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ORIGINAL RESEARCH



## Expression of CD94 by *ex vivo*-differentiated NK cells correlates with the *in vitro* and *in vivo* acquisition of cytotoxic features

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### ABSTRACT

The administration of *ex vivo*-expanded Natural Killer (NK) cells in leukemia therapy is still challenging, in part due to the difficulty to generate in sufficient quantities fully mature and functional NK cells and identification of surface markers indicative of NK maturation and functionality is therefore needed. Here, based on the analysis of surface receptors of *ex vivo*-expanded NK cells, we identified CD94 as a surface marker correlating with high lytic potential against leukemic cell lines and immunological synapse formation. CD94-positive *ex vivo*-expanded NK cells displayed higher expression of NKG2 receptors and the adhesion molecule LFA-1, as compared with their CD94-negative counterparts. We also tested the *in vivo* anti-leukemic capacity of *ex vivo*-expanded NK cells against patient-derived acute myeloid leukemia cells. Although no anti-leukemic effect was detected, we noticed that only CD94-positive *ex vivo*-expanded NK cells were detected in leukemic mice at the end of the 2-week treatment. Moreover, flow cytometry analysis showed a subpopulation harboring CD94 (NK) and CD34 (leukemic cells) double staining, indicative of conjugate formation. Therefore surface expression of CD94 on *ex vivo*-differentiated NK cells emerged as an indicator of *in vitro* and *in vivo* killer cell functionality.

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CD94; leukemia; natural cytotoxicity; NK cells; NK receptors

## Introduction

The first breakthrough in immunotherapy for Acute Myeloid Leukemia (AML) treatment lies in Hematopoietic Stem Cell Transplantation (HSCT).<sup>1,2</sup> In fact, the most important curative effect of HSCT relies on the graft-versus-leukemia (GvL) effect, which is an immune response of the donor's lymphocytes against the residual disease in the recipient.<sup>3-5</sup> Therefore, one strategy for enhancing the GvL effect is to give donor T lymphocyte infusions after allogeneic HSCT.<sup>6,7</sup> However, the beneficial effect of this adoptive transfer of lymphocytes is limited by graft-vs.-host disease (GVHD)<sup>8</sup>: the allogeneic T lymphocytes will attack not only residual leukemic cells, but also host cells resulting in severe organ damages.<sup>9</sup>

Different from T cells, NK cells are CD3<sup>-</sup>CD56<sup>+</sup> lymphocytes that belong to the innate immune system and do not require pre-stimulation to exert their effect or functions.<sup>10,11</sup> Most importantly, they can exert a GvL effect<sup>12-14</sup> without attacking host cells<sup>15,16</sup> which makes them the ideal candidate

for adoptive immunotherapy<sup>17,18</sup> in the treatment of acute leukemia<sup>19</sup> especially in the post-transplant setting.<sup>20</sup>

NK cells from healthy donors are not easily available, therefore methods for generating allogeneic, cancer-reactive NK cells are being developed.<sup>21</sup> *Ex vivo* expansion of NK cells from haematopoietic stem cells (HSC) is one promising approach,<sup>22-25</sup> with umbilical cord blood (UCB) being used as readily available source of CD34<sup>+</sup>haematopoietic progenitor cells (HPC).<sup>24,26-32</sup> However, despite these *ex vivo*-generated NK cells (evNK) display toxicity against some well established leukemic cell lines such as K562 cells, their lytic potential may not be as efficient as that of NK from healthy donors and fully mature state based on expression of NK receptors and markers is not reached. Using a described previously cytokine-based culture method in the presence of HOXB4-transduced stromal MS-5 cells,<sup>33,34</sup> we show that the lytic potential of *ex vivo* differentiated NK cells (evNK) is associated with the surface expression of CD94 *in vitro*. We further characterized these cells by investigating their

NK receptor panel and their *in vivo* toxicity, biodistribution, and anti-leukemic potential after adoptive transfer in immunodeficient mice engrafted with primary acute myeloid leukemia (AML) cells (patient-derived xenograft model). We demonstrated that *in vitro* CD94 expression on evNK is associated with higher lytic potential, higher expression of NKG2 receptors and LFA-1 molecule, and higher ability to form immunological synapses with leukemic target cells, and that only CD94<sup>+</sup> NK cells form *in vivo* conjugates with leukemic cells.

## Material and methods

### *In vitro* generation of human NK cells from UCB-derived CD34<sup>+</sup>-HPC

Umbilical cord blood samples were collected from healthy newborns with mothers' consent in accordance with the institutional review boards of the Etablissement Français du Sang, Créteil France, and the Institut National de la Santé et de la Recherche Médicale, Paris, France. Mononuclear cells were isolated from UCB by a Ficoll method. CD34<sup>+</sup> cells were further isolated using a dextran/ficoll based procedure followed by immuno-magnetic separation (MACS, Miltenyi Biotec) (purity  $\geq 80\%$ ) and transferred into 12-well plates ( $25 \times 10^3$  cells/well) in a co-culture system using feeder murine MS-5 cells engineered to actively secrete the human HOXB4 protein, as described previously.<sup>33,34</sup> CD34<sup>+</sup> cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C for 4 weeks in RPMI-1640 media containing 10% pooled human serum (Jacques Boy), 5% horse serum (Stem Cell Technology), 1% Penicilline-Streptomycin (Invitrogen) and the following cytokines: human recombinant Stem Cell Factor (SCF, 50 ng/mL), interleukin-2 (IL-2, 200 UI/mL), IL-7 (20 ng/mL), IL-15 (20 ng/mL), and FLT-3 (50 ng/mL) (all from Miltenyi Biotec). Cells were splitted with new media and cytokines twice a week. After 4 weeks, CD56<sup>+</sup>-evNK cells were isolated using the MACS system (Miltenyi Biotec) (purity  $\geq 90\%$ ). CD56<sup>+</sup>-evNK cells were subsequently cultured in RPMI-1640 media added with 10% pooled human serum (Jacques Boy), 5% Horse Serum (Stem Cell Technology), 1% Penicilline-Streptomycin (Invitrogen), and IL-2 (200 UI/mL, Miltenyi).

### Isolation of NK cells from healthy donor-peripheral blood

NK from healthy donors (NKhd) were obtained from fresh apheresis products after Ficoll and CD56 purification using the MACS system (Miltenyi Biotec) (purity  $\geq 90\%$ ). CD56<sup>+</sup>-NKhd were subsequently cultured in RPMI-1640 media added with 10% pooled human serum (Jacques Boy), 5% Horse Serum (Stem Cell Technology), 1% Penicilline-Streptomycin, and IL-2 (200 UI/mL, Miltenyi Biotec).

### Culture of leukemic and stromal MS-5 cell lines

K562, U937, and HL-60 cells were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. Mouse stromal cells MS5-HOXB4 cells were cultured in MEM medium (Invitrogen) supplemented with 10% fetal bovine

serum and 1% Penicillin-Streptomycin. All cells were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

### Chromium (Cr<sup>51</sup>) release assay

The cytotoxic activity of the *ex-vivo* differentiated NK cells (evNK) was measured by a conventional 4-hour <sup>51</sup>Cr-release assay in round-bottom 96-well plates. The K562, U937, HL-60 cell lines and patient-derived acute myeloid leukemia cells were used as targets ( $10^3$  cells/well). Experiments were performed in triplicate. Data were expressed as the percentage of <sup>51</sup>Cr release from target cells, calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release)  $\times$  100.

### Flow cytometry analysis

Flow cytometry analysis for evNK phenotyping was performed using a C6 cytometer and data were processed using FlowJo software. CD94-based cell sorting was performed on an ARIA cytometer. Monoclonal anti-human antibodies recognizing the following surface markers were from Biolegend: anti-CD337-PE (NKp30), -CD336-PE (NKp44), -CD335-PE (NK p46), -CD16-PE, -CD161-PE, -CD226-PE (DNAM1). The following anti-human antibodies were from Miltenyi Biotec: anti-CD56-APC, -CD7-PE, -CD45RA-PE, -CD94-FITC, -CD117-PE, -CD158A-PE (KIR2DL1), -CD158B-PE (KIR2DL2/DL3), -CD158E-PE (KIR3DL1), -CD158I-PE (KIR2DS4), -NKG2A-PE, -NKG2D-PE.

### Analysis of immune synapse formation

K562, U937, and HL-60 leukemia cells were spread on poly-L-lysine-coated coverslips for 2 hours at 37°C. CD94-positive and -negative evNK cells were then added at 2:1 effector-to-target ratio. After a co-culture of 30 min, cells were fixed (4% PFA, 30 min), permeabilized (0.1% Triton, 20 min), blocked with 10% FBS for 20 min, and stained with rhodamine-phalloidine (1/500, Molecular Probes). Coverslips were washed 3 times in PBS and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) before imaging (IX83 microscope; Olympus) and analysis (CellSense Dimension software; Olympus). Percentage of NK forming immune synapses with target leukemic cells was calculated as (number of NK involved in immune synapses)/(total NK number)  $\times$  100.

### UCB derived-NK cell adoptive transfer in leukemic NSG-mice

Immunodeficient NOD/SCID IL2-R $\gamma^{-/-}$  (NSG) mice (6–8 weeks old) were bred and housed under specific pathogen-free conditions at the animal facility of Gustave Roussy Cancer Center. All animal experiments were conducted in accordance with institutional and national guidelines under the permit number E-94-076-11.

For leukemic engraftment of NSG mice, CD3-depleted mononuclear cells of a freshly diagnosed AML patient<sup>35</sup> (source: peripheral blood; Table 1) were used after informed consent and approval by local Research Ethics Committees of

**Table 1.** Characteristics of AML patient.

| UPN      | Sex  | Age (years) | pri/sec | FAB classification | Karyotype                | WBC (G/l) | % Blastes | Hb(g/dl) | PLT (G/l) |
|----------|------|-------------|---------|--------------------|--------------------------|-----------|-----------|----------|-----------|
| 2014-042 | Male | 49          | Primary | M2 relapse         | 46XY, del(20)q12(7)/(27) | 50,47     | 60%       | 7,6      | 24        |

UPN: patient number; Pri/sec: primary/secondary; FAB: French-american-british classification; WBC: white blood cell count; Hb: hemoglobin; PLT: platelets; G/l:  $10^9/l$ .

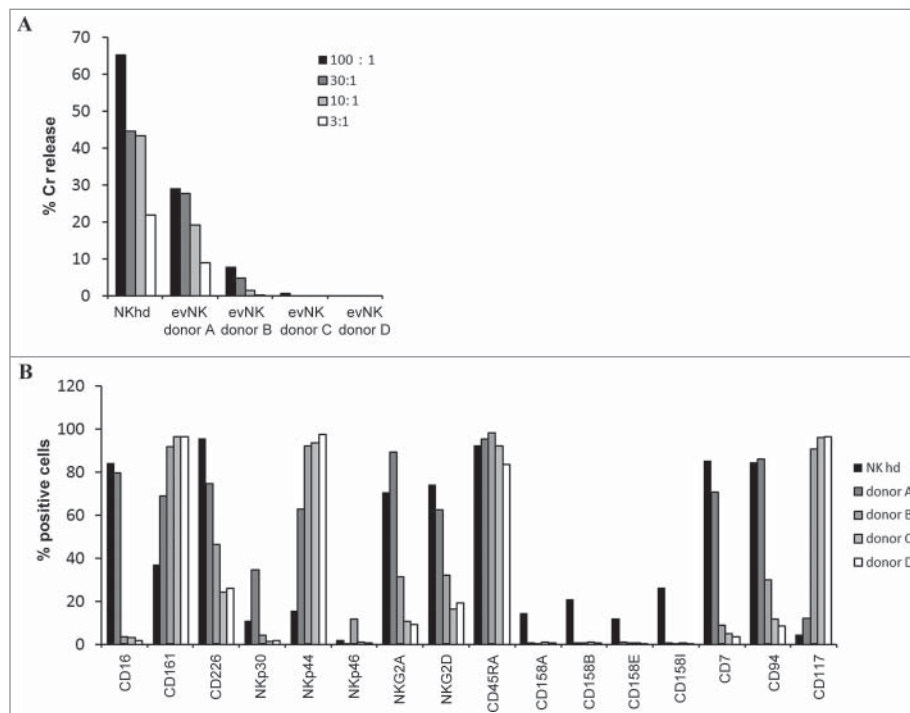
Saint-Antoine hospital (Paris, France).  $2.10^6$  AML cells were intravenously injected (retro-orbital venous sinus) to mice 24 h after irradiation with an X-Ray source (dose: 2.5 Gy). After 31 days, mononuclear cells collected from AML engrafted-mice blood were counted and quadruple-stained with anti-mouse CD45, anti-human CD45, anti-human CD19, anti-human CD33 (all from BD PharMingen). Stained cells were analyzed on a FACScanto I cytometer, and the presence of a single CD34<sup>+</sup> population in the human CD45<sup>+</sup> population was considered as AML engraftment.<sup>36</sup>

Treatment was initiated when human leukemic cells in the mice blood reached 3%, which was achieved 31 d after the initial injection of AML cells. Mice were then randomized into 3 groups of treatment: evNK ( $n = 6$ ), NKhd ( $n = 6$ ), and control ( $n = 5$ ). Each 3–4 d during 14 days,  $5.10^6$  evNK or  $5 \times 10^6$  NKhd were intravenously injected per mouse, each in 200  $\mu$ l PBS, via the retro-orbital venous sinus and under isoflurane gas-anesthesia. Namely, each NK-treated mouse received  $10^7$  NK per week. In parallel, control animals received 200  $\mu$ l PBS. All mice received interleukin-15 (0.5  $\mu$ g/mouse in 200  $\mu$ l PBS) via intraperitoneal injection concomitantly with NK or PBS treatment.<sup>37</sup> Mice were monitored once a week by weight assessment and blood collection for determination of leukemic burden. Mice were killed by cervical dislocation

after 2 weeks of treatment. In addition, for each group, 1 mouse was kept alive for 2 additional weeks without any treatment to detect a possible delayed effect of NK immunotherapy. At sacrifice, bone marrow (BM) and spleens were analyzed for total cell number and the presence of leukemic cells and NK cells. Flow cytometry analysis was performed using: anti-mouse CD45, anti-human CD45, anti-human CD34, anti-human CD34, and anti-human CD56 (all from BD PharMingen). White blood cell (WBC) count was determined on a Melet Schloesing MS9–5V hematology analyzer (Melet Schloesing Laboratoires). BM and spleen cell numbers were counted using a Sysmex counter (Sysmex) according to the manufacturer's instructions. The absolute number of human leukemic cells in the BM and spleen was calculated by the equation: total cell number  $\times$  % of human CD45<sup>+</sup> CD34<sup>+</sup> cells. The absolute number of NK cells in the BM and spleen was calculated by the equation: total cell number  $\times$  % of human CD45<sup>+</sup> CD56<sup>+</sup> cells.

### Statistical analyses

*In vitro* data are expressed as mean or mean  $\pm$  SD. *In vivo* data were analyzed with GraphPad Prism. Two-tailed unpaired student *t* test was used for single comparisons.



**Figure 1.** Heterogeneity of cytotoxic potential and surface receptor expression among evNK issued from different UCB-donors. (A) Cytotoxic activity of evNK issued from 4 different donors against K562 target cells. Cytotoxicity was determined by a conventional 4-hr  $^{51}\text{Cr}$ -release assay at different E:T ratios (100:1, 30:1, 10:1, 3:1). (B) Flow cytometry analysis of the surface expression of the indicated NK receptor on evNK derived from UCB-donors of Fig. 1A. Percentage of positive cells was measured on CD56-purified evNK cells.



Statistically significant differences are indicated by asterisks (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$ ).

## Results

### **CD94 surface expression by ex vivo differentiated natural killer correlates with their cytotoxic activity**

We aimed to identify a functional marker that would reflect the lytic potential of evNK. Therefore, we selected 4 samples of evNK (issued from 4 different UCB-donors) that displayed variability in their cytotoxic activity against K562 cells after 4 weeks of *in vitro* co-culture with MS5 stromal cells and cytokines. As shown in Fig. 1A, percentage of lysis varied from 0 to 28% between the 4 samples of evNK. A phenotypic analysis was performed by flow cytometry on these evNK and NK cells from healthy donors were used as a reference. We evaluated the expression NK cell-associated antigens including the receptor for the Fc portion of IgG CD16, the inhibiting receptors CD161, NKG2A, the activating receptors CD226, NKp30, NKp44, NKp46, NKG2D, the KIR receptors CD158A, CD158B, CD158E, CD158I, the memory receptor CD45RA, the lineage marker CD7, and receptors reported to be associated with NK maturation including CD94 and CD117.<sup>38,39</sup>

As shown in Fig. 1B, only NKhd expressed detectable levels of KIR receptors. Apart from KIR receptors, donor A-evNK and NKhd displayed similar expression levels for CD16, NKG2A, NKG2D, CD7, CD94, and CD117 receptors. All NK showed a comparable level for CD45RA. Various expression levels were found for CD226, NKp30, NKp44, and NKp46. Comparison with the lytic potential of evNK indicates a direct correlation with the expression level of the surface differentiation marker CD94 and an inverse correlation with the expression level of CD117 (Fig. 1B).

To confirm a potential link between CD94 expression and cytotoxic activity of evNK, we performed a CD94-based cell sorting on evNK cells derived from 3 additional UCB-donors at the end of the 4 week-co-culture and compared the cytolytic activity of CD94<sup>+</sup> and CD94<sup>-</sup>evNK against the leukemic cell lines K562, U937 and HL-60. As shown in Fig. 2 A, B, C, CD94<sup>+</sup>-evNK from the 3 UCB-donors showed a consistent higher cytotoxicity against K562 (Fig. 2A), U937 (Fig. 2C) and HL-60 (Fig. 2C) cell lines as compared with CD94<sup>-</sup>-evNK.

### **CD94<sup>+</sup>-evNK cells display higher expression of LFA-A1 and NKG2 receptors than CD94<sup>-</sup>**

To better delineate the role of CD94 in evNK cytolytic functions, CD94<sup>+</sup>- and CD94<sup>-</sup>-evNK issued from CD56<sup>+</sup>-evNK from 3 UCB-donors were sorted by flow cytometry and analyzed for the expression of NK receptors including CD16, CD161, CD226, NKp30, NKp44, NKp46, NKG2A, NKG2C, NKG2D, CD7, CD45RA, and the adhesion molecule LFA-A1 recently reported to be associated with CD94 expression and NK maturation.<sup>40</sup> As shown in Fig. 3, CD94<sup>+</sup>- and CD94<sup>-</sup>-evNK expressed comparable levels of CD161 and NKp44, but differed with respect to CD16, CD226, NKp30, NKp46, NKG2A, NKG2C, NKG2D, CD7, CD45RA, and LFA-A1 receptors that were consistently higher expressed in CD94<sup>+</sup>-

than in CD94<sup>-</sup>evNK (Fig. 3A, B, C). Of note, since CD94 forms heterodimers with NKG2 receptors, it is expected to find increased expression of NKG2A, C, D receptors on CD94<sup>+</sup>-evNK.

### **CD94<sup>+</sup>-evNK and not CD94<sup>-</sup>-evNK form immune synapses with leukemic cell lines**

To further investigate the role of CD94 in evNK cytolytic functions, we studied the ability of CD94<sup>+</sup>- and CD94<sup>-</sup>-evNK to form immune synapses with K562, U937, and HL-60 target cells. NK and target cells co-cultures were stained to visualize F-actin accumulation at the immune synapse, the first critical event for NK immune synapse formation and functions.<sup>41</sup> Clearly, CD94<sup>+</sup>-evNK were more able to form synapses with K562, U937, and HL-60 than CD94<sup>-</sup>-evNK as shown in Fig. 4 (Fig. 4A and 4B). We observed consistently more F-actin accumulation and polarization within synaptic zone between CD94<sup>+</sup>-evNK and leukemic cells than between CD94<sup>-</sup>-evNK and leukemic cells. This suggests that CD94<sup>+</sup>-evNK are capable of interacting with leukemic cells whereas CD94<sup>-</sup>-evNK are not.

This interaction is not due to an interaction of CD94 with its ligand HLA-E on target cells.<sup>42-44</sup> Indeed, K562, U937 and HL-60 cells were negative for HLA-E expression by flow cytometry (data not shown). However, we cannot exclude a contribution of LFA-1<sup>45</sup> since these target cells strongly expressed the LFA-A1 receptor ICAM-1<sup>46</sup> (data not shown).

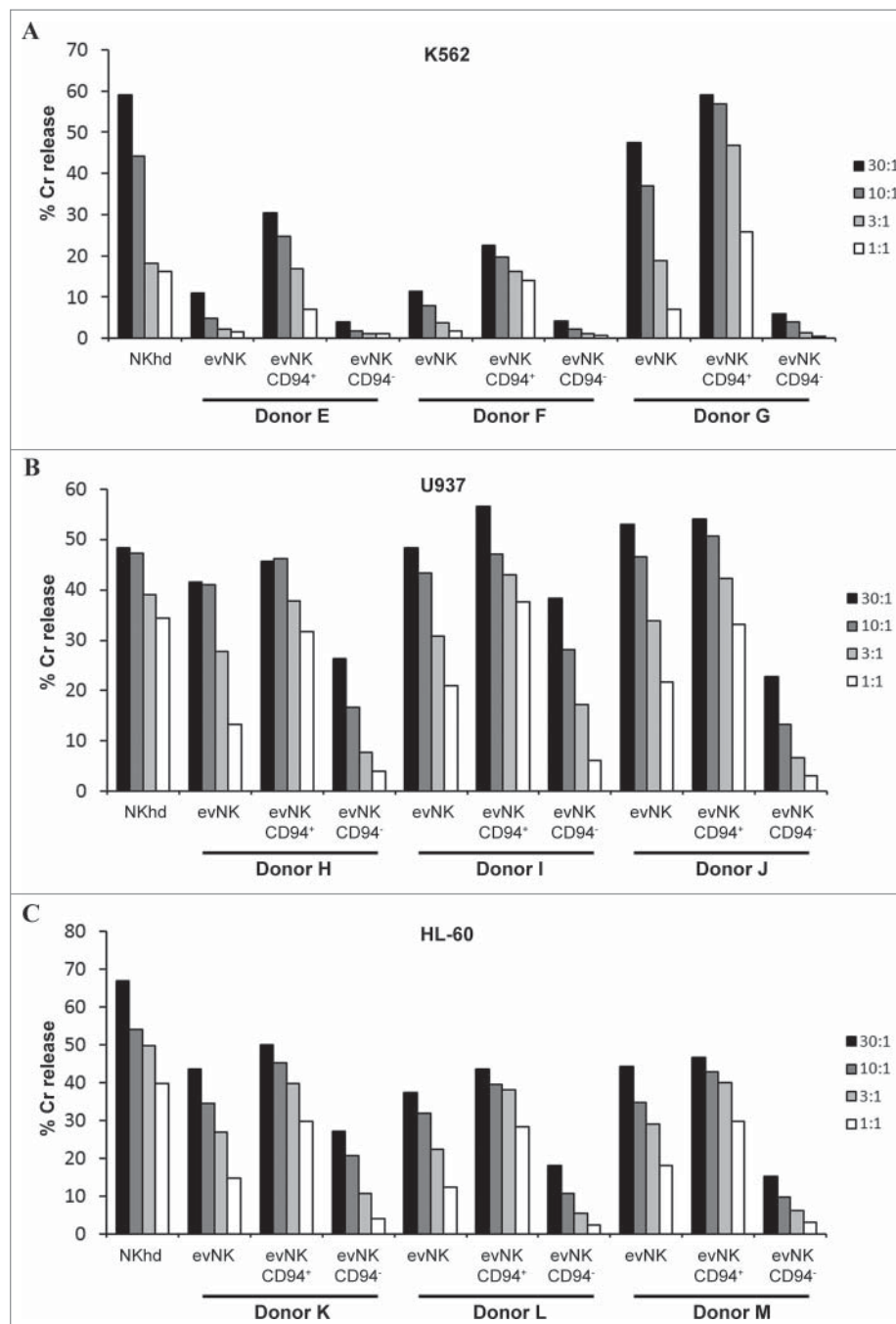
### **CD94<sup>+</sup> cells form conjugates with primary leukemic cells in vivo following adoptive transfer of evNK in leukemic NSG mice**

We first evaluated *in vitro* the cytotoxicity of evNK and NKhd against patient-derived AML cells, using K562 cells as positive control for NKhd and evNK cytotoxicity. AML cells were found to be completely resistant to evNK and NKhd as compared with K562 cells (Fig. 5). We continued using these AML cells for adoptive transfer, questioning whether the *in vivo* mouse environment would alter the cytotoxic response of NK cells or the sensitivity of AML cells.

To evaluate *in vivo* the toxicity, tissue distribution and anti-leukemic potential of evNK, AML-engrafted immunodeficient mice were adoptively transferred with evNK and NKhd were used as reference. After 2 weeks of treatment, there was no evidence of toxicity of evNKs or NKhd cells in mice since any death nor did weight loss occur among treated mice (data not shown). Organ distribution analysis showed that evNK and NKhd preferentially circulated in the blood and rested in the spleen with a minority of cells reaching the BM (Fig. 6). However, NKhds reached higher levels than evNKs (Fig. 6).

EvNK and NKhd did not show anti-leukemic activity since the absolute numbers of leukemic cells in AML-engrafted mice after the 2 week-treatment did not decrease (Fig. 7). This correlated with *in vitro* data (Fig. 5).

Interestingly, phenotypic analysis of blood from evNK-injected mice revealed the presence of a population stained with CD56<sup>+</sup> and CD34<sup>+</sup> (Fig. 8A). This population was not present in the leukemic mice not injected with NK (Fig. 8C),

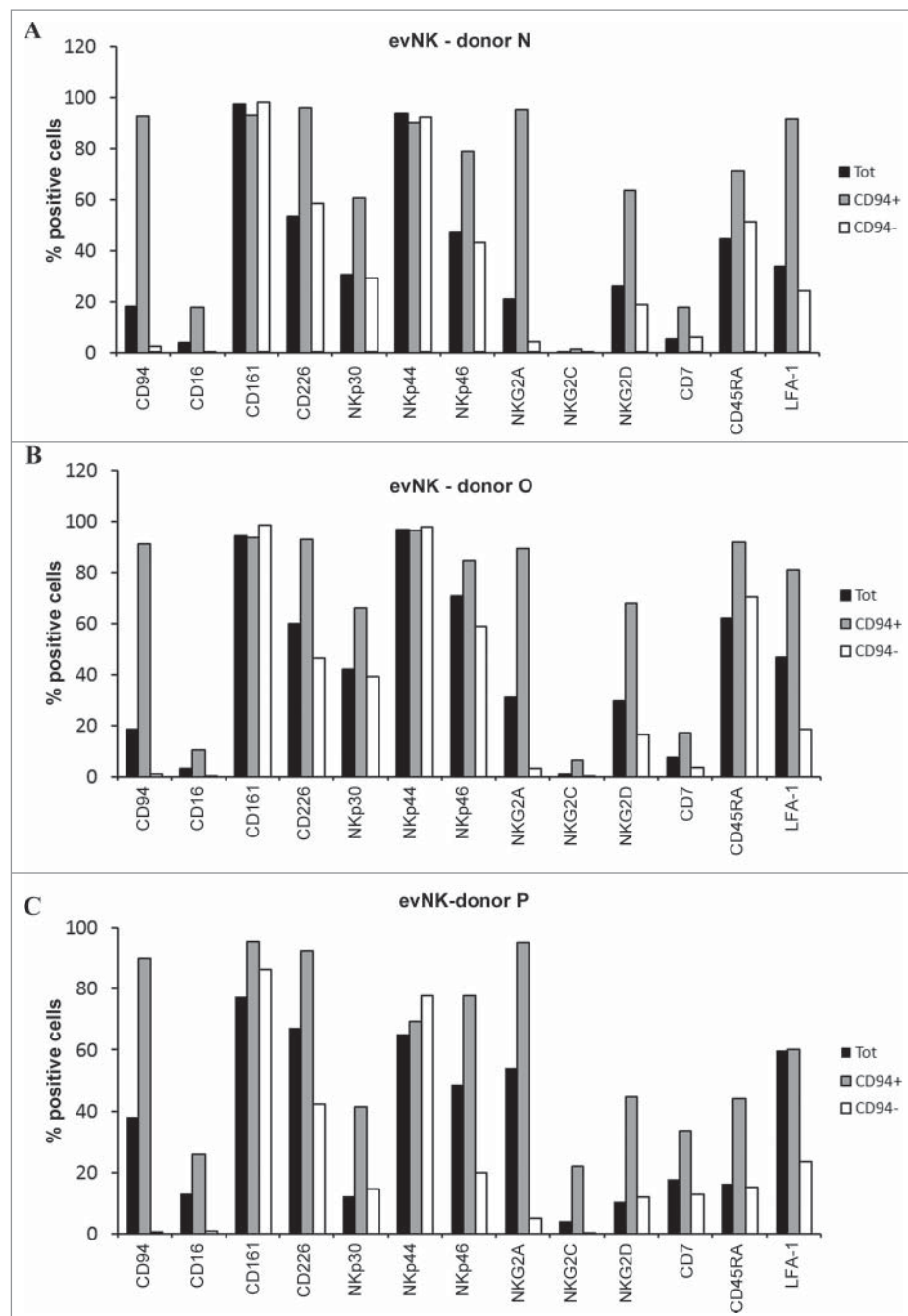


**Figure 2.** CD94 surface expression on evNK correlates with higher lytic potential. Cytotoxic activity of evNK derived from different UCB-donors was measured against K562 (A), U937 (B), and HL-60 (C) target cells at different E:T ratios (30:1, 10:1, 3:1, 1:1) by a conventional 4-hr Cr<sup>51</sup>-release assay. EvNK were either sorted based on CD94 surface expression (evNK CD94<sup>+</sup>; evNK CD94<sup>-</sup>) or not (evNK).

suggesting conjugate formation between NK and leukemic cells *in vivo*. This “double positive” population also expressed CD94. Of note, the entire evNK cells found *in vivo* 2 weeks after the adoptive transfer expressed the CD94 receptor (Fig. 8A) whereas no more than 20% of the injected evNK were positive for this marker (data not shown). Immunophenotyping of NKhd found in mice blood at 2 weeks also showed the double-stained population and contained the same proportions of CD94<sup>+</sup> cells (Fig. 8B).

As mentioned previously (see Mat. and Meth. Section), one mouse from each treatment group was kept alive for 2

additional weeks without NK administration to detect an eventual delayed anti-leukemic effect of NK. At sacrifice (at day 28 after the first treatment injection), there was no delayed effect of NK immunotherapy (Fig. 9A). In fact, absolute numbers of human CD45<sup>+</sup>CD34<sup>+</sup> leukemic cells were similar across the 3 treatment groups as shown in Fig. 9A. Strikingly, we noticed that evNK maintained their level in blood, spleen, and BM whereas NKhd levels decreased to reach the same level as evNK cells in organs. Absolute numbers of both types of NKs were very low in the blood 4 weeks after adoptive transfer (Fig. 9B). Remarkably, almost all the remaining evNK displayed high



**Figure 3.** CD94-positive evNK display higher expression of NKG2 receptors and LFA-1. (A) (B) (C) Comparative flow cytometry analysis of surface expression of the indicated NK receptors on evNK derived from 3 UCB donors and sorted (CD94<sup>+</sup>; CD94<sup>-</sup>) or not (Tot) based on CD94 surface expression. Percentage of positive cells was measured on CD56-purified evNK cells (Tot), CD56-purified then CD94-positive sorted evNK cells (CD94<sup>+</sup>), and CD56-purified then CD94-negative sorted evNK cells (CD94<sup>-</sup>).

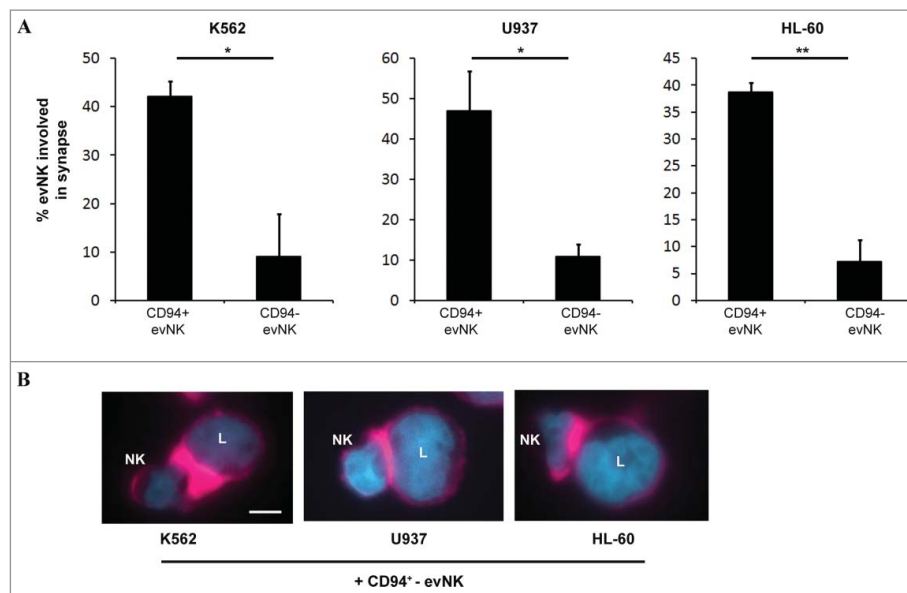
level of expression of CD94 (Fig. 10A) just as NKhd (Fig. 10B), raising the hypothesis that CD94-positive evNK are long-living cells.

## Discussion

Our study and others<sup>24-32</sup> confirm that generation of functional human NK cells derived from CD34<sup>+</sup> cells isolated from freshly collected UCB units is feasible. However, parameters involved in generating fully functional and mature NK cells remain a subject of investigations.

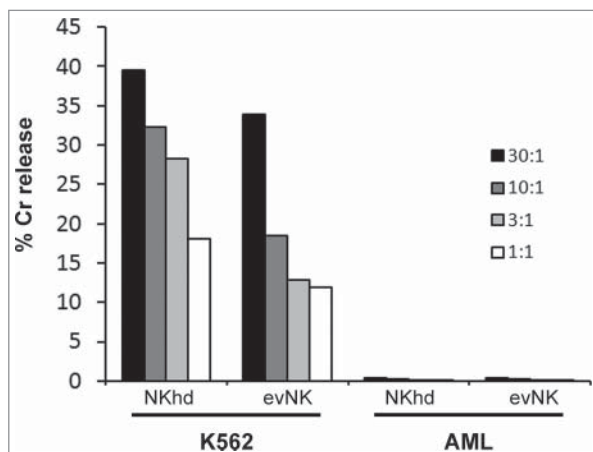
Here we first show that at the term of the 4-week differentiation process, evNK display variability in their cytotoxic function and the expression of NK receptors despite their acquired CD56 expression. In particular, NK receptor associated with lymphocyte lineage (CD7) and NK maturation such as CD94 and CD117 were differentially expressed, which may reflect different kinetics associated with UCB-donors.

We also show that the expression of the CD94 receptor on evNK is associated with a high cytotoxic potential in these cells. CD94<sup>+</sup>-evNK displayed a more mature phenotype characterized by a higher expression of CD16, CD226, Nkp30, Nkp46,



**Figure 4.** CD94-positive evNK are efficient to form immune synapses with target leukemic cells unlike CD94-negative ones. (A) Quantification of CD94-positive and CD94-negative evNK involved in immune synapse formation with K562, U937, and HL-60 target cells. EvNK were sorted based on CD94 expression and CD94-positive and -negative evNK were subsequently co-cultured for 30 min with target leukemic cells. Results are means of 2 independent experiments and are expressed as percent of evNK cells involved in immune synapses/total evNK. \* $p < 0.05$ . (B) Representative illustration of immune synapse formation between K562, U937, or HL-60 leukemic cells and CD94<sup>+</sup>-evNK. Immune synapses are visualized by F-actin staining with rhodamine-phalloidine (purple). DAPI (blue) is used for nuclei labeling. Scale bars, 5  $\mu$ m. L: leukemic cells; NK: NK cells.

NKG2A, NKG2C, NKG2D, CD7, CD45RA, and LFA-A1 receptors. Acquisition of CD94 by evNK seems to illustrate a developmental stage in NK differentiation that correlates with the acquisition of a more mature and functional phenotype. Our results parallel those of Freud et al. who studied NKs freshly isolated from human secondary lymphoid tissues (SLT) and depicted 4 differentiation stages for human NK cells in SLT, the last and more mature stage (stage 4) being characterized by CD94 expression and CD117 loss.<sup>39</sup> In reference to this classification, donors C and D may correspond to stage 3 (CD94<sup>+</sup>/CD117<sup>+</sup>).<sup>39</sup> Grzywacz et al. also proposed that CD94 expression on evNK corresponds to a more mature stage of differentiation associated with the acquisition of natural cytotoxicity and IFN $\gamma$  production.<sup>38</sup>



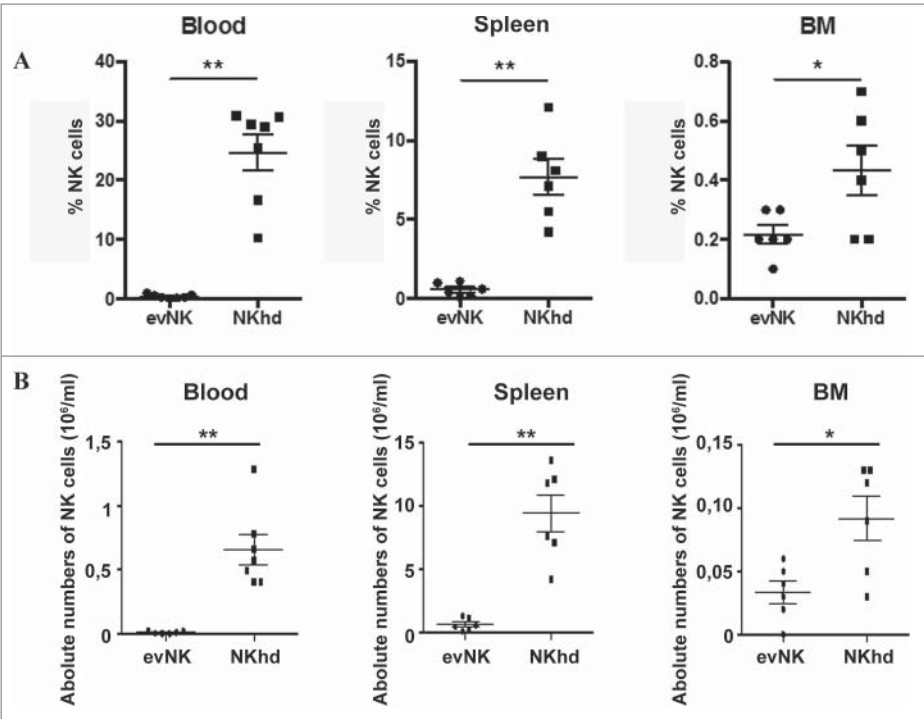
**Figure 5.** Patient-derived AML cells are resistant to evNK and NKhd. Cytotoxic activity of evNK and NKhd was measured against patient-derived AML cells at different E:T ratios (30:1, 10:1, 3:1, 1:1). K562 cells were used as positive control of NK cytotoxicity.

CD94<sup>+</sup>-evNK form significantly more synapses with leukemic target cells as compared with CD94<sup>-</sup>-evNK. Since K562 target cells did not express the CD94/NKG2 ligand HLA-E, CD94 may not directly drive interactions with target cells. Rather, since LFA-1 is known to be important for NK synapse formation,<sup>45</sup> the increased LFA-1 expression on CD94<sup>+</sup>-evNK may be involved in their interaction with leukemic target cells that display high levels of ICAM-1, the LFA-1 receptor.<sup>46</sup> In addition, LFA-1 expression has been associated with late development stage of NK cells.<sup>40</sup> Blockade of CD94 will shed light on its exact function in synapse formations. CD94 expression in evNK may also be associated with a global transcriptional profile associated with more mature stages of evNK compatible with target cell recognition and interaction.

Which factors govern the acquisition of CD94 by evNK are not known, nor yet we know which cytokine combination or concentration to use to increase CD94 rates in evNK population. A role for IL-12 is likely since IL-12 has been involved in CD94 upregulation on evNK.<sup>47</sup> In addition, Boieri et al. have recently reported that IL-12 and IL-18, in association with IL-15, are able to increase the lytic potential of NK.<sup>37,48</sup> Future studies are warranted to refine culture parameters to optimize the rate of CD94<sup>+</sup>-evNK.

In mice, the role of CD94 is still a subject of debate. A study by Orr et al., who generated C57Bl/6 CD94<sup>-</sup> deficient mice by backcrossing CD94-deleted I29/SvJ embryonic cells to C57Bl/6 genome, concluded that NK cells derived from CD94-deficient mice develop normally and efficiently kill NK cell-susceptible YAC tumor and splenocyte target cells.<sup>49</sup> In another hand, Yu et al. demonstrated that CD94<sup>+</sup> NK derived from wild type C57Bl/6 mice were more cytolytic than their CD94<sup>-</sup> counterpart.<sup>50</sup> These discrepancies between these studies were suggested to be related to the differential Ly49 inhibitory

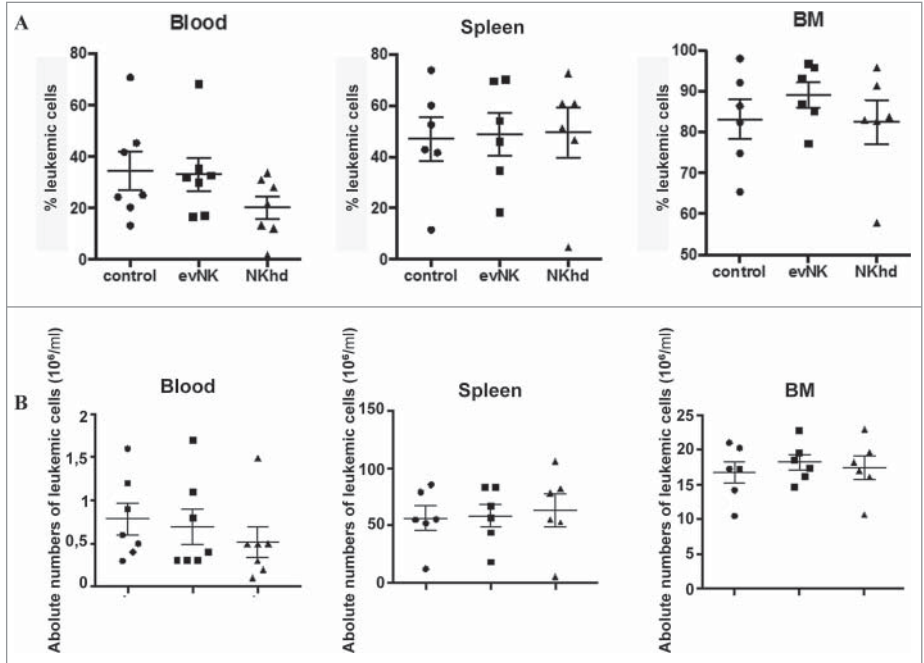




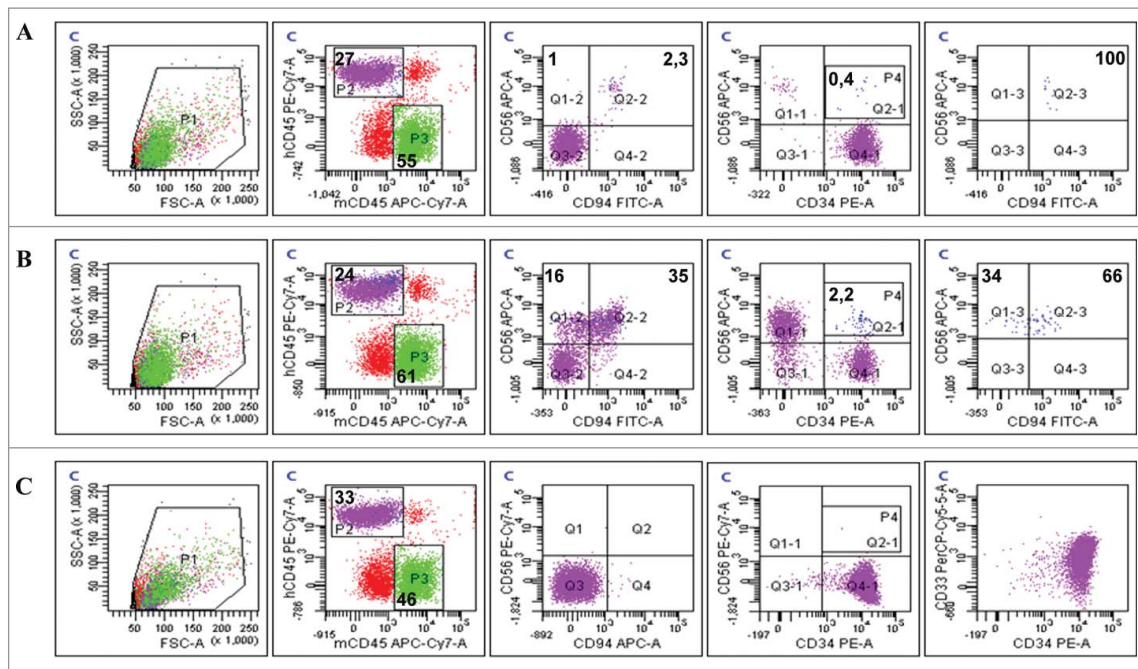
**Figure 6.** Organ distribution of evNK and NKhd in AML-engrafted mice.  $5.10^6$  evNK or NKhd were intravenously injected per mouse each 3–4 d for 14 d in AML-engrafted NSG mice. Percentages (A) and absolute numbers (B) of evNK and NKhd isolated from blood, spleen, and BM of AML-engrafted mice. EvNK and NKhd were identified using anti-human CD45 and CD56 antibodies. Mouse cells were excluded using anti-mouse CD45 antibody.  $n = 6\text{--}7$  for evNK or NKhd groups.

repertoire expressed by NK from transgenic CD94-deficient C57Bl/6NK cells (containing the I29/SvJ haplotype) and NK from wild type C57Bl/6 NK cells. Further studies are still warranted to uncover the role of mouse CD94 receptor in NK cell-mediated toxicity.

This study also aimed to evaluate the toxicity, biodistribution and anti-leukemic potential of evNK following adoptive transfer in immune-deficient mice. EvNK were not toxic in accordance with other studies of adoptive transfer of UCB-derived NK cells in mice.<sup>31,37</sup>



**Figure 7.** Absence of anti-leukemic effect of evNK and NKhd in AML-engrafted mice. Percentages (A) and absolute numbers (B) of AML cells isolated from blood, spleen, and BM of AML-engrafted NSG mice at day 14 after evNK and NKhd intravenous injection. AML were identified using anti-human CD45 and CD34 antibodies. Mouse cells were excluded using anti-mouse CD45 antibody.  $n = 6\text{--}7$  for control, evNK, or NKhd groups.

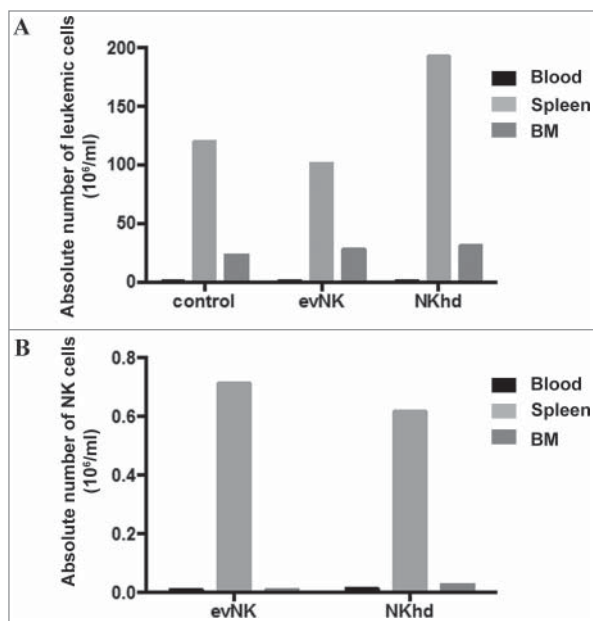


**Figure 8.** CD94-positive evNK interact with AML cells. Representative dot plots of CD56, CD94, and CD34 expression in blood isolated from AML-engrafted NSG mice injected with either evNK (A) or NKhd (B) at day 14 after NK cell intravenous injection. Untreated AML-engrafted mice were used as control (C). Anti-human CD45, CD56 and CD94 antibodies were used for NK cell identification. Anti-human CD45 and CD34 antibodies were used for AML cell identification. Bold numbers represent the percentage of cells in the corresponding quadrant. The percentages indicated in the third to fifth columns are calculated within human cells (P2 gate, hCD45<sup>+</sup>).

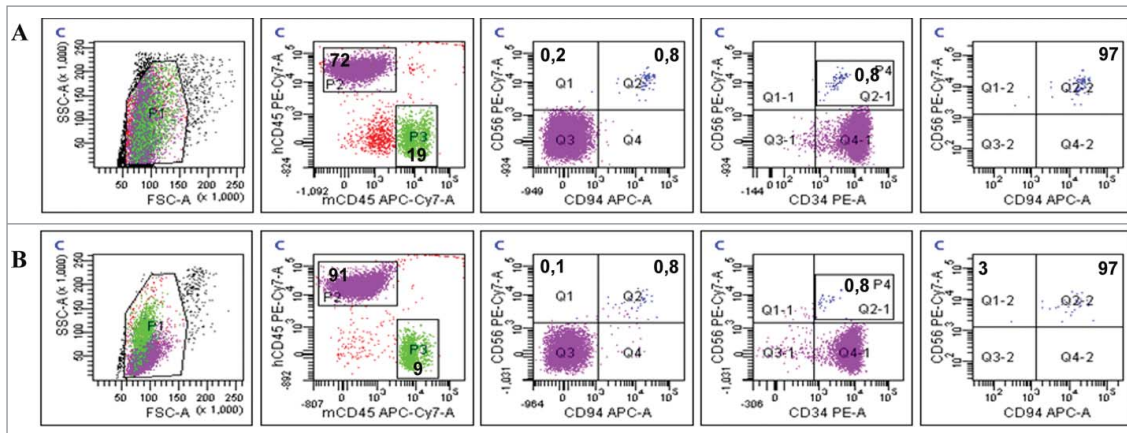
Concerning organ distribution, BM remained a sanctuary for leukemic cells but not for both evNK and NKhd. This separated distribution of AML and NK cells may account for the absence of anti-leukemic effect. Therefore, it is of interest to understand why NK cells are driven away from BM. That could be due to changes in chemokine microenvironment in BM induced by AML and resulting in driving NK outside this organ. This phenomenon was described in multiple myeloma-

bearing mice.<sup>51</sup> Also, the CXCR4 receptor, reported to retain NK cells in the BM,<sup>52</sup> may be lacking in evNK and NKhd. A study of the panel of chemokines/chemokine receptors of evNK cells and AML cells should be performed to help understanding NK biodistribution.<sup>53,54</sup>

Only 2 previous studies by Cany et al. evaluated adoptive transfer of UCB-derived evNK in leukemic mice. The authors used mice engrafted with K562 cells which are constitutively sensitive to NK lysis, and showed that treatment with UCB-NK cells potently inhibited progression of K562 leukemic cells, corroborating *in vitro* data.<sup>31,37</sup> Here we used a patient-derived AML strain for mice engraftment that was resistant *in vitro* to NK-mediated lysis questioning the impact of mice environment on AML response to NK. EvNK as well as NKhd intra-venous transfer did not decrease leukemic cell numbers in mice in the different organs, demonstrating that mice environment did not impact on NK lytic potential or AML sensitivity. But, at the end of NK treatment after mice sacrifice, we identified in mice blood a subpopulation of cells double-stained for the human leukemic marker CD34 and the human NK cell marker CD56. This suggests conjugate formation between leukemic and NK cells. Since this phenomenon did not lead to killing of leukemic cells by NK, additional elucidation of AML resistance mechanisms to NK lysis is needed. Interestingly, all the evNK cells analyzed after 2 weeks of treatment expressed the CD94 whereas no more than 20% of the injected evNK were positive for this marker. Whether it was by *in vivo* selection of CD94<sup>+</sup> evNK or by *in vivo* differentiation is not known and should be investigated. CD94 expression on all NK cells was maintained after 4 weeks, raising the hypothesis that CD94 positive NKs could be long living cells. This phenomenon of long-lasting NKs was described after human CMV reactivation, where the viral infection promoted the expansion of a cytolytic,



**Figure 9.** Absence of delayed anti-leukemic effect by evNK of NKhd at day 28 post-NK injection. Absolute numbers of AML (A) and evNK/NKhd (B) cells in the blood, spleen, and BM of AML-engrafted NSG mice at day 28 after the first NK intravenous injection. n = 1 for control, evNK, and NKhd groups.



**Figure 10.** Residual evNK at day 28 are CD94-positive. Representative dot plots of CD56, CD94 and CD34 expression in blood isolated from AML-engrafted NSG mice injected with either evNK (A) or NKhd (B) at day 28 after the first NK cell intravenous injection. Anti-human CD45, CD56 and CD94 antibodies were used for NK identification. Anti-human CD45 and CD34 antibodies were used for AML identification. Bold numbers represent the percentage of cells in the corresponding quadrant. The percentages indicated in the third to fifth columns are calculated within human cells (P2 gate, hCD45<sup>+</sup>).

urable subtype of NK cells, which was termed by the authors as “adaptive NK cells.” These cells expressed NKG2C, had a specific methylation profile and played a role in preventing leukemia relapse.<sup>55</sup>

In conclusion, evNK differentiation from UCB-CD34<sup>+</sup> cells is feasible and yields functional NK, and CD94 was found to be a marker of *in vitro* and *in vivo* functionality. Nevertheless, further understanding of the role of CD94 in the cytotoxicity displayed by evNK is needed, as well as the culture parameters that govern its expression by evNK. As for therapeutic implications, cell sorting by CD94 selection may represent an interesting option in the future to select highly functional evNK cells before adoptive transfer.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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