5$ to about $5x10^5$ or about $1.5x10^5$ to about $3.5x10^5$ or about $2x10^5$ to about $2.5x10^5$ cells/mL). The human pluripotent stem cells may be cultured for any suitable amount of time before the first differentiation step, e.g., one to seven days (such as one to four days or one to three days), to provide a sufficient number of human pluripotent stem cells suitable for differentiation. The method then comprises differentiating the human pluripotent stem cells to generate precursor cells capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives. In various aspects, the pluripotent stem cells are differentiated to precursor cells using embryoid bodies (EBs). See, e.g., Itskovitz-Eldor et al., Mol Med., 6(2): 88-95 (2000), hereby incorporated by reference in its entirety. EBs are aggregates or clusters of multipotent cells. EBs may be employed as an intermediate stage in the production of precursor cells with the potential of further differentiating into hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives (or as an intermediate stage in the production of hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives themselves). In various aspects, the method does not comprise differentiating the human pluripotent stem cells to hemangioblast or hemangio-colony forming cells as precursor cells.

[0089] The differentiation step to produce precursor cells comprises culturing the human PSCs under conditions suitable for producing the precursor cells (e.g., using EBs). In exemplary aspects, the human pluripotent stem cells are cultured in media comprising cytokines that promote the formation of EBs. In various aspects, the media comprises at least one of bone morphogenic protein 4 (BMP4), vascular endothelial growth factor (VEGF), or stem cell factor (SCF). Combinations of BMP4, SCF, and VEGF are contemplated, including use of all three cytokines to induce differentiation. The cytokines may be used in any suitable amount. For example, about 1 ng/mL to about 40 ng/mL of BMP4 may be provided (e.g., about 5 ng/mL to about 35 ng/mL, about 10 ng/mL to about 30 ng/mL, about 15 ng/mL to about 25 ng/mL, about 16 ng/mL, about 17 ng/mL, about 18 ng/mL, about 19 ng/mL, about 20 ng/mL, about 21 ng/mL, about 22 ng/mL, about 23 ng/mL, or about 24 ng/mL of BMP4 may be provided). Optionally, about 1 ng/mL to about 40 ng/mL of VEGF may be provided (e.g., about 5 ng/mL to about 35 ng/mL, about 10 ng/mL to about 30 ng/mL, about 15 ng/mL to about 25 ng/mL, about 16 ng/mL, about 17 ng/mL, about 18 ng/mL, about 19 ng/mL, about 20 ng/mL, about 21 ng/mL, about 22 ng/mL, about 23 ng/mL, or about 24 ng/mL of VEGF is provided). Also, optionally, about 10 ng/mL to about 60 ng/mL of

SCF may be provided (e.g., about 25 ng/mL to about 55 ng/mL, about 30 ng/mL to about 50 ng/mL, about 35 ng/mL to about 45 ng/mL, about 36 ng/mL, about 37 ng/mL, about 38 ng/mL, about 39 ng/mL, about 40 ng/mL, about 41 ng/mL, about 42 ng/mL, about 43 ng/mL, or about 44 ng/mL of SCF is provided). In various aspects, the method comprises exposing the human pluripotent stem cells to fibroblast growth factor (FGF, such as bFGF); alternatively, the method does not comprise the use of FGF (e.g., bFGF). The differentiation step may be performed for any suitable amount of time, e.g., about one day to about 21 days, such as about one day to about 14 days, about 7 days to about 18 days, about 10 days to about 14 days, or about 14 days. In various aspects, the first differentiation step is performed up to about 14 days. Media may be changed periodically during the differentiation step culture period. Generally, at least a portion of the media (e.g., half the media) is exchanged every other day until the end of the differentiation step. The conditions described herein result in high quality EBs, which lead to improved differentiation and NK cell yield.

[0090] The method further comprises differentiating the precursor cells (e.g., using EBs) to NK cells under feeder free, optionally serum-free, conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L (i.e., step (c) or second differentiation step). In various aspects, precursor cells are harvested and replated in a vessel suitable for growth, expansion, and differentiation. The harvested cells are cultured in media comprising SCF (e.g., about 5 ng/mL to about 40 ng/mL, about 10 ng/mL to about 35 ng/mL, about 15 ng/mL to about 30 ng/mL, about 15 ng/mL to about 25 ng/mL, about 16 ng/mL, about 17 ng/mL, about 18 ng/mL, about 19 ng/mL, about 20 ng/mL, about 21 ng/mL, about 22 ng/mL, about 23 ng/mL, or about 24 ng/mL of SCF), IL-7 (e.g., about 5 ng/mL to about 40 ng/mL, about 10 ng/mL to about 35 ng/mL, about 15 ng/mL to about 30 ng/mL, about 15 ng/mL to about 25 ng/mL, about 16 ng/mL, about 17 ng/mL, about 18 ng/mL, about 19 ng/mL, about 20 ng/mL, about 21 ng/mL, about 22 ng/mL, about 23 ng/mL, or about 24 ng/mL of IL-7), IL-15 (e.g., about 1 ng/mL to about 30 ng/mL, about 5 ng/mL to about 20 ng/mL, about 7 ng/mL to about 15 ng/mL, about 9 ng/mL to about 12 ng/mL, about 10 ng/mL of IL-15), and Flt3L (e.g., about 1 ng/mL to about 30 ng/mL, about 5 ng/mL to about 20 ng/mL, about 7 ng/mL to about 15 ng/mL, about 9 ng/mL to about 12 ng/mL, or about 10 ng/mL of Flt3L). The second differentiation step may be performed for any suitable amount of time, e.g., about one day to about 35 days, such as one day to about 21 days, about 7 days to about 32 days, about 10 days to about 28 days, about 14 days to about 25 days, or about 21 days. In various aspects, this step is performed up to about 21 days. Media may be changed periodically during the second differentiation step culture period. In some aspects, the culture is not disturbed for an initial period of time (e.g., one, two, three, or four days calculated from the start of the differentiation step), although this is not required. At least a portion of the media (e.g., half the media) may be exchanged every other day, twice a week, once a week, etc. until the end of the differentiation step.

[0091] In various aspects, IL-3 is included in the media during the second differentiation step. In this regard, about 1 ng/mL to about 10 ng/mL of IL-3 is optionally provided (e.g., about 3 ng/mL to about 8 ng/mL, about 4 ng/mL to about 6 ng/mL, about 2 ng/mL, about 3 ng/mL, about 4 ng/mL, about 5 ng/mL, about 6 ng/mL, about 7 ng/mL, about 8 ng/mL, or about 9 ng/mL of IL-3 is provided). IL-3 is optionally only provided for a portion of the

second differentiation step culture period, e.g., only for the first one, two, three, four, five, six, or seven days of culture, and is not included in the media for the remaining time of the second (NK) differentiation step.

[0092] In a preferred aspect of the disclosure, the culture media of the second differentiation step (step (c), differentiation of precursor cells to NK cells) comprises UM171. Any suitable amount of UM171 may be employed to achieve a desired effect; typically about 10 nM to about 60 nM (e.g., about 15 nM to about 55 nM, about 20 nM to about 50 nM, about 25 nM to about 45 nM, about 30 nM to about 40 nM, about 31 nM, about 32 nM, about 33 nM, about 34 nM, about 35 nM, about 36 nM, about 37 nM, about 38 nM, or about 39 nM) is employed. UM171 is a pyrimido-indole derivative that significantly increases the yield of NK cells (i.e., NK cell expansion) and reduces variability in the resulting NK population. Thus, a substantially homogenous population of NK cells can be achieved without the need for purification or filtration of the final NK cell product. The term "homogenous" refers to a population of cells wherein each cell is the same or substantially the same as the other cells in the population (i.e., expresses one or more of the same cell surface markers indicative of NK cells, such as CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and/or NKp44). In various aspects of the method, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more of the cells of the resulting human pluripotent stem cell-derived engineered NK cell population are the same or substantially the same as other cells in the population (e.g., display one or more of the cell surface markers above). In this regard, the disclosure contemplates a method which does not comprise a purification, isolation, or a filtration step following differentiation of the precursor cells to NK cells (or optionally following the step of culturing the NK cells to promote activation) to remove undesired cell types. In various aspects, UM171 is present only in the culture media of step (c) and is not present in the culture media of any other step(s) of the method (although this is not required).

[0093] The method of the disclosure optionally comprises a further culture step (step (d)) to promote activation and/or expansion of the NK cells. While this step is not required, it may be desirable to provide a population of NK cells with particularly desirable characteristics (e.g., improved cytotoxicity, improved persistence *in vivo*, improved engraftment, etc.). The activation step comprises culturing the NK cells (from the second differentiation step, step (c)) in a culture media comprising IL-15 and IL-18. IL-15 and IL-18 may be provided in any suitable amount, including, for example, about 1 ng/mL to about 30 ng/mL, about 5 ng/mL to about 20 ng/mL, about 7 ng/mL to about 15 ng/mL, about 9 ng/mL to about 12 ng/mL, or about 10 ng/mL of each of IL-15 and IL-18. Optionally, the NK cells are cultured in the presence of IL-15, IL-18, and IL-21 during at least a portion of the activation step (step (d)). Suitable amounts of IL-21 include, but are not limited to, about 1 ng/mL to about 30 ng/mL, about 5 ng/mL to about 20 ng/mL, about 7 ng/mL to about 15 ng/mL, about 9 ng/mL to about 12 ng/mL, or about 10 ng/mL of IL-21). In various aspects, culture media utilized in the activation step does not comprise IL-12.

[0094] In various aspects, the NK cells are cultured in the presence of an antibody that binds to DNAM-1 (DNAX accessory molecule, CD222), an antibody that binds to OX40, an antibody that binds to NKG2D, an antibody that binds to 2B4, an antibody that binds to NKp30, an antibody that binds to NKp46, or a combination

of any of the foregoing antibodies. For example, step (d) may comprise culturing the NK cells in the presence of an antibody that binds NKp30. NKp30 is also referenced as CD337 and is a 30 kD type I transmembrane protein which is a stimulatory receptor on NK cells. Alternatively or in addition, step (d) may comprise culturing the NK cell in the presence of an antibody that binds 2B4. 2B4, also referenced as CD244, is a member of the signaling lymphocyte activation molecule (SLAM) family that mediates non-MHC restricted killing. Alternatively or in addition, step (d) may comprise culturing the NK cell in the presence of an antibody that binds DNAM-1. DNAM-1 is also referenced as CD226 and is a 65 kD glycoprotein member of the Ig-superfamily expressed on the surface of NK cells, T cells, and monocytes/macrophages. For example, step (d) may comprise culturing the NK cell in the presence of an antibody that binds NKp30 and an antibody that binds 2B4. In a further example, step (d) may comprise culturing the NK cell in the presence of an antibody that binds NKp30 and an antibody that binds DNAM-1. Anti-NKp30 antibodies, 2B4 antibodies, and anti-DNAM-1 antibodies are commercially available from, e.g., SinoBiological (Wayne, PA) and BioLegend (San Diego, CA). In various aspects, step (d) may comprise culturing the NK cells in the presence of a ligand that binds DNAM-1, a ligand that binds OX40 (e.g., OX40L), a ligand that binds NKG2D (e.g., MICA or ULBP1), a ligand that binds 2B4 (CD244), a ligand that binds NKp30 (e.g., B7H6), and/or a ligand that binds NKp46, to activate the NK cell. A combination of any of the foregoing ligands may be provided during the culturing step. The antibody, antibodies, ligand, or ligands may be provided in the culture media or may be coated on the vessel.

[0095] The activation step (step (d)) may be performed for any suitable amount of time, e.g., one, two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, or 14 days. In various aspects, step (d) is performed for about five days to about 10 days. Additionally, the activation step may be performed in multiple vessels to accommodate proliferation of the NK cell population. For example, the activation step may comprise culturing the NK cells in one vessel (e.g., a flask, such as T75 flask) for an initial period of time (e.g., one, two, three, or four days), then transferring the cells to a larger vessel (e.g., a bioreactor) for a second period of time (e.g., one, two, three, or four days). Also, in various aspects, IL-21 is provided for only a portion of the activation step, when present at all. In this regard, the method may comprise inclusion of IL-21 in the culture media for an initial period time (e.g., one or more of days 0-4 of the activation step), but not include IL-21 in the culture media for the remainder of the culture period.

[0096] The NK cells are cultured in a vessel (e.g., flask or bioreactor) suitable for growth, expansion, and activation of NK cells. The vessel is optionally coated with a substance which promotes cell adhesion or one or more matrix component, such as laminin or laminin fragments, entactin, collagen, gelatin, vitronectin, fibronectin, Synthemax® (Corning Incorporated), Matrigel®, polylysine, thrombospondin, ProNectin-F™, and the like. An exemplary matrix component is RetroNectin®, a 63-kD fragment of recombinant human fibronectin fragment also referenced in the art as rFN-CH-296. Optionally, a Notch ligand is provided, such as DLL4. In various aspects, the vessel is coated with a Notch ligand, e.g., DLL4, and RetroNectin®. The DLL4 and RetroNectin® may be provided in any suitable amount, including relative amounts resulting in a DLL4: RetroNectin® ratio of 5:1, 4:1,

3:1, 2:1, 1:1, 1:2, 1:3, 1:4, or 1:5. Coated vessels may be employed for any of the method steps disclosed herein, including step (c) and/or step (d).

[0097] Remarkably, it has been determined that the activation step (step (d)) may be performed multiple times without negatively impacting the characteristics of the NK cell population (e.g., without significantly reducing the functionality of the cells). If desired, step (d) may be repeated up to five times (i.e., repeated twice, three times, four times, or five times) to further expand and/or activate the NK cells.

[0098] In various aspects of the method, IL-2 and/or IL-6 is not included in the culture media of one or more (e.g., any) steps of the method.

[0099] The disclosure further provides a method of increasing the purity of a population of NK cells derived from pluripotent stem cells. The method comprises differentiating embryoid bodies (i.e., differentiating cells within embryoid bodies) to NK cells under feeder free, optionally serum-free, conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L and in the presence of UM171. In various aspects, the culture media further comprises IL-3. Optionally, the method is performed in a vessel coated with RetroNectin® and DLL4. Also optionally, the method is performed for about one day to about 21 days. The result of the method is a substantially pure population of CD56+, CD45+ NK cells (i.e., a population of NK cells with low levels of unwanted cell types (contaminating cells)). The method allows for the production of PSC-derived NK cells without the need for extensive further purification of the resulting cell population to obtain a substantially pure population of NK cells. In various aspects, the method results in a population of cells wherein at least about 75% of the cells are CD56+, CD45+ NK cells (e.g., at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the cells are CD56+, CD45+ NK cells).

[00100] The method of the disclosure results in a robust population of NK cells suitable for use in, e.g., therapeutic applications. For example, the NK cells optionally express one or more cell surface markers selected from the group consisting of CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and NKp44, including any combination of the cell surface markers. In a preferred aspect, the NK cells express at least CD56. The NK cells also optionally express an engineered non-classical HLA class I protein associated with B2M, e.g., HLA-E, HLA-F, and/or HLA-G, as described above. The NK cells also may be engineered to express a CAR (e.g., a universal CAR), a FcR, a non-natural NKG2D receptor, or other type of receptor (e.g., as described above). The resulting NK cell population also preferably exhibits the characteristics described above, e.g., are capable of engrafting into a subject following administration and optionally remaining engrafted for at least 20 days, capable of inducing ADCC and/or NCC of a cancer cell, and capable of decreasing tumor load. In various aspects, the method results in production of at least 10^9, at least 10^{10}, or at least 10^{11} CD56-positive NK cells.

[00101] The following example is given merely to illustrate the present invention and not in any way to limit its scope.

EXAMPLES

[00102] The examples describe exemplary methods of producing human pluripotent stem cell-derived engineered natural killer (NK) cells under feeder-free, serum-free conditions.

Example 1: Engineered Human Pluripotent Stem Cells

[00103] Human pluripotent stem cells (PSCs) (NIH2, NIH3 and W1C1) were genetically engineered to (i) disrupt the B2M gene resulting in the abrogation of the HLA class I protein, and (ii) express an exogenous single chain fusion HLA-E ("UDC I-PSCs"). The UDC I-PSCs were further genetically engineered with a knockout mutation of a HLA class II related gene, RFXANK, resulting in disruption of the HLA class II protein ("UDC I/II-PSCs"). Alternatively, the UDC I-PSCs or UDC I/II-PSCs were further genetically engineered to express a gene encoding an Fc receptor/CD16 ("FcR-UDC I-PSCs" or "FcR-UDC I/II-PSCs"). UDC I-PSCs, UDC I/II-PSCs, and/or FcR-UDC I-PSCs are collectively referred to herein as "UDC PSCs." Differentiated cells are referenced herein as, e.g., "FcR-UDC I-NK" or "FcR-UDC I/II-NK."

Example 2: NK Differentiation from Parental and Engineered Human Pluripotent Stem Cells

[00104] Parental and engineered UDC PSCs (collectively, "PSCs") were thawed, expanded, and cultured in mTeSR™ (STEMCELL Technologies, Cambridge, MA) feeder-free, animal component-free media for up to seven days on vitronectin. The PSCs were dissociated into single cells and transferred into microwell plates and cultured in STEMdiff™ APEL™2 and Gibco® PFHM-II media (STEMCELL Technologies, Cambridge, MA; ThermoFisher Scientific, Waltham, MA) for fourteen days to differentiate into precursor cells (via embryoid bodies) capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives. The media was supplemented with BMP4 (20 ng/mL), VEGF (20 ng/mL), and SCF (40 ng/mL). Half media changes were performed every other day starting on day 3. The embryoid bodies were replated on DLL4/Retronectin-coated T75 flasks to differentiate into NK cells. The embryoid bodies were cultured under feeder free conditions in differentiation media comprising SCF (20 ng/mL), IL-7 (20 ng/mL), IL-15 (10 ng/mL), Flt3L (10 ng/mL), IL-3 (5 mg/mL) and 35 nM UM171 for 21 days in modified DMEM/F12. Half media changes were performed twice a week. The resulting NK cells ("UDC-NK") were washed and harvested. The UDC-NK cells were activated and expanded by culturing the UDC-NK cells in flasks coated with anti-NKp30 antibodies, anti-DNAM-1 antibodies, and Retronectin in CTS™ NK-Xpander™ or RPMI-1640 medium comprising IL-15 (10 ng/mL), IL-18 (10 ng/mL), and IL-21 (10 ng/mL) for three days, after which the UDC-NK cells were transferred to a G-REX® or spinner flask bioreactor and cultured in the presence of IL-15 and IL-18 for another three days. A representative schematic of the NK differentiation process is shown in Figure 1.

[00105] The method produced a substantially homogenous population of UDC NK cells regardless of parental cell line or engineered UDC PSC. Starting with about 4 x 10^6 iPSC, at the beginning of Step 0 (as illustrated in Figure 1), approximately 5 x 10^{10} of NK cells were produced, up to a 10,000-fold expansion. The addition of UM171 improved UDC-NK cell yield during NK differentiation as shown in Figures 2A-2C. Further, across multiple production runs and gene-edited clones, at least 90% of cells were CD56-and CD45-positive without

requiring any purification step to remove undesired cell types (see Figure 7A). Both UDC I-PSC and FcR-UDC I-PSC produced at least 90% $CD45^{+}CD56^{+}$ NK cells. In addition, as shown in Figures 3A & 3B, activation and expansion of NK cells resulted in consistent, high cell yield across PSC cell line (NIH3 and W1C1) and gene edited clones (UDC I-PSC, UDC I/II-PSC, and FcR-UDC I-PSC).

[00106] For the activation and expansion of the UDC-NK cell (Step 3 or step (d)), combinations of NK agonists were tested (see Figure 4). Single NK agonists were also tested and showed moderate NK activation. The UDC-NK cells (Step 2) showed robust expansion and high cell viability. As shown in Figures 5A-5B, across four different cell seeding densities, the UDC NK cells (Step 2) cultured on Retronectin, anti-NKp30 antibodies, anti-DNAM-1 antibodies with media comprising IL-15, IL-18, and IL-21 demonstrated upwards of 16- to 32-fold expansion, as compared to Step 2 control which only had 3- to 4-fold expansion, and greater than 90% cell viability.

[00107] An analysis of the cell population subset and cellular impurities at the end of the NK differentiation process show very low levels of CD3+ T cells and little, if any, remaining PSCs. Table 1 provides the details of the cell population subsets/impurities.

TABLE 1

Cell Phenotype	Cell Type	% of Cells Expressing		Batches
		Mean ± s.d.	Range	
CD45+	Hematopoietic Cells	99.5 ± 0.8	98.0 – 99.9	5
CD45+ CD56+	NK Cells	96.7 ± 1.8	93.9 – 98.7	5
CD45+ CD56- CD11b+	Myeloid Cells	2.00 ± 0.70	1.14 – 2.87	5
CD45+ CD56- CD15+	Granulocytes	0.50 ± 1.06	0.007 – 2.40	5
CD45+ CD56- CD33+	Myeloid Lineage Cells	0.12 ± 0.09	0.04 – 0.27	5
CD45+ CD56- CD14+	Monocytes	0.02 ± 0.02	0.001 – 0.04	5
CD45+ CD56- CD3+	T Cells	0.01 ± 0.01	0.001 – 0.030	5
CD45+ CD56+ CD3+	Possible NKT Cells	0.14 ± 0.18	ND – 0.33	5
CD45+ CD56- CD19+	B Cells	0.015 ± 0.010	0.001 – 0.030	5
CD45+ CD56- CD34+	Hematopoietic Stem and Progenitor Cells	0.011 ± 0.013	ND – 0.030	5
SSEA3+ Tra-181+	PSC	0.0002 ± 0.0004	ND – 0.001	5

ND = not detected

[00108] The UDC-NK cells of the invention were on average approximately 18% larger by volume compared to peripheral blood NK cells (PB-NK) as demonstrated by flow cytometry (see Figure 6).

Example 3: *In Vitro* Characterization of UDC PSC-derived NK Cells

[00109] The UDC-NK cells derived from the UDC I-PSC, UDC I/II-PSC, and UDC I-FcR-PSC expressed the expected cell phenotype across different gene edited clones. As shown in Figure 7A, the parental- and UDC PSC-derived UDC I-PSC-derived NK cells all had a high positivity for $CD45^{+}CD56^{+}$. UDC I-PSC-derived NK cells (UDC I-NK cells) failed to express HLA class I, but expressed HLA class II and HLA-E, and wild-type levels of CD16 (FcR). See Figures 7B-7E. FcR-UDC I-PSC-derived UDC-NK (FcR-UDC I-NK cells) cells had similar expression profile, except expressed higher levels of CD16 as compared to wild-type. Lastly, UDC I/II-PSC-derived UDC-NK cells (UDC I/II-NK cells) failed to express both HLA class I or class II protein, expressed HLA-E, and express wild-type levels of CD16. This data demonstrates that the NK cells derived from the gene edited clones maintained the gene edits after differentiation from the PSCs. The expected cell phenotype was also demonstrated across different PSC cell lines (NIH3 and W1C1) (see Figures 8A-8B). Based at least upon on the foregoing, the NK differentiation process disclosed herein is robust across different gene edited clones as well as PSC cell lines.

[00110] The UDC-NK cells expressed common NK markers and other biomarkers similar to PB-NK. As shown in Figure 9C, common NK markers, e.g., NKp30, NKp46, NKG2D, DNAM-1, 2B4, NKG2A, and CD94, with some exceptions, were expressed on FcR-UDC I-NK cells (both Step 2 and Step 3 illustrated in Figure 1, i.e., step (b) and (c), respectively) like expanded PB-NK cells (PB-NK cells were expanded with the Step 3 of the NK differentiation protocol). Similarly, T cell marker CD8 was commonly expressed in FcR-UDC I-NK cells and expanded PB-NK but not CD4, TCRαβ, or TCRγδ, and little to no CD3. Figure 9B. The lack of TCRαβ plays an important role in safety.

[00111] The UDC-NK cells expressed several activation markers and little, if any, exhaustion or "memory-like" markers. In Figures 10A-10C, FcR-UDC I-NK cells (Step 2 and Step 3) and expanded PB-NK were measured for activation, exhaustion, and "memory-like" markers. Apart from CD69, activation markers were, for the most part, similar between FcR-UDC I-NK cells and expanded PB-NK. FcR-UDC I-NK cells showed higher levels of CD69 compared to expanded PB-NK. Unlike expanded PB-NK, FcR-UDC I-NK cells showed little, if any, exhaustion or "memory-like" markers. Expanded PB-NK had higher levels of exhaustion markers TIGIT, LAG3, KLRG1 and ILT2, and higher levels of memory-like marker NKG2C. Differential expression of cytokine and chemokine receptors (see Figures 11A-11B) and adhesion/homing, costimulatory, and apoptotic markers (see Figures 12A-12C) are shown for FcR-UDC I-NK cells and expanded PB-NK.

[00112] The UDC-NK cells also demonstrated natural cellular cytotoxicity (NCC) activity across multiple runs, gene edited clones, and PSC cell lines. Using a 2-hour europium release assay, NCC activity of UDC-NK cells was measured by their ability to kill K562 tumor target cells. In Figure 13, 1 x 10^4 K562-GFP tumor cells were added per well and a corresponding number of UDC-NK cells were added per the E:T ratio. The results demonstrate consistent and similar *in vitro* NCC activity (i.e., specific lysis) of the UDC-NK cells against K562 tumor cells regardless of PSC cell line or gene edited clones across multiple runs. Further, FcR-UDC I-NK cells

exhibited similar NCC activity to expanded PB-NK; at the same time, no cytotoxicity nor fratricide was observed against normal allogeneic peripheral blood mononuclear cells (PBMC) or FcR-UDC I-NK cells themselves respectively (see Figure 16). Surprisingly, these UDC-NK cells demonstrated serial NCC activity against K562 tumor cells such that the UDC-NK cells were capable of killing freshly added K562 tumor cells in a dose-dependent manner (see Figure 17). IncuCyte serial killing assay was performed using 1.5 x 10^4 (3:1 E:T) or 5 x 10^4 (10:1 E:T) UDC-NK cells (FcR-UDC I-NK) (c.3C1). Every 24 hours, 5 x 10^3 K562-GFP target cells added for a total of 6 times. Wells were imaged every 3 hours for 6 days.

[00113] Similar to NCC, the UDC-NK cells exhibit potent antibody-dependent cellular cytotoxicity (ADCC) against a broad range of $CD20^+$ tumor cell lines. Using a 4-hour flow-based ADCC assay, tumor cells were labeled with cell trace violet and antibodies were dosed at 1 µg/ml. FcR-UDC I-NK cells showed an increase in ADCC activity in conjunction with Rituximab against three (3) tumor cell lines (Daudi, Ramos, and Raji) as compared to a control IgG1 antibody (see Figures 18A-18C). In contrast, expanded PB-NK cells plus Rituximab showed no increase in ADCC activity against Raji tumor cells as compared to the control IgG1 antibody (see Figure 18D). Further, the FcR-UDC I-NK cells also demonstrated NCC and ADCC activity against a broad range of B lymphoma cell lines (see Figure 19). These results demonstrate ADCC activity of UDC-NK cells against a broad range of $CD20^+$ and B lymphoma cell lines.

[00114] The UDC-NK cells produced cytokine profiles indicative of active NK cells capable of inducing ADCC after stimulation with Rituximab-coated Raji tumor cells. Using a standard cytokine secretion assay, 1 x 10^5 FcR-UDC I-NK cells/well were co-cultured with Rituximab-coated Raji tumor cells at a 1:1 E:T ratio for 25 hours. Cytokine secretion was measured using a 15-Plex Cytokine Luminex immunoassay (see Figure 20A). Moreover, FcR-UDC I-NK cells did not elevate cytokines/chemokines secretion following co-culture with allogeneic PBMCs demonstrating specificity of the FcR-UDC I-NK cells (see Figures 20B-E and 21A-D).

Example 4: *In Vivo* UDC-PSC-derived NK Cells Persistence and Distribution

[00115] The UDC-NK cells also exhibited persistence *in vivo* in the blood, spleen, and bone marrow in the absence of antigen. In NSG mice, 4 x 10^6 FcR-UDC I-NK cells (c.3C1) were injected intravenously (i.v.) followed by IL-2 (30,000 IU, 3x/wk.) and IL-15 (100 ng, daily for 7 days). For each tissue, the percentage of FcR-UDC I-NK cells within lymphocytes/WBC was determined by flow cytometry and staining for human CD45 and human CD56. Total cell counts were determined using a flow cytometric method (per mL blood, per spleen, per BM cells in 2 femurs + 2 tibia), and the % FcR-UDC I-NK was applied to this number to calculate the number of FcR-UDC I-NK in each tissue. As shown in Figures 22A-22G, FcR-UDC I-NK cells persisted *in vivo* in the absence of antigen for greater than 10 days post i.v. injection. Further, FcR-UDC I-NK cells distributed to multiple tissues: lung > liver > spleen > blood > bone marrow (see Figures 23A-23E).

Example 5: *In Vivo* Anti-Tumor Efficacy Studies Using UDC-NK Cells in a Raji i.p. Tumor Model

[00116] The efficacy of the UDC-NK cells against tumors *in vivo* was studied in a Raji intraperitoneal (i.p.) tumor model. In a representative study, NSG mice were divided into six group (n = 7 or 8) as shown in Table 2 below.

TABLE 2

Group No.	Treatment
1	Untreated
2	Rituximab alone
3	FcR-UDC-NK cells alone
4	Expanded PB-NK alone
5	FcR-UDC-NK cells + Rituximab
6	Expanded PB-NK + Rituximab

[00117] In Figure 24, NSG mice were irradiated and injected i.p. with Raji (Burkitt's lymphoma) cells (3 x 10^5 cells). Raji cells are resistant to NK cells in the absence of an anti-CD20 antibody. At day four, 5x10^6 FcR-UDC I-NK cells and/or Rituximab (300 µg) was administered to the NSG mice. At day 7, a second dose of Rituximab (300 µg) was administered. NSG mice were administered daily doses of IL-15 (100 ng) for 7 days, and IL-2 (30,000 IU) was administered 3x/week for 3 weeks. The NSG mice were imaged and assessed for survival, BLI (tumor burden), and body weight and other health conditions at various days through day 121. As shown in Figures 25A-25B, the combination of FcR-UDC I-NK and Rituximab significantly prolonged survival of lymphoma-bearing NSG mice, beyond that observed using Rituximab or FcR-UDC I-NK cells alone (see Figure 25A). The efficacy was similar between FcR-UDC I-NK cells plus Rituximab and PB-NK cells plus Rituximab (see Figure 25B). However, the responder rate was approximately 50%.

[00118] In another study, mice were divided into five group (n = 8) as shown in Table 3 below.

TABLE 3

Group No.	Treatment
1	Untreated
2	Rituximab alone (Day 2)
3	FcR-UDC-NK cells alone (Day 2, 6, and 10)
4	FcR-UDC-NK cells + Rituximab (Day 2) and FcR-UDC-NK cells alone (Day 6 and 10)
5	FcR-UDC-NK cells + Rituximab (Day 2) + FcR-UDC-NK cells alone (Day 10 and 16)

[00119] In Figure 26, NSG mice were irradiated and injected i.p. with Raji (Burkitt's lymphoma) cells (2 x 10^5 cells). At day 2, 10 x 10^6 FcR-UDC I-NK cells and/or 100 µg Rituximab was administered to the mice. At day 6, 10, and/or 16, 10 x 10^6 FcR-UDC I-NK cells were administered i.p. to the mice. Mice were administered daily doses of IL-15 (100 ng) and IL-2 (30,000 IU) 3x/week from day 2 through 21 days post last NK dose. The NSG mice were imaged and assessed for survival, BLI (tumor burden), and body weight and other health conditions at various days through day 86. FcR-UDC I-NK and Rituximab significantly prolonged survival of lymphoma-bearing mice, beyond that observed using Rituximab or FcR-UDC-NK cells alone (see Figure 27B). All mice treated with FcR-UDC-NK plus Rituximab responded to treatment and increasing the interval between doses prolonged the duration of the response (see Figure 27A).

Example 6: *In Vivo* Anti-Tumor Efficacy Studies Using UDC-NK Cells in a Raji i.v. Tumor Model

[00120] The efficacy of the UDC-NK cells against tumors *in vivo* was studied in a Raji i.v. tumor model. The Raji i.v. model is a more aggressive, disseminated model and represents a more clinically relevant, physiological model of lymphoma. In a representative study using NOGf mice (FcResolv NOG) that have mouse FcgR knocked out, NOGf mice were divided into three groups as shown in Table 4 below.

TABLE 4

Group No.	Treatment	No. of Mice
1	Untreated	8
2	Rituximab alone (Day 1, 8, 15)	8
3	FcR-UDC-NK cells + Rituximab (Days 1, 8, and 15) and FcR-UDC-NK cells alone (Day 4)	8

[00121] In Figure 28A, NOGf mice were injected i.v. with Raji cells (1 x 10^5 cells), at Day 1 with Rituximab (100 µg, i.p.) or in combination with FcR-UDC I-NK cells (10 x 10^6, i.v.). At Day 8 and 15 Rituximab alone (100 µg, i.p.) or in combination with FcR-UDC I-NK cells (10 x 10^6, i.v.) were administered to the mice. For group 3, FcR-UDC I-NK cells alone (10 x 10^6, i.v.) were administered to the mice on day 4. Mice were administered daily doses of IL-15 (100 ng, i.p.). As shown in Figure 28B, the combination of FcR-UDC I-NK plus Rituximab achieved a significantly greater reduction of tumor burden compared to Rituximab alone as well as untreated.

[00122] In another study, mice were divided into four group (n = 8) as shown in Table 5 below.

TABLE 5

Group No.	Treatment	No. of Mice
1	Untreated	8
2	Rituximab alone (Days 1, 8, 11)	8
3	3M FcR-UDC I-NK cells + Rituximab (Days 1, 8, and 11) and 3M FcR-UDCI-NK cells alone (Day 4)	8

4	15M FcR-UDC I-NK cells + Rituximab (Days 1 and 11), 15M FcR-UDC I-NK cells alone (Day 4), and Rituximab alone (Day 8)	8

[00123] In Figure 29A, NOGf mice engineered to express human IL-15 were injected i.v. with Raji cells (1 x 10^5 cells), at Day 1 with Rituximab alone (100 μg, i.p.) or in combination with FcR-UDC I-NK cells (3 x 10^6 or 15 x 10^6). At Day 4, FcR-UDC I-NK cells were given alone (3 x 10^6 or 15 x 10^6, i.v.). At Day 8 and 11, Rituximab alone (50 μg, i.p.) was administered or was administered in combination with 3 x 10^6 FcR-UDC I-NK cells (i.v.), or on Day 11 Rituximab was administered in combination with 15 x 10^6 FcR-UDC I-NK cells (i.v.). As shown in Figure 29B, the combination of 15 x 10^6 FcR-UDC I-NK cells + Rituximab achieved the greatest reduction in tumor burden.

[00124] Example 7: Anti-Tumor Efficacy Studies Using UDC-NK Cells and an Anti-Claudin 6 (CLDN6) Antibody

[00125] Antibody-dependent cell cytotoxicity (ADCC) was evaluated using the UDC-NK cells described herein in combination with anti-CLDN6 antibodies against the CLDN6+ ovarian cancer cell line PA-1. Briefly, in a four-hour flow-based ADCC assay, CellTrace Violet-labeled PA-1 cells were co-cultured with FcR-UDC I-NK cells in the presence of titrated concentrations of anti-CLDN6 antibodies, ASP1650 (also known as IMAB027) and ASP1893 mAb. As shown in Figure 30, the FcR-UDC I-NK cells demonstrated enhanced cytotoxicity against PA-1 cells in an anti-CLDN6 antibody dose-dependent manner. The EC50 using antibody ASP1893 mAb (humanized IgG1) was about 4.32 ng/mL, and the EC50 using antibody ASP1650 (chimeric IgG1) was about 8.49 ng/mL.

[00126] A tumor cell line panel was also tested for surface CLDN6 expression and categorized into CLDN6hi, CLDN6mid, CLDN6low, and CLDN6- groups. The FcR-UDC I-NK cells were tested in a four-hour flow-based ADCC assay against a panel of tumor cell lines with variable levels of surface CLDN6. As shown in Figures 31A-31M, FcR-UDC I-NK cells demonstrated ADCC activity against CLDN6+ tumor cells in a CLDN6 expression-dependent manner. Interestingly, FcR-UDC I-NK cells showed natural cytotoxicity (NCC) without anti-CLDN6 antibodies against a broad range of tumor cell lines, regardless of CLDN6 surface expression.

[00127] TNF-α and IFN-γ production by UDC-NK cells after stimulation with anti-CLDN6 antibody-coated tumor cells also was examined. Briefly, 10^5 FcR-UDC I-NK cells and 10^5 tumor cells were co-cultured in a 96-well plate in the presence or absence of 1 μg/mL of anti-CLDN6 antibody (ASP1893 mAb) or control hIgG1 antibody. 24 hours after co-culture, culture supernatants were harvested for TNF-α and IFN-γ measurements by ELISA (R&D Systems). Figures 32A-32B show that FcR-UDC I-NK cells exhibited elevated TNF-α and IFN-γ secretions after stimulation with a panel of tumor cell lines compared to the basal cytokine levels without stimulation. FcR-UDC I-NK cells demonstrated superior cytokine production upon stimulation with CLDN6+ tumor cells and ASP1893 mAb.

[00128] The efficacy of the UDC-NK cells against tumors *in vivo* was studied in a CLDN6+ PA-1 cell xenograft mouse tumor model. In a representative study, mice were divided into four group (n = 6 or 7) as shown in Table 6 below. Briefly, 5 x 10^6 PA-1 cells were engrafted into the flank of NOGf mice engineered to express human IL-15 subcutaneously. 13 days after tumor engraftment, mice were treated with 7.5 x 10^6 cryopreserved FcR-UDC I-NK cells i.v. with or without anti-CLDN6 (300 µg ASP1650) i.p. as illustrated in Figure 33. Cryopreserved FcR-UDC-I-NK cells were thawed and immediately injected to mice. Figures 34A-34B show that combination treatment with FcR-UDC I-NK cells and anti-CLDN6 antibody demonstrated statistically significant anti-tumor efficacy and improved overall survival. Monotherapy of FcR-UDC I-NK cells or anti-CLDN6 antibody showed no significant anti-tumor efficacy.

TABLE 6

Group No.	Treatment	No. of mice
1	Untreated	7
2	Anti-CLDN6 Ab alone	6
3	7.5M UDC-NK	7
4	7.5M UDC-NK + anti-CLDN6 Ab	7

[00129] The above example demonstrates that UDC-NK cells exhibit natural cytotoxicity *in vitro* without anti-CLDN6 antibodies against a broad range of tumor cell lines, regardless of CLDN6 surface expression. The example further demonstrates that UDC-NK cells of the disclosure exhibit potent anti-tumor activity *in vitro* and *in vivo* when used in conjunction with an anti-CLDN6 antibody.

[00130] In another study, a PA-1 cell xenograft mouse tumor model using NOGf mice expressing human IL-15 was employed. As illustrated in Figure 35A, 7.5 x 10^6 FcR-UDC I-NK cells were administered i.v. and/or anti-CLDN6 antibody (300 µg) was administered i.p. The combination treatment of FcR-UDC-NK cells and anti-CLDN6 antibody resulted in tumor growth inhibition (Figure 35B) and prolonged the survival (Figure 35C). Greater than 50% survival rate was observed at about 50 days in mice administered FcR-UDC 1-NK cells + anti-CLDN6 antibody.

[00131] Example 8: Anti-Tumor Studies Using UDC-NK Cells and an Anti-Claudin 18.2 (CLDN18.2) Antibody

[00132] This example demonstrates that UDC-NK cells demonstrate anti-tumor activity when used in combination with an anti-CLDN18.2 antibody, Zolbetuximab. Briefly, ADCC assays were performed as a four-hour co-culture with FcR-UDC I-NK cells and BxPC3-CLDN18.2-Luc tumor target cells, similar to the assay described in Example 7. Zolbetuximab was present at 1 µg/mL. The results are illustrated in Figure 36A, which shows Zolbetuximab-dependent killing of CLDN18.2-expressing tumor cells. The anti-tumor effect of FcR-UDC I/II-NK cells in combination with anti-claudin 18.2 antibody (Zolbetuximab) against BxPC3-CLDN18.2-Luciferase tumor cells is illustrated in Figure 36B, which demonstrates that the highest level of cell killing was achieved using FcR-UDC I/II-NK cells in combination with Zolbetuximab.

[00133] A cytokine secretion assay also was performed using methods similar to those in Example 7. BxPC3-CLDN18.2-Luc cells were cultured with FcR-UDC I-NK cells of the disclosure, FcR-UDC I-NK cells and human IgG, or FcR-UDC I-NK cells and Zolbetuximab. Supernatants were collected after a 24-hour co-culture in the presence of IL-15 (10 ng/mL), and the level of interferon-gamma or tumor necrosis factor-alpha was examined. Zolbetuximab was present at 1 μg/mL. The results are illustrated in Figures 37A-37B. Cytokine secretion by FcR-UDC I-NK cells was greatly increased in samples comprising Zolbetuximab and BxPC3-CLDN18.2-Luc tumor target cells.

[00134] The examples provided herein establish that the engineered NK cells of the disclosure exhibit anti-tumor activity against a broad range of tumor types, including lymphoma, ovarian cancer, and pancreatic cancer. The examples report that NK cells exhibit ant-tumor activity alone, and anti-tumor activity is potentiated by use of antibody constructs against tumor antigens (such as CLDN6, CLDN18.2, and CD20). While the working examples utilized tumor cells to demonstrate the cell-killing activity of the engineered NK cells of the disclosure, it will be appreciated that the data may be extrapolated to additional cell types.

[00135] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

WHAT IS CLAIMED:

1. A population of engineered natural killer (NK) cells, wherein the NK cells express at least one polypeptide selected from the group consisting of human leukocyte antigen E (HLA-E), human leukocyte antigen F (HLA-F), and human leukocyte antigen G (HLA-G);

wherein the NK cells comprise a genetically engineered disruption of one or more copies of endogenous β-2 microglobulin (B2M); and

wherein the NK cells comprise a genetically engineered disruption of one or more copies of a human leukocyte antigen (HLA) class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein (RFXANK), regulatory factor 5 (RFX5), regulatory factor X associated protein (RFXAP), and class II transactivator (CIITA).

2. The population of engineered NK cells of claim 1, wherein the NK cells further comprise a nucleic acid molecule encoding a suicide gene product.

3. The population of engineered NK cells of claim 2, wherein NK cells comprise a herpes simplex virus thymidine kinase (TK) suicide gene.

4. The population of engineered NK cells of any one of claims 1-3, wherein the NK cells are derived from pluripotent stem cells.

5. The population of engineered NK cells of claim 4, wherein the pluripotent stem cells are embryonic stem cells.

6. The population of engineered NK cells of claim 4, wherein the pluripotent stem cells are induced pluripotent stem cells.

7. The population of engineered NK cells of any one of claims 1-6, wherein the NK cells express one or more cell surface markers selected from the group consisting of CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and NKp44.

8. The population of engineered NK cells of any one of claims 1-7, wherein the population of engineered NK cells is capable of engrafting into a subject following administration.

9. The population of engineered NK cells of claim 8, wherein at least 50% of the population of engineered NK cells is capable of engrafting into a target tissue of the subject.

10. The population of engineered NK cells of claim 8 or claim 9, wherein the population of engineered NK cells is capable of remaining engrafted in the target tissue of the subject for at least 20 days following administration.

11. The population of engineered NK cells of any one of claims 8-10, wherein the target tissue is blood or bone marrow.

12. The population of engineered NK cells of any one of claims 1-11, wherein the NK cells are capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of a cancer cell.

13. The population of engineered NK cells of any one of claims 1-12, wherein the NK cells are capable of inducing natural cell cytotoxicity (NCC) of a cancer cell.

14. The population of engineered NK cells of claim 12 or claim 13, wherein the NK cells decrease tumor load by at least 10%.

15. The population of engineered NK cells of any one of claims 1-14, wherein the NK cells have at least about a 5% increase in cell volume as compared to peripheral blood NK cells.

16. The population of engineered NK cells of any one of claims 1-15, wherein the cells express a chimeric antigen receptor or an Fc receptor.

17. The population of engineered NK cells of any one of claims 1-16, wherein the NK cells comprise a genetically engineered disruption of all copies of endogenous B2M.

18. The population of engineered NK cells of any one of claims 1-17, wherein the NK cells comprise a genetically engineered disruption of all copies of an HLA class II-related gene selected from the group consisting of RFXANK, RFX5, RFXAP, and CIITA.

19. A method of producing human pluripotent stem cell-derived engineered natural killer (NK) cells under feeder free conditions, the method comprising

(a) culturing human pluripotent stem cells genetically engineered to disrupt one or more copies of endogenous β-2 microglobulin (B2M) and one or more copies of a human leukocyte antigen (HLA) class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein (RFXANK), regulatory factor 5 (RFX5), regulatory factor X associated protein (RFXAP), and class II transactivator (CIITA);

(b) differentiating the human pluripotent stem cells to precursor cells capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives;

(c) differentiating the precursor cells to NK cells under feeder free conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L; and

(d) culturing the NK cells in a culture media comprising IL-15 and IL-18, and in the presence of an antibody or ligand that binds a receptor on NK cells to promote activation of the NK cells,

thereby generating human pluripotent stem cell-derived engineered NK cells.

20. A method of producing human pluripotent stem cell-derived engineered natural killer (NK) cells under feeder free conditions, the method comprising

(a) culturing human pluripotent stem cells genetically engineered to disrupt one or more copies of endogenous β-2 microglobulin (B2M) and one or more copies of a human leukocyte antigen (HLA) class II-related gene selected from the group consisting of regulatory factor X associated ankyrin containing protein (RFXANK), regulatory factor 5 (RFX5), regulatory factor X associated protein (RFXAP), and class II transactivator (CIITA);

(b) differentiating the human pluripotent stem cells using embryoid bodies to precursor cells capable of differentiating to hematopoietic cell types, endothelial cell types, and/or mesodermal derivatives; and

(c) differentiating the precursor cells to NK cells under feeder free conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L;

thereby generating human pluripotent stem cell-derived engineered NK cells.

21. The method of claim 20, further comprising (d) culturing the NK cells in a culture media comprising IL-15 and IL-18, and in the presence of an antibody or ligand that binds a receptor on an NK cell to promote activation of the NK cells.

22. The method of claim 19 or claim 21, wherein step (d) comprises culturing the NK cells in the presence of an antibody that binds to DNAM-1, an antibody that binds to OX40, an antibody that binds to NKG2D, an antibody that binds to 2B4, an antibody that binds to NKp30, or an antibody that binds to NKp46, or a combination thereof.

23. The method of claim 22, wherein step (d) comprises culturing the NK cells in the presence of an antibody that binds NKp30 and an antibody that binds DNAM-1.

24. The method of claim 19 or claim 21, wherein step (d) comprises culturing the NK cells in the presence of a ligand that binds to DNAM-1, a ligand that binds to OX40, a ligand that binds to NKG2D, a ligand that binds to 2B4, a ligand that binds to NKp30, or a ligand that binds to NKp46, or a combination thereof.

25. The method of claim 24, wherein step (d) comprises culturing the NK cells in the presence of a ligand that binds NKp30 and a ligand that binds DNAM-1.

26. The method of any one of claims 19 or 21-25, wherein the culture media in step (d) further comprises IL-21.

27. The method of any one of claims 19-26, wherein the human pluripotent stem cells of step (a) further comprise a nucleic acid molecule encoding a suicide gene product.

28. The method of claim 27, wherein the NK cells comprise a herpes simplex virus thymidine kinase (TK) suicide gene.

29. The method of any one of claims 19-28, wherein the human pluripotent stem cells are human embryonic stem cells.

30. The method of any one of claims 19-28, wherein the human pluripotent stem cells are human induced pluripotent stem cells

31. The method of any one of claims 19-30, wherein the culture media of step (c) further comprises UM171.

32. The method of any one of claims 19-31, wherein the NK cells express one or more cell surface markers selected from the group consisting of CD56, CD45, CD25, DNAM-1, NKp30, NKG2D, and NKp44.

33. The method of any one of claims 19-32, wherein the NK cells express at least one polypeptide selected from the group consisting of human leukocyte antigen E (HLA-E), human leukocyte antigen F (HLA-F), and human leukocyte antigen G (HLA-G).

34. The method of any one of claims 19-33, wherein the NK cells are capable of engrafting into a subject following administration.

35. The method of claim 34, wherein at least 50% of the NK cells are capable of engrafting into the subject.

36. The method of claim 34 or claim 35, wherein the NK cells are capable of remaining engrafted in the subject for at least 20 days following administration.

37. The method of any one of claims 34-36, wherein the NK cells are capable of engrafting into the blood and bone marrow.

38. The method of any one of claims 19-37, wherein the NK cells are capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of a cancer cell.

39. The method of any one of claims 19-38, wherein the NK cells are capable of inducing natural cell cytotoxicity (NCC) of a cancer cell.

40. The method of claim 38 or claim 39, wherein the NK cells decrease tumor load by at least 10%.

41. The method of any one of claims 19 and 21-40, wherein step (d) is repeated up to five times.

42. A method of increasing the purity of a population of NK cells derived from pluripotent stem cells, wherein the method comprises differentiating embryoid bodies to NK cells under feeder free conditions in a culture media comprising SCF, IL-7, IL-15, and Flt3L and in the presence of UM171.

43. The method of claim 42, wherein the culture media further comprises IL-3.

44. The method of claim 42 or claim 43, wherein the method is performed in a vessel coated with a recombinant human fibronectin fragment and DLL4.

45. The method of any one of claims 42-44, wherein the method is performed for about one day to about 21 days.

46. A pharmaceutical composition comprising the population of engineered NK cells of any one of claims 1-18, and a pharmaceutically acceptable carrier.

47. A method of treating a disease or disorder in a subject in need thereof, the method comprising administering to the subject an effective amount of the population of engineered NK cells of any one of claims 1-18, or the pharmaceutical composition of claim 46, thereby treating the disease or disorder in the subject.

48. The method of claim 47, wherein the disease or disorder is cancer.

49. The method of claim 47 or claim 48, wherein the method further comprises administering one or more cytokines to the subject.

50. The method of claim 49, wherein the one or more cytokines are IL-15 and/or IL-2.

51. The method of any one of claims 47-50, wherein the administering increases survival time of the subject by at least 20 days relative to survival time of the subject in the absence of administering the population of engineered NK cells.

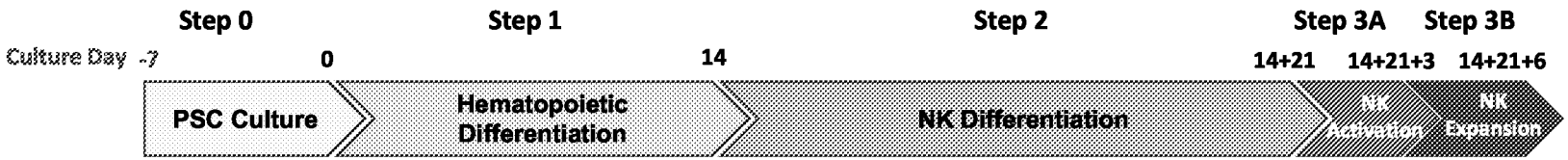
52. The method of any one of claims 47-50, wherein the administering decreases tumor load in the subject.

53. The method of any one of claims 47-52, wherein the method further comprises administering one or more antibodies to the subject.

54. The method of claim 47, wherein the disease or disorder is an autoimmune disorder.

55. The method of claim 54, wherein the method further comprises administering one or more antibodies to the subject.

FIG. 1



	Step 0	Step 1	Step 2	Step 3A	Step 3B
Culture Surface	Cell passage onto Vitronectin	Single cell passage onto Aggrewell800	EBs onto DLL4/RTN-coated plates	Anti-NKp30 Anti-DNAM-1 Retronectin	
Medium/ Cytokines	mTeSR media	APEL-2 media 20 ng/mL BMP4 20 ng/mL VEGF 40 ng/mL SCF	Modified DMEM/F12 7.5% Human AB serum 20 ng/mL SCF 10 ng/mL Flt3L 20 ng/mL IL-7 10 ng/mL IL-15 5 ng/mL IL-3 (first week) 35nM UM171	NK-Xpander Medium or RPMI-1640 7.5% Human AB serum 10 ng/ml IL-15 10 ng/ml IL-18 10 ng/ml IL-21	NK-Xpander Medium or RPMI-1640 7.5% Human AB serum 10 ng/ml IL-15 10 ng/ml IL-18

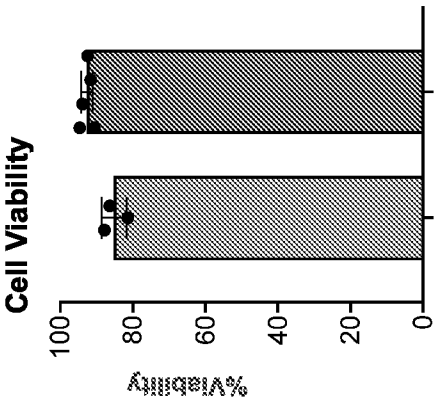


FIG. 2C

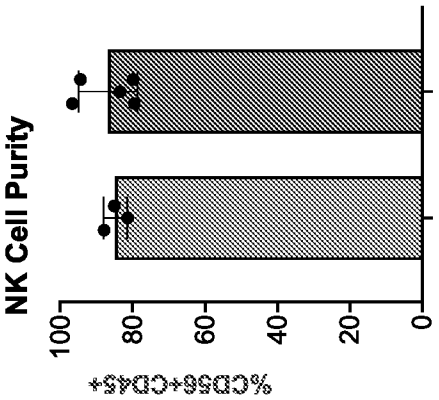


FIG. 2B

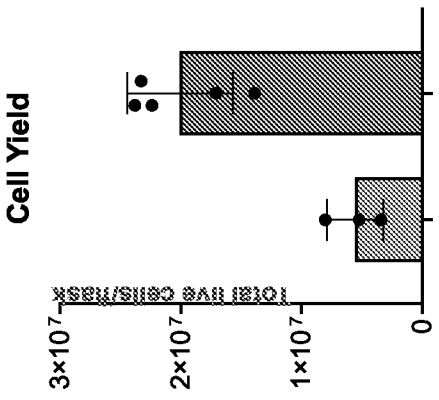


FIG. 2A

FIG. 3A

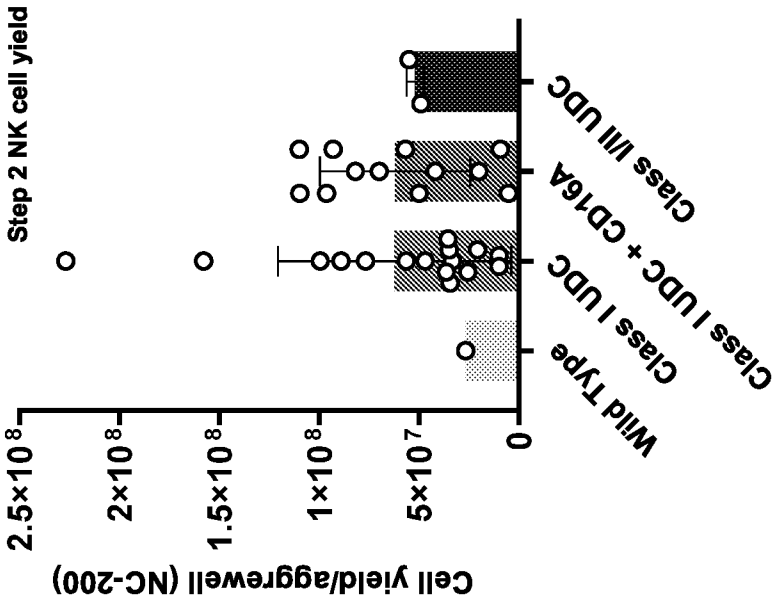

Step 2 NK cell yield
Cell yield/aggrewell (NC-200)
2.5×10^8
2×10^8
1.5×10^8
1×10^8
5×10^7
0
Wild Type
Class I UDC
Class I UDC + CD16A
Class I/II UDC

FIG. 3B

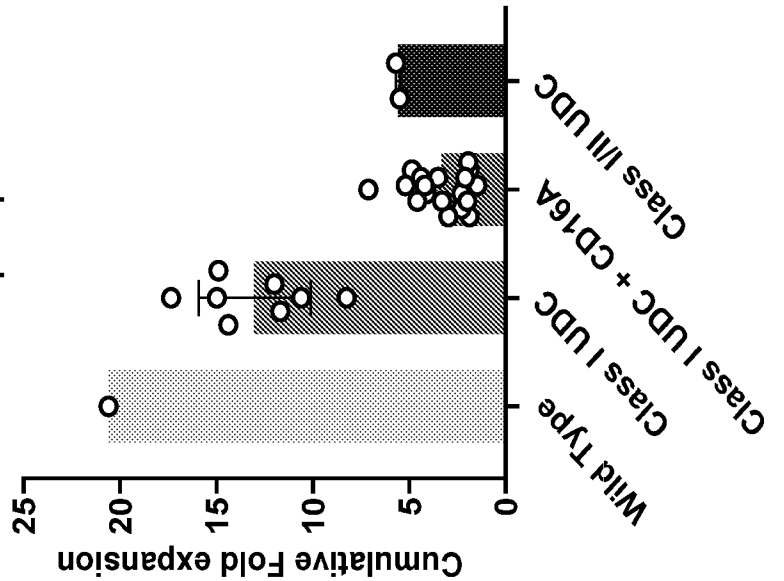

Step 3 Expansion
Cumulative Fold expansion
25
20
15
10
5
0
Wild Type
Class I UDC
Class I UDC + CD16A
Class I/II UDC

FIG. 3C

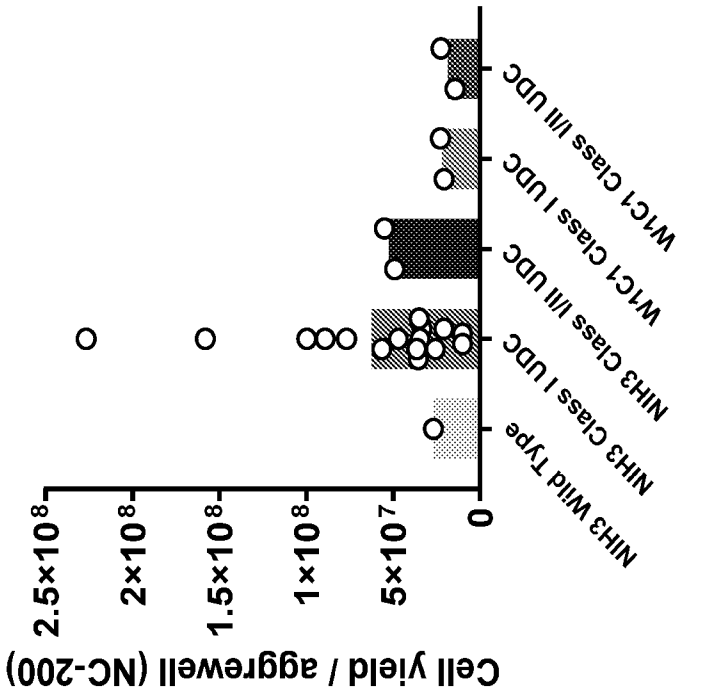

Step2 Average cell yield
Cell yield / aggrewell (NC-200)
2.5×10^8
2×10^8
1.5×10^8
1×10^8
5×10^7
0
NIH3 Wild Type
NIH3 Class I UDC
NIH3 Class I/II UDC
WiC1 Class I UDC
WiC1 Class I/II UDC

FIG. 3D

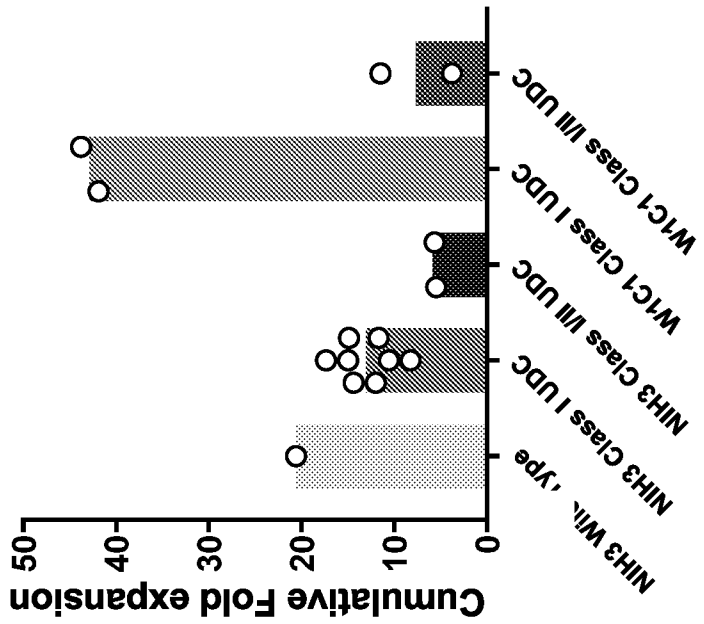

Step3 Fold Expansion
Cumulative Fold expansion
50
40
30
20
10
0
NIH3 Wild Type
NIH3 Class I UDC
NIH3 Class I/II UDC
WiC1 Class I UDC
WiC1 Class I/II UDC

FIG. 4

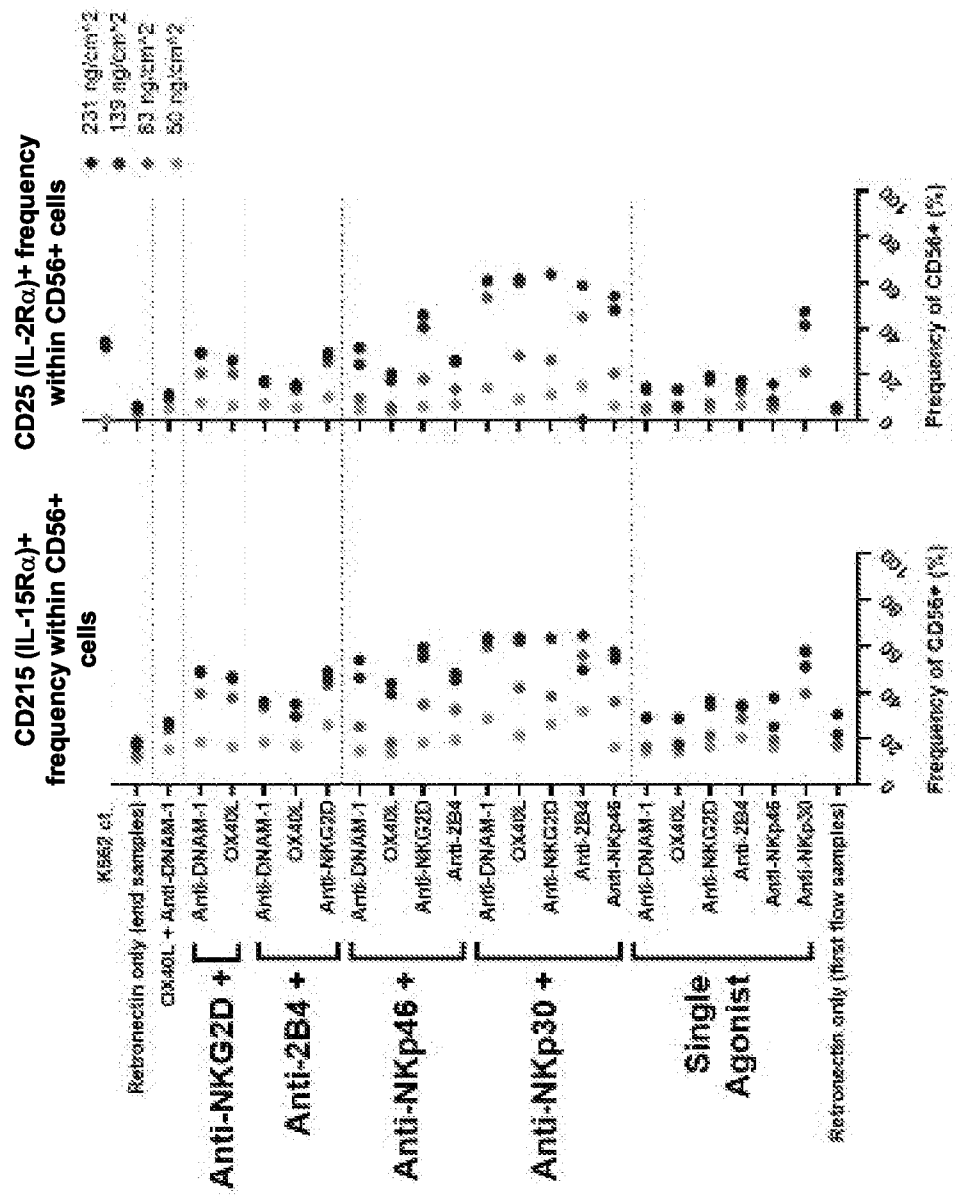

CD215 (IL-15Rα)+ frequency within CD56+ cells
CD25 (IL-2Rα)+ frequency within CD56+ cells
231 ng/cm^2
139 ng/cm^2
83 ng/cm^2
50 ng/cm^2
K562 cl.
Retronectin only (seed samples)
CD40L + Anti-DNAM-1
Anti-NKG2D +
Anti-2B4 +
Anti-NKp46 +
Anti-NKp30 +
Single Agonist
Anti-DNAM-1
CD40L
Anti-NKG2D
Anti-2B4
Anti-NKp46
Anti-NKp30
Retronectin only (first flow samples)
Frequency of CD56+ (%)
Frequency of CD56+ (%)

FIG. 6

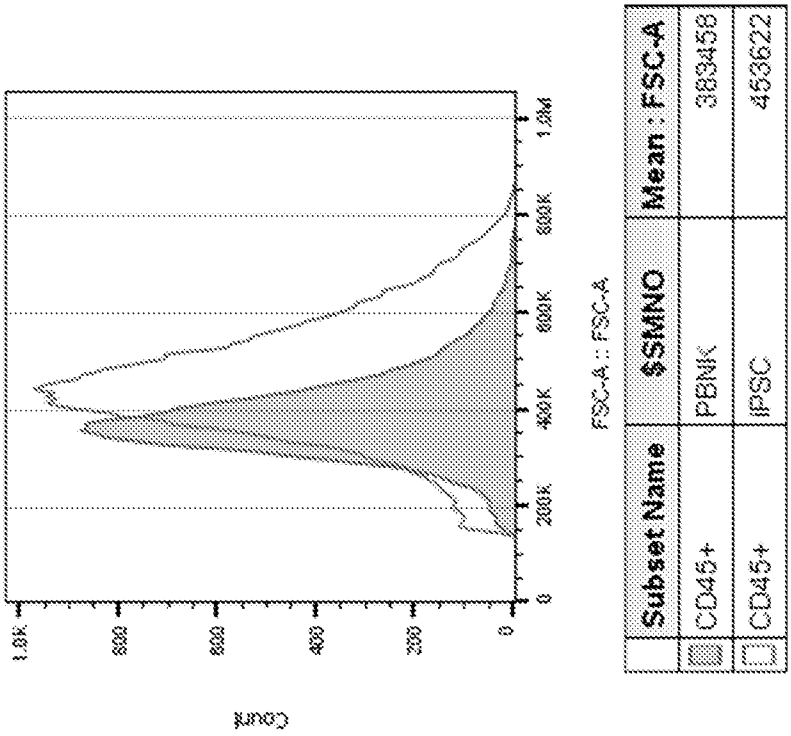


FIG. 5A

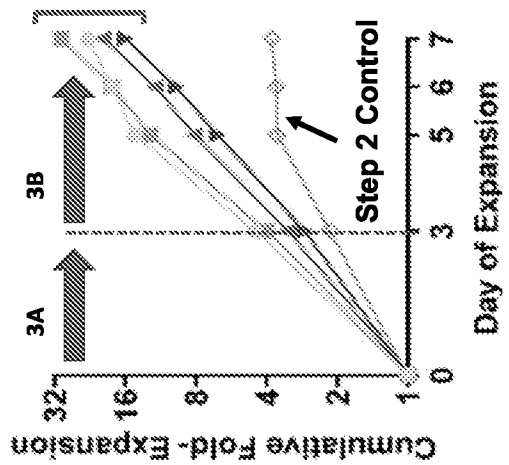


FIG. 5B

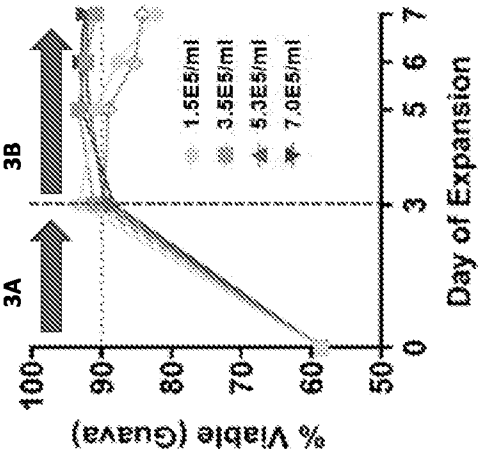


FIG. 7A

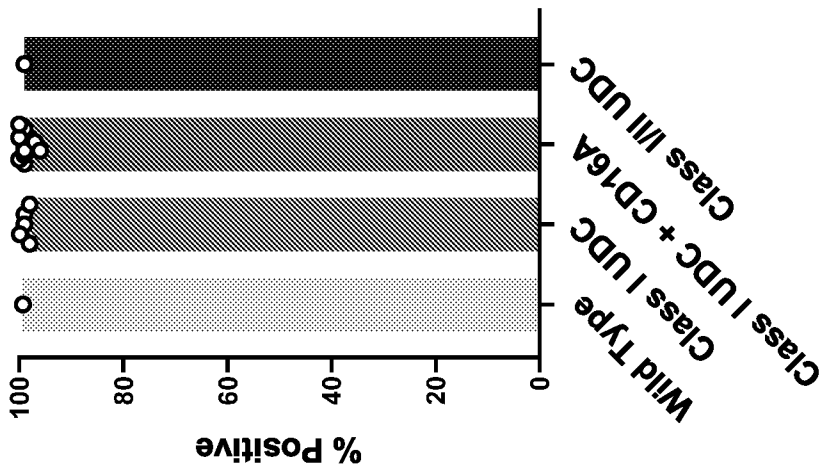


FIG. 7B

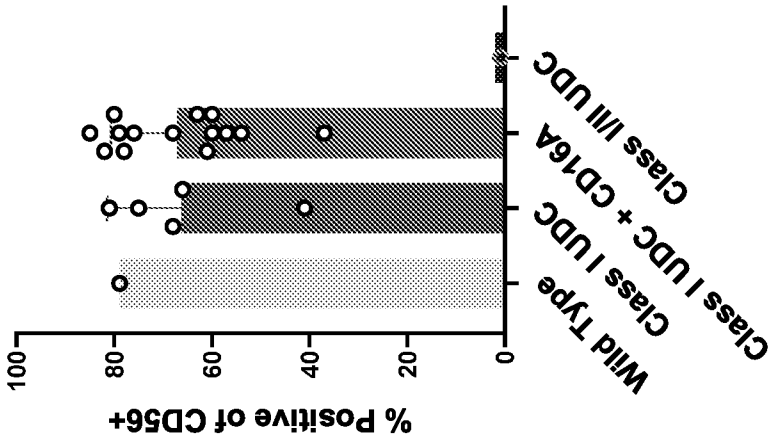
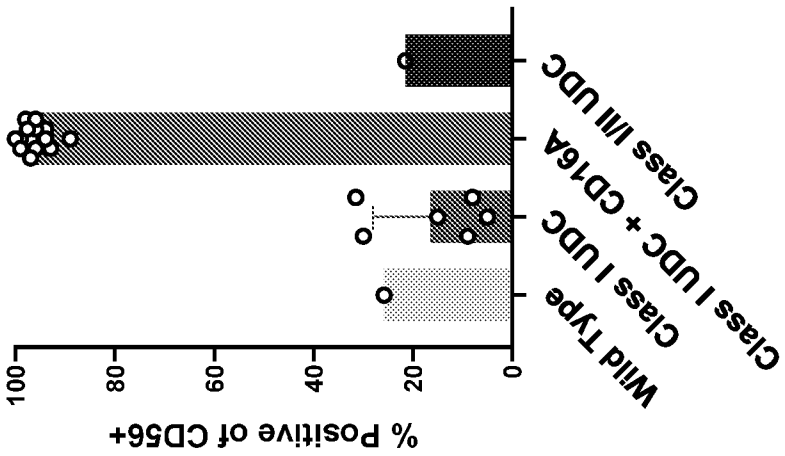


FIG. 7C



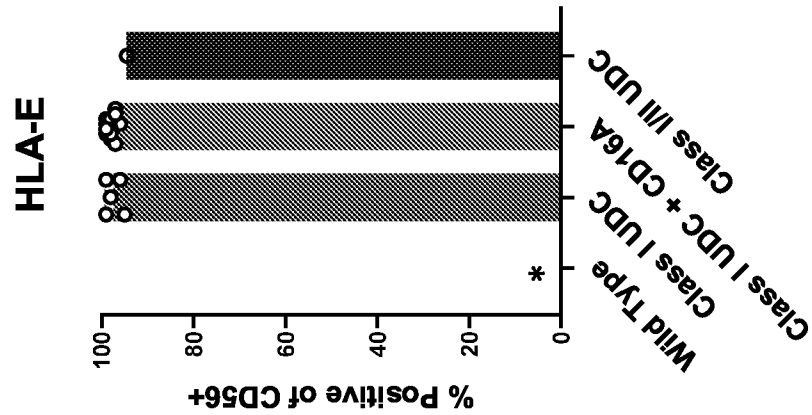

HLA-E
% Positive of CD56+
100
80
60
40
20
0
*
Wild Type
Class I UDC
Class I UDC + CD16A
Class I/II UDC

FIG. 7E

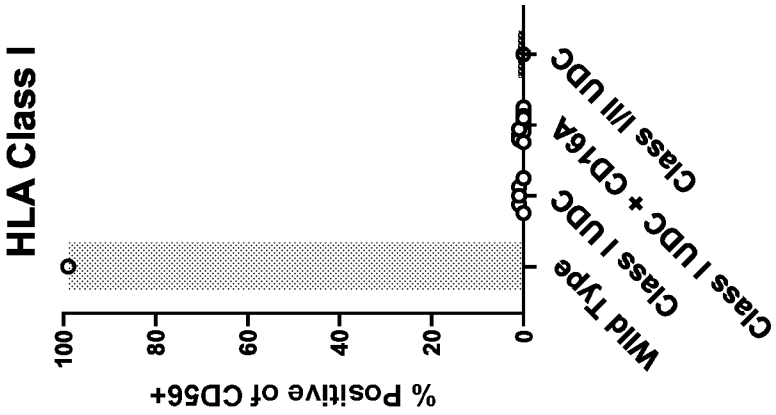

HLA Class I
% Positive of CD56+
100
80
60
40
20
0
Wild Type
Class I UDC
Class I UDC + CD16A
Class I/II UDC

FIG. 7D

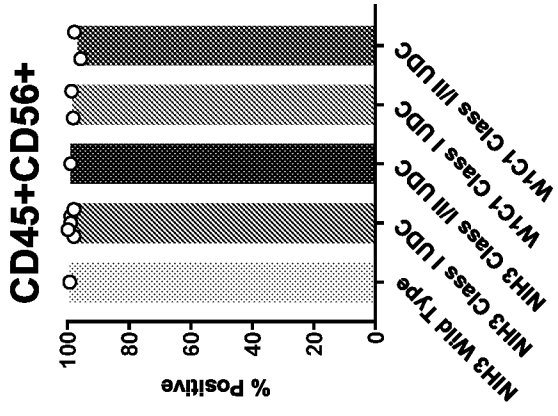


FIG. 8A

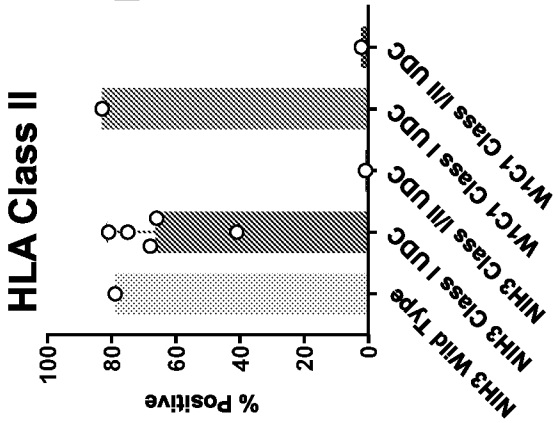


FIG. 8B

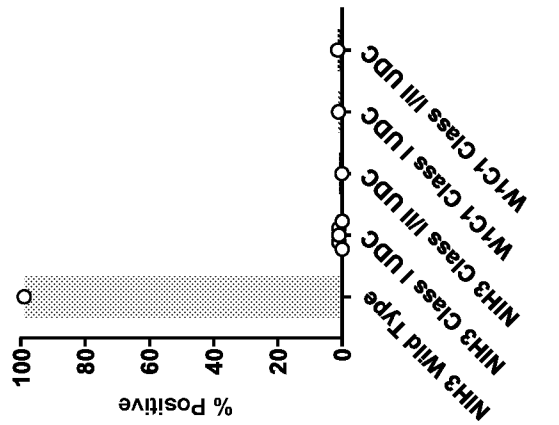


FIG. 8C

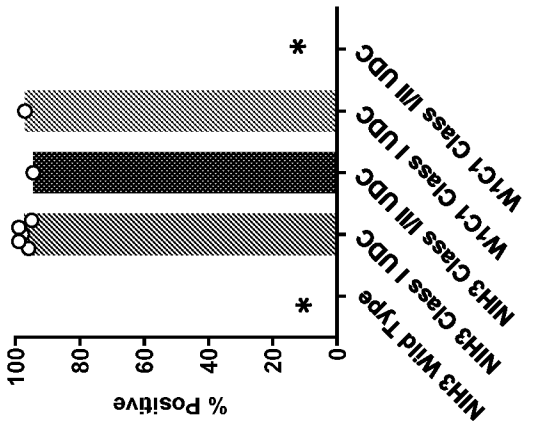


FIG. 8D

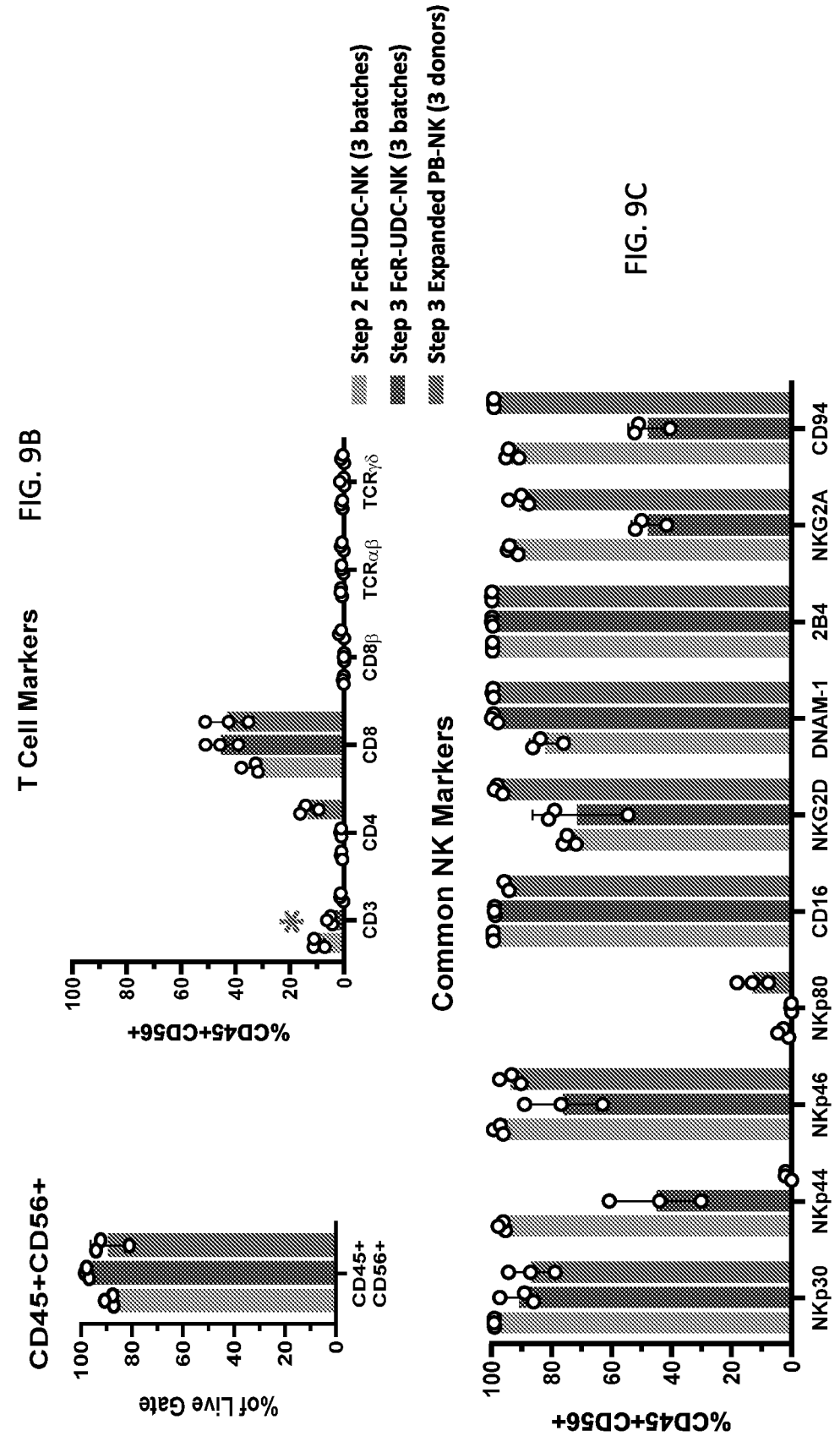


FIG. 9A
CD45+CD56+
%of Live Gate
CD45+ CD56+
FIG. 9B
T Cell Markers
%CD45+CD56+
CD3
CD4
CD8
CD8β
TCRαβ
TCRγδ
Step 2 FcR-UDC-NK (3 batches)
Step 3 FcR-UDC-NK (3 batches)
Step 3 Expanded PB-NK (3 donors)
FIG. 9C
Common NK Markers
%CD45+CD56+
NKp30
NKp44
NKp46
NKp80
CD16
NKG2D
DNAM-1
2B4
NKG2A
CD94

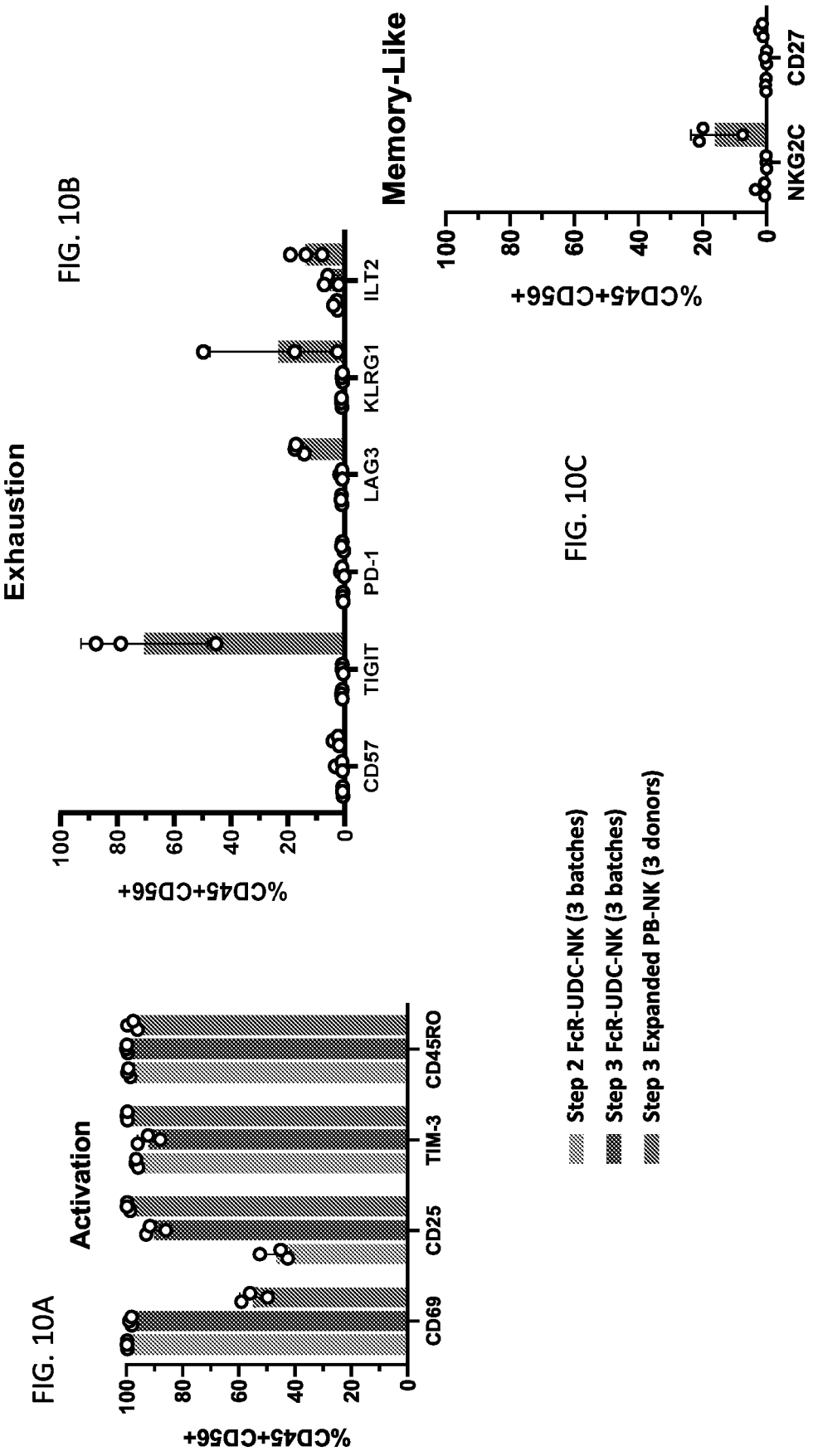


FIG. 10A
Activation
%CD45+CD56+
100
80
60
40
20
0
CD69
CD25
TIM-3
CD45RO
FIG. 10B
Exhaustion
%CD45+CD56+
100
80
60
40
20
0
CD57
TIGIT
PD-1
LAG3
KLRG1
ILT2
Memory-Like
%CD45+CD56+
100
80
60
40
20
0
NKG2C
CD27
FIG. 10C
Step 2 FcR-UDC-NK (3 batches)
Step 3 FcR-UDC-NK (3 batches)
Step 3 Expanded PB-NK (3 donors)

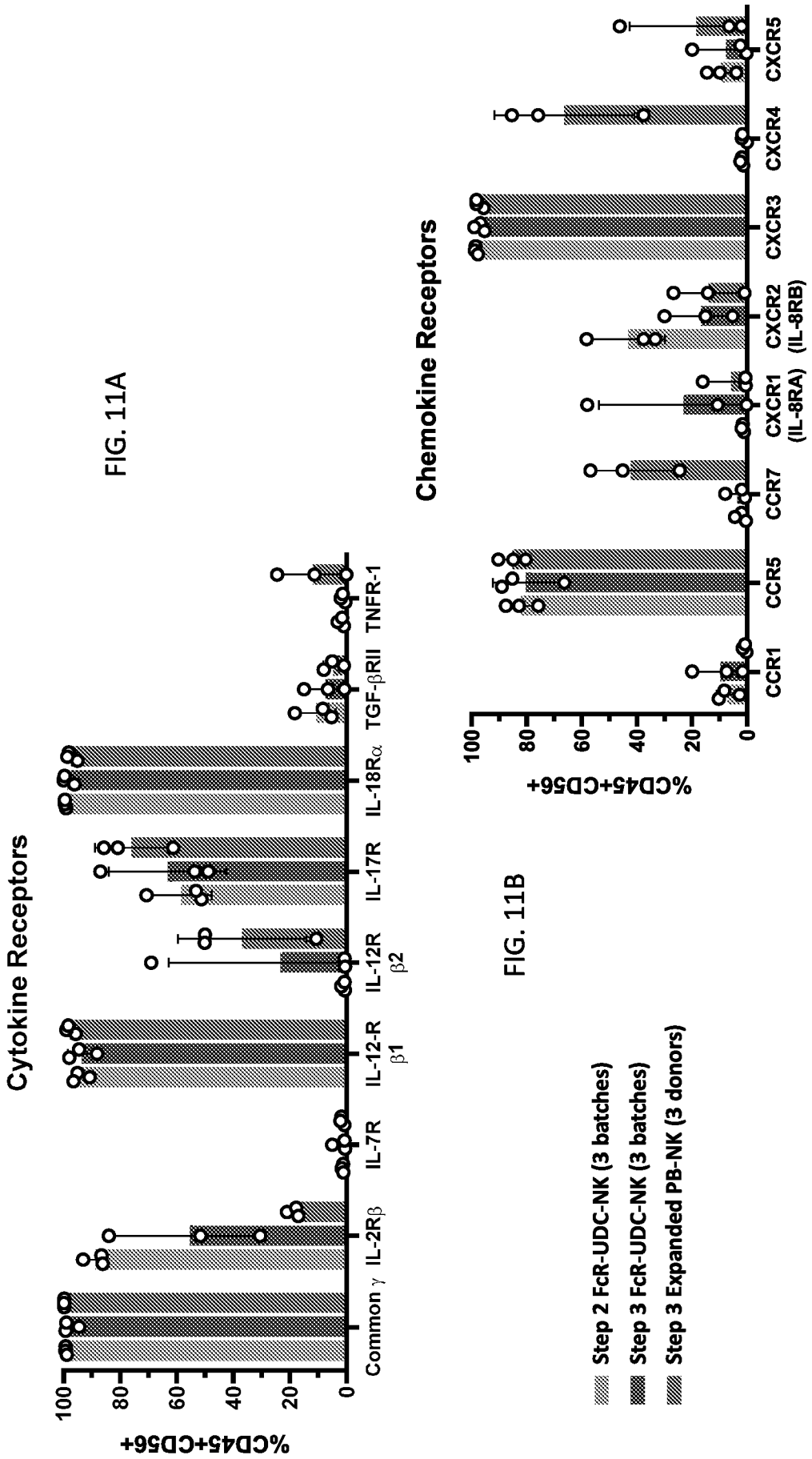


FIG. 11A

FIG. 11B

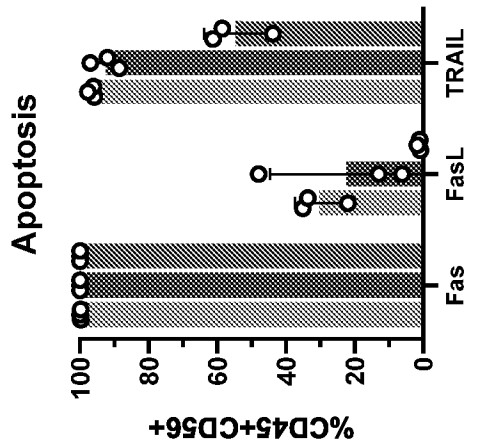


FIG. 12B

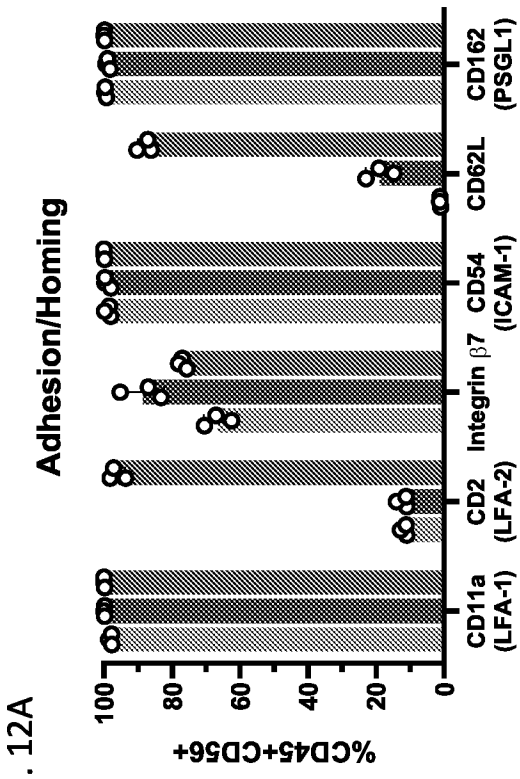


FIG. 12A

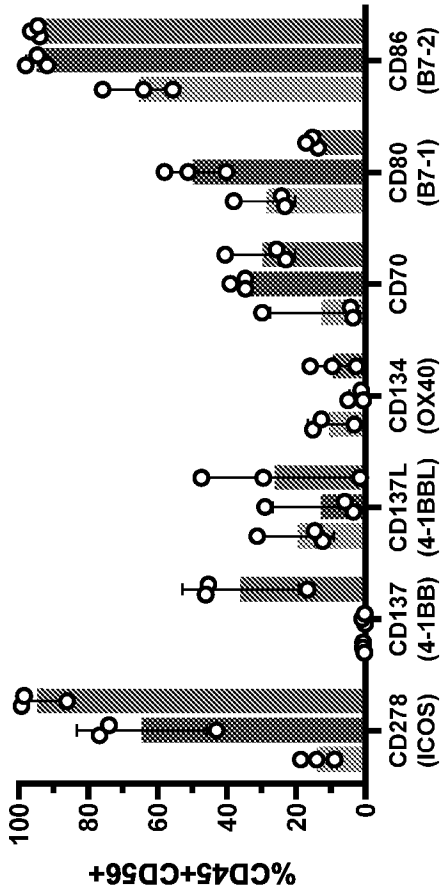


FIG. 12C

Step 2 FcR-UDC-NK (3 batches)
Step 3 FcR-UDC-NK (3 batches)
Step 3 Expanded PB-NK (3 donors)

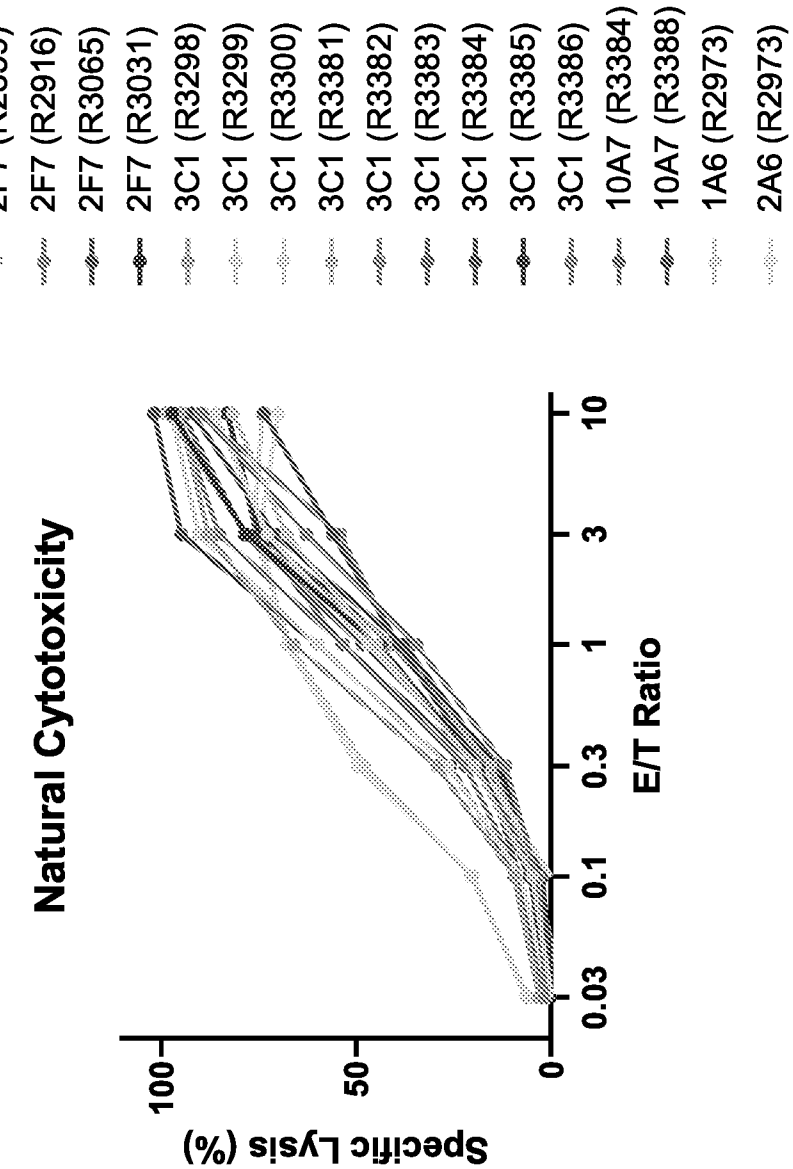

2F7 (R2859)
2F7 (R2916)
2F7 (R3065)
2F7 (R3031)
3C1 (R3298)
3C1 (R3299)
3C1 (R3300)
3C1 (R3381)
3C1 (R3382)
3C1 (R3383)
3C1 (R3384)
3C1 (R3385)
3C1 (R3386)
10A7 (R3384)
10A7 (R3388)
1A6 (R2973)
2A6 (R2973)
Natural Cytotoxicity
Specific Lysis (%)
100
50
0
0.03
0.1
0.3
1
3
10
E/T Ratio

FIG. 13

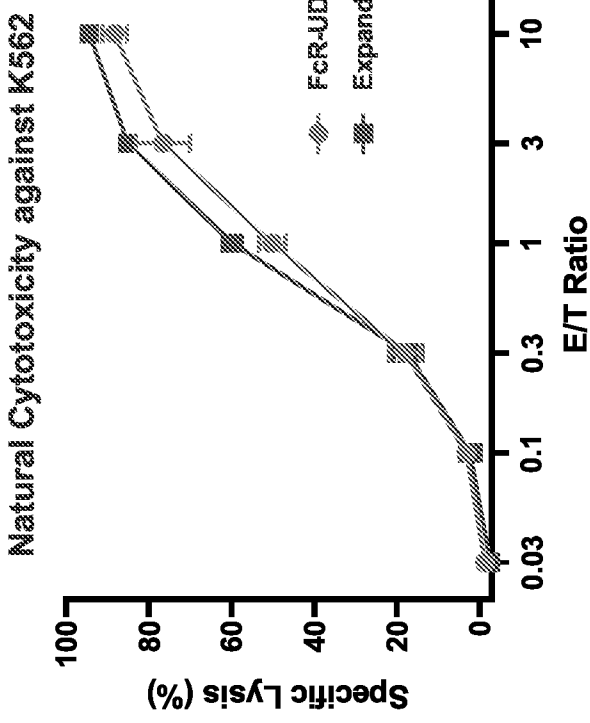

Natural Cytotoxicity against K562
Specific Lysis (%)
100
80
60
40
20
0
0.03
0.1
0.3
1
3
10
E/T Ratio
FcR-UDC-NK
Expanded PB-NK

FIG. 15

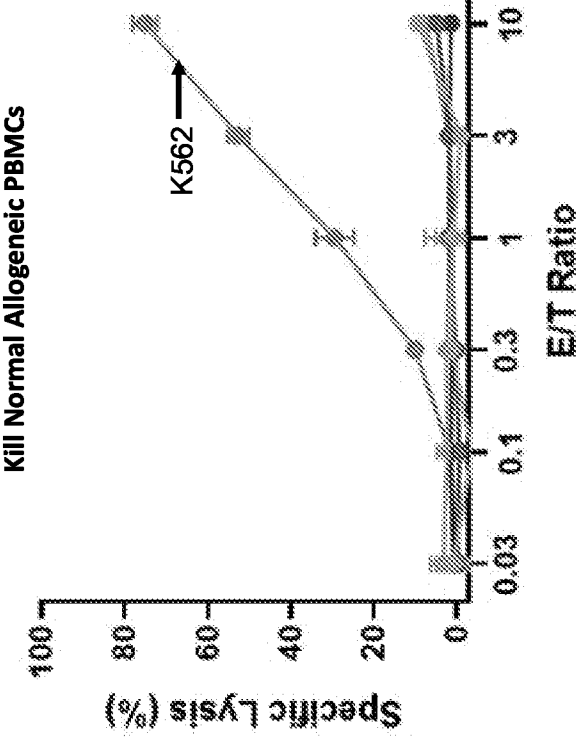
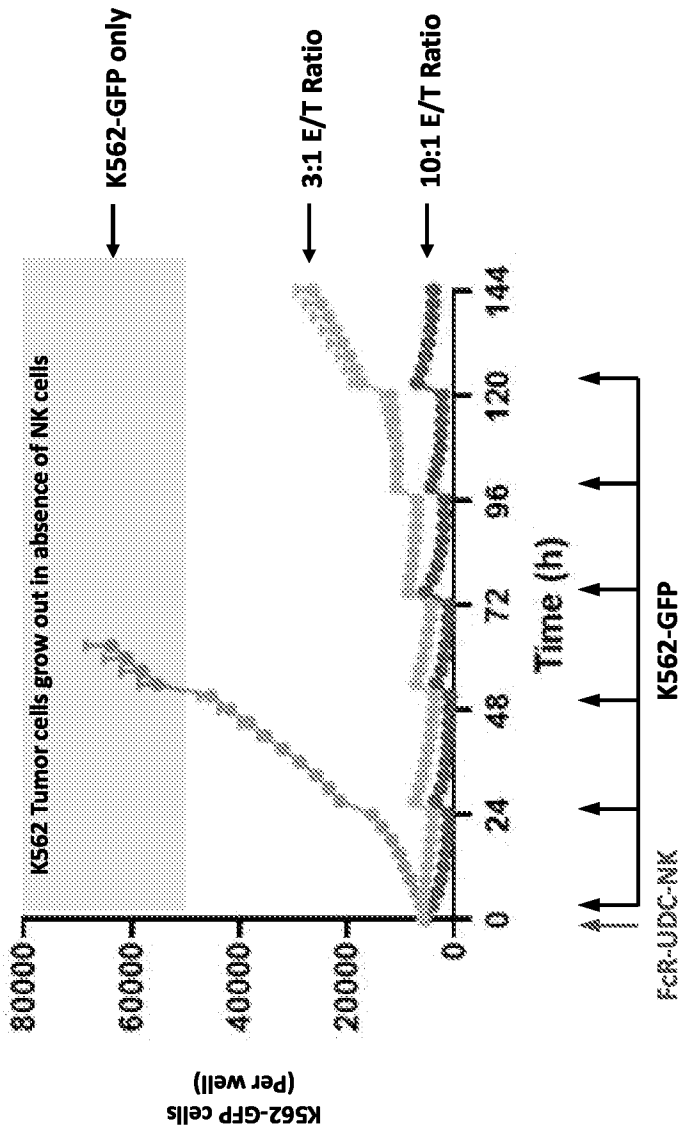

FcR-UDC-NK Cells Do Not Exhibit Fratricide or Kill Normal Allogeneic PBMCs
Specific Lysis (%)
100
80
60
40
20
0
0.03
0.1
0.3
1
3
10
E/T Ratio
K562
K562
PBMC Donor#398
PBMC Donor#403
PBMC Donor#2089
FCR-UDC-NK

FIG. 16

FIG. 17


K562-GFP only
3:1 E/T Ratio
10:1 E/T Ratio
K562 Tumor cells grow out in absence of NK cells
K562-GFP cells (Per well)
80000
60000
40000
20000
0
0
24
48
72
96
120
144
Time (h)
FcR-UDC-NK
K562-GFP

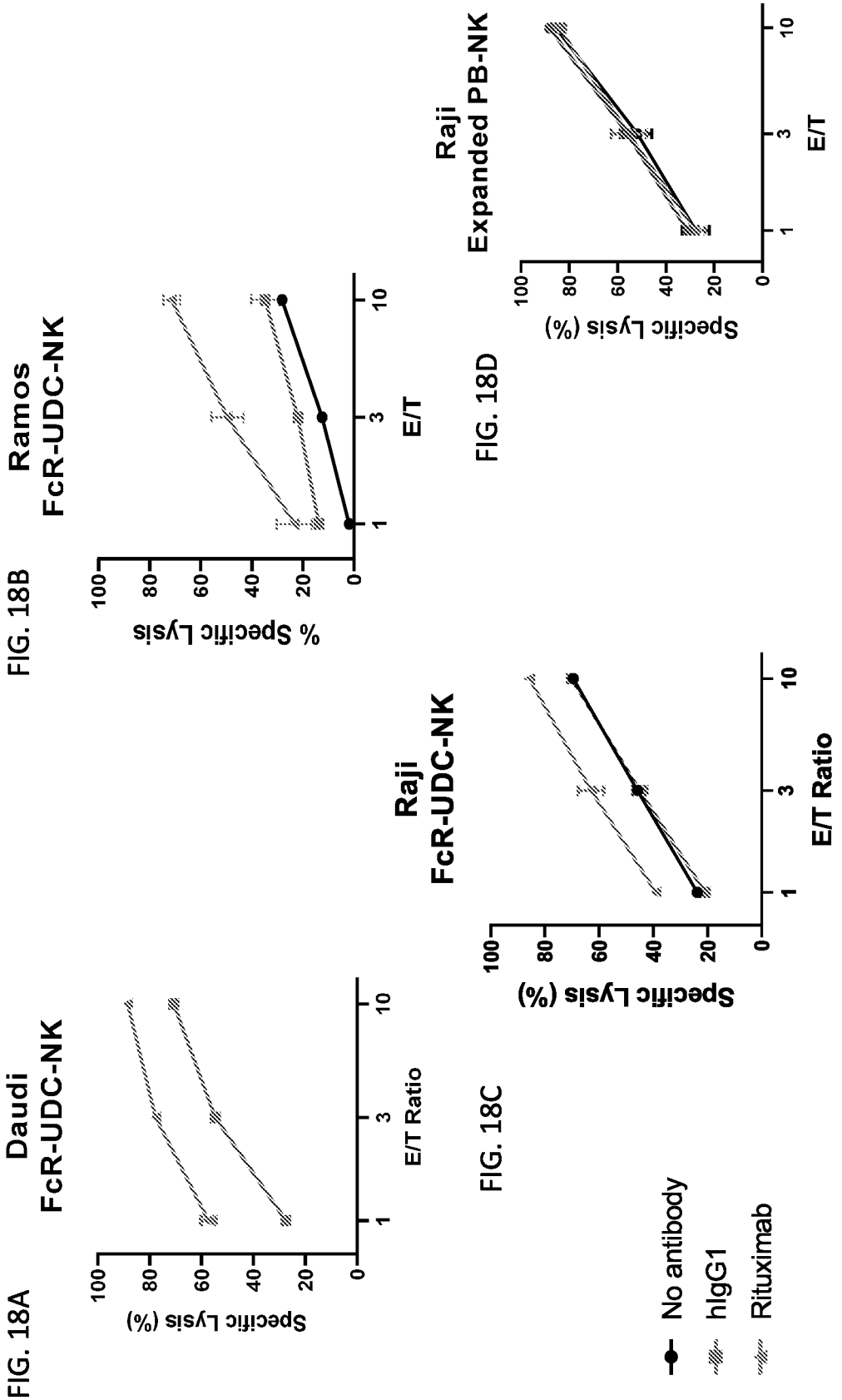


FIG. 18A
Daudi
FcR-UDC-NK
Specific Lysis (%)
E/T Ratio
FIG. 18B
Ramos
FcR-UDC-NK
% Specific Lysis
E/T
FIG. 18C
Raji
FcR-UDC-NK
Specific Lysis (%)
E/T Ratio
FIG. 18D
Raji
Expanded PB-NK
Specific Lysis (%)
E/T
No antibody
hIgG1
Rituximab

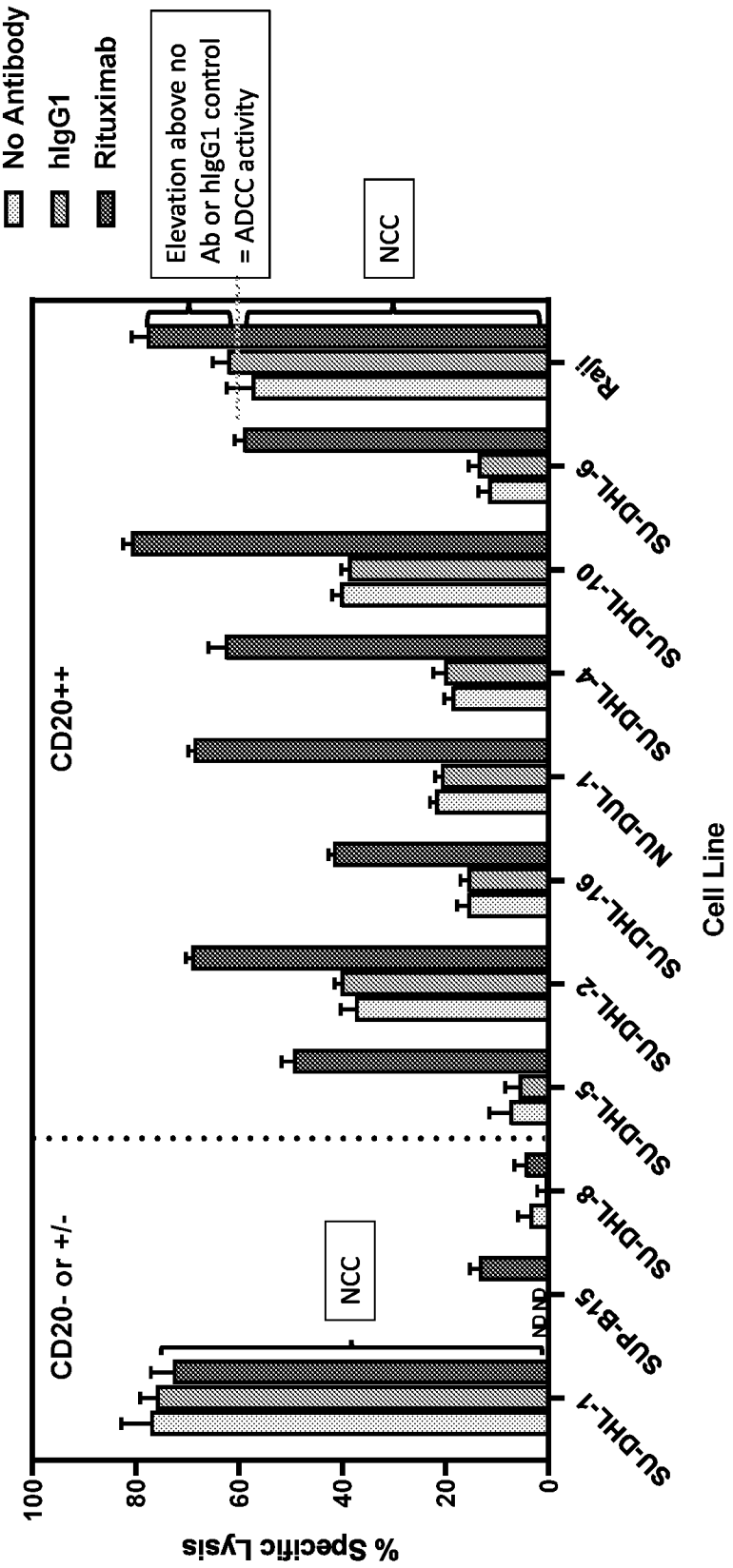
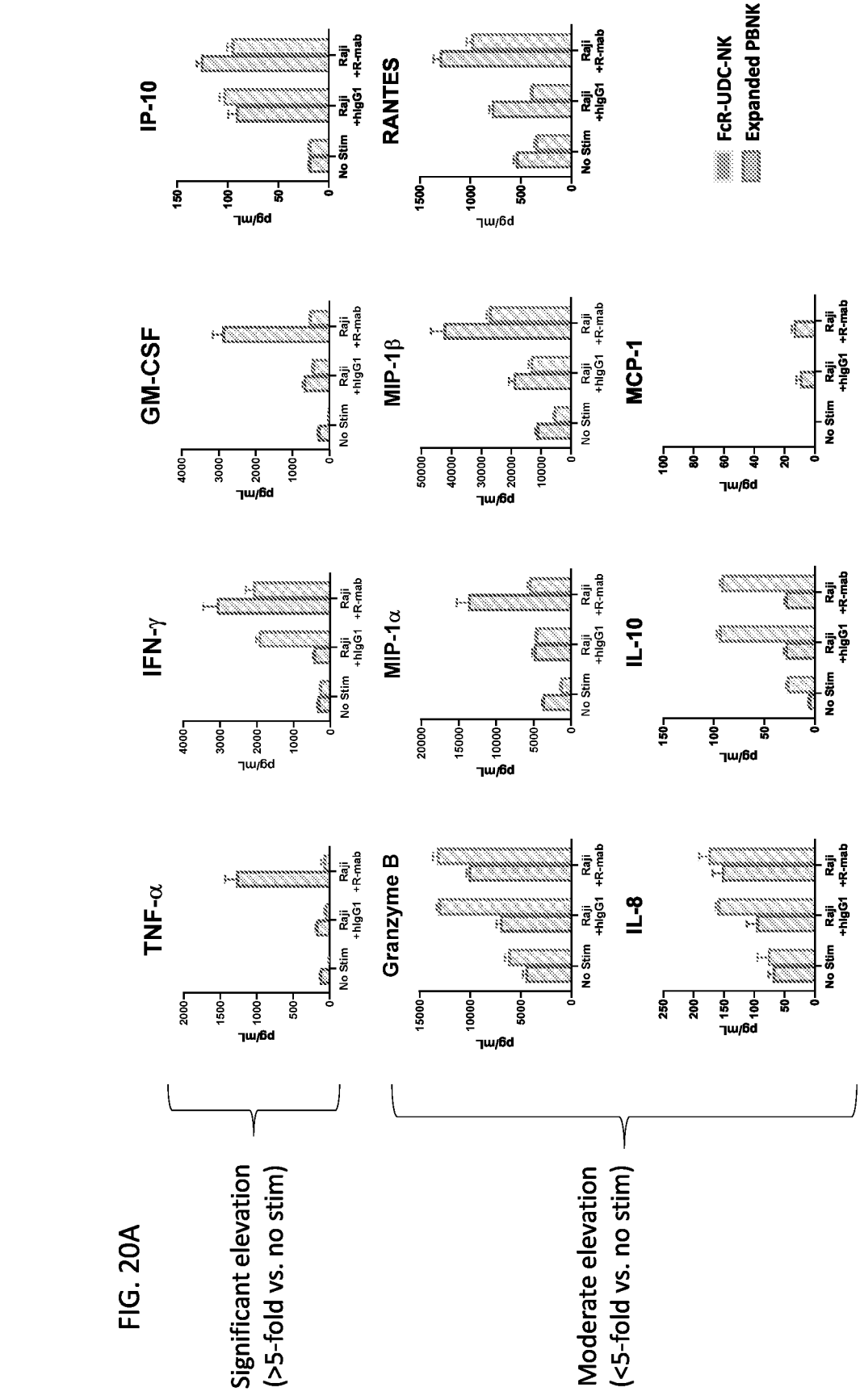

No Antibody
hIgG1
Rituximab
Elevation above no Ab or hIgG1 control = ADCC activity
NCC
CD20- or +/-
CD20++
% Specific Lysis
0
20
40
60
80
100
SU-DHL-1
SUP-B15
SU-DHL-8
SU-DHL-5
SU-DHL-2
SU-DHL-16
NU-DUL-1
SU-DHL-4
SU-DHL-10
SU-DHL-6
Raji
Cell Line

FIG. 19

FIG. 20A


Significant elevation (>5-fold vs. no stim)
Moderate elevation (<5-fold vs. no stim)
TNF-α
IFN-γ
GM-CSF
IP-10
Granzyme B
MIP-1α
MIP-1β
RANTES
IL-8
IL-10
MCP-1
pg/mL
No Stim
Raji
Raji +hIgG1
Raji +R-mab
FcR-UDC-NK
Expanded PBNK

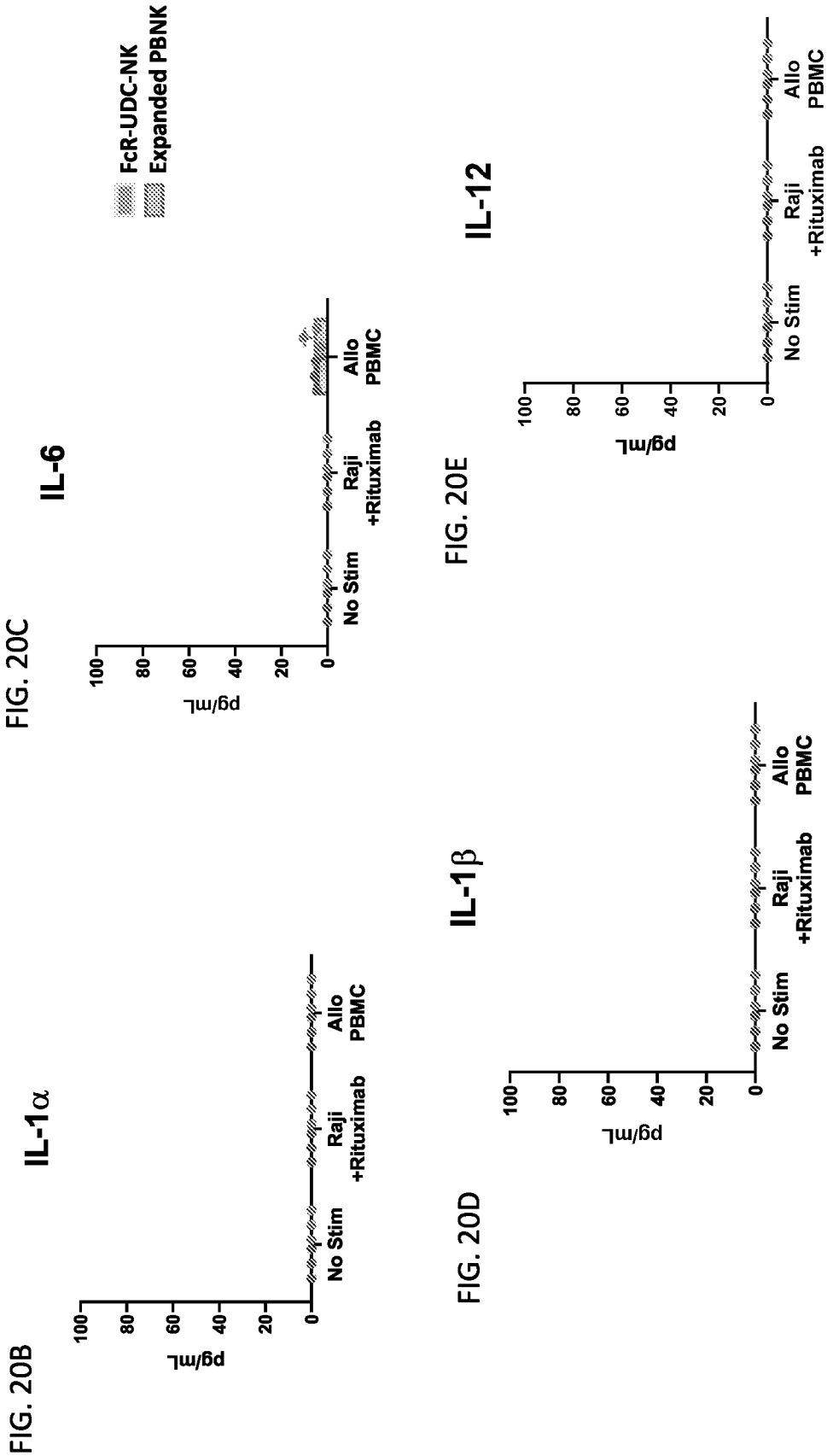


FIG. 20B
IL-1α
pg/mL
100
80
60
40
20
0
No Stim
Raji
Raji +Rituximab
Allo PBMC
FIG. 20C
IL-6
FcR-UDC-NK
Expanded PBNK
pg/mL
100
80
60
40
20
0
No Stim
Raji
Raji +Rituximab
Allo PBMC
FIG. 20D
IL-1β
pg/mL
100
80
60
40
20
0
No Stim
Raji
Raji +Rituximab
Allo PBMC
FIG. 20E
IL-12
pg/mL
100
80
60
40
20
0
No Stim
Raji
Raji +Rituximab
Allo PBMC

FIG. 21A

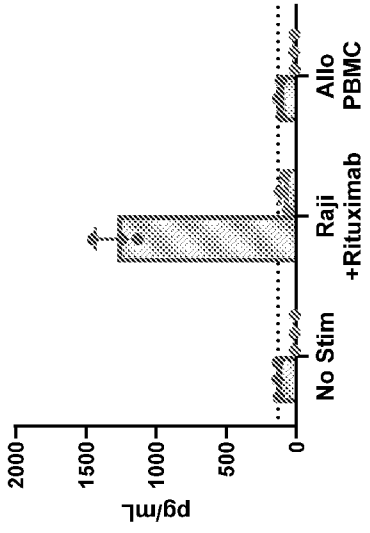


FIG. 21B

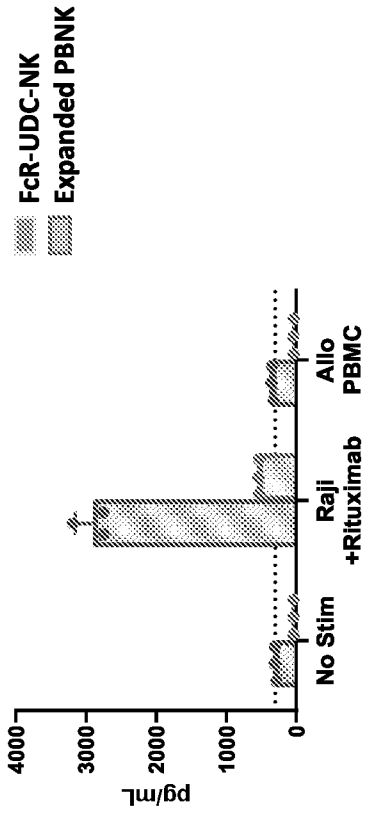


FIG. 21C

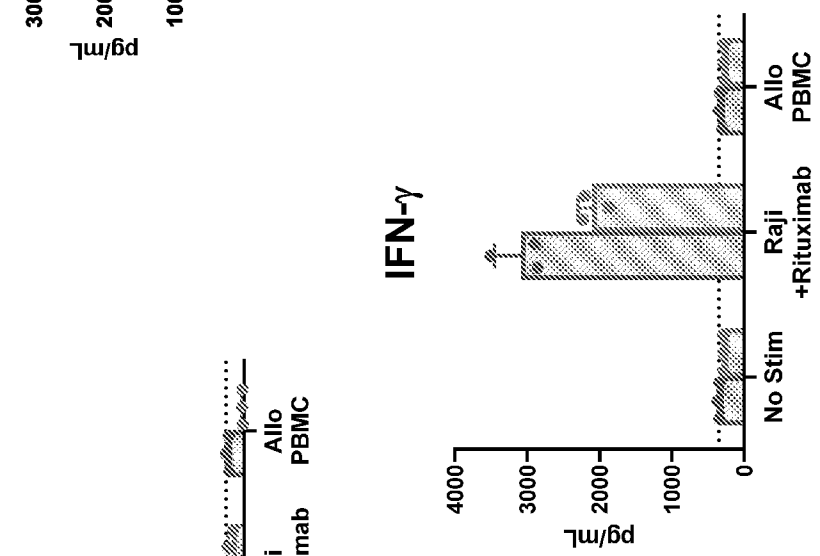
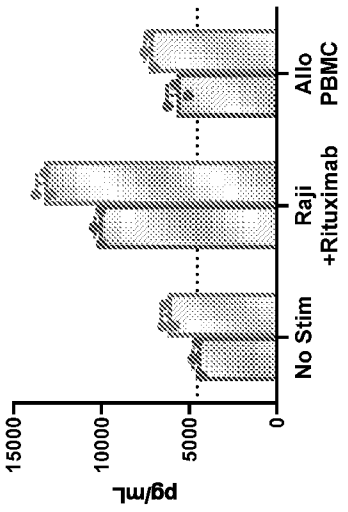
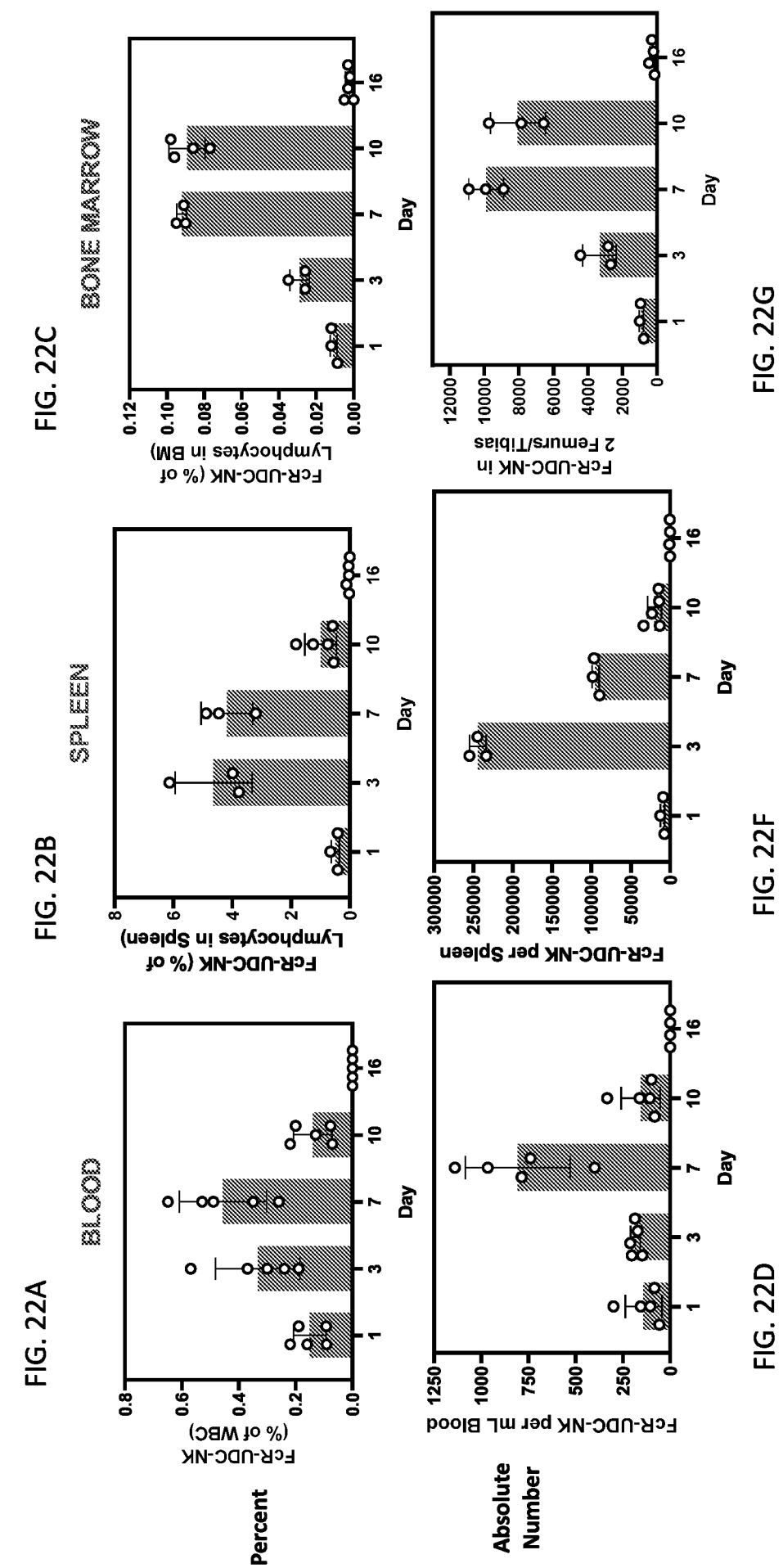


FIG. 21D




BLOOD
FcR-UDC-NK (% of WBC)
Percent
Day
FIG. 22A
SPLEEN
FcR-UDC-NK (% of Lymphocytes in Spleen)
Day
FIG. 22B
BONE MARROW
FcR-UDC-NK (% of Lymphocytes in BM)
Day
FIG. 22C
FcR-UDC-NK per mL Blood
Absolute Number
Day
FIG. 22D
FcR-UDC-NK per Spleen
Day
FIG. 22F
FcR-UDC-NK in 2 Femurs/Tibias
Day
FIG. 22G

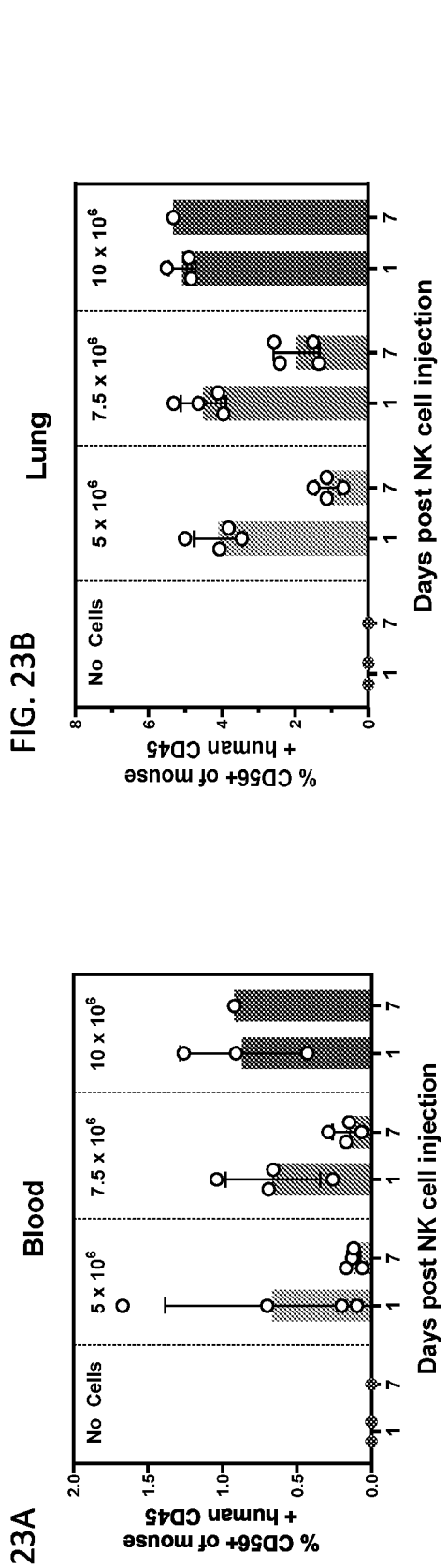


FIG. 23A

FIG. 23B

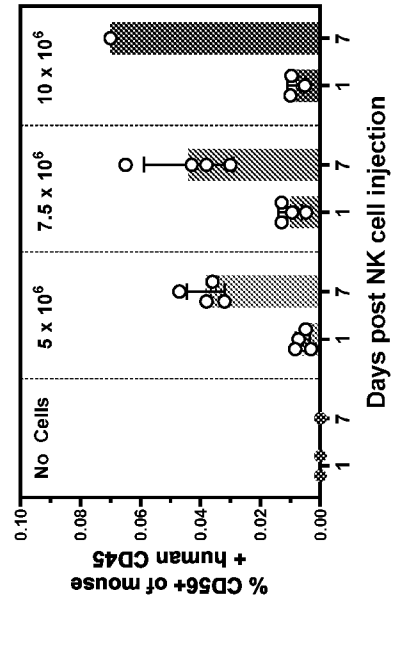


FIG. 23C

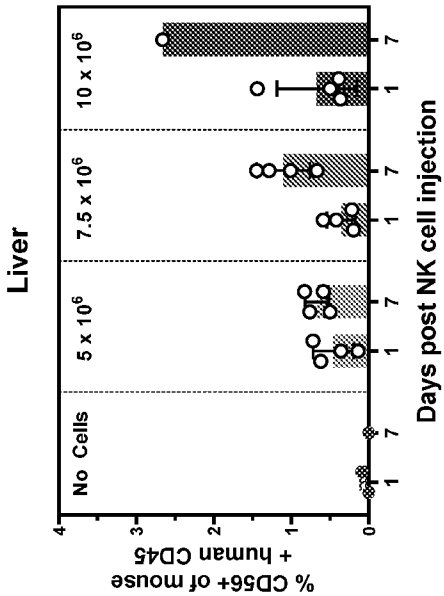


FIG. 23D

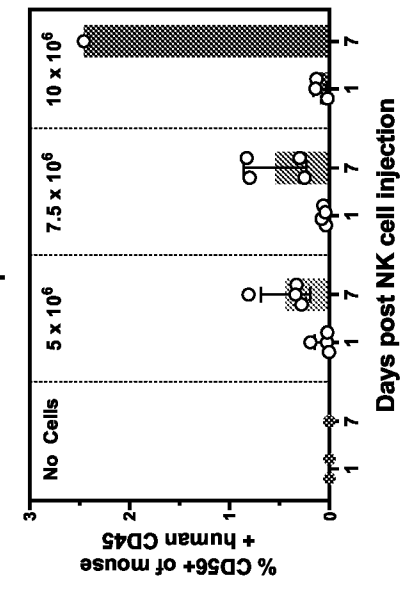


FIG. 23E

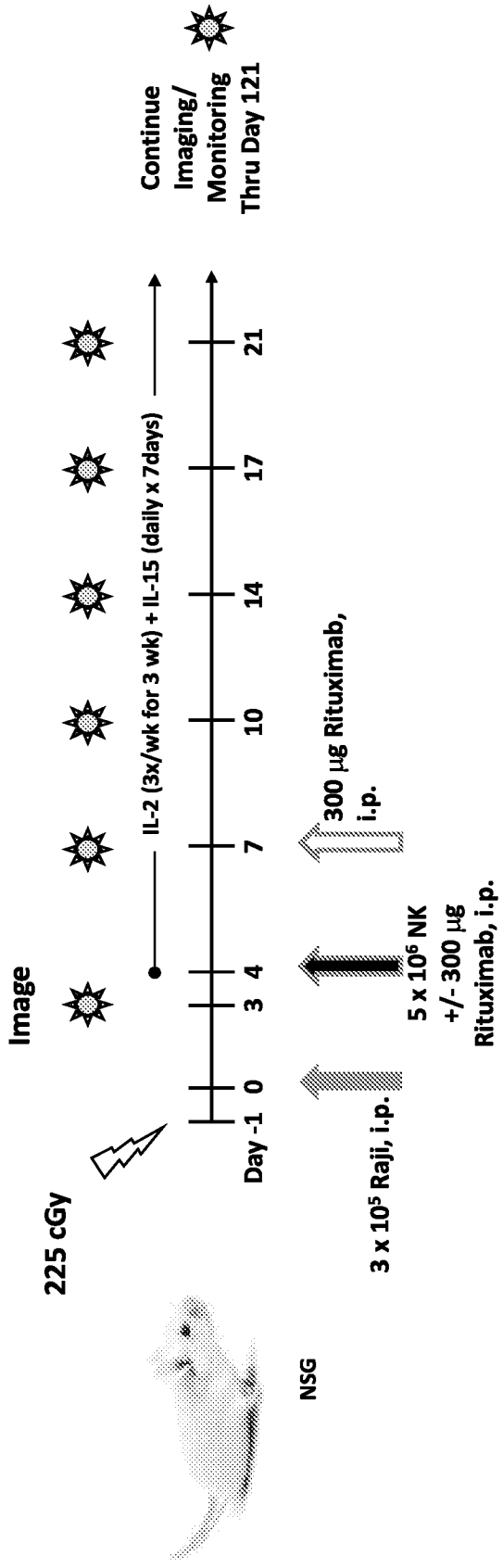


Image
225 cGy
IL-2 (3x/wk for 3 wk) + IL-15 (daily x 7days)
Continue Imaging/ Monitoring Thru Day 121
Day -1 0 3 4 7 10 14 17 21
3 x 10^5 Raji, i.p.
5 x 10^6 NK +/- 300 µg Rituximab, i.p.
300 µg Rituximab, i.p.
NSG

FIG. 24

FIG. 25A

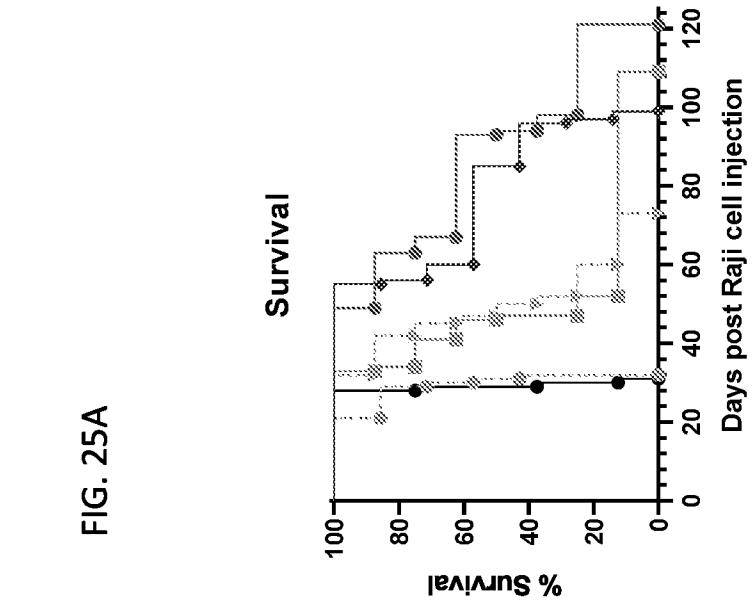


FIG. 25B

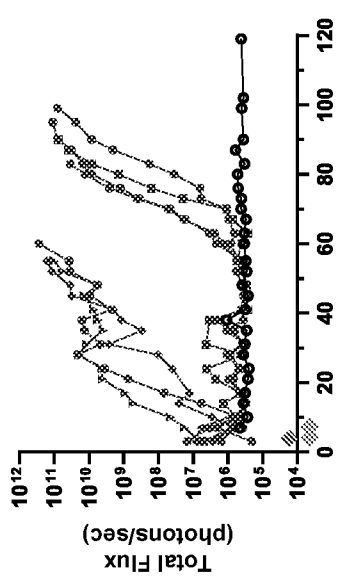
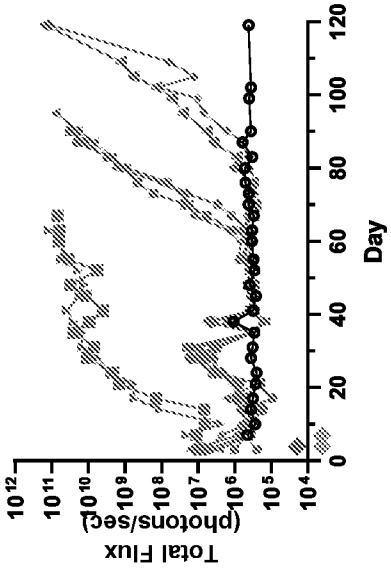


FIG. 25C



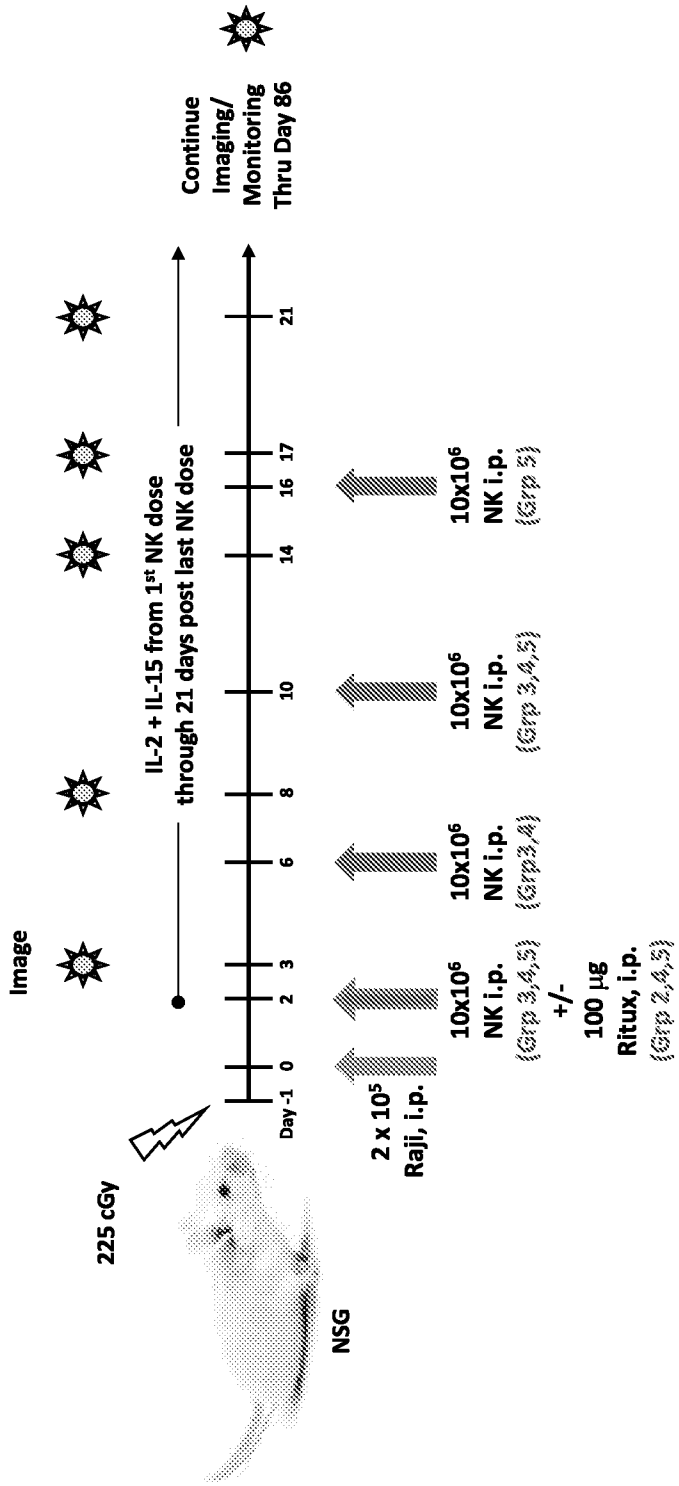


Image
225 cGy
NSG
Day -1
0
2
3
6
8
10
14
16
17
21
2 x 10^5 Raji, i.p.
10x10^6 NK i.p. (Grp 3,4,5)
+/- 100 µg Rituxi, i.p. (Grp 2,4,5)
10x10^6 NK i.p. (Grp3,4)
10x10^6 NK i.p. (Grp 3,4,5)
10x10^6 NK i.p. (Grp 5)
IL-2 + IL-15 from 1st NK dose through 21 days post last NK dose
Continue Imaging/ Monitoring Thru Day 86

FIG. 26

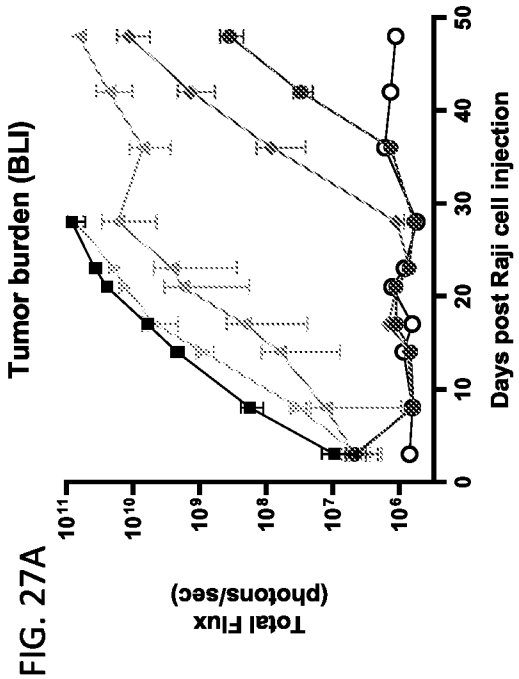


FIG. 27A

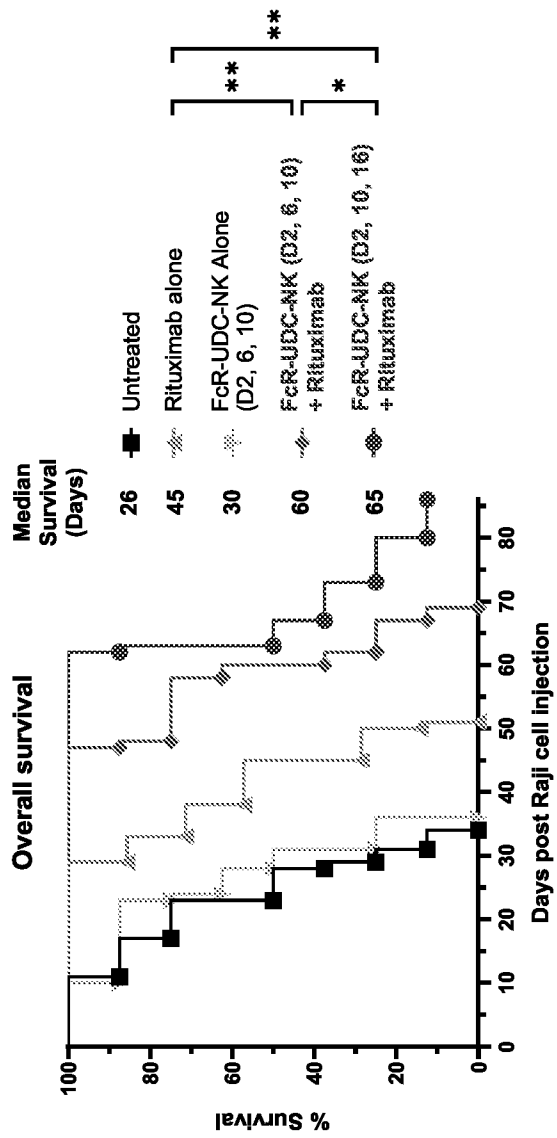


FIG. 27B

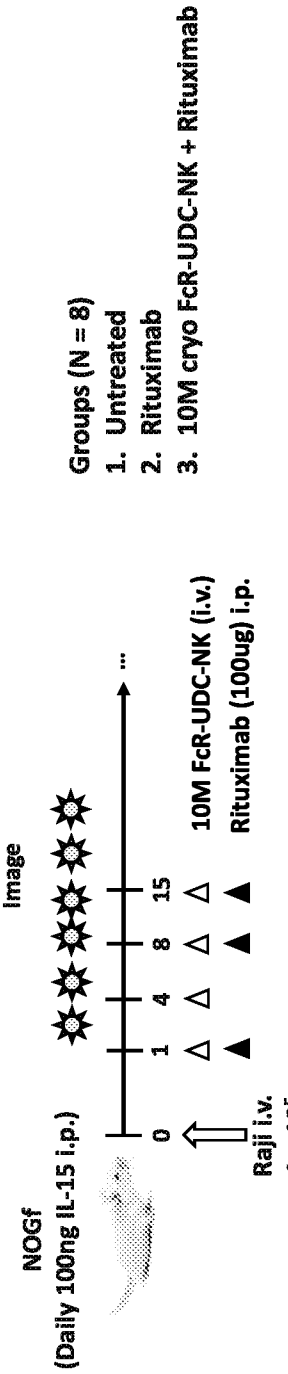


FIG. 28A

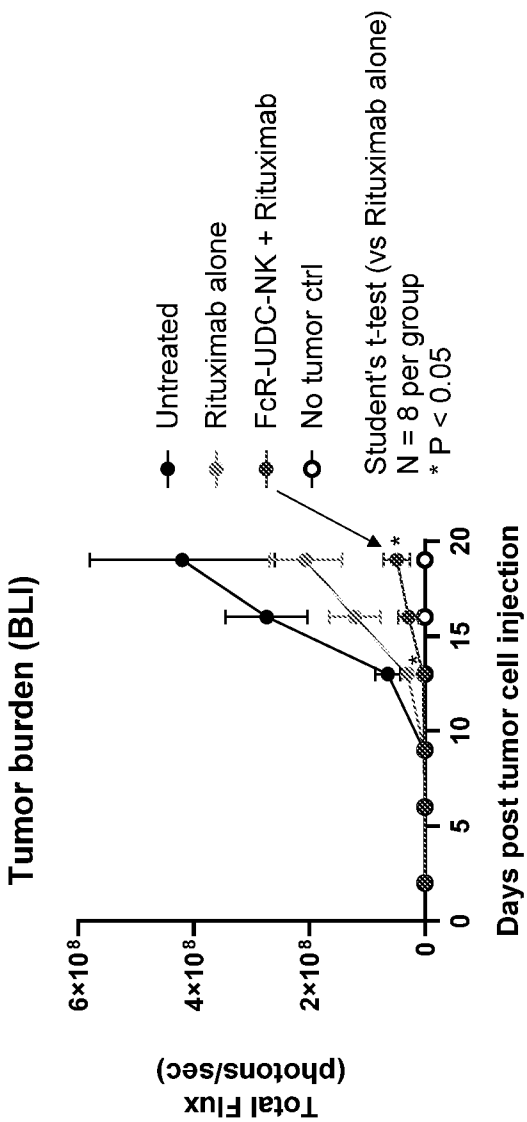


FIG. 28B

FIG. 29A

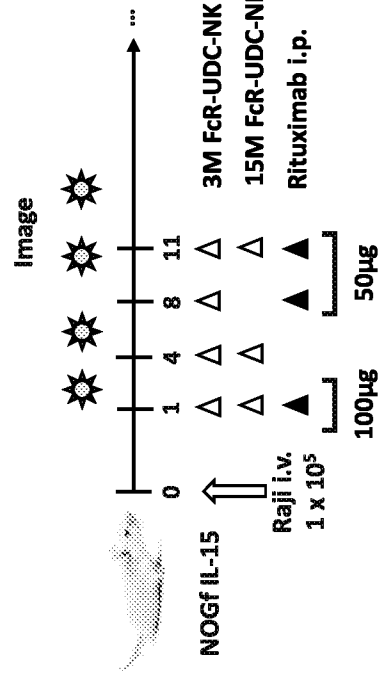
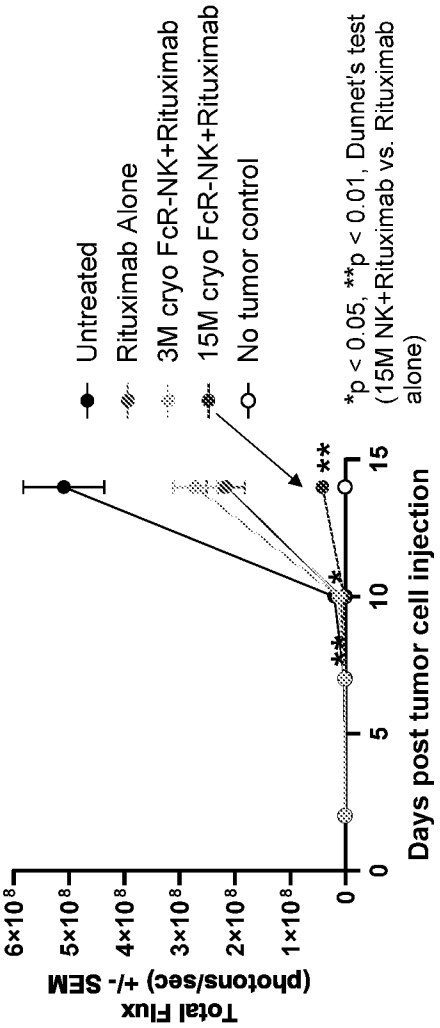


FIG. 29B



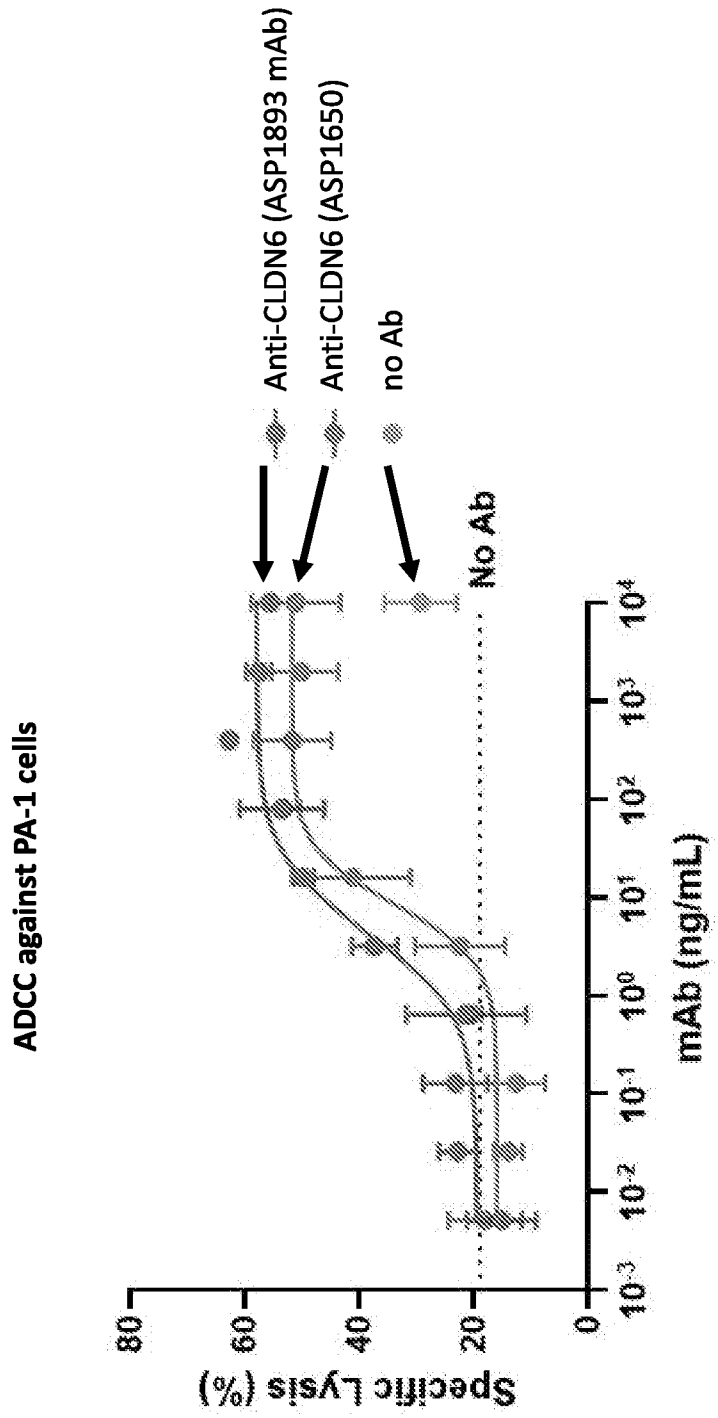

ADCC against PA-1 cells
Anti-CLDN6 (ASP1893 mAb)
Anti-CLDN6 (ASP1650)
no Ab
No Ab
Specific Lysis (%)
0
20
40
60
80
mAb (ng/mL)
10-3
10-2
10-1
100
101
102
103
104

FIG. 30

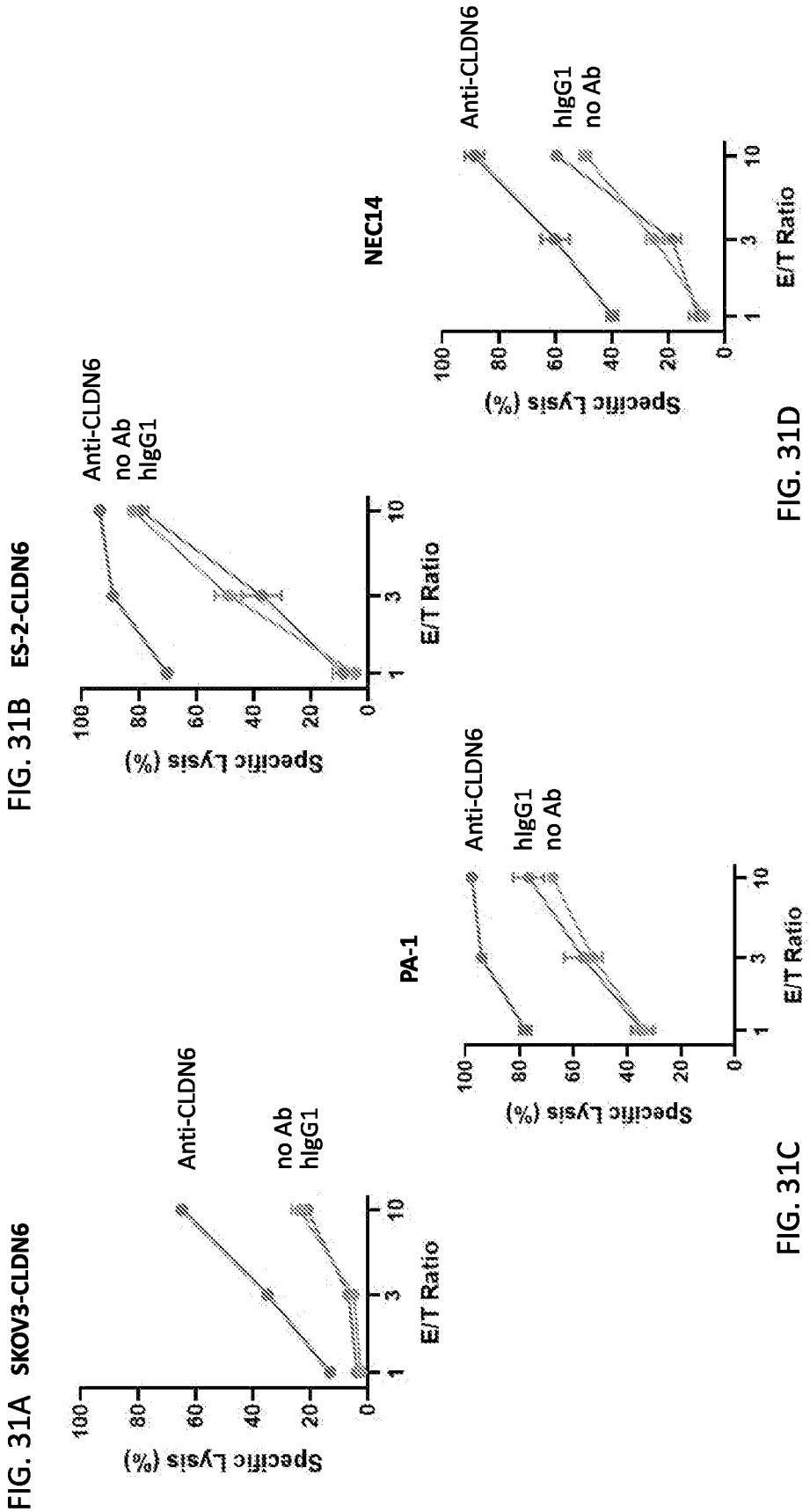


FIG. 31A

FIG. 31B

FIG. 31C

FIG. 31D

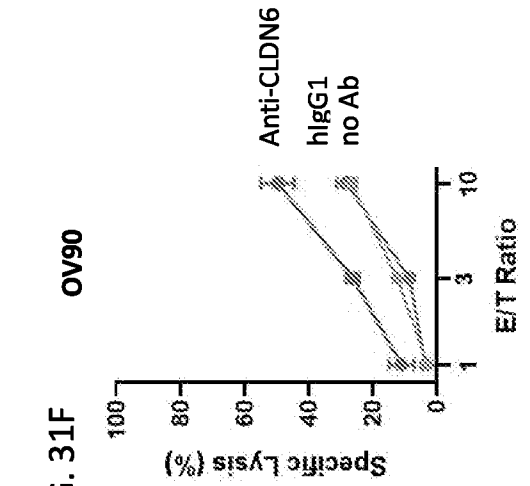


FIG. 31F

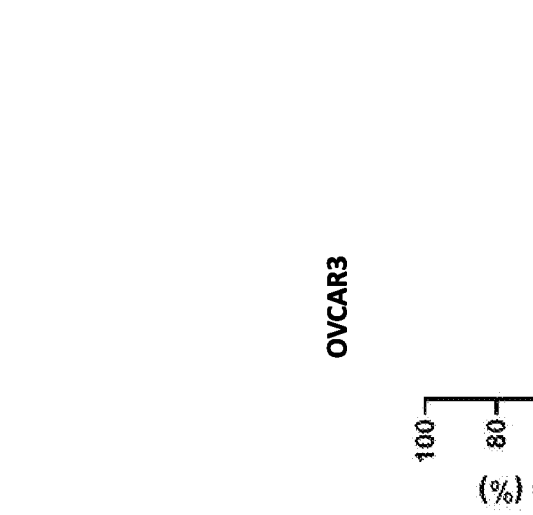


FIG. 31G

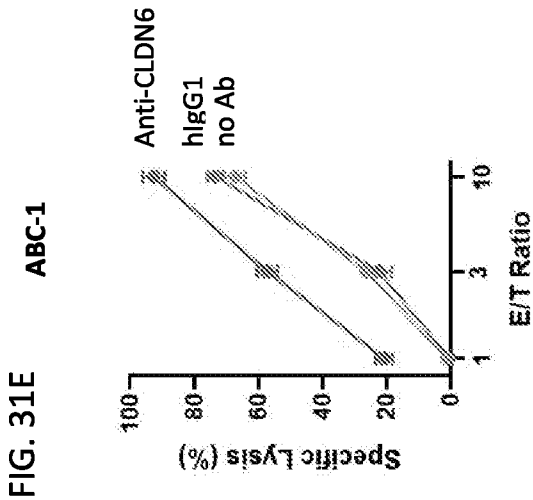


FIG. 31E

FIG. 31I

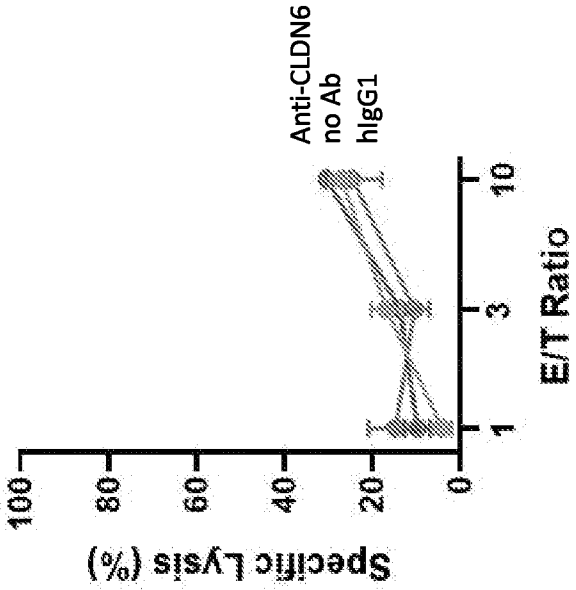
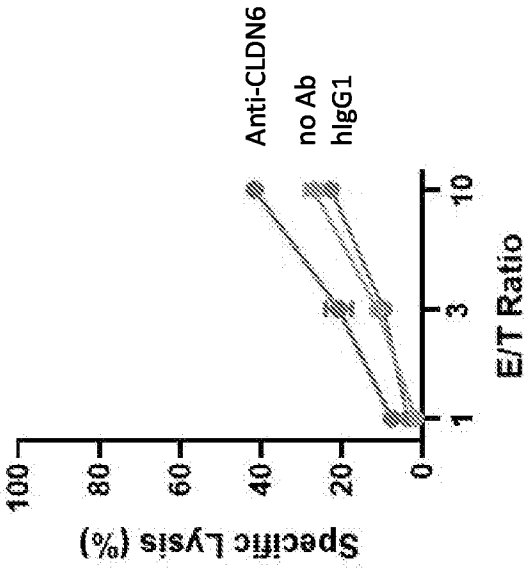


FIG. 31H



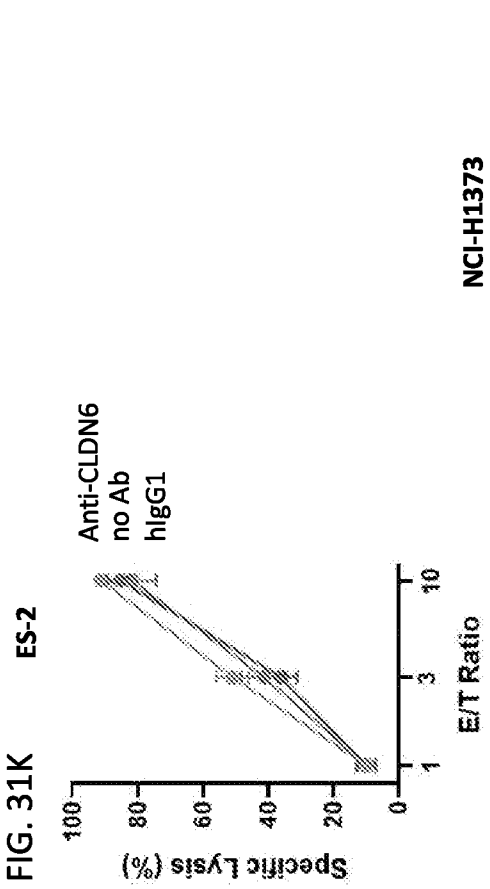


FIG. 31J

FIG. 31K

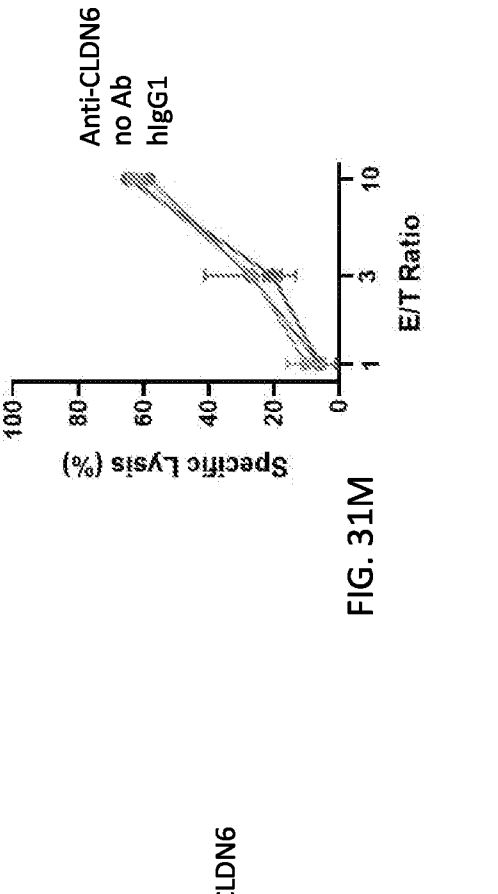


FIG. 31L

FIG. 31M

FIG. 32A

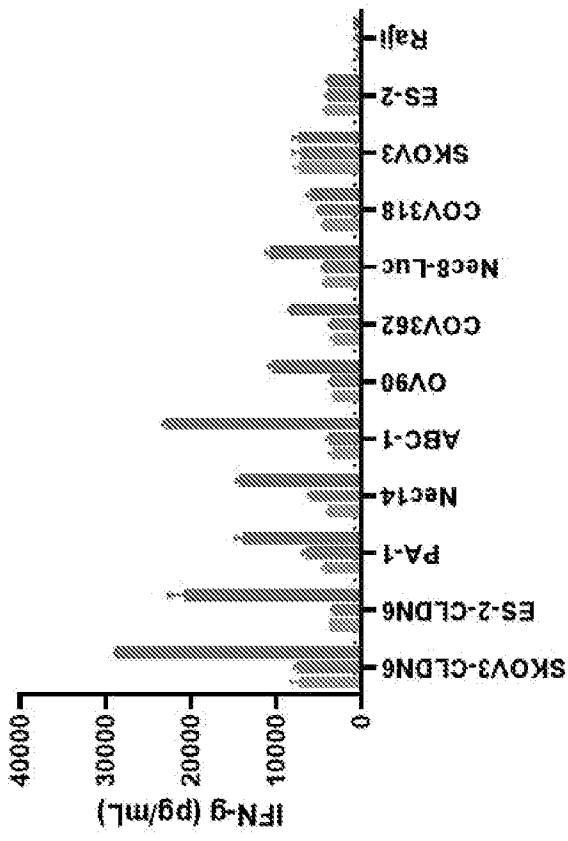


FIG. 32B

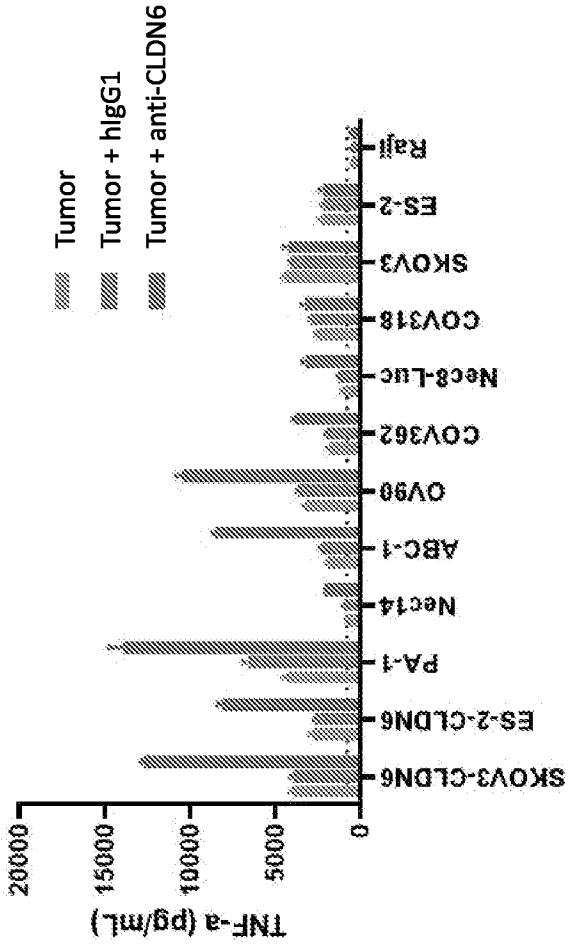


FIG. 33

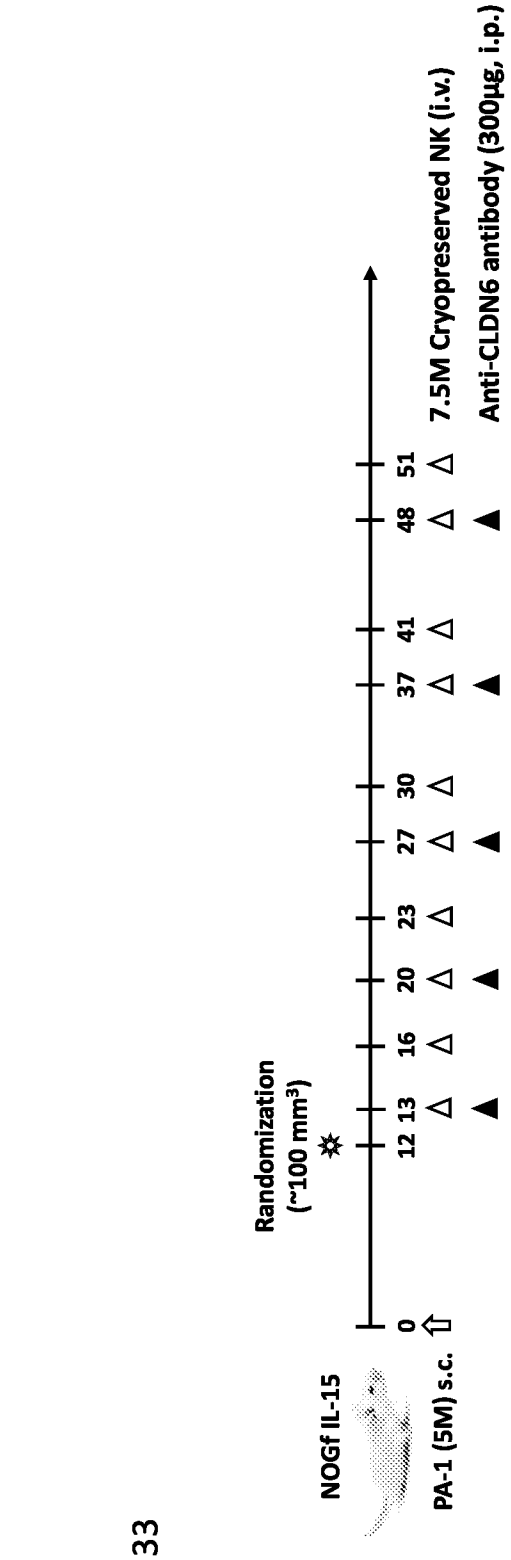

NOGf IL-15
PA-1 (5M) s.c.
0
Randomization
(~100 mm³)
12 13 16 20 23 27 30 37 41 48 51
7.5M Cryopreserved NK (i.v.)
Anti-CLDN6 antibody (300µg, i.p.)

FIG. 34A

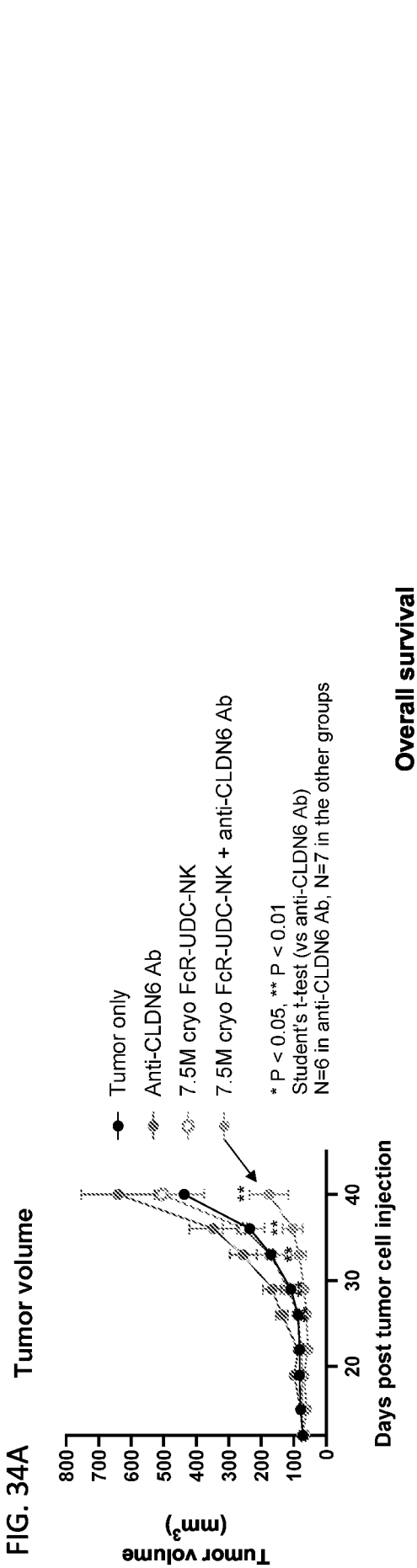


FIG. 34B

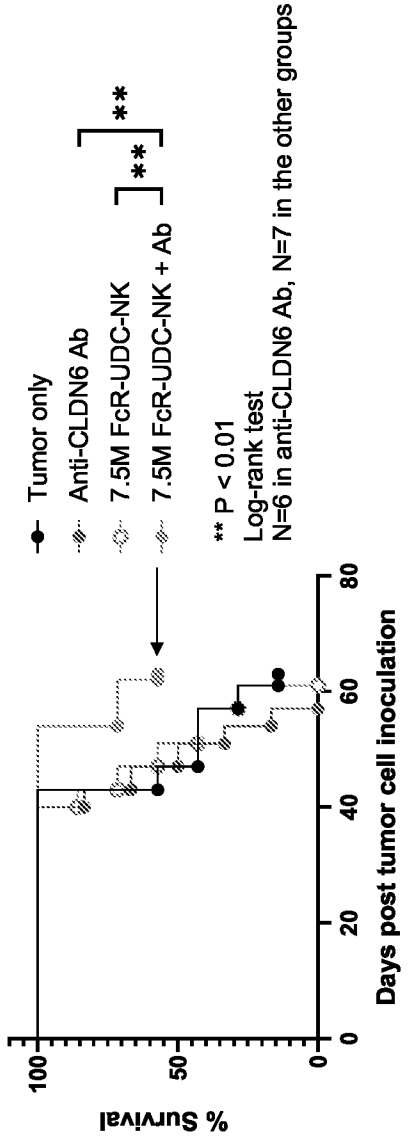


FIG. 35A

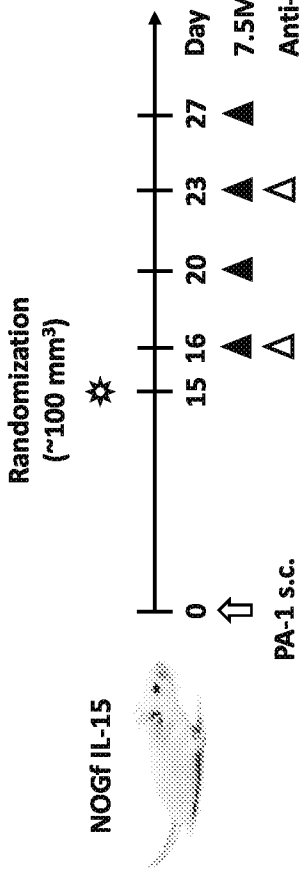


FIG. 35B

FIG. 35C

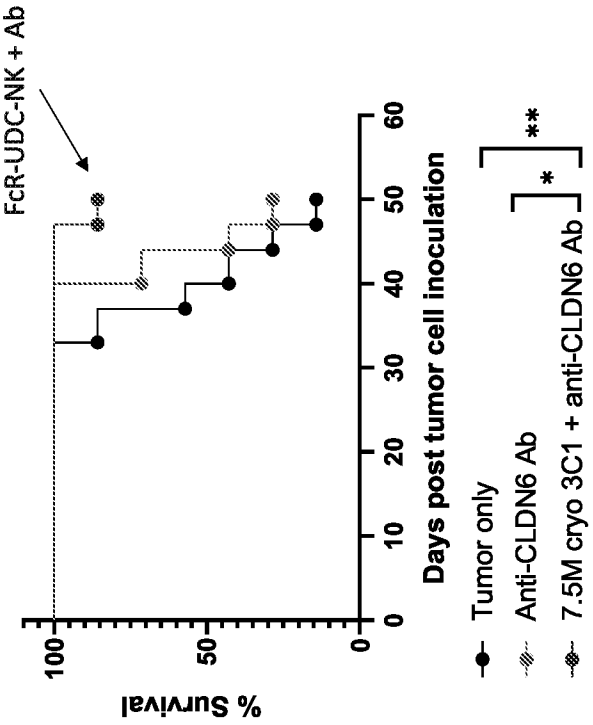


FIG. 36A

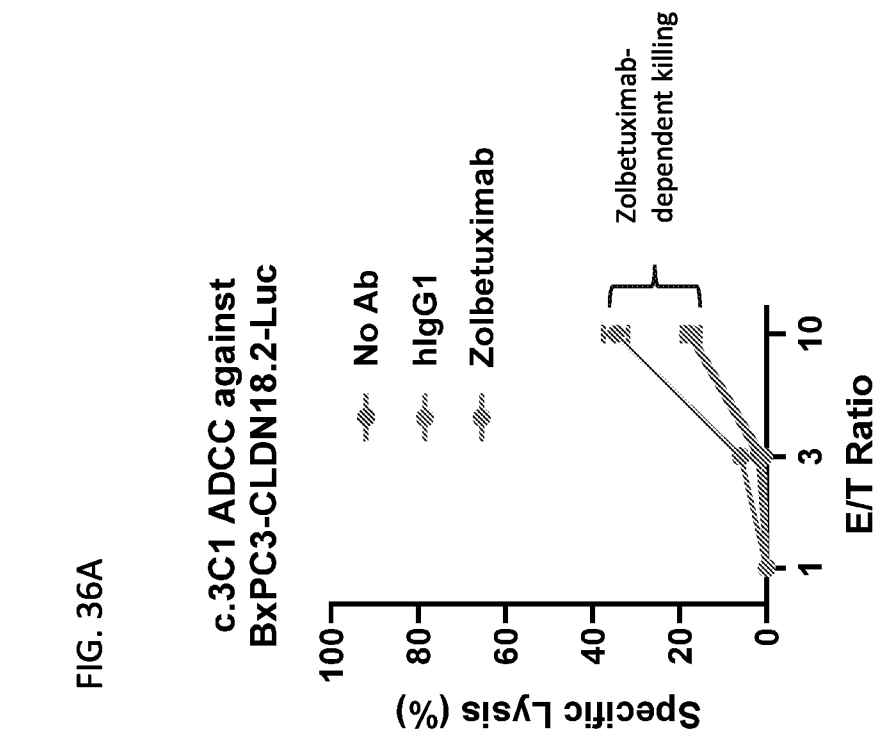


FIG. 36B

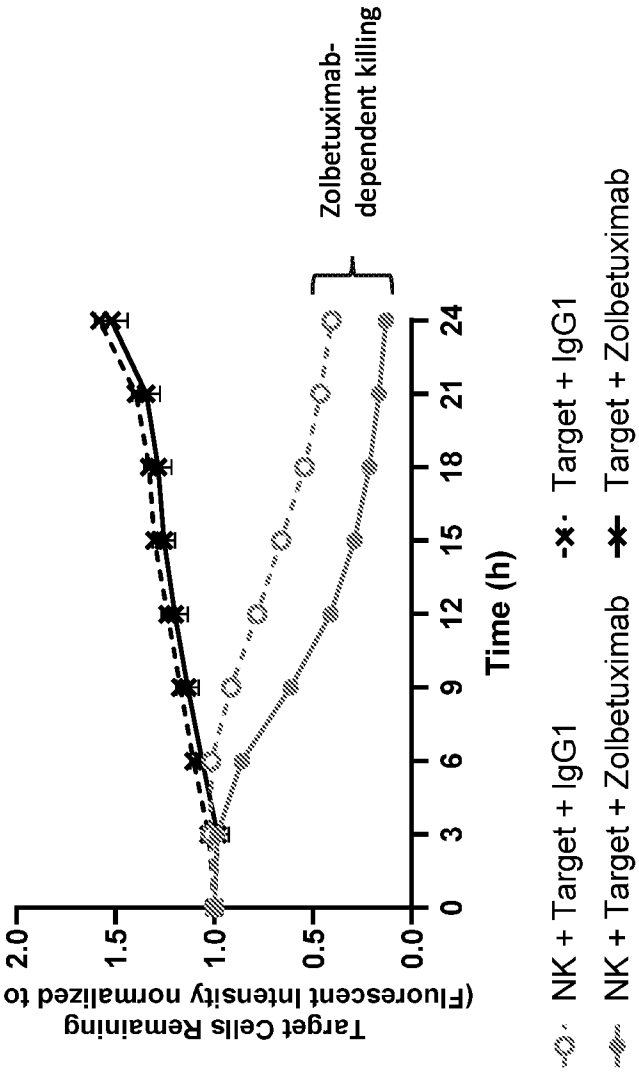


FIG. 37A

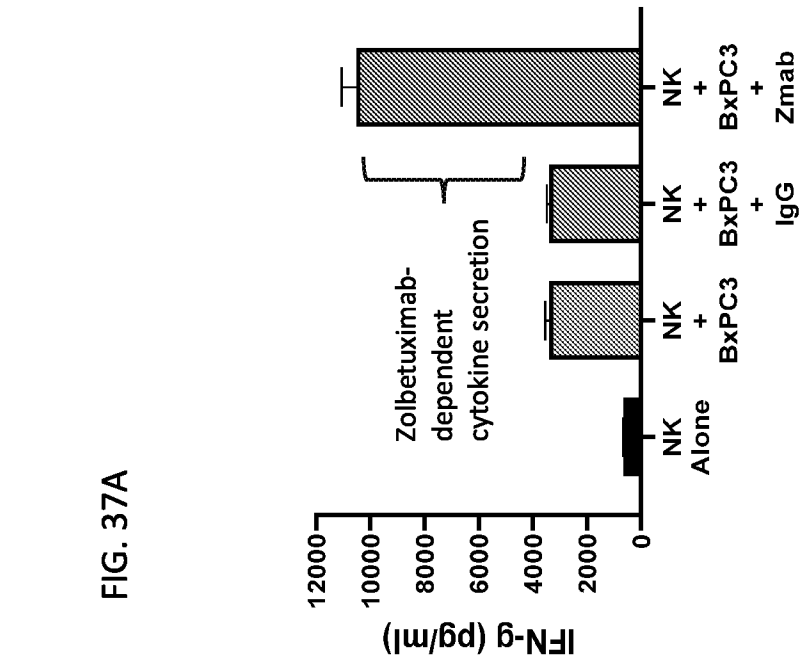
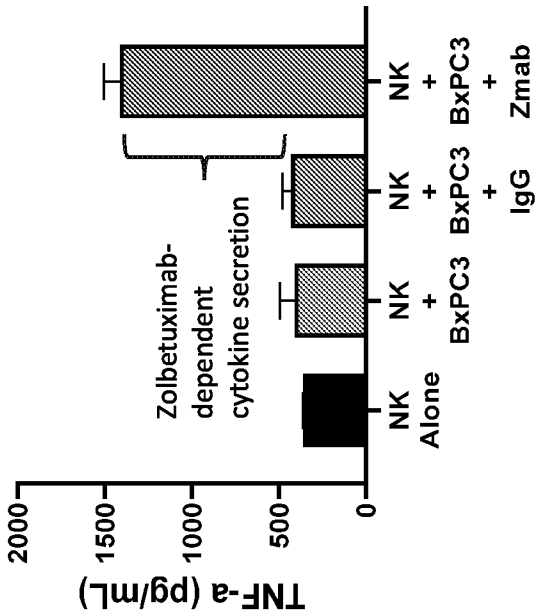


FIG. 37B



INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2023/063330

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0783 A61K35/17 A61K39/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2022/169700 A1 (KYRYCHENKO VIKTORIIA [US] ET AL) 2 June 2022 (2022-06-02) paragraph [0003] - paragraph [0006] paragraph [0127] paragraph [0179] paragraph [0332] - paragraph [0334] ----- -/--	1-13, 16-18, 20, 27-30, 32-39, 46-48

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

[x] See patent family annex.

* Special categories of cited documents :

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 2024	02/05/2024

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2023/063330

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GURUNG BUDDHA ET AL: "Empowering iPSC-derived iNK cells with multiple gene edits to improve persistence and anti-tumor efficacy", REGULAR AND YOUNG INVESTIGATOR AWARD ABSTRACTS, 1 November 2022 (2022-11-01), pages A280-A280, XP093151635, DOI: 10.1136/jitc-2022-SITC2022.0265 abstract -& GURUNG BUDDHA ET AL: "265?Empowering iPSC-derived iNK cells with multiple gene edits to improve persistence and anti-tumor efficacy", J. IMMUNOTHER. CANCER, vol. 10, no. 2, 7 November 2022 (2022-11-07), pages A280-A280, XP093074333, DOI: 10.1136/jitc-2022-SITC2022.0265 -----	1-13,18
Y	SARAH COOLEY: "First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia", BLOOD ADVANCES, vol. 3, no. 13, 9 July 2019 (2019-07-09), pages 1970-1980, XP093152223, ISSN: 2473-9529, DOI: 10.1182/bloodadvances.2018028332 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6616260/pdf/advances028332.pdf> page 1971, left-hand column; figures; tables -----	49,50
Y	DEMAGALHAES-SILVERMAN M ET AL: "Posttransplant adoptive immunotherapy with activated natural killer cells inpatients with metastatic breast cancer", JOURNAL OF IMMUNOTHERAPY, LIPPINCOTT WILLIAMS & WILKINS, US, vol. 23, no. 1, 1 January 2000 (2000-01-01), pages 154-160, XP009089991, ISSN: 1524-9557 page 155, right-hand column ----- -/--	49,50

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2023/063330

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GERHARD HAMILTON: "The Impact of NK Cell-Based Therapeutics for the Treatment of Lung Cancer for Biologics: Targets and Therapy", BIOLOGICS: TARGETS & THERAPY, vol. Volume 15, 1 July 2021 (2021-07-01), pages 265-277, XP093152196, US ISSN: 1177-5491, DOI: 10.2147/BTT.S290305 Retrieved from the Internet: URL:https://www.dovepress.com/getfile.php?fileID=71409> figure 2	49,50
Y	ZIQING CHEN: "Strategies to Augment Natural Killer (NK) Cell Activity against Solid Tumors", CANCERS, vol. 11, no. 7, 23 July 2019 (2019-07-23), page 1040, XP093152247, CH ISSN: 2072-6694, DOI: 10.3390/cancers11071040 table 1	49,50
X,P	WO 2023/215724 A1 (FACTOR BIOSCIENCE INC [US]) 9 November 2023 (2023-11-09) page 33, line 5 - line 10; claims; figure 43a page 30, line 1 - line 14	1-8, 16-18, 46,47
A	LANZA ROBERT ET AL: "Engineering universal cells that evade immune detection", NATURE REVIEWS IMMUNOLOGY, NATURE PUBLISHING GROUP UK, LONDON, vol. 19, no. 12, 15 August 2019 (2019-08-15), pages 723-733, XP036929449, ISSN: 1474-1733, DOI: 10.1038/S41577-019-0200-1 [retrieved on 2019-08-15]	1-55

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2023/063330

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2023/063330

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2022169700 A1	02-06-2022	EP 4251741 A1	04-10-2023
		TW 202237826 A	01-10-2022
		US 2022169700 A1	02-06-2022
		US 2022169988 A1	02-06-2022
		US 2023130564 A1	27-04-2023
		US 2023227532 A1	20-07-2023
		WO 2022113056 A1	02-06-2022
WO 2023215724 A1	09-11-2023	NONE	