Efficacy of third-party chimeric antigen receptor modified peripheral blood natural killer cells for adoptive cell therapy of B Cell Precursor Acute Lymphoblastic Leukemia.

Quintarelli C[§1,2], Sivori S[§3], Caruso S[§1], Carlomagno S[§4], Falco M[5], Boffa I[1], Orlando D[1], Guercio M[1], Abbaszadeh Z[1], Sinibaldi M[1], Di Cecca S[1], Camera A[1], Cembrola B[1], Pitisci A[1], Andreani M[1], Vinti L[1], Gattari S[1], Del Bufalo F[1], Algeri M[1], Li Pira G[1], Moseley A[6], De Angelis B[1], Moretta L*[7], Locatelli F*[1,8].

1) Department of Onco-Haematology and Cell and Gene Therapy, Bambino Gesù Children's Hospital, IRCCS, Rome, 00142, Italy
2) Federico II University of Naples, Department of Clinical Medicine and Surgery, Naples, 81100, Italy
3) University of Genoa, Department of Experimental Medicine (DIMES) and Centre of Excellence for Biomedical Research (CEBR) – Genoa , 16145, Italy
4) University of Genoa, Department of Experimental Medicine (DIMES) – Genoa , 16145, Italy
5) Istituto G Gaslini, Laboratorio di Immunologia Clinica e Sperimentale, Dipartimento di Ricerca e Diagnostica – Genoa, 16147, Italy
6) Sandhill Therapeutics, Dallas Texas, 75231, USA
7) Department of Immunology, Bambino Gesù Children's Hospital, IRCCS, Rome, 00142, Italy
8) Department of Pediatrics, Sapienza, University of Rome, Italy

[§]Co-First

***Co-last**

Corresponding Authors:
Locatelli F, franco.locatelli@opbg.net
De Angelis B, biagio.deangelis@opbg.net

Abstract

We developed an innovative and efficient, feeder-free culture method to genetically modify and expand peripheral blood-derived NK cells with high proliferative capacity, while preserving the responsiveness of their native activating receptors. Activated peripheral blood NK cells were efficiently transduced by a retroviral vector carrying a second-generation CAR targeting CD19. CAR expression was demonstrated across the different NK subsets. CAR.CD19-NK cells display higher anti-leukemic activity towards $CD19^+$ cell lines and primary blasts obtained from patients with B-cell precursor ALL compared to un-modified NK cells. *In vivo* animal model data showed that the anti-leukemia activity of CAR.CD19-NK cell is superimposable to that of CAR-T cells, with a lower toxicity profile. These data support the feasibility of generating feeder-free expanded, genetically-modified peripheral blood NK cells for effective 'off-the-shelf' immuno-gene-therapy, while their innate alloreactivity can be safely harnessed to potentiate allogeneic cell therapy.

Introduction

Natural killer (NK) cells are an integral component of the innate immune system and play an important role in the host response against viral infections and cancers, including leukemia (1, 2). NK cells are both “serial killers” capable of killing multiple targets without requiring antigen recognition, as well as efficient producers of soluble factors important for regulating both innate and adaptive immune responses (3).

Increased knowledge of NK cellular and molecular biology has renewed the interest in NK cell-based antitumor therapies, and recent efforts have been made to exploit the high potential of these cells in clinical practice. The use of chimeric antigen receptors (CAR) represents a promising approach to enhance NK cell function against tumor cells. Like T cells, NK cells can be genetically modified to express CARs that bind tumor-associated cell surface antigens, mediate specific recognition and subsequent lysis of cancer cells (4-10). Moreover, since NK cells do not cause graft-versus-host disease (GvHD), they represent an "off-the-shelf" product, potentially eliminating the challenges of patient-specific products that plague current CAR-T cell therapies (11, 12).

Historically, there has been significant difficulty in expanding and transducing primary NK cells from peripheral blood (PB) of healthy donors at clinical scale using a feeder-free culture method. While there are many ongoing clinical trials for CAR-modified, expanded peripheral blood T cells, only a small number of clinical trials explore the use of CAR-NK cells. These trials have been limited to CAR-NK cells produced from transformed NK cell lines (5-7, 13, 14) and cord-blood derived NK cells grown on transformed feeder cells,(15, 16) due to the great difficulty in generating, expanding and transducing feeder-free primary NK cells from peripheral blood (PB) of healthy donors. However, CAR-modified primary PB-derived NK cells could represent a feasible, safe, “off-the-shelf” CAR product for widespread treatment of solid and hematologic malignancies. Furthermore, insight into the effects of CAR expression on phenotypic/functional features of engineered NK cells, as well as on the regulation of native NK receptor balance and function is still limited. In this regard, it is important to take into consideration that the activity of NK cells is the result of a fine balance between inhibitory and activating receptors belonging to different families, namely killer-cell immunoglobulin-like receptors (KIRs)(17), C-type lectin-like receptors (NKG2)(18, 19), natural cytotoxicity receptors (NCRs)(20-23), and various co-stimulatory receptors(24). To prevent autoreactivity, human NK cells express inhibitory receptors, such as inhibitory KIRs (iKIRs) and CD94/NKG2A(25), which are specific for self-HLA class-I molecules(25-27). During cancer progression, transformed cells often reduce, or even lose, the

expression of HLA-class I molecules on their surface, thus evading T-cell recognition and killing, while still allowing activation of NK-mediated killing.

In the present study, we have demonstrated the significant advantage of genetic modification of NK cells with CAR targeting CD19-expressing leukemia cells, showing that all of the "native" NK co-receptors were still functional after genetic modification. Using an in vivo mouse model of human lymphoma, we show that CAR-modified, PB NK cells are able to mediate strong antitumor responses against B-cell malignancies, without inducing toxicity.

Material and Methods

Plasmid construction and retrovirus production.

Retroviral plasmid has been designed to carry the cassette of a second generation CAR with specificity for CD19. Single-chain variable fragment (scFv), derived from a murine antibody of IgG (FMC63) class, was linked via an optimized human CD8 hinge-transmembrane domain, to the costimulatory domains 4-1BB (CD137) and CD3-ζ. As a trackable marker, we add a peptide derived from the human phosphoglycoprotein CD34 (ΔCD34).

An additional retroviral vector encoding eGFP-Firefly-Luciferase (eGFP-FFLuc) was used in selected experiments to label tumor cells (DAUDI-Luc.GFP and BV173-FF-Luc.GFP) for *in vitro* and *in vivo* studies.

Generation and expansion of CAR-modified NK cells.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor's buffy coat or leukapheresis (OPBG Hospital, Rome, Italy) by a density-gradient technique (Ficool-Histopaque (Eurobio;France); the healthy donors had signed a written informed consent, in accordance with rules set by the Institutional Review Board of OPBG (Approval of Ethical Committee N_969/2015 prot. N_669LB). $CD56^{+}$ $CD3^{neg}$ NK cells, isolated with an NK isolation Kit (Miltenyi Biotec, Inc., San Diego, CA, USA), and expanded with NK Cell Activation/Expansion Kit (adapted protocol from Navarro et al.[46]) (Miltenyi Biotec, Inc., San Diego, CA, USA) and recombinant human interleukin 2 (IL2, 500 U/ml; R&D; USA) or recombinant interleukin 15 (IL15 10 U/ml; R&D; USA). Activated NK cells were transduced in 24-well plates pre-coated with recombinant human RetroNectin (Takara-Bio. Inc; Japan) using retroviral supernatant. Enriched NK cells were cultured in GMP-compliant media (NK MACS Miltenyi Biotec, Inc., San Diego, CA, USA).

Phenotypic analysis

For flow-cytometry analyses, cells were incubated with appropriate mAbs (described in supplemental Material) followed by PE- or FITC- or APC or APC-Cy7.7 -conjugated isotype-specific goat anti-mouse secondary reagents (Southern Biotechnology Associated, Birmingham, AL; Invitrogen Corporation, Carlsbad, CA; Jackson ImmunoResearch Laboratories, Suffolk, UK) and/or fluorochrome-conjugated mAbs. Flow-cytometry analyses were performed on FACSVerse (Becton Dickinson & Co, Mountain View, CA) and data were analyzed by FacsSuite software.

CAR expression and immunophenotype of NK transduced cells

The expression of CAR.CD19 on NK cells was detected with an anti-CD34 and was evaluated over time, as indicated, in association with CD56 and CD3 specific mAb and isotype control. Samples were analyzed with a BD LSRFortessa X-20. Data were analyzed using the FACSDiva software (BD Biosciences). Un-manipulated PB-derived NK cells were analyzed in PBMC, and gating strategy is reported in Supplemental Material.

Cell lines

Daudi, BV173, LCL 721.221 cell line and $CD19^{neg}$ Karpass 299 were obtained from LGC Standards-ATCC and were maintained in culture in RPMI 10% fetal bovine serum (Hyclone, Thermo Scientific, Pittsburgh, PA) and 2 mM Gluta-Max (Invitrogen, California, USA). Cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C. All cell lines were routinely tested for mycoplasma and for surface expression of target antigens.

Co-culture assay

For functional analysis, NT-NK and CAR.CD19-NK cells were co-cultured ($0.5x10^6$ cells/well) for 7 days with target cell lines in 24 well-plates at an effector:tumor cell ratio (E:T) 1:1. The antitumor effect was evaluated in flow-cytometry assay assessing residual tumor cells. The co-culture media was collected after 24 hours to evaluate cytokine secretion by standard enzyme-linked immunosorbent assay ELISA; R&D Systems (Minneapolis, MN)]

CD107a assay and Chromium release assay.

CD107a assay has been performed as previously described and summarized in Supplemental Material, as read-out for reverse Ab-dependent cellular cytotoxicity (ADCC) assays, and in masking experiments. In order to evaluate NT-NK and CAR.CD19-NK cytotoxic activity, we used a 4-hour ^{51}Cr release as previously described, and summarized in Supplemental Material.

Patient details

For *in vitro* studies of NK-CAR cytotoxicity, primary BM samples were collected at diagnosis from 6 pediatric patients with B-cell precursor Acute Lymphoblastic Leukemia (ALL). Patient HLA characteristics are summarized in Figure 5.

***KIR* gene repertoire, *KIR*-ligand analyses and NK alloreactivity.**

KIR gene repertoire and *KIR*-ligand analyses have been performed as previously described,[47,48] and better specified in Supplemental Material.

Xenogenic *in vivo* leukemia models

NOD/SCID IL-2Rγnull (NSG) xenograft mice were infused with DAUDI cells to assess *in vivo* the antitumor effect of CAR-transduced NK cells. Mouse experiments were conducted in compliance with the ethical international, EU and national requirements and were approved by the Italian Health Ministry (N°88/2016-PR). NGS mice (5 weeks old; The Jackson Laboratory, USA) were inoculated with Firefly Luciferase-labeled Daudi cells (FF-Daudi) ($0.25x10^6$) on day -3. Mice were injected with $10x10^6$ NT-NK or CAR.CD19-NK cells through *i.v.* injection on day 0, and subjected to weekly bioluminescence imaging (IVIS System, Perkin Elmer, USA). Signal quantitation of photons/second was performed as previously described.[49,50] NT-NK or CAR.CD19-NK expansion were monitored by peripheral blood collection and analyzed by flow BD LSR Fortessa X-20 cytometry. Data were analyzed using the FACSDiva software (BD Biosciences).

Statistical analysis

Wilcoxon or Mann-Whitney non-parametric tests were employed. The statistical significances (p value: *$p< 0.05$, **$p\leq0.01$, *** $p\leq0.001$) are indicated. Graphic representations and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Results

PB-derived NK cells can be extensively expanded in a feeder-free manner and efficiently transduced to stably expressing a CAR.CD19 construct

$CD3^{neg}$ $CD56^{+}$ cells were purified from either buffy coat or leukapheresis with high purity (80.95% ± 8.77% $CD56^{+}$ cells; n=13; Supplemental Figure 1A). The selected culture conditions were able to preferentially expand NK cells over other contaminating cells, including $CD3^{+}$ T cells and $CD3^{+}$/$CD56^{+}$ NKT cells (Supplemental Figure 1A).

On day +4, activated NK cells were genetically modified with a retroviral vector expressing a second-generation CAR.CD19(33) (CAR.CD19-NK cells). We did not observe a significant difference in NK-cell proliferation after CAR transduction compared to un-modified NK cells (NT-NK cells) (n=12; Figure 1A and Supplemental Figure 1B). The culture yielded high-fold expansion of NK cells over 30 days [$6.88x10^3$ ± $4.76x10^3$ fold (range $3.84x10^3$-$1.39x10^4$) and $6.70x10^4$±$1.08x10^5$ fold (range $2.48x10^3$-$1.91x10^5$) for NT-NK and CAR.CD19-NK cells, respectively; Figure 1A], with negligible difference among NK donors (Figure 1A). In addition, we also observed that the same rate of expansion could be obtained through the use of IL-2 or IL-15 (Supplemental Figure 1B), as well as the use of bioreactors plating a low rate of cells for cm^2 (data not shown). The median CAR-NK transduction efficiency on day +3 of culture after gene transfer was 27.78 (range, 14.6–50%; n=8; Figure 1B shows an exemplifying donor; Figure 1C shows the average of 8 independent CAR-NK products). Moreover, we observed that CAR expression is stable over time for at least 30 days (Figure 1C) in culture.

Distribution of CAR.CD19 on different KIR/NKG2A NK cell subsets

We studied the distribution of CAR.CD19 expression across specific NK-cell subsets with the aim of determining if gene transfer could promote the development/growth of peculiar subpopulations during *in vitro* culture (Figure 2 and Supplemental Figure 2)(34). To this end, we analyzed the four NK cell subsets identifiable on the basis of NKG2A and KIR expression (i.e. $NKG2A^{-}$ KIR^{-}, $NKG2A^{+}$ KIR^{-}, $NKG2A^{+}$ KIR^{+} and $NKG2A^{-}$ KIR^{+} NK cell subsets) in NT-NK, as well as in NK cells exposed to the retroviral vector (CAR.CD19-NK cells), either expressing or not CAR.CD19 ($CAR.CD19^{+}$ or $CAR.CD19^{neg}$-NK cells, respectively). As shown in Figure 2A, CAR.CD19 expression was observed in all four NK cell subsets, with no particular preference for a given subset's differentiation stage. Moreover, we also report the analysis of individual KIR expression in NT-NK and CAR.CD19-NK cells,

showing that CAR expression is achieved in all the analyzed subsets with any significant difference compared to NT-NK or CAR.CD19^{neg}-NK cells (Supplemental Figure 2A).

Flow-cytometry analysis of the activating CD94/NKG2C receptor, in terms of percentage and distribution, showed that this receptor was similarly expressed on CAR.CD19^{+}-NK cells and NT-NK cells (Figure 2B-C). Moreover, a higher percentage of CD94/NKG2C was detected in those donors in whom this marker was clearly detectable on PB resting NK cells (PB-NK cells) (Supplemental Figure 2B, donor #2). A fraction of expanded NK cells was also characterized by the surface expression of LIR-1; this molecule showed a slight trend to increase on CAR.CD19^{+}-NK cells during cell culture (Figure 2B-C). Thus, PB-derived NK cells, expanded without cellular feeder addition, showed a high grade of heterogeneity, in terms of relevant NK-cell markers and receptors, indicating that the method is able to genetically modify and expand NK cells, independent of the cell maturation stage.

Surprisingly, although unmanipulated PB-NK cells expressed high percentages of CD57^{+} NK cells, NT-NK and CAR.CD19^{+}-NK did not, even after *in vitro* activation and prolonged *in vitro* expansion in IL2 (Figure 2B-C) or IL15 (Supplemental Figure 3). Remarkably, the percentage of terminally differentiated KIR^{+} LIR-1^{+} NKG2A^{-} CD57^{+} NK subset (35, 36) among NT-NK and CAR.CD19-NK cells was negligible. Notably, a very low percentage of NT-NK and CAR.CD19^{+}-NK cells expressed the PD-1 inhibitory receptor even after prolonged cell culture in IL2 (Figure 2B-C) or in IL15 (Supplemental Figure 3).

Transduction with CAR.CD19 enhances *in vitro* NK cytotoxicity against CD19^{+} tumor cell lines.

We tested whether genetically modifying NK cells to express CAR.CD19 enhanced their anti-leukemia activity against CD19-expressing tumors compared to expanded NT-NK cells from the same donors. CAR.CD19-NK cells and NT-NK cells were co-incubated with CD19^{+} 221, DAUDI and BV173 cell lines (n=7; Figure 3A) at the effector:target (E:T) ratio of 1:1 and the anti-leukemia activity was evaluated using a 5-day long-term co-culture assay. Across all CD19^{+} tested cell lines, *ex vivo*-expanded CAR-transduced NK cells exerted superior killing of leukemia cells compared to *ex vivo*-expanded NT-NK cells (Figure 3A is representative of a single donor and show the reduction of leukemia cells mediated by different CAR.NK drug products obtained from 7 different healthy donors). No anti-tumor activity was observed for either NT-NK or CAR.CD19-NK cells against the CD19 negative KARPAS 299 cell line, also known to be an NK-resistant lymphoma cell line(37) (Figure 3A). As shown in Figure 3B, we also demonstrated a significant increase of degranulation activity (as assessed by CD107a assay) against all tested CD19^{+} leukemic cell lines by CAR.CD19-NK cells compared to NT-NK cells. These data

strongly correlated with cytokine quantification, since both IFN-γ and TNF-α were produced by CAR.CD19-NK cells during 24 hours of co-culture with $CD19^+$ leukemia targets (Figure 3C). Moreover, we evaluated whether the effect of CAR.NK mediated killing could be improved in combination with ADCC propriety of the NK cells. Indeed, we observed that the use of an anti-CD20 monoclonal antibody (namely, Rituximab) significantly increased the degranulation level in CAR.$CD19^+$-NK cells against the $CD19^+$ $CD20^+$ 221 cell line (n=3; Supplement Figure 4).

PB CAR.CD19-NK cells retain the expression and function of their native activating receptors

To evaluate whether *ex-vivo* expanded, NT-NK and genetically modified CAR-NK cells could retain their full array of native receptors, we compared the surface expression of a wide set of NK cell markers on PB-NK, NT-NK or CAR.$CD19^+$-NK cells. In particular, the surface expression of CD16 (FcγRIIIa), NCRs (i.e. NKp46, NKp30 and NKp44), NKG2D, activating co-receptors (e.g. DNAM-1 and 2B4) and adhesion molecules (such as LFA-1 and CD2) was assessed by flow-cytometry analysis. As shown in Figure 4A (in one representative donor) and in Supplemental Figure 5 (data from 8 different donors), upon *ex-vivo* expansion, NT-NK and CAR.$CD19^+$-NK cells expressed comparable surface densities of the above-mentioned molecules. As expected, the surface densities of NKp46, NKp30, NKp44, NKG2D, CD2, LFA-1 and 2B4 substantially increased after *ex-vivo* stimulation and expansion as compared to those of un-manipulated PB-derived NK cells. Notably, *in vitro* expanded NT-NK cells, as well as CAR.$CD19^+$-NK cells, conserved a significant fraction of $CD16^+$ $CD56^+$ cells (average of 71.89%±13.47% and 71.10%±14.41%, respectively, Figure 4A and Supplemental Figure 5), important for the preservation of ADCC properties in the genetically modified NK cells.

In addition, the activating receptors and co-receptors preserved their activity, as shown by reverse ADCC assays, in which NT- and CAR.CD19-NK cells were stimulated by specific antibodies (Figure 4B).

CAR.CD19-mediated activity does not require the engagement of activating NK receptors

Since NK cell-mediated killing of 221 cells is known to be dependent on engagement of NKp46, CD2 and 2B4, we also evaluated whether the engagement of these activating receptors/co-receptors was required for 221 cell killing by CAR.CD19-NK cells. As shown in Figure 4C, the use of an antibody mix blocking NKp46, CD2 and 2B4 receptors (Mix #1) induced a significant reduction of NT-NK degranulation against 221 cells [average CD107a% equal to 4.69%±4.23% vs 13.85%±8.19% in the control condition of isotype (CTR) and 11.09%±8.88% in the condition of un-specific antibody mix blocking NKp30, NKG2D, DNAM-1 (Mix #2); p=0.04]. In contrast, the mAb-mediated blockade of

NKp46, CD2 and 2B4 did not significantly affect degranulation of CAR.CD19-NK cells when incubated with the 221 cell line, showing that CAR.CD19 activity was independent of the engagement of native activating NK receptor-coreceptors. Analogous trend was observed using DAUDI cell line as target (Figure 4C).

CAR.CD19-NK cells efficiently recognize primary ALL blasts.

Previous studies have reported that primary ALL blasts are relatively resistant to lysis by allogeneic NK cells and the NK-cell line NK92.(38) Therefore, *in vitro*-expanded CAR.CD19-NK cells were tested against a panel of blasts obtained from 6 pediatric, primary B-cell precursor (Bcp)-ALL bone marrow samples (Figure 5 and 6). All samples were collected at the time of diagnosis and had a percentage of blasts >60%.

Based on the presence/absence of NK alloreactivity between effector and target, two different long-term co-culture assays (5 days) were performed. In the first set of experiments, CAR.CD19-NK and NT-NK cells derived from a donor characterized by alloreactive NK cell subsets were challenged with 4 different primary Bcp-ALL samples (n=2 missing HLA-C C1, KIR2DL2/L3 ligand and n=2 missing HLA-C C2, KIR2DL1 ligand) (Figure 5 and 6A), whereas in the second set of experiments CAR.CD19-NK and NT-NK cells derived from three different donors were challenged with one single primary Bcp-ALL sample (expressing all KIR-ligands) (Figure 5 and 6B). In the presence of NK alloreactivity, primary NK or NT-NK cells mediated significant, although partial, elimination of the primary leukemia cells, whereas CAR.CD19-NK cells were able to almost completely eliminate the target, independent of NK alloreactivity (Figure 5C showing the reactivity against the single Bcp-ALL sample and Figure 6A showing the average of the data for the statistical significance). Only in one case (ALL#1), after co-culture with CAR.CD19-NK cells, a very low percentage (5.9%) of residual leukemia cells was observed (Figure 5C). Notably, flow-cytometry analysis showed that, differently from NT-NK, CAR.CD19-NK recognized and lysed all leukemic cells expressing high CD19 level and that the few residual leukemia cells spared were characterized by an extremely dim expression of the target antigen CD19 (Supplemental Figure 6A-B). Interestingly, we demonstrated that the in vitro activation/expansion did not modify the anti-tumor activity of allogeneic primary NK cells, as shown in Figure 6A and Supplemental Figure 7 (representing the data of anti-leukemic activity of NK cells from three additional HDs tested against ALL#4).

To more fully investigate the CAR.CD19-NK capability to lyse Bcp-ALL expressing all KIR ligands, we performed short-term cytotoxic assays (4h ^{51}Cr assay). In particular, two primary Bcp-ALL samples (ALL#4 and ALL#5) were challenged in two independent experiments with NT-NK or CAR.CD19-NK cells derived from three different donors. In the absence of NK-mediated alloreactivity, CAR.CD19-NK cells exerted a significantly higher lysis of primary Bcp-ALL blasts (n=2; ALL #5 and ALL#4 in Figure 6C) compared to NT-NK cells.

To confirm these data, we analysed CD107a degranulation of NT-NK and CAR.CD19-NK cell subsets expressing only the HLA-specific inhibitory receptor KIR3DL1 against HLA-B*51 transfected 221 cell line (221-B51 cells expressing KIR3DL1 ligand). Two different NK cell subsets expressing NKG2A or KIR2DL2/L3 were used as control (Supplemental Figure 8A). Our data indicated that KIR3DL1$^+$ CAR.CD19-NK subset was characterized by a significant increment of CD107a expression when compared to KIR3DL1$^+$ NT-NK cells (p=0.008. Supplemental Figure 8A). These data correlated with cytokine quantification, since both IFN-γ and TNF-α were produced by CAR.CD19-NK cells after 3 hours of co-culture with HLA-B*51 transfected 221 cell line (Supplemental Figure 8B).

CAR.CD19 NK cells exert anti-leukemic activity *in vivo* in the absence of toxicity

We challenged the anti-leukemic activity of CAR.CD19-NK cells in a DAUDI xenograft immunodeficient mouse model. Mice received one intravenous infusion (0.25× 10^6/mouse) of DAUDI-FF cell line. At leukemia establishment (day 0), mice were treated with control *ex vivo*-expanded NT-NK cells or expanded CAR.CD19-NK cells (10×10^6/mouse; 10 mice per group). Tumor growth was monitored by measuring changes in tumor bioluminescence over time. As expected, tumour bioluminescence increased rapidly in mice treated with control NT-NK cells (Figure 7A-B). By contrast, infusion of CAR.CD19-NK cells led to improved tumor control and significant prolongation of leukemia-free survival (LFS) compared to NT-NK (p=0.001, Figure 7C). Notably, by applying two consecutive infusions of effector cells (Day 0 and Day 14; 10x10^6/mouse), we confirmed the anti-leukemia activity of CAR.CD19-NK cells (Figure 8A) in the absence of toxicity as shown by overall survival (OS) data with 80% of the mice surviving at day 60 (end of the experiment; Figure 8B). A control group of mice (n=5), treated with two consecutive infusion of CAR.CD19-T cells (Day 0 and Day 14; 10x10^6/mouse), experienced a similar kinetics of leukemia control (Figure 8A) as in mice treated with CAR.CD19-NK cells. However, all the mice infused with CAR.CD19-T cells were dead at Day 50 after infusion of the effector cells, after developing toxicity related to the release of human cytokines, including IFN-γ and TNF-α (Figure 8C) detected at Day 35 in mouse peripheral blood. At this time-point, mice receiving T

effector cells (both control NT-T and CAR.CD19-T cells) showed a significant expansion of effector cells (Supplemental Figure 9) concurrently to the elevation of plasma concentration of inflammatory cytokines as compared to that detected in mice infused with NK cells. Therefore, OS of the mice treated with CAR.CD19-NK cells was superior to that of mice receiving CAR.CD19-T cells (median 32 days, range 32-50; p=0.001; Figure 8B). Notably, NT-NK cells exerted a higher anti-leukemia activity compared to NT-T cells (p=0.018), as shown both by bioluminescence imaging at early time points (Figure 8A) and OS data (Figure 8B).

DISCUSSION

NK cells are important effector cells in cell-based cancer immunotherapy, particularly in the control of hematological malignancies.(1, 2) NK cells are efficient serial killers and they can survive in patients for several weeks to months as demonstrated by different clinical trials with adoptively transferred allogeneic NK cells.(1, 39, 40)

While gene modification of NK cells CAR can enhance NK-cell tumor targeting and elimination of different malignancies and enhance their function, the efficiency of gene transfer in mature NK cells has been shown to be limited.(9, 41) Indeed, CAR can enhance NK-cell tumor targeting and elimination of different malignancies. Some CAR-engineered NK cell lines are currently used in clinical trials (NCT00900809 and NCT00990717 NCT02944162 and NCT02742727), however, the use of these CAR-engineered NK cell lines as clinical products may raise concerns. The necessary irradiation before infusion may limit the *in vivo* function and persistence of transformed NK cell lines and these NK cell lines are often latently infected with the Epstein-Barr virus and present multiple cytogenetic abnormalities.(10) Surprisingly, CAR-engineered NK cell lines exert low anti-tumor activity against tumor cells expressing low levels of CAR-targeted molecules and often do not express several typical NK cell activating receptors that are important for triggering NK-cell effector functions (e.g., NK-92 cell line does not express CD16, NKp44 and NKp46). The cytotoxic activity of CAR.CD20-NK-92 cells against NK-cell resistant primary chronic lymphocytic leukemia (CLL) cells has been shown to be superior to ADCC induced in NK-92 expressing FcγRIII by anti-CD20 monoclonal antibodies (rituximab or ofatumumab)(42) Notably, NK-92 cells require CD16 transfection in order to mediate ADCC. However, PB-NK cells are better equipped than the NK-92 cell line for use in association with the administration of tumor-targeting antibodies capable of activating NK-mediated ADCC, since PB-NK cells express the main activating receptors (NCRs, NKG2D, DNAM-1, CD16).

Despite the many advantages of donor-derived NK cells, there are potential impediments to the successful clinical use of donor-derived CAR NK cells. In particular, the use of NK cells as an allogenic adoptive platform may be limited by the short half-life of the cells after infusion, especially in the presence of a functional immune system of the recipient. However, like with the current autologous CAR-T cell approach,(43, 44) the drug product infusion could be associated to lymphodepleting regimes. This could have a double advantage, namely both to reduce the alloreactivity of patient immune system against donor-derived NK cells, and to impair inhibitory effects mediated by myeloid suppressor or T-regulatory cells.

Moreover, until recently, genetic engineering of NK cells, even with viral platforms, had proved to be challenging, with reports of <10% transduction efficiency for primary CB- or PB-derived NK cells (45, 46). CB-derived NK cells have been showed to express a relatively higher percentage of inhibitory receptors (CD94/NKG2A and killer-cell immunoglobulin-like receptors) and less adhesion molecules,(47) as well as lower KIRs expression than PB-NK cells, indicating an immature phenotype. Furthermore, although the frequencies of NK cells present in CB is greater than PB,(48) low numbers of CB NK cells are obtained as a result of the limited volume of an CB unit, this representing a major obstacle in obtaining sufficient numbers of NK cells for clinical application of large third-party bank allogenic product.(49)

Therefore, PB-derived NK cells represent a promising alternative to NK-cell lines, as well as CB-derived NK cells for CAR engineering.

We have established a robust, feeder-free, bovine serum-free protocol to generate high-purity, functional, and expandable PB-NK and PB-CAR-NK cells from widely available donor-derived leukapheresis products or PBMCs. The CAR-NK cells express a broad number of relevant NK cell markers and receptors, indicating that the established method is able to genetically modify and expand heterogeneous NK cells, independent of their maturation stage and cytokine-induced activation.

The capability to manufacture a large number of PB-derived NK or PB-CAR-NK cells is a prerequisite for their translation into clinical use. We demonstrated that with a feeder-free, fetal bovine serum-free approach, an optimal rate of proliferation was obtained with both NK and CAR-NK cells, with up to 13,000-fold expansion achieved over 30 days, although the proliferating curve did not reach a plateau at the end of the culture period. These encouraging data also demonstrate the feasibility of manufacturing an “off-the-shelf” NK cell bank that may serve many different recipients.

Although characterized by a high degree of proliferation, the generated NK cells preserve a phenotype compatible with non-fully differentiated cells, with a sizeable percentage of cells with a KIR-negative

phenotype, an important finding for their clinical allogeneic use. Indeed, the PB-CAR-NK cells are characterized by a limited expression of CD57, a molecule that is known to define a subpopulation of KIR+ LIR-1+ NKG2A-negative terminally differentiated NK cells, characterized by retained cytolitic activity, but poor responsiveness to cytokine stimulation, as well as limited persistence over time.(35, 36) Moreover, the CAR-NK cells express a very low percentage of the PD-1 receptor, an inhibitory checkpoint capable of compromising the effector functions of NK cells against tumors expressing PD-1 ligands. (50)

It is also important to consider that therapeutic approaches based on the use of CAR-NK cells could allow potentiating NK-cell activity even against tumor cells not expressing activating ligands responsible for NK cytotoxicity.

CAR-NK cells exerted a high degree of anti-leukemia activity toward all the different $CD19^+$ cell lines tested. Notably, we observed a significant reactivity of CAR.CD19-NK cells also against BV173 cell line, which, unlike 221 and DAUDI cells, is characterized by high expression of classical HLA class I molecules that could inhibit NK cell function. These data obtained using cell lines were also corroborated by experiments in which donor-derived, KIR-characterized NK and CAR-NK cells were challenged with patient-derived HLA-characterized Bcp-ALL cells. We clearly demonstrated that leukemia control was also achieved in the absence of NK alloreactivity. Remarkably, although the maximum antitumor activity was observed using CAR.CD19-NK cells as effector cells, *ex vivo* expanded donor-derived PB-NK cells also exert leukemia control independently of CAR transduction, as demonstrated by the significant killing of primary leukemia targets exerted by NT-NK cells, that preserved the anti-tumor activity of allogeneic primary NK cells even after extended *in vitro* expansion. This “natural” killing effect represents a further potential advantage of NK cells over T cells in CAR-based 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 potentially less likely than with CAR-T cells.

The advantage of NK cellular adoptive platform over the more conventional T cell-based one was also proved in *in vivo* xenogenic mouse model. In particular, a single CAR.CD19-NK cell systemic infusion was capable of inducing significant tumor control in immunodeficient leukemia-bearing mice, with respect to NT-NK cells. Moreover, in the mouse model, the choice of a double-dose treatment scheme was manageable and safe only in mice receiving NT-NK and CAR.CD19-NK cells, in which we did not observed long term persistence or expansion in the absence of cytokine support. In contrast, all CAR-T treated mice died by day 60, with the sign of weight and hair loss, as well as plasma level elevation of the human pro-inflammatory cytokines IFN-γ and TNF-α, associated to a massive in vivo expansion.

Notably, despite the lack of high level of NK cell persistence, leukemia-bearing mice treated with NT-NK cells show a superior OS compared to those receiving NT-T cells, thus highlighting the synergy between tumor targeting of a specific CAR and the naturally expressed receptors on NK cells.

In conclusion, we describe a novel approach of allogeneic adoptive cell transfer using engineered PB-derived NK cells. CAR.CD19-NK cells with high transduction efficiency are generated using a clinical grade approach that could be easily applied on a large scale by using either manual or bioreactor-based procedures. The PB-NK cell platform has shown high *in vitro* and *in vivo* anti-leukemia activity against both immortalized and primary Bcp-ALL cells, and the data of the *in vivo* model resulted into a pivotal understanding of the CAR.NK therapy safety profile.

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Competing Interests

The authors have no conflicting financial interests.

Author Contributions

L. M. and F.L. share Last authorship, C.Q, S.S., S.Caru. and S.Carl. share First authorship of this paper.
C.Q., S.S., A. M., F.L., L.M. and B.D.A. designed experimental studies, supervised the project conduction, analysed the data and wrote the manuscript.
S.Caru, S.Carl. M. G., Z. A., S. D. C., M. S., performed the *in vitro* experiments.
S.Caru, B.D.A, A. C., B. C., performed the *in vivo* experiments.
A. P. supported the patient sample processing
D.O. and I.B., cloned the retroviral vector.
F.M, S.S. and S.Carl. performed Kir analysis and NK phenotype analysis
M. A. performed HLA typing of patient sample
L.M. and F.L., provide scientific advice and expertise in the immunotherapy field
G.L.P provided expertise in the optimization of the process for clinical translation
S.G., L.V., F.D.B., A.M. and F.L. provide patient's samples, medical advice and expertise in the field of onco-haematology.
S. Caru., S. Carl, B.D.A., C. Q and S.S. contributed to the study design and to the analysis of experimental data.

1. Locatelli F, Pende D, Falco M, Della Chiesa M, Moretta A, Moretta L. NK Cells Mediate a Crucial Graft-versus-Leukemia Effect in Haploidentical-HSCT to Cure High-Risk Acute Leukemia. Trends in immunology. 2018;39(7):577-90.
2. Suen WC, Lee WY, Leung KT, Pan XH, Li G. Natural Killer Cell-Based Cancer Immunotherapy: A Review on 10 Years Completed Clinical Trials. Cancer Invest. 2018;36(8):431-57.
3. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science. 2011;331(6013):44-9.
4. Chiossone L, Dumas PY, Vienne M, Vivier E. Natural killer cells and other innate lymphoid cells in cancer. Nat Rev Immunol. 2018;18(11):671-88.
5. Chen X, Han J, Chu J, Zhang L, Zhang J, Chen C, et al. A combinational therapy of EGFR-CAR NK cells and oncolytic herpes simplex virus 1 for breast cancer brain metastases. Oncotarget. 2016;7(19):27764-77.
6. Han J, Chu J, Keung Chan W, Zhang J, Wang Y, Cohen JB, et al. CAR-Engineered NK Cells Targeting Wild-Type EGFR and EGFRvIII Enhance Killing of Glioblastoma and Patient-Derived Glioblastoma Stem Cells. Sci Rep. 2015;5:11483.
7. Zhang C, Burger MC, Jennewein L, Genssler S, Schonfeld K, Zeiner P, et al. ErbB2/HER2-Specific NK Cells for Targeted Therapy of Glioblastoma. J Natl Cancer Inst. 2016;108(5).
8. Burga RA, Nguyen T, Zulovich J, Madonna S, Ylisastigui L, Fernandes R, et al. Improving efficacy of cancer immunotherapy by genetic modification of natural killer cells. Cytotherapy. 2016;18(11):1410-21.
9. Carlsten M, Childs RW. Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications. Frontiers in immunology. 2015;6:266.
10. Rezvani K, Rouce RH. The Application of Natural Killer Cell Immunotherapy for the Treatment of Cancer. Frontiers in immunology. 2015;6:578.
11. Klingemann H. Are natural killer cells superior CAR drivers? Oncoimmunology. 2014;3:e28147.
12. Liu D, Tian S, Zhang K, Xiong W, Lubaki NM, Chen Z, et al. Chimeric antigen receptor (CAR)-modified natural killer cell-based immunotherapy and immunological synapse formation in cancer and HIV. Protein Cell. 2017;8(12):861-77.
13. Romanski A, Uherek C, Bug G, Seifried E, Klingemann H, Wels WS, et al. CD19-CAR engineered NK-92 cells are sufficient to overcome NK cell resistance in B-cell malignancies. J Cell Mol Med. 2016;20(7):1287-94.
14. Zhang C, Oberoi P, Oelsner S, Waldmann A, Lindner A, Tonn T, et al. Chimeric Antigen Receptor-Engineered NK-92 Cells: An Off-the-Shelf Cellular Therapeutic for Targeted Elimination of Cancer Cells and Induction of Protective Antitumor Immunity. Frontiers in immunology. 2017;8:533.
15. Liu E, Tong Y, Dotti G, Shaim H, Savoldo B, Mukherjee M, et al. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. Leukemia. 2018;32(2):520-31.
16. Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, et al. Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity. PloS one. 2013;8(10):e76781.
17. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, et al. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol. 1996;14:619-48.

18. Sivori S, Vitale M, Sanseverino L, Parolini S, Barbaresi M, Bottino C, et al. Inhibitory CD94 molecules identified by the Z199 monoclonal antibody recognize different HLA-class I molecules. Transplant Proc. 1996;28(6):3199-203.
19. Sivori S, Vitale M, Bottino C, Marcenaro E, Sanseverino L, Parolini S, et al. CD94 functions as a natural killer cell inhibitory receptor for different HLA class I alleles: identification of the inhibitory form of CD94 by the use of novel monoclonal antibodies. Eur J Immunol. 1996;26(10):2487-92.
20. Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med. 1997;186(7):1129-36.
21. Pende D, Parolini S, Pessino A, Sivori S, Augugliaro R, Morelli L, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190(10):1505-16.
22. Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med. 1998;187(12):2065-72.
23. Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol. 1999;29(5):1656-66.
24. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.
25. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391(6669):795-9.
26. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005;5(3):201-14.
27. Biassoni R, Cantoni C, Pende D, Sivori S, Parolini S, Vitale M, et al. Human natural killer cell receptors and co-receptors. Immunol Rev. 2001;181:203-14.
28. Gras Navarro A, Kmiecik J, Leiss L, Zelkowski M, Engelsen A, Bruserud O, et al. NK cells with KIR2DS2 immunogenotype have a functional activation advantage to efficiently kill glioblastoma and prolong animal survival. Journal of immunology. 2014;193(12):6192-206.
29. Bono M, Pende D, Bertaina A, Moretta A, Della Chiesa M, Sivori S, et al. Analysis of KIR3DP1 Polymorphism Provides Relevant Information on Centromeric KIR Gene Content. Journal of immunology. 2018;201(5):1460-7.
30. Della Chiesa M, Falco M, Bertaina A, Muccio L, Alicata C, Frassoni F, et al. Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in patients transplanted with NKG2C-/- umbilical cord blood. Journal of immunology. 2014;192(4):1471-9.
31. Quintarelli C, Orlando D, Boffa I, Guercio M, Polito VA, Petretto A, et al. Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma. Oncoimmunology. 2018;7(6):e1433518.
32. Orlando D, Miele E, De Angelis B, Guercio M, Boffa I, Sinibaldi M, et al. Adoptive Immunotherapy Using PRAME-Specific T Cells in Medulloblastoma. Cancer research. 2018;78(12):3337-49.

33. Diaconu I, Ballard B, Zhang M, Chen Y, West J, Dotti G, et al. Inducible Caspase-9 Selectively Modulates the Toxicities of CD19-Specific Chimeric Antigen Receptor-Modified T Cells. Mol Ther. 2017;25(3):580-92.
34. Della Chiesa M, Pesce S, Muccio L, Carlomagno S, Sivori S, Moretta A, et al. Features of Memory-Like and PD-1(+) Human NK Cell Subsets. Frontiers in immunology. 2016;7:351.
35. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010;116(19):3853-64.
36. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood. 2010;116(19):3865-74.
37. Pinz KG, Yakaboski E, Jares A, Liu H, Firor AE, Chen KH, et al. Targeting T-cell malignancies using anti-CD4 CAR NK-92 cells. Oncotarget. 2017;8(68):112783-96.
38. Godal R, Bachanova V, Gleason M, McCullar V, Yun GH, Cooley S, et al. Natural killer cell killing of acute myelogenous leukemia and acute lymphoblastic leukemia blasts by killer cell immunoglobulin-like receptor-negative natural killer cells after NKG2A and LIR-1 blockade. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2010;16(5):612-21.
39. Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood. 2011;118(12):3273-9.
40. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(6):955-9.
41. Matosevic S. Viral and Nonviral Engineering of Natural Killer Cells as Emerging Adoptive Cancer Immunotherapies. Journal of immunology research. 2018;2018:4054815.
42. Boissel L, Betancur-Boissel M, Lu W, Krause DS, Van Etten RA, Wels WS, et al. Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity. Oncoimmunology. 2013;2(10):e26527.
43. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. The New England journal of medicine. 2018;378(5):439-48.
44. Park JH, Riviere I, Gonen M, Wang X, Senechal B, Curran KJ, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. The New England journal of medicine. 2018;378(5):449-59.
45. Boissel L, Betancur M, Lu W, Wels WS, Marino T, Van Etten RA, et al. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. Leuk Lymphoma. 2012;53(5):958-65.
46. Sutlu T, Nystrom S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther. 2012;23(10):1090-100.
47. Verneris MR, Miller JS. The phenotypic and functional characteristics of umbilical cord blood and peripheral blood natural killer cells. British journal of haematology. 2009;147(2):185-91.

48. Luevano M, Daryouzeh M, Alnabhan R, Querol S, Khakoo S, Madrigal A, et al. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. Human immunology. 2012;73(3):248-57.
49. Sarvaria A, Jawdat D, Madrigal JA, Saudemont A. Umbilical Cord Blood Natural Killer Cells, Their Characteristics, and Potential Clinical Applications. Frontiers in immunology. 2017;8:329.
50. Pesce S, Greppi M, Tabellini G, Rampinelli F, Parolini S, Olive D, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. J Allergy Clin Immunol. 2017;139(1):335-46 e3.

Figure Legends

Figure 1. Peripheral blood-derived NK cells *in vitro* expanded in a feeder-free manner and efficiently transduced with retroviral vector carrying second generation CAR.CD19. (A) Fold expansion of NT-NK (grey line), CAR.CD19-NK (black line) cells in the presence of IL-2. Data from 12 heathy donors (HDs) are expressed as average ± SD (left panel) or single NK cell donor (right panel). (B) CAR.CD19 is efficiently expressed after retroviral transduction in both $CD56^+$ $CD16^{neg}$ and $CD56^+$ $CD16^+$ NK cells. Plots of a representative donor at Day 30 after transduction are shown. CAR expression was assessed by the use of anti-CD34 (APC) mAb in combination with anti-CD56 (BV510) and anti-CD16 (PerCpCy5.5.) mAbs. Grey dots identify the NK-cell subpopulation that did not show CAR expression ($CAR.CD19^{neg}$ NK cells), whereas black dots identify the NK subpopulation with positive CAR expression ($CAR.CD19^+$ NK cells). (C) CAR.CD19 is steadily expressed in NK cells over extensive *in vitro* culture (up to 30 days). Data from 8 HDs for which CAR expression was monitored over time are expressed as average $\pm$ SD.

Figure 2. Flow-cytometry analysis of feeder-free NK cell subsets *in vitro* expanded and genetically modified with CAR.CD19. (A) Percentages of $NKG2A^-$ KIR^- (white), $NKG2A^+$ KIR^- (light grey), $NKG2A^+$ KIR^+ (dark grey) and $NKG2A^-$ KIR^+ (black) NK cell subsets detectable in un-transduced (NT-NK) and in NK cells exposed to the retroviral vector and resulting negative ($CAR.CD19^{neg}$-NK) or positive ($CAR.CD19^+$-NK) for the CAR.CD19 expression after 20 days of culture in IL-2. Data represents average of 10 different healthy donors. (B-C) The percentage of $NKG2C^+$, $LIR\text{-}1^+$, $CD57^+$, and $PD\text{-}1^+$ cells were analyzed in NT-NK cells (grey bars) or $CAR.CD19^+$-NK cells (black bars) after 20 and 60 days of *in vitro* culture in the presence of IL-2, and compared to unmanipulated PBMC-derived NK cells (PB-NK, white bars). Data from 5 donors are expressed as average $\pm$ SD.* $p<0.05$

Figure 3. Functional analysis of NT-NK and CAR.CD19-NK cells. (A) $CD19^+$ tumor cell lines (i.e. 221, DAUDI, BV173) and the $CD19^{neg}$ control cell line (KARPAS 299) were co-cultured for 5 days with NT-NK or CAR.CD19-NK cells at the effector:target ratio 1:1. Flow-cytometry analysis of a representative experiment shows the percentage of residual tumor cells (either $CD19^+$ or $CD19^{neg}$) and $CD56^+$ cells at the end of the culture time. Long term co-cultures were performed in 7 independent experiments, in which $CD19^+$ tumor cell lines (i.e. 221, DAUDI, BV173) and the $CD19^{neg}$ control cell line (KARPAS 299) were co-cultured for 5 days with NT-NK (grey bars) or CAR.CD19-NK cells (black

bars) at the effector:target ratio 1:1. Data are shown as average ± SD. (B) CD107a expression was measured in NT-NK (grey bars) or CAR.CD19-NK cells (black bars) in response to $CD19^+$ tumor cell lines (i.e. 221, DAUDI, BV173) and the $CD19^{neg}$ control cell line (KARPAS 299). Degranulation assay was performed in 6 independent experiments and data are shown as average ± SD. (C) IFN-γ and TNF-α were measured by ELISA assay in 24hrs culture supernatant of NT-NK (grey bars) or CAR.CD19-NK cells (black bars) in response to $CD19^+$ tumor cell lines (i.e. 221, DAUDI, BV173) or the $CD19^{neg}$ control cell line (KARPAS 299). Cytokine analysis was performed in 6 independent experiments and data are shown as average ± SD. * $p<0.05$ ** $p<0.01$.

Figure 4. **CAR.CD19 activity is independent of the engagement of activating NK receptors. (**A) The surface expression of CD16, natural activating receptors (NKp46, NKp30, NKp44), co-receptors (NKG2D, DNAM-1, 2B4) and adhesion molecules (CD2 and LFA-1) was assessed by flow-cytometry analysis. Histogram plots obtained from the analysis of a representative donor are shown in A (PB-NK cells, black line; NT-NK cells, red line; CAR.$CD19^+$-NK cells, blue line). (B) Percentage of $CD107a^+$ NT-NK (grey bars) and CAR.$CD19^+$-NK (black bars) cells upon engagement of the indicated molecules in reverse ADCC assays against the $Fc\gamma R^+$ P815 cell line are shown. Data are representative of 8 independent experiments as average ± SD. (C) Percentage of $CD107a^+$ NT-NK and CAR.$CD19^+$-NK cells against 221 or DAUDI cell lines either in the absence (grey bars) or in the presence of 2 different mixture of mAbs capable for masking specific activating NK receptors. Mix1: anti-NKp46+anti-CD2+anti-2B4 (vertical-lined bars), mix2: anti-NKp30+anti-NKG2D+anti-DNAM-1 (horizontal-lined bars). Data are representative of 5 independent experiments and shown as average ± SD. *$p<0.05$, **$p<0.01$, *** $p\leq0.001$

Figure 5. Analysis of NK alloreactivity. (A) Analyses of *KIR* repertoire and *KIR-L* genes characterizing effector NK cells. By the use of an SSP-PCR approach, *KIR* gene profiles were analyzed for either the presence (grey boxes) or the absence (white boxes) of the indicated *KIR* genes. The gene order was established on *KIR* haplotype published sequences. S3 and S5 in the boxes indicate the presence of *KIR2DS3* and *KIR2DS5*, respectively. In *KIR2DS4* boxes, F indicates the presence of an allele coding for membrane bound receptor, while D indicates allele(s) coding for putative soluble receptor(s). *KIR3DL1* alleles coding for surface/functional receptors or for polypeptides retained into the cell are reported with surface (Sur) and no surface (No s), respectively. Dark grey boxes indicate educated KIRs. Recombination hotspot site, located between centromeric and telomeric regions, is indicated with •.

Based on this, we have performed the analysis of effector/target pairs. In particular, according to KIR/KIR-L mismatched in effector-versus-target direction model, presence/absence of NK alloreactivity was established on the basis of the results reported in panels A and B. Type of alloreactivity (C1 or C2), KIR relevant for the presence of NK alloreactivity, and percentage of alloreactive NK cell subsets (in both NT-NK and CAR.CD19-NK cells) are reported. Analysis of KIR2DS1-mediated alloreactivity. In KIR2DS1 E/U column, Yes indicates the presence of KIR2DS1 gene in a C1/x donor and presence of KIR2DS1 ligand (i.e. HLA-C C2) on leukemic blasts. Percentages of $KIR2DS1^+$ $NKG2A^+$ NK cell subsets (in both NT-NK and CAR.CD19-NK cells) are also indicated. (B) Analysis of KIR-L allotypes present on leukemia blasts. HLA class I high resolution typing of leukemic blasts was converted into KIR-L. (C) Leukemic $CD19^+$ cells obtained from bone marrow samples of ALL #1, ALL #2, ALL #3 and ALL #6 (white bars represent leukaemia cells alone) were co-cultured for 5 days with primary NK (striped bars), NT-NK (grey bars) or CAR.CD19-NK (black bars) from a KIR alloreactive NK HD (HD #4). Residual $CD19^+$ cells are shown at the end of the co-culture period.

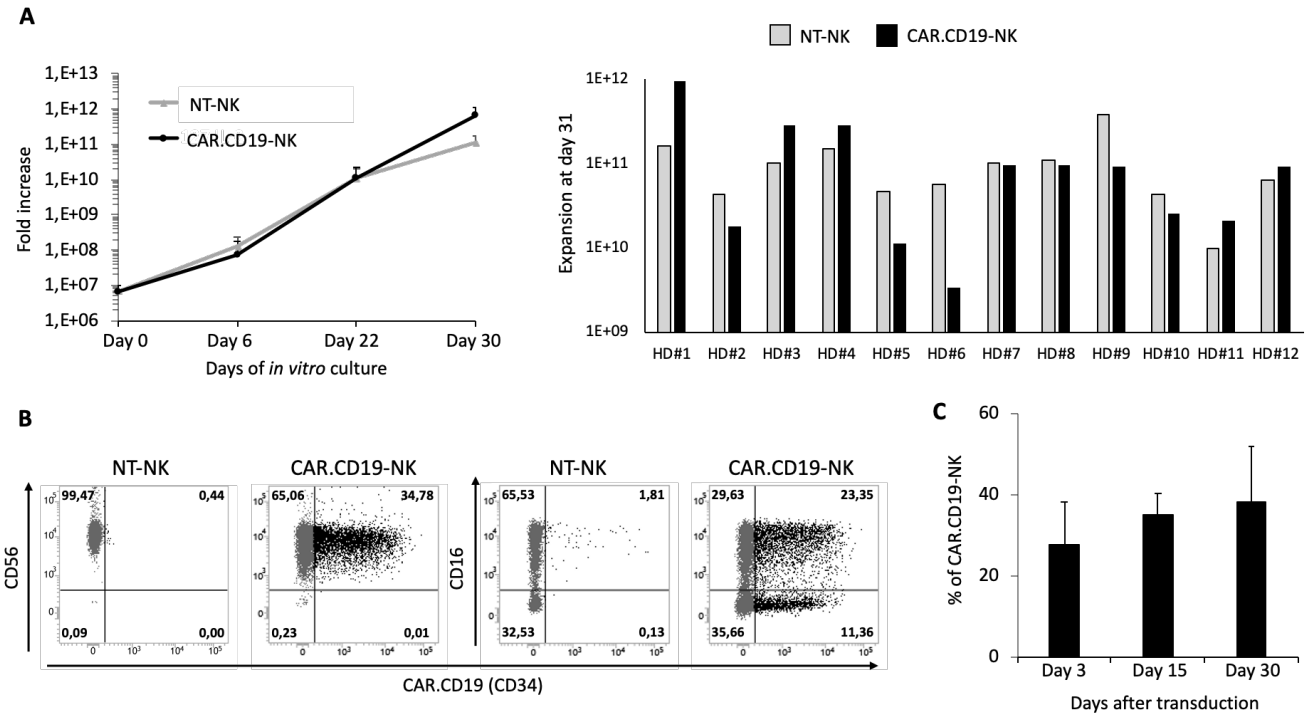
Figure 6. CAR.CD19-NK cells efficiently recognize and kill primary ALL blasts. (A) Leukemic $CD19^+$ cells obtained from bone marrow samples of four patients (ALL #1, ALL #2, ALL #3 and ALL #6) affected by Bcp-ALL (dot filled white bars represent leukaemia cells alone) were co-cultured for 5 days with primary NK cells (white bar), NT-NK (grey bar) or CAR.CD19-NK (black bar) derived from HD characterized by alloreactive NK subsets. Co-culture was performed at the effector:target ratio 1:1. Data of residual $CD19^+$ tumor cells is shown as average $\pm$ SD. (B) Leukemic $CD19^+$ cells from bone marrow samples of one patient (ALL #4) affected by Bcp-ALL (dot filled white bars represent leukaemia cells alone) were co-cultured with NT-NK (grey bars) or CAR.CD19-NK (black bars) from three HDs missing alloreactive NK subsets. Co-culture was performed at the effector:target ratio of 1:1. Data of residual $CD19^+$ tumor cells is shown as average $\pm$ SD. (C) *In vitro* ^{51}Cr cytotoxic assay was performed considering as a target leukemic $CD19^+$ cells obtained from bone marrow samples of one Bcp-ALL patient (ALL #5, panel on the left; ALL #4, panel on the right) and as effectors NT-NK (grey lines) or CAR.CD19-NK (black lines) from three HDs missing alloreactive NK subsets. *$p<0.05$, *** $p\leq0.001$

Figure 7. *In vivo* xenogenic mouse model shows that CAR.CD19-NK cells allow improved tumor control and significant prolongation of leukaemia-free survival as compared to NT-NK. NSG mice were systemically infused with DAUDI-FF-Luc.GFP cells and treated with NT-NK and CAR.CD19-NK cells. (A) Time course of *in vivo* bioluminescence imaging of the treated NSG mice from day 0 (day of

effector cell infusion) to day 75 (end of experiment). (B) Bioluminescence analysis of each leukemia-bearing mouse treated with NT-NK (red line, 5 mice) and CAR.CD19-NK (blue line, 5 mice) cells from day 3 to day 75. (C) 80-day probability of leukemia-free survival (LFS) of NSG mice systemically infused with DAUDI-FF-Luc.GFP cells after adoptive transfer of NT-NK (red bars) or CAR.19-NK cells (blue bars). *** $p \leq 0.001$

Figure 8. CAR.CD19-NK cells show equal anti-leukaemia activity, but lower toxicity, than CAR.CD19-T cells. NSG mice were systemically infused with DAUDI-FF-Luc.GFP cells and treated with NT-T, CAR.CD19-T, NT-NK, and CAR.CD19-NK cells. (A) Time course of *in vivo* bioluminescence imaging of the treated NSG mice from day 0 (day of effector cell infusion) to day 28. Graph shows bioluminescence analysis of leukaemia bearing mice treated with NT-T (black line; 4 mice), CAR.CD19-T (blue line; 5 mice), NT-NK (red line; 5 mice) and CAR.CD19-NK (green line; 5 mice) cells. Data are shown as average $\pm$ SD. Arrows indicate days of infusion. (B) 60-day probability of overall survival (OS) of leukaemia bearing mice treated with two consecutive adoptive transfer of NT-T (black line), CAR.CD19-T (blue line), NT-NK (red line) and CAR.CD19-NK cells (green line). Statistical analysis of 60 days overall survival of leukemia bearing mice treated with two consecutive adoptive transfer of NT-T, CAR.CD19-T, NT-NK and CAR.CD19-NK cells. (C) Cytokine concentration was measured in NSG mice serum bearing DAUDI-FF-Luc.GFP cells and treated with NT-T (black bars), NT-NK (red line bars), CAR.CD19-T (green bars), and CAR.CD19-NK (blue line bars) cells at day 7 and at 25 days after second effector cells infusion. IFN-γ and TNF-α were measured by ELISA assay and data are shown as average $\pm$ SD. *$p<0.05$, **$p<0.01$.

Figure 1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35,66
11,36
CAR.CD19 (CD34)
C
% of CAR.CD19-NK
60
40
20
0
Day 3
Day 15
Day 30
Days after transduction

Figure 2

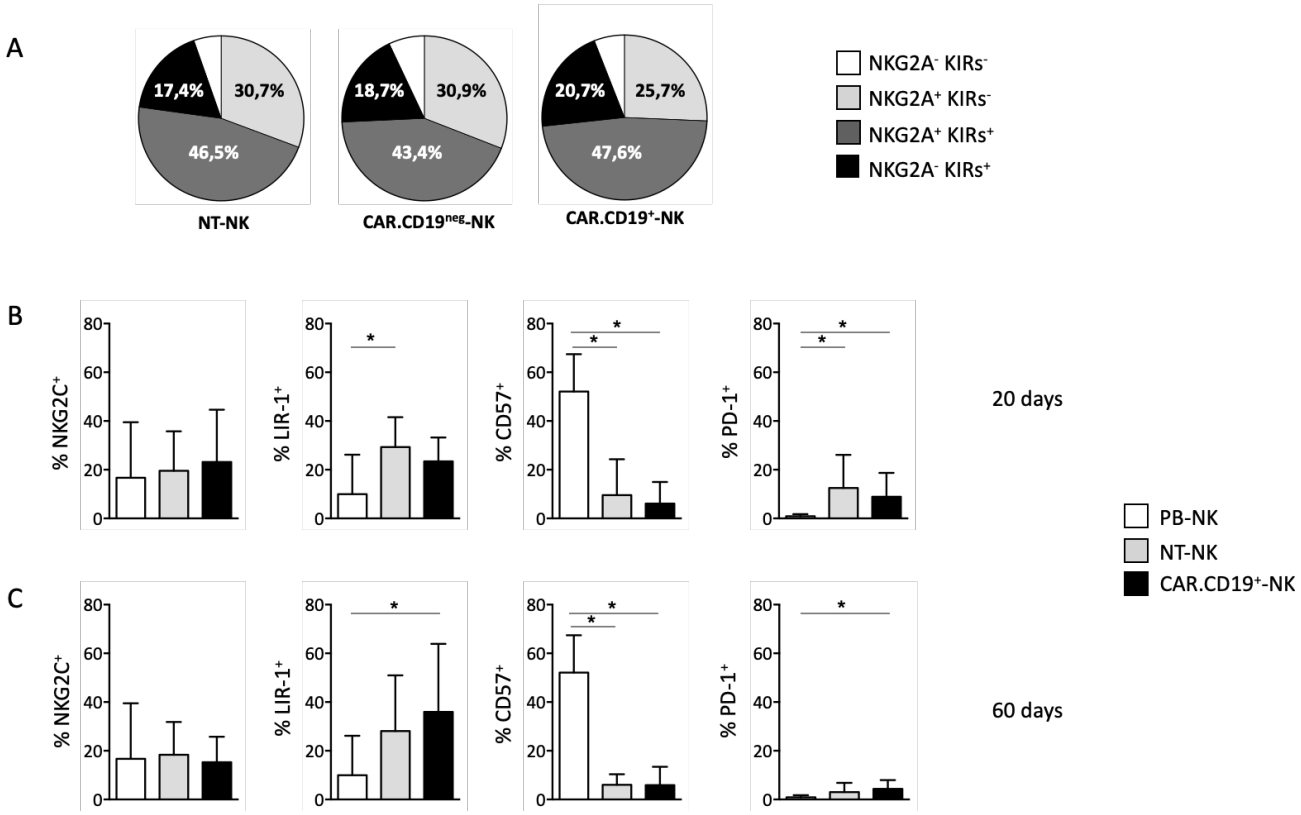

A
17,4%
30,7%
46,5%
NT-NK
18,7%
30,9%
43,4%
CAR.CD19neg-NK
20,7%
25,7%
47,6%
CAR.CD19+-NK
NKG2A- KIRs-
NKG2A+ KIRs-
NKG2A+ KIRs+
NKG2A- KIRs+
B
% NKG2C+
% LIR-1+
% CD57+
% PD-1+
20 days
C
60 days
PB-NK
NT-NK
CAR.CD19+-NK

Figure 3

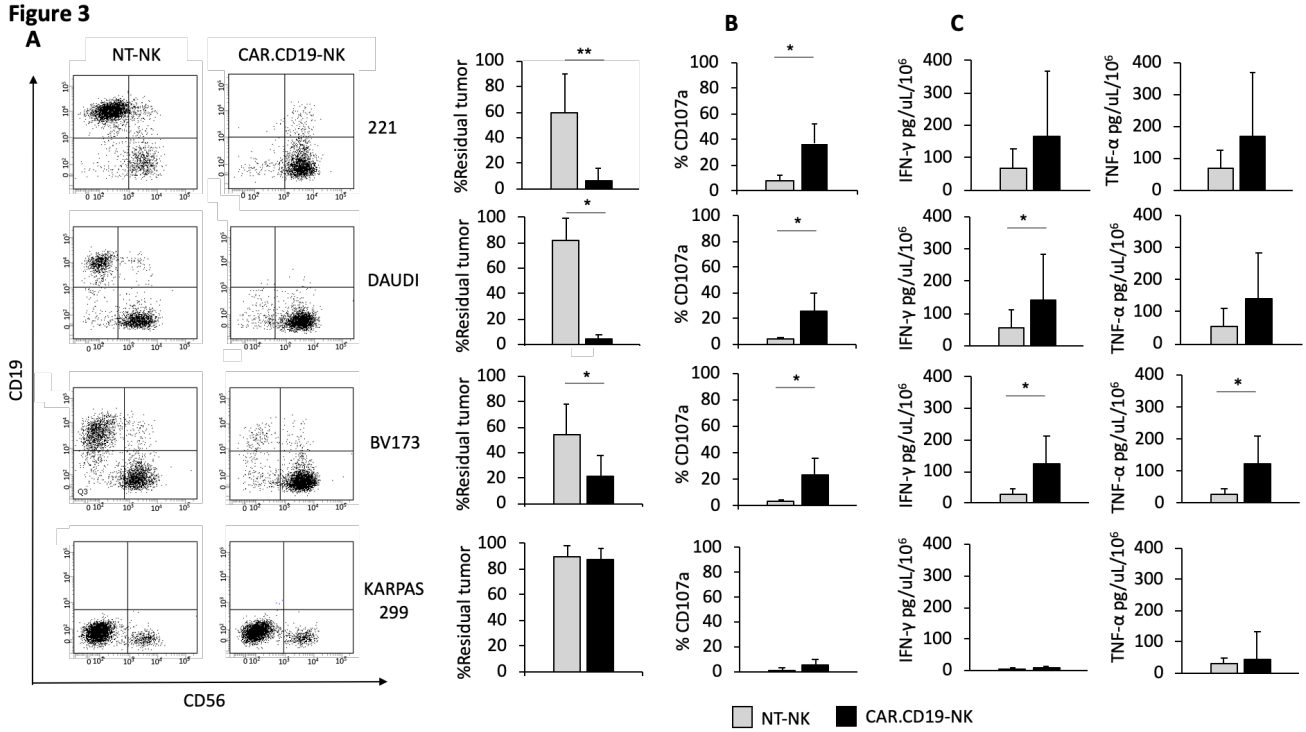

A
NT-NK
CAR.CD19-NK
221
DAUDI
BV173
KARPAS 299
CD19
CD56
B
C
%Residual tumor
% CD107a
IFN-γ pg/uL/10⁶
TNF-α pg/uL/10⁶
NT-NK
CAR.CD19-NK

Figure 4

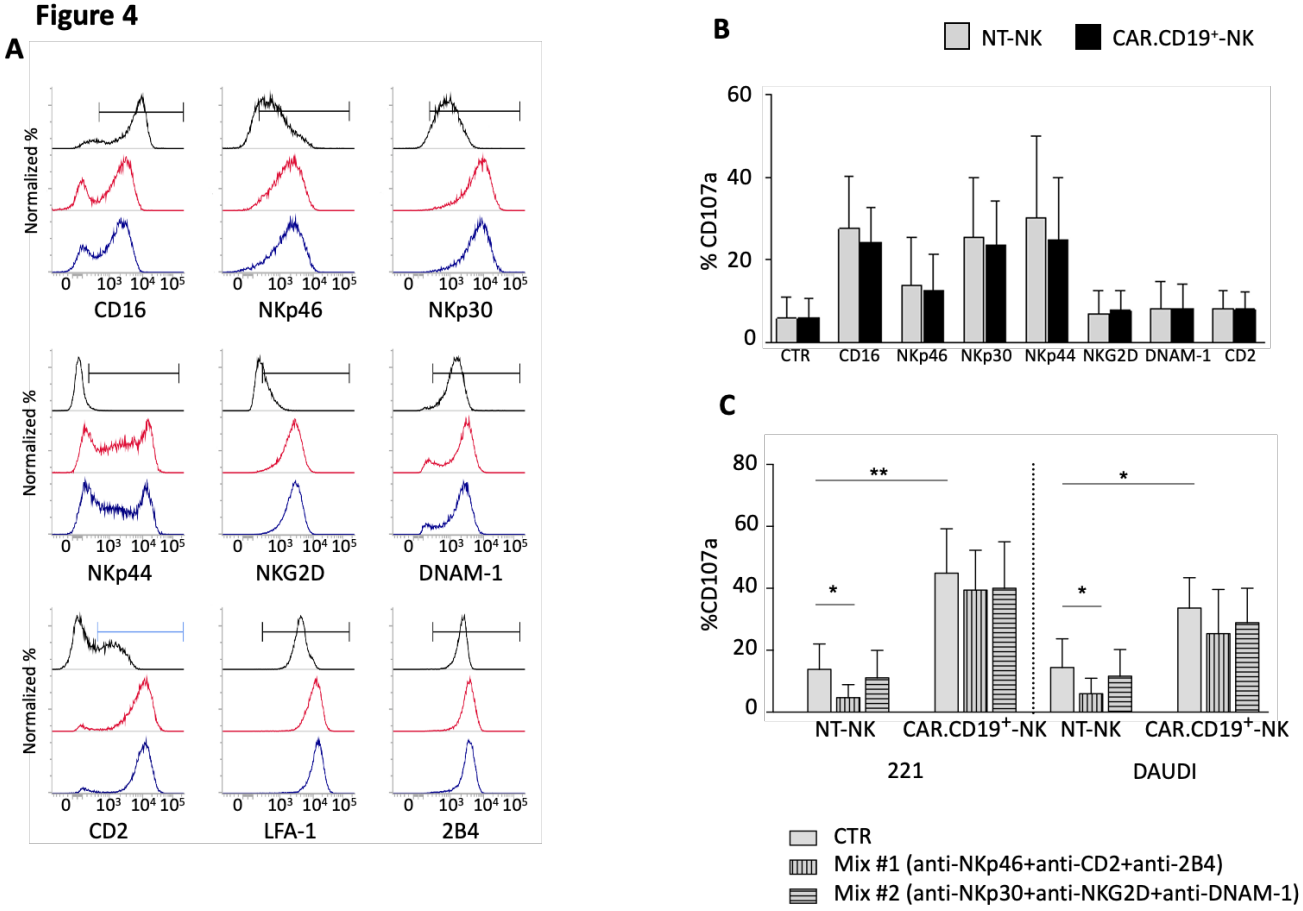

A
Normalized %
0 10³ 10⁴ 10⁵
CD16
NKp46
NKp30
NKp44
NKG2D
DNAM-1
CD2
LFA-1
2B4
B
NT-NK
CAR.CD19⁺-NK
% CD107a
0
20
40
60
CTR
CD16
NKp46
NKp30
NKp44
NKG2D
DNAM-1
CD2
C
%CD107a
0
20
40
60
80
**
*
*
*
NT-NK
CAR.CD19⁺-NK
NT-NK
CAR.CD19⁺-NK
221
DAUDI
CTR
Mix #1 (anti-NKp46+anti-CD2+anti-2B4)
Mix #2 (anti-NKp30+anti-NKG2D+anti-DNAM-1)

Figure 5

A

	KIR gene repertoire																KIR-L				
	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/S5	2DP1	2DL1	3DP1	•	2DL4	3DL1	3DS1	2DL5A	2DS3/S5	2DS1	2DS4	3DL2	genotype	C1	C2	Bw4 (T)	Bw4 (I)	A Bw4
HD#1						S3						Sur			S3				B/X					*24
HD#2												Sur							A/A					
HD#3												Sur			S5				B/X					
HD#4												No s			S5				B/X					

		NK alloreactivity				KIR2DS1-mediated alloreactivity		
		Effector				Effector		
Effector	Target	Yes/No, type	relevant KIR	NT-NK % subset	CAR.CD19-NK % subset	KIR2DS1 E/U	NT-NK % subset	CAR.CD19-NK % subset
HD#4	ALL#1	Yes, C1	2DL3	5,62	3,73	Yes	5,03	4,2
HD#4	ALL#3	Yes, C1	2DL3	5,62	3,73	Yes	5,03	4,2
HD#4	ALL#2	Yes, C2	2DL1	1,3	1,3	No	n.a.	n.a.
HD#4	ALL#6	Yes, C2	2DL1	1,3	1,3	No	n.a.	n.a.
HD#1	ALL#4	No	n.a.	n.a.	n.a.	Yes	0,5	0,5
HD#2	ALL#4	No	n.a.	n.a.	n.a.	No	n.a.	n.a.
HD#3	ALL#4	No	n.a.	n.a.	n.a.	Yes	0,9	1,9
HD#1	ALL#5	No	n.a.	n.a.	n.a.	Yes	0,5	0,5
HD#2	ALL#5	No	n.a.	n.a.	n.a.	No	n.a.	n.a.
HD#3	ALL#5	No	n.a.	n.a.	n.a.	Yes	0,9	1,9

B

	KIR-L				
	C1	C2	Bw4 (T)	Bw4 (I)	A Bw4
ALL#1		*04:01,*06:02	*47:01		*24:03
ALL#2	*03:03,*16:01		*44:03		
ALL#3		*04:01, *06:02		*57:01	
ALL#4	*12:03	*15:02			*24:02
ALL#5	*12:02	*15:02		*52:02	
ALL#6	*07:02,*12:03				*24:02

C

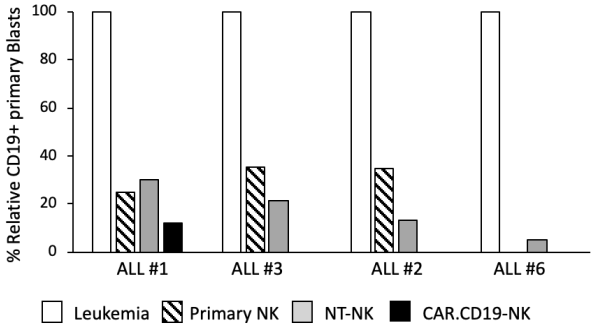


Figure 6

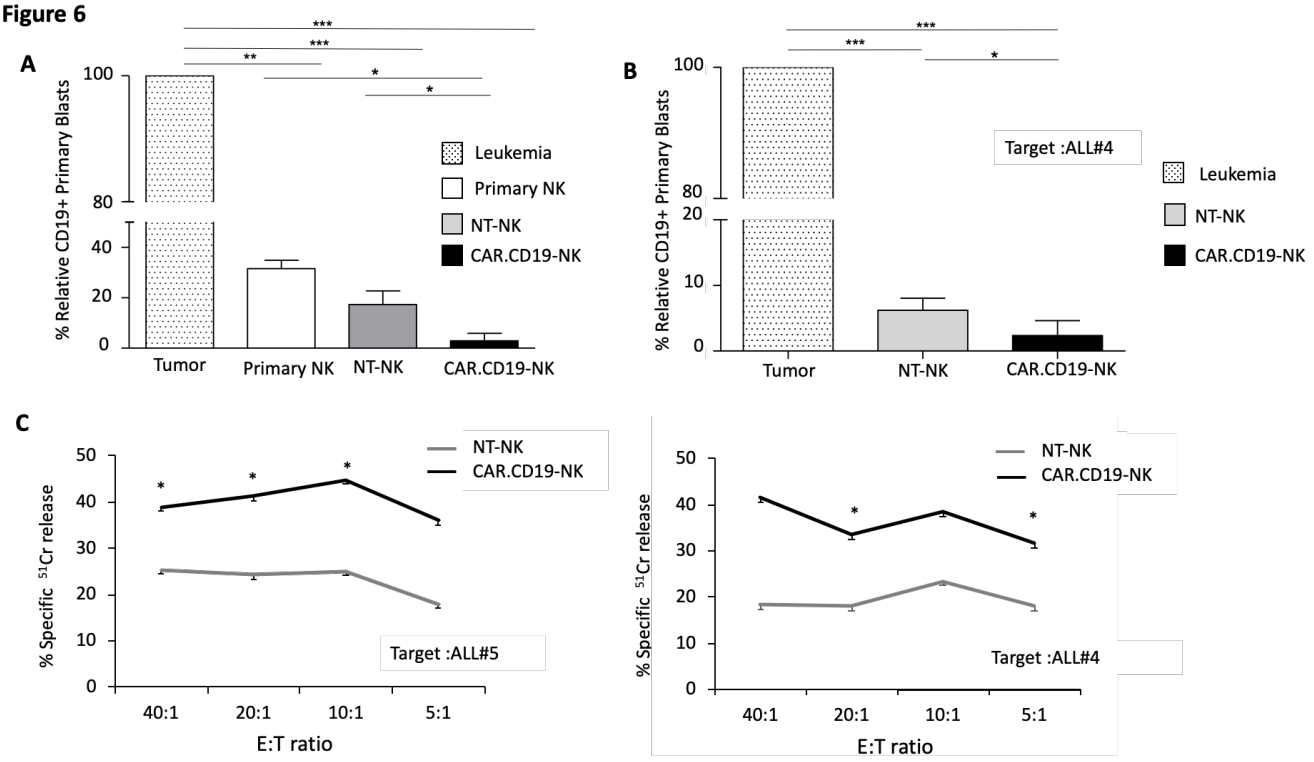

A
% Relative CD19+ Primary Blasts
100
80
40
20
0
***
***
**
*
*
Leukemia
Primary NK
NT-NK
CAR.CD19-NK
Tumor
Primary NK
NT-NK
CAR.CD19-NK
B
% Relative CD19+ Primary Blasts
100
80
20
10
0
***
***
*
Target :ALL#4
Leukemia
NT-NK
CAR.CD19-NK
Tumor
NT-NK
CAR.CD19-NK
C
% Specific 51Cr release
50
40
30
20
10
0
NT-NK
CAR.CD19-NK
Target :ALL#5
40:1
20:1
10:1
5:1
E:T ratio
% Specific 51Cr release
NT-NK
CAR.CD19-NK
Target :ALL#4
40:1
20:1
10:1
5:1
E:T ratio

Figure 7

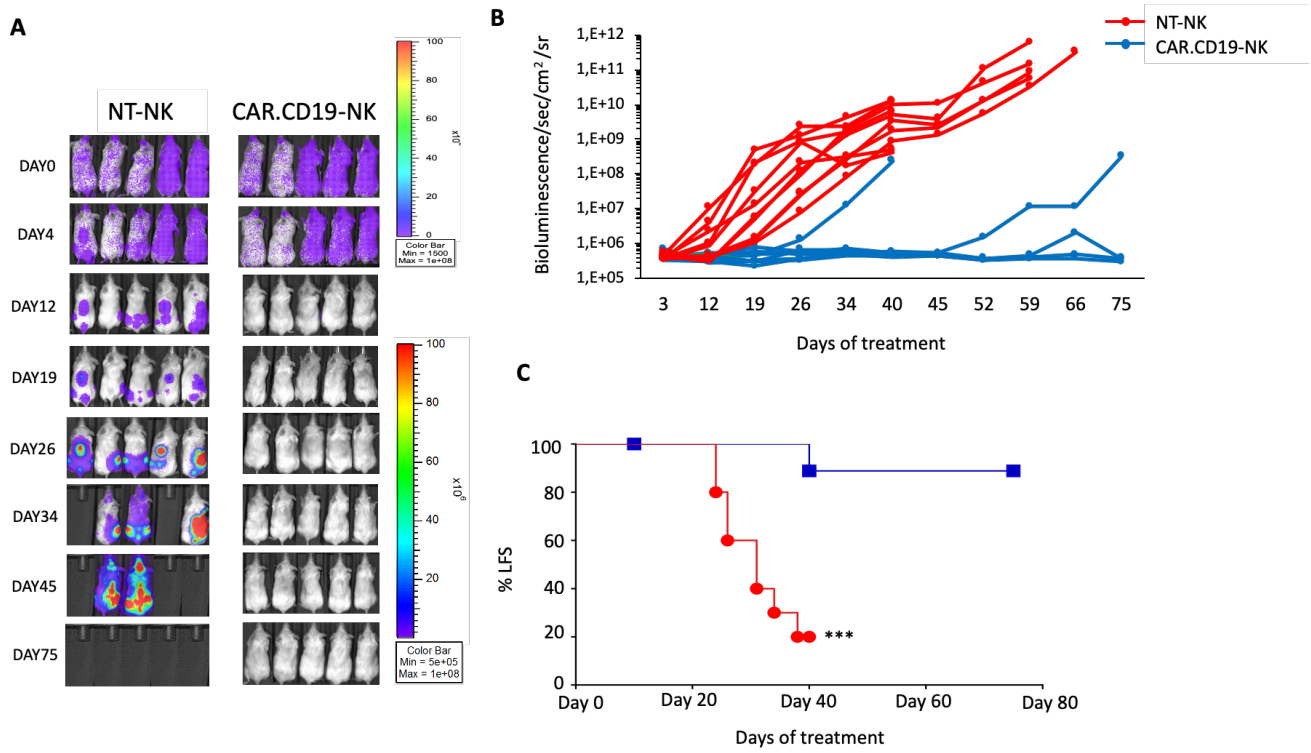
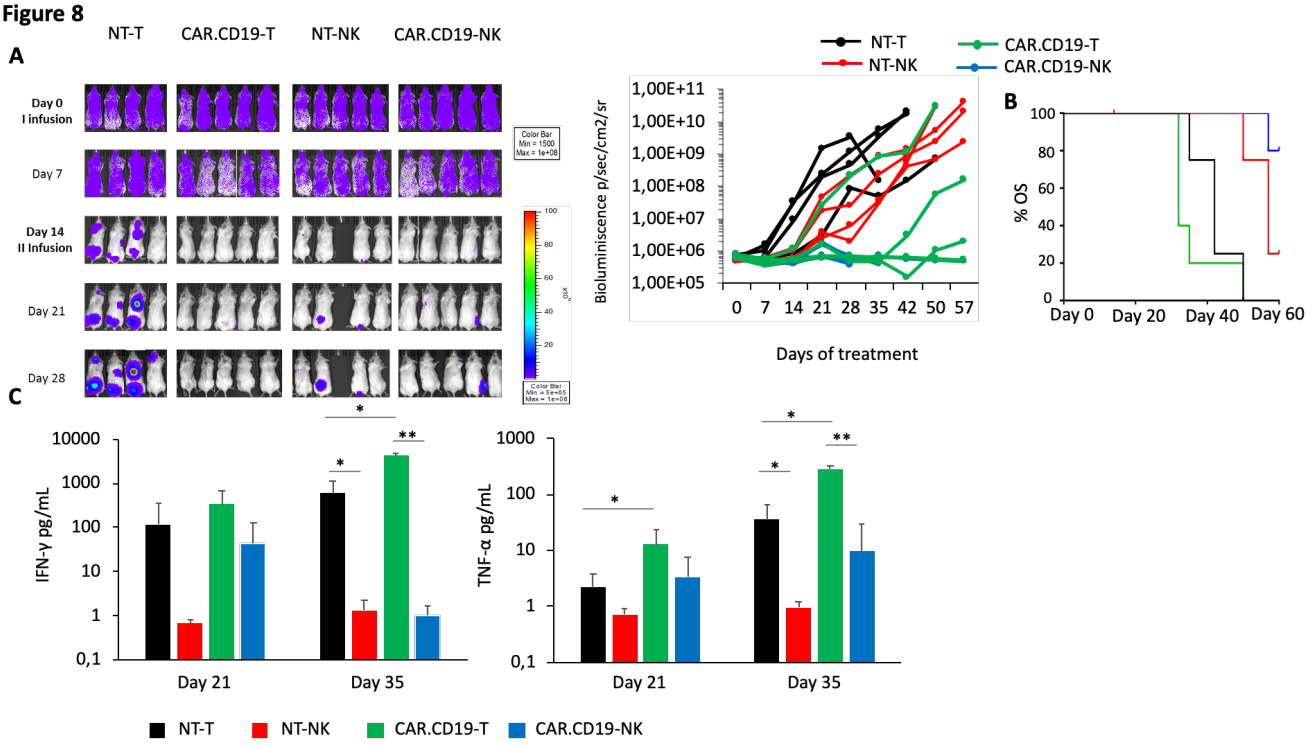

A
NT-NK
CAR.CD19-NK
DAY0
DAY4
DAY12
DAY19
DAY26
DAY34
DAY45
DAY75
Color Bar
Min = 1500
Max = 1e+08
Color Bar
Min = 5e+05
Max = 1e+08
B
NT-NK
CAR.CD19-NK
Bioluminescence/sec/cm²/sr
1,E+12
1,E+11
1,E+10
1,E+09
1,E+08
1,E+07
1,E+06
1,E+05
3
12
19
26
34
40
45
52
59
66
75
Days of treatment
C
% LFS
100
80
60
40
20
0
***
Day 0
Day 20
Day 40
Day 60
Day 80
Days of treatment

Figure 8



	p value
NT-T vs NT-NK	0,018
NT-T vs CAR.CD19-T	n.s.
NT-NK vs CAR.CD19-NK	n.s.
CAR.CD19-T vs CAR.CD19-NK	0,001
NT-T vs CAR.CD19-NK	0,003
NT-NK vs CAR.CD19-T	0,01