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Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent anti-tumor activity

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Abstract

Chimeric antigen receptors (CARs) have been used to redirect the specificity of autologous T-cells against leukemia and lymphoma with promising clinical results.(1–3) Extending this approach to allogeneic T-cells is problematic as they carry a significant risk of graft-versus-host disease (GVHD).

Natural killer (NK) cells are highly cytotoxic effectors, killing their targets in a non-antigen specific manner without causing GVHD. Cord blood (CB) offers an attractive, allogeneic, off-the-self source of NK cells for immunotherapy. We transduced CB-derived NK cells with a retroviral vector incorporating the genes for CAR-CD19, IL-15 and inducible caspase-9-based suicide gene (iC9), and demonstrated efficient killing of CD19-expressing cell lines and primary leukemia cells in vitro, with dramatic prolongation of survival in a xenograft Raji lymphoma murine model. IL-15 production by the transduced CB-NK cells critically improved their function. Moreover, iC9/CAR.19/IL-15 CB-NK cells were readily eliminated upon pharmacologic activation of the iC9 suicide gene. In conclusion, we have developed a novel approach to immunotherapy using

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engineered CB-derived NK cells which are easy to produce, exhibit striking efficacy and incorporate safety measures to limit toxicity. This approach should greatly improve the logistics of delivering this therapy to large numbers of patients, a major limitation to current CAR-T cell therapies.

INTRODUCTION

Chimeric antigen receptors (CARs) that redirect the specificity of autologous T-cells against lymphoid malignancies have produced striking clinical results.(1–6) Nonetheless, CAR-modified T-cells have a number of limitations. The generation of an autologous product for each individual patient is logistically cumbersome and restrictive for widespread clinical use. The manufacturing of CAR T-cells often takes several weeks, making it impractical for patients with rapidly advancing disease. Furthermore, it is not always possible to generate clinically relevant doses of CAR T-cells from heavily pre-treated, often lymphopenic patients. A previously collected allogeneic product could overcome these limitations; however, allogeneic T-cells (even if HLA-matched) carry a risk of graft-versus-host disease (GVHD),(7) mediated through their native αβ T-cell receptor.

Natural killer (NK) cells provide an attractive alternative to T-cells for CAR engineering. NK cells do not cause GVHD,(8, 9) and thus open opportunities to produce an off-the-shelf product for immediate clinical use. Moreover, as engineered NK cells should also retain their full array of native receptors, they have the potential to exert cytotoxicity(10) through mechanisms other than that dictated by the specificity of the CAR, which in principle could reduce the risk of relapse mediated by loss of CAR-targeted antigen, as reported for CAR-T cell therapy.(11)

Functional NK cells can be derived from several sources.(9, 12, 13) Autologous NK cells can be reproducibly generated in vitro, but have limited activity against autologous tumors,(14, 15) which may not be overcome by CAR engineering. Cord blood (CB) is a readily available source of allogeneic NK cells with clear advantages. CB is available as an off-the-shelf frozen product, an advantage that has been bolstered by methods to generate large numbers of highly functional NK cells from frozen CB units ex vivo.(16) The generation of CAR-transduced NK cells from frozen CB units stored in large global CB bank inventories holds promise for widespread scalability that cannot be replicated with individual adult donors who require screening and leukapheresis. However, a major disadvantage of NK cells is their lack of persistence after adoptive transfer in the absence of cytokine support.(17) Finally, CAR-engineered NK cells may also exert potentially serious toxicity, such as cytokine release syndrome (CRS) or off-tumor/on-target toxicity, as reported with CAR T-cells.(18)

Here, we present a novel approach to the generation of CAR-CD19+ NK cells that we believe addresses the limitations described above. We genetically modified NK cells with a retroviral vector (iC9/CAR.19/IL15) that (i) incorporates the gene for CAR.19 to redirect their specificity; (ii) ectopically produces IL-15, to support their survival and proliferation,(19, 20) and (iii) expresses a suicide gene, inducible caspase-9 (iC9), that can be pharmacologically activated to eliminate transduced cells.(21) We investigated whether these

genetic modifications would enable CB NK cells to persist in sufficient numbers to effectively kill B-cell malignancies.

METHODS

Cell lines

K562-based feeder cells expressing membrane-bound IL-21 and CD137-ligand (Clone 9.mbIL21)(16) were generously provided by Laurence Cooper, MD Anderson Cancer Center (MDACC). Clone 9.mbIL21 cells co-express CD64/FcγRI, CD86/B7-2, CD137L/4-1BBL, truncated CD19, and membrane-bound IL-21 was recently reported to promote peripheral blood and CB NK cell expansion (16, 22). Raji (Burkitt lymphoma cell line) and K562 (erythroleukemia cell line) were purchased from ATCC (Manassa, VA).

Patient details

Primary CLL cells from 6 patients were used for in vitro studies of NK-CAR cytotoxicity. Patient characteristics are summarized in Supplementary Table 1.

Plasmid construction and retrovirus production

The retroviral vectors encoding iC9.CAR19.CD28-zeta-2A-IL-15 and firefly luciferase (FFLuc) have been described.(19, 23) Transient retroviral supernatants were produced as previously described.(23)

Generation of CAR-modified NK cells

CB units for research were provided by the MDACC CB Bank and peripheral blood mononuclear cells (PBMCs) were collected from CLL patients following informed consent, under IRB-approved protocols. CB and PBMCs were isolated by a density-gradient technique (Ficoll-Histopaque; Sigma). CD56+ NK cells, purified with an NK isolation kit (Miltenyi Biotec, Inc., San Diego, CA), were stimulated with irradiated (100 Gy) Clone 9 (2:1 feeder cell:NK ratio) and recombinant human IL-2 (Proleukin, 200 U/mL; Chiron, Emeryville, CA) in complete Serum-free Stem Cell Growth Medium (SCGM) (CellGenix GmbH, Freiburg, Germany) on day 0. Activated NK cells were transduced with retroviral supernatants on day +4 in human fibronectin-coated plates (Clontech Laboratories, Inc., Mountain View, CA). Five days later (day +9), NK cells were stimulated again with irradiated Clone 9 and IL-2. On day +14, CAR-transduced NK cells were harvested for use.

CAR expression and immunophenotype of transduced cells

Transduced CB-NK cells were stained with Alexa-Fluor647 affinity-purified $F(ab')_2$ fragment goat anti-human IgG (H+L) antibody (CAR Ab) (Jackson ImmunoResearch, West Grove, PA) for CAR expression (see supplementary material for details of antibodies used to phenotype NK cells).

IL-15 cytokine secretion

IL-15 production was measured with the human IL-15 Quantikine ELISA kit (R&D) following the manufacturer's instructions.

Intracellular cytokine production

On day 14 of culture, control non-transduced (NT) and CAR-transduced CB-NK cells (0.25×10^6 cells/well) were cocultured for 5 hours in 96-well plates with purified CLL cells, Raji cells, or K562 targets (positive control) at an effector: tumor cell ratio (E:T) of 5:1. CD107a degranulation and intracellular cytokine production were measured as previously described.(14) (See supplementary material for assay details).

NK cell proliferation and cytotoxicity assays

To evaluate for autonomous NK cell growth, we maintained control NT and iC9/CAR.19/IL15$^+$ NK cells in SCGM without stimulation or addition of exogenous cytokines. Cells were cultured for 42 days, and counted using trypan blue exclusion every 3 days.

To assess cytotoxicity, CAR-transduced and NT-NK cells were cocultured with ^{51}Cr-labeled CLL, Raji and K562 targets (positive control) at multiple E:T ratios; cytotoxicity was measured by ^{51}Cr-release as previously described,(14) the findings are reported as specific lysis relative to K562 targets.(24) For HLA-blocking experiments, the anti-HLA-ABC clone W6/32 (Biolegend) was used.

Confocal microscopy and measurement of MTOC polarization

Conjugates were imaged by sequential scanning with a Leica TCS SP8 laser scanning confocal microscope as previously described.(25) Details are included in the supplementary material.

Xenogeneic lymphoma models

To assess the persistence and anti-tumor effect of CAR-transduced CB-NK cells in vivo, we used a NOD/SCID IL-2Rγnull (NSG) xenograft model, with the aggressive NK-resistant Raji cell line. Mouse experiments were performed in accordance with NIH recommendations under protocols approved by the Institutional Animal Care and Use Committee.

Anti-tumor effect of CAR-transduced CB-NK cells

NSG mice (10–12 weeks old; Jackson Laboratories, ME) were irradiated with 300 cGy and inoculated intravenously (i.v.) with FFLuc-labeled Raji cells (2×10^5) on day 0. Where indicated, 10×10^6 fresh, aAPC-expanded NT or CAR-transduced CB-NK cells were injected through the tail vein on days 0 and 7. Mice were subjected to weekly bioluminescence imaging (BLI; Xenogen-IVIS 200 Imaging system; Caliper, Waltham, MA). Signal quantitation in photons/second (p/s) was performed by determining the photon flux rate within standardized regions of interest using Living Image software (Caliper).(16) In selected experiments, mice received NT-NK cells or CB-NK cells transduced with CAR.19 (lacking IL-15 in the construct) plus low dose recombinant human IL-15 (Miltenyi Biotech) intra-peritoneally (i.p.) at the dose of 0.5 μg/mouse (i.e. 2.500 Units/mouse) on the day of NK cell infusion and thereafter every 2–3 days for 2 weeks, following established protocols. (26).

Trafficking, persistence and expansion of transduced vs. NT-NK cells were measured by flow cytometry.

Activation of suicide gene in vitro and validation in vivo

The small molecule dimerizer AP1903 (10nM), generously provided by Bellicum Pharmaceuticals, Inc. (Houston, Texas), was added to NK cell cultures for 4 hours. The elimination of transduced cells was evaluated by Annexin-V/7-AAD staining. The efficacy of the suicide gene was tested in vivo by treating tumor-bearing mice that had received iC9/CAR.19/IL15^{+} NK cells with two doses of AP1903 (50 μg each) intraperitoneally (i.p.), 2 days apart.(19)

Karyotyping and single nucleotide polymorphism (SNP) microarray analysis

Standard karyotyping and SNP analysis were performed in the MDACC Cytogenetics Laboratory (See supplementary material for details).

Pathologic analysis

Mice were euthanized and necropsied 10 months after the first treatment, at the age of 13 months. Blood samples were collected immediately after euthanasia and analyzed with Advia 120. Formalin-fixed tissues were embedded in paraffin blocks, cut into 4-μm thick sections, H&E-stained and examined microscopically by a trained pathologist.

Statistical analysis

Student's *t* test was used to compare quantitative differences (mean ± SD) between samples; p values were two sided and $p<0.05$ was considered significant. For all bioluminescence experiments, intensity signals were summarized as means±SD at baseline and at multiple subsequent timepoints for each group of mice.(16) Probabilities of survival were calculated using the Kaplan Meier method.

RESULTS

CB-NK cells can be stably transduced with a retroviral vector to express iC9/CAR.CD19/IL-15

Two million NK cells were isolated from banked CB units and cultured with Clone 9 and IL-2 for 14 days (non-transduced [NT] control) or transduced on day +4 with a retroviral vector expressing iC9/CAR.19/IL15 and cultured for an additional 10 days (see Methods). NK cell viability on day 4 after transduction was ≥95% in all cases. The median CAR-NK transduction efficiency on day 14 of culture was 66.6% (range, 47.8–87.4%; n=18) (Supplementary Fig. 1A). Table 1 summarizes data on fold-expansion and absolute CAR-NK counts from 5 different CB units. After 14 days of culture, the median NK cell expansion was 2222-fold (range 564–7370).

We studied the stability of CAR expression over time by culturing CAR-transduced CB-NK cells from 6 different CB units for a total of 7 weeks. CAR expression remained stable over this interval, as determined by flow cytometry every 10–14 days. In addition, there was no significant difference in the expression level of CAR when NK cells were transduced with

iC9/CAR.19/IL-15 or CAR19 (lacking IL-15). Representative FACS plots and a summary of the data are presented in Supplementary Fig 1B–C.

Transduction with iC9/CAR.19/IL-15 enhances CB-NK cytotoxicity against CD19+ tumor targets in vitro

We tested whether engineering CB-NK cells to express iC9/CAR.19/IL15 enhanced their cytotoxicity against CD19-expressing tumors compared to expanded, non-transduced (NT) CB-NK cells. iC9/CAR.19/IL15 CB-NK cells and NT CB-NK cells cultured for 14 days were co-incubated with Raji (n=18) (Fig 1A) or primary CLL cells (n=6) (Fig 1B) at different E:T ratios and their cytotoxicity was tested using a standard ^{51}Cr-release assay. Across all E:T ratio, CAR-transduced CB-NK cells exerted superior killing of Raji and CLL cells compared to NT-NK cells. CAR-transduced NK cells were equally efficient as NT-NK cells in killing K562 targets (Fig 1A,B), indicating that the enhanced killing of CD19 targets by the transduced cells is mediated by the CAR receptor and not related to a non-specific enhancement in NK cytotoxicity.

To confirm that the enhanced cytotoxic activity against CD19 targets is derived from the iC9/CAR.19/IL15-expressing fraction of the product, we measured CD107a degranulation and IFN-γ and TNF-α response to Raji and CLL targets (n=8). As shown in Fig 1C, the CAR-positive NK cells were the main source of IFN-γ, TNF-α and CD107a in response to CD19+ targets, compared to the CAR-negative fraction, while the CAR-positive and CAR-negative fractions showed similar effector function against K562 cells.

CB-derived iC9/CAR.19/IL15-transduced NK cells have superior cytotoxicity against primary CLL targets, compared to CLL patient-derived NK cells transduced with the same vector

We generated both NT and iC9/CAR.19/IL15-transduced NK cells from 2 CLL patients using the methodology described above and tested their ability to lyse autologous CLL cells in 3 independent experiments. We also compared their cytotoxicity with those of CB-derived iC9/CAR.19/IL15-transduced and NT-NK cells. Expanded NT-NK cells from CLL patients and CB were equally poorly cytotoxic against CLL targets (Supplementary Fig 2A). However, whereas CAR-transduced CB-NK cells could efficiently kill CLL cells, expression of the same vector by NK cells from patients only modestly increased their cytotoxicity against autologous CLL targets, suggesting that transduced NK cells from CLL patients will be less effective immunotherapy than healthy CAR-transduced CB cells. To investigate whether an inhibitory effect of KIR/self-HLA interaction could have impaired the cytotoxicity of patient-derived CAR-NK cells against autologous CLL cells, we repeated the experiments in the presence or absence of HLA class-I blocking (Supplementary Fig 2B). This intervention only partially improved the cytotoxicity of patient-derived CAR-transduced NK cells against autologous targets. CLL cells express high levels of HLA-E (Supplementary Figure 2C), the ligand for the inhibitory receptor NKG2A. Thus, to determine if an inhibitory effect of NKG2A/HLA-E interaction could influence patient-derived CAR-NK killing of autologous CLL cells, we repeated the experiments in the presence or absence of an NKG2A blocking antibody (clone Z199, Beckman Coulter). As shown in Supplementary Figure 2E, NKG2A blocking significantly improved the ability of

both NT-NK and CAR.19-transduced NK cells to recognize and kill primary CLL cells, without significantly influencing their cytotoxicity against K562 cells (Supplementary Figure 2D). Taken together, these data suggest that multiple mechanisms likely contribute to the relative inability of CAR-transduced patient-derived NK cells to kill autologous targets.

iC9/CAR.19/IL15-transduced CB-NK cells form a stronger immunologic synapse with CLL targets compared to CB-NK cells transduced with CAR.CD19 (without IL-15) or patient-derived iC9/CAR.19/IL15-transduced NK cells

To gain insight into the specific mechanisms by which iC9/CAR.19/IL15-transduced CB-NK cells mediate superior cytotoxicity and the contribution of IL-15 to this effect, we performed qualitative and quantitative assessments of immunologic synapse formation in human NK cells. We first asked whether CAR molecules accumulate at the immunologic synapse (IS) between iC9/CAR.19/IL15-transduced CB-NK cells and CLL targets and whether this recruitment was CD19 antigen-specific. Using confocal microscopy, we observed significantly higher accumulation of CAR molecules at the IS formed between CAR.19/IL15 CB-NK cells and CLL targets (Fig 2A–C) compared to the diffuse presence of CARs at the IS with K562 targets (Fig 2B,C), indicating that CAR molecules participate in IS formation in a CD19-dependent manner.

Increased polarization of the microtubule-organizing center (MTOC) is an essential step in the final stages of NK cell-mediated cytotoxicity and exocytosis of lytic granules at the IS. (27) Thus, we used confocal microscopy to assess the MTOC polarization of NT CB-NK cells, iC9/CAR.19/IL15$^+$ CB-NK, iC9/CAR.19/IL15$^+$ CLL patient-derived NK cells and CAR.19-transduced CB-NK (lacking IL-15) in experiments with CLL targets. MTOC polarization was quantified by measuring the distance between the pericentrin-defined MTOC to the IS for at least 15 conjugates, including all four groups of NK cells and CLL targets. MTOC polarization against K562 targets was used as control as NK cells form a “natural” synapse with K562 targets through multiple activating receptors on their surface. As shown in Fig 2C and Supplementary Fig. 3, MTOC was significantly closer to the IS in iC9/CAR.19/IL15-transduced CB-NK cells compared to any of the remaining groups (Fig 2C, left panel). CLL patient-derived iC9/CAR.19/IL15 NK cells did show improved polarization compared to NT-CB NK cells, but this gain was still significantly less than that seen with CB-NK cells transduced with iC9/CAR.19/IL15. No differences in MTOC polarization were observed among the NK cell groups in the presence of a non-CD19-presenting K562 target (Fig 2C, right panel). These findings provide a mechanistic basis for the enhanced antitumor activity of iC9/CAR.19/IL15-transduced CB-NK cells; namely, engagement of CARs on transduced NK cells with CD19 on target cells results in increased polarization of the MTOC, and superior killing.

IL-15 by iC9/CAR.19/IL15+ CB-NK cells is produced predominantly in response to CD19+ targets and does not induce NK cell anergy

NT CB-NK and iC9/CAR.19/IL15$^+$ CB-NK lymphocytes expanded for 14 days were cultured with or without CLL cells, and IL-15 release was measured at 24, 48 and 72 hours. IL-15 was undetectable in supernatants collected from NT CB-NK cells cultured alone or with CLL targets. By contrast, iC9/CAR.19/IL15$^+$ CB-NK cells produced small amounts of

IL-15 in the absence of antigen stimulation (mean 15.05 pg/mL/10^6 cells, range 6.2–23.47), which significantly increased with antigen stimulation (mean 27.61 pg/mL/10^6 cells, range 15.82–38.18) (Fig. 3A), in keeping with enhanced proliferation of iC9/CAR.19/IL-15+ CB NK cells in response to CLL cells in culture (Supplementary Fig. 4).

To investigate the potential of IL-15 to induce NK anergy, we comprehensively characterized the NK cell phenotype, including expression of activating and inhibitory receptors, exhaustion markers, chemokine receptors and transcription factors on expanded NK products by multiparameter flow cytometry. The heatmap in Fig 3B summarizes the average expression levels of markers from 3 independent CB-NK expansion and transduction experiments. Ex vivo expansion drove the maturation of both NT-NK and iC9/CAR.19/IL15$^+$ as evidenced by expression of CD16 and KIRs, (Fig. 3B and Supplementary Fig. 5) with no selectivity in the subsets of NK cell transduced with the CAR vector. iC9/CAR.19/IL15$^+$ CB-NK cells expanded for 2 weeks showed no signs of exhaustion, such as downregulation of eomesodermin and T-bet,(28), or upregulation of KLRG1, and in fact exhibited a phenotype similar to that of NT-NK cells. Moreover, in contrast to a previous report in murine NK cells that sustained stimulation with IL-15/IL-15R-α complexes induces dysfunction,(29) human iC9/CAR.19/IL15-transduced CB-NK cells proliferated as efficiently as NT CB-NK cells and followed a similar kinetic of in vitro expansion (Fig. 3C).

iC9/CAR.19/IL15-tranduced CB-NK cells exert enhanced anti-tumor activity in vivo

We used our Raji xenograft model to study the in vivo antitumor activity of iC9/CAR.19/IL15-transduced CB-NK cells and the contribution of IL-15 to this effect. Mice received one i.v infusion (10×10^6/mouse) of control NT CB-NK cells, iC9/CAR.19/IL15-transduced CB-NK cells or CAR.19 CB-NK cells lacking IL-15 (5 mice per group). Tumor growth was monitored by measuring changes in tumor bioluminescence over time. Tumor bioluminescence increased rapidly in mice treated with control NT CB-NK cells (Fig. 4A,B). By contrast, infusion of either CAR.19$^+$ or iC9/CAR.19/IL15$^+$ CB-NK cells led to improved tumor control and significant prolongation of survival compared to NT CB-NK or CAR.19 CB-NK cells lacking IL-15 (and p=0.001 and p=0.044, respectively) (Fig. 4C). Notably, iC9/CAR.19/IL15$^+$ CB-NK cells controlled tumor expansion (Fig. 4A) and prolonged survival (Fig. 4C) better than the CB-NK cells transduced with CAR.CD19 without IL-15 (p=0.044), underscoring the vital contribution of IL-15 to enhanced antitumor activity. We also asked if intraperitoneal administration of low doses of recombinant human IL-15 could support the survival potential and anti-tumor efficacy of CAR.19 CB-NK cells (lacking IL15 in the construct). A dose of 0.5 μg/mouse (*i.e.* 2.500 Units/mouse) administered on the day of NK cell infusion and every 2–3 days thereafter resulted in significant expansion of CAR.19 NK cells and control of tumor progression (Supplementary Fig 6A–C); however, it was associated with significant toxicity and early mortality treatment-related mortality (Supplementary Fig 6D).

We next asked if increasing the dose of CB-NK cells could enhance anti-tumor activity by administering two i.v infusions (10×10^6 cells each, 5–7 days apart) of control NT or iC9/CAR.19/IL15$^+$ NK cells (5 mice per group). None of the mice receiving iC9/CAR.19/IL15$^+$ CB-NK cells died of lymphoma (Fig. 4D). However, 3 mice died on days 11, 14 and 16 after

infusion of the cells from complications related to the release of inflammatory cytokines, including high levels of TNF-α (median 999.2 pg/mL), IL-1β (median 1271.4 pg/mL) and IL-18 (5570 pg/mL) detected in serum at the time of death. These data indicate that activated NK cells transduced with iC9/CAR.19/IL15 may also cause CRS similar to that of CAR T-cells, with the potential to cause toxicity in humans.

IL-15 enhances the proliferation, persistence and homing of CAR.CD19-transduced CB-NK cells in a xenograft NSG mouse model of Raji lymphoma

To examine the contribution of IL-15 to the proliferation, persistence and homing of CAR-NK, NSG mice engrafted with Raji lymphoma were treated with two i.v infusions (10×10^6 cells each, 5–7 days apart) of NT CB-NK cells (control), CB-NK cells transduced with iC9/CAR.19/IL15 or CAR.19 (lacking IL-15) as described in Methods (5 mice per group). On day +21 post-NK infusion, mice were sacrificed. High frequencies of CAR-expressing NK cell were identified in blood, bone marrow, liver and spleen of mice treated with iC9/CAR.19/IL15 (Fig. 4E), indicating proliferation and successful homing of CAR-NK cells to sites of disease. Notably, there was no evidence of human CD19+ cells in any of the organs examined, consistent with efficient control of tumor by the iC9/CAR.19/IL15 CB-NK cells. By contrast, in mice treated with either CAR.19 CB-NK cells (lacking IL15) or NT CB-NK cells, proliferation or homing to sites of disease was more limited. Furthermore, CD19+ tumor cells were detected at high frequencies in blood and organs of the mice, suggesting that CAR.19 CB-NK cells lacking IL-15 are capable of controling the tumor for only a short period of time. Moreover, in mice receiving two infusions of iC9/CAR.19/IL15$^+$ CB-NK cells, the cells expanded over time and could be detected up to 68 days post infusion, after which their numbers receded (Fig. 4F). These data indicate that IL-15 in the CAR construct endows CB-NK cells with the capacity to proliferate and persist in vivo in an antigen-driven manner.

iC9/CAR.19/IL15-transduced CB-NK cells do not show signs of dysregulated growth either in vitro or in vivo

To investigate the possibility that the IL-15 gene in the vector may result in autonomous or dysregulated growth of transduced CB-NK cells, we cultured iC9/CAR.19/IL15-transduced CB-NK cells in media without the addition of exogenous IL-2 or clone 9.mbIL21 for 42 days (n=5). Cultured iC9/CAR.19/IL15-transduced CB-NK cells did not show any signs of abnormal growth over 6 weeks (Fig 5A), after which the cells stopped expanding. Karyotyping and SNP microarray analyses of CAR-transduced NK cells after up to 22 weeks of culture (n=7) did not reveal any chromosomal alterations or evidence of genetic instability (data not shown).

Nine mice treated with CB-NK cells transduced with iC9/CAR.19/IL15 (n=5) or CAR.CD19 (lacking *IL-15*) (n=4) were followed for at least 10 months and then sacrificed. The hematologic parameters were within normal ranges (Supplementary Table 2), with no evidence of lymphocytic leukemia in either treatment group (Fig 5B).

iC9/CAR.19/IL15^{+} CB-NK cells are eliminated by activation of the suicide gene with a small-molecule dimerizer

To counteract excessive toxicity mediated by the release of inflammatory cytokines by transduced CB-NK cells, we incorporated a suicide gene (iC9) into our construct.(21) The addition of as little as 10 nM of the small molecule dimerizer AP1903 to cultures of iC9/CAR.19/IL15-transduced CB-NK cells induced apoptosis/necrosis of transduced NK cells within 4 hours but had no effect on the viability of NT CB-NK cells (Fig. 6A–B). The suicide gene was also effective in vivo. Mice engrafted with Raji tumor received iC9/CAR.19/IL15-transduced CB-NK cells. Mice were then either treated with the dimerizer or not (n=5 mice per group) and were sacrificed 3 days later. Administration of the small-molecule dimerizer resulted in a striking reduction in iC9/CAR.19/IL15-transduced CB-NK cells in the blood and tissues of the treated mice (Fig 6C).

DISCUSSION

We have developed a novel approach to engineering NK cells with potent antitumor activity by transducing CB-derived NK cells with a retroviral vector encoding a CAR against CD19, IL-15 (a cytokine crucial for NK cell persistence), and the inducible caspase-9 suicide gene. NK cells transduced with this vector form strong immunologic synapses with CD19-positive targets and effectively kill CD19-expressing leukemia/lymphoma cell lines as well as primary CLL cells. Moreover, when the iC9/CAR.19/IL15-transduced CB-NK cells were infused into an NSG mouse model of Raji lymphoma, they proliferated rapidly in vivo and homed to sites of disease, where they mediated potent antitumor responses.

We chose CD19 as the target for our studies, as proof-of-principle, based on the striking clinical efficacy shown by CAR.CD19+ T-cells against B-lineage cancers(1–6). The signaling domain of our construct contains CD28 and CD3ζ. CD3ζ is crucial for both T and NK cell signaling and activation.(30, 31) While CD28 is well recognized as an important costimulatory molecule for T cell activation,(32) its role in NK cell activation is less clear; however, human fetal NK cells and a number of NK cell lines express CD28 and can kill CD80/CD86-expressing tumor targets, supporting a role for CD28 in the activation of NK cells.(33–35) Further, CD28 ligation in NK cells enhances NK cell killing of its target by phosphorylating ERK2.(27) In our study, NK cells transduced with a CAR incorporating CD28 showed marked antitumor activity, both in vitro and in vivo, although other costimulatory domains, such as 4-1BB,(17) may improve results further.

CAR-NK cells also exert cytotoxicity that is non-CAR.CD19-mediated, as demonstrated by the modest killing of tumor targets by non-transduced NK cells. This could represent an advantage for NK cells over T-cells in CAR-driven immunotherapy, as the intrinsic capacity of NK cells to recognize and target tumor cells remains intact, making disease escape through downregulation of the CAR target antigen less likely than it is with CAR-T cells. One could potentially exploit this property by selecting donors for NK-CAR production based on KIR-ligand mismatch with the recipient, or haplotype B KIR gene content, as shown in the setting of stem cell transplantation.(8, 13, 36–38) Using readily accessible CB units and GMP-compliant procedures for robust expansion (19), it is feasible to generate multiple clinical doses of CAR-NK cells from a single CB unit (Table 1).

Mature NK cells have a short lifespan with poor in vivo persistence both in humans and in mice(39, 40). Although recent data support the existence of long-lived memory NK cells in mice (41, 42) and possibly in humans(43–45), the absence of a reliable and stable marker (or set of markers) to define memory NK cells hinders their selection for immunotherapy. This poses a major limitation on their use for adoptive therapy, as in vivo persistence of effector cells are crucial for sustained clinical responses.(46) We therefore incorporated in our construct the gene encoding IL-15, a cytokine that drives NK cell expansion and persistence. (19, 20, 47) This modification led to ectopic production of IL-15, which was predominantly antigen-driven, and to more robust activation of NK cells with enhanced in vivo proliferation, persistence and anti-tumor activity than that seen with CAR.19-transduced NK cells lacking IL-15. Although the latter could mediate an antitumor response, the effect was only transient, further emphasizing the importance of in vivo persistence of CAR-expressing NK cells for effective and durable antitumor immunity. We also examined if exogenous administration of IL-15 could support the in vivo proliferation and anti-tumor activity of CAR19-transduced NK cells, thus, overcoming the requirement to include IL-15 in the construct. However, IL-15, even when administered at a low dose of 0.5 μg/mouse every 2–3 days(26) was associated with significant toxicity when administered in combination with CAR.CD19-transduced CB-NK cells (but not non-transduced NK cells), supporting our strategy to include IL-15 in the construct. It is conceivable that ectopic IL-15 production could lead to abnormal NK-cell proliferation or leukemia transformation.(48) In the present study only picogram quantities of IL15 were produced by our CAR-transduced CB-NK cells, without evidence of autonomous growth in vitro or leukemic transformation in vivo.

Severe toxicity, including on-target/off-tumor effects and CRS is a major clinical limitation of CAR-T cell therapy.(49) These concerns may also be relevant to CAR-NK cells. Human NK cells predominantly produce IFN-γ, IL-3 and GM-CSF (50), which may result in a different pattern and kinetic of CRS. In our study infusion of a higher number of iC9/CAR. 19/IL15-transduced CB-NK cells was associated with a systemic inflammatory response and toxic death in a number of mice. To counteract these potential toxicities, we equipped CAR-modified NK cells with an inducible suicide gene(21) and showed that pharmacologic activation of this molecule could rapidly and efficiently eliminate the gene-modified NK cells.

In conclusion, we have developed a novel approach to immunotherapy using engineered CB-derived NK cells. The iC9/CAR.19/IL15-transduced CB-NK cells are relatively easy to produce, show striking efficacy both in vitro and in vivo, and incorporate safety measures that are designed to limit toxicity. Clinical trials of these CAR-NK cells will begin shortly at our center.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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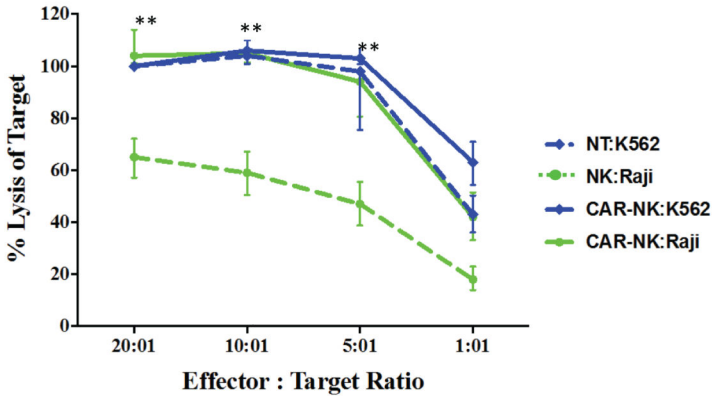
1A



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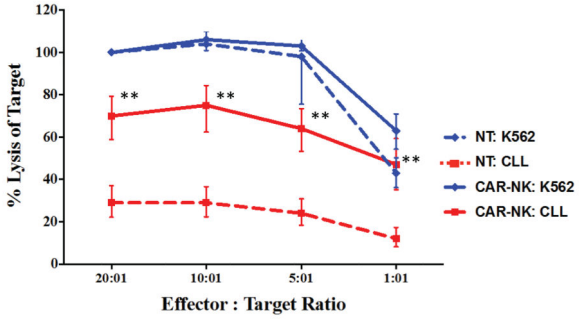


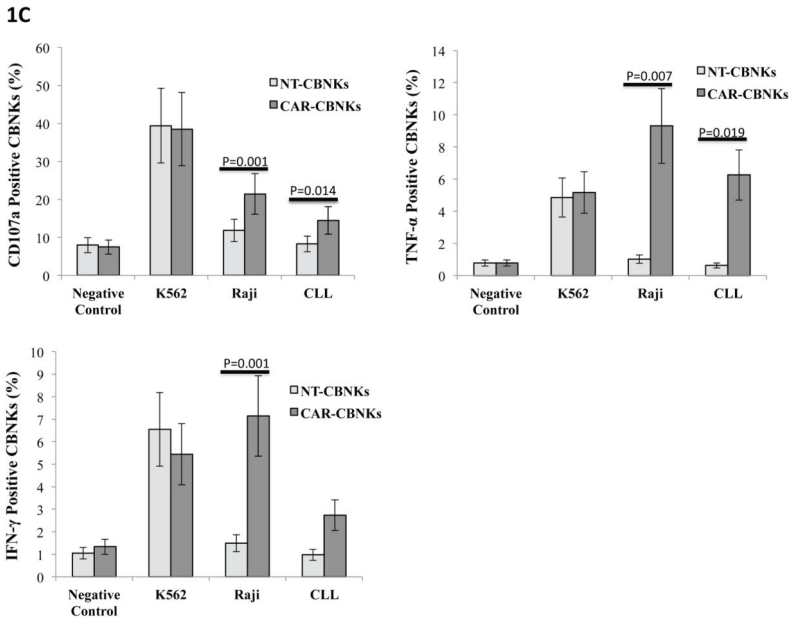


Fig. 1. Anti-tumor function of CB-NK cells transduced with the iC9/CAR.19/IL15 vector

Panels A and B summarize the cytotoxic activity of iC9/CAR.19/IL15-transduced CB-NK cells (CAR, solid lines) vs. NT CB-NK cells (broken lines), as measured by ^{51}Cr release assay, against Raji (n=18) (**A**) and primary CLL cells (n=6) (**B**). CAR transduced NK cell kill CD19 expressing targets more efficiently than non-transduced (NT) ex vivo expanded and activated NK cells ($p<0.001$, all comparisons). CAR-transduced NK cells (solid blue line) were equally efficient as NT NK cells (broken blue line) in killing K562 targets. Data are presented as specific lysis relative to K562 targets(20) to correct for inter-donor variability in killing. (**C**) Cytokine production and CD107a degranulation by flow cytometry in gated CAR positive vs. CAR negative NK cells in response to different stimuli in 8 independent experiments, showing that CAR transduction of CB-NK cells significantly increased their cytokine effector response (IFN-γ and TNF-α production) and CD107a degranulation to the CD19-expressing Raji cell line and primary CLL cells. The effector function of both iC9/CAR.19/IL15-transduced CB-NK-CAR and non-transduced NK cells against K562 was similar.

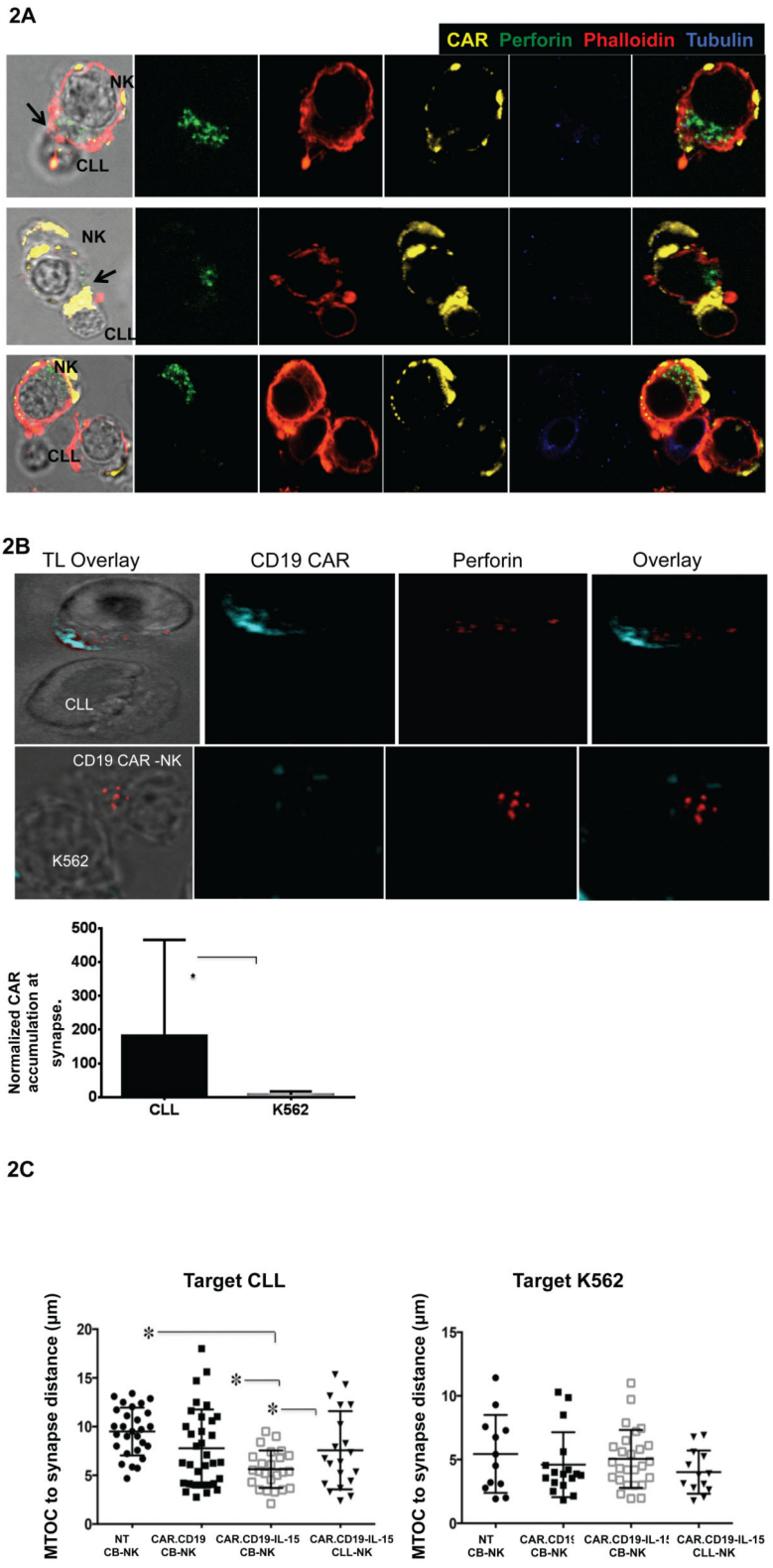
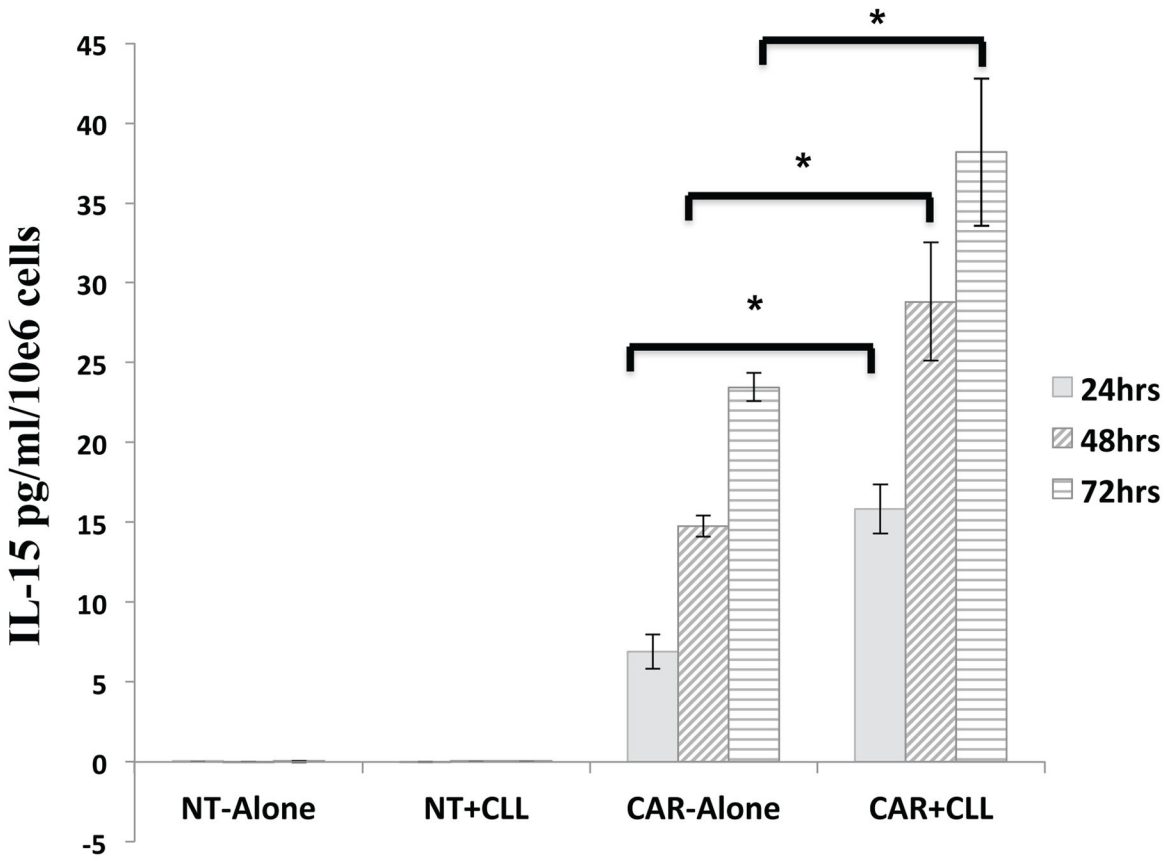
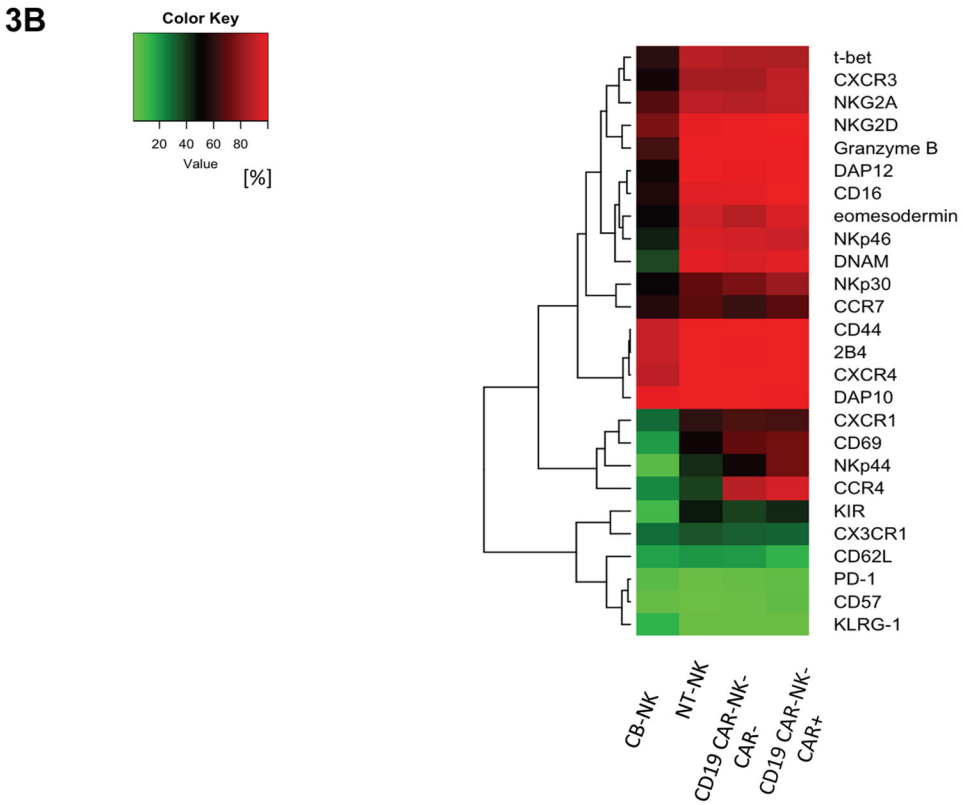


Fig. 2. Assessments of immunologic synapse formation and function in human iC9/CAR.19/IL15-transduced CB-NK cells

(**A**) Confocal microscopy showing representative synapse images of CB-NK cells (transduced with iC9/CAR.19/IL15) conjugated to primary CLL cells. Conjugates were stained with anti-perforin (green), phalloidin-F-actin (red) and anti-CD19-CAR (yellow). Note formation of immunological synapse (black arrow; left panels). (**B- top panel**) Confocal representative images (original magnification ×100) demonstrating that CD19-specific CAR on NK cells preferentially accumulates at the CLL (target) cell synapse and not at the K562 (non-target) cell synapse. Cells were imaged in Z stacks covering the entire volume of the immunological synapse. Imaging was performed on a Leica TCS SP8 confocal microscope using a 100X oil objective. Images were acquired with Imaris software (Bitplane). Transmitted light (TL) overlay, single-color anti–CD19 CAR (blue), anti-perforin (red) and an overlay of all stains are shown. (**B- bottom panel**) summarizes data on the accumulation of CD19-specific CARs at the immunologic synapse between CB-NK cells transduced with iC9/CAR.19/IL15 vector with CLL cells (CD19 positive) vs. K562 targets (CD19 negative). *p= 0.02. There was significantly more accumulation of CD19-specific CARs at the immunologic synapse with CLL cells compared to K562 targets. **(C)** iC9/CAR.19/IL15-transduced CB NK cells, CAR.19-transduced CB NK cells (without IL-15) and CLL patient-derived NK cells transduced with iC9/CAR.19/IL15 were assessed and compared with non-transduced NK cells for their ability to polarize lytic granules and MTOC to CLL targets (left panel) vs. K562 cells (right) (measured by distance from the MTOC to the immune synapse). Results from two independent experiments are shown; each data point represents a single immunologic synapse. Cells were imaged as a Z stack on a Leica TCS SP8 laser scanning microscope. Images were acquired with Volocity software (PerkinElmer). The asterisk indicates statistical significance ($P < 0.05$ by Student's t test) vs. the control or another CAR construct.

3A





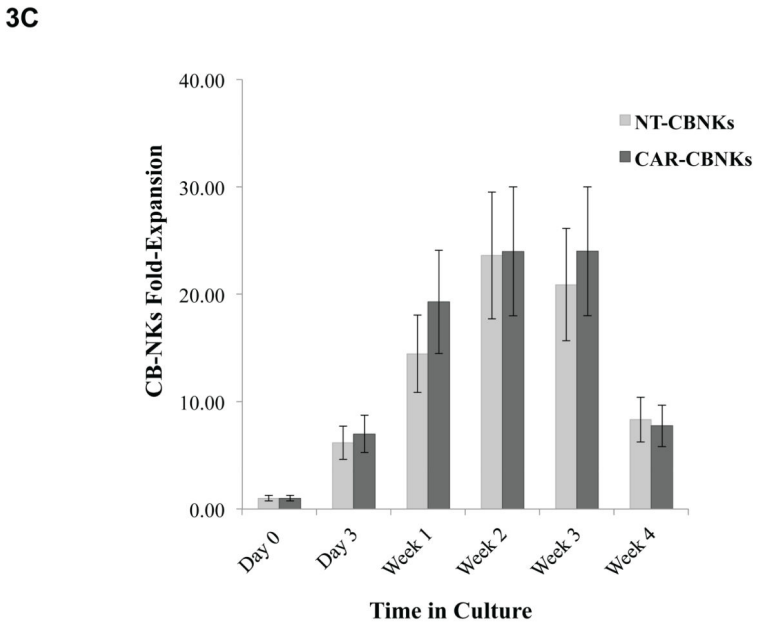
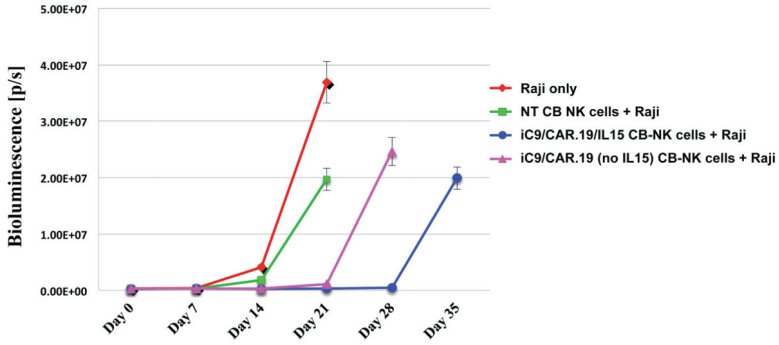


Fig. 3. IL-15 production and phenotype of iC9/CAR.19/IL15-transduced CB-NK cells

(A) IL-15 production by NT-NK cells or CAR transduced NK cells cultured in the presence or absence of CLL targets for 24h, 48h or 72hs in 4 independent experiments. For each time point, secretion of IL-15 by iC9/CAR.19/IL15-transduced CB NK cells was greater in the presence of antigenic stimulation in the form of CLL targets compared to iC9/CAR.19/IL15-

transduced CB NK cells cultured alone ($p<0.05$ in all 3 cases). **(B)** CB-NK cell phenotype based on the average expression of 25 markers, including NK cell receptors, transcription factors, adaptor molecules, homing receptors and markers of exhaustion, in triplicate experiments. MFI or the percentages of positive cells were submitted to a hierarchical clustering program to generate a global view of marker expression in iC9/CAR.19/IL15-transduced NK vs. non-transduced (NT) CB-NK cells (n=3 independent NK expansion and transduction experiments using different CB units). The transduced cells lacked any phenotypic evidence of exhaustion and maintained a phenotype similar to that of NT CB-NK cells. (**C**) Proliferative capacity of CAR-transduced vs. NT CB-NK expansion in response to in vitro stimulation with clone 9 and IL-2 (200 iU/mL). The kinetics of iC9/CAR.19/IL15 NK fold expansion in vitro was similar to NT-NKs (starting from 2 $x10^6$ CB-NK cells; N=5).

4A



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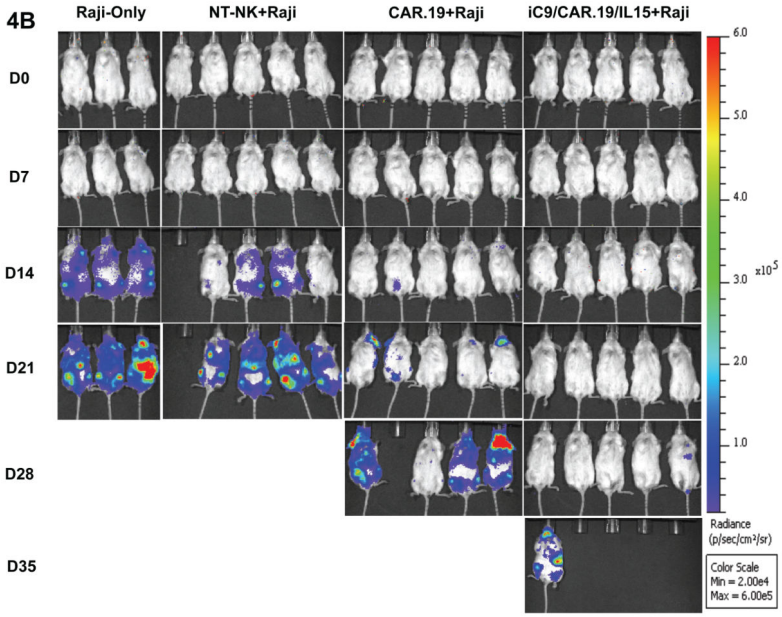

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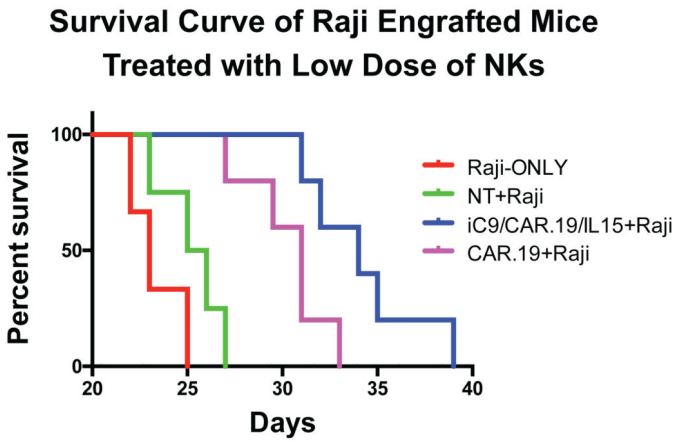
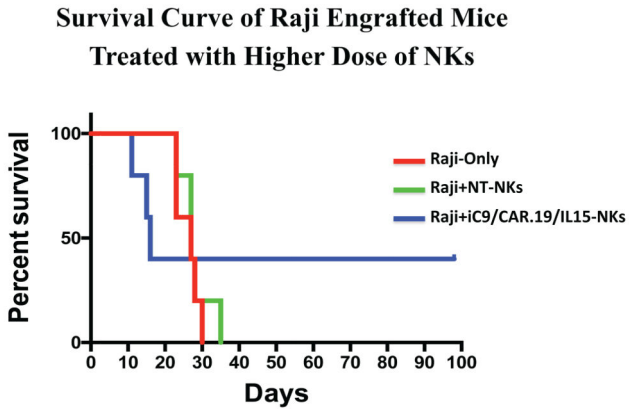


***P*-values:**

- ***p*= 0.044 (iC9/CAR.CD19/IL15 + Raji vs CAR.CD19 + Raji)**
- ***p*= 0.001 (iC9/CAR.CD19/IL15 + Raji vs NT-NKs + Raji)**
- ***p*= 0.006 (CAR.CD19 + Raji vs NT-NKs + Raji)**
- ***p*= 0.182 (NT-NKs + Raji vs Raji-Only)**

4D



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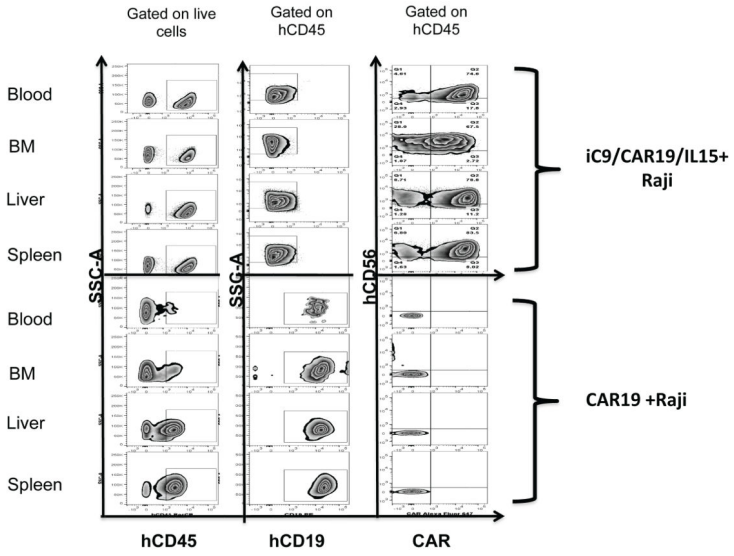

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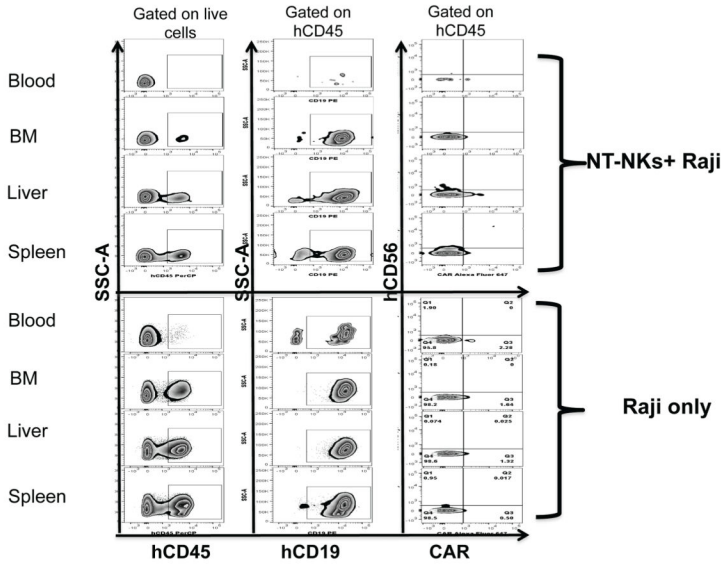


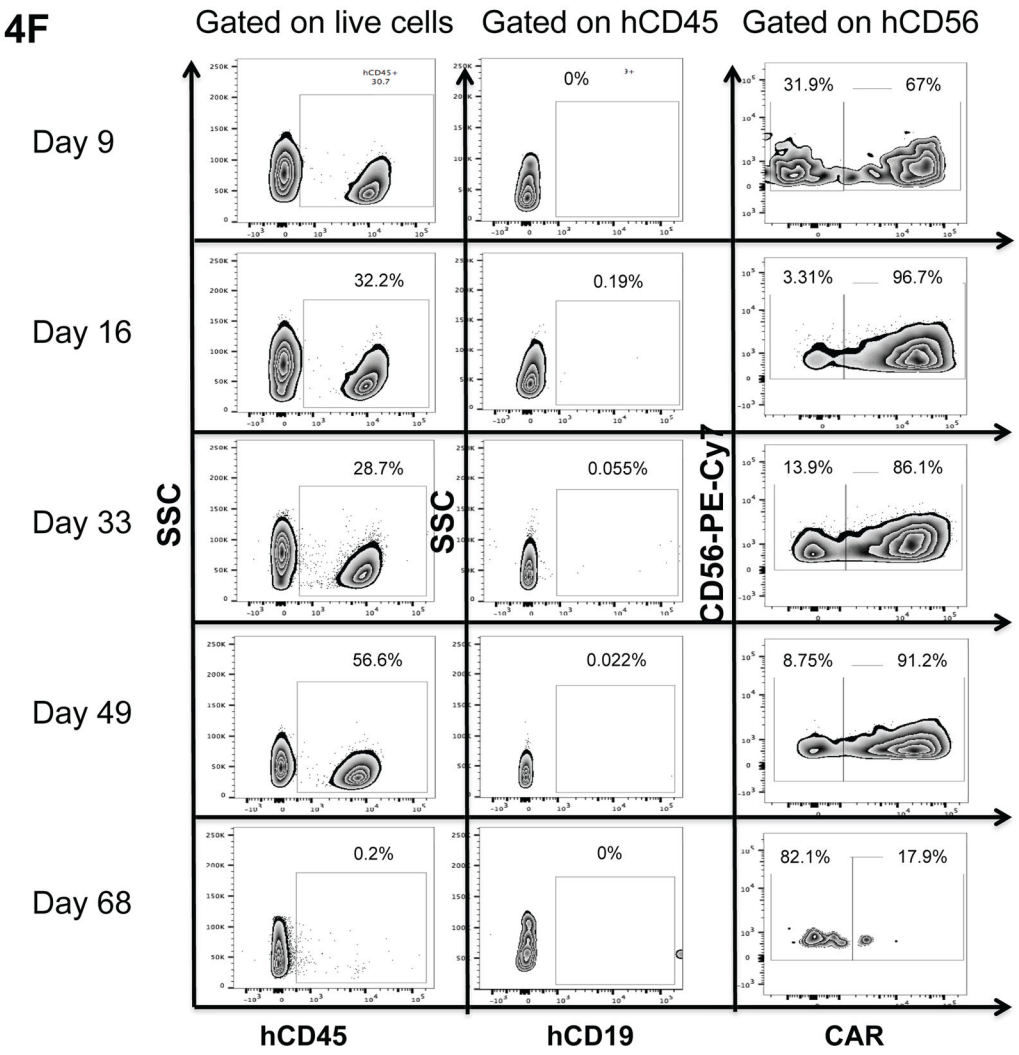
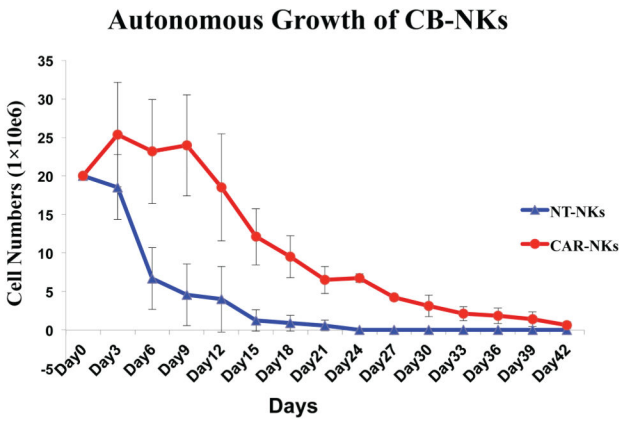


Fig. 4. In vivo homing, proliferation and antitumor activity of iC9/CAR.19/IL15-transduced CB-NK cells in NSG Raji mouse model

(**A**) Bioluminescence imaging was used to monitor the growth of FFluc-labeled Raji tumor cells in NSG mice. The plot summarizes the bioluminescence data from 4 groups of mice treated with Raji alone, or Raji plus one dose (10×10^6) of iC9/CAR.19/IL15 CB-NK cells, CAR.19 (no IL-15) CB-NK cells or NT CB-NK cells (5 mice per group). Infusion of one dose of 10×10^6 iC9/CAR.19/IL15-transduced CB-NK cells into NSG mice engrafted with FFluc-labeled Raji cells results in superior control of tumor (blue line) compared with NT CB-NK cells (green line) and NK cells transduced with CAR.19 lacking IL-15 (pink line). (**B**) BLI figures of the experiments described in panel A. Colors indicate intensity of luminescence (red, highest; blue, lowest). (**C**) Kaplan Meier plots showing the probability of survival for the 4 groups of mice described in Panel A (5 mice per group). Mice treated with a single dose of 10×10^6 iC9/CAR.19/IL15-transduced CB NK cells (blue line) had significantly better survival than mice receiving CB-NK cells that were either not transduced (green line) (p=0.001) or transduced with a CAR.CD19 construct lacking IL-15 (pink line) (p=0.044). (**D**) Kaplan Meier plots showing that infusion of two doses (10×10^6 each, 5 – 7

days apart) of iC9/CAR.19/IL15-transduced CB-NK cells in NSG mice engrafted with Raji cells resulted in better survival, but was associated with early toxicity. P values were computed using the log rank test. (**E**) NSG mice were treated with Raji cells alone or Raji plus two doses (10×10^6 each, 5 – 7 days apart) of iC9/CAR.19/IL15 CB-NK cells, CAR.19 (no IL-15) CB-NK cells or NT CB-NK cells (n=5 mice per group). Mice were sacrificed on day +21 post-infusion and peripheral blood, bone marrow (BM), liver and spleen were harvested and analyzed by flow cytometry for expression of human (h)CD45, hCD19, hCD56 and CAR. Representative FACS plots are presented. iC9/CAR.19/IL15 transduced CB-NK cells home to sites of disease (liver, spleen, BM) more efficiently than CAR.19 transduced CB-NK cells or NT-NK cells and control disease. (**F**) Mice that received Raji cells plus two doses (10×10^6 each, 5 – 7 days apart) of iC9/CAR.19/IL15 CB-NK cells were monitored over time by weekly blood collection for expansion of CAR-expressing NK cells. Serial measurement of CAR expressing NK cells in the peripheral blood of mice by flow cytometry shows iC9/CAR.19/IL15$^+$ CB-NK cells expand over time and could be detected up to 68 days post infusion.

5A



5B

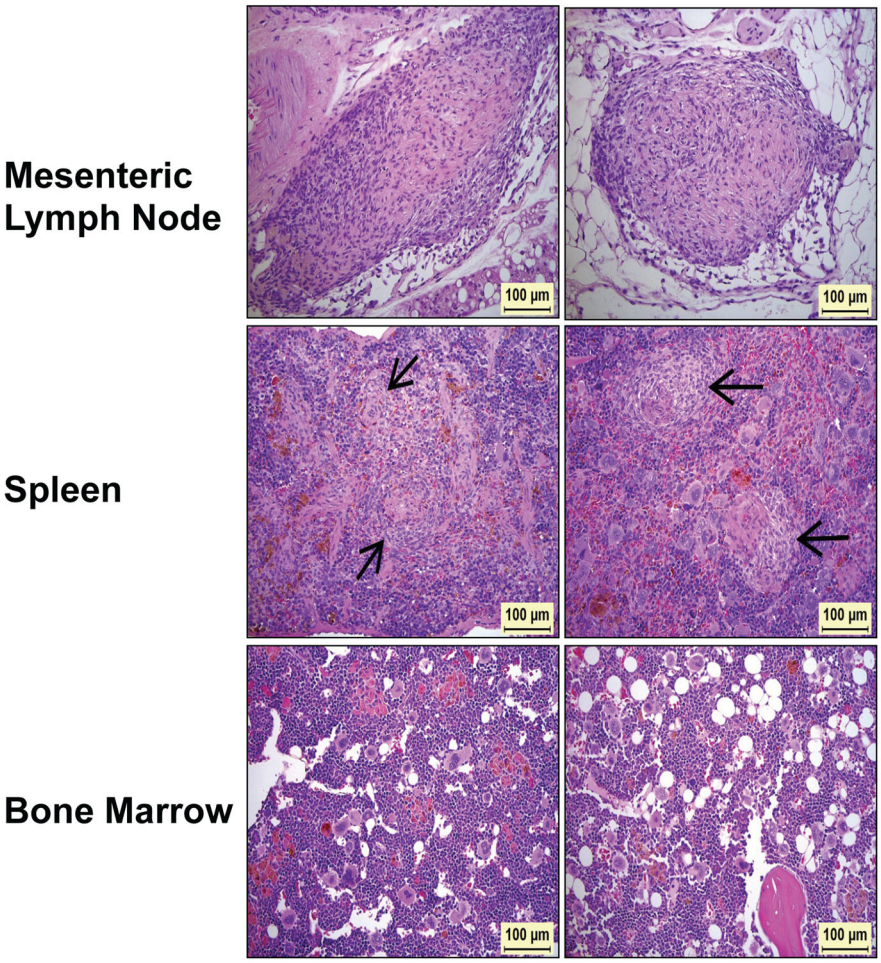
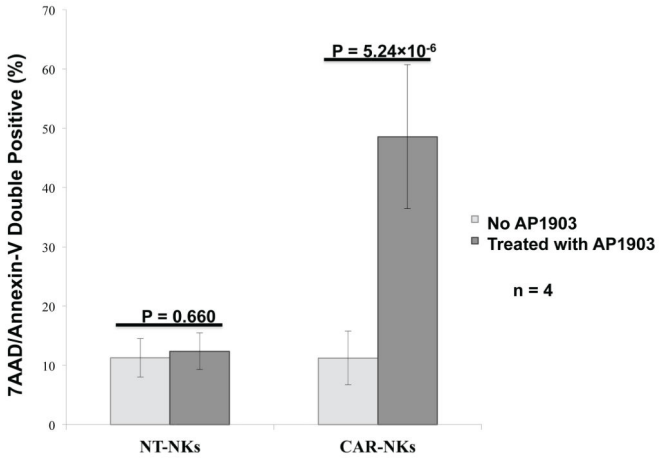


Fig. 5. IL-15-transduced CB-NK cells lack signs of autonomous or dysregulated growth

(A) iC9/CAR.19/IL15-transduced CB NK and NT CB NK cells were put in culture without cytokines or exogenous stimulation to assess their growth over time. iC9/CAR.19/IL15-transduced CB NK cells stop expanding within 6 weeks of in vitro culture with no evidence of autonomous growth. **(B)** NSG mice 10 months after treatment with CB-NK cells transduced with iC9/CAR.19/IL15 or CAR.19 (no IL15) were sacrificed and examined for evidence of NK dysregulated growth or leukemia/lymphoma. Photomicrographs of mesenteric lymph nodes show vestigial lymphoid tissue with no lymphocytic infiltration. Images of the spleen show rudimentary periarteriolar lymphoid tissue devoid of lymphocytes (black arrows) and is surrounded by hematopoietic tissue comprising of erythroid and myeloid series cells in different stages of development, including megakaryocytes and hemosiderin-laden macrophages. Bone marrow contains normal hematopoietic cells and no abnormal lymphocytes. H&E stain, magnification x200. The micrographs are from two representative groups of NSG mice treated with iC9/CAR.19/IL15-transduced CB-NK cells.

6A



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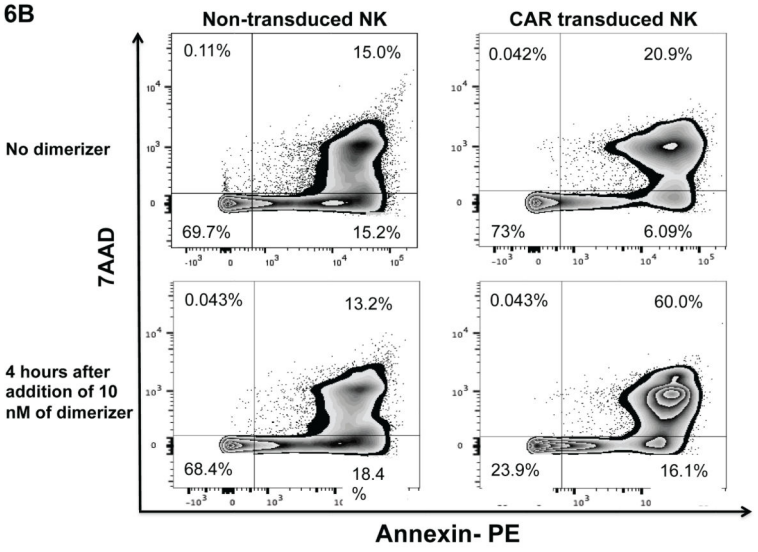

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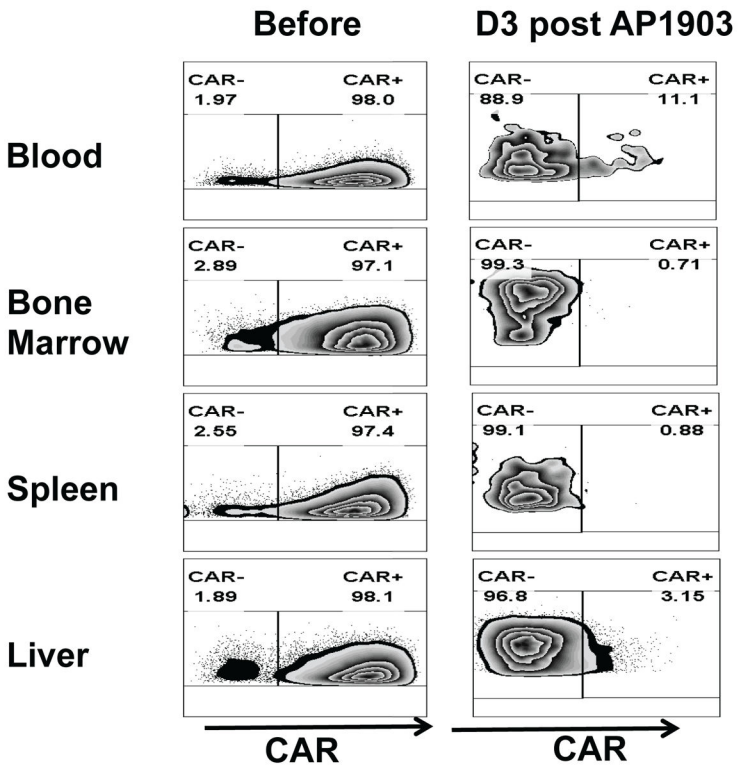

Fig. 6. Activation of inducible caspase-9 suicide gene eliminates iC9/CAR.19/IL15^{+} CB-NK cells

(**A**) Addition of 10 nM of AP1903 to cultures of iC9-CAR-IL15+ CB-NK cells induced apoptosis/necrosis of transgenic cells within 4 hours as assessed by annexin-V-7AAD staining in 4 independent experiments. The dimerizer did not induce apoptosis in NT NK cells. (**B**) A representative FACS plot of the experiment described in Panel A is presented. (**C**) NSG mice engrafted with Raji cells and infused with iC9/CAR.19/IL15^{+} CB-NK cells were treated 10–14 days later with two doses of the AP1903 dimerizer (50 μg) i.p. 2 days apart. FACS plots from a representative experiment are presented. iC9/CAR.19/IL15-expressing NK cells were substantially reduced in all organs tested 3 days later as measured by the frequencies of CAR-positive NK cells in blood, bone marrow, spleen and liver of animals by flow cytometry.

Table 1

Characteristics of iC9/CAR.19/IL15-transduced CB-NK cells generated from 5 different CB units after 14 days of culture.

	Starting Cell Number (×10e6)	NK Fold Expansion	NK Absolute Count at Day 14 (×10e6)	CAR Transduction Efficiency (%)	iC9/CAR.CD19/IL-15 NK cells absolute Count at Day 14 (×10e6)
CAR-CBNK#1	2	564.3	1128.65	66.6	751.7
CAR-CBNK#2	2	843.7	1687.4	87.4	1474.7
CAR-CBNK#3	2	7369.6	15279.2	64.4	9839.8
CAR-CBNK#4	2	2514.3	5028.5	47.8	2403.6
CAR-CBNK#5	2	2221.8	4443.6	67.5	2999.4
Median	**2**	**2221.8**	**4443.6**	**66.6**	**2403.6**