CD56\(^{bright}\)CD16\(^{-}\) Killer Ig-Like Receptor\(^{-}\)NK Cells Display Longer Telomeres and Acquire Features of CD56\(^{dim}\) NK Cells upon Activation

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CD56\textsuperscript{bright}CD16\textsuperscript{−} Killer Ig-Like Receptor\textsuperscript{−} NK Cells Display Longer Telomeres and Acquire Features of CD56\textsuperscript{dim} NK Cells upon Activation\textsuperscript{1}

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Human NK cells can be divided into CD56\textsuperscript{dim}CD16\textsuperscript{+} killer Ig-like receptors (KIR)\textsuperscript{+−} and CD56\textsuperscript{bright}CD16\textsuperscript{+} KIR\textsuperscript{−} subsets that have been characterized extensively regarding their different functions, phenotype, and tissue localization. Nonetheless, the developmental relationship between these two NK cell subsets remains controversial. We report that, upon cytokine activation, peripheral blood (PB)-CD56\textsuperscript{bright} NK cells mainly gain the signature of CD56\textsuperscript{dim} NK cells. Remarkably, KIR can be induced not only on CD56\textsuperscript{bright}, but also on CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells, and their expression correlates with lower proliferative response. In addition, we demonstrate for the first time that PB-CD56\textsuperscript{dim} display shorter telomeres than PB- and lymph node (LN)-derived CD56\textsuperscript{bright} NK cells. Along this line, although human NK cells collected from nonreactive LN display almost no KIR and CD16 expression, NK cells derived from highly reactive LN, efferent lymph, and PB express significant amounts of KIR and CD16, implying that CD56\textsuperscript{bright} NK cells could acquire these molecules in the LN during inflammation and then circulate through the efferent lymph into PB as KIR\textsuperscript{−}CD16\textsuperscript{−} NK cells. Altogether, our results suggest that CD56\textsuperscript{bright}CD16\textsuperscript{−} KIR\textsuperscript{−} and CD56\textsuperscript{dim}CD16\textsuperscript{+} KIR\textsuperscript{+−} NK cells correspond to sequential steps of differentiation and support the hypothesis that secondary lymphoid organs can be sites of NK cell final maturation and self-tolerance acquisition during immune reaction. The Journal of Immunology, 2007, 178: 4947–4955.

For many years, NK cells have been regarded as a homogeneous lymphocyte population characterized by strong cytotoxic capability. More recently, NK cells have been shown to comprise various subsets that differ in function, phenotype, and tissue localization. The majority of peripheral blood (PB)\textsuperscript{1} NK cells (≥95%) belong to the CD56\textsuperscript{dim}CD16\textsuperscript{+} cytotoxic subset (1, 2) and express homing receptors for inflamed peripheral sites and lytic granules to rapidly mediate cytotoxicity (1, 3). The remaining PB-NK cells (≤5%) are represented by CD56\textsuperscript{bright}CD16\textsuperscript{+} cells (2), which conversely express very low levels of lytic granules, secrete larger amounts of IFN-γ and TNF, and proliferate much more vigorously than CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells upon activation (4). Phenotypically, CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells, unlike CD56\textsuperscript{dim}CD16\textsuperscript{+}, express the receptor for stem cell factor (c-kit or CD117), the α-chain of IL-7R, and secondary lymphoid organ (SLO) homing markers, namely CCR7, CD62L, and CXCR3 (2, 4, 5). Notably, the MHC class I allele-specific killer Ig-like receptors (KIR) are expressed on a considerable fraction of CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells, whereas the CD56\textsuperscript{bright}CD16\textsuperscript{+} NK subset lacks KIR (1). Recent reports have shown that a substantial amount of human NK cells resides in SLO, representing up to 5% of mononuclear cells in noninflamed lymph nodes (LN) (6, 7). As it might be predicted from their homing receptor expression, CD56\textsuperscript{bright} NK cells are enriched in all SLO analyzed to date (LN, tonsils, and spleen) (7). Like PB-CD56\textsuperscript{bright} NK cells, SLO-NK cells exhibit no KIR or CD16 expression and poor cytolytic activity. However, SLO-NK cells promptly acquire cytotoxic ability and the expression of most inhibitory and activating receptors upon IL-2 stimulation (7). Therefore, activation converts SLO-NK cells into cytotoxic effectors analogous to blood CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells. Whether upon activation PB-CD56\textsuperscript{bright} NK cells also may convert into KIR\textsuperscript{−}CD16\textsuperscript{−} NK cells remains to date to be established. In this regard, controversial hypotheses have been proposed recently for the developmental relationship between these two NK cell subsets because CD56\textsuperscript{bright} KIR\textsuperscript{−} CD16\textsuperscript{−} NK cells have been suggested either to represent precursors of CD56\textsuperscript{dim} KIR\textsuperscript{−−} CD16\textsuperscript{−} cells or to be derived from CD56\textsuperscript{dim} KIR\textsuperscript{++−} CD16\textsuperscript{−} cells (8–10). Studies addressing this question have been
hampered by the lack of CD56 in mice and by missing information regarding the site of terminal NK cell differentiation.

In our present study, we investigate whether PB-CD56bright NK cells give rise in vitro and in vivo to cells akin of CD56dim NK cells and whether SLO can be sites of NK cell maturation. All of the observations collected along our present study support the hypothesis that CD56dim cells derive from the CD56bright NK cell subset and that this differentiation can take place during immune activation in inflamed peripheral tissues, such as reactive LN.

Materials and Methods

Cell preparations and cultures

Whole blood and leukocyte concentrates were collected from healthy donors. LN, thoracic duct lymph, and blood were obtained from patients who underwent surgery for cancer resection. All sample collections were obtained after donor informed consent and approval by our Institutional Ethics Committee. When histological evaluation of LN was required, LN were incised immediately after removal and cut into two parts, one of which was paraffin embedded to perform histology, whereas the other was processed for single-cell isolation. For isolation of LN single cells, LN were mechanically dissociated and then treated with enzymes, as previously described (7). NK cells were enriched from PB and LN by negative selection using NK Cell Isolation Kit II (Miltenyi Biotec). To obtain highly purified CD56bright and CD56dim NK cell subsets, cells were FACS sorted directly or after MACS-negative selection using FACSAria (BD Biosciences), according to manufacturer instructions. Briefly, a single-cell suspension (either PB-CD56dim, PB-CD56bright, LN-NK cells, naive, or memory CD4+ T cells) was obtained and mixed with control cells (i.e., the 1301 cell line), which display very long telomeres. Mixed cell suspension DNA was denatured for 10 min at 82°C either in hybridization solution without probe or in hybridization solution containing fluorescein-conjugated PNA telomere probe. Hybridization took place in the dark at room temperature overnight and was followed by two washes at 40°C. After propidium iodide staining, flow cytometric analysis was performed gating on G0/G1 cells. The relative telomere length (RTL) value was calculated as the ratio between the telomere signal of each sample and the control cells (1301 cell line) with correction for the DNA index of G0/G1 cells. This correction was performed to standardize the number of telomere ends per cell and thereby telomere length per chromosome.

Quantitative determination of telomerocyte activity was performed on highly purified CD56bright and CD56dim NK cells by a photometric enzyme immunoassay (Telo TAGGG Telomerase PCR ELISAkit, Roche), according to the manufacturer instructions.

Statistical analysis

Statistics were calculated using Student’s t test or Mann-Whitney U test.

Results

PB-CD56bright NK cells can express KIR and CD16 upon cytokine stimulation

Some reports have shown that CD16 can be down-regulated on PB-CD56dim NK cells (9, 10), suggesting that CD56bright NK cells might represent activated CD56dim NK cells. Nevertheless, down-regulation of KIR expression from PB-CD56dim KIR+ NK cells has never been reported to date. To identify variations of KIR expression on the distinct PB-NK cell subsets, NK cells were sorted with high purity after staining with anti-CD56, anti-CD3, and a combination of anti-KIR (anti-KIR2DL3/L2/S2, anti-KIR2DL1/S1, anti-KIR3DL1/L2/S1) Abs. CFSE-labeled CD56bright (excluding the few KIR+) events, CD56dim KIR+, and CD56dim KIR− NK cells were cultured in the presence of IL-2, IL-12, or IL-15 and analyzed for KIR surface expression. As shown in Fig. 1A, both CD56bright KIR+ (top row) and CD56dim KIR− NK cells (middle row) exhibited de novo expression of KIR on a significant proportion of cells in response to IL-2 (and IL-15; data not shown) and in lower proportion in response to IL-12. In contrast, none of these stimuli was able to down-regulate KIR expression on sorted CD56dim KIR+ NK cells, which was conversely up-regulated after IL-2 stimulation (Fig. 1A, bottom row).

As previously shown, CD56bright NK cells displayed a higher proliferative response to IL-2, IL-15, and IL-12 compared with total CD56dim NK cells. Nonetheless, the comparison of the proliferative ability at day 5 of CD56bright in response to 100 IU/ml IL-2 (percentage of mean proliferation ± SEM = 92.5 ± 1.6%) with CD56dim KIR+ (67.7 ± 9.5%) or KIR− NK cells (26.4 ± 5.8%) showed that CD56bright proliferate slightly more than CD56dim KIR− NK cells (p < 0.05), whereas CD56dim KIR+ proliferate significantly less compared not only to CD56bright (p ≤ 0.002), but also to KIR− NK cells (p ≤ 0.015) (Fig. 1A), suggesting that expression of KIR might correlate with a terminally differentiated phenotype. Notably, the lower proliferative capacity of KIR− NK cells excludes the possibility that rare contaminating KIR+ NK cells could overgrow CD56bright and CD56dim KIR− NK cells and be responsible for KIR expression among KIR− NK cells.
To investigate more in detail which KIR could be up-regulated on the surface of CD56\textsuperscript{bright} or CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells, NK cell subsets were sorted, as previously described, and analyzed for single KIR expression after 5-day culture in IL-2. As shown in Fig. 1B, each KIR expressed ex vivo on CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells (left column) was up-regulated on the surface of CD56\textsuperscript{bright} KIR\textsuperscript{−} or CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells (right columns). Moreover, in donors in which staining of single KIR was performed, mRNA expression of KIR2D and KIR3D ORF (see Materials and Methods) and of their activating or inhibitory counterparts was also analyzed in the different NK cell subsets directly after sorting or after 5-day culture in the presence of IL-2. As shown in Fig. 1C, IL-2 was able to induce mRNA expression of both inhibitory and activating KIR on CD56\textsuperscript{bright} and CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells. The signal detectable in CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells for all KIR ORF mRNA at day 0 after sorting appears to be mainly related to activating rather than inhibitory KIR. Indeed, this could be due to the use of primers recognizing not only KIR2DL2/L3/S2, KIR2DL1/S1, and KIR3DL1/L2/S1 (stained for FACS sorting), but also KIR2DS3/S4/S5, which could not be included in the sorting of KIR\textsuperscript{−} or KIR\textsuperscript{+} NK cells.

To investigate modulation of CD16 expression, NK cells were sorted after staining with anti-CD56, anti-CD3, and anti-CD16 Abs. CFSE-labeled CD56\textsuperscript{bright} and CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells were cultured, as described in Fig. 1A, and analyzed for CD16 surface expression. As shown in Fig. 2, significant CD16 up-regulation occurs on the majority of CD56\textsuperscript{bright} CD16\textsuperscript{−} NK cells after IL-2 (and IL-15; data not shown) stimulation, whereas, as for KIR induction, IL-12 was less efficient. Partial down-regulation of CD16 expression occurs on CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells in the presence of IL-2, as previously reported (9), as well as when cells were left for 5 days in medium alone, although they have been sorted previously with very high purity for CD16 expression (Fig. 1D).
CD56bright NK cells acquire signature of CD56dim NK cells upon cytokine activation

In addition to CD16 and KIR, many other molecules have been described to be differentially expressed in CD56bright and CD56dim NK cells. Although analysis of CD56bright vs CD56dim vs IL-2-activated CD56dim NK cell gene signature has been performed (5), no extensive analysis exists concerning modulation of PB-CD56bright NK cell phenotype, in particular during proliferation. To test the hypothesis that, upon cytokine activation, CD56bright can acquire the signature of CD56dim NK cells, CFSE-labeled PB-CD56bright and CD56dim NK cells were cultured with IL-2, IL-12, or IL-15 for 5 days and then analyzed for the expression of markers that are mutually expressed on one of the two subsets, i.e., IL-7Rα, c-kit, CXCR3, CCR7, CD62L (mainly expressed on CD56bright NK cells), granzyme A, and granzyme B (almost confined to CD56dim NK cells). Modulation of the expression of each molecule was evaluated comparing its mean fluorescence intensity (MFI) among cells that have undergone extensive proliferation after 5-day cytokine stimulation with the MFI among resting cells kept in medium (Fig. 3, A and B, first column) for the same time period. Notably, expression of each molecule analyzed ex vivo did not significantly change after 5-day culture in medium alone, i.e., without cytokines (data not shown). As shown in Fig. 3A, CD56bright NK cells were able to down-regulate surface expression of IL-7Rα, c-kit, CXCR3, and CCR7 after IL-2 and IL-12 (and IL-15; data not shown) stimulation in proliferating cells compared with resting cells kept in medium for the same time interval. Notably, down-regulation of cell surface expression of these markers is not necessarily related to proliferation, because MFI decreased already in nondividing cells cultured in the presence of cytokines when compared with the ones kept in medium only. CD62L expression on CD56bright NK cells was strongly down-regulated after IL-2 (and IL-15; data not shown) activation, although even up-regulated by IL-12 independently of proliferation.

CD56bright NK cells derived from PB and LN display longer telomeres than PB-CD56bright NK cells

To address whether CD56bright NK cells represent an earlier differentiation step of CD56dim NK cells, we evaluated telomere length in NK cell subsets isolated ex vivo from PB and LN. The measurement of telomere length has been widely used for assessing the proliferative history of distinct cell subsets, among which are naive and memory T cells (12–17). In most normal somatic cells, telomere sequences are lost during replication, and therefore telomere length inversely correlates with cell age. In our study, we used flow-FISH because this technique allows the assessment of telomere length with greater sensitivity than traditional methodologies (14, 15).

As shown in Fig. 4, in seven donors analyzed, sorted PB-CD56dim NK cells displayed significantly shorter telomere length than autologous PB-CD56bright NK cells (p ≤ 0.01), with a mean telomere shortening of 15.3% in the CD56dim compared with the CD56bright NK cells (Fig. 4, B and C). As a comparison, we assessed telomere length difference also in naive CD45RA+CD45RO−CD27+ and memory CD45RA−
CD45RO⁺CD4⁺ T cells derived from PB of two donors analyzed in Fig. 4, B and C, for NK cell subsets. Fig. 4D shows that telomere shortening in memory compared with naive CD4⁺ T cells matches up with the one observed in CD56dim NK cells (mean percentage of proliferation ± SEM = 90.7 ± 3.6 for IL-2 and 53.4 ± 7.1 for IL-12) or CD56bright NK cells (mean percentage of proliferation ± SEM = 66.6 ± 7.2 for IL-2 and 34.1 ± 7.3 for IL-12) are due to the fact that a distinct donor was used for each marker. To avoid flow cytometer compensation incoerences due to counterstaining with CFSE, fluorescence intensities and compensation were set based on isotype controls, in which MFI in proliferating and resting cells remains constant. Notably, due to the use of two versions of Diva Software (3.0 and 4.1.2) on LSRII, dot plots with two different logarithmic scales are shown. For each molecule analyzed, one representative experiment of three is shown.

**FIGURE 3.** PB-CD56bright NK cells acquire signature of CD56dim NK cells upon cytokine activation. Sorted CD56bright (A) or CD56dim NK cells (B) were CFSE labeled and cultured in the presence of medium, IL-2, or IL-12. Because a small fraction of CD56dim NK cells can express CD62L, for this experiment (fifth row), CD56dim NK cells were negatively sorted for CD62L. At day 5, cells were stained with their correspondent mAbs, and modulation of the indicated surface or intracellular molecule was evaluated comparing MFI among resting (right gate) and proliferating (left gate) cells, as depicted in each dot plot. Slight differences in CFSE intensity and in the proliferative ability of CD56bright (mean percentage of proliferation ± SEM = 90.7 ± 3.6 for IL-2 and 53.4 ± 7.1 for IL-12) or CD56dim NK cells (mean percentage of proliferation ± SEM = 66.6 ± 7.2 for IL-2 and 34.1 ± 7.3 for IL-12) are due to the fact that a distinct donor was used for each marker. To avoid flow cytometer compensation inconveniences due to counterstaining with CFSE, fluorescence intensities and compensation were set based on isotype controls, in which MFI in proliferating and resting cells remains constant. Notably, due to the use of two versions of Diva Software (3.0 and 4.1.2) on LSRII, dot plots with two different logarithmic scales are shown. For each molecule analyzed, one representative experiment of three is shown.

CD45RO⁺CD4⁺ T cells derived from PB of two donors analyzed in Fig. 4, B and C, for NK cell subsets. Fig. 4D shows that telomere shortening in memory compared with naive CD4⁺ T cells matches up with the one observed in CD56dim compared with CD56bright NK cells (24.3 and 15.4%, respectively). Notably, CD56bright NK cells displayed RTL similar to naive T cells, whereas CD56dim NK cells to memory T cells. In addition, comparative analysis of telomere length in PB-CD56dim NK cells and autologous LN-NK cells, which are predominantly CD56bright NK cells, revealed that PB-CD56dim NK cells significantly displayed shorter telomeres than LN-NK cells (p < 0.04), with a mean telomere shortening of 14.5% in the PB-CD56dim compared with the LN-CD56bright NK cells (Fig. 4, E and F). A similar degree of telomere cutback was observed when LN-NK cells were sorted and cultured for as long as 3 wk in the presence of 100 IU/ml IL-2 (Fig. 4G).

Quantitative determination of telomerase activity was also evaluated on highly purified CD56bright or CD56dim NK cells for assessing putative different ability in telomere maintenance between the two cell subsets. The analysis of enzyme basal activity in PB-NK cells derived from three distinct donors did not show any difference between the two cell subsets (data not shown).

These results indicated that CD56bright NK cells have undergone a considerable smaller number of cell divisions in vivo as compared with CD56dim and might therefore represent an upstream developmental stage of NK cells.

**NK cells isolated from efferent lymph, but not LN-NK cells, express CD16 and KIR**

It has been proposed recently that SLO might represent sites of differentiation for NK cells (7, 8), which would eventually colonize blood and other peripheral tissues. We hypothesized that if SLO were indeed sites of NK cell differentiation, NK cells leaving LN should be different from NK cells resident in LN. To this aim, we analyzed in parallel NK cells isolated from the efferent lymph system (i.e., thoracic duct) and from autologous LN. Consistent with previous reports (6, 7), NK cells harbored in nonreactive LN displayed low or no KIR and CD16 expression. In contrast, a significant proportion of NK cells collected from the efferent lymph of the thoracic duct expressed KIR and CD16, although the latter to a lower extent than their blood counterpart (Fig. 5A). CD56 expression was still bright as compared with PB, suggesting that this marker might be down-regulated in vivo at later time points, as also indicated by our results obtained in vitro. These data cannot rule out the possibility that, conversely, a small percentage of CD56dim NK cells expressing KIR and CD16 enters the LN, expands in situ, and then leaves LN via efferent lymph. Nonetheless, considering the lower proliferative ability and chemokine receptor
expression of CD56<sup>dim</sup>CD16<sup>+</sup> KIR<sup>+</sup> NK cells, we favor the hypothesis that NK cells can acquire de novo expression of relevant functional molecules in LN and then circulate to PB through the efferent lymph.

In vivo expression of KIR in LN-NK cells correlates with LN-paracortical/follicular hyperplasia

The latter observation that NK cells emigrating from, but not resident in LN, also express KIR and CD16 (Fig. 5A) prompted us to speculate that only reactive LN might be site of NK cell activation and KIR and CD16 acquisition. We therefore analyzed NK cells isolated from 28 LN of 14 individual donors with the aim of investigating whether KIR and CD16 expression might be present in nonreactive LN, also express KIR and CD16 (Fig. 5). The latter observation that NK cells emigrating from, but not resident in LN-paracortical/follicular hyperplasia express KIR and CD16. In vivo expression of KIR in LN-NK cells correlates with LN inflammatory status and NK cell KIR or CD16 expression, whereas the other was processed for single-cell isolation and analyzed by flow cytometry.
reactive LN. Remarkably, a significant percentage of NK cells expressing KIR was detectable only in LN characterized by paracortical/follicular hyperplasia (mean percentage ± SEM: 7.3 ± 0.3; Fig. 5C), which is characterized by the presence of secondary follicles and lymphocyte proliferation (Fig. 5B, iii–vi). NK cells isolated from nonreactive LN (Fig. 5B, i and ii) or LN with sinus hyperplasia (characterized by an increased number of macrophages) showed low or no expression of KIR (mean percentage ± SEM: 1.75 ± 0.2; Fig. 5C). Similarly to KIR, CD16 expression also correlated with LN-paracortical/follicular hyperplasia (data not shown). Because of the striking association of KIR and CD16 expression with LN-paracortical/follicular hyperplasia (p ≤ 0.002) and according to previous reports demonstrating that PB-NK cells can reach, but promptly leave, inflamed LN within 72 h (18), we are tempted to speculate that KIR expression in LN NK cells might represent a de novo induction of these molecules occurring on LN resident CD56bright NK cells in the course of an inflammatory immune response, characterized by the abundant presence of different cytokines (e.g., IL-15, IL-12, and IL-2).

Discussion

Despite progress in understanding NK cell specificity for target cells, less is known about stages of NK cell maturation, expansion, and site of differentiation, especially in the human system. This study sheds some light on the final stages of human NK cell differentiation, showing that after cytokine activation, in particular IL-2 and IL-15, CD56bright NK cells could acquire the signature of CD56dim NK cells, i.e., KIR+ CD16− IL-7Rα+ c-kit− CXCR3− CCR7−, CD62L−, whereas CD56dimCD16+ KIR− NK cells substantially maintain their features of terminally differentiated cells. Within the molecules acquired by CD56bright NK cells, KIR are of great interest: in contrast to CD16 expression, which has been shown to be differentially modulated in several experimental models (9, 10), once a NK cell has acquired its specific set of KIR, the expression remains stable, as shown in NK cell clones under various cell culture conditions and activation stimuli (19). KIR expression is actually regulated by epigenetic mechanisms, and it had been shown to date that it could be acquired in vitro only using methyltransferase inhibitors (19). Indeed, our data demonstrate that both CD56bright KIR+ and CD56dim KIR− NK cells could acquire KIR expression on a subset of cells after cytokine stimulation. This result is apparently in contrast to a previous publication in which it was claimed that no KIR or CD16 up-regulation was occurring on CD56bright NK cells after IL-2 stimulation (1). One possible explanation for this discrepancy might rely on the different experimental conditions (e.g., IL-2 concentration). Conversely, the time frame analyzed is comparable and, by performing a kinetic of KIR expression in CD56bright NK cells stimulated with IL-2 up to 40 days after sorting, we could observe that the percentage of up-regulated KIR remains constant (data not shown).

The evidence that KIR are molecules inducible on NK cells raises interest about the mechanisms by which this process can occur, because this understanding might help to clarify the mechanisms underlying NK cell tolerance to self. Recent studies have changed our view on how NK cell self-tolerance is achieved, showing that NK cells, which do not express inhibitory receptors recognizing self-MHC, do exist. Nonetheless, only NK cells expressing inhibitory receptors that recognize self-MHC are competent, whereas those that do not display an anergic phenotype (20–24). In this context, our finding that KIR can be induced by cytokines is of great interest because it suggests that NK cell self-tolerance might be a dynamic process probably related to cell differentiation: cytokines produced during an inflammatory response by dendritic cells (DCs) or T cells would induce NK cell differentiation and generation of new competent NK cells. Along this line, it has been shown also that Ly49 molecules can be modulated on NK cells after cytokine stimulation, suggesting a similar scenario in mice (25). Our findings about KIR as inducible receptors might also have relevant implications for manipulating NK cell self-tolerance in clinical settings such as organ transplantation, in which KIR specificity has already been shown to be relevant for tumor rejection (26).

KIR+ NK cells showed lower ability to proliferate in response to cytokines compared not only to CD56bright, but also to CD56dim KIR+. This surprising finding was not due to a mAb-staining artifact (e.g., inhibition of proliferation via triggering of inhibitory receptors by anti-KIR mAb) because staining or not total CD56dim NK cells (mixture of KIR+ and KIR− NK cells) with anti-KIR mAb combination used for our sorting procedure did not influence at all NK cell proliferation (data not shown). One possible explanation of the lower CD56dim KIR+ NK cell proliferation might be due to KIR binding in cis or in trans to surface MHC class I molecules expressed on NK cells, which could result in inhibition of proliferation after stimulation, as it has been shown for cis binding of Ly49 in mice (27). Nonetheless, we tend to exclude this hypothesis because, also in this case, staining of KIR with mAb should influence KIR ability to bind MHC class I molecules and resulting in less inhibition. Given all these observations, we favor the hypothesis that CD56bright and CD56dim NK cells do not belong to two distinct subsets, each one displaying dissimilar proliferative features, but rather that CD56bright represent an earlier stage of NK cell development and that KIR acquisition correlates with a terminal step of NK cell maturation, as it has been speculated already both for NK cells and CD8+ KIR+ T cells (28, 29). CD56bright NK cells also down-regulated the expression of cytochrome receptors such as the stem cell factor receptor CD117 (c-kit) and CD127 (IL-7Rα) during cytokine-induced proliferation. The progressive loss of receptors that are selectively expressed not only by CD56bright, but also by NK cell immature precursors in humans and in mice (30, 31), is also very suggestive of a differentiation process from an early to a more advanced stage at which NK cells do not require stem cell factor or IL-7 signaling any longer. Accordingly, mouse Mac1− NK cells displaying an immature phenotype tend to express c-kit, which is then absent in mature Mac1− NK cells (31). Down-regulation of SLO homing molecules such as CD62L, CXCR3, and CCR7 on CD56bright NK cells upon cytokine stimulation is consistent with the hypothesis that these NK cells would leave SLO after their activation. If CD56bright NK cells represented an earlier developmental step of NK cell differentiation, they should have undergone a lower number of proliferative events in vivo. Consistent with this hypothesis, CD56bright NK cells display longer telomeres than CD56dim NK cells. In this regard, we could demonstrate that CD56bright exhibit the same characteristics of naive T cells, i.e., longer telomeres compared with memory T cells, according to previous reports (17).

Although not providing the definitive proof that CD56bright are the precursors of CD56dim NK cells, these findings definitely rule out the hypothesis that CD56bright are derived from CD56dim NK cells and strengthen their close functional and molecular resemblance with naive T cells.

Ex vivo analysis of human NK cells from different compartments revealed that nonreactive LN contain almost exclusively CD56bright KIR− CD16− NK cells, whereas a significant NK cell expression of KIR and CD16 is present in highly inflamed LN and in the efferent lymph. These data suggest that CD56bright KIR− CD16− NK cells can acquire KIR and CD16 in inflamed LN and then circulate via the efferent lymph in PB as KIR+ CD16+ NK cells. The assumption that cytokines released during inflammation...
can mobilize NK cells from SLO to PB is also supported by previous studies reporting that rIL-2 therapy for human cancer results in a striking increase of CD56brightCD16+ NK cells in PB (32). Because these NK cells are not cycling (33), it is conceivable that they are mobilized from extravascular tissues, rather than directly proliferating in the blood. It could be envisaged that in steady state or very early during an immune response, CD56bright KIR+ NK cells can be recruited into LN (18, 34), whereas later on during inflammation mature NK cells leave LN and then circulate in PB to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR+CD16+ NK cells in inflamed LN (and in the effluent lymph) might be due to selective migration of this subset into LN and not to CD56bright differentiation into CD56dim NK cells. Nonetheless, because it has been shown that NK cell recruitment into inflamed LN occurs via CD62L and CXCR3 (18), KIR+CD16+ NK cells, which are generally CD62L− and CXCR3−, should be less prone to migrate to this site.

Other studies have also suggested that LN may represent a key site for NK cell development (8, 30). In fact, it has been shown recently that four different developmental stages of human NK cell precursors are present in LN and that differentiation from these precursors to mature CD56bright NK cells can be mediated by cytokines and supported by stromal cells (8, 30). However, from these previous observations, it is not yet clear to what extent NK cell differentiation in SLO might account for the total mature NK cell compartment in the body, as most NK cells in human PB are CD56dim. We now suggest that also the final maturation of CD56bright into CD56dim might occur in SLO, further supporting the hypothesis that CD56dim NK cells might correspond to the terminally differentiated stage of human NK cell development. Although reactive SLO might represent an important site of NK cell differentiation and maturation, such developmental processes could also take place in other inflamed tissues, where both CD56bright and CD56dim NK cells can be found (35). Whatever the case, it would be of great interest to investigate which cell type resident in the LN is essential to induce NK cell proliferation and maturation. DCs are interesting candidates because NK cells and DCs are colocalized in LN paracortex and medulla, and have been shown to interact together over extended times (34, 36). Because we have shown previously that both myeloid and plasmacytoid DCs can induce selective expansion of CD56bright NK cells (36–38), it would be important to determine whether DCs can induce not only CD56bright proliferation, but also differentiation into CD56dim NK cells. To this aim, whether DCs can induce KIR and CD16 expression on proliferating CD56bright NK cells should be analyzed. It is also not clear at which phase of an immune response NK cell final maturation may happen. Both DC (IL-15 and to some extent IL-12)- and T cell (IL-2)-derived cytokines can induce this differentiation step in vitro. If DC-derived cytokines were primarily involved in NK cell maturation in vivo, this process could take place in the very early phase of an innate immune response before T cell clonal expansion. However, considering the effect of IL-2 in vitro and the significant in vivo association between KIR/CD16 expression and paracortical/follicular hyperplasia, in which extensive lymphocyte proliferation occurs, it is conceivable that NK cell terminal differentiation could take place or be enhanced later on during an immune response, when proliferating naive T cells start to produce high amounts of IL-2.

In conclusion, our data provide new evidence supporting the hypothesis that CD56bright may give raise to CD56dim NK cells, and envisage a scenario in which NK cell final maturation and acquisition of competence might occur in SLO during an inflammatory response.

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Disclosures

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References


