T and NK cell immunity after hematopoietic stem cell transplantation

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Chapter 7

Human lymphoid tissues harbor a distinct CD69^{+}CXCR6^{+} natural killer cell population

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Abstract

Knowledge on human NK cells is mainly based on conventional CD56^{bright} and CD56^{dim} NK cells from blood. However, most cellular immune interactions occur in lymphoid organs. Based on the co-expression of CD69 and CXCR6, we identified a major third NK cell subset in lymphoid tissues. This population represents 30-60% of NK cells in marrow, spleen and lymph node and is absent from blood. CD69^{+}CXCR6^{+} lymphoid tissue NK cells have an intermediate expression of CD56 and high expression of NKp46 and ICAM-1. In contrast to circulating NK cells, they have a bi-modal expression of the activating receptor DNAM-1. CD69^{+}CXCR6^{+} NK cells neither express the early markers c-kit and IL7Rα, nor express *KIRs* or other late-differentiation markers. After cytokine stimulation, CD69^{+}CXCR6^{+} NK cells produce interferon-γ at levels comparable to CD56^{dim} NK cells. They constitutively express perforin, but require pre-activation to express granzyme B and exert cytotoxicity. After hematopoietic stem cell transplantation, CD69^{+}CXCR6^{+} lymphoid tissue NK cells do not exhibit the hyper-expansion as observed for both conventional NK cell populations.

CD69^{+}CXCR6^{+} NK cells constitute a separate NK cell population with a distinct phenotype and function. The identification of this NK cell population in lymphoid tissues provides tools to further evaluate the cellular interactions and role of NK cells in human immunity.

Introduction

As effector lymphocytes of the innate immune system, natural killer cells contribute to the first line of immune defense through the lysis of virus-infected and malignant cells. Furthermore, NK cells play an important role in the initiation, enhancement and regulation of immune responses, directly through the secretion of cytokines and chemokines as well as via their interaction with other innate immune cells [84;85;114;115;251-254].

Human NK cells are usually categorized in two main subsets: $CD56^{bright}CD16^{+/-}$ ($CD56^{bright}$) and $CD56^{dim}CD16^{+}$ ($CD56^{dim}$) NK cells [95;254]. $CD56^{bright}$ NK cells are considered to be the precursors of $CD56^{dim}$ NK cells and represent 5-15% of NK cells in blood [97;100]. $CD56^{bright}$ NK cells express low levels of c-kit (CD117) and typically express the inhibitory receptor CD94/NKG2A [97;98;100]. $CD56^{dim}$ NK cells loose the expression of NKG2A and acquire KIRs and CD57 as they continue to differentiate [99]. Both NK cell subsets produce IFN-γ upon monokine stimulation [101-103]. $CD56^{dim}$ NK cells constitutively express high levels of perforin and granzyme B, resulting in efficient lysis of target cells without prior stimulation [101]. In contrast, $CD56^{bright}$ NK cells express perforin but require pre-activation to exert cytotoxicity [95;100;254].

Most research on human NK cells is performed with circulating cells. However, the majority of NK cells is localized in lymphoid tissues [255]. Data obtained from murine experiments indicate lymphoid tissues as the primary site for cellular interactions of NK cells with other immune cells [85;114;253]. In humans, $CD56^{bright}$ NK cells are enriched in marrow and spleen, and compromise the vast majority of mature NK cells in lymph node [90;107;108;256;257]. Still, the mechanisms involved in the localization of human NK cells in lymphoid organs are largely unknown. Chemokine receptor expression patterns can provide information about human NK cell trafficking [107;258]. To investigate potential mechanisms of NK cell localization in lymphoid tissues, we compared the expression of chemokine receptors and adhesion molecules on circulating NK cells with NK cells in marrow, spleen and lymph node. Using this approach, we identified a major $CD69^{+}CXCR6^{+}$ NK cell population in lymphoid tissues. This population of lymphoid tissue NK cells was not found in the circulation and had a distinct phenotype and function compared to conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Material and Methods

Tissues and ethics statement

With approval of the institutional review board (protocols P00.068 and P01.028), residual paired blood and bone marrow samples from 17 healthy bone marrow donors and 6 HSCT recipients were analyzed after informed consent was provided. In HSCT recipients, NK cell reconstitution was evaluated in marrow of pediatric acute leukemia patients who received an unmanipulated bone marrow (n=4) or peripheral blood stem cell (n=2) transplantation from a HLA-matched unrelated donor after myeloablative conditioning. MNC were isolated and samples were analyzed

immediately (9 HD) or after cryopreservation (8 HD, all HSCT). Small parts of splenic tissue from Dutch solid organ transplant donors (n=7) were obtained during explantation surgery as part of the standard diagnostic procedure for HLA-typing. In accordance with the Dutch law for organ donation, residual splenic tissue could be used for scientific research anonymously. Omental lymph nodes (n=3) were collected as leftover material from bariatric surgery and used anonymously in accordance with Dutch national ethical and professional guidelines (http://www.federa.org). Splenic tissues and lymph nodes were stored in University of Wisconsin solution at 4°C and processed within <12h after surgery. Tissues were dispersed through a 70 µm cell strainer, MNC were isolated and samples were analyzed immediately.

Flow cytometry and cell sorting

For flow cytometry, MNC were subjected to a three step staining procedure. All antibodies used are listed in Table 7.S1. MNC were first incubated with unconjugated antibodies, washed twice and stained with fluorochrome-labeled secondary antibodies. For the third step, MNC were stained with directly labeled antibodies in FACS buffer containing 5% (v/v) mouse serum (Seralab, London, UK). Prior to analysis, DAPI (25 ng/ml, Sigma-Aldrich) was added. Data were acquired on a LSR II flow cytometer (Becton Dickinson (BD), Franklin Lakes, NJ, US) using FACS Diva Software (v6.1, BD). For FACS purification, NK cells were pre- enriched using the MACS untouched NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. Enriched NK cells were stained for cell surface markers (Table 7.S1). Based on the expression of CD56 and CD69, conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells and lymphoid tissue NK cells were sorted on an ARIAIII cell sorter (BD).

Gating strategy and data analysis

The gating strategy is depicted in Figure 7.S1A and Figure 7.1A-B. NK cells were defined as living $CD45^{+}CD19^{-}CD3^{-}CD7^{+}$ lymphocytes expressing CD56 and/or CD16 but not high levels of CD117. Using this strategy, type 3 innate lymphoid cells or immature NK cells ─mainly present in lymph node─ were excluded [91;259]. $CD69^{+}CXCR6^{+}$ lymphoid tissue NK cells were first selected by gating cells with the combined expression of CD69 and CXCR6. From the remaining NK cells, the conventional $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets were defined based on the expression levels of CD56 and CD16. We excluded $CD56^{dim}CD16^{-}$ NK cells from further phenotypic characterization as they formed a separate population in cryopreserved but not in fresh MNC [237]. Flow cytometric data were analyzed using Kaluza software (v1.3, Beckman Coulter, Brea, CA, US) and density plots were created with FACS Diva Software.

In vitro stimulation and intracellular staining

MNC from paired blood and marrow (n=3) or spleen (n=3) were cultured in AIM-V (Life Technologies) with 10% fetal calf serum (GE Hyclone, Logan, UT, US) for 16-18h. Cells were either unstimulated (medium) or stimulated with recombinant human IL12 (10ng/ml, Peprotech, Rocky Hill, NJ, US), IL15 (10ng/ml, Cellgenix, Freiburg, Germany), IL18 (20ng/ml, MBL International, Woborn, MA, US) or combinations of these monokines. Brefeldin A (5µg/ml,

Sigma-Aldrich, IFN-γ assay) or medium (perforin/granzyme B assay) was added during the last 4h of culture. MNC from marrow (n=4) were cultured overnight, followed by a 6h co-culture with K562 cells and/or IL12+IL18. BD golgistop (1:1500, BD) was added during the last 4h of culture. Cells were harvested and stained for cell surface markers, fixed and permeabilized using a paraformaldehyde/saponin based protocol as previously described [244] and stained for intracellular IFN-γ or perforin and granzyme B (Table 7.S1). Since CD69 cannot be used as discriminative marker in activated NK cells, the combination of CD56, CD54 and CXCR6 was used to define lymphoid tissue NK cells after in vitro stimulation as alternative for CD69-CXCR6. The definition of ltNK cells based on CD56, CD54 and CXCR6 expression after short-term culture was validated by flow cytometry after in vitro stimulation of FACS purified NK cell populations (Figure 7.S2).

Interferon-γ, degranulation and chromium release assays

$1x10^4$ FACS purified lymphoid tissue NK, $CD56^{dim}$ or $CD56^{bright}$ NK cells from marrow (n=3) were cultured in 96-well round bottom plates (Greiner Bio-One, Kremsmünster, Austria) for 18h in 100 µl AIM-V with or without IL12, IL15 and IL18. Supernatant was harvested after 18h and the concentration of IFN-γ was assessed using a Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturers' instructions.

MNC from paired blood and marrow (n=6) or spleen (n=4) were cultured overnight in the absence or presence of IL15 (10ng/ml). The next day, CD107a FITC (BD) was added, followed by K562 cells (ratio 1:1) or culture medium. After one hour, BD golgistop was added and 4h later, cells were harvested and stained for cell surface markers (Table 7.S1). Again, the combination of CD56, CD54 and CXCR6 were used to define lymphoid tissue NK cells.

After FACS purification of NK cell subsets from marrow (n=2) or spleen (n=1), sufficient cell numbers to perform a chromium release assay were only obtained for lymphoid tissue NK and $CD56^{dim}$ NK cells. After overnight culture in the absence or presence of IL15 (10ng/ml), their cytotoxicity against K562 cells was evaluated in a 4h chromium release assay as previously described [242].

Statistics

For each cell surface marker, Z-scores were calculated using the average expression (percentage or geomean of fluorescence intensity) of each NK cell subset per tissue. A heat map was generated from the collective data by unsupervised hierarchical clustering using MultiExperimentViewer (v4.9) software [260]. Statistical tests were performed and graphs were built in GraphPad Prism software (v6.07, GraphPad, La Jolla, CA, US). Paired t tests were applied for the comparison of NK cell populations within the same tissue and between paired blood and marrow samples. Unpaired t tests were used to compare NK cell populations between different tissues. P values <0.05 were considered statistically significant. This threshold was adjusted using Bonferroni correction to correct for multiple testing of phenotypic markers.

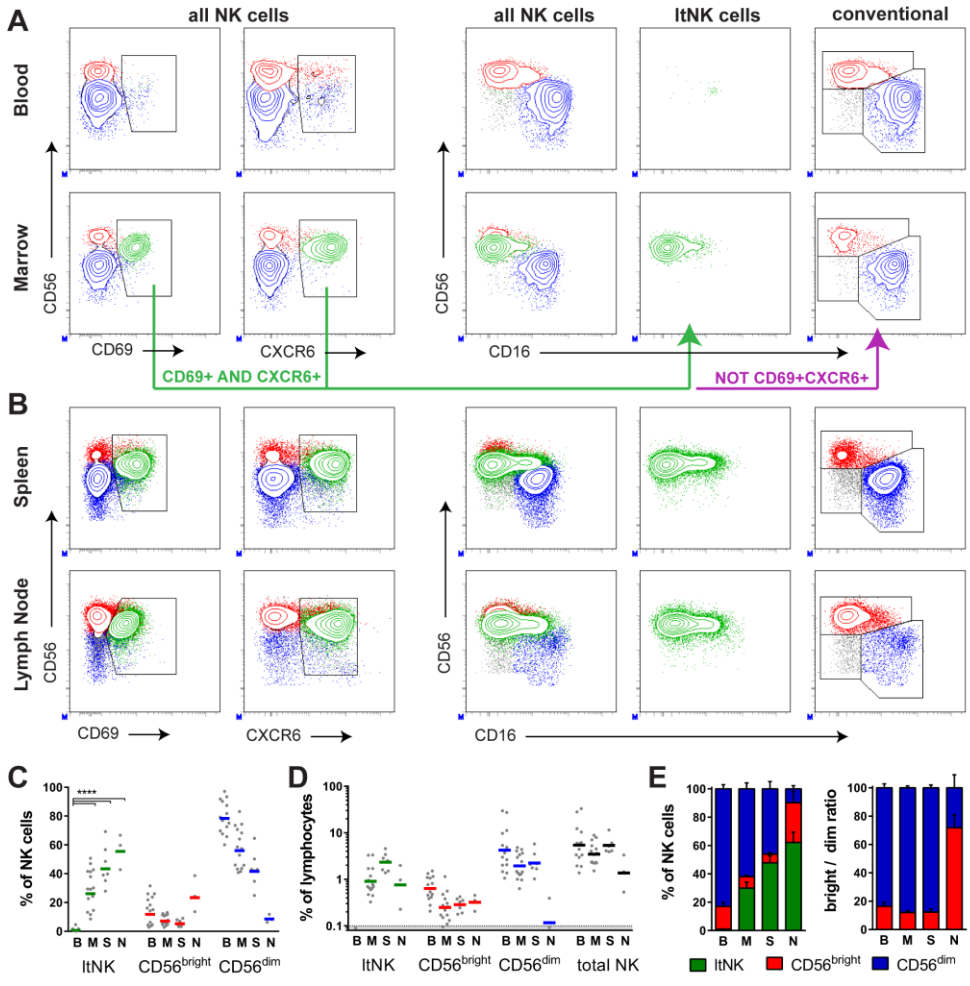

Figure 7.1. Identification of lymphoid tissue NK cells.

(A-B) Flow cytometric analysis of NK cells from blood, marrow, spleen and lymph node. NK cells were defined as living $CD45^{+}CD19^{-}CD3^{-}CD7^{+}$ lymphocytes expressing CD56 and/or CD16, but not high levels of CD117 (see Methods for gating strategy). Within total NK cells, $CD69^{+}CXCR6^{+}$ lymphoid tissue NK cells (ltNK, green) were first selected by gating cells with the combined expression of CD69 and CXCR6. From the remaining NK cells, the expression levels of CD56 and CD16 were used to define the conventional $CD56^{bright}CD16^{+/-}$ (red) and $CD56^{dim}CD16^{+}$ (blue) NK cell populations.

(C-D) Percentage of ltNK and conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells in blood (B, n=17), marrow (M, n=17), spleen (S, n=7) and lymph node (N, n=3) expressed as (C) percentage of total NK cells and (D) percentage of total lymphocytes. Lines: geomean. Shaded area: below limit of detection. Statistics: paired t test (blood vs. marrow) or unpaired t test (other comparisons). ****: p-value <0.0001.

(E) Ratio of all NK cell subsets (left panel) and ratio between conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells (right panel) in blood, marrow, spleen and lymph node. Bars: mean +/- SEM.

Results

Identification of CD69^{+}CXCR6^{+} lymphoid tissue NK cells

We evaluated human NK cells in blood and marrow to identify tissue specific expression patterns of chemokine receptors, adhesion molecules and NK cell receptors. A major NK cell population, identified by the combined expression of CD69 and CXCR6, was present in marrow in addition to the conventional CD56^{bright} and CD56^{dim} NK cell subsets (Figure 7.1A). The CD69^{+}CXCR6^{+} NK cell population was also observed in spleen and lymph node, but absent from blood (Figure 7.1A-B). Therefore, this population is further denoted as lymphoid tissue NK (ltNK) cells. In blood, single positive CXCR6^{+} CD56^{bright} (mean 12%) and CD56^{dim} (mean 5%) NK cells were present, while very few circulating NK cells expressed CD69 (Figure 7.S1B).

CD69^{+}CXCR6^{+} lymphoid tissue NK cells represented 9-51% (mean 29%) of NK cells in marrow, 28-69% (mean 45%) of NK cells in spleen and 43-67% (mean 56%) of NK cells in lymph node (Figure 7.1C). The relative contribution of NK cells to the lymphocyte compartment varied between the investigated tissues. LtNK cells represented 0.9%, 2.3% and 0.8% (geomean) of lymphocytes in marrow, spleen and lymph node, respectively (Figure 7.1D).

Although CD56^{bright} NK cells are considered to be enriched in lymphoid tissues, the ratio between conventional CD56^{bright} and CD56^{dim} NK cells in marrow and spleen turned out to be comparable to blood when ltNK cells were regarded as a separate population. In lymph node, however, CD56^{bright} NK cells remained the predominant NK cell population when ltNK cells were analyzed separately (Figure 7.1E).

Phenotype of lymphoid tissue NK cells

To further define the position of ltNK cells in NK cell development and differentiation, we evaluated their cell surface receptor expression profile in comparison to the conventional NK cell subsets (Figure 7.2A-B; Figure 7.S1B). For each NK cell subset, the expression profile of cell surface markers was similar in all tissues analyzed (Figure 7.2B; Figure 7.S1B). Compared to the conventional NK cells, ltNK cells expressed intermediate levels of CD56 and 4-43% (mean 18%) of ltNK cells expressed CD16. Besides CD69 and CXCR6, ltNK cells expressed high levels of the natural cytotoxicity receptor NKp46 and ICAM-1 (CD54). TNF receptor superfamily member CD27 was expressed by a large proportion (mean 66%) of ltNK cells and the chemokine receptor CCR5 was expressed by 18-71% (mean 37%) of ltNK cells (Figure 7.2; Figure 7.S1B).

Subsequently, we evaluated the expression of NK cell differentiation markers. In contrast to CD56^{bright} NK cells, ltNK cells did not express the early differentiation markers c-kit (CD117) and IL7Rα (CD127), distinguishing ltNK cells from immature NK cells and type 3 innate lymphoid cells (ILC) [91;259]. Furthermore, like CD56^{bright} and CD56^{dim} NK cells, ltNK cells expressed the transcription factor EOMES (data not shown). The inhibitory receptor NKG2A, which is lost during NK cell differentiation [95;254], was expressed by a mean of 62% of ltNK cells, between the proportion of NKG2A^{+} CD56^{bright} and NKG2A^{+} CD56^{dim} NK cells (Figure 7.2; Figure 7.S1B). LtNK cells did not express CX3CR1, which is expressed by all CD56^{dim} NK cells. Only a small proportion of ltNK cells expressed KIRs, KLRG1, CD57 and NKG2C (mean 5%, 4%, 0% and

2%, respectively, Figure 7.2; Figure 7.S1B). Together, these data indicate that ltNK cells have a mature phenotype but do not express markers acquired late in differentiation.

The activation markers NKp30 and NKG2D were uniformly expressed on all NK cell subsets. In contrast, ltNK cells expressed the activating receptor DNAM-1 in a bimodal fashion. Whereas DNAM-1 was expressed by all circulating NK cells, only 20-70% (mean 42%) of ltNK cells expressed DNAM-1 (Figure 7.2; Figure 7.S1B).

Next, we evaluated the expression of molecules associated with tissue localization of NK cells. LtNK cells did not express the adhesion molecule CD49a, distinguishing them from memory-like liver resident NK cells [261]. In contrast to tonsillar NK cells [262;263], ltNK cells did not express the adhesion molecule CD103. Furthermore, the natural cytotoxicity receptor NKp44 was only expressed by a small minority of ltNK cells. Finally, the expression of chemokine receptor CXCR4 did not differ from the conventional NK cell subsets (Figure 7.2B; Figure 7.S1B). Together, based on these phenotypic criteria, ltNK cells represent a mature NK cell population which is uniform between the tissues and distinct from both conventional NK cell subsets.

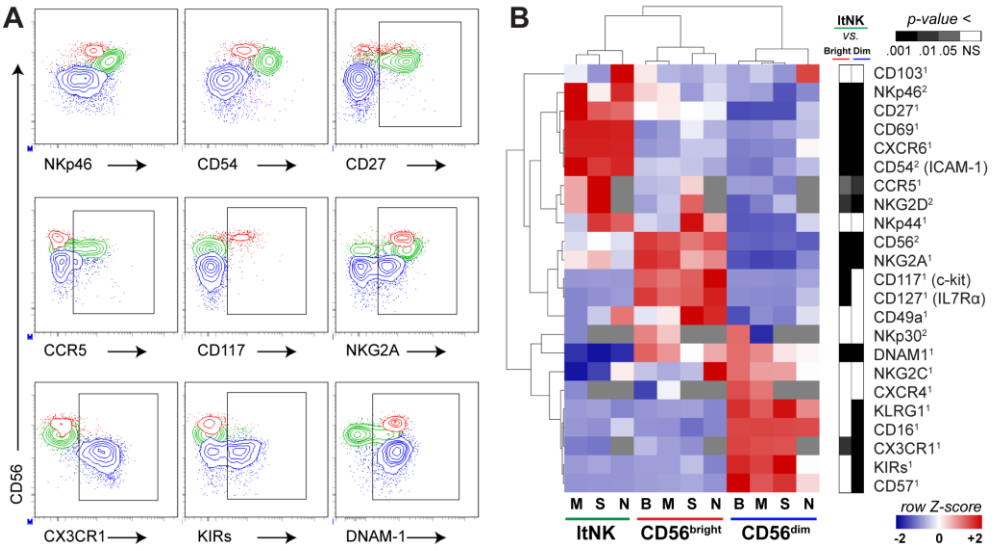

Figure 7.2. Phenotype of NK cell subsets.

(A) Selection of density plots showing cell surface marker expression on NK cells from marrow of a representative donor (HD17). Subsets: ltNK cells (green), CD56^{bright} NK cells (red) and CD56^{dim} NK cells (blue).

(B) Heat map generated from collective flow cytometry data by unsupervised hierarchical clustering. Z-scores were calculated using the average expression ([1]: percentage of subset or [2]: geomean of fluorescence intensity) of each cell surface marker on ltNK cells, CD56^{bright} and CD56^{dim} NK cells from blood (B, n=10-17), marrow (M, n=10-17), spleen (S, n=5-7) and lymph node (N, n=3). The side bar represents p-values of the comparison of ltNK cells with CD56^{bright} (left) and CD56^{dim} NK cells (right) from marrow. Statistics: paired t test. P-values <0.001 (black) remain statistically significant after Bonferroni correction for multiple testing. For gating strategy and bulk flow cytometry data: see Methods and Figure 7.S1B.

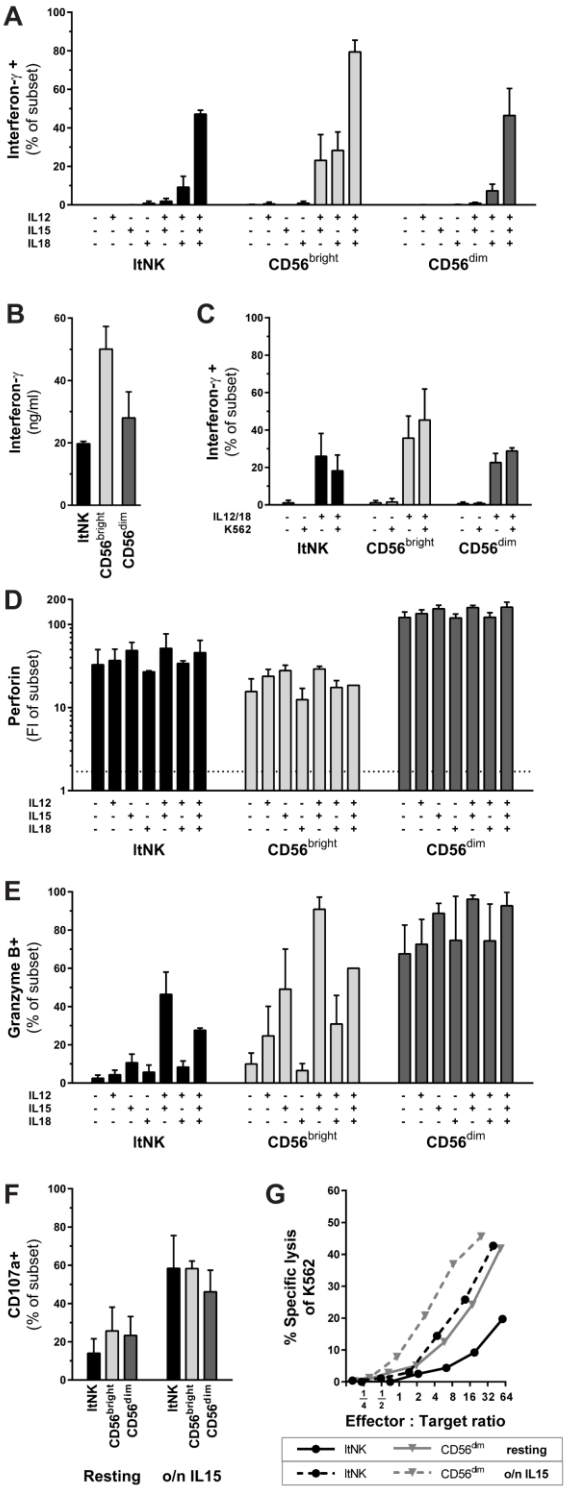

Figure 7.3. Functional properties of NK cell subsets.

(A, C) Intracellular expression of interferon-γ of lymphoid tissue NK cells (ltNK), $CD56^{bright}CD16^{+/-}$ NK cells and $CD56^{dim}CD16^{+}$ NK cells. Mononuclear cells (MNC) from marrow (n=3) were cultured for 16-18h in absence or presence of (combinations of) IL12, IL15 or IL18. For (C), MNC from marrow (n=4) were cultured overnight, K562 cells and cytokines (IL12+IL18) were added for the last 6h of culture.

(B) Interferon-γ production of FACS purified ltNK, $CD56^{bright}$ and $CD56^{dim}$ NK cells from marrow (n=3) upon 16-18h stimulation with IL12, IL15 and IL18. Shown is the interferon-γ concentration in supernatant after subtraction of unstimulated cells.

(D-E) Intracellular expression of perforin and granzyme B of ltNK, $CD56^{bright}$ and $CD56^{dim}$ NK cells. MNC from marrow (n=3) were cultured for 16-18h in absence or presence of (combinations of) IL12, IL15 or IL18. FI: geomean of Fluorescence Intensity. Dotted line: FI of B cells (negative control).

(F) Degranulation of ltNK, $CD56^{bright}$ and $CD56^{dim}$ NK cells. Resting and overnight (o/n) IL15 activated MNC from marrow (n=6) were co-cultured with K562 cells for 4 hours. Shown is the percentage of $CD107a^{+}$ NK cells after subtraction of CD107a expression of NK cells cultured without K562 cells. Bars: mean +/- SD (A-F). See Figure 7.S3 for representative flow cytometry plots and Methods for gating strategy.

(G) Specific lysis of K562 cells in chromium release assay by resting (solid lines) and o/n IL15 activated (dashed lines) FACS purified ltNK cells (black, circles) and $CD56^{dim}$ NK cells (grey, triangles) from marrow. Representative graph of three experiments.

Functional properties of lymphoid tissue NK cells

To determine the functional properties of ltNK cells, we compared their cytokine production and cytotoxicity with the conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells. Results obtained with marrow derived NK cells are described here, but similar results were obtained with the NK cells from spleen and blood (Figure 7.S3).

After overnight stimulation with IL12+IL15, IL12+IL18 and IL12+IL15+IL18, respectively, a mean of 2%, 9% and 47% of ltNK cells produced IFN-γ, which was comparable to the cytokine production of $CD56^{dim}$ NK cells. In contrast, 23%, 28% and 79% (mean) of the $CD56^{bright}$ NK cells produced IFN-γ upon these stimuli (ltNK vs. $CD56^{dim}$: NS, ltNK vs. $CD56^{bright}$: $p<0.0001$, Figure 7.3A and Figure 7.S3A). This was confirmed by an IFN-γ secretion of 20, 50 and 28 ng/ml after IL12+IL15+IL18 stimulation of FACS purified ltNK, $CD56^{bright}$ and $CD56^{dim}$ NK cells (ltNK vs. $CD56^{bright}$: $p=0.019$, ltNK vs. $CD56^{dim}$: NS, Figure 7.3B).

Co-culture of resting NK cells with K562 cells did not result in detectable intracellular IFN-γ production. However, in the presence of IL12 and IL18, co-culture with K562 cells resulted in a (non-significant) increase in IFN-γ production by $CD56^{bright}$ and $CD56^{dim}$ NK cells compared to cytokines alone. In contrast, IFN-γ production of IL12+IL18 activated ltNK cells was not increased upon co-culture with K562 cells (Figure 7.3C).

To assess the cytolytic potential of ltNK cells, we first evaluated the expression of perforin and granzyme B. In resting state, ltNK cells expressed perforin at levels intermediate to $CD56^{bright}$ and $CD56^{dim}$ NK cells (ltNK vs. $CD56^{bright}$: $p<0.01$, ltNK vs. $CD56^{dim}$: $p<0.0001$, Figure 7.3D). In contrast to $CD56^{dim}$ NK cells, resting ltNK and $CD56^{bright}$ NK cells hardly expressed granzyme B (ltNK vs. $CD56^{bright}$: NS, ltNK vs. $CD56^{dim}$: $p<0.0001$, Figure 7.3E). After overnight activation by IL12 and IL15, 46% (mean) of ltNK cells expressed granzyme B whereas the expression of granzyme B was upregulated in the majority of $CD56^{bright}$ NK cells (Figure 7.3D-E; Figure 7.S3B). Although with a slightly higher perforin and lower granzyme B expression, the cytolytic protein expression profile of ltNK was most comparable to $CD56^{bright}$ NK cells.

In CD107a based degranulation assays, a mean of 13% of resting ltNK cells externalized CD107a upon co-culture with K562 cells. Degranulation was increased to 60% after prior overnight IL15 activation. Both in rest and after pre-activation, the proportion of ltNK cells that externalized CD107a did not differ significantly from both $CD56^{bright}$ and $CD56^{dim}$ NK cells (Figure 7.3F; Figure 7.S3C).

Finally, we evaluated the lysis of K562 cells using FACS purified ltNK and $CD56^{dim}$ NK cells. Compared to $CD56^{dim}$ NK cells, ltNK cells had a low cytotoxicity in resting state. In line with the cytolytic protein expression profile and degranulation, their cytolytic capacity increased after overnight IL15 activation (Figure 7.3G). Altogether, ltNK cells produce IFN-γ at levels comparable to $CD56^{dim}$ NK cells. In line with the functional properties of $CD56^{bright}$ NK cells, ltNK cells have a low natural cytotoxicity which is enhanced upon short-term activation.

Reconstitution of lymphoid tissue NK cells after hematopoietic stem cell transplantation

Next, we evaluated the reconstitution of NK cell subsets in bone marrow after hematopoietic stem cell transplantation. Expressed as a percentage of NK cells, ltNK cells gradually increased towards healthy donor levels in the year after HSCT (Figure 7.4A). However, expressed semi-quantitatively as a percentage of lymphocytes, ltNK cells reached healthy donor levels at the third month after HSCT to remain stable thereafter (Figure 7.4B). This reconstitution profile differed significantly from the hyper-expansive reconstitution of conventional NK cells. The reconstitution of CD56^{bright} and CD56^{dim} NK cells in marrow mirrored their reconstitution in blood (data not shown and [216;237]). Conventional NK cells reached the highest levels at 1 month (CD56^{bright}) and 3-6 months (CD56^{dim}) after HSCT and gradually decreased to healthy donor levels at one year after HSCT. In conclusion, ltNK cells are not subjected to the hyper-expansion early after HSCT as observed for both conventional NK cell populations.

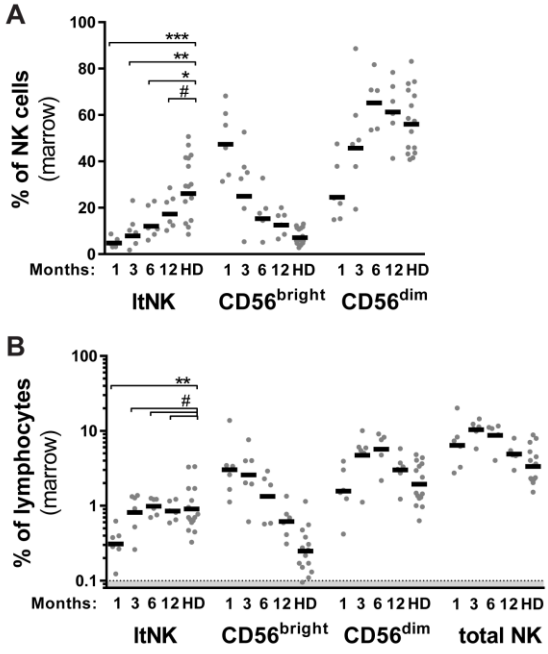

Figure 7.4. Reconstitution of NK cell subsets in marrow after hematopoietic stem cell transplantation.
Reconstitution of lymphoid tissue NK cells (ltNK) cells and conventional CD56^{bright} and CD56^{dim} NK cells in marrow at 1, 3, 6 and 12 months after pediatric allogeneic hematopoietic stem cell transplantation (HSCT), expressed as **(A)** percentage of total NK cells and **(B)** percentage of total lymphocytes. Lines: geomean. Shaded area: below limit of detection. Statistics: unpaired t test comparing the % of ltNK cells between HSCT recipients (n=6) and healthy donors (HD, n=17). For % of lymphocytes, data were log-transformed. #: p-value ≥0.05, *: p<0.05, **: p<0.01, ***: p<0.001. See Methods for gating strategy.

Discussion

Based on the co-expression of the tissue retention marker CD69 and chemokine receptor CXCR6, we identified a novel NK cell population in human lymphoid tissues (ltNK). With respect to their surface marker expression profile, these cells constitute a population which is distinct from the conventional CD56^{bright} and CD56^{dim} NK cell subsets. Functionally, ltNK cells share features with both CD56^{bright} and CD56^{dim} NK cells. In addition, ltNK cells have their own reconstitution kinetics after HSCT.

Lymphoid tissues are often considered to be enriched for CD56^{bright} NK cells. However, this is only based on the expression pattern of CD56 and CD16 [90;107;108;256;257]. Our data provide evidence that ltNK cells are not just a subpopulation of either of the conventional NK cell populations. The expression of the C-type lectin CD69 on ltNK cells is in line with their tissue residency. Although initially identified as an early lymphocyte activation marker [264-266], CD69 has been recognized to play an important role in tissue retention [267;268]. In B- and T-cells, the expression of CD69 is inversely correlated with the expression of sphingosine-1-phosphate receptor 1, thereby inhibiting lymphocyte egress from tissues along the gradient of sphingosine-1-phosphate [267;268].

The G-protein coupled chemokine receptor CXCR6 is often associated with liver resident lymphocytes [269], but is also enriched on bone marrow resident CD69^{+} memory T cells [270]. A recent report showed that the majority of CD56^{bright} NK cells in human liver expresses CD69 or CXCR6 [271]. Murine CXCR6^{+}CD49a^{+} liver resident NK cells display memory-like features; however, only few NK cells in human liver display the homologous CD49a^{+}NKG2C^{+}KIR^{+} phenotype [261;272;273]. Similar to most liver NK cells, lymphoid tissue NK cells do not express CD49a, NKG2C or KIRs. As a consequence, it cannot be excluded that a population with a phenotype comparable to ltNK cells might also be present in human liver. Whether CXCR6 is associated with memory generation in human NK cells requires further investigation.

Strikingly, ltNK cells have a bimodal expression pattern of the activating NK cell receptor DNAM-1. DNAM-1 is involved in adhesion and immune synapse formation and has been shown to play an important role in the control of cytotoxicity [274-277]. A bimodal expression pattern of DNAM-1 on human NK cells has only been reported in bone marrow of myelodysplastic syndrome patients and in liver [271;278]. The high surface expression intensity of the activating NK cell receptor NKp46 on ltNK cells is in line with the description of NKp46^{bright} NK cells in lymph node and spleen [257]. Moreover, an enrichment of CD27^{+} NK cells was previously reported in human spleen [279]. Although these observations are likely to be a representation of the presence of ltNK cells in these organs, we are the first to use specific markers to discriminate the tissue resident NK cell population from conventional NK cells.

Like conventional NK cells, ltNK cells can be distinghuised from helper-ILC1 through their expression of perforin and transcription factor EOMES, as well as from ILC2 and ILC3 by their lack of CD117 and CD127 expression [89]. Lymphoid tissue NK cells produced relatively low levels of IFN-γ, similar to CD56^{dim} NK cells and required prior stimulation to exert cytotoxicity. Together with the low expression of DNAM-1 [276;277], this gives the impression that ltNK cells may be a hypo-functional NK cell population. Possibly, these cells have an immunomodulatory function within the lymphoid tissues to bridge innate and adaptive immunity, rather than that they

exert one of the classical NK cell functions. In addition to their distinct phenotype and functional profile, the reconstitution pattern of ltNK cells after HSCT differs from that of the conventional NK cell subsets. Together, these arguments argue against the hypothesis that ltNK cells only represent a transient tissue equivalent of $CD56^{bright}$ or $CD56^{dim}$ cells trafficking through tissues. Our data do not allow for firm conclusions regarding the ontogeny of ltNK cells and their relation to the conventional NK cell populations. The lack of both very early (CD117, CD127) and late (CD57, KLRG1, NKG2C) NK cell receptors suggest that ltNK cells could represent a developmental intermediate between $CD56^{bright}$ and $CD56^{dim}$ NK cells. However, the distinct expression pattern of the markers NKp46, CD54 and DNAM-1 argues against this linear relationship. Alternatively, ltNK cells may constitute a separate tissue specific NK cell lineage, and develop independently from the circulating NK cells.

While knowledge on human NK cell biology is mainly derived from circulating $CD56^{bright}$ and $CD56^{dim}$ NK cells, lymphoid tissues contain the majority of human NK cells [255]. Importantly, our data indicate that lymphoid tissues are populated by a third distinct population of NK cells in addition to the conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells. The identification of this specific lymphoid tissue resident NK cell population provides tools to further study cellular interactions of these NK cells in the lymphoid tissue microenvironment and their role in the regulation of immune responses.

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Supplemental data

CD designation	Alternative name	Fluorochrome	Type	Clone	Company	Catalog#	Staining step
CD184	CXCR4	Unconjugated	m-IgG2b	44716	R&D	MAB172	Primary
CD186	CXCR6	Unconjugated	m-IgG2b	56811	R&D	MAB699	Primary
CD195	CCR5	Unconjugated	m-IgG2b	45531	R&D	MAB182	Primary
n/a	Isotype m-IgG2b	Unconjugated	m-IgG2b	DAK-GO9	DAKO	X0944	Primary
n/a	Goat α m-IgG2b	AF488	polyclonal		Invitrogen	A21141	Secondary
n/a	Goat α m-IgG2b	AF647	polyclonal		Invitrogen	A21242	Secondary
n/a	Goat α m-IgG2b	PE	polyclonal		Southern	1092-09	Secondary
CD003	CD3	BV421	m-IgG1	UCHT1	BD	562426	Direct
CD004	CD4	PE-Cy5.5	m-IgG1	13B8.2	BC	B16491	Direct
CD007	GP40	AF700	m-IgG1	M-T701	BD	561603	Direct
CD008	CD8	BV510	m-IgG1	SK1	BD	563919	Direct
CD014	CD14	PE	m-IgG1	MφP9	BD	342408	Direct
CD014	CD14	PE-Cy7	mIgG2a	M5E2	BD	557742	Direct
CD016	FcγRIII	BV711	m-IgG1	3G8	BD	563127	Direct
CD019	CD19	BV510	m-IgG1	SJ25C1	BD	562947	Direct
CD019	CD19	APC	m-IgG1	J3.119	BC	IM2470	Direct
CD020	MS4A1	PE-Cy7	m-IgG1	L27	BD	335828	Direct
CD027	TNFRSF7	APC	m-IgG1	L128	BD	337169	Direct
CD033	SIGLEC3	PE	m-IgG1	P67.6	BD	345799	Direct
CD045	PTPRC	PE-Cy5.5	m-IgG1	J33	BC	A54139	Direct
CD045	PTPRC	FITC	m-IgG1	2D1	BD	342408	Direct
CD049a	ITGA1	PE	m-IgG1	TS2/7	Biolegend	328304	Direct
CD054	ICAM1	PE	m-IgG1	HA58	BD	555511	Direct
CD056	NCAM1	ECD	m-IgG1	N901	BC	A82943	Direct
CD056	NCAM1	ECD	m-IgG1	N901	BC	A82943	Direct
CD057	B3GAT1	FITC	m-IgM	HNK1	BD	347393	Direct
CD069	EA-1	FITC	m-IgG1	L78	BD	347823	Direct
CD069	EA-1	PE-Cy7	m-IgG1	L78	BD	347823	Direct
CD103	ITGAE	FITC	m-IgG1	BER-ACT8	DAKO	F7138	Direct
CD117	c-kit	PE	m-IgG1	104D2	BD	332785	Direct
CD127	IL7rα	FITC	m-IgG1	eBioRDR5	eBioscience	11-1278	Direct
CD158a/h[1]	KIR2DL1 DS1	PE[1]	m-IgG1	EB6	BC	A09778	Direct
CD158b/j[1]	KIR2DL2/3 DS2	PE[1]	m-IgG1	GL183	BC	IM2278U	Direct
CD158e[1]	KIR3DL1	PE[1]	m-IgG1	DX9	BD	555967	Direct
CD158i[1]	KIR2DS4	PE[1]	m-IgG1	FES172	BC	IM3337	Direct
CD159a	NKG2A	PE	m-IgG2b	z199	BC	IM3291U	Direct
CD159c	NKG2C	APC	m-IgG1	134591	R&D	FAB138A	Direct
CD226	DNAM1	FITC	m-IgG1	DX11	BD	559788	Direct
CD235a	GPA	PE	m-IgG1	11E4B-7-6	BC	A07792	Direct
CD314	NKG2D	APC	m-IgG1	1D11	BD	558071	Direct
CD335	NKp46	PE	m-IgG1	BAB281	BC	IM3711	Direct
CD336	NKp44	PE	m-IgG1	Z231	BC	IM3710	Direct
n/a	KLRG1[2]	AF488	m-IgG2a	13F12F2	n/a[2]	n/a[2]	Direct
n/a	CX3CR1	PE	r-IgG2b	2A9-1	Biolegend	341604	Direct
n/a	Isotype m-IgG1	APC	m-IgG1	15H6	BC	731586	Direct
n/a	Isotype m-IgG1	FITC	m-IgG1	15H6	BC	731583	Direct
n/a	Isotype m-IgM	FITC	m-IgM	R4A3-22-12	BC	6602434	Direct
n/a	Isotype m-IgG1	PE	m-IgG1	X40	BD	345816	Direct
n/a	Isotype m-IgG2a	PE	m-IgG2a	X39	BD	349053	Direct
n/a	Granzyme B	AF700	m-IgG1	GB11	BD	557971	Intracellular
n/a	Interferon-γ	FITC	m-IgG1	4S.B3	BD	554551	Intracellular
n/a	Perforin	FITC	m-IgG2b	delta G9	Ancell	358-040	Intracellular
CD107a	LAMP1	FITC	m-IgG1	H4A3	BD	555800	In Culture

Table 7.S1 (previous page). Antibodies used for flow cytometry.
[1]All four PE-conjugated α-KIR antibodies were combined in a pan-KIR staining.
[2]α-KLRG1 antibody was kindly provided by Prof. H. Pircher (Universitätsklinikum Freiburg, Freiburg, Germany)
CD: Cluster of differentiation. n/a: not applicable. m: mouse, r: rat.
Fluorochromes: AF: Alexa Fluor, APC: Allophycocyanin, BV: Brilliant Violet, ECD: Energy Coupled Dye (=Phycoerythrin-Texas Red conjugate), FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PE-Cy5.5: Phycoerythrin-Cyanine5.5 conjugate, PE-Cy7: Phycoerythrin-Cyanine7 conjugate.
Companies: Ancell: Ancell Corporation (Bayport, MN, USA), BC: Beckman Coulter (Brea, CA, USA), BD: Becton Dickinson Biosciences (San Jose, CA, USA), Biolegend: Biolegend (San Diego, CA, USA), DAKO: Dako Denmark, (Glostrup, Denmark), eBioscience: eBioscience (San Diego, CA, USA), Invitrogen: Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA), R&D: R&D Systems (Minneapolis, MN, USA), Southern: Southern Biotech (Birmingham, AL, USA).

Figure 7.S1 (next pages).
(A) Gating strategy for NK cells
First, DAPI negative, non-doublet, CD45+CD19-CD3-CD7+ lymphocytes were selected (gate 1-6). Then, NK cells were defined as cells expressing CD56 and/or CD16 but not high levels of CD117 (gate 7-8). Using this strategy, type 3 innate lymphoid cells (ILC3) or immature NK cells (mainly present in lymph node, see ref 24 and 25) were excluded. Shown are density plots from marrow of a representative donor (HD17)
(B) Flow cytometry data of cell surface marker expression.
Left panels summarize the expression of surface markers on lymphoid tissue NK (ltNK, green) cells as well as conventional CD56bright (red) and CD56dim (blue) NK cells from blood (B, n=10-17, open bars), marrow (M, n=10-17, diagonally hatched bars), spleen (S, n=5-7, horizontally hatched bars) and lymph node (N, n=3, checkered bars). Expression: % positive (bimodal expression) or geomean of fluorescence intensity (FI, continuous expression). Bars: mean +/- SD. x: subset not present in blood, na: not assessed. Dashed line: isotype control FI.
In the right panels, a representative density plot displays the expression of each cell surface marker (X-axis) plotted against CD56 (Y-axis) on NK cells from marrow. Density plots are from healthy donor 16 (HD16), except for NKG2D (HD15), NKG2C (HD18), NKp30 (HD22), CXCR4 and CCR5 (HD6). See methods, Figure 7.S1A and Figure 7.1 for gating strategy.

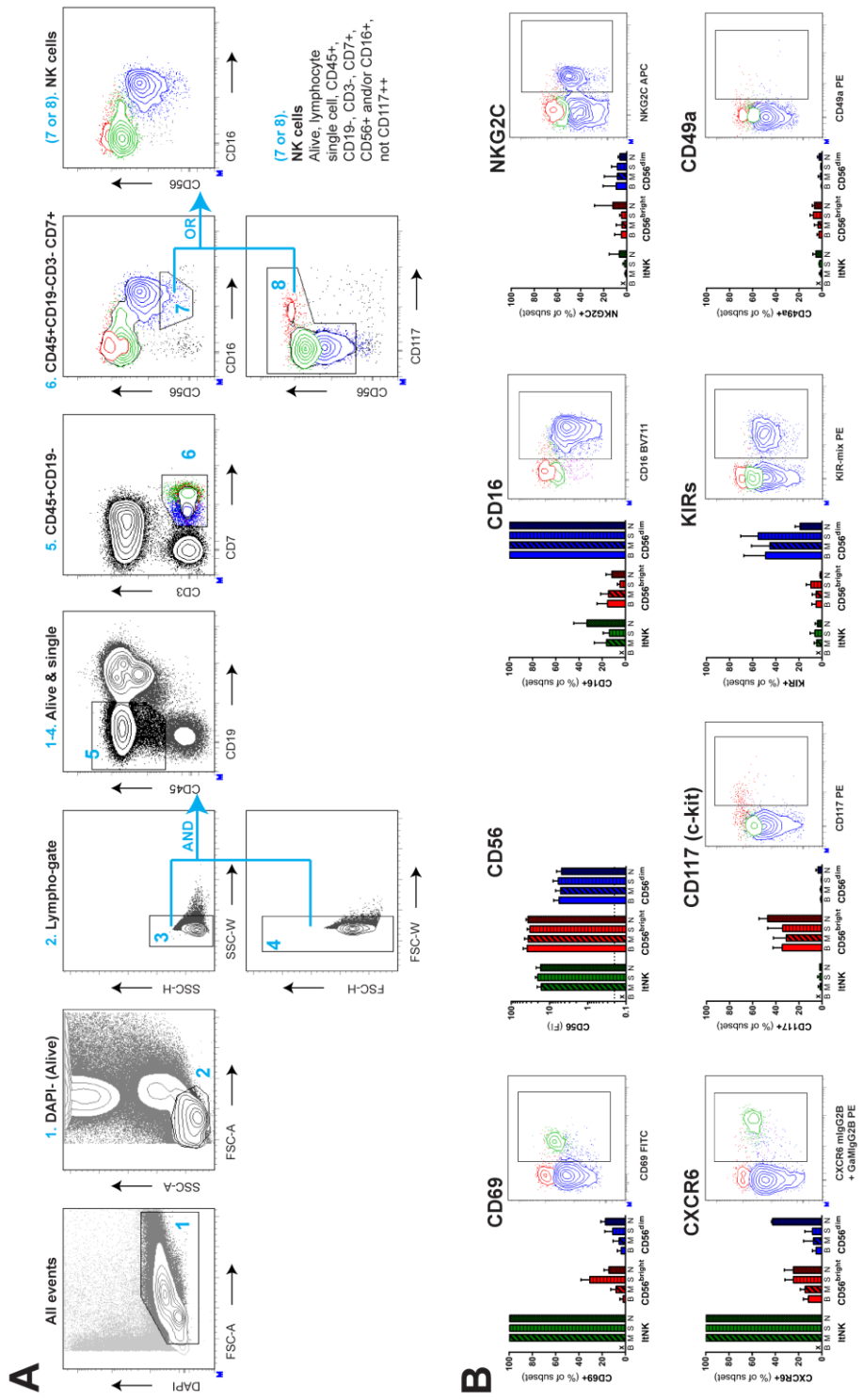
A
All events
1. DAPI- (Alive)
2. Lympho-gate
1-4. Alive & single
5. CD45+CD19-
6. CD45+CD19-CD3- CD7+
(7 or 8). NK cells
(7 or 8).
NK cells
Alive, lymphocyte
single cell, CD45+,
CD19-, CD3-, CD7+,
CD56+ and/or CD16+,
not CD117++
AND
OR
B
CD69
CD56
CD16
NKG2C
CD49a
KIRs
CD117 (c-kit)
CXCR6

Figure 7.S1.

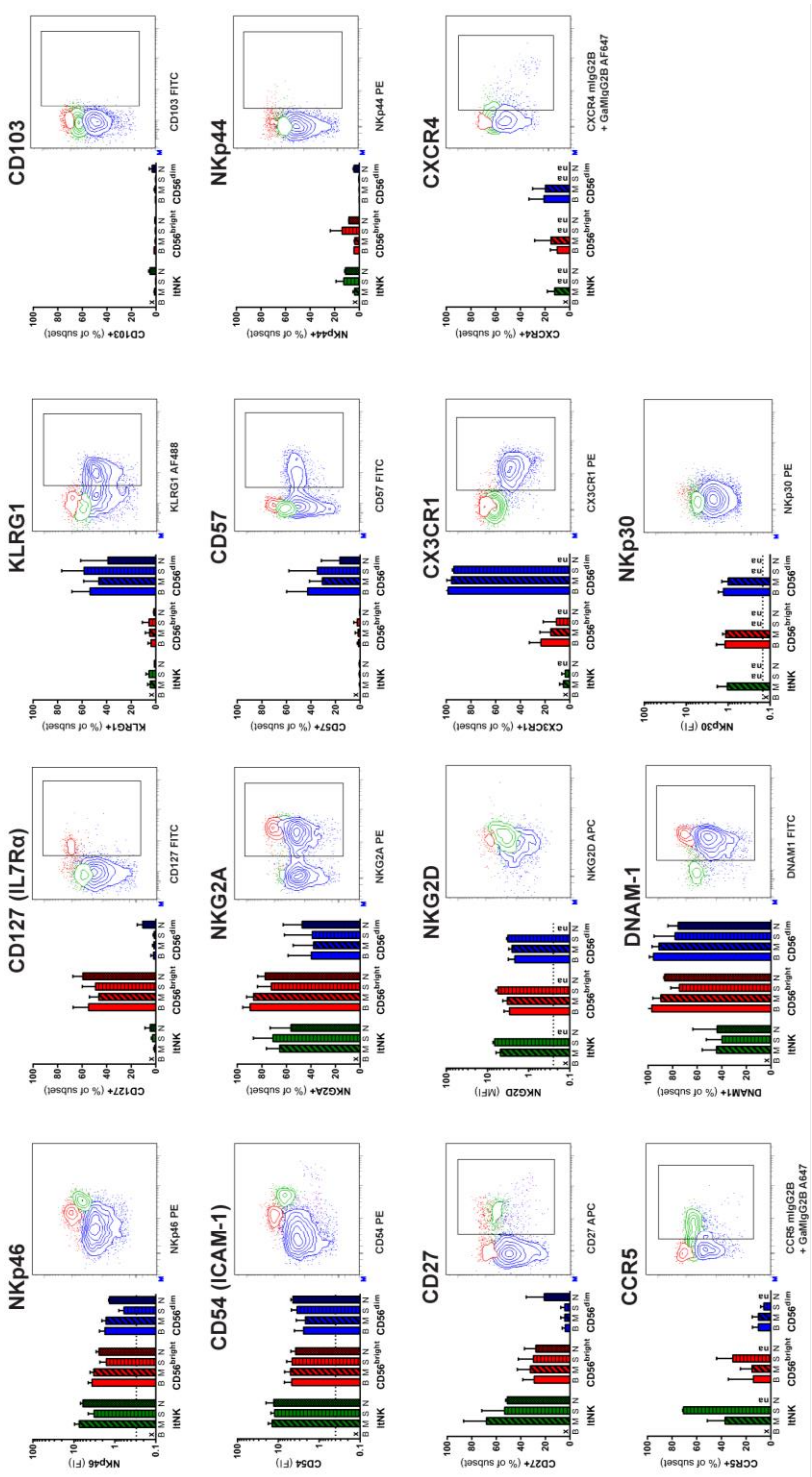

Figure 7.S1 (continued).

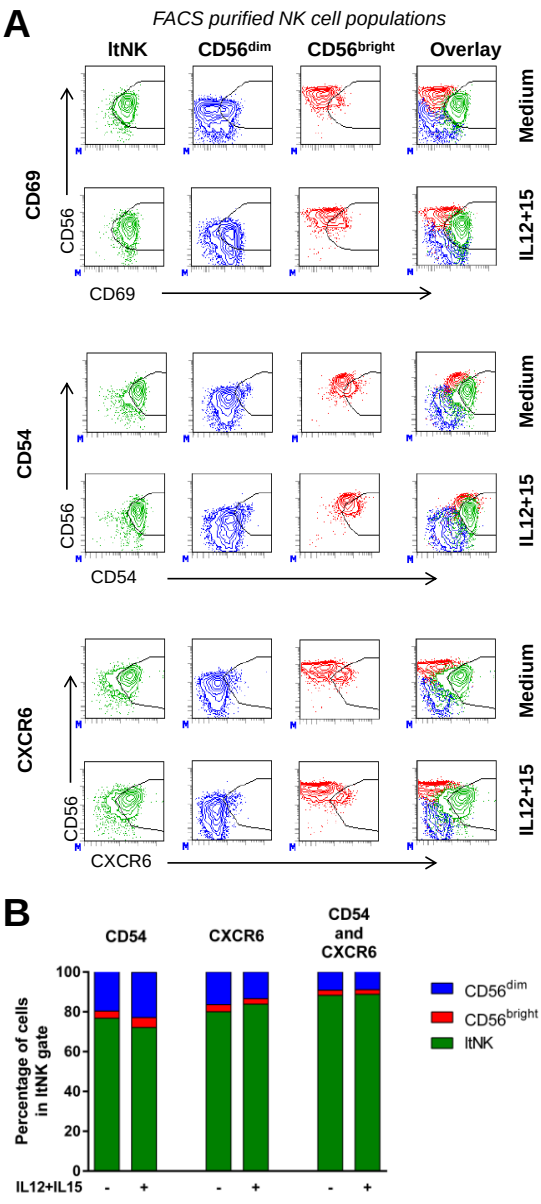

Figure 7.S2. Validation of the use of CXCR6 and CD54 to identify ltNK cells after culture.

MACS-enriched NK cells from marrow were stained for cell surface markers and the three NK cell populations were FACS-sorted (see methods). Sorted NK cell populations were cultured for 18h with or without cytokines and stained for the expression of CD56 (PE-Cy5.5, BC), CD69 (PE-Cy7, BD), CD54 (PE, BD) and CXCR6.

(A) Individual density plots display the expression of CD69 (top), CD54 (middle) and CXCR6 (bottom) of FACS purified NK cell populations in rest (medium) or after activation (IL12+IL15). In the right column, composed overlay figures are shown. Gates are drawn as applied for the functional (intracellular staining) experiments.

(B) The percentage of contamination by $CD56^{bright}$ and $CD56^{dim}$ NK cells included within the lymphoid tissue NK cell (ltNK) gate if only CD54, only CXCR6 or a combination of CD54 and CXCR6 are used to define ltNK cells. Percentages were calculated from the original composition. The combined use of CD56, CD54 and CXCR6 to define ltNK cells results in 90% purity.

Figure 7.S3 (next page). Functional properties of NK cell subsets from blood, marrow and spleen.

Intracellular expression of **(A)** interferon- γ and **(B)** perforin and granzyme B of NK cells from blood (n=3), marrow (n=3) and spleen (n=3) cultured for 16-18h in absence or presence of (combinations of) IL12, IL15 or IL18. Representative density plots display NK cells from marrow of HD6. **(C)** Degranulation (CD107a externalization) of resting and overnight (o/n) IL15 activated NK cells from blood (n=6), marrow (n=6) and spleen (n=4) cultured for 4h with or without K562 cells. Representative density plots display NK cells from marrow of HD7. Evaluated subsets: lymphoid tissue NK (ltNK) cells (green), CD56brightCD16+/- NK cells (red) and CD56dimCD16+ NK cells (blue). Bars: mean +/- SD. See Methods for gating strategy.

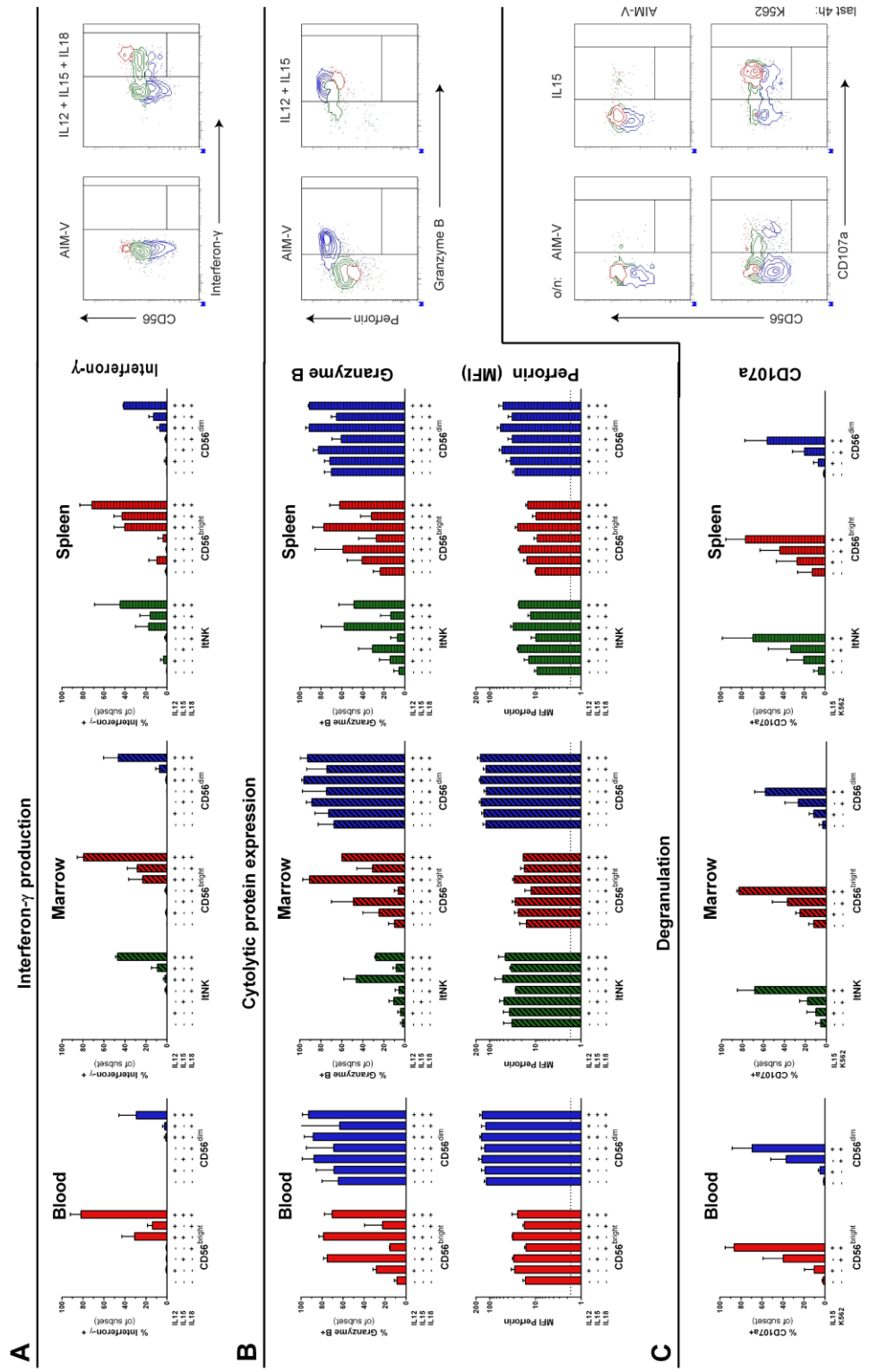

Figure 7.S3. Functional properties of NK cell subsets from blood, marrow and spleen.

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