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CD56\textsuperscript{bright}CD16\textsuperscript{+} NK Cells: A Functional Intermediate Stage of NK Cell Differentiation

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Human NK cells comprise two main subsets, CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells, which differ in function, phenotype, and tissue localization. To further dissect the differentiation from CD56\textsuperscript{bright} to CD56\textsuperscript{dim} cells, we performed ex vivo and in vitro experiments demonstrating that the CD56\textsuperscript{bright}/CD16\textsuperscript{+} cells are an intermediate stage of NK cell maturation. We observed that the maximal frequency of the CD56\textsuperscript{bright}/CD16\textsuperscript{+} subset among NK cells, following unrelated cord blood transplantation, occurs later than this of the CD56\textsuperscript{bright}/CD16\textsuperscript{−} subset. We next performed an extensive phenotypic and functional analysis of CD56\textsuperscript{bright}/CD16\textsuperscript{+} cells in healthy donors, which displayed a phenotypic intermediary profile between CD56\textsuperscript{bright}/CD16\textsuperscript{−} and CD56\textsuperscript{dim}/CD16\textsuperscript{−} NK cells. We also demonstrated that CD56\textsuperscript{bright}/CD16\textsuperscript{+} NK cells were fully able to kill target cells, both by Ab-dependent cell cytotoxicity (ADCC) and direct lysis, as compared with CD56\textsuperscript{bright}/CD16\textsuperscript{−} cells. Importantly, in vitro differentiation experiments revealed that autologous T cells specifically encourage the differentiation from CD56\textsuperscript{bright}/CD16\textsuperscript{−} to CD56\textsuperscript{bright}/CD16\textsuperscript{+} cells. Finally, further investigations performed in elderly patients clearly showed that both CD56\textsuperscript{bright}/CD16\textsuperscript{+} and CD56\textsuperscript{dim}/CD16\textsuperscript{−} mature subsets were substantially increased in older individuals, whereas the CD56\textsuperscript{bright}/CD16\textsuperscript{−} precursor subset was decreased. Altogether, these data provide evidence that the CD56\textsuperscript{bright}/CD16\textsuperscript{+} NK cell subset is a functional intermediate between the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells and is generated in the presence of autologous T CD3\textsuperscript{+} cells.

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NK cells were originally described for their ability to kill target cells without prior stimulation (1). It is currently recognized that these cells have potent antileukemic and antiviral activities (2, 3). Human NK cells are identified by the CD3\textsuperscript{−}CD56\textsuperscript{+} phenotype. Different NK cell subpopulations are determined according to the presence and density of CD56 and CD16 (Fc\textsuperscript{γ}RIII) surface molecules. Low-density CD56 (CD56\textsuperscript{dim}) subsets constitute >90% of peripheral blood NK cells, which also express perforin and killer Ig-like receptors (KIR). These cells, which express the CD16 marker, are involved in Ab-dependent cellular cytotoxicity (ADCC). The subset of CD56\textsuperscript{bright} NK cells, which are rare in blood but predominant in lymph nodes and other tissues, do not express perforin and KIR (4). This CD56\textsuperscript{bright} subset exhibits immuno-regulatory functions through the secretion of various cytokines (i.e., IFN-γ, TNF-α, or IL-10) in response to monokine stimulation. In contrast, the CD56\textsuperscript{dim} cells are highly cytotoxic and preferentially produce cytokines after recognition of target cells (5, 6).

It is currently accepted that CD56\textsuperscript{bright} NK cells are the precursors of the CD56\textsuperscript{dim} NK cells, a concept originally hypothesized by Lanier et al. (7) in 1986. Although in vitro differentiation assays could not directly confirm this theory, many observations supported it. Indeed, the CD56\textsuperscript{bright} population displays longer telomeres than the CD56\textsuperscript{dim} NK cells, suggesting that they have proliferated less (8). In contrast with CD56\textsuperscript{dim} cells, CD56\textsuperscript{bright} cells express high levels of CD117 but do not express CD57, two receptors expressed on progenitor and senescent cells, respectively (9–11). NK cell differentiation from hematopoietic stem cell precursors primarily gives rise to CD56\textsuperscript{bright}/CD16\textsuperscript{−} KIR\textsuperscript{−} NK cells (12–14). The CD56\textsuperscript{bright} NK cells are the first lymphocytes, which appear following hematopoietic stem cell transplantations (HSCT) (15–18). Thus, HSCT is a good in vivo model to study hematopoietic cell differentiation, in particular NK cells. Dulphy et al. (15) have recently described an unusual CD56\textsuperscript{bright}/CD16\textsuperscript{+} population that was transiently increased 3 mo after identical HSCT. This subset was originally described in healthy individuals (19).

The aim of the current study was to describe more precisely the CD56\textsuperscript{bright}/CD16\textsuperscript{+} NK subset and determine its role during NK cell differentiation. Our analysis of their function, phenotype, and frequencies during aging or after unrelated cord blood transplantation (UCBT), together with in vitro NK differentiation studies, strongly suggest that the CD56\textsuperscript{bright}/CD16\textsuperscript{+} NK cell subset is a functional intermediate between the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells. Furthermore, we demonstrate that CD16 acquisition occurs in the presence of autologous T CD3\textsuperscript{+} cells.

Materials and Methods

Patients and donors

Twenty-five patients (median age 43.5 y) underwent UCBT between 2005 and 2008 at either the Pitié-Salpêtrière or Hôtel Dieu hospitals (Paris,
France) for high-risk hematopoietic malignancies. The cohort of these patients, mainly of Caucasian origin (24 out of 25), was previously described (18). Briefly, acute myeloid leukemia was the most common diagnosis (14 out of 25). All patients received a reduced-intensity conditioning regimen; 20 patients received the Minneapolis protocol containing cyclophosphamide 50 mg/kg at day 6, fludarabine 200 mg/m² for 5 d, and total body irradiation 2 Gy. For the last five patients, total body irradiation was replaced by 140 mg/m² melphalan. Cyclosorpine and mycophenolate mofetil were given 3 d before transplantation to intensify the immunosuppressive conditioning and prevent graft-versus-host disease. Generic HLA-A, HLA-B, and allelic HLA-DR typing was used in the matching strategy; most of the cord blood pairs were 4 out of 6 matches. Adult controls and cord blood samples were obtained, respectively, from the Etablissement Français du sang and the obstetrics department of the Pitie-Salpêtrière Hospital. All adult donors used for the phenotyping, functional and differentiation assays were aged between 18 and 60 y old.

The rheumatology and gerontology departments of Pitie-Salpêtrière or Charles-Foix Hospitals furnished 33 samples from individuals aged ≥60 y, 17 aged 60–80 y (mean age 68.8 ± 7.2 y), and 26 >80 y (mean age 87.1 ± 4.9 y). All volunteers affirmatively stated, and their medical records confirmed, that they had no infectious, malignant, or autoimmune diseases during the 6 mo before the study and were without acute illnesses at the time of the sampling, but could present treatment for classical age-related pathologies as previously described (20). Patients and donors provided informed consent in compliance with the ethics committee guidelines before peripheral blood samples were collected for the study.

Flow cytometry

Phenotypes were realized on whole blood. Cells were stained using the appropriate Ab mixture: anti-CD3 (UCHT1; Beckman Coulter), anti-CD56 (N901; Beckman Coulter), anti-CD16 (3G8; Beckman Coulter; or VEP13; Miltenyi Biotec); anti-CD57 (anti-CD57-H4A3; BD Biosciences); anti-CD127 (anti-CD127/KIR2DS1; BD Biosciences); anti-KIR2DL3/KIR2DL2/KIR2DS1 (CD183; Beckman Coulter); anti-CD134/KIR3DL1/KIR3DL3 (227; Beckman Coulter); anti-CD159a/NKG2A (Z199; Beckman Coulter); anti-CD55/IG-like transcript-2 (ITL-2) (HP-F1; Beckman Coulter); anti-CD8a (T8; Beckman Coulter), anti-CD117 (104D2D1; Beckman Coulter), anti-CD161 (B1; Beckman Coulter); anti-CD122 (R34.34; Beckman Coulter); anti-CD262-L (MEL-14; BD Biosciences), and anti-CX3CR1 (2A9-1; Biolegend). FACS lysing solution (BD Biosciences) was used to lyse erythrocytes. Intracellular stainings were performed after permeabilization (0.1% saponin, 0.5% BSA, PBS 1× solution) using appropriate Abs: anti-granzyme A (CB9; BD Biosciences), anti-granzyme K (GM6C3; Santa Cruz Biotechnology), anti-perforin (2B7; Beckman Coulter), anti-KIR2DL1 (HP-F1; Beckman Coulter); anti-CD16 (VEP13; Miltenyi Biotec); anti-CD94 (HP-3D9; BD Biosciences); anti-KIR2DL1/B (GB11; Abcam). Acquisitions were performed on FC500 (Beckman Coulter), LSRII, or FACSCanTo (BD Biosciences) flow cytometers, depending on the experiments.

Cell sorting

NK cells from freshly isolated PBMCs were negatively sorted using the MACS NK Cells Isolation kit (reference number 130.092.657; Miltenyi Biotec). NK cells were stained with an anti-CD3-ECD (UCHT1; Beckman Coulter), anti-CD56 (N901; Beckman Coulter), anti-CD16 (3G8; Beckman Coulter; or VEP13; Miltenyi Biotec); anti-CD57 (anti-CD57-H4A3; BD Biosciences); anti-KIR2DL1/KIR2DS1 (EB6B; Beckman Coulter); anti-KIR2DL2/KIR2DL3/KIR2DS1 (CD183; Beckman Coulter); anti-KIR3DL1/KIR3DS1 (227; Beckman Coulter); anti-CD159a/NKG2A (Z199; Beckman Coulter); anti-CD55/IG-like transcript-2 (ITL-2) (HP-F1; Beckman Coulter); anti-CD8a (T8; Beckman Coulter), anti-CD117 (104D2D1; Beckman Coulter), anti-CD161 (B1; Beckman Coulter); anti-CD122 (R34.34; Beckman Coulter); anti-CD262-L (MEL-14; BD Biosciences), and anti-CX3CR1 (2A9-1; Biolegend). FACS lysing solution (BD Biosciences) was used to lyse erythrocytes. Intracellular stainings were performed after permeabilization (0.1% saponin, 0.5% BSA, PBS 1× solution) using appropriate Abs: anti-granzyme A (CB9; BD Biosciences), anti-granzyme K (GM6C3; Santa Cruz Biotechnology), anti-perforin (2B7; Beckman Coulter), anti-KIR2DL1/B (GB11; Abcam). Acquisitions were performed on FC500 (Beckman Coulter), LSRII, or FACSCanTo (BD Biosciences) flow cytometers, depending on the experiments.

NK degranulation, ADCC, and cytolytic assays

NK cell subset’s cytolytic activity was assessed in standard 4-h [106Cr] release assays against K562 or RAJI target cells with a 5:1 E:T ratio, as described (18). ADCC experiments were performed against RAJI cells in the presence of 1 μg/ml anti-CD20 (rituximab; Roche). Degranulation assays were tested by CD107a-PC5 (H4A3; BD Biosciences) detection, as described (18). Importantly, staining with the nonblocking anti-CD16 mAb VEP13 clone was performed before the CD107a assays.

Intracellular analysis of IFN-γ production

PBMCs were first stained with CD16-FITC (clone VEP-13) and incubated for 6 h in the presence of 10 ng/ml IL-12 and 100 ng/ml IL-18 at 37°C and 5% CO2. Cells were thereafter stained for CD3 and CD56, fixed (BD Cell Fix; BD Biosciences), and permeobilized (PBS/0.5% BSA/0.1% saponin) before staining for intracellular IFN-γ (B27; BD Biosciences) expression. Acquisitions were performed on an LSRII (BD Biosciences) flow cytometer.

In vitro differentiation assays

Ten thousand FACS-sorted CD56+CD16+ or CD56+CD16+ NK cells were cultured in RPMI 1640 (Life Technologies), supplemented with 10% human serum AB (BioWest), nonessential amino acids (1×; Life Technologies), antibiotics/antimycotic (1×; Life Technologies), and IL-2 (102 U/ml, proleukin; Roche). Cultures were performed in 96-well U-bottom plates (BD Falcon). Depending on the assay, 106 autologous CD3+ T cells were added to NK cell cultures. In some assays, CD3+ T cells were replaced by CD3+CD4+, CD3+CD8+, or CD3+CD56+ lymphocyte T subsets. When specified, T cells were physically separated from NK cells with transwells (eight-well strip insert; Nunc). Cytokine and culture media were renewed every 2 or 3 d. NK cells were characterized at indicated time points using the same panel of Abs as those used for the cell sorting.

Statistics

All statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA). Nonparametric Wilcoxon and Mann–Whitney tests were used for paired and unpaired data comparisons, respectively. Repeated-measures ANOVA with Tukey posttest for p value calculation were performed for multiple comparisons of paired data. Kruskal–Wallis test with the Dunn posttest for p value calculation was performed for multiple comparisons of independent groups. Significance is defined by a p value <0.05 using two-tailed tests. *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Kinetics of CD56+CD16+ NK cell repopulation following UCBT

The phenotypic characterization of NK cells following UCBT revealed that the CD56+CD16+ NK cell subset is highly expressed among whole NK cells following the transplantation. The CD56+CD16+ subset increases very rapidly just after neutrophil engraftment, at 1-mo post-UCBT, and then gradually decreases during the time examined (Fig. 1A). Interestingly, CD56+CD16+ NK cells were present at 1 mo posttransplantation, but increased to their maximum frequency 2 mo after UCBT and remained stable during the following 12 mo (Fig. 1B). These observations suggested that the CD56+CD16+ cells are generated early posttransplantation and may be the precursors of the CD56+CD16+ subset.

Phenotypic characterization of CD56+CD16+, CD56+CD16−, and CD56dimCD16+ NK cells in healthy individuals

To precisely determine the role of the CD56+CD16+ NK cells, we performed an extensive phenotypic comparison with CD56+CD16− and CD56dimCD16+ NK cells in healthy donors. We firstly observed that CD56+CD16− and CD56+CD16+ NK cells expressed numerous receptors in common with CD56dimCD16+ cells, such as NKp30, NKp46, DNAM-1, 2B4, LAIR-1, and NKG2D (data not shown). However, some markers discriminated CD56+ from CD56dim NK cells, regardless of CD16 expression, such as CD94, NKG2A, CD127, CD27, CD62L, ILT-2, granzyme B, granzyme K, and perforin (Supplemental Fig. 1). More importantly, Fig. 2 shows that CD117 and CD25 were highly expressed in CD56+CD16− NK cells, and their expression progressively decreased in CD56+CD16+ and CD56dimCD16+ NK cells. Conversely, the expression of CD8, granzyme A, CX3CR1, and pan-KIR progressively accumulated when looking sequentially at the NK cell subsets from CD56+CD16+ to CD56dim cells. Of note, high expression of KIR in the CD56+CD16+ subset compared with the CD56+CD16− subset was also demonstrated individually for each KIR tested: KIR2DL1/D51, KIR2DL2/DL3/D2, and KIR3DL1 (Supplemental Fig. 2). Together, these data suggest that CD56+CD16+ cells may be an intermediate between CD56+CD16− and CD56dimCD16+ NK cells.
CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells are fully functional for cytolytic function

To characterize functional properties of NK cells, regardless of CD16 expression, preliminary experiments were performed to determine appropriate experimental conditions. Indeed, CD16 is quickly downmodulated after encounter with K562 or RAJI target cells, which renders discrimination of the different subsets after degranulation assays impossible (Supplemental Fig. 3) (21, 22). Furthermore, the anti-CD16 3G8 clone is a blocking Ab of ADCC, which does not allow cell sorting for [51Cr] release assays (data not shown). For these reasons, we performed all experiments in the presence of the anti-CD16 mAb VEP13 clone, which does not block ADCC. Fig. 3A shows that in the presence of the anti-CD16 mAb VEP13 clone, the expression of CD16 on NK cells is preserved after encountering of target cells, which allows analysis of NK subsets after degranulation assays (Fig. 3A).

We next compared the cytolytic capacities of CD56\textsuperscript{bright}CD16\textsuperscript{+}, CD56\textsuperscript{bright}CD16\textsuperscript{−}, and CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cell subsets in the presence of VEP13 anti-CD16 mAb. Fig. 3B shows that the CD56\textsuperscript{bright} NK cells, expressing or not CD16, displayed similar degranulation ability against K562 target cells (p < 0.001 in both cases). However, chromium release assays revealed that CD56\textsuperscript{bright}CD16\textsuperscript{+} cells were significantly less cytotoxic than CD56\textsuperscript{bright}CD16\textsuperscript{−} (p < 0.01) and CD56\textsuperscript{dim}CD16\textsuperscript{+} (p < 0.05) NK cell subsets against K562 cells, whereas CD56\textsuperscript{bright}CD16\textsuperscript{+} and CD56\textsuperscript{dim}CD16\textsuperscript{−} cells had similar direct cytolytic function (Fig. 3C). The high lytic ability of CD56\textsuperscript{bright}CD16\textsuperscript{+} cells was confirmed by ADCC assays against RAJI cells covered with anti-CD20 mAb (rituximab). Indeed, both in degranulation and chromium release assays, CD56\textsuperscript{bright}CD16\textsuperscript{+} cells were as efficient as CD56\textsuperscript{dim}CD16\textsuperscript{−} cells (Fig. 3E, 3F). In contrast, CD56\textsuperscript{bright}CD16\textsuperscript{−} cells could neither degranulate nor kill RAJI target cells in the presence of anti-CD20 mAb, in accordance with their lack of CD16 expression. Additionally, we demonstrated after IL-12/IL-18 stimulation that both CD56\textsuperscript{bright}CD16\textsuperscript{−} and CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cell subsets produce large and equivalent amounts of IFN-γ compared with the low production of the CD56\textsuperscript{dim} subset (Supplemental Fig. 4). Together, these data show that CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells contain more cytotoxic properties than CD56\textsuperscript{bright}CD16\textsuperscript{−} cells but also maintain the full ability to produce IFN-γ after cytokines stimulation.

CD3\textsuperscript{+} T cells drive CD16 acquisition and subsequent ADCC ability of CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells

We next performed in vitro differentiation assays to further characterize the CD56\textsuperscript{bright}CD16\textsuperscript{−} to CD56\textsuperscript{bright}CD16\textsuperscript{+} differentiation. These experiments were performed with highly purified CD56\textsuperscript{bright}CD16\textsuperscript{−} cells. Fig. 4A (left panels) shows that IL-2 alone was not able to drive CD16 expression on CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells after 7 or 14 d of culture. By contrast, in the presence of autologous purified CD3\textsuperscript{+} T cells, a significant fraction of CD56\textsuperscript{bright}CD16\textsuperscript{−} cells acquired CD16 (Fig. 4A, middle panels). To determine the role of the cell–cell contact requirements, similar experiments were performed in transwell plates. As shown in Fig. 4A (right panels), in the absence of cellular contacts, the CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells showed reduced CD16 acquisition. We next evaluated the efficacy of CD4\textsuperscript{+}, CD8\textsuperscript{+}, and CD3\textsuperscript{+}CD56\textsuperscript{+} T cell subsets to induce CD16 acquisition by CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells. In the presence of all of these CD3\textsuperscript{+} T cell subsets, similar proportions of NK cells expressing CD16 were observed (Fig. 4B). Importantly, kinetic studies revealed that in the presence CD3\textsuperscript{+} T cells, expression of CD16 increased until day 14, when it reached a maximum level of between 25 and 30% (Fig. 4C). In an attempt to increase CD16\textsuperscript{+} expression on NK cells, we next performed experiments in the presence of autologous dendritic cells (DC). Immature DCs (iDC) were derived from purified CD14 monocytes in the presence of IL-4 and GM-CSF, whereas mature DCs (mDC) were obtained from iDC pulsed with LPS or bacillus Calmette–Gue´rin. Both iDC and mDC were cultured with or without CD3\textsuperscript{+} T cells to induce CD16 expression on CD56\textsuperscript{bright}CD16\textsuperscript{−} cells. Unfortunately, the presence of iDC or mDC had no effect on CD16 expression (data not shown).

To determine the differentiation state of CD16\textsuperscript{+} cells generated in vitro in the presence of CD3\textsuperscript{+} T cells, we studied the expression of major differentiation markers including KIR, NKG2A, ILT-2, and CD62L (Fig. 4D). We demonstrated that CD16\textsuperscript{+} NK cells expressed a significantly higher level of KIR than the CD16\textsuperscript{−} subset at all culture times examined, although this level remains low (~10%). Concomitantly, NKG2A remained highly expressed on both subsets, whereas CD62L and ILT-2 decreased and increased, respectively, during the culture, independently of CD16 expression.
We further assessed cytolytic function acquisition by CD56<sup>bright</sup>CD16<sup>−</sup> NK cells after in vitro coculture with T cells. We tested direct cytotoxicity and ADCC ability of CD56<sup>bright</sup> NK cell subsets after 28 d of culture. Fig. 4E shows that both NK cell subsets generated in vitro were able to degranulate against K562 target cells. More interestingly, degranulation against RAJI target cells covered with anti-CD20 mAb was restricted to the CD16-expressing subset.

These data suggest that T cells drive CD16 expression on CD56<sup>bright</sup>CD16<sup>−</sup> NK cells and their subsequent ability to perform ADCC.

**In vitro differentiation of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells**

Next, a similar strategy was used to determine in vitro differentiation of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells in the presence of CD3<sup>+</sup> T cells. Fig. 5A shows that CD16 expression disappeared on half of the cells during the first days of culture and thereafter prog-
obtained for NKG2A, CD62L, and ILT-2 (Fig. 5).

**CD107 expression** (presence of anti-CD16 VEP-13 Ab clone. Box and whiskers plots of donors in the presence of CD3+ T cells, similar profiles were differentially increased to ∼80% of the cells after 28 d of culture. When differentiation markers were examined on purified CD56bright CD16+ or CD56brightCD16+ NK cells (from matched healthy donors) in the presence of CD3+ T cells, similar profiles were obtained for NKG2A, CD62L, and ILT-2 (Fig. 5B). In contrast, higher KIR expression was observed in in vitro-cultured CD56brightCD16+ cells compared with CD56brightCD16− NK cells (Fig. 5B).

**Aging is associated with the accumulation of CD16* NK cells among the CD56bright subset**

Finally, we performed an analysis of CD16 expression among NK cells during aging. It is already known that the aging process is associated with fewer CD56bright NK cells and an accumulation of CD56dim NK cells in peripheral blood (20, 23). We showed that the percentage of CD56bright NK cells expressing CD16 increases linearly with aging (Fig. 6A). Indeed, when comparing people’s aging with <60 and >80 y old, we observed that older persons expressed 2–3-fold more CD16 in their CD56bright compartment (p < 0.001). Thus, younger donors expressed CD16 on ∼25% of CD56bright NK cells, whereas elderly people >80 y old expressed this marker on ∼50–75% of these cells. Fig. 6B and 6C show that this change is principally due to a decrease of the CD56bright CD16− (p < 0.01) and an increase of CD56brightCD16+ cell counts (p < 0.01). These data clearly show that old age is associated with fewer CD56brightCD16− cells and an accumulation of both CD56brightCD16+ and CD56dimCD16+ NK cells in the periphery.

**Discussion**

The present study presents evidence that CD56brightCD16+ NK cells are a functional differentiation intermediate between CD56bright and CD56dim cells. A recent paper of Dulphy et al. (15) showed that 3 mo after matched HSCT, the frequency of CD56brightCD16− NK cells was largely increased among NK cells. In this study, we confirm this observation and additionally show that the maximum level of CD56brightCD16− into NK cells is observed later than that of CD56brightCD16+. This suggests that the CD56brightCD16+ NK cells may be an intermediate between CD56brightCD16− and CD56dim CD16− NK cell subsets. Concomitantly, in elderly individuals, more CD56dimCD16+ cells associated with aging were previously reported (20, 23, 24). More importantly, in this study, we have determined the percentage of CD56bright cells expressing CD16 and observed that it is inversely associated with aging. These data suggest that old age favors an accumulation of more mature NK cell subsets, such as CD56brightCD16+ and CD56dimCD16− cells, in detriment to the CD56brightCD16+ precursor cells, as previously described for T and B cells (25). It was observed in older individuals both increasing of soluble IL-2R, which downregulated IL-2 activity (26), and IL-12p40 homodimers, which could act as an IL-12 antagonist (27). This could suggest that the disturbance of specific cytokine signaling could partially block the final maturation of NK cells at a stage of CD56brightCD16+ NK cells, which are accumulated in older subjects.

To minimize this effect due to particular clinical situations, the main experiments of this study were performed in healthy donors. The CD56brightCD16+ NK cell subpopulation represents 1.7 ± 1.6% of NK cells. An extensive phenotypic analysis revealed few differences between CD56brightCD16+ and CD56brightCD16+ NK cells. CD56brightCD16+ NK cells showed intermediate expression levels of CD25, CD117, CD8, CX3CR1, KIRs, and granzyme A between the CD56brightCD16− and the CD56dimCD16− cells. This was in accordance with Caligiuri’s model (28), which predicts CD117 loss and KIR acquisition during stage 4 (CD56bright) to stage 5 (CD56dim) transition. Specific cytolytic molecules also revealed the maturity of CD56brightCD16+ NK cells. Indeed, the CD56brightCD16− NK cells expressed almost exclusively granzyme K, whereas CD56brightCD16+ additionally overexpressed granzyme A. Finally, CD56dimCD16+ NK cells lost granzyme K.

**FIGURE 3.** CD56brightCD16+ NK cells efficiently kill target cells. A, Representative samples of CD107a expression on CD56bright and CD56dim NK cells in absence (w/o target) or in the presence of K562 or RAJI (plus rituximab) target cells. Analysis is gated on CD3+ NK cells in absence (w/o target) or in the presence of K562 or RAJI (plus rituximab) target cells. Chromium killing assays are performed at a 5:1 E:T ratio (20,000 effectors:4,000 targets). *p < 0.05, **p < 0.01, ***p < 0.001.
and expressed abundant levels of granzyme A, granzyme B, and perforin. This succession of granzymes in NK cell subsets is similar to what is observed during memory CD8+ T cells differentiation (29).

Importantly, we show that CD56 bright CD16+ and CD56 dim CD16+ cells have similar cytotoxic functions, greater than those of CD56^{bright}CD16^{−} precursor cells. Furthermore, the progressive increase of CX3CR1 expression on CD56^{bright}CD16^{−}, CD56^{bright}CD16^{+}, and CD56^{dim}CD16^{+} NK cell subsets suggests an evolution in the acquisition of migratory properties to inflammations sites (30). Remarkably, we also found that both CD56^{bright}CD16^{−} and CD56^{bright}CD16^{+} cells displayed equivalent high abilities to

**FIGURE 4.** T cells permit CD16 expression and subsequent acquisition of ADCC ability by CD56^{bright}CD16^{−} NK cells. A, Purified CD56^{bright}CD16^{−} NK cells were cultured with IL-2 alone (IL-2) in the presence of IL-2 plus autologous T cells (IL-2 + CD3) or in a Transwell assay (IL-2 + TW-CD3). Expression of CD16 was monitored 7 and 14 d after the beginning of culture and gated on CD3^{+} CD56^{+} NK cells. B, Representative patterns of three independent experiments realized on purified CD56^{bright}CD16^{−} NK cells cultured in the presence of autologous, purified CD4^{+}CD56^{−}, CD8^{+}CD56^{−}, or CD3^{+}CD56^{+} T cells in direct contact (Mix) or in a Transwell assay. Experiments were performed at 14 d postculture and gated on CD3^{+} CD56^{+} NK cells. C, Box and whiskers plots of 12 different cultures (left panel) and representative pictures (right panel) of CD16 expression on CD56^{bright}CD16^{−} NK cells coculture with CD3^{+} T cells at different time points postculture. D, Box and whiskers plots of differentiation markers expression on CD16^{−} (white box) and CD16^{+} (gray box) NK cell subsets occurring after cocultures of CD56^{bright}CD16^{−} with CD3^{+} T cells during 7 (d7), 14 (d14), and 28 d (d28). Analyses were based on CD3^{+} CD56^{+} gated NK cells of 12 different cultures. E, CD107a degranulation assays of cultured CD56^{bright}CD16^{−} NK cells against K562 or RAJI ± 1 μg/ml rituximab target cells. Degranulation assays were performed after 28 d of coculture with autologous CD3^{+} T cells. These data are representative of three independent experiments. *p < 0.05, **p < 0.01.
produce IFN-γ after an IL-12/IL-18 stimulation compared with CD56dimCD16+ cells. Altogether, these data demonstrate that CD56brightCD16− NK cells share functional properties of the CD56brightCD16− and CD56dimCD16− subsets and suggest that CD16 acquisition could be used as a measure of NK cells’ functional maturity.

These ex vivo data were confirmed by in vitro NK cell differentiation experiments. Our data show that culture of CD56bright

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FIGURE 5. Modulation of NK receptor on CD56brightCD16− or CD56brightCD16+ NK cells cultured in the presence of autologous T cells. A. Box and whiskers plots of 12 different cultures (left panel) and representative pictures (right panel) of CD16 expression on CD56brightCD16− NK cells after 0, 7 (d7), 14 (d14), and 28 d (d28) of cocultures with CD3+ T cells. Analysis are based on CD3+CD56+ gated NK cells. B. Evolution of differentiation markers on CD56brightCD16− or CD56brightCD16+ NK cells after 7 (d7), 14 (d14), and 28 d (d28) of coculture of with CD3+ T cells. Data are represented as mean and SD of three independent cultures. *p < 0.05, **p < 0.01, ***p < 0.001.
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FIGURE 6. Old age is associated with a decrease of CD56brightCD16+ cells and an accumulation of CD56brightCD16− and CD56dimCD16+ NK cell subsets. A. Expression of CD16 on CD56bright NK cell subsets from donors <60 (n = 29; 18 < x < 60) and between 60 and 80 (n = 22; 60 < x < 80), or >80 (n = 17; x > 80) y old. B. Representative patterns of two independent donors gated on CD3+CD56+ NK cells. C. Absolute values of CD56brightCD16+, CD56brightCD16−, and CD56dimCD16+ NK cell subsets from donors ranged between 18 and 60 (n = 20; 18 < x < 60), between 60 and 80 (n = 22; 60 < x < 80), or >80 (n = 17; x > 80) y old. *p < 0.05, **p < 0.01, ***p < 0.001.
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CD16− cells with IL-2 alone is not sufficient to induce CD16 expression, as previously reported (19). This contrasts with results reported by two other groups, describing an overexpression of CD16 after culture with IL-2 alone (4, 8). This discrepancy could be explained by cell-sorting conditions, as these groups sorted all CD56bright NK cells, regardless of CD16 expression. However, our results are in line with the findings of in vitro NK cell differentiation from CD34+ stem cells (31). Freud et al. (31) showed that in medium containing IL-2 or IL-15, it was only possible to generate a CD56bright-like subset, expressing no CD16 and KIR, but high amounts of CD117.

More importantly, we provide solid evidence that CD3+ T cells are a key component to drive acquisition of functional CD16 during the CD56brightCD16− to CD56brightCD16+ transition. These data are in line with Fehniger et al. (32), who showed that CD56bright NK cells were found in the parafollicular T area of lymph nodes in direct contact with CD3+ T cells. Additionally, Freud et al. (31) demonstrated that autologous activated T cells were able to induce the differentiation of CD34dimCD45RA+ integrin β7+ stem cells into CD56bright NK cells. More recently, we have shown that after haploidentical HSCT, NK cell reconstitution was positively influenced by the amount of T cells contained in the graft (33). This work also showed that NK–T cell interactions seemed to drive CD16 acquisition, although a slight expression of this marker was also detected in transwells. This suggests that CD56brightCD16− cells gave rise to CD56brightCD16+ not only under the influence of CD3+ T cell contacts, but also from T cell-derived cytokines. Our results also indicate that these CD16− NK cells expressed significantly more KIR than the CD16− subpopulation. However, in CD56bright CD16− cells, this low KIR expression is coupled to high levels of NKG2A, suggesting that they are closer to the CD56brightCD16+ than the CD56dimCD16− NK cell subset. Intriguingly, ILT-2, a marker specifically expressed on the CD56dimCD16− subset (34), is similarly expressed on CD16− and CD16+ NK cells after in vitro culture. This could reflect long-lasting activation/proliferation instead of a differentiation of cells, as previously shown for CD62L (35). As expected, purified CD56brightCD16− NK cells retained a greater ability to express CD16 and KIR after culture in the presence of autologous IL-2–activated CD3+ T cells from primitive CD34+CD33− bone marrow progenitors.

Our data strongly support the model of NK cell differentiation as multistep processes occurring in distinct NK cell compartments and stages. Our findings could have important implications for the development of therapeutic NK cell products and clinical trials, as they provide insights into the potential activation and differentiation pathways that can be exploited to generate novel NK cell products with desired functional properties.

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