# Multifunctional human CD56$^{low}$ CD16$^{low}$ natural killer cells are the prominent subset in bone marrow of both healthy pediatric donors and leukemic patients

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## ABSTRACT

We phenotypically and functionally characterized a distinct CD56$^{low}$ natural killer cell subset based on CD16 expression levels in bone marrow and peripheral blood of healthy children and pediatric patients with acute lymphoblastic leukemia. Our findings demonstrate for the first time that CD56$^{low}$CD16$^{low}$ natural killer cells are more abundant in bone marrow than in peripheral blood and that their frequency is further increased in children with acute lymphoblastic leukemia. Bone marrow and peripheral blood CD56$^{low}$CD16$^{low}$ natural killer cells compared with CD56$^{low}$CD16$^{high}$ natural killer cells express lower levels of killer inhibitory receptors, higher levels of CD27, CD127, CD122, CD25, but undetectable levels of CD57, suggesting that they have a higher proliferative and differentiation potential. Moreover, CD56$^{low}$CD16$^{low}$ natural killer cells display higher levels of CXCR4 and undetectable levels of CX3CR1 and can be consistently and rapidly mobilized in peripheral blood in response to CXCR4 antagonist. Unlike CD56$^{low}$CD16$^{high}$, both bone marrow and peripheral blood CD56$^{low}$CD16$^{low}$ natural killer cells release IFNγ following cytokine stimulation, and represent the major cytotoxic natural killer cell population against K562 or acute lymphoblastic leukemia target cells. All these data suggest that CD56$^{low}$CD16$^{low}$ natural killer cells are multifunctional cells, and that the presence of hematologic malignancies affects their frequency and functional ability at both tumor site and in the periphery.

## Introduction

Natural killer (NK) cells are innate lymphocytes known to be important players in the early phase of immune defense against certain microbial infections and tumor growth. They represent a highly specialized effector population, capable of mediating cellular cytotoxicity and secreting several chemokines and cytokines.[1-3]

Natural killer cells differentiate primarily in the bone marrow (BM) from a lymphoid precursor, but final maturation of NK-cell progenitors can also occur in the periphery, and the existence of a thymic pathway of NK-cell differentiation has been described.[4,5] Mature NK cells mainly circulate in peripheral blood (PB), but are also resident in several lymphoid and non-lymphoid organs, including the decidua, where they are the most prominent population in early pregnancy.[6] During maturation, NK cells acquire a number of inhibitory receptors, as well as several activating or co-stimulatory molecules.[7,8] The inhibitory receptors mostly recognize MHC class I molecules and belong to two distinct groups: the killer cell immunoglobulin-like receptor (KIR) family, which comprises receptors for human leukocyte antigen (HLA)-A, -B, -C alleles, and C-type lectin receptors, such as CD94/NKG2A, which binds to non-classical HLA-class I molecule, HLA-E. Both receptor families include an activating counterpart with similar specificity, but different ligand affinity. The engagement of these receptors is also important for the acquisition of functional competence during NK-cell development through a process defined as NK-cell education or licensing.[9,10] The best studied NK-cell activating receptor is the low affinity Fc-γ receptor IIIA (CD16) responsible for antibody-dependent cellular cytotoxicity (ADCC).[11] Other activating receptors that trigger natural killing, often in combination, include NKp44, NKp46 and NKp30 Ig-like molecules, collectively termed natural cytotoxicity receptors (NCR), and DNAM-1 (CD226).[12-14] NKG2D is another important activating receptor that recognizes self proteins up-regulated on stressed or damaged cells.[15] The expression of both activating and inhibitory receptors is highly regulated during NK-cell differentiation and activation, and some of them are selectively expressed on distinct NK-cell subsets. Thus, based on receptor repertoire and expression levels, phenotypically distinct NK-cell populations have been identified in different tissues, and likely represent specialized NK-cell subsets capable of mediating different functions and endowed with distinct migratory properties.[16,17] Two major subsets of human PB NK cells have been widely reported: CD56$^{low}$CD16$^{high}$ NK cells, which represent approximately 90% of PB NK cells and are the principal cytotoxic NK-cell population, and CD56$^{high}$CD16$^{+/-}$ cells, which represent 10% of PB NK cells and more abundantly secrete immunoregulatory cytokines.[16] However, recent evidence indicates that PB CD56$^{low}$CD16$^{neg}$ cells are responsible for natural cytotoxicity against human leukemia and lymphoma cells.[18]

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CD56$^{high}$CD16$^{+/-}$ NK cells originate from CD34$^{+}$ hematopoietic precursors through phenotypically distinct stages, whereas the CD56$^{low}$CD16$^{high}$ NK-cell population can originate from the CD56$^{high}$ subset, upon interaction with peripheral fibroblasts.[19] Moreover, based on the surface density of CD94 and CD62L, functional intermediates between CD56$^{high}$ and CD56$^{low}$ have also been described.[20-22] This sequential differentiation pathway is supported by the observation that CD56$^{high}$ NK cells have longer telomeres than CD56$^{low}$ NK cells, that they predominate in PB earlier after hematopoietic stem cell (HSC) transplantation, and that they differentiate into CD56$^{low}$ in humanized mice engrafted with human HSCs in the presence of human IL-15, a cytokine capable of inducing NK-cell proliferation and differentiation.[20,23-24]

Furthermore, it is well established that mature human CD56$^{low}$ NK cells display marked phenotypic and functional heterogeneity. Indeed, lymph node and tonsil CD56$^{low}$ NK cells are functionally and phenotypically different from PB CD56$^{low}$ NK cells, in that they are negative for CD16, KIRs, perforin, and for most NCR that are acquired after IL-2 stimulation.[25,26]

Unlike the well-defined stages of BM NK-cell development in the mouse, in humans the information on NK-cell development in BM is rather limited. Indeed, while four NK-cell developmental intermediates have been described, both phenotypically and functionally, in human lymph nodes and tonsils,[25,27] no evidence on the effector functions of the four different NK-cell subsets so far reported in the BM[28] and their relation to the PB counterpart has been provided.

In this study, we analyzed the CD56$^{low}$ NK-cell terminal differentiation in BM and PB of healthy pediatric BM donors by evaluating the phenotype and the effector functions of NK-cell subsets identified on the basis of the expression levels of the two major markers of NK-cell lineage CD56 and CD16, namely: CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{high}$ and CD56$^{low}$CD16$^{low}$ cells. We also assessed the presence of these NK-cell subsets in BM and PB of age-matched patients affected by acute lymphoblastic leukemia (ALL) in order to evaluate whether the presence of lymphoid blasts can shape NK-cell subset distribution at tumor site and in the periphery. Finally, we investigated the effect of G-CSF administered either alone or in combination with a CXCR4 antagonist, on the distribution of PB NK-cell subsets. Our results indicate that CD56$^{low}$CD16$^{low}$ NK cells are a unique subset endowed with multifunctional activity in BM and PB, and we suggest that they can represent an intermediate differentiation stage between CD56$^{high}$CD16$^{+/-}$ and CD56$^{low}$CD16$^{high}$ NK cells. Moreover, we also show that the frequency of distinct NK-cell subsets is affected by the presence of leukemia cells both at tumor site and in the periphery.

## Methods

### Cell source

Peripheral blood and BM cells were obtained from 16 pediatric healthy donors who donated BM for transplantation at Bambino Gesù Children's Hospital, Rome, Italy, and from 19 children with acute lymphoblastic leukemia (ALL) (5 T-cell and 14 B-cell precursor ALL) at diagnosis (*Online Supplementary Table S1*). PB mononuclear cells (PBMC) were obtained from 9 adult donors given G-CSF only (12 μg/kg/day for 5 consecutive days) and from 6 donors receiving G-CSF plus plerixafor (240 μg/kg in single dose) for HSC mobilization.

The study was approved by the institutional ethics committees and informed assent/consent was obtained from donors, patients and/or their legal guardians.

### Multicolor immunofluorescence, cytofluorimetric analysis and cell sorting

Freshly isolated PBMC and BM cells were stained using the appropriate antibody (Ab) combination and subjected to cytofluorimetric analysis. Intracellular staining with appropriate mAb was performed after fixation with 1% paraformaldehyde and permeabilization (0.5% saponin, 1% FCS) (*Online Supplementary Table S1*).

Sample acquisition was performed on FACSCantoII (BD Biosciences, San Jose, CA, USA) flow cytometer, and cytofluorimetric analysis was performed with FlowJo 9.2.3 (TreeStar, Ashland, OR, USA).

For cell sorting, PBMCs, freshly isolated by Lymphoprep (Nycomed AS, Oslo, Norway) gradient centrifugation, were stained with the appropriate mAb, NK-cell subsets were sorted by FACSAria (BD Biosciences) and used for cytotoxicity and differentiation assays.

### Degranulation assay and IFNγ production

Freshly isolated NK-cell subsets from PB or BM were co-cultured with K562 cells or ALL blasts at 1:1 effector/target (E/T) ratio for 3 h, in the presence of 50 μM monensin (BD Biosciences) for the last 2 h, and degranulation was assessed by evaluating CD107a expression.

In some experiments, NK cells were co-cultured with the FcγR+ murine mastocytoma cell line, P815, in the presence of mAbs directed against the relevant activating NK-cell receptors. Degranulation was assessed upon 2 h-culture at 37°C.

To assess intracellular IFNγ production, cells were incubated with IL-12 (25 ng/mL) plus IL-15 (50 ng/mL) (PeproTech, London, UK) at 37°C. After 1 h, 10 μg/mL brefeldin A were added, and cells were incubated for an additional 12 h. Cells were subsequently fixed, permeabilized, stained with anti-IFNγ-APC, and analyzed by flow cytometry. To evaluate the ability of NK-cell subsets to degranulate and produce IFNγ FACS-sorted NK-cell subsets were stimulated with K562 target cells for 6 h, and analyzed for the co-expression of CD107a and IFNγ, as described above.

### Cytotoxicity assay

The MHC class I negative human erythroleukemia cell line K562 was used as target for natural cytotoxicity. In some experiments, the cytotoxic potential of FACS-sorted NK-cell subsets was evaluated by $^{51}$Cr release assay, performed as previously described.[29]

### In vitro differentiation assay

FACS-sorted CD56$^{high}$CD16$^{+/-}$ or CD56$^{low}$CD16$^{low}$ or CD56$^{low}$CD16$^{high}$ NK cells were cultured in RPMI 1640 (EuroClone, Pero, Milano, Italy), supplemented with 10% FCS (EuroClone), antibiotic, L-glutamine and IL-12 (25 ng/mL) plus IL-15 (25 ng/mL). NK-cell subsets were characterized at different time points by immunofluorescence and flow cytometric analysis.

### Statistical analysis

*t*-test or Mann-Whitney U test were used to compare independent groups; paired *t*-test or Wilcoxon matched test were used to compare matched groups. Statistical analyses were performed using PRISM 6.0 (GraphPad, La Jolla, CA, USA).

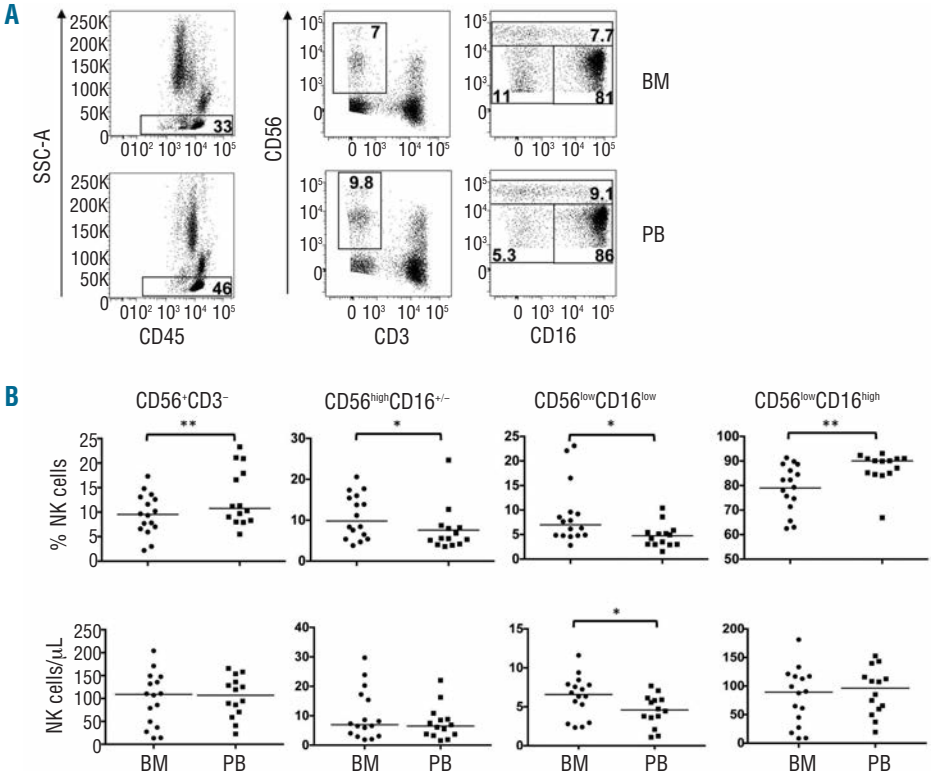


**Figure 1.** Natural killer NK-cell subset distribution in bone marrow (BM) and peripheral blood (PB) from healthy pediatric donors. (A) Representative dot plots of NK-cell subsets gated on $CD56^{+}CD3^{-}$ and dissected based on the CD56 and CD16 expression levels. (B) Percentage and absolute cell number/μL of total NK cells among lymphocytes and of $CD56^{high}CD16^{+/-}$, $CD56^{low}CD16^{low}$ and $CD56^{low}CD16^{high}$ NK-cell subsets in BM (circles) and PB (squares). BM: n=16; PB: n=14. $^{**}P<0.005$; $^{*}P<0.05$.

## Results

### *Distribution of NK-cell subsets in BM and PB from healthy pediatric donors and children with ALL*

In order to better characterize the last stages of human BM NK-cell differentiation, we first analyzed the distribution of distinct NK-cell subsets in BM and PB of pediatric healthy BM donors. We dissected the mature $CD56^{+}CD3^{-}$ NK-cell compartment, based on the expression levels of CD56 and CD16, into three distinct subsets: $CD56^{high}CD16^{+/-}$, the $CD56^{low}CD16^{high}$ and the $CD56^{low}CD16^{low}$ NK cells. According to this classification, analysis of BM and PB NK cells revealed that the $CD56^{high}CD16^{+/-}$ NK-cell subset is more abundant in the BM (12.9±6.4%) than in the PB (9±6%). Similarly, the $CD56^{low}CD16^{low}$ NK-cell subset is significantly more abundant in the BM than in the PB, both in frequency (9.6±5% in the BM *vs.* 4.7±3% in the PB) and in absolute number. By contrast, the frequency of $CD56^{low}CD16^{high}$ NK cells is higher in PB (85.6±8.4%) than in BM (74.6±12.5%) (Figure 1).

In order to investigate whether the presence of ALL blasts can affect NK-cell subset distribution, we evaluated the proportion of the three NK-cell subsets in BM and PB samples collected from age-matched children with ALL at diagnosis. In children with ALL, the absolute number of total NK cells, $CD56^{low}CD16^{low}$ and $CD56^{high}CD16^{+/-}$ NK-cell subsets were significantly more abundant in the BM and PB with respect to healthy donors, whereas no major differences were observed in the absolute number of $CD56^{low}CD16^{high}$ NK cells (Figure 2A and B).

Collectively, these observations indicate that $CD56^{low}CD16^{low}$ NK cells are more abundantly present in the BM as compared to the PB in physiological conditions, and that the presence of lymphoid blasts markedly influences the distribution of distinct NK-cell subsets.

### *$CD56^{low}CD16^{low}$ NK cells exhibit a distinct NK-cell phenotype*

We further characterized the phenotypic profile of $CD56^{low}CD16^{low}$ NK cells in the BM and PB from pediatric healthy donors. First, the expression of different NK-cell activating receptors was evaluated as percentage of positive cells (Figure 3) and mean fluorescence intensity (MFI) (*data not shown*). We found that $CD56^{low}CD16^{low}$ NK cells express intermediate levels of NKG2D with respect to $CD56^{high}CD16^{+/-}$ and $CD56^{low}CD16^{high}$ NK-cell subsets, with no major differences between BM and PB NK cells. With regard to the expression of the NCR, similar levels of NKp46 were found on both $CD56^{low}CD16^{low}$ and on $CD56^{low}CD16^{high}$ NK cells both in BM and PB, whereas higher levels were observed on $CD56^{high}CD16^{+/-}$ NK cells. By contrast, NKp44, an activating receptor preferentially expressed on stimulated or tissue resident NK cells, was present at low levels only in BM $CD56^{high}CD16^{+/-}$ and $CD56^{low}CD16^{low}$ NK cells. In addition, we found that $CD56^{low}CD16^{low}$ NK cells express lower levels of the co-stimulatory and adhesion molecule DNAM-1 as compared to $CD56^{high}CD16^{+/-}$ and $CD56^{low}CD16^{high}$ NK-cell subsets, with no major differences observed between BM and PB compartments (Figure 3A).

We then evaluated the expression profile of the receptors for the MHC class I molecules associated with NK-cell education and acquisition of killing capability.[30] As expected, most $CD56^{low}CD16^{high}$ NK cells express CD158a (KIR2DL1), CD158b (KIR2DL2), CD158e1 (KIR3DL1), NKG2A and NKG2C, while $CD56^{high}CD16^{+/-}$ cells are positive only for the C-type lectin receptor NKG2A, which is

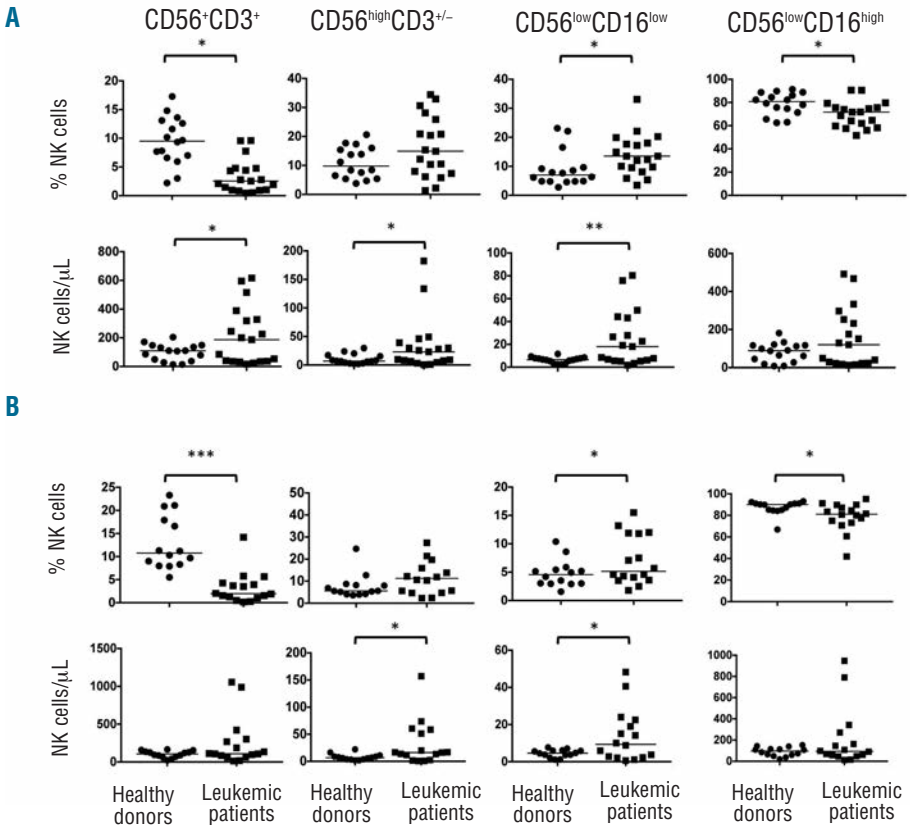


**Figure 2.** Natural killer (NK)-cell subset distribution in bone marrow (BM) and peripheral blood (PB) from pediatric leukemic patients. Percentage and absolute cell number/μl of total NK cells among lymphocytes and of $CD56^{high}CD16^{+/-}$, $CD56^{low}CD16^{low}$ and $CD56^{low}CD16^{high}$ NK-cell subsets in BM (A, upper panels) and PB (B, bottom panels) from 19 pediatric leukemic patients (squares) and 16 healthy pediatric donors (circles). ***$P$<0.0005; *$P$<0.05.

expressed on this subset at higher levels than on $CD56^{low}CD16^{high}$ and $CD56^{low}CD16^{low}$ NK cells. Notably, only very few $CD56^{low}CD16^{low}$ NK cells in PB express CD158b receptor, most of them being negative for CD158a, CD158e1 and NKG2C (Figure 3B).

In parallel, we also investigated the expression profile of several cytokine receptors on PB and BM NK-cell subsets, namely the alpha(α)-chain of the IL-2R, CD25, and the beta(β)-chain of IL-2R and IL-15R, CD122. We observed that CD25 was significantly more expressed on $CD56^{high}CD16^{+/-}$ and on $CD56^{low}CD16^{low}$ NK cells than on $CD56^{low}CD16^{high}$ NK cells, while CD122 was found predominantly on BM-derived $CD56^{high}CD16^{+/-}$ NK cells, with similar but lower levels on both the $CD56^{low}$ NK-cell subsets (Figure 3C).

In addition, we looked at the expression of the α-chain of IL-7R, CD127, a receptor preferentially found at higher levels on pre-pro NK cells and immature NK cells.[31] We found that BM and PB $CD56^{high}CD16^{+/-}$ and $CD56^{low}CD16^{low}$ NK-cell subsets express high levels of CD127, which is undetectable on $CD56^{low}CD16^{high}$ cells (Figure 3C).

Based on the pivotal role played by IL-15 on NK-cell development, homeostasis and activation, and on the role of other activating cytokines, such as IL-2, in the regulation of NK-cell activation and acquisition of effector functions at different steps of immune responses,[32-35] our results suggest that $CD56^{high}CD16^{+/-}$ and $CD56^{low}CD16^{low}$ NK cells more capably survive, proliferate and undergo activation in response to IL-15 and IL-2. In agreement with these results, we observed that sorted $CD56^{low}CD16^{low}$ NK cells can up-regulate the expression of CD56 after 7-day exposure to IL-15 plus IL-12, thus acquiring a phenotype similar to that of $CD56^{high}CD16^{+/-}$ NK cells (Figure 4).

Finally, we investigated the expression of molecules associated with the maturation/differentiation process, both on BM and PB NK cells. We found that CD161, a marker of NK-cell lineage commitment,[36] is highly expressed on BM $CD56^{high}CD16^{+/-}$ and at intermediate levels on $CD56^{low}CD16^{low}$ and $CD56^{low}CD16^{high}$ NK cells, with no major differences between BM and PB compartments. By contrast, the expression of CD57, a marker of senescent or terminally differentiated NK cells,[37,38] was more abundant on $CD56^{low}CD16^{high}$ and marginally on $CD56^{low}CD16^{low}$ and $CD56^{high}CD16^{+/-}$ NK cells. Moreover, in both BM and PB compartments, the expression of CD27, a TNF-R family member which is a marker known to identify distinct stages of mouse BM NK-cell development,[39,40] was high on $CD56^{high}CD16^{+/-}$, lower on $CD56^{low}CD16^{low}$, and undetectable on $CD56^{low}CD16^{high}$NK cells (Figure 3D). Altogether, these results suggest that, like $CD56^{high}CD16^{+/-}$ NK cells, $CD56^{low}CD16^{low}$ NK cells represent a less mature stage than $CD56^{low}CD16^{high}$ NK cells.

### Chemokine and adhesion receptor profile on BM and PB NK-cell subsets

In order to elucidate the homing properties of BM and PB NK-cell subsets, we analyzed the expression pattern of chemokine and adhesion receptors crucial for controlling lymphocyte trafficking. As shown in Figure 5A, both in BM and PB, $CD56^{low}CD16^{low}$, NK cells have increased expression levels of the receptor for CXCL12/SDF-1, CXCR4, while they exhibit only low levels of the receptor for CXCL10/IP10, CXCR3, and of the receptor for

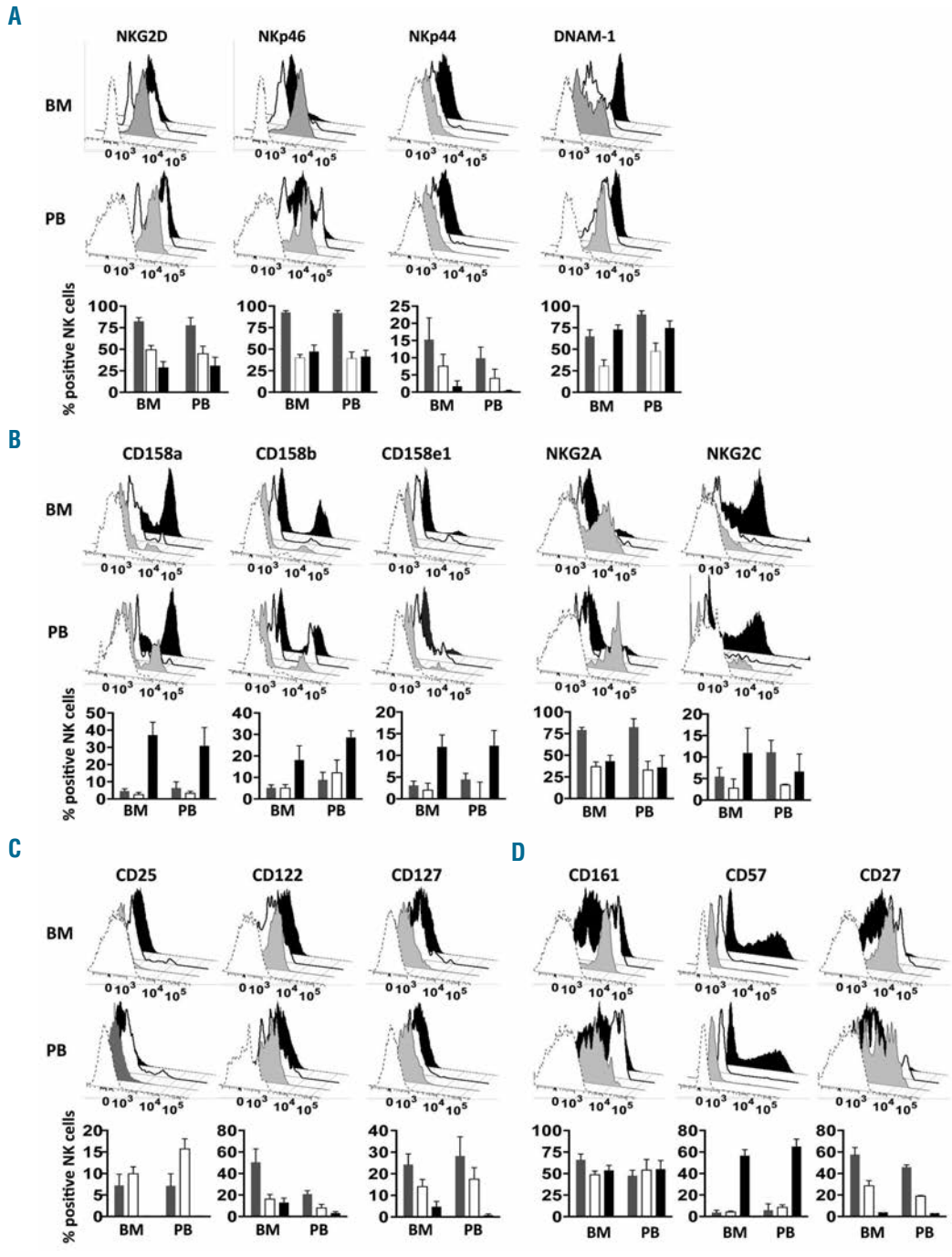


Figure 3. Phenotypic characterization of human bone marrow (BM) and peripheral blood (PB) natural killer (NK)-cell subsets defined by CD56 and CD16 expression levels. The CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets were gated on CD56$^{+}$CD3$^{-}$ NK cells and analyzed for the expression of activating (A), MHC class I-specific (B), cytokine (C), and differentiation (D) receptors. A representative histogram for the expression of each marker in BM and PB is shown. Dotted histogram represents isotype-matched control Ig. Mean value+SD of the percentage of positive cells in BM and PB of 5 independent experiments is shown.

CX3CL1/Fractalkine, CX3CR1. In addition, both in BM and PB, with respect to CD56$^{low}$CD16$^{high}$ NK cells, CD56$^{low}$CD16$^{low}$ NK cells display increased expression levels of the adhesion molecule CD62L, a molecule expressed on resting, but not on activated, NK cells, and capable of guiding lymphocyte migration. Collectively, these data suggest that the CD56$^{low}$CD16$^{low}$ NK-cell subset has a preferential tropism for lymph nodes instead of inflamed tissues. Moreover, in accordance with the literature, we found that CD56$^{low}$CD16$^{high}$ NK cells display higher expression levels of CX3CR1 and lower expression levels of CD62L. This supports the notion that this NK-cell subset might preferentially migrate to inflamed tissues in response to Fractalkine.[41,42] Both in BM and PB, CD56$^{high}$CD16$^{+/-}$ NK cells displayed higher levels of CD62L, intermediate to low levels of CXCR4 and

CX3CR1, and undetectable levels of CXCR3, suggesting that these cells mainly recirculate in the periphery.

In agreement with the higher expression levels of CXCR4 on CD56$^{low}$CD16$^{low}$ NK cells, we observed that these cells can be mobilized more efficiently when plerixafor, a CXCR4 antagonist, is added to G-CSF for HSC mobilization (Figure 5B).[43] These results suggest that CD56$^{low}$CD16$^{low}$ NK cells have a different trafficking behavior and that, because of the higher expression of CXCR4, they may be more efficiently retained in BM as compared to CD56$^{low}$CD16$^{high}$ NK cells.

### *CD56$^{low}$CD16$^{low}$ NK cells display higher degranulating capacity and ability to produce IFN within the CD56$^{low}$ NK-cell subset: analysis of BM and PB of pediatric healthy donors and leukemic patients*

To assess the functional properties of BM and PB NK-cell subsets, we first evaluated their ability to degranulate upon binding to HLA class-I deficient K562 target cells (in the absence of exogenously added cytokines). Interestingly, upon binding to K562 cells, BM and PB CD56$^{low}$CD16$^{low}$ NK cells have the highest degranulation potential, as evaluated by the percentage of CD107a positive cells. By contrast, freshly isolated BM or PB CD56$^{low}$CD16$^{high}$ and CD56$^{high}$CD16$^{+/-}$ NK cells poorly degranulated upon interaction with K562 cells (Figure 6A). Similar results were observed at different E:T ratios (*data not shown*). These data were further confirmed using sorted PB NK-cell subsets in a classical $^{51}$Cr-release assay against K562 target (Figure 6B). These results also indicate that the higher cytotoxic activity attributed to the CD56$^{low}$ NK-cell population as compared to CD56$^{high}$ NK cells[16,17] (*Online Supplementary Figure S1*) is mainly confined to the CD56$^{low}$CD16$^{low}$ NK cells. Moreover, by performing a reverse ADCC assay, we found that CD56$^{low}$CD16$^{low}$ NK cells degranulated in response to stimulation with anti-NKp46 mAb, used either alone or in combination with anti-DNAM-1 or anti-NKG2D mAb, similarly to CD56$^{high}$CD16$^{+/-}$ NK cells. By contrast, CD56$^{low}$CD16$^{high}$ NK cells, either from BM or PB, expressed lower levels of CD107a upon triggering of NKp46, DNAM-1 or NKG2D receptors (Figure 6C and D) independently of the expression levels of these activating receptors.

We further assessed the ability to produce IFNγ in response to overnight stimulation with IL-12 plus IL-15. The results obtained indicate that PB CD56$^{high}$CD16$^{+/-}$ and CD56$^{low}$CD16$^{low}$ NK-cell subsets are the major producers of IFNγ, while the percentage of IFNγ-producing PB or BM CD56$^{low}$CD16$^{high}$ NK cells is lower (Figure 7A). Moreover, CD56$^{low}$CD16$^{low}$and CD56$^{high}$CD16$^{+/-}$ degranulating NK cells are also the major IFNγ-producing cells upon pre-treatment with low doses of cytokines followed by binding to K562 target cells (Figure 7B).

These results indicate that CD56$^{low}$CD16$^{low}$ NK cells may represent a unique subset of NK cells equipped with both high degranulation capacity and the ability to produce IFNγ.

We also assayed the degranulation ability of NK-cell subsets isolated from BM or PB of children with ALL at diagnosis. We observed that, unlike healthy donors, in these subjects all NK-cell subsets poorly degranulate in response to K562 target cells (Figure 8A). Degranulation assay was performed using as source of effector cells the PB NK-cell subsets from 2 haploidentical HSC donors, and as target the leukemic blasts of the corresponding recipients. Interestingly, we observed that, in both cases, the CD56$^{low}$CD16$^{low}$ NK cells are the only population capable of degranulation and that they are, therefore, endowed with the ability to kill leukemic blasts (Figure 8B).

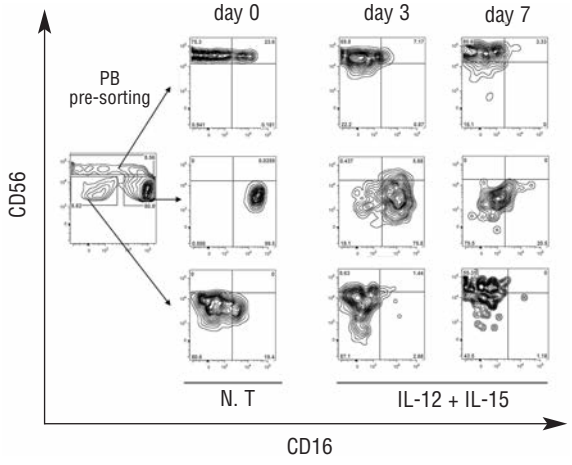


**Figure 4.** CD56$^{low}$CD16$^{low}$ peripheral blood (PB) natural killer (NK) cells up-regulate CD56 upon IL-12 plus IL-15 *in vitro* treatment. CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{low}$ and CD56$^{low}$CD16$^{high}$ sorted PB NK-cell subsets were cultured with IL-12 (25 ng/mL) and IL-15 (25 ng/mL) for seven days. NK-cell phenotypic profile was analyzed on day 3 and 7 of culture. Results of 1 of 3 independent experiments are shown.

No changes in the expression levels of CD56 and CD16 were observed upon NK-cell binding to target cells (*data not shown*).

## Discussion

In this study, we identified a subset of CD56$^{low}$ NK cells characterized by low expression of CD16 that are prevalent in the BM both of healthy children and of pediatric patients with ALL, and that that are endowed with potent killer and IFNγ producing capacity.

The expression of inhibitory receptors belonging to NKG2 and KIR families reveals that, unlike CD56$^{low}$CD16$^{high}$ NK cells, CD16$^{low}$ NK cells in both BM and PB display lower levels of KIR that are acquired at late stages of NK-cell differentiation, thus suggesting that these cells have not yet completed their development. Accordingly, the CD56$^{low}$CD16$^{low}$ NK-cell subset displays higher levels of CD27, the expression of which has been associated with an earlier differentiation stage both in humans and mice,[39,40,44] and with lower levels of the CD57 senescence marker than CD56$^{low}$CD16$^{high}$ NK cells.[37,38] In addition, CD56$^{low}$CD16$^{low}$ NK cells, differently from CD56$^{low}$CD16$^{high}$NK cells, express CD25, CD122 and CD127, the receptor chains for IL-2/IL-15 and IL-7 cytokines, respectively, which play a major role in controlling NK-cell development, homeostasis, survival and activation. This finding further supports the idea that they have higher proliferative and differentiation potential. In addition, in accordance with the higher expression of CD122, we found that sorted PB CD56$^{low}$CD16$^{low}$ NK cells up-regulate CD56 following 7-day exposure to IL-15 plus IL-12, as previously reported.[45]

The analysis of chemokine receptors on BM and PB CD56$^{low}$CD16$^{low}$ NK cells reveals that this subset exhibits higher expression of CXCR4, whereas CX3CR1 is unde-

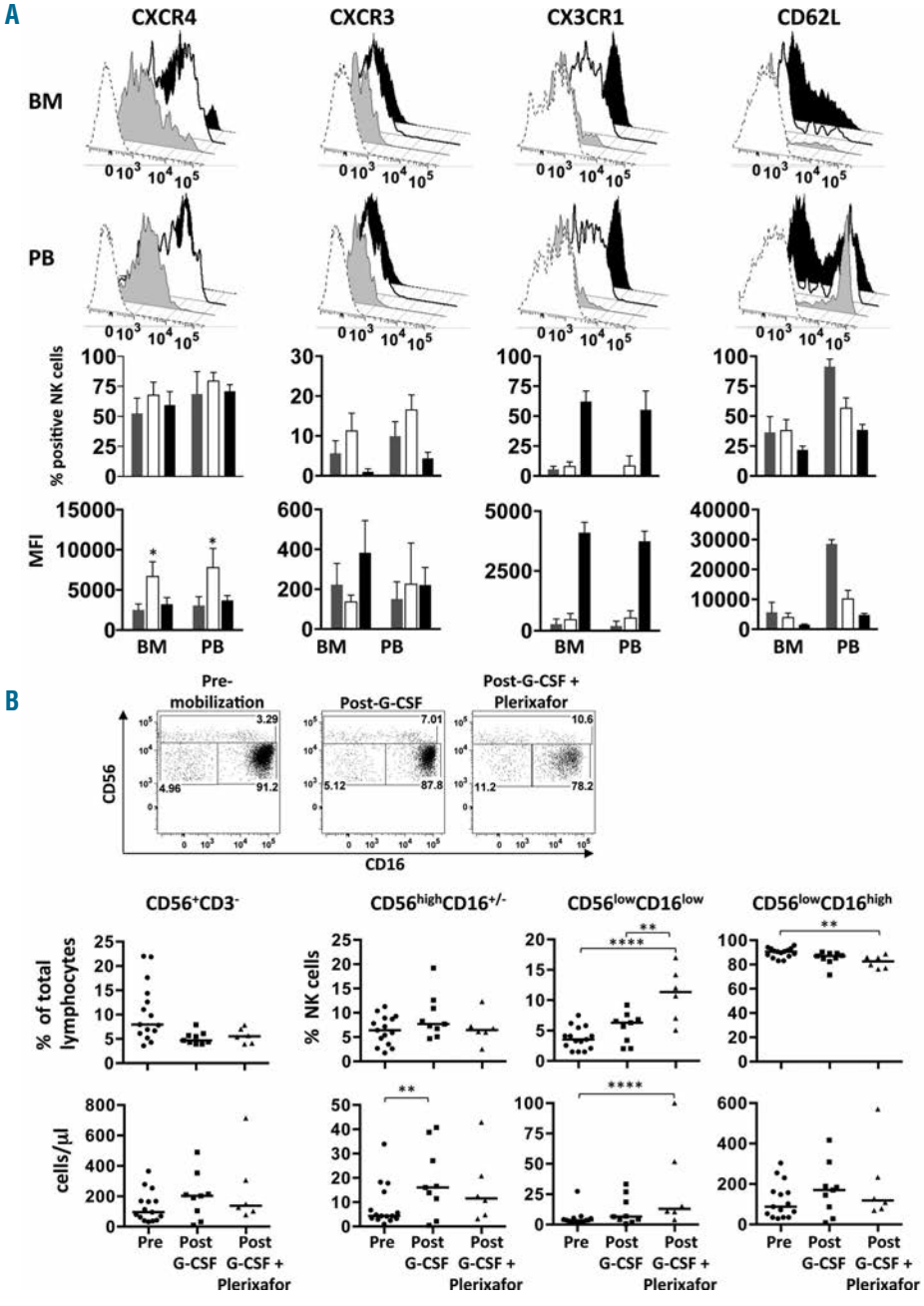


**Figure 5.** Chemokine receptor profile on bone marrow (BM) and peripheral blood (PB) CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{low}$ and CD56$^{low}$CD16$^{high}$ natural killer (NK)-cell subsets and their distribution in the PB before and after hematopoietic stem cell (HSC) mobilization. (A) Representative histograms of chemokine receptor expression on CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets, gated on CD56$^{+}$CD3$^{-}$ NK cells. Dotted histogram represents isotype-matched control Ig. Mean value+SD of the percentage of positive cells and the mean fluorescence intensity (MFI) in BM and PB of 5 independent experiments is shown. (B) Percentage and absolute cell number/µL of total NK cells and CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{low}$ and CD56$^{low}$CD16$^{high}$ NK-cell subsets in PB from untreated (circle), G-CSF (square) or G-CSF plus Plerixafor (triangle) mobilized donors of allogeneic HSC. Upper panels show a representative cytofluorimetric profile of NK-cell subsets gated on CD56$^{+}$CD3$^{-}$ and dissected based on the CD56 and CD16 expression levels before and after mobilization with G-CSF or G-CSF plus Plerixafor. ****$P$<0.00005; **$P$<0.005.

tectable. Again, the expression pattern of these chemokine receptors is consistent with a more immature phenotype, since CXCR4 expression was described to decline during BM NK-cell development in mice,[42,46] and CX3CR1 is predominant on terminally differentiated mouse and human NK cells.[42,47] Notably, higher expression levels of CXCR4 on CD56$^{low}$CD16$^{low}$ NK cells, as compared to the other two NK-cell subsets, paralleled their preferential BM localization, and their capacity to be more efficiently mobilized in response to the combined treatment with G-CSF and plerixafor.

Our findings also indicate that, among the CD56$^{low}$ NK cells, the CD16$^{low}$ subset displays the highest degranulation/cytotoxic potential upon binding to K562 target cells or triggering of activating receptors. In addition, in response to cytokine stimulation, BM and PB CD16$^{low}$ NK cells were found to produce IFNγ at levels comparable to the major cytokine producer, the CD56$^{high}$ NK-cell subset.

In the light of the phenotypic profile and the multifunctional ability of the CD56$^{low}$CD16$^{low}$ NK cell-subset described here, we suggest that these cells partially overlap with the multifunctional PB NK-cell subset recently identified based on the high CD62L expression levels[21] and with the KIR$^{low}$ CD94/NKG2A$^{high}$ NK-cell subset endowed with highly proliferating and IFNγ producing ability upon cytokine stimulation;[22] this further substantiates the heterogeneity of their composition.

There is evidence that CD56$^{low}$CD16$^{high}$ NK cells can undergo downmodulation of CD16 expression after mitogenic stimulation or co-culture with malignant targets, resulting in rapid modulation of their activation status and effector function.[48,49] Moreover, CD16 loss is also associated with downregulation of CD62L expression, this being attributable to the activation of the metalloprotease ADAM17.[48,49] Therefore, we cannot exclude the possibility that the CD56$^{low}$CD16$^{low}$ NK cells arise from *in vivo* activa-

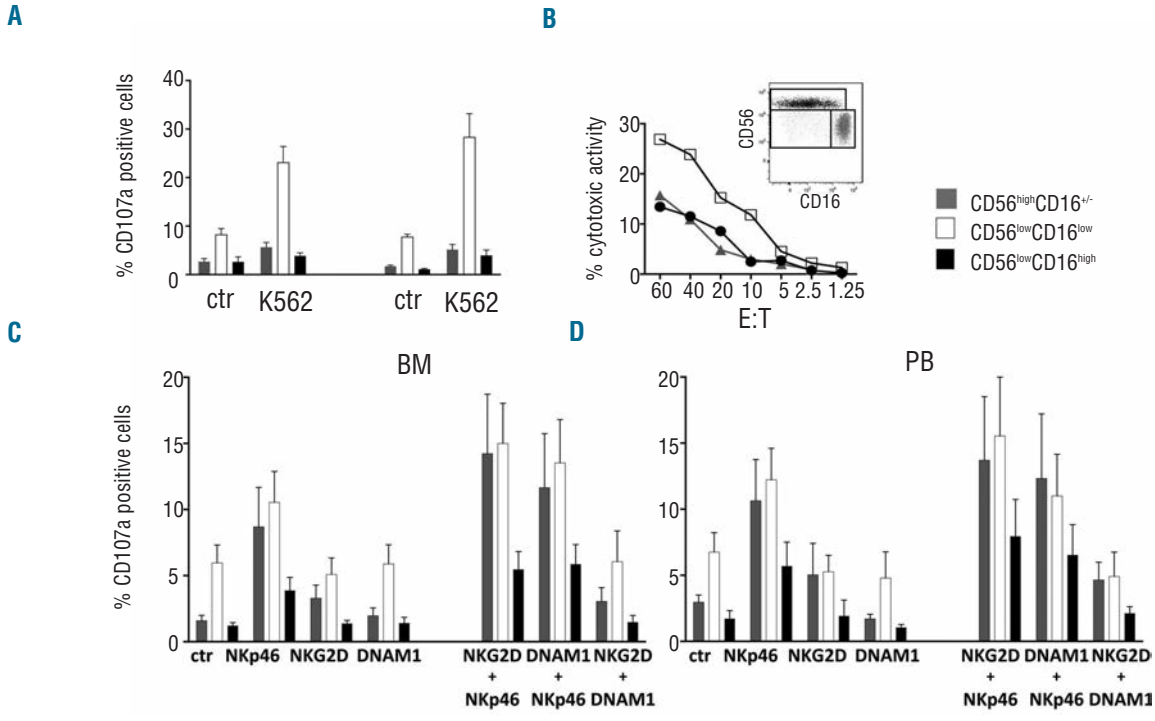


**Figure 6.** Cytotoxic activity of peripheral blood (PB) and bone marrow (BM) CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{low}$ and CD56$^{low}$CD16$^{high}$ natural killer (NK)-cell subsets. BM and PB from healthy donors were co-cultured with K562 and degranulation ability of CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets, gated on CD56$^{+}$CD3$^{-}$, was assessed by evaluating the percentage of CD107a positive cells (A). Mean value+SD of 5 independent experiments is shown. CD56$^{high}$CD16$^{+/-}$ (black line), CD56$^{low}$CD16$^{low}$ (dark gray line) and CD56$^{low}$CD16$^{high}$ (light gray line) sorted NK-cell subsets from PB of healthy donors according to the expression levels of CD56 and CD16 (inset), was tested against 1x10$^{3}$ $^{51}$Cr-labeled K562 target cells in a classical NK-cell cytotoxic assay at different E:T ratios. All experimental groups were analyzed in triplicate (B). NK cells from BM (C) or PB (D) were stimulated with P815 target cells pre-coated with anti-NKG2D, anti-NKp46, anti-DNAM1 alone or in combination. Degranulation of CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$ CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets was assessed by measuring the percentage of CD107a positive cells. Mean value+SD of 5 independent experiments is shown.

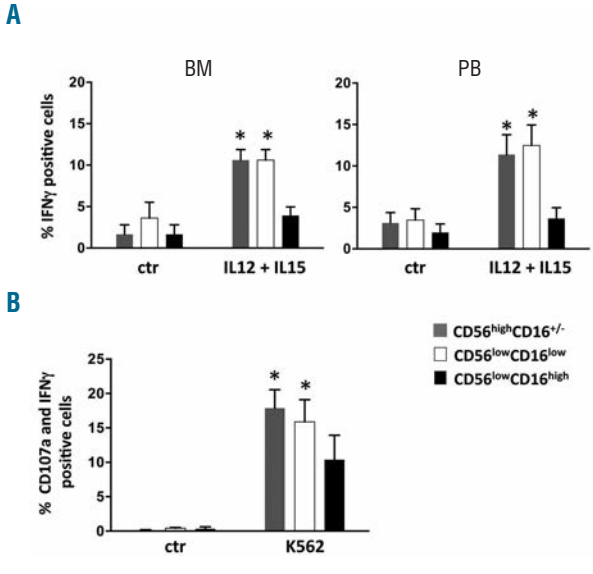


**Figure 7.** Effector functions of bone marrow (BM) and peripheral blood (PB) CD56$^{high}$CD16$^{+/-}$, CD56$^{low}$CD16$^{low}$ and CD56$^{low}$CD16$^{high}$ natural killer (NK)-cell subsets following cytokine stimulation. (A) Freshly isolated cells from BM or PB of healthy pediatric donors were stimulated with IL-12 (25 ng/mL) plus IL-15 (50 ng/mL). The percentage of IFNγ positive CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets were assessed by cytofluorimetric analysis. *$P$<0.05. (B) NK-cell subsets sorted from PB of healthy donor according to the expression levels of CD56 and CD16, were stimulated for 18 h with IL-12 (2 ng/mL) plus IL-15 (5 ng/mL) and then assayed for their ability to degranulate and to produce IFNγ upon binding to K562 target cells (E:T ratio=1:1). The percentage of IFNγ and CD107a positive CD56$^{high}$CD16$^{+/-}$ (gray histogram), CD56$^{low}$CD16$^{low}$ (white histogram) and CD56$^{low}$CD16$^{high}$ (black histogram) NK-cell subsets was assessed by cytofluorimetric analysis. Mean value+SE obtained from 3 independent experiments is shown. *$P$<0.05. $P$ values were calculated by comparing stimulated cells with controls.

tion of the CD56$^{low}$CD16$^{high}$ subset. However, the CD56$^{low}$CD16$^{low}$ NK cells we described still express higher levels of CD62L, supporting the idea that these cells likely represent a distinct NK-cell subset.

Overall, our results suggest that CD56$^{low}$CD16$^{low}$ NK cells represent an intermediate state between CD56$^{high}$ and CD56$^{low}$CD16$^{high}$ NK cells, as has been proposed for the CD94$^{high}$CD56$^{low}$ NK-cell subset.[22] In addition, based on their unique functional properties, we suggest that NK-cell effector functions are acquired either before or independently from the acquisition of a terminally differentiated phenotype. Moreover, our data support the notion that the differentiation of CD56$^{low}$ NK cells may proceed in a non-linear way, passing through many phenotypically and functionally distinct intermediate stages.[25] The similar phenotypic and functional profile we observed in BM and PB CD56$^{low}$CD16$^{low}$ NK cells suggest that they probably represent a population that can complete its differentiation pathway either in BM or in other peripheral compartments.

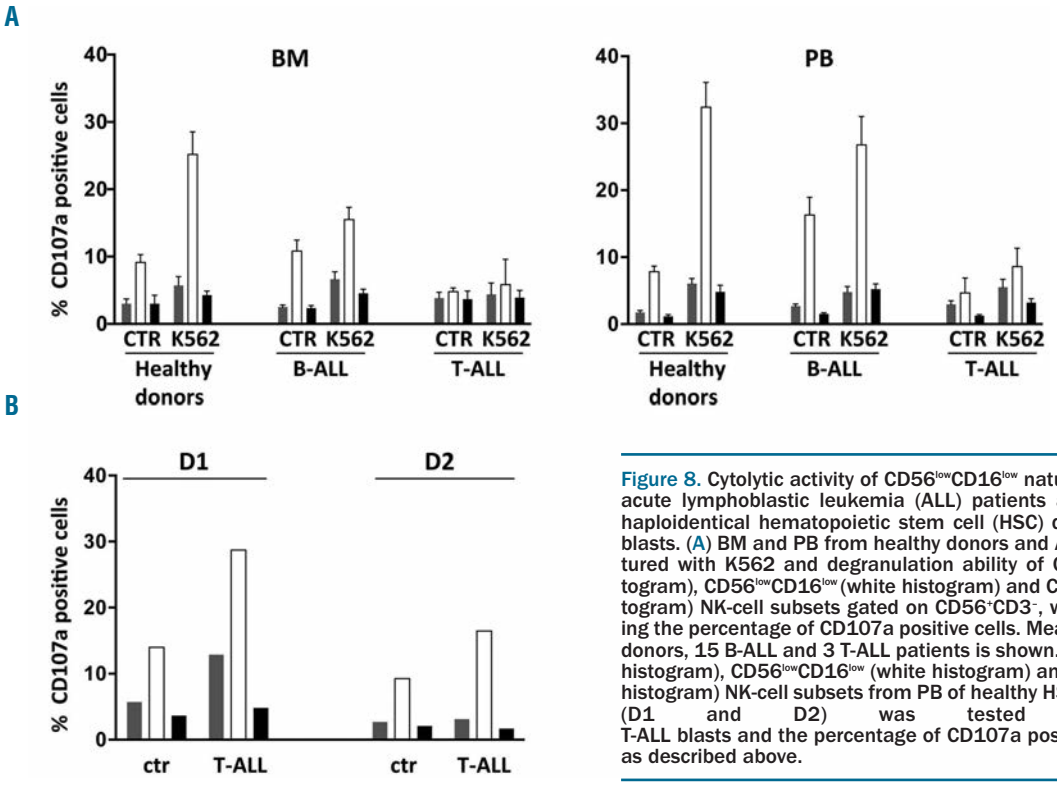


Figure 8. Cytolytic activity of CD56lowCD16low natural killer (NK) cells from acute lymphoblastic leukemia (ALL) patients against K562 and from haploidentical hematopoietic stem cell (HSC) donors against leukemic blasts. (A) BM and PB from healthy donors and ALL patients were co-cultured with K562 and degranulation ability of CD56highCD16+/- (gray histogram), CD56lowCD16low (white histogram) and CD56lowCD16high (black histogram) NK-cell subsets gated on CD56+CD3-, was assessed by evaluating the percentage of CD107a positive cells. Mean value+SD of 5 healthy donors, 15 B-ALL and 3 T-ALL patients is shown. (B) CD56highCD16+/- (gray histogram), CD56lowCD16low (white histogram) and CD56low CD16high (black histogram) NK-cell subsets from PB of healthy HSC haploidentical donors (D1 and D2) was tested against recipient T-ALL blasts and the percentage of CD107a positive cells was evaluated as described above.

Our findings also provide information on the distribution and functional ability of BM and PB NK-cell subsets in pediatric patients affected by ALL, the most frequent childhood neoplasm. CD56lowCD16lowNK cells are present at higher frequency in both BM and PB, even if these cells are not fully functional, as previously reported for other leukemic malignancies.[50] Based on their multifunctional ability, it is conceivable that CD56lowCD16low NK cells might play a crucial role in controlling tumor growth and progression. Moreover, our data indicate that CD56lowD16low NK cells are the only population capable of anti-leukemic activity and are, therefore, extremely important for patients undergoing hematopoietic stem cell transplantation.

We believe that the identification and functional characterization of this NK-cell subset represents an important advance in understanding human NK-cell development and may have important implications in clarifying the role of NK cells under pathological conditions. In addition, in view of the fact that the CD56lowCD16low NK-cell subset is highly multifunctional and can be rapidly mobilized from BM, we propose that it could be the object of preferential collection and selection for approaches of adoptive immunotherapy in patients with NK cell-susceptible hematologic malignancies. Further studies could also clarify the relationship between emergence and persistence of this subset during post-graft reconstitution and maintenance of remission.

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### Authorship and Disclosures

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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