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(54) Title: CELL LINE OF LYMPHOCYTES COMPRISING GAMMA-DELTA T CELLS, COMPOSITION AND PRODUCTION METHOD THEREOF

(57) Abstract: The present invention relates to a cell line of lymphocytes comprising γδ T cells, composition and production method thereof, and medical use namely for the use in medicine, namely in cancer immunotherapy. The cell line comprise a sample of human peripheral blood Vδ1$^+$ γδ T cells expressing functional natural cytotoxicity receptors (NCRs). These Vδ1$^+$ NCR$^+$ T lymphocytes can directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells. The present invention shows that human Vδ1$^+$ NCR$^+$ T cells can be differentiated and expanded from total γδ peripheral blood lymphocytes (PBLs), upon regular in vitro or ex vivo stimulation with γδTOK agonists and yc-family cytokines. This subset surprisingly expresses NKp30, NKp44 and NKp46, and high levels of Granzyme B that associate with highly enhanced cytotoxicity against lymphoid leukemias.

D E S C R I P T I O N

"CELL LINE OF LYMPHOCYTES COMPRISING GAMMA-DELTA T CELLS, COMPOSITION AND PRODUCTION METHOD THEREOF"

Technical field of the invention

The invention relates to a cell line of lymphocytes comprising γδ T cells, composition and production method thereof, and medical use namely for the use in medicine, namely in cancer immunotherapy.

Background

Tumors develop in hosts endowed with a highly complex immune system that includes various lymphocyte subsets capable of recognizing and destroying transformed cells. It is now widely accepted that, while lymphocytes may constantly patrol tumor formation, cancer cells develop molecular strategies to evade immune surveillance, which are competitively selected under the pressure of the host immune system. This dynamic process, termed "cancer immunoediting", is thought to constitute a major obstacle to cancer immunotherapy.

Among multiple immune evasion mechanisms, it was recently shown that leukemia and lymphoma primary samples often down-regulate the non-classical MHC (major histocompatibility complex) protein, ULBP1, which is critical for recognition of hematological tumors by γδ T-cells expressing the counter-receptor NKG2D (Lanca, T. *et al.*, "The MHC class Ib protein ULBP1 is a nonredundant

determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity", *Blood* 115:2407-2411; 2010).

γδ T-cells are innate-like lymphocytes that account for 1-10% of peripheral blood lymphocytes (PBL) of healthy individuals and are capable of targeting a significant fraction of hematological tumor cell lines tested in the laboratory. However, it was demonstrated that many lymphoid leukemia primary samples are resistant to fully-activated Vγ9Vδ2 T-cells (Lanca, T. *et al.*, "The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity", *Blood* 115:2407-2411, 2010 ; Gomes, A.Q. *et al.*, "Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells", *Haematologica* 95:1397-1404, 2010), the dominant subset of γδ PBLs. Furthermore, clinical trials involving the *in vivo* administration of activators of Vγ9Vδ2 T-cells have shown limited success, with objective responses restricted to 10-33% of patients with either hematological or solid tumors. Even more modest has been the outcome of trials involving the adoptive transfer of activated/ expanded $V\delta2^+$ cells, since no objective responses have been reported. In fact, the simple *ex vivo* expansion of autologous $V\delta2^+$ T-cells, whose surveillance the tumor managed to escape *in vivo*, may be condemned to little therapeutic effect upon re-injection into the patient.

Cancer immunotherapy relies on tumor cell recognition by cytotoxic lymphocytes. γδ T-cells are a population of MHC-unrestricted killer lymphocytes that play critical roles in

various animal tumor models. This notwithstanding, it was showed that a large proportion of human hematological tumors is resistant to γδ peripheral blood lymphocytes (PBLs) activated with specific agonists to the highly prevalent Vγ9Vδ2 T-cell receptor (TCR). This likely constitutes an important limitation to current γδ-T-cell-mediated immunotherapy.

Therefore, it is critical to invest in strategies that endow γδ T-cells with additional recognition machinery to detect tumors that have resisted the natural components present *in vivo*.

Natural cytotoxicity receptors were identified by A. Moretta and co-workers over a decade ago, and were shown to play critical synergistic roles in the anti-tumor functions of Natural Killer (NK) cells. In fact, NKp30 and NKp46 are widely considered to be two of the most specific NK cell markers.

The document written by von Lilienfeld-Toal, M., J. Nattermann, et al. (2006). "Activated gammadelta T cells express the natural cytotoxicity receptor natural killer p 44 and show cytotoxic activity against myeloma cells." Clin Exp Immunol 144(3): 528-533. discloses the expression of a NK receptor on peripheral blood γδ T lymphocytes. However, disclosed γδ T cells are different from those stated in the present invention. The document also reveals, contrary to the present invention, that NKp30 is not expressed in such γδ T cells. Some of differences are:

- The treatment (IFN-γ, TNF-α + anti-CD3 monoclonal antibody + IL-1β + IL-2 + IL-15);

- The phenotype of γδ T cells (NKp30$^-$ and NKp46$^-$; moreover,62% of γδ T cells belong to the Vδ2$^+$ subtype;
- Less than 20% of the γδ T cells are CD56$^+$;
- Less than 20% of the γδ T cells are CD8$^+$;
- The expression levels of NKp44 are not modulated by stimulation of the γδTCR/CD3 complex (thus, different molecular mechanisms are involved that induce NKp44 expression on those cells)

This document describes peripheral blood γδ T cells expressing NKp44. NKp44 was functional and was involved in the recognition and elimination of tumor cells (myeloma cells). However, only 8 ± 7% of γδ T cells expressed NKp44 and most (62%) of γδ T cells belonged to the Vδ2$^+$ subtype (they were Vδ1$^-$). NKp30 and NKp46 were not expressed by γδ T cells, and less than 20% of these cells were CD8$^+$ or CD56$^+$.

Thus, the γδ T cell line described is totally different from our identified γδ T cell line: typically more than 95% of γδ T cells in our cell line belong to the Vδ1$^+$ subtype and a high percentage of these Vδ1$^+$ γδ T cells (typically more than 30%) express functional NKp44. Importantly, NKp44 demonstrate synergism with NKp30 to greatly enhance the ability to recognize and kill tumor cells (Figure 5B). This cooperation between NCR is critical to obtain the desired effect (elimination of cancer disease), and is absent in the previously identified NKp44$^+$ γδ T cells.

The document written by Rey, J., C. Veuillen, et al. (2009). "Natural killer and gammadelta T cells in haematological malignancies: enhancing the immune effectors." Trends Mol Med **15**(6): 275-284. discloses

the common expression of the NKG2D receptor on NK cells and on γδ T cells. This document also states that the expansion and activation of γδ T cells can generate anti-tumor responses. However, contrary to the present invention the γδ cells are $NKp30^-$ and $NKp46^-$.

The document WO 00/44893 (Palmetto Richland Memorial Hos) discloses a method of treatment of leukemia based on the administration of substantially purified γδ T lymphocytes. The method comprises the preparation of lymphocyte activation and expansion thereof. The main differences are:

- The treatment (plate-bound immobilized anti-TCR antibodies + irradiated "feeder" tumor B-cells);
- The γδ T cells ($NKp30^-$, $NKp44^-$, $NKp46^-$, $CD8^-$).

The document WO 2011/053750 (Emory University) discloses a method of reducing cancer in a patient, comprising the steps of isolating a population of cytotoxic cells, like γδ T cells; administration of the therapeutic agent and the cytotoxic cells. The main difference to present invention is that γδ T cells are genetically modified to resist chemotherapeutic agents.

Summary

The present invention relates to a cell line of peripheral blood lymphocytes comprising or consisting of $V\delta1^+$ γδ T cells expressing functional natural cytotoxicity receptors

(NCR). In a more preferred embodiment, the natural citotoxicity receptors comprise or consist of NKp30.

In a more preferred embodiment, the cell line disclosed could further comprise $V\delta2^+$ $\gamma\delta$ T cells.

Another more preferred embodiment of the cell line disclosed, the natural cytotoxicity receptors could further comprise NKp44, NKp46, or mixtures thereof.

Another preferred embodiment of the cell line disclosed, the cell line could express granzime B.

The disclosed subject matter further includes a composition with the said cell line. In a preferred embodiment, the composition is an injectable. In a preferred embodiment the injectable composition comprises a cell population composed of more than 80%, namely more than 80%, 85%, 90%, 95%, of functional $V\delta1^+$ $\gamma\delta$ T cells expressing functional natural cytotoxicity receptors, more preferably the natural citotoxicity receptors comprise or consist of NKp30 and, wherein it comprises more than 100 million of $V\delta1^+$ $\gamma\delta$ T cells expressing functional natural cytotoxicity receptors. Preferably the composition also comprises a pharmaceutically acceptable agent or carrier and, more preferably, a stabilizing agent, namely as human serum albumin. The cells are preferably autologous, that is to say, derived from a same biological preparation (or from a same donor). More preferably, they are obtained by a method such as method described by disclosed subject matter.

Another aspect of the disclosed subject matter is the use in medicine of composition that comprises cells of the cell line disclosed in the present invention.

In a more preferred embodiment, the composition disclosed in the present invention could be used in autologous or heterologous adoptive cell therapy, tumor or cancer treatment, tumor or cancer immunotherapy, and/or leukemia treatment, or for treatment of acute lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, multiple myeloma, burkitt's lymphoma, follicular lymphoma, breast carcinoma, lung carcinoma, prostate carcinoma, colon carcinoma, bladder carcinoma, renal cell carcinoma, or skin melanoma, among others.

In a more preferred embodiment, the composition disclosed in the present invention could be used in the treatment of a viral infection. Namely, when the virus treated is from the family *Herpesviridae* or *Retroviridae,* among others.

The disclosed subject matter further includes a method of producing the cell line described which comprises isolating γδ PBLs. In a preferred embodiment, the starting sample could be blood including peripheral blood sample or fractions thereof, including buffy coat cells, mononuclear cells and low density mononuclear cells. The cells may be obtained from a starting sample of blood using techniques known in the art such as density gradient centrifugation. Total γδ PBLs can be isolated via positive selection with magnetic-labeled anti-TCR$\gamma\delta^{+}$ antibodies or through depletion/elimination of TCR$\gamma\delta^{(-)}$ cells. γδ PBL can be maintained in any suitable mamalian cell culture medium such as RPMI 1640 or IMDM.

The disclosed subject matter further includes a method of cultivating these cells in an adequate culture medium in the presence of γδTCR agonists, preferably by regular addition (more preferably continuous adition) of said agonists, preferably soluble or immobilized, and in the presence of at least one γc-cytokine, preferably by regular addition (more preferably continuous adition) of said cytokine or cytokines. The said γδTCR agonists and γc-cytokines are added to said culture of cells at the time of the culturing and also throughout the culturing period, preferably every 3-6 days, so that the concentration of γδTCR agonists and γc-cytokines in the said culture is always typically more than zero.

In a preferred embodiment, the addition of said γδTCR agonists and γc-cytokines could be carried out until at least 40% of the cells express natural cytotoxicity receptors, more preferably at least 50%, 60%, 70%, 75%, 80%, 85%, 95%. In a more preferred embodiment, the addition of said γδTCR agonists and γc-cytokines could be carried out until it is achieved more than 50 million, more than 100 million, more than 200 million of viable and functional cells that express natural cytotoxicity receptors, namely the natural citotoxicity receptors comprise or consist of NKp30.

In a more preferred embodiment of the disclosed method, the addition of said γδTCR agonists and γc-cytokines could be carried out until at least 40% of the cells express NKp30,

more preferably at least 50%, 60%, 70%, 75%, 80%, 85%, 95%, 100%.

In other preferred embodiment of the disclosed method, the γδTCR agonist can be any soluble or immobilized molecule or compound that is able to activate, stimulate or trigger the γδTCR/CD3 receptor complex expressed in all $V\delta1^+$ γδ PBL, including, but not limited to, plant lectins (including phytohemagglutinin-PHA), anti-CD3 monoclonal antibodies (including OKT3 mAb), anti-γδTCR monoclonal antibodies or mixtures thereof. Other agonistic antibodies to the $V\delta1^+$ γδTCR can be used. The term "antibodies" include monoclonal antibodies, polyclonal antibodies, antibody fragments, single chain antibodies, single chain variable fragments and recombinantly produced binding partners. More preferably, the range of γδTCR agonist concentration could vary between 0,01 - 100 µg/ml, even more preferably between 1-5 µg/ml.

In a more preferred embodiment of the disclosed method the said "γc-cytokine" means common cytokine receptor gamma-chain family of cytokines, preferably interleukin, namely IL-2, IL-4, IL-7, IL-9, IL-12, IL-15, IL-21 or mixtures thereof, among others. The interleukin used may be of human or animal origin, preferably of human origin. It may be a wild-type protein or any biologically active fragment or variant, that is, to say, capable of binding its receptor and inducing activation of γδ T cells in the conditions of the method according to the invention. More preferably, the cytokines may be in soluble form, fusioned or complexed with another molecule, such as for example a peptide,polypeptide or biologically active protein. Preferably, a human recombinant γc cytokine is used. More

preferably, the range of interleukin concentration could vary between 1-10000 U/ml, even more preferably between 100-1000 U/ml.

In other preferred embodiment of the disclosed method the regular addition of said γδTCR agonists and γc-cytokines could be performed for 2-60 days, more preferably between 9-25 days, even more preferably between 10-15 days, namely 11, 12, 13, 14 days.

General Description of the invention

The present invention describes a cell line of peripheral blood lymphocytes comprising γδ T cells, composition and production method thereof, preferentially expressing NKp30, NKp44 and NKp46, for use in medicine, namely in cancer immunotherapy.

The present invention discloses a novel subset of $V\delta2^{(-)}$ $V\delta1^{+}$ γδ PBLs expressing natural cytotoxicity receptors (NCRs) that directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells. Is shown that NCR^{+} $V\delta1^{+}$ T-cells can be differentiated and expanded from total γδ PBL upon stimulation with γc-cytokines and pan-TCR agonists, through a process that requires functional PI-3K/ AKT signaling. Surprisingly, this subset stably expresses NKp30, NKp44 and NKp46, and high levels of Granzyme B that associates with enhanced cytotoxicity against lymphoid leukemia cells. Specific gain-of-function and loss-of-function experiments demonstrate that NKp30 and NKp44, but not NKp46, play non-redundant and synergistic roles in TCR-independent leukemia cell recognition. Thus, NCR^{+} $V\delta1^{+}$ T-cells constitute an

inducible and specialized killer lymphocyte population for adoptive cell immunotherapy of human cancer.

Description of the figures

The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of invention.

Figure 1a, 1b, 1c- Enhanced anti-leukemia cytotoxicity of γδ PBL cultures activated with pan-T-cell mitogen. (A) γδ peripheral blood lymphocytes (γδ PBLs) were MACS-sorted from the peripheral blood of healthy volunteers (left panel), and stimulated with either HMB-PP/ IL-2 or PHA/ IL-2 for 4-19 days. Activation was evaluated by flow cytometry for CD69 upregulation (middle panels; levels in freshly-isolated control cells are shaded) and CFSE dilution (right panels; dotted line indicates initial CFSE levels). (B-C) Pre-activated (for 14 days, as in A) γδ PBL were co-incubated with DDAOse-labeled leukemia cells for 3 hours. Tumor cell lysis was evaluated by Annexin-V staining using flow cytometry. (B) Representative results of 6 different donors for the Bv173 leukemia cell line. Percentages refer to Annexin-V^+ tumor cells. Basal tumor cell apoptosis (in the absence of γδ PBL) was <5%. (C) Summary of the results of 6 different donors with 4 leukemia target cell lines. Error bars represent SD (n=6, *p<0.05; **p<0.01). (D) Real-time PCR quantification of GzmB mRNA levels in freshly-isolated, HMB-PP/ IL-2-activated and PHA/ IL-2-activated γδ PBL. Data in this figure are representative of 2-3 independent experiments with similar results.

Figure 2 a, 2b, 2c – Induction of Natural Cytotoxicity Receptor expression in γδ PBLs activated with pan-T-cell mitogen. γδ PBLs were cultured as described in Figure 1 for 4-19 days, and analyzed by flow cytometry for surface expression of various NK receptors. (A) Results for NKG2D, 2B4 and DNAM-1 in 10-day cultures activated either with HMB-PP/ IL-2 (grey) or PHA/ IL-2 (black), derived from 6 independent healthy donors. Error bars represent SD (n=6; $p>0.05$). (B) Expression of NKp30 in the same cultures of (A). FACS plots correspond to cultures derived from 3 individual donors. Percentages refer to $NKp30^{+}$ γδ PBLs. Isotype control staining is presented in Figure 7. (C-D) Real-time PCR quantification of Nkp44 (C) and Nkp46 (D) mRNA levels in freshly-isolated, HMB-PP/ IL-2-activated and PHA/ IL-2-activated γδ PBL. (E) Evolution of the percentage of $NKp30^{+}$ cells in the cultures described in (A), analyzed up to day 19. Error bars represent SD (n=5). (F) Induction of NKp30 expression in total γδ PBL or in FACS-sorted (>99% pure) $NKp30^{(-)}$ γδ PBL from PHA/ IL-2-activated γδ PBL cultures (as in B). Cells were stimulated with PHA/ IL-2 for 14 days. Data in this figure are representative of 2-4 independent experiments with similar results.

Figure 3a, 3b – Natural Cytotoxicity Receptors are selectively expressed on proliferating $V\delta1^{+}$ T-cells. (A) γδ PBLs were labeled with CFSE and cultured as described in Figure 1, or in the absence of T cell mitogens (i.e., IL-2 alone). Flow cytometry analysis of CFSE dilution and Vδ2 TCR expression after 7 days in culture. (B) Percentage of $V\delta1^{+}$ or $V\delta2^{+}$ cells among total γδ PBLs cultured up to 19 days with PHA/ IL-2. Error bars represent SD (n=3). (C)

NKp30 expression in PHA/ IL-2-activated γδ PBL subsets. Vδ1$^+$ or Vδ2$^+$ cells were FACS-sorted from peripheral blood, labeled with CFSE and cultured with PHA/ IL-2 for 7 days. Percentages refer to NKp30$^+$ cells within each cell division (according to CFSE levels and indicated by vertical rectangles). (D) Expression of NKp30, NKp44 and NKp46 in Vδ1$^+$ T-cells after 19 days of PHA/ IL-2 stimulation. Isotype mAb control stainings are also shown. Data in this figure are representative of 2-3 independent experiments with similar results.

Figure 4a, 4b – AKT-dependent γc cytokine and TCR signals induce NKp30 expression in Vδ1$^+$ T-cells. (A-B) Flow cytometry analysis of NKp30 expression on pre-gated Vδ1$^+$ T-cells from γδ PBL cultures after 7 days in the presence of IL-2 or IL-15, alone or in combination with PHA or OKT3 (anti-CD3 mAb). (C) Effect of blocking anti-TCRγδ mAb on NKp30 induction in PHA/ IL-2-activated γδ PBLs. Shaded grey are pre-gated NKp30$^+$ cells in 7-day control cultures. (D) Effect of chemical inhibitors LY294002 and UO126 on NKp30 induction in PHA/ IL-2-activated γδ PBLs, pre-labeled with CFSE. Data in this figure are representative of 2-3 independent experiments with similar results.

Figure 5 - NKp30 and NKp44 mediate tumor cell killing by NCR$^+$ γδ PBLs. (A) Functional evaluation of NKp30, NKp44 and NKp46 using specific monoclonal antibodies in a 4-hour ^{51}Cr release redirected killing assay (at 2:1 effector:target ratio) of the FcγR$^+$ P815 target cell line by γδ PBLs activated and expanded with PHA/ IL-2. Data are presented as mean and SD of 8 independent experiments performed in

triplicate (*p<0.05, ***p<0.001; ns = not statistically significant). (B) Effect of blocking antibodies to NKp30, NKp44 and NKp46 (or control IgG1 isotype control) on killing assays of the leukemia cell line Molt-4 by γδ PBLs activated and expanded with PHA/ IL-2. Where noted, blocking anti-TCRγδ mAb (TCR) was also added. Tumor cell death was assessed by Annexin-V staining as described in Figure 1. Error bars represent SD (n=3, **p<0.01; ***p<0.001).

Figure 6a, 6b – NCR+ γδ PBLs are a stable subset endowed with enhanced cytotoxicity against primary lymphocytic leukemias. $NKp30^{+}$ and $NKp30^{(-)}$γδ PBLs were FACS-sorted from 14-day PHA/ IL-2-activated cultures. (A) Re-analysis of NKp30 expression in the purified populations. (B) Real-time PCR quantification of Nkp44 (left) and Nkp46 (right) mRNA levels in $NKp30^{(-)}$ or $NKp30^{+}$ γδ T-cells, compared to freshly-isolated γδ PBLs. Error bars represent SD (n=3). (C) Sorted $NKp30^{+}$ γδ PBLs were cultured in the presence of IL-2. Analysis of NKp30, NKp44 and NKp46 expression after 14 days. (D) $NKp30^{(-)}$ or $NKp30^{+}$ γδ T-cells, or freshly-isolated γδ PBLs, were used in killing assays with the leukemia cell line Bv173 (as in Figure 1). Tumor cell death was evaluated by Annexin-V staining (n=3, *p<0.05). (E) Real-time PCR quantification of GzmB mRNA levels in freshly-isolated, $NKp30^{(-)}$ or $NKp30^{+}$ γδ T-cells. Error bars represent SD (n=3). (F-G) Representative plots (F) and data summary (G) for 5 primary B-cell chronic lymphocytic leukemia samples that were used in killing assays (as in Figure 1) with γδ PBLs obtained from 6 distinct donors and activated with either HMB-PP/ IL-2 or PHA/ IL-2. $NCR^{(-)}$γδ

PBL from HMB-PP/ IL-2-activated cultures (grey bars) were compared to $NCR^{(+)}\gamma\delta$ PBL from PHA/ IL-2-activated cultures (black bars). Error bars represent SD (n=6, *p<0.05; **p<0.01; ***p<0.001).

Figure 7 – Isotype control staining for NKp30 in PHA/ IL-2-activated γδ PBLs. Flow cytometry IgG1 staining in gated $TCR\gamma\delta^{+}$ cells from PBL cultures described in Figures 1-2. Data are representative of 6 independent experiments.

Figure 8 – Vγ9Vδ2 PBLs are not preferentially susceptible to PHA-induced apoptosis. γδ PBLs were cultured with HMB-PP/ IL-2 or PHA/ IL-2 as described in Figures 1 and 3. Apoptotic Annexin-V^{+} cells within pre-gated $V\delta2^{+}$ cells were analyzed after 7 days in culture by flow cytometry.

Figure 9 – $V\delta1^{+}$ T-cell enrichment in γδ PBL cultures activated with PHA/ IL-2. γδ PBLs were cultured with PHA/ IL-2 for 19 days, as described in Figures 1 and 3, and analyzed by flow cytometry for Vδ1 TCR and CD3 expression.

Figure 10 - Freshly-isolated γδ PBLs do not express NKp30. Flow cytometry data for NKp30 and Vδ1 TCR expression in γδ PBLs freshly-isolated from two healthy donors. Data are representative of fifteen different healthy donors.

Figure 11 - TCR plus IL-2 signals induce NKp30 expression in proliferating $V\delta1^{+}$ T-cells. NKp30 expression in $V\delta1^{+}$ T-cells, FACS-sorted from peripheral blood, labeled with CFSE and cultured with or without OKT3 mAb and IL-2 for 7 days.

Figure 12 – Increased expression of CD56 in NCR^+ $V\delta1^+$ PBLs. Flow cytometry data for CD56 and CD27 expression in $V\delta1^+$ PBLs activated with PHA/ IL-2 for 19 days and gated on either $NKp30^{(-)}$ or $NKp30^+$ cells.

Figure 13 – B7h6 is not overexpressed in most lymphoid leukemia samples. Real-time quantitative PCR data for B7h6 expression on (A) acute lymphoblastic leukemia cell lines; (B) T-cell acute lymphoblastic leukemia patient samples; (C) B-cell chronic lymphocytic leukemia patient samples. Healthy fresh PBMC are shown as reference (dashed line). B7h6 levels were normalized to the housekeeping genes Gusb and Psmb6 and are expressed in arbitrary units. Error bars represent SD of triplicate measurements.

Figure 14 – Phenotype of γδ PBLs in PHA/ IL-2-activated cultures. γδ PBLs were cultured with PHA/ IL-2 for 19 days, as described in Figures 1 and 3, and analyzed by flow cytometry for (A) Vδ1 TCR and CD3 expression; (B) CD11c and CD8 expression. PHA/ IL-2-activated cells are depicted in full line.

Figure 15 – $NKp30^+$ γδ PBLs are highly cytotoxic against cancer cells from diverse tissue origins. γδ peripheral blood lymphocytes (γδ PBLs) were MACS-sorted from the peripheral blood of healthy volunteers, and stimulated with PHA/ IL-2 for 2 weeks. $NKp30^+$ γδ PBLs were further FACS-sorted and used in a 3h killing assays with a panel of tumor cell lines: Acute lymphoblastic leukemia – ALL (RS4-11), Acute myelogenous leukemia – AML (HL-60), Chronic myelogenous leukemia – CML (K562), Chronic lymphocytic

leukemia - CLL (MEC-1), Myeloma (KMM1), Burkitt's lymphoma (RAJI), Follicular lymphoma (DOHH2), Breast carcinoma (MDA231), Lung carcinoma (NCI-H520), Prostate carcinoma (PC3), Colon carcinoma (HCT116), Bladder carcinoma (UM-UC-3), Renal cell carcinoma (VMRC-RCW), Skin Melanoma (MEL-1). Tumor cell death was evaluated by Annexin-V staining (n=3, *p<0.05).

Detailed description of the invention

The disclosed subject matter relates to a cell line of peripheral blood lymphocytes comprising γδ T cells, composition and production method thereof and use in medicine, namely in cancer treatment. These γδ T cells express the Vδ1$^+$ T cell receptor (TCR) and functional natural cytotoxicity receptors (NCRs). NCRs are up regulated in Vδ1$^+$ T cells by AKT-dependent signals provided synergistically by γc-cytokines and Vδ1$^+$ TCR.

The invention discloses a novel Vδ1$^+$ PBL subset capable of targeting hematological tumors highly resistant to fully-activated Vγ9Vδ2 PBLs. This Vδ1$^+$ population owe its specialized killer function to induced expression of natural cytotoxicity receptors, which have been mostly regarded as NK-specific markers. Is shown that, although neither Vδ1$^+$ nor Vδ2$^+$ cells express NCRs constitutively, these can be selectively upregulated in Vδ1$^+$ cells by AKT-dependent signals provided synergistically by γ_c-family cytokines and Vδ1$^+$ TCR. It is also shown that NKp30 and NKp44 are both functional in NCR$^+$ Vδ1$^+$ PBLs and critically contribute to their enhanced targeting of lymphocytic leukemia cells.

The disclosed subject matter relates to the combination of γc cytokines and mitogenic (PHA or OKT3) stimuli, to induce NCR expression in a sizeable $V\delta1^+$ PBL subset that is endowed with increased cytolytic activity against hematological tumors. Although PHA is a non-physiological T-cell mitogen, is demonstrated that its effect on NCR induction was fully mimicked by cross-linking the TCR/CD3 complex on $V\delta1^+$ PBL. Thus, NCR induction is coupled to TCR-mediated proliferation of $V\delta1^+$ cells, while also requiring γ_c cytokine signals.

Among inducible NCRs, NKp30 is the most important for the anti-tumor activity of $V\delta1^+$ T-cells, based on the proportion of cells that express it (Figure 3D); the higher enhancement in $V\delta1^+$ T-cell cytotoxicity upon NKp30 triggering (Figure 5A); and the significant reduction in leukemia cell killing upon NKp30 blockade (Figure 5B). This notwithstanding, NKp44 (but not NKp46) is also functional in NCR^+ $V\delta1^+$ cells (Figure 5A), and appears to synergize with NKp30 for enhanced tumor targeting (Figure 5B).

Both NKp30 and NKp44 have been implicated in human NK cell recognition of viral-infected cells. As for tumors, antibody-mediated blocking experiments demonstrated important roles in myeloma and melanoma cell targeting. Moreover, lack of NCR expression has been clinically correlated with poor survival in AML patients.

$V\delta1^+$ T-cells are the predominant γδ T-cell subset during fetal/ early life, when they are already able to respond to viral infection. In adults, $V\delta1^+$ T-cell expansions have been associated with CMV infection, HIV-1 infection, and tumors of either epithelial or hematopoietic origin. An

attractive prospect for adoptive transfer of activated $V\delta1^+$ T-cells is that they may display particularly good capacity to home to tissues, since, contrary to their circulating $V\delta2^+$ counterparts, $V\delta1^+$ cells are preferentially tissue-associated lymphocytes (Groh, Spies, Science 1998). Interestingly, the abundance of $V\delta1^+$ T-cells at mucosal surfaces has been attributed to IL-15, which induces chromatin modifications that control TCR gene rearrangement.

The present invention describes that NCR^+ $V\delta1^+$ cells are capable of targeting primary lymphoid leukemic cells, which is particularly interesting taking into account that $V\delta1^+$ T-cells have been previously reported to be inefficient killers of primary leukemia or lymphoma cells. The present invention also discloses the preferential expansion of $V\delta1^+$ T-cells (among $\gamma\delta$ PBL) upon PHA treatment *in vitro* (Figure 3B). Since this is not due to selective apoptosis of the dominant $V\delta2^+$ counterparts (Figure 8), it must derive from a proliferative advantage of $V\delta1^+$ cells when receiving PHA-dependent TCR signals (Figures 3A-B). It was previously observed that $V\delta1^+$ T-cells express significantly higher levels of the CD27 coreceptor (when compared to $V\delta2^+$ cells). In both humans and mice, CD27 stimulation enhances Cyclin D2 expression and promotes $\gamma\delta$ T-cell proliferation *in vitro* and *in vivo*.

The invention describes that efficient induction of NCR expression on $V\delta1^+$ cells depends on TCR stimulation; in its absence, γ_c cytokines can only effect a very modest upregulation of NCR expression (Figures 4A-B). Thus, TCR

signals are upstream of NCR-mediated tumor cell recognition by NCR^+ $V\delta1^+$ lymphocytes.

In a clinical perspective, this invention describes a method to induce NCR *ex vivo*, and a very feasible way to expand and inject large numbers of cells into patients. The activation status of these cells could potentially be maintained *in vivo*, via administration of low doses of IL-2, which appears to be sufficient to sustain NCR expression.

PHA / IL-2 activated $NKp30^+$ $\gamma\delta$ T cells preferentially express CD11c and CD8. CD11c is an adhesion molecule usually expressed in other lymphocytes and has been shown to be involved in the recognition of tumor cells.

MATERIALS AND METHODS

Isolation of human peripheral blood γδ *T-cells*. Peripheral blood was collected from anonymous healthy volunteers, diluted 1:1 (v/v) with PBS (preferably, Invitrogen Gibco) and centrifuged preferably, in Ficol-Paque (preferably, Histopaque-1077, Sigma) in a volume ratio of 1:3 (1 part of ficol for 3 of diluted blood) for 30 minutes at 1500 rpm and 25°C. The interface containing mononuclear cells was collected and washed (in PBS), and γδ T-cells were isolated (to above 95% purity) by magnetic cell sorting via positive selection (preferably, with a FITC-labeled anti-TCRγδ antibody) or via negative selection (with a cocktail of Biotin-labelled antibodies; preferably, Miltenyi Biotec).

Cell culture. Isolated γδ PBLs were cultured at 10^6 cells/mL at 37°C, 5% CO^2 in round-bottom 96 well plates with RPMI 1640 with 2 mM L-Glutamine (preferably, Invitrogen Gibco) supplemented with 10% foetal bovine serum (preferably, Invitrogen Gibco), 1 mM Sodium Pyruvate (Invitrogen Gibco), 50 mg/mL of penicillin/streptomycin (preferably, Invitrogen Gibco). The cells were expanded in the presence of 100 U/ml of rhIL-2 (preferably, Roche Applied Science), with or without 10 nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate; preferably, Echelon Biosciences) and 1µg/ml of Phytohemagglutinin (PHA; preferably,Sigma-Aldrich). Cells were washed and the culture medium was replaced every 5-6 days. To study the induction of NKp30 expression, γδ PBLs were cultured in the presence or absence of 100 U/ml of rhIL2 (preferably, Roche Applied Science), 1µg/ml of soluble anti-CD3 antibody (preferably, eBioscience, clone OKT3) and 20ng/ml of rhIL-

15 (preferably, Biolegend). For TCR blockade, freshly-isolated γδPBL were CFSE-labeled and then incubated for 7 days with anti-TCRγδ (preferably, Beckman Coulter, clone IMMU510) diluted 1:20 in complete medium supplemented with 1µg/ml PHA and 100 U/ml rhIL2. To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126 and the PI-3K inhibitor LY294002 (preferably, both from Calbiochem) were added at 10 mM for a 2-hour incubation period and then maintained in culture with 100 U/ml rhIL2 and 1µg/ml PHA for 7 days.

Flow cytometry cell sorting. For sorting of γδ PBL based on the expression of NKp30 and Vδ1$^+$ TCR, cells from PHA/ IL-2-activated cultures were stained with anti-NKp30 (preferably, Biolegend, clone P30-15), anti-Vδ1 (preferably, Thermo Fisher Scientific, clone TS8.2) and sorted on a FACSAria cell sorter (preferably, BD Biosciences).

Leukemia patient samples. B-cell chronic lymphocytic leukemia cells were obtained from the peripheral blood of patients at presentation, after informed consent and institutional review board approval (Instituto Português de Oncologia de Lisboa, Portugal). Samples were enriched by density centrifugation over Ficol-Paque and then washed twice in 10% RPMI 1640 (as above).

In vitro tumor killing assays. All tumor cell lines were cultured in complete 10% RPMI 1640 (as above), maintained at 10^5 up to 10^6 cells/mL by dilution and splitting 1:3 every 3-4 days. For cytotoxicity assays, magnetically purified γδ PBL were pre-activated for 7-19 days in the

presence of IL-2 (100 U/mL) and either 1µg/mL PHA or 10 nM HMB-PP. For receptor blocking, γδ PBLs were incubated for 2h with the blocking antibodies anti-NKp30 (clone F252), anti-NKp44 (clone KS38), anti-NKp46 (clone KL247) or anti-TCRγδ (Beckman Coulter, clone IMMU510). The blocking antibodies were maintained in the culture medium during the killing assays. Tumor cell lines or leukemia primary samples were stained with CellTrace Far Red DDAO-SE (1 µM ; Molecular Probes, preferably, Invitrogen) and each $3x10^4$ tumor cells were incubated with $3x10^5$ γδ T-cells in RPMI, for 3 hrs at 37°C and 5% CO^2 on a round-bottom 96 well plate. Cells were then stained with Annexin V-FITC (preferably, BD Biosciences) and analyzed by flow cytometry. For the redirected killing assays, PHA/IL-2-activated γδ PBL were incubated for 4 hrs with the NCR agonists anti-NKp30 (clone AZ20), anti-Nkp44 (clone Z231) or anti-NKp46 (clone Bab281) during a standard ^{51}Cr release assay.

Flow cytometry analysis. Cells were labeled with fluorescent monoclonal antibodies: anti-CD3-PerCP-Cy5.5 (preferably, eBioscience, clone OKT3), anti-TCRγδ-FITC (preferably, eBioscience, clone B1.1), anti-CD69-PE (preferably, BD Pharmingen, clone FN50), anti-NKG2D-PE/Cy7 (Biolegend, clone 1D11), anti-2B4-APC (preferably, Biolegend, clone C1.7), anti-DNAM-1-Alexa-Fluor647 (Biolegend, clone DX11), anti-NKp30-APC (preferably, Biolegend, clone P30-15), anti-Vδ2 TCR -PE (preferably, Biolegend, clone B6), anti-NKp44-APC (preferably, Biolegend, clone P44-8), anti-NKp46-AlexaFluor647 (preferably, Biolegend, clone 9E2), anti-Vδ1 TCR -FITC (preferably, Thermo Fisher Scientific, clone TS8.2), anti-

NKp30-PE (preferably, Biolegend, clone P30-15), anti-Mouse IgG1κ-APC Isotype Ctrl (preferably, Biolegend, clone MOPC-21), anti-Mouse IgG1κ-PE Isotype Ctrl (preferably, Biolegend, clone MOPC-21), anti-CD27-APC/Cy7 (preferably, Biolegend, clone O323), anti-CD56-APC (preferably, Biolegend, clone HCD56). Cell proliferation was measured by following a standard CFSE staining protocol (preferably, CellTrace CFSE Cell Proliferation Kit, Invitrogen; final concentration 0.5 mM), while apoptosis was assessed by AnnexinV-FITC (preferably, BD Pharmingen) staining. Cells were analyzed on a FACSCanto flow cytometer (preferably, BD Biosciences).

RNA isolation and cDNA production. Total RNA was extracted using the RNeasy Mini Kit according to manufacture's protocol (preferably, Qiagen, Hilden, Germany). Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (preferably, Agilent Technologies, Palo Alto, CA). Total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II first strand synthesis reagents (preferably, Invitrogen).

Real-time quantitative PCR (qPCR). qPCR was performed on ABI Prism 7500 FAST Sequence Detection System using SYBR Green detection system (preferably, both from Applied Biosystems). Primers were designed using Primer3 v.0.4.0 online program (http://primer3.sourceforge.net). For each transcript, quantification was done using the calibration curve method. β_2-microglobulin (*b2m*), Glucoronidase beta (*Gusb*) and proteasome subunit beta type 6 (*Psmb6*) were used

as housekeeping controls for normalization of gene expression. The following primers were used:

- *b2m* – forward CTAT CCAG CGTA CTCC AAAG ATTC, reverse CTTG CTGA AAGA CAAG TCTG AATG;
- *Psmb6* – forward GGCG GCTA CCTT ACTA GCTG, reverse AAAC TGCA CGGC CATG ATA;
- *Gusb* – forward TGCA GCGG TGTA CTTC TG, reverse CCTT GACA GAGA TCTG GTAA TCA;
- B7-H6 – forward TCAC CAAG AGGC ATTC CGAC CT, reverse ACCA CCTC ACAT CGGT ACTC TC;
- *Nkp44* – forward CCGT CAGA TTCT ATCT GGTG GT, reverse CACA CAGC TCTG GGTC TGG;
- *Nkp46* – forward AAGA CCCC ACCT TTCC TGA, reverse TGCT GGCT CGCT CTCT AGT;
- *Gzmb*– forward GGGG GACC CAGA GATT AAAA, reverse CCAT TGTT TCGT CCAT AGGA G.

All samples were run in triplicate and repeated three times. Analysis of the qPCR results was performed using the ABI SDS v1.1 sequence analysis software (Applied Biosystems).

Statistical analysis. Differences between subpopulations

were assessed using Student's t-test and is indicated when significant as *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

RESULTS

Enhanced cytotoxicity of γδ PBL cultures activated with pan-T-cell mitogen

Was compared the anti-tumor killing capacity of γδ PBL cultures (always maintained in the presence of IL-2) activated either with PHA, a plant lectin that acts as a potent T-cell mitogen, or the specific Vγ9Vδ2 TCR agonist HMB-PP (Morita, C.T., Jin, C., Sarikonda, G., and Wang, H. 2007. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215:59-76). Although both regimens were similarly efficient at activating γδ PBLs, as evaluated by cell proliferation and CD69 upregulation (Figure 1A), it was noted that samples activated with PHA were consistently better killers of hematopoietic tumor cell lines than samples (of the same donor origin) stimulated with HMB-PP (Figure 1B-C). This was valid across all donors tested (Figure 1B) and was associated with higher expression of Granzyme B (Figure 1D), a key component of the lymphocyte cytolytic machinery. Of note, freshly-isolated γδ PBLs, which lack Granzyme B expression (Figure 1D), displayed very poor anti-leukemia cytotoxicity (<10% killing), as previously reported.

The superior cytotoxic function of PHA-stimulated γδ PBL cultures was a surprising finding, since is shown that HMB-PP is a very potent activator of the highly dominant Vγ9Vδ2

PBL subset (Morita, C.T., Jin, C., Sarikonda, G., and Wang, H. 2007. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215:59-76). Compared to HMB-PP-activated γδ PBL, PHA-stimulated cultures displayed improved cytotoxicity against various resistant leukemia cell lines, such as Bv-173, REH or HPB-ALL (Figures 1B-C), which has been shown to lack expression of the critical NKG2D ligand ULBP1. These data demonstrate that the pan-T-cell mitogen PHA is capable of increasing the cytolytic potential of medium-term (1-3 weeks) γδ PBL cultures, which could be of great value for adoptive cell immunotherapy.

Induction of NCR expression on γδ PBLs activated with pan-T-cell mitogen

Was investigated the mechanism(s) underlying the enhanced cytotoxicity of PHA-activated γδ PBL cultures. The above data could be explained by the differential expression of receptors such as NKG2D or DNAM-1 or 2B4. All previously were shown to participate in tumor cell recognition by killer lymphocytes. However, none of these candidates was differentially expressed between PHA- and HMB-PP-activated γδ PBL cultures (Figure 2A). By contrast, and unexpectedly, the natural cytotoxicity receptor NKp30, an important trigger of NK cell cytotoxicity, was specifically found on PHA-stimulated γδ PBLs (Figure 2B; Figure 7). Furthermore, the other NCR family members, NKp44 and NKp46, were also selectively expressed in these samples (Figures 2C-D; see bellow).

The proportion of NKp30^{+} cells increased steadily with culture time (Figure 2E), suggesting an association of NKp30 induction with cell proliferation. Although unlikely due to the very low background in fresh samples (Figure 2E), it was possible that a minute subset constitutively expressing NKp30 could be preferentially expanded in PHA-stimulated γδ PBL cultures. To address this, were performed experiments with highly (>99%) FACS-purified NKp30$^{(-)}$cells, which demonstrated that NKp30$^{(-)}$ cells were able to acquire NKp30 expression as efficiently as unsorted cells upon PHA+IL-2 stimulation (Figure 2F). Moreover, under such conditions, NKp30$^{(-)}$ and NKp30^{+} cells proliferated to similar extent, further arguing against preferential expansion of NKp30^{+} cells under such conditions. These results suggest that NKp30 expression is induced *de novo* upon γδ PBL activation by PHA/IL-2 treatment, which is coupled to cell proliferation.

NCRs are selectively expressed by proliferating Vδ1^{+} T-cells

Considering that HMB-PP had been shown to be an optimal agonist of Vγ9Vδ2 cells, although, by contrast with HMB-PP, treatment with PHA preferentially expanded Vδ2$^{(-)}$ cells among γδ PBL (Figure 3A). This was not due to differences in Vδ2^{+} cell apoptosis in the two experimental conditions (Figure 8). The most likely Vδ2$^{(-)}$ population to expand so significantly (Figure 3A) were Vδ1^{+} cells, since other subsets are very rare in the peripheral blood of healthy adults. When Vδ1 versus Vδ2 TCR usage was assessed, a dramatic Vδ1^{+} cell enrichment was found in PHA-activated

cultures (>80% of all γδ T-cells after 19 days) (Figure 3B; Figure 9). Conversely, and as described, HMB-PP-activated cultures were progressively dominated by $V\delta2^+$ cells (Figure 3A).

The induction of NKp30 expression was examined in parallel cultures of isolated $V\delta1^+$ or $V\delta2^+$ cells, which were stimulated with PHA+IL-2. While neither freshly isolated $V\delta1^+$ nor $V\delta2^+$ cells expressed NKp30 (Figure 10), this NCR was strongly induced (upon PHA+IL-2 treatment) in $V\delta1^+$ but not $V\delta2^+$ cells (Figure 3C). Moreover, by following CFSE dilution was demonstrated a striking accumulation of $NKp30^+$ cells with progressive division of $V\delta1^+$ cells (Figure 3C). These data suggest that activation of $V\delta1^+$ cells in PHA/IL-2 cultures induces NKp30 expression concomitantly with cell proliferation.

Whereas high percentages (>50%) of $NKp30^+$ cells were usually detected after 2-3 weeks in culture, NKp44 (~30%) and NKp46 (<20%) were expressed in lower proportions of $V\delta1^+$ cells (Figure 3D). Furthermore, most of $NKp44^+$ or $NKp46^+$ $V\delta1^+$ cells also expressed NKp30 (Figure 3D). Therefore is considered NKp30 as the most informative marker of the inducible NCR^+ $V\delta1^+$ subset.

NCR induction requires AKT-dependent γc cytokine and TCR signals

Was dissected the specific signals required for the differentiation of NCR^+ $V\delta1^+$ T-cells. First, the two components of the activation protocol, IL-2 and PHA, were dissociated. IL-2, or its related γ_c cytokine, IL-15, alone

were sufficient to induce some NKp30 expression, but the effect was modest when compared to PHA/ IL-2 (or PHA/ IL-15) combinations (Figure 4A). On the other hand, PHA alone was not able to keep the cultures viable, consistent with the critical role of γ_c cytokines in the survival of γδ T-cells, particularly upon activation/ proliferation.

Although PHA has been a widely used T-cell mitogen, it is also a non-physiological compound capable of cross-linking a series of surface receptors, including the TCR. Thus, the molecular mediator of PHA stimulation could be the $V\delta1^+$ TCR complex. Was compared the ability of PHA and the OKT3 mAb, which specifically cross-links CD3ε chains of the TCR complex, to induce NKp30 expression (when combined with IL-2 or IL-15) in $V\delta1^+$ T-cells. OKT3 was fully capable of mimicking PHA in these assays (Figures 4A-B), thus inducing NKp30 in proliferating $V\delta1^+$ T-cells (Figure 11). Moreover, TCRγδ blockade in PHA/IL-2 cultures prevented NKp30 induction (Figure 4C). These data suggest that PHA treatment provides TCR signals to induce NCR expression on $V\delta1^+$ PBL. Moreover, the differences between cytokine alone or combination treatments with OKT3 (or PHA) highlight a marked synergy between γ_c cytokine and TCR signals in this process (Figures 4A-B).

To further explore the molecular mechanisms of NCR induction, was employed chemical inhibitors of key signal transduction pathways downstream of γ_c cytokine receptors and/ or TCR signaling. While blocking JAK signaling triggered extensive cell death before any NCR induction, co-incubation with the PI-3K/ AKT inhibitor LY294002 specifically prevented NKp30 induction in proliferating $V\delta1^+$ T-cells (Figure 4D). AKT is involved in transducing

both γ_c cytokine and TCR signals), including TCR$\gamma\delta$ signals). By contrast, the MAPK/ Erk inhibitor UO126 had no detectable effect on NKp30 induction in proliferating V$\delta1^+$ T-cells (Figure 4D). Importantly, the selective effect of LY294002 dissociated NCR induction from cell proliferation, thus demonstrating that V$\delta1^+$ T-cell proliferation is necessary (Figure 3C; Figure 11) but not sufficient (Figure 4D) to induce NKp30 expression. Collectively, these data demonstrate that AKT-dependent γ_c cytokine and TCR signals synergize to induce NKp30 expression in V$\delta1^+$ T-cells.

Functional NKp30 and NKp44 trigger tumor cell killing by V$\delta1^+$ PBLs

Were undertooked gain- and loss-of-function experiments to evaluate the impact of NCR modulation on V$\delta1^+$ enriched (>80%; Figure 9A) PBL cultures, which expressed NCRs at levels similar to those in Figure 3D. First, by using a reverse Ab-dependent cytotoxicity assay, was showed that cross-linking of NKp30 or NKp44, but not NKp46, produced highly significant increases in lysis of the P815 tumor cell targets (Figure 5A). These data demonstrate that induced NKp30 and NKp44 are functional and mediate tumor cell killing. To assess if they played non-redundant roles in targeting leukemia cells, was performed receptor blockade experiments using NCR-specific mAbs. Was observed significant reductions in tumor cell lysis upon NKp30 and NKp44 blockade (Figure 5B). As expected from the results in Figure 5A, NKp46 blockade did not affect tumor cell killing. Interestingly, a synergistic effect between NKp30 and NKp44 was also clearly observed. Of note, TCR$\gamma\delta$

blockade in any setting (alone or in combination with anti-NCR mAbs) was a neutral event during the killing assay (Figure 5B). These data suggest that leukemia cell targeting by NCR^+ $V\delta1^+$ PBLs is a TCR-independent event mostly mediated by the synergistic function of NKp30 and NKp44.

NCR^+ $V\delta1^+$ PBL are specialized killers that target resistant primary lymphocytic leukemias

To fully characterize the anti-tumor potential of NCR^+ $V\delta1^+$ PBL, $NKp30^+$ cells were FACS-sorted to high degree of purity (>99%) (Figure 6A) and a series of functional assays were performed. As expected (Figure 3D), sorted $NKp30^+$ cells also expressed NKp44 and NKp46 (Figure 6B), and the three NCRs were largely stable on the surface of the purified cells when cultured for two weeks with IL-2 alone (Figure 6C). These data demonstrate the feasible expansion of a stable NCR^+ $V\delta1^+$ T-cell subset.

When the cytotoxic function of $NKp30^+$ cells was assessed, an increased targeting of the resistant leukemia cell line Bv173 (among others) was observed (in comparison with $NKp30^{(-)}$ counterparts) (Figure 6D). This correlated with higher expression of Granzyme B (Figure 6E). Moreover, NKp30 expression also associated with higher degree of CD56 expression (Figure 12), which has been previously linked to cytotoxicity of human lymphocytes, including $V\delta2^+$ T-cells.

Finally, were performed functional killing assays with primary samples obtained from chronic lymphocytic leukemia patients. Importantly, HMB-PP/IL-2-activated $\gamma\delta$ PBLs do not express NCRs (Figure 2B). Was compared their anti-tumor

cytolytic activity with that of NCR^+ cells isolated from $\gamma\delta$ PBL cultures activated with PHA/ IL-2. Was observed that NCR^+ $\gamma\delta$ PBLs, obtained from 6 different donors, were consistently more efficient at eliminating primary B-CLL cells (Figures 6F-G).

The invention is of course not in any way restricted to the embodiments described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof without departing from the basic idea of the invention as defined in the appended claims.

The following claims set out particular embodiments of the invention.

C L A I M S

1. Cell line of lymphocytes comprising $V\delta1^+$ $\gamma\delta$ T cells expressing functional natural cytotoxicity receptors.

2. Cell line according to claim 1 consisting of $V\delta1^+$ $\gamma\delta$ T cells expressing functional natural cytotoxicity receptors.

3. Cell line according to any one of the previous claims further comprising $V\delta2^+$ $\gamma\delta$ T cells.

4. Cell line according to any one of the previous claims wherein the natural cytotoxicity receptors comprise NKp30.

5. Cell line according to any one of the previous claims wherein the said lymphocytes are from a peripheral blood sample.

6. Cell line according to any one of the previous claims wherein the natural cytotoxicity receptors comprise NKp44.

7. Cell line according to any one of the previous claims wherein the natural cytotoxicity receptors comprise NKp46.

8. Cell line according to any one of the previous claims comprising cells expressing granzime B.

9. Composition comprising cells of the cell line according to any one of the previous claims.

10. Composition according to any one of the previous claims wherein the composition is an injectable.

11. Composition according to any one of the previous claims for the use in medicine.

12. Composition according to the previous claim for use in autologous or heterologous adoptive cell therapy, tumor or cancer treatment, tumor or cancer immunotherapy, and/or leukemia treatment.

13. Composition according to claim 11 for use in treatment of acute lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, multiple myeloma, burkitt's lymphoma, follicular lymphoma, breast carcinoma, lung carcinoma, prostate carcinoma, colon carcinoma, bladder carcinoma, renal cell carcinoma, or skin melanoma.

14. Composition according to claim 11 for use in treatment of a viral infection.

15. Composition according to the previous claim wherein the virus is from the family *Herpesviridae* or *Retroviridae*.

16. A method of producing a cell line described in any of the claims 1-8 which comprises isolating γδ PBLs and cultivating these cells in adequate culture

medium, with addition of γδTCR agonists and at least one γ_c-family cytokine.

17. Method according to claim 16 wherein the regular addition of said γδTCR agonists and γ_c-cytokines is carried out until at least 40% of the cells express natural cytotoxicity receptors.

18. Method according to the previous claim wherein the regular addition of said γδTCR agonists and γ_c-cytokines is carried out until at least 40% of the cells express NKp30.

19. Method according to any of the claims 16 - 18 wherein the γδTCR agonist is phytohemagglutinin-PHA, anti-CD3 monoclonal antibody, anti-γδTCR monoclonal antibody or mixtures thereof.

20. Method according to the previous claim wherein the range of γδTCR agonist concentration is 0,01 - 100 µg/ml.

21. Method according to any of the claims 16 - 20 wherein the γ_c-cytokine is IL-2, IL-4, IL-9, IL-12, IL-15, IL-21 or mixtures thereof.

22. Method according to the previous claim wherein the range of γ_c-cytokine concentration is 1-10000 U/ml.

23. Method according to the previous claim wherein the range of γ_c-cytokine concentration is 100-1000 U/ml.

24. Method according to the claims 16-23 wherein the time range of the addition of said γδTCR agonists and γ_c-cytokines is 2-60 days.

25. Method according to the previous claim wherein the time range of the addition of said γδTCR agonists and γ_c-cytokines is 9-25 days.

26. Method according to the previous claim wherein the time range of the addition of said γδTCR agonists and γ_c-cytokines is 10-15 days.

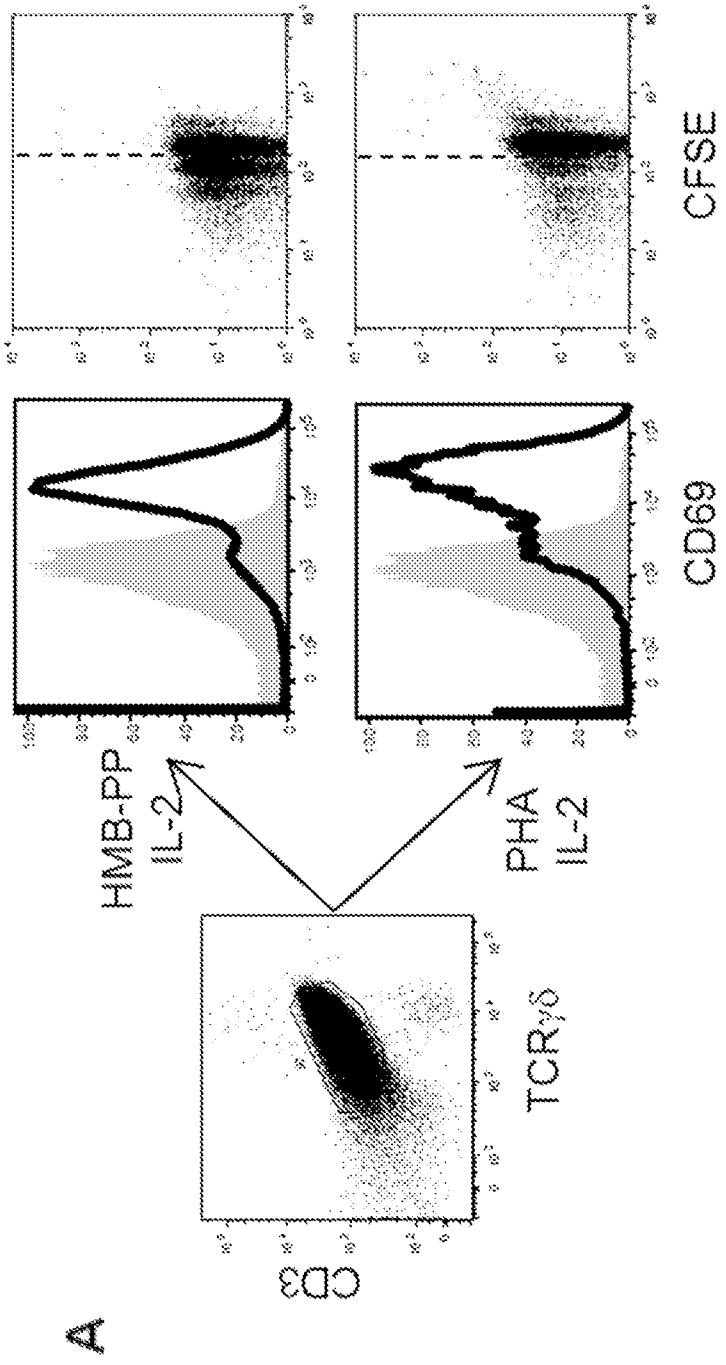
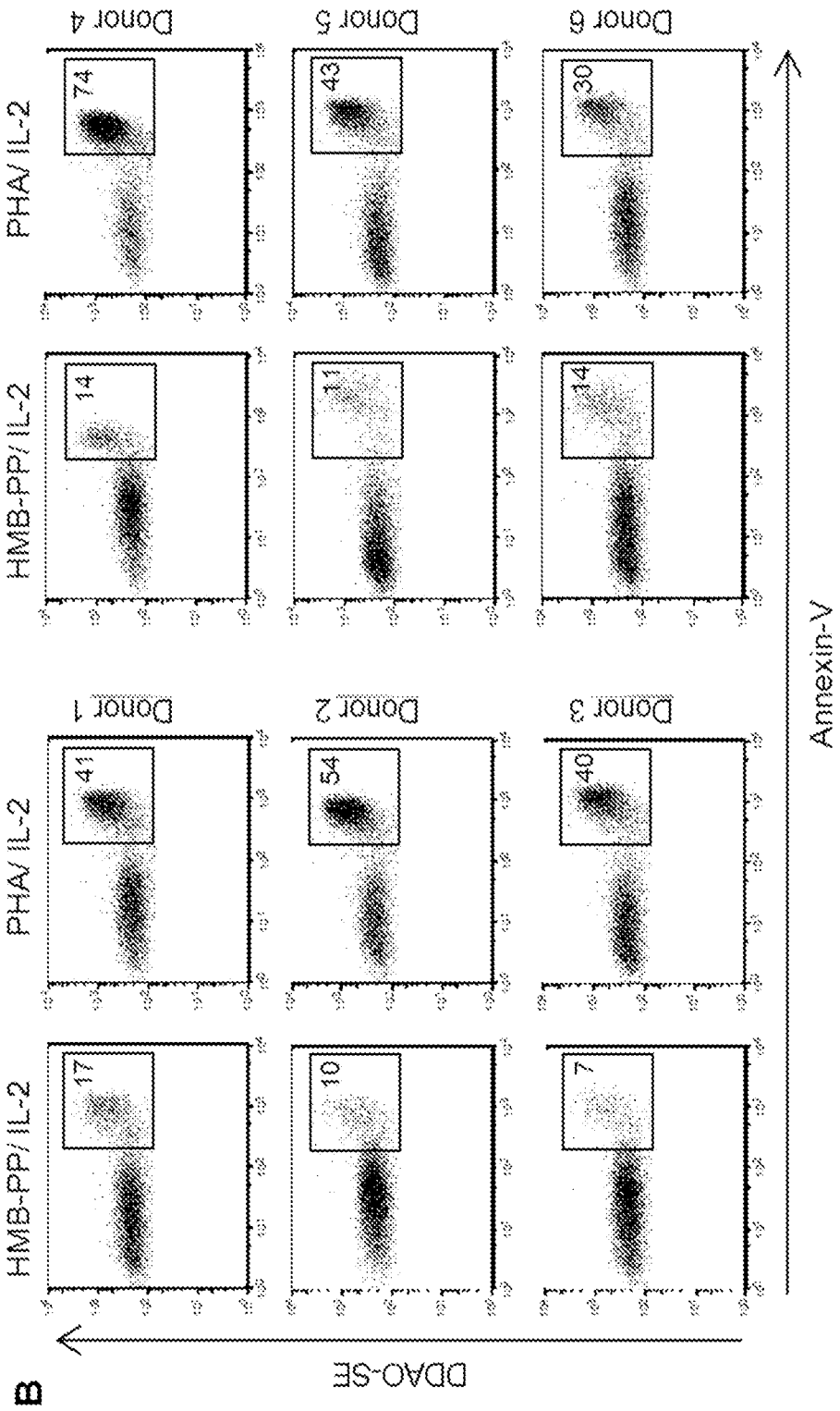


Figure 1a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Annexin-V
HMB-PP/ IL-2
PHA/ IL-2
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Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
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54
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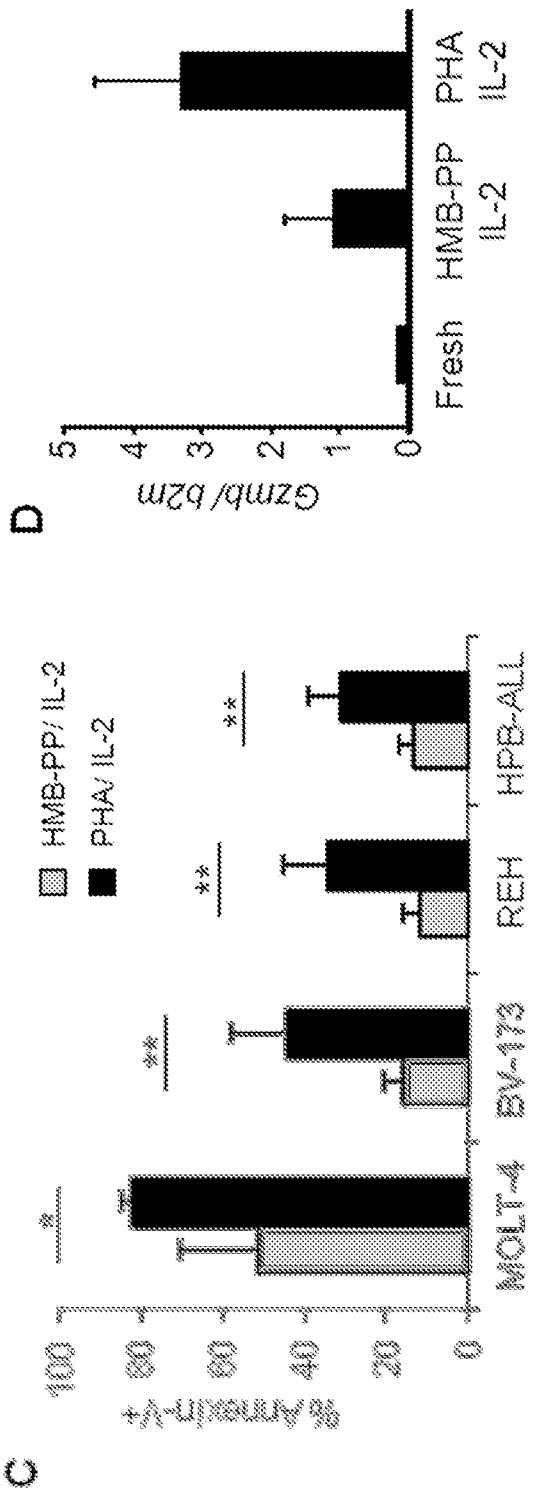
14
11
14
74
43
30

Figure 1b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Gzmb/ b2m
0
1
2
3
4
5
Fresh
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PHA IL-2
C
% Annexin-V+
0
20
40
60
80
100
HMB-PP/ IL-2
PHA/ IL-2
*
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**
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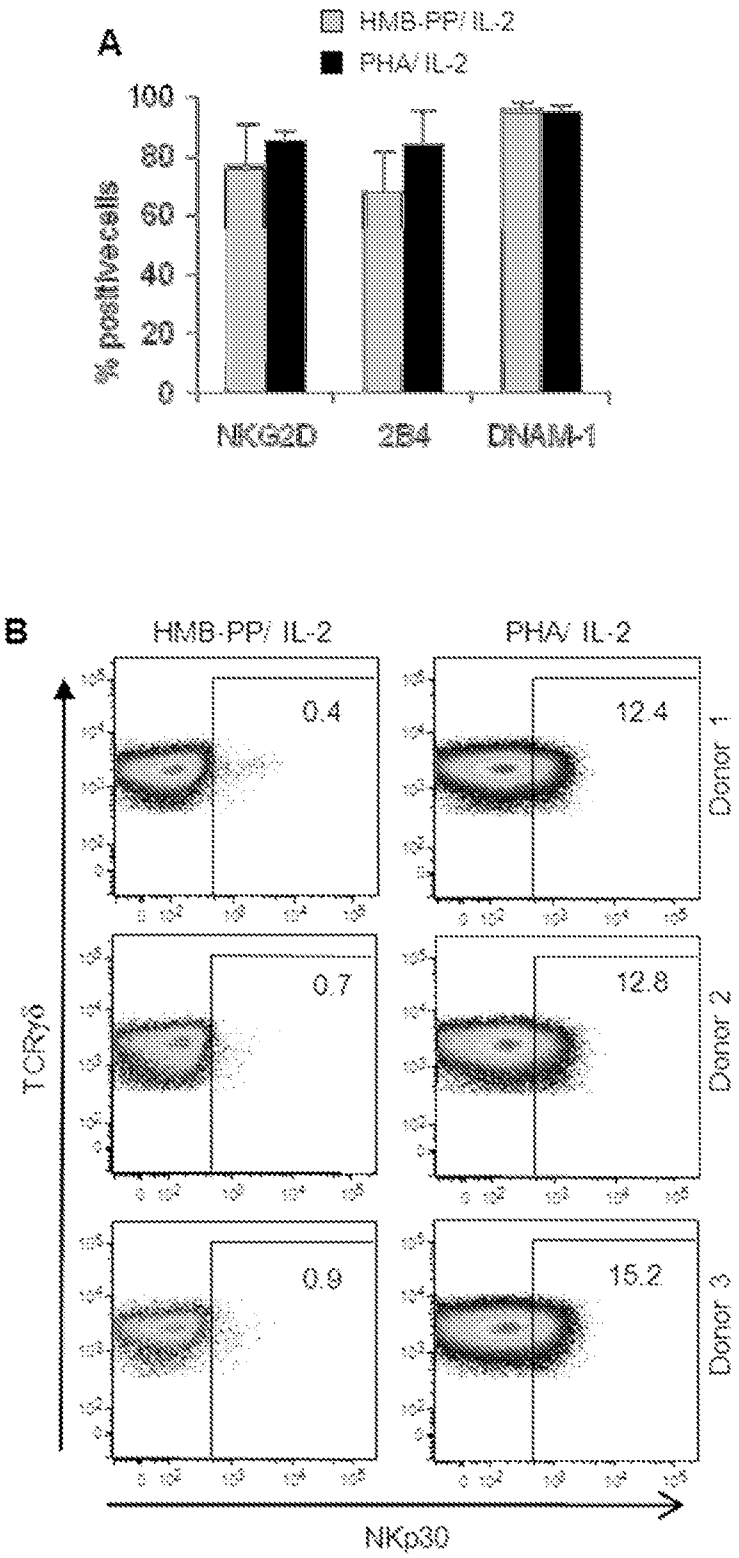
MOLT-4
BV-173
REH
HPB-ALL

Figure 1c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HMB-PP/ IL-2
PHA/ IL-2
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100
80
60
40
20
0
NKG2D
2B4
DNAM-1
B
HMB-PP/ IL-2
PHA/ IL-2
0.4
12.4
0.7
12.8
0.9
15.2
Donor 1
Donor 2
Donor 3
TCRγδ
NKp30

Figure 2a

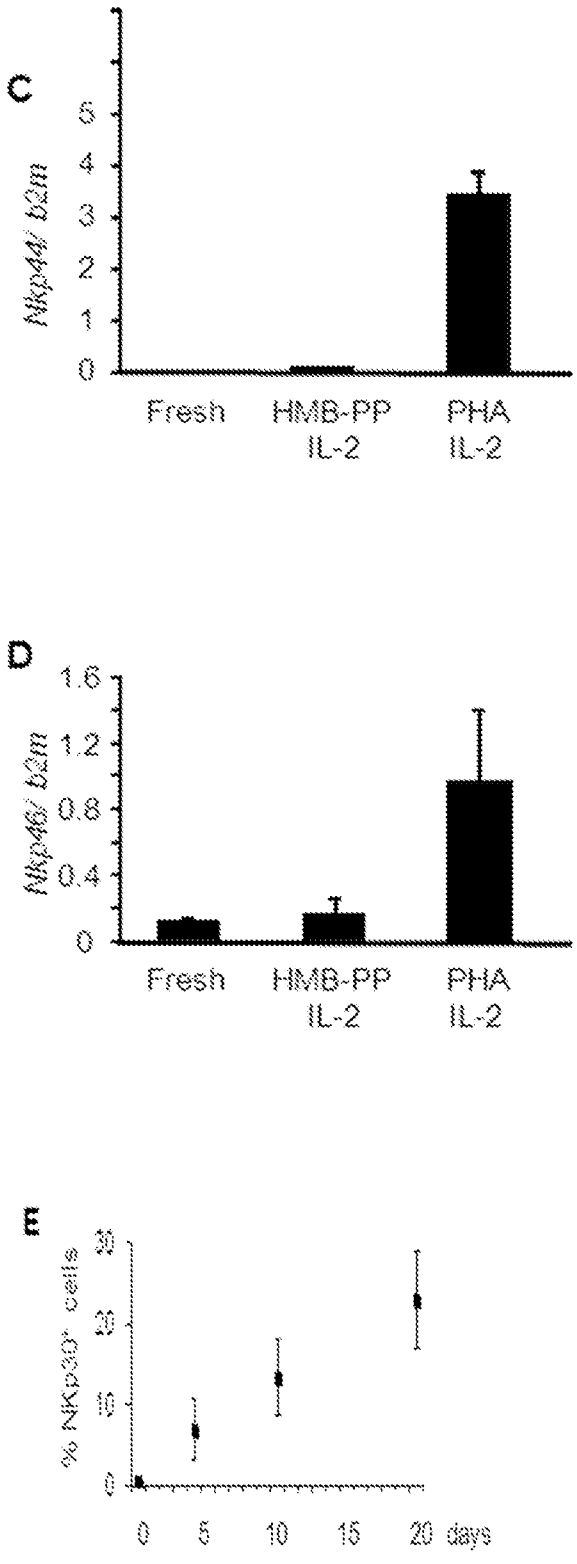

C
Nkp44/ b2m
0
1
2
3
4
5
Fresh
HMB-PP IL-2
PHA IL-2
D
Nkp46/ b2m
0
0.4
0.8
1.2
1.6
Fresh
HMB-PP IL-2
PHA IL-2
E
% NKp30+ cells
0
10
20
30
0
5
10
15
20 days

Figure 2b

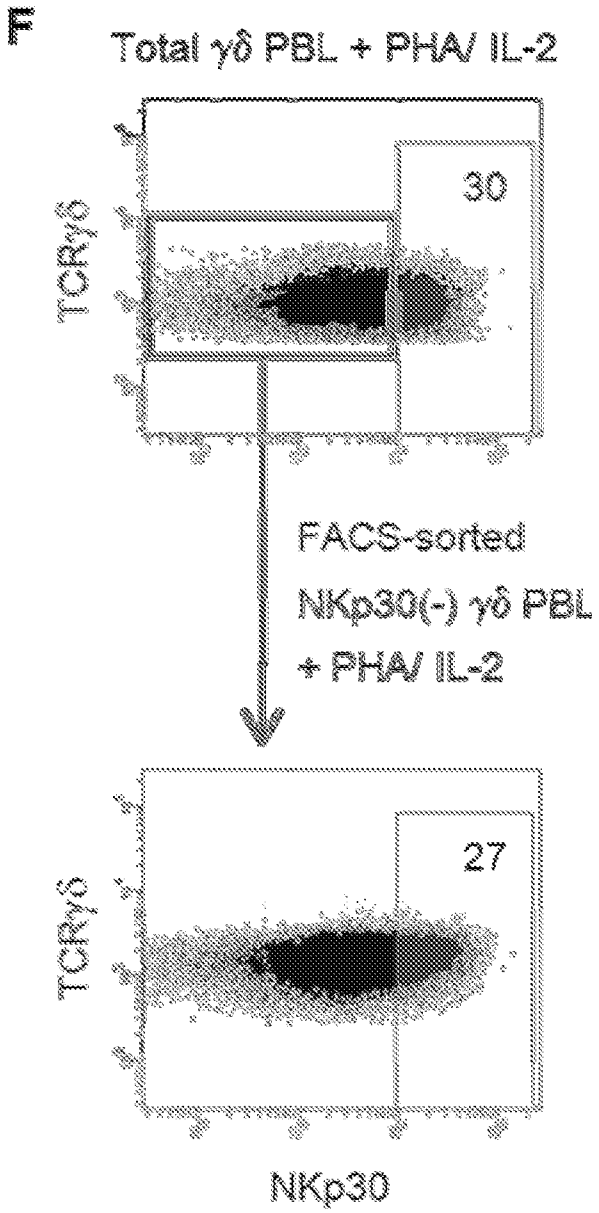
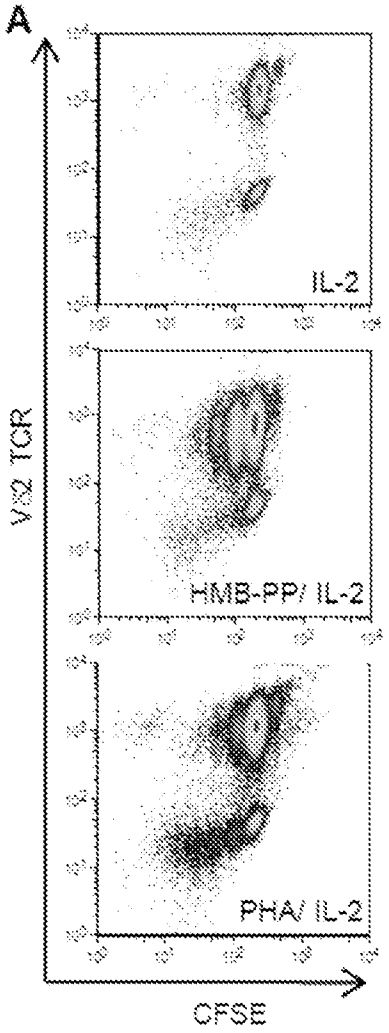

F
Total γδ PBL + PHA/ IL-2
30
TCRγδ
FACS-sorted
NKp30(-) γδ PBL
+ PHA/ IL-2
27
TCRγδ
NKp30

Figure 2c


A
IL-2
HMB-PP/ IL-2
PHA/ IL-2
Vδ2 TCR
CFSE

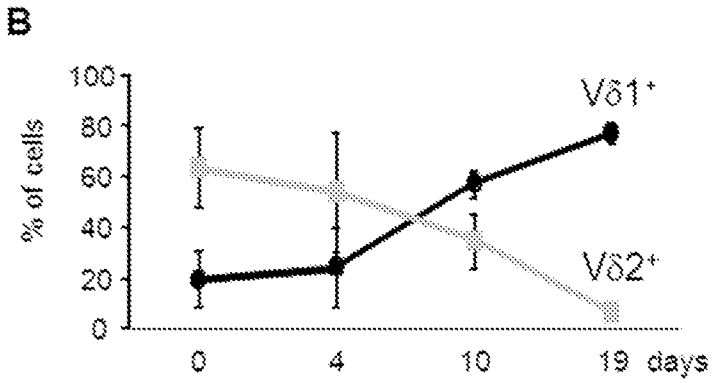
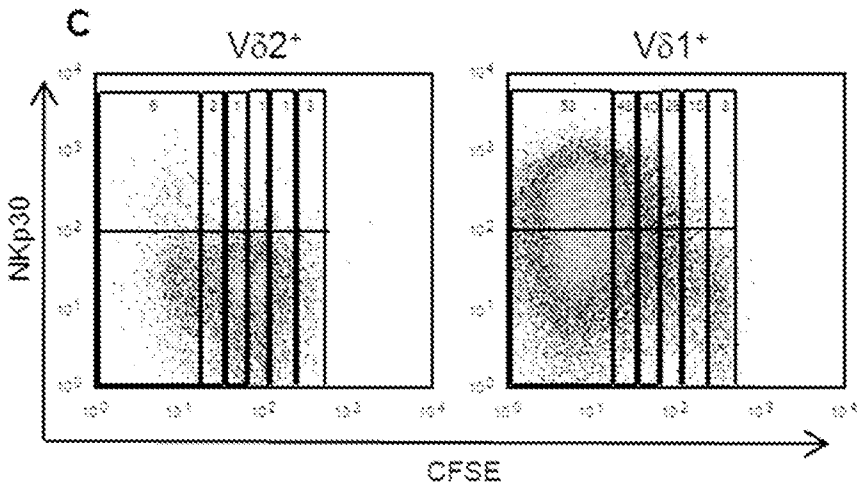

B
% of cells
100
80
60
40
20
0
Vδ1+
Vδ2+
0
4
10
19 days

Figure 3a


C
Vδ2+
Vδ1+
NKp30
CFSE

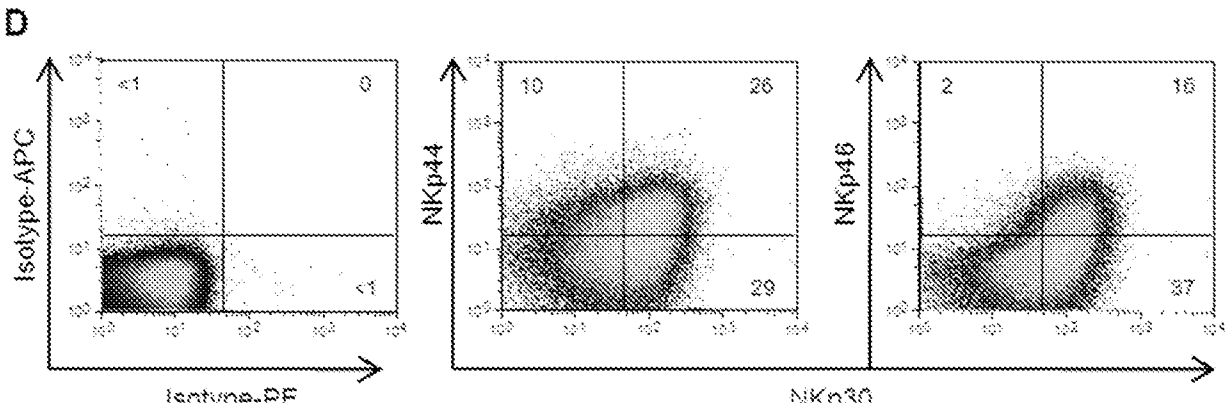

D
Isotype-APC
<1
0
<1
NKp44
10
26
29
NKp46
2
16
37

Figure 3b

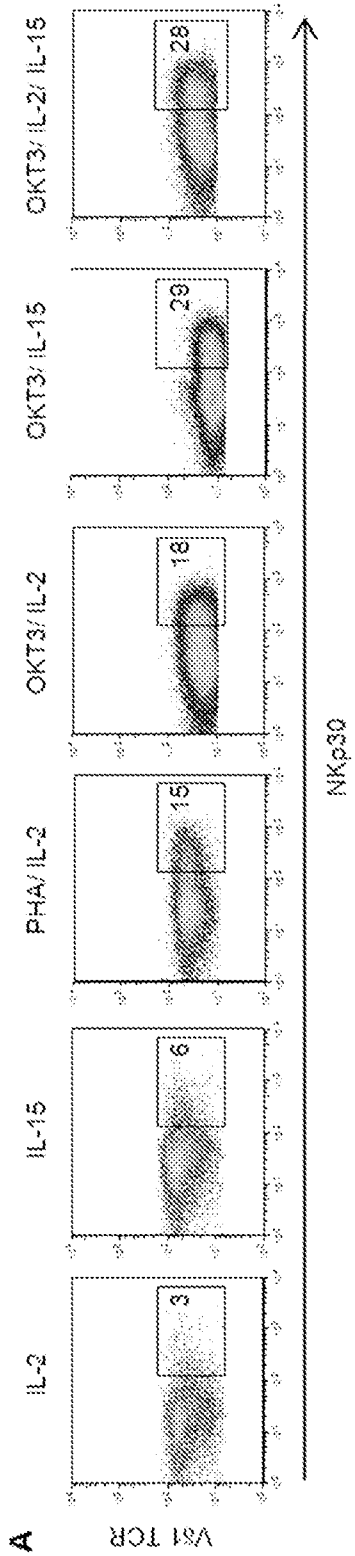

A
Vδ1 TCR
NKp30
IL-2
3
IL-15
6
PHA/ IL-2
15
OKT3/ IL-2
18
OKT3/ IL-15
29
OKT3/ IL-2/ IL-15
28

Figure 4a

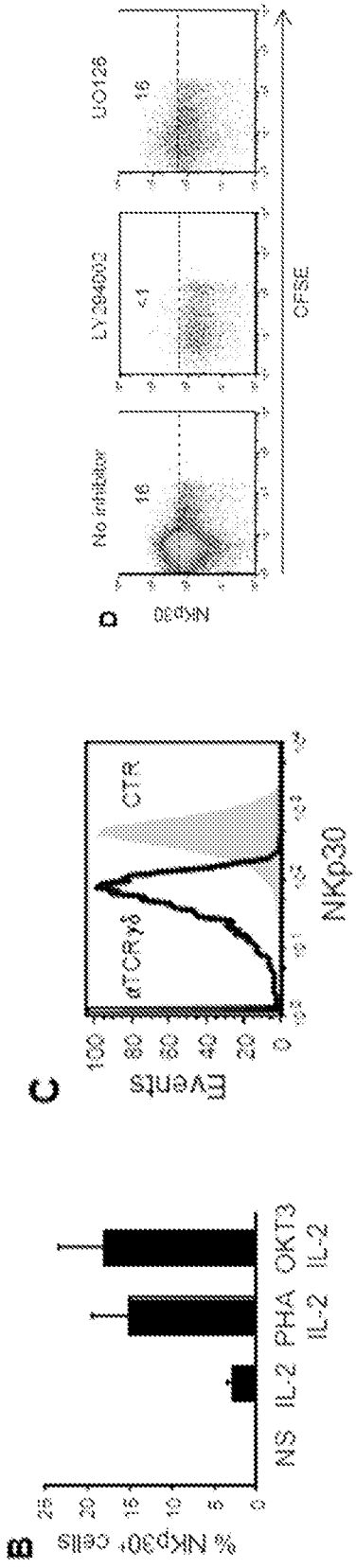

a
NKp30
No inhibitor
16
LY294002
<1
U0126
16
CFSE
c
Events
100
80
60
40
20
0
CTR
αTCRγδ
NKp30
b
% NKp30+ cells
25
20
15
10
5
0
NS
IL-2
PHA
IL-2
OKT3
IL-2

Figure 4b

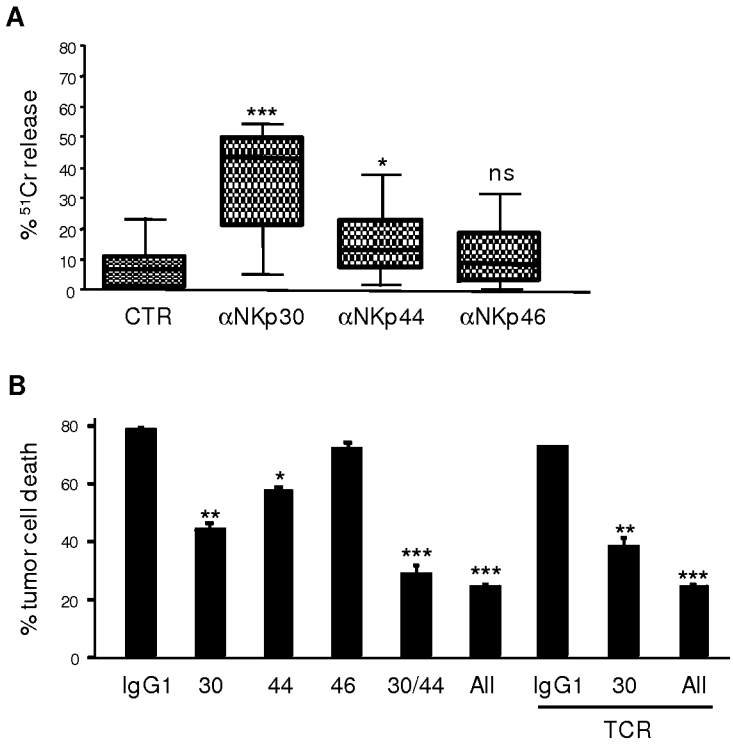


Figure 5

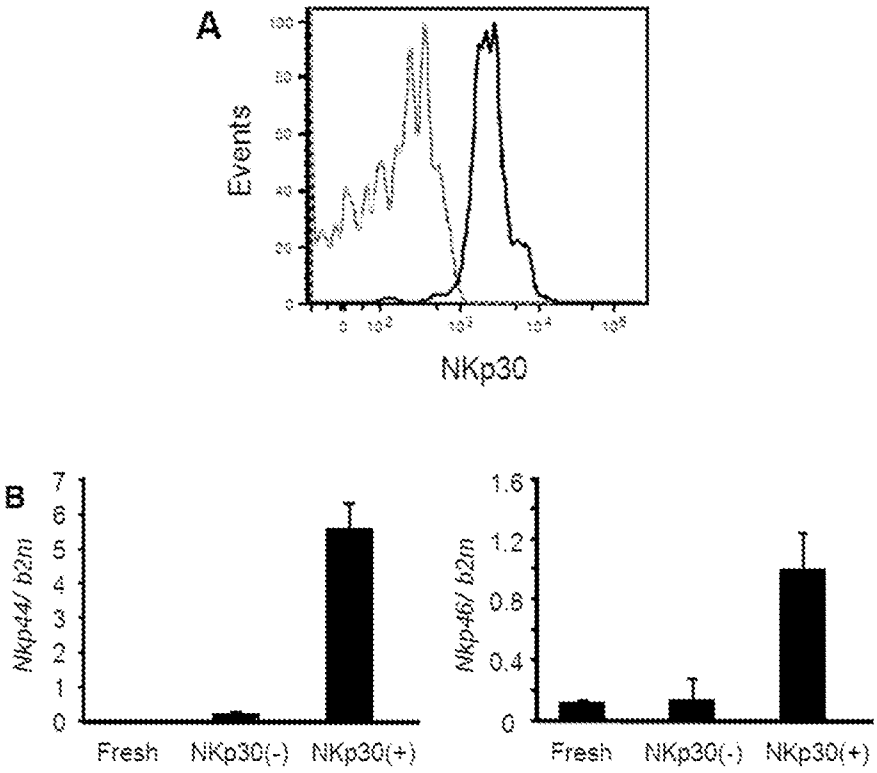


Figure 6a

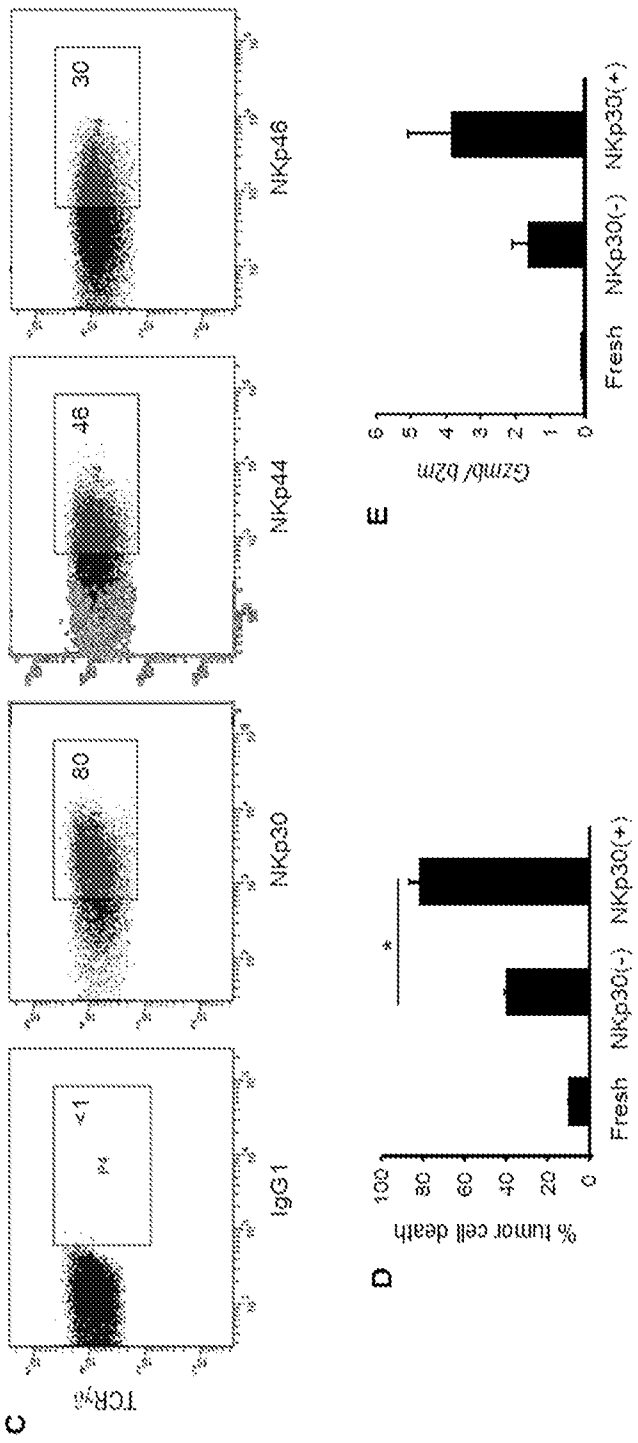

C
TCRγδ
IgG1
<1
NKp30
80
NKp44
46
NKp46
30
D
% tumor cell death
Fresh
NKp30(-)
NKp30(+)
*
E
Granzyme B/zm
Fresh
NKp30(-)
NKp30(+)

Figure 6b

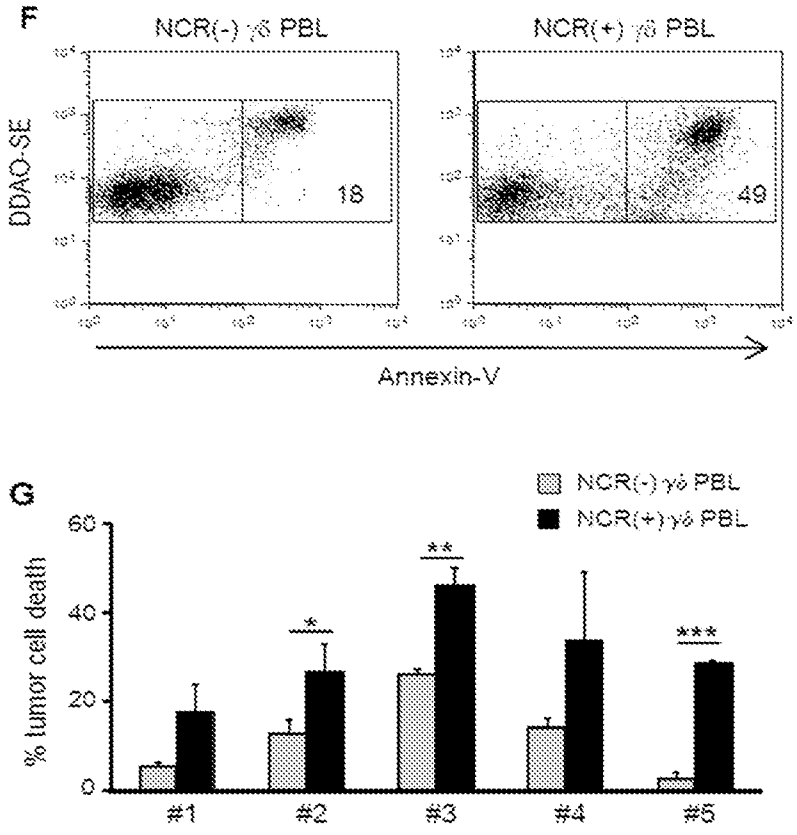


Figure 6

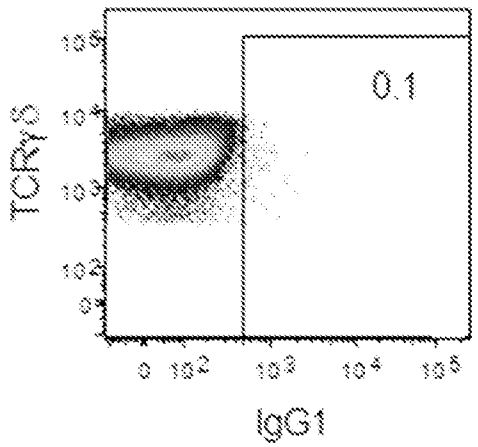


Figure 7

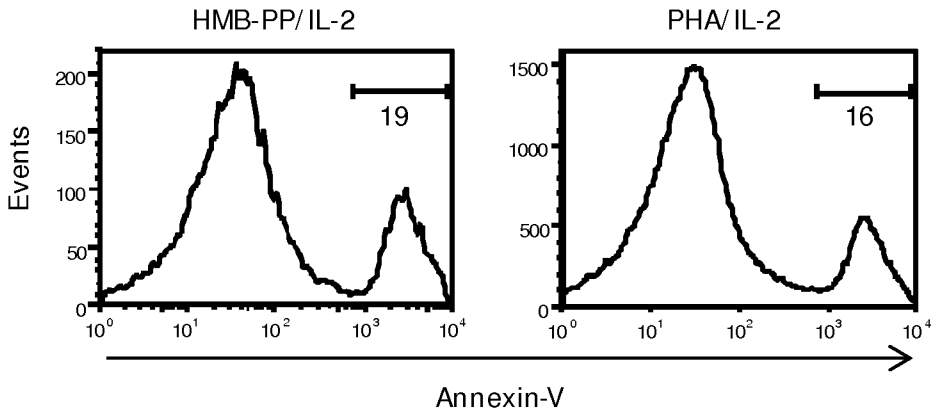


Figure 8

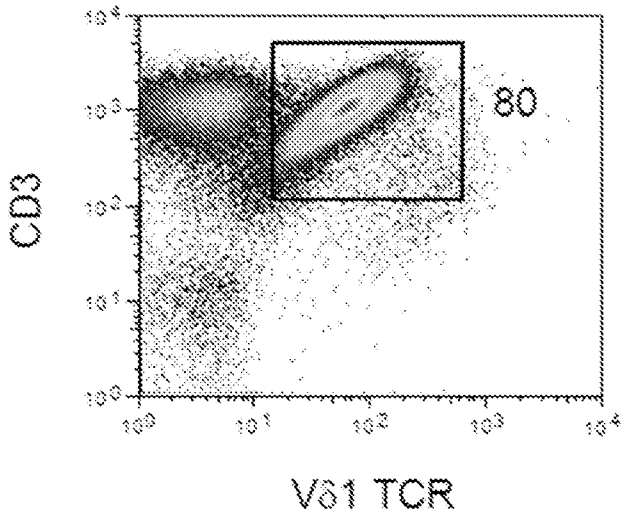


Figure 9

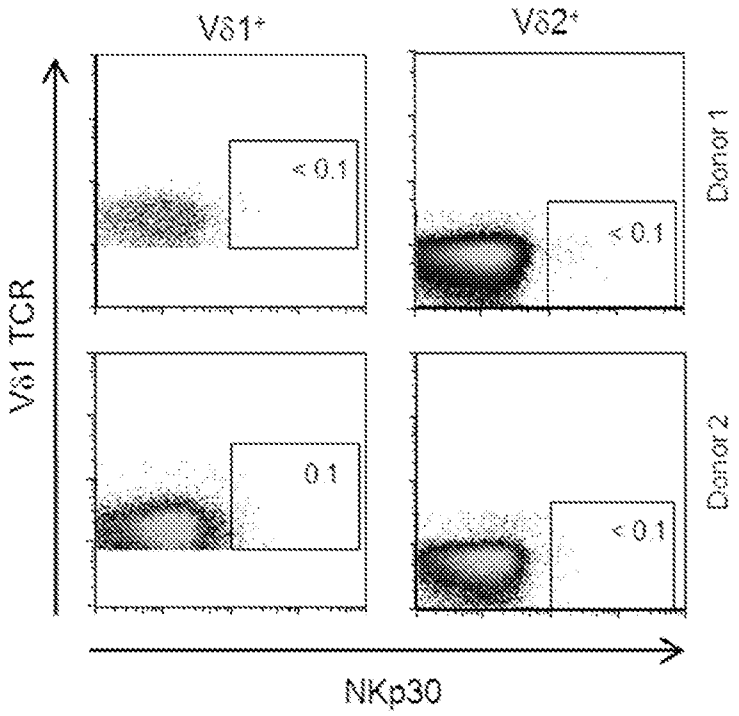


Figure 10

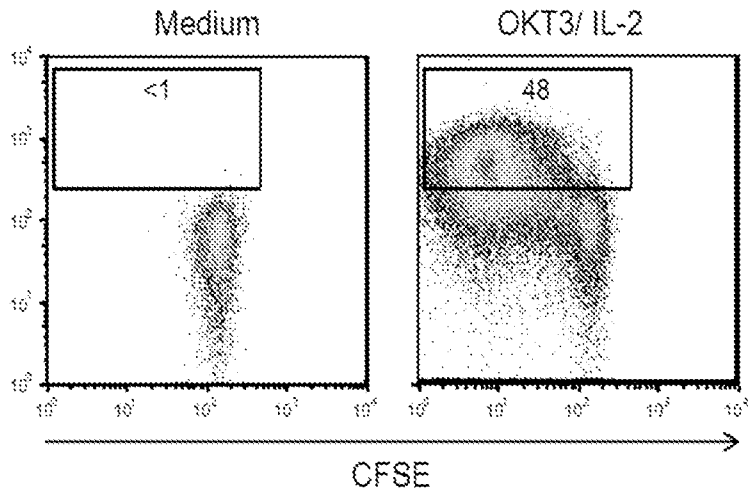


Figure 11

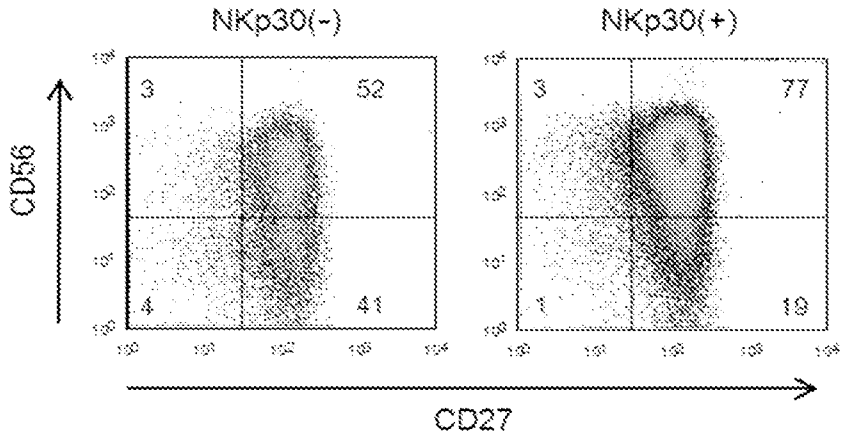


Figure 12

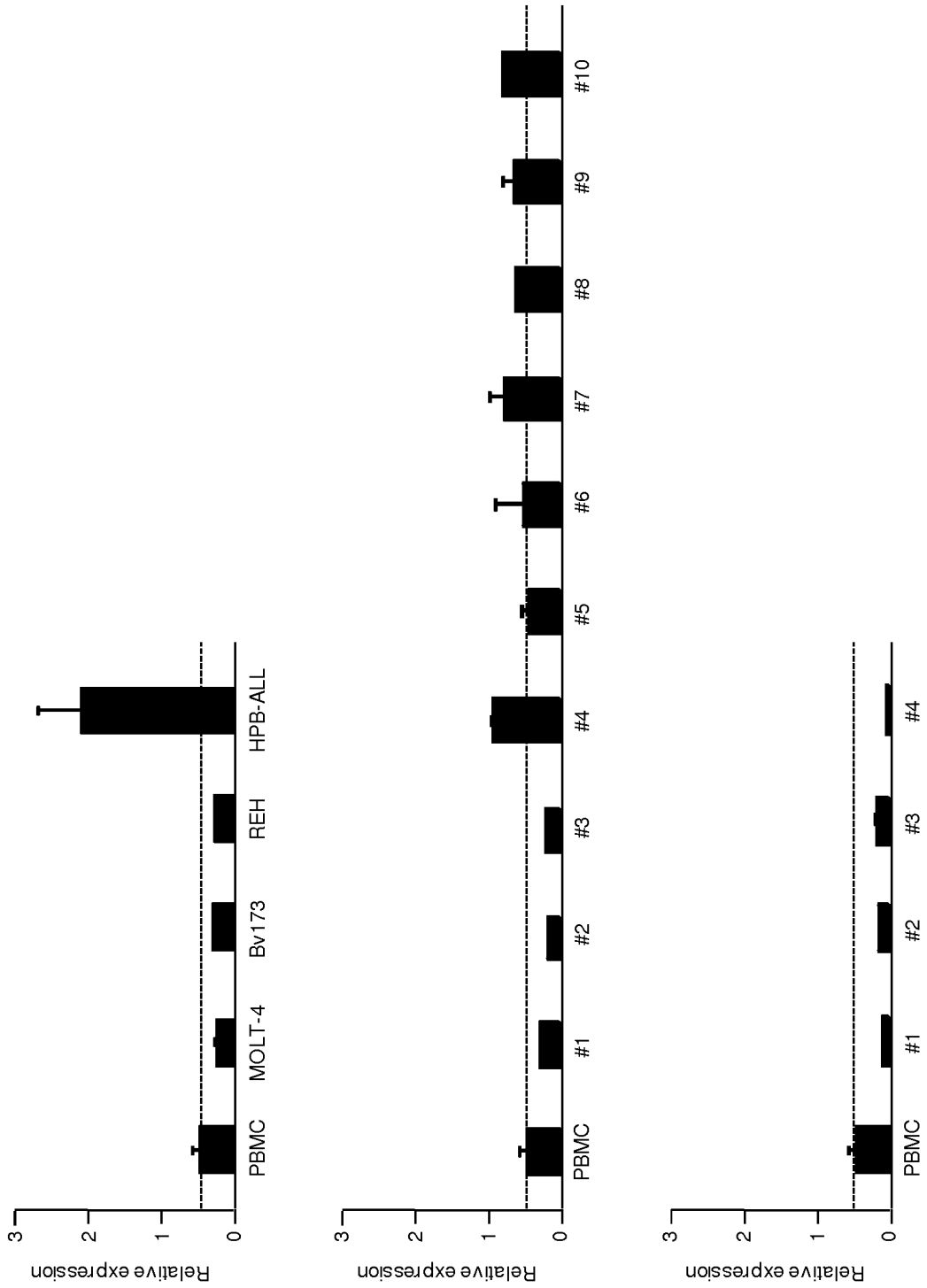

Relative expression
0
1
2
3
PBMC
MOLT-4
Bv173
REH
HPB-ALL
PBMC
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
PBMC
#1
#2
#3
#4

Figure 13

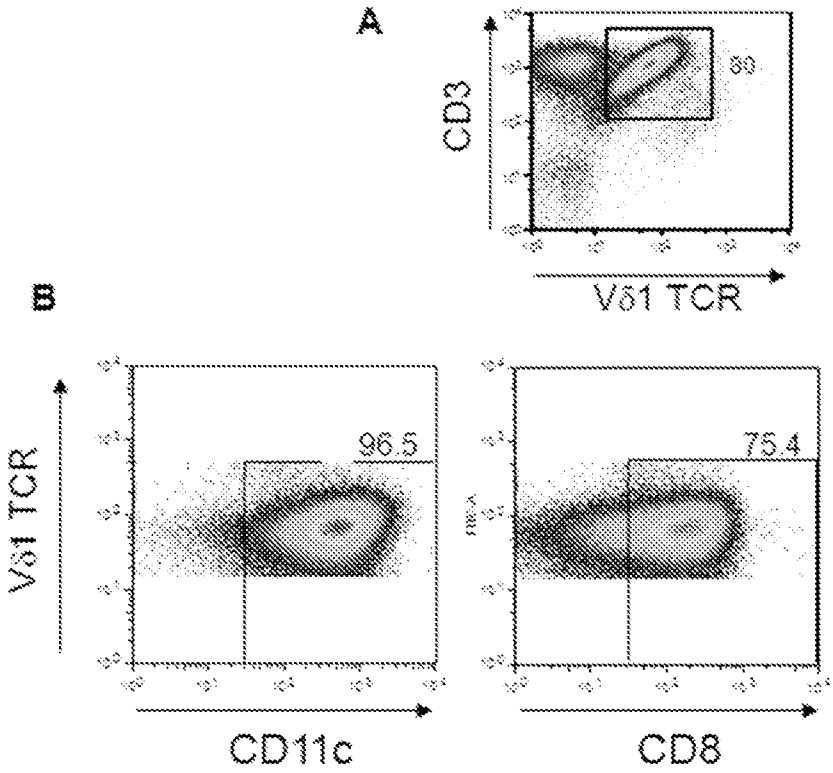


Figure 14

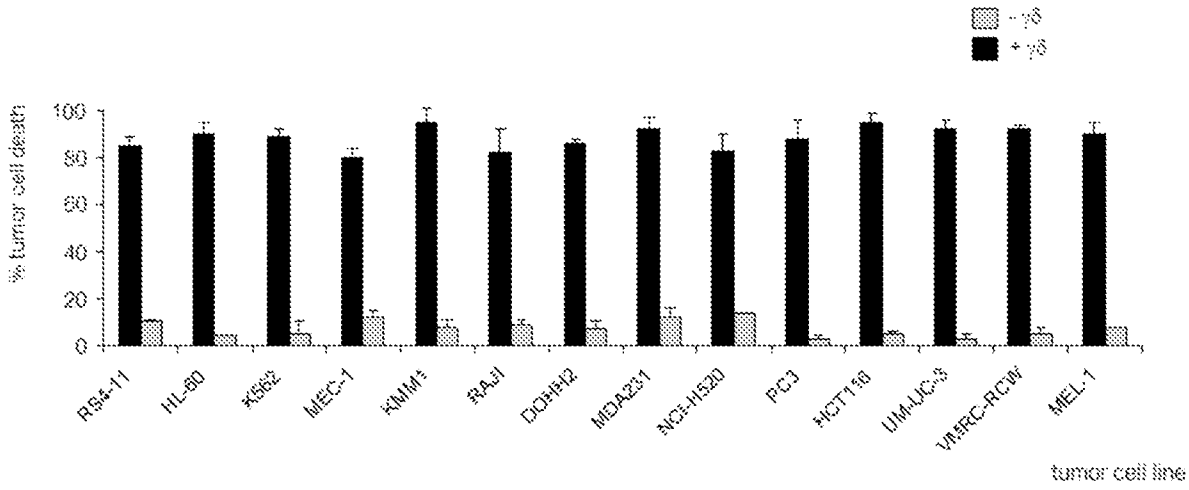


Figure 15