CD56<sup>bright</sup>CD16<sup>−</sup> Killer Ig-Like Receptor<sup>−</sup> NK Cells Display Longer Telomeres and Acquire Features of CD56<sup>dim</sup> NK Cells upon Activation

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*J Immunol* 2007; 178:4947-4955;
doi: 10.4049/jimmunol.178.8.4947
http://www.jimmunol.org/content/178/8/4947

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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)-NK cells (≥95%) belong to the CD56\textsuperscript{bright}CD16\textsuperscript{−} cytoplasmic...
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-CD56\textsuperscript{bright} NK cells give rise in vitro and in vivo to cells akin of CD56\textsuperscript{dim} 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 CD56\textsuperscript{dim} cells derive from the CD56\textsuperscript{bright} 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 CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subsets, cells were FACS sorted directly or after MACS-negative selection using FACSARia (BD Biosciences), according to the lack of CD3 and the expression of CD56 and additional markers, when indicated in the text. For isolation of naive and memory CD4\textsuperscript{+} T cells, CD4\textsuperscript{+} T cells were first positively selected using CD4 Microbeads (Miltenyi Biotec) and then FACS sorted after staining with anti-CD4, anti-CD45RA, anti-CD45RO, and anti-CD27 mAbs. All sorted subsets used for the experiments always displayed purity above 97%. The medium used throughout experiments was RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Invitrogen Life Technologies) and 1% antibiotic mixture (5 mg/ml penicillin, 5 mg/ml streptomycin stock solution). Unless differently indicated in the text, 10\textsuperscript{5} NK cells were cultured in 96-well round-bottom plates (Costar) in the presence or absence of the following cytokines: 100 IU/ml IL-2 (Roche), 25 ng/ml IL-12, or 25 ng/ml IL-15 (R&D Systems). To evaluate proliferation, cells were labeled with CFSE (Molecular Probes), as previously described (7).

Flow cytometric phenotypic analysis

The following Abs, anti-CD56 PE Cy7 (NCAM16.2), anti-CD16 allophycocyanin (YB5.8), anti-CD16 PE or allophycocyanin Cy7 (clone 3G8), anti-CD262L PE or allophycocyanin (Dreg56), anti-CCCR3 allophycocyanin (1C6/CCCR3), anti-granzyme A PE (CB9), anti-CD45RA PE, and anti-CD45RO PE FITC, were purchased from BD Pharmingen. Anti-granzyme B PE (GB12) was purchased from Caluag Laboratories. Anti-CD56 PE or allophycocyanin (AF12-7H5) was purchased from Miltenyi Biotec. Anti-CD27 PE (R34.34) was purchased from Beckman Coulter. Anti-KIR3DL1 PE (DX9) was purchased from Abcam. For CCR7 staining, cells were first incubated with anti-PE CCR7 mAb (IgG2a, clone 150503; R&D Systems) and afterward with a biotin-conjugated mAb directed against IgG2a (Southern Biotechnol Associates). Anti-CD3 (OKT3), anti-CD4 (TT1), and anti-CD27 (2E4) mAbs were produced and conjugated in our laboratory either with FITC, PE, or Cy5. Anti-KIR2DL2/L3/S2 (GL183), anti-KIR2DL1/L1/S1 (EB6), and anti-KIR3DL1/L1/S1 (Az158) mAbs were also produced and conjugated in our laboratory either with FITC, Cy5, or biotin. When cells were stained with biotin-conjugated mAbs, streptavidin-Pacific Blue or streptavidin-Alexa647 (Molecular Probes) were used as secondary reagents. For phenotypic analysis, data were acquired on a FACScalibur or LSRII flow cytometer, the latter using Diva Software 3.0 and 4.1.2 (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

RT-PCR analysis of KIR transcripts

Total RNA was extracted from CD56\textsuperscript{bright}, CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells at day 0 directly after sorting or after a 5-day culture in the presence of 100 IU/ml IL-2 using RNeasy micro kit (Qiagen), according to manufacturer’s instruction. cDNA synthesis was performed on ~500 ng of RNA using oligo(dT) primers. Three different sets of primers were used in this study. Anti-KIR2DL2 PE (R34.34) was purchased from Beckman Coulter. GOT TTT GAG GAC GGG CTG allowed the amplification of the KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, and KIR3DS1 open reading frame (ORF). The sets of primers common upC and common upPE allowed the amplification of a segment of activating and inhibitory KIR transcripts, respectively, as previously described (11). The PCR products were resolved into 0.8% agarose gel.

Analysis of telomere length and telomerase activity

Analysis of telomere length was performed using a quantitative flow-fluoroescence in situ hybridization (FISH) methodology that employs a fluorescein-conjugated peptide nucleic acid (PNA) probe (Telomere PNA Kit/FITC for Flow Cytometry; DakoCytomation), according to manufacturer’s instructions. Briefly, a single-cell suspension (either PB-CD56\textsuperscript{bright}, PB-CD56\textsuperscript{dim}, LN-NK cells, naive, or memory CD4\textsuperscript{+} 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 telomerase activity was performed on highly purified CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells by a photometric enzyme immunoassay (Telo TAGGG Telomerase PCR ELISA\textsuperscript{kit}, Roche), according to the manufacturer instructions.

Statistical analysis

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

Results

PB-CD56\textsuperscript{bright} NK cells can express KIR and CD16 upon cytokine stimulation

Some reports have shown that CD16 can be down-regulated on PB-CD56\textsuperscript{dim} NK cells (9, 10), suggesting that CD56\textsuperscript{bright} NK cells might represent activated CD56\textsuperscript{dim} NK cells. Nevertheless, down-regulation of KIR expression from PB-CD56\textsuperscript{dim} KIR\textsuperscript{−} 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 for high purity after staining with anti-CD56, anti-CD3, and a combination of anti-KIR (anti-KIR2DL2/L3/S2, anti-KIR2DL1/S1, anti-KIR3DL1/L1/S1) Abs. CFSE-labeled CD56\textsuperscript{bright} (excluding the few KIR\textsuperscript{+} events), CD56\textsuperscript{dim} KIR\textsuperscript{−}, and CD56\textsuperscript{dim} KIR\textsuperscript{+} 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 CD56\textsuperscript{bright} KIR\textsuperscript{−} (top row) and CD56\textsuperscript{dim} KIR\textsuperscript{−} 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 CD56\textsuperscript{dim} KIR\textsuperscript{+} NK cells, which was conversely up-regulated after IL-2 stimulation (Fig. 1A, bottom row). As previously shown, CD56\textsuperscript{bright} NK cells displayed a higher proliferative response to IL-2, IL-15, and IL-12 compared with total CD56\textsuperscript{dim} NK cells. Nonetheless, the comparison of the proliferative ability at day 5 of CD56\textsuperscript{bright} in response to 100 IU/ml IL-2 (percentage of mean proliferation \pm SEM = 92.5 \pm 1.6%) with CD56\textsuperscript{dim} KIR\textsuperscript{−} (67.7 \pm 9.5%) or KIR\textsuperscript{+} NK cells (26.4 \pm 5.8%) showed that CD56\textsuperscript{bright} proliferate slightly more than CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells (p < 0.05), whereas CD56\textsuperscript{dim} KIR\textsuperscript{+} proliferate significantly less compared not only to CD56\textsuperscript{bright} (p \geq 0.002), but also to KIR\textsuperscript{−} NK cells (p \geq 0.015) (Fig. 1A), suggesting that expression of KIR might correlate with a terminally differentiated phenotype. Notably, the lower proliferative capacity of KIR\textsuperscript{−} NK cells excludes the possibility that rare contaminating NK cells could overgrow CD56\textsuperscript{bright} and CD56\textsuperscript{dim} KIR\textsuperscript{−} NK cells and be responsible for KIR expression among KIR\textsuperscript{−} NK cells.
To investigate more in detail which KIR could be up-regulated on the surface of CD56bright or CD56dim KIR− 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 CD56dim KIR+ NK cells (left column) was up-regulated on the surface of CD56bright KIR− or CD56dim KIR− 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. Analysis was performed directly after sorting or after a 5-day culture in the presence of IL-2. One representative experiment of two is shown.

To investigate modulation of CD16 expression, NK cells were sorted after staining with anti-CD56, anti-CD3, and anti-CD16 Abs. CFSE-labeled CD56bright CD16+ and CD56dim CD16+ NK cells were cultured, as described in Fig. 1A, and analyzed for CD16 surface expression. Fig. 2 shows that significant CD16 up-regulation occurs on the majority of CD56bright CD16+ 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 CD56dim CD16+ 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.
CD56<sup>bright</sup> GAIN CD56<sup>dim</sup> NK CELL FEATURES UPON ACTIVATION

In addition to CD16 and KIR, many other molecules have been described to be differentially expressed in CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells. Although analysis of CD56<sup>bright</sup> vs CD56<sup>dim</sup> vs IL-2-activated CD56<sup>dim</sup> NK cell gene signature has been performed (5), no extensive analysis exists concerning modulation of PB-CD56<sup>dim</sup> NK cells. In addition, we show that CD56<sup>dim</sup> KIR<sup>+</sup> NK cells cannot down-regulate KIR under any tested stimulus and display lower proliferative ability compared not only to CD56<sup>bright</sup>, but also CD56<sup>dim</sup> KIR<sup>+</sup> NK cells, suggesting that KIR acquisition might represent a late maturation stage of NK cell differentiation.

CD56<sup>bright</sup> NK cells acquire signature of CD56<sup>dim</sup> NK cells upon cytokine activation

To test the hypothesis that, upon cytokine activation, CD56<sup>bright</sup> NK cells (Fig. 3A, and data not shown for IL-15), whereas the same molecules remained rather stable in CD56<sup>dim</sup> NK cells (Fig. 3B). Stimulation with IL-18 alone or in combination with IL-2 was conversely not able to significantly modulate the expression of any of the markers analyzed, neither on CD56<sup>bright</sup> or CD56<sup>dim</sup> NK cells, apart from CCR7, which, as previously described (10), was up-regulated on CD56<sup>dim</sup> NK cells. Notably, CD83 which has also been shown to be up-regulated on NK cells after IL-18 stimulation (10), could be induced not only on CD56<sup>dim</sup>, but also on CD56<sup>bright</sup> NK cells (data not shown).

In regard to the expression of CD56, we did not expect any down-regulation of this molecule on CD56<sup>bright</sup> NK cells, because it is well known that high levels of this molecule can also be induced and maintained in all NK cells during activation. Nevertheless, we observed that LN-NK cells that have been stimulated with IL-2 down-regulated their level of CD56 expression following IL-2 withdrawal, whereas KIR expression was maintained (data not shown).

CD56<sup>bright</sup> NK cells derived from PB and LN display longer telomeres than PB-CD56<sup>dim</sup> NK cells

To address whether CD56<sup>bright</sup> NK cells represent an earlier differentiation step of CD56<sup>dim</sup> 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-CD56<sup>dim</sup> NK cells displayed significantly shorter telomere length than autologous PB-CD56<sup>bright</sup> NK cells (p ≤ 0.01), with a mean telomere shortening of 15.3% in the CD56<sup>dim</sup> compared with the CD56<sup>bright</sup> NK cells (Fig. 4, B and C). As a comparison, we assessed telomere length difference also in naive CD45RA<sup>+</sup>CD45RO<sup>+</sup>CD27<sup>+</sup> and memory CD45RA<sup>−</sup>

FIGURE 2. PB-CD56<sup>bright</sup> NK cells up-regulate CD16 expression upon cytokine activation. CD56<sup>bright</sup>CD16<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells were sorted, CFSE labeled, and cultured in the presence or absence of IL-2 or IL-12. At day 5, modulation of surface CD16 expression was evaluated after staining with anti-CD16 Ab. Percentages of CD16<sup>+</sup> cells are indicated in each dot plot. One representative experiment of five is shown.

Day 0-sorted

Medium

IL-2

IL-12

CD56<sup>bright</sup>

CD56<sup>dim</sup>
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 (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.

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 considerably 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 CD56dimCD16+ KIR+ 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

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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 varying 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 cytokine 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 Mac1bright 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 to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues.

CD16 expression on proliferating CD56bright NK cells should be challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues. Although this hypothesis is very challenging, we cannot exclude that the presence of KIR to reach inflamed tissues.

To this aim, whether DCs can induce KIR and envisage a scenario in which NK cell final maturation and acquisition of competence might occur in SLO during an inflammatory response.

Acknowledgments

We acknowledge Siegfried Kohler for critical reading of the manuscript and discussion; Daniele Marras for inspired critical suggestions; and Toralf Kaiser, Katharina Raba, and Ennio Albani for cell sorting.

Disclosures

The authors have no financial conflict of interest.

References


