CD27 Defines Phenotypically and Functionally Different Human NK Cell Subsets

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*J Immunol* 2008; 180:3739-3745; doi: 10.4049/jimmunol.180.6.3739

http://www.jimmunol.org/content/180/6/3739
CD27 Defines Phenotypically and Functionally Different Human NK Cell Subsets

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The absence of the TNF-receptor family member CD27 marks the stable acquisition of cytolytic effector functions by both CD4+ and CD8+ T cells. We found that the majority of circulating human NK cells was CD27+. These cells were largely CD56dim, contained high levels of perforin and granzyme B, and were able to exert strong cytotoxic activity. In contrast, circulating CD27+ NK cells were mostly CD56dim/bright, had significant lower levels of perforin and granzyme B, and had a low cytolytic potential. Primary and secondary lymphoid organs were markedly enriched for CD27+ NK cells. When correlating the expression of CD27 to recently defined developmental stages of NK cells in tonsil, we observed that CD27 was exclusively found on mature CD94+, stage 4 NK cells. On these cells, regulation of CD27 expression appeared to be controlled by the common γ-chain cytokine IL-15, and down-regulation of CD27 was specifically induced by its ligand, CD70. Thus, the absence of CD27 expression allows the definition of cytotoxic effector cells within the known mature NK cell subsets in humans. The Journal of Immunology, 2008, 180: 3739–3745.

Natural killer (NK) cells are key mediators in the first line of defense of the innate immune response against invading pathogens and act before the induction of adaptive immune responses. Characterized by the absence of a TCR/CD3 complex and the expression of CD56, NK cells comprise ~15% of human peripheral blood lymphocytes. The activation of NK cells appears to be tightly regulated by a balance between inhibitory and activating signals that derive from receptors belonging to either Ig or C-type lectin superfamilies (1, 2). The ligands for activating NK cell receptors are up-regulated on nucleated cells in response to infection, cellular activation, or stress (3–5). Down-modulation of MHC class I on tumor cells and on virus-infected cells reduces the activity of MHC-specific inhibitory NK receptors and thereby increases the sensitivity of these cells for NK cell-mediated killing (6). Using the balanced interplay between activating and inhibitory receptors, NK cells have a pivotal role in the innate immune response against viruses and cancer. Killing of target cells by NK cells is accomplished by a variety of effector mechanisms, including the release of granules containing perforin and granzymes (7) and the binding of CD95-ligand to its receptor on the target cell (8). In addition to their role in the direct elimination of potentially harmful cells, NK cells are able to secrete immunoregulatory cytokines such as IFN-γ and TNF-α (9).

Until recently, NK cells were considered to be a functionally homogeneous population. It has now become clear that both murine and human NK cells can be divided into functional subsets (10–12). In humans, ~90% of peripheral blood NK cells are CD56dim. CD56dim NK cells express moderate to high levels of FcγRIIIA (CD16) and perforin and have high cytotoxic capability. CD56bright NK cells, in contrast, are mainly CD16low, are poorly cytolytic, and probably function as immunoregulatory cells by the secretion of cytokines. In contrast to circulating NK cells, NK cells within lymph nodes are enriched for the CD56bright phenotype. This localization may be the consequence of the expression pattern of homing receptors on NK cell subsets, because CD56dim NK cells express homing markers for inflamed peripheral sites, whereas CD56bright NK cells display homing markers for secondary lymphoid organs (13). In addition, elegant recent studies have provided evidence that secondary lymphoid tissue (SLT) is a principal site of human NK cell maturation, which can be accurately monitored by combinations of phenotypic markers (14).

T cell subsets can be discriminated by the differential expression of a variety of membrane molecules, including the TNF-receptor family member CD27. Loss of CD27 on both murine and human CD8+ T cells is associated with a high cytotoxic capacity (15–17). Moreover, the small fraction of human CD4+ T cells that has cytolytic potential lacks CD27 (18). Although it has been reported that CD27 expression varies on murine NK cells (19), little is known on the expression and function of this receptor on human NK cells. We here show that on human NK cells, lack of CD27 is associated with high cytolytic function and, in combination with CD56, allows a better definition of cytotoxic effector cells. Moreover, we demonstrate that CD27 becomes up-regulated during the late stage of NK cell maturation in secondary lymphoid organs, and that subsequent loss of CD27 expression from these cells is subject to regulation by the common γ-chain cytokine IL-15.

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Received for publication July 17, 2007. Accepted for publication January 11, 2008.

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3 Abbreviations used in this paper: SLT, secondary lymphoid tissue; rh, recombinant human.
CD3+, CD56dimCD27+, and CD56brightCD27+. These sorted cell fractions were either used in cytotoxicity assays or in cytokine-induced cultures as described below.

**NK cell cytotoxicity assay**

K562, a human leukemic cell line that is highly sensitive to killing by NK cells, was prepared by labeling these cells with 51Cr (Amersham Biosciences) for 1 h at 37°C, 5% CO2. 51Cr-labeled target cells were then incubated in triplicates in culture medium with effector cells (NK cells) at various effector:target (E:T) ratios for 4 h at 37°C, 5% CO2. Supernatants were harvested and counted in a gamma counter (Packard) or a beta counter (Bran + Luebbe). Percentage specific lysis was calculated by subtracting the spontaneous release from the experimental release, and dividing by 100.

**Stimulation of the cells**

CD27- or CD27+ sorted NK cells were cultured for 8 days in 24-well plates at a concentration of 0.5–1 × 10⁶ cells/ml in culture medium in the presence or absence of recombinant human rhIL-15 (10 ng/ml; R&D Systems) or rhIL-21 (25 ng/ml; kindly provided by ZymoGenetics) for 5–12 days. The stimulation index was calculated by dividing the number of recovered NK cells at the end of the culture period by the amount of input cells. The effects of blocking CD27-CD70 signaling were studied by stimulation with CFSE-labeled PBMCs with IL-15 (10 ng/ml) in presence or absence of 40 μg/ml CD70 mAb (clone 2F2) for 7 days. NK cells were identified by gating on the CD8dim fraction. Used concentrations of cytokines and mAbs were based on titration experiments (IL-15 and 2F2) or manufacturer’s recommendation (IL-21).

**CFSE labeling**

PBMCs were resuspended in PBS at a final concentration of 5–10 × 10⁶ cells/ml and labeled with 2.5 μM final concentration of CFSE (Molecular Probes) for 8 min by shaking at 37°C. Cells were washed and subsequently cultured in supplemented IMDM as described above.

**Detection of cytokine expression**

NK cell subsets were sorted on the basis of CD56 and CD27 expression as described above. Sorted cells were cultured for 72 h in 96-well plates at a concentration of 2 × 10⁵ cells/ml in culture medium containing 20 ng/ml rhIL-15 (10 ng/ml; R&D Systems) or rhIL-18 (10 ng/ml; R&D Systems) (21) for 5 days. The effects of blocking CD27-CD70 signaling were studied by stimulation with CFSE-labeled PBMCs with IL-15 (10 ng/ml) in presence or absence of 40 μg/ml CD70 mAb (clone 2F2) for 5 days. Cell supernatants were removed and analyzed in a gamma counter for 5 days. Supernatants were harvested and counted in a gamma counter (Packard) or a beta counter (Bran + Luebbe). Percentage specific lysis was calculated by subtracting the spontaneous release from the experimental release, and dividing by 100.

**Statistical analysis**

The two-tailed Mann-Whitney U test was used for analysis of differences between groups. Values of p < 0.05 were considered statistically significant.

**Table I. Relation between CD56 and CD27 expression on circulating NK cell subsets**

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<tr>
<th>CD56dim</th>
<th>CD56bright</th>
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<tr>
<td>CD27+</td>
<td>94 ± 3%</td>
</tr>
<tr>
<td>CD27+</td>
<td>56 ± 11%</td>
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*CD27+ and CD27- peripheral blood NK cells were analyzed for CD56 expression (n = 10). Numbers indicate the proportion of positive cells ± SD.
either CD56dim or CD56bright (Fig. 1 and Table I). In agreement with earlier findings, CD56 dim NK cells were CD16+ and CD94+ (data not shown). Based on the expression of CD34, NK cells appeared to be CD56dim, whereas CD27+ NK cells were either CD56dim or CD56bright (Fig. 1 and Table I). In agreement with earlier findings, CD56dim NK cells were CD16+ and the highest expression of CD16 was detected on the CD27+ NK cells. Thus, CD27+ NK cells were largely contained within the CD56dimCD16+ NK cell subset.

Secondary lymphoid organs are enriched for CD27+ NK cells

Exploring the percentages of CD27+ NK cells within different lymphoid organs revealed marked differences as depicted in Fig. 2. Frequencies of CD27+ NK cells in bone marrow (14 ± 6%) were slightly higher than those observed in peripheral blood. In line with the increased presence of CD56bright NK cells in lymphoid organs (23, 24), we detected significantly higher frequencies of CD27+ NK cells in spleen and tonsil (37 ± 16% and 34 ± 5%, respectively), the majority being CD56bright (data not shown). Based on the expression of CD34, CD117, and CD94, four successive developmental stages of NK cells have recently been described in SLT, i.e., CD34+CD117+CD94− (stage 1), CD34+CD117+CD94+ (stage 2), CD34+CD117+CD94+ (stage 3), and CD34+CD117+CD94+ (stage 4) (14). Progression through these stages is associated with a gradual up-

Results

CD27 is expressed on a subset of circulating NK cells

Because functional T cell subsets can be discriminated on the basis of CD27 expression (22), we determined whether this marker could also be used to separate human NK cell subsets. The majority of peripheral blood NK cells was CD27−, but in all donors (n = 15), ~6% of the CD3−CD56− cells expressed CD27 (Fig. 1 and data not shown). It has previously been shown that human NK cell subsets can be separated in CD56dim and CD56bright populations (10). The vast majority of CD27− NK cells appeared to be CD56dim, whereas CD27+ NK cells were either CD56dim or CD56bright (Fig. 1 and Table I). In agreement with earlier findings, CD56dim NK cells were CD16+ and the highest expression of CD16 was detected on the CD27− NK cells. Thus, CD27+ NK cells were largely contained within the CD56dimCD16+ NK cell subset.

Expression of NK cell receptors differs between CD27+ and CD27− NK cells

Next we analyzed whether CD27+ and CD27− NK cells differed in the expression of NK cell receptors, the major determinants of NK cell activation (Fig. 4 and Table II). Whereas fractions of circulating CD27+ NK cells expressed CD158a, CD158b, or NKB1, these percentages were considerably lower in the CD27− pool. A similar difference between CD27+ and CD27− subsets was found in the spleen, whereas the expression of these markers on tonsillar NK cells was, in agreement with the premature character of most CD27− cells (14), low irrespective of their CD27 phenotype (Table II). In addition, the amount (as measured by mean fluorescence intensity (MFI)) of regulation of CD56. As shown in Fig. 3, CD27 expression was exclusively found on the most mature CD34+CD94+ cells (stage 4), whereas premature CD34+CD94−/+ cells (stages 1–3) were CD27−. Notably, also, about two thirds of the mature CD94+ NK cells were CD27− (63 ± 14%, n = 3).

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both CD94 and NKp46 expression was relatively high on CD27+ NK cells from all sites.

NKp44, which becomes up-regulated in response to stimulation (25), could hardly be detected on circulating or splenic NK cells, however, was elevated in comparison with peripheral blood NK cells. The expression of NKp44 on tonsillar CD27+ cells, indicating that these populations largely contain resting NK cells, was low (data not shown).

Circulating CD27− NK cells have potent cytotoxic capacity

Loss of CD27 on circulating CD8+ T cells marks the stable acquisition of cytolytic effector function (15). Killing of target cells by NK cells is mainly accomplished by the coordinated release of cytolytic mediators containing perforin and granzymes, which induce apoptosis of the target cell (26). Peripheral blood and splenic CD27− NK cells contained significantly higher levels of perforin and granzyme B compared with their CD27+ counterparts (Fig. 5). Importantly, also within the cytolytic CD56dim subset, the absence of CD27 marked cells with the highest expression of both perforin and granzyme B. In tonsil, no perforinbright NK cells were found and the number granzyme B− cells was low (data not shown).

The differential expression of cytolytic mediators was reflected by a 4-fold higher cytotoxicity of sorted CD27− NK cells toward the NK cell-sensitive cell line K562 compared with CD27+ NK cells (Fig. 6A). The difference between CD27− and CD27+ NK cells accords well with previously published data because CD27− NK cells largely reside within the cytolytic CD56dim fraction (11). In addition, inclusion of CD27 expression facilitated definition of the cytotoxic capacity of NK cells within both the CD56dim and the CD56bright fractions (Fig. 6B).

Analysis of cytokine production in sorted NK cell subsets, stimulated for 72 h with IL-12 in combination either IL-15 or IL-21, confirmed the well-established capacity of CD56bright cells to produce high amounts of IFN-γ (21). Interestingly, within both the

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<tr>
<th>Peripheral Blood</th>
<th>Spleen</th>
<th>Tonsil</th>
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<tr>
<td>CD27−</td>
<td>CD27+</td>
<td>p</td>
</tr>
<tr>
<td>CD158a</td>
<td>26 ± 10%</td>
<td>5 ± 2%</td>
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<tr>
<td>CD158b</td>
<td>34 ± 8%</td>
<td>8 ± 1%</td>
</tr>
<tr>
<td>NK1</td>
<td>22 ± 12%</td>
<td>5 ± 3%</td>
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<tr>
<td>NK2D</td>
<td>18 ± 2</td>
<td>26 ± 4</td>
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<tr>
<td>CD94</td>
<td>128 ± 91</td>
<td>289 ± 128</td>
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<tr>
<td>NKp46</td>
<td>144 ± 50</td>
<td>491 ± 199</td>
</tr>
<tr>
<td>NK44</td>
<td>1 ± 1%</td>
<td>3 ± 1%</td>
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*p CD27+ and CD27− NK cells from peripheral blood (n = 10), spleen (n = 5), and tonsil (n = 4) were analyzed for NK cell receptor expression. Numbers ± SD indicate the proportion of positive cells within the CD27+ and CD27− fractions (CD158a, CD158b, NK1, and NKp44) or the MFI of the cells expressing the receptor within the CD27+ and CD27− fractions (NK2D, CD94, and NKp46). ns, Not significant.

**FIGURE 5.** Circulating and splenic CD27− NK cells contain high levels of perforin and granzyme B. Peripheral blood NK cells were subdivided into subsets based on CD27 and CD56 expression (see Fig. 1). In each subset, perforin and granzyme B expression was analyzed. A, Perforinbright cells and Granzyme B+ cells were identified using a histogram on lymphocytes. B, Perforin and granzyme B expression of different NK cell subsets for three donors in peripheral blood (PB) and spleen. Values are mean percentages ± SD.
CD56^dim and the CD56^bright population, CD27^+ cells produced greater levels of IFN-γ (Fig. 7).

CD27 expression on NK cells is influenced by cytokines and the availability of CD70

The above data showed that although the most mature NK cells in SLT express CD27, the majority of the circulating NK cells is CD27^-. To examine the possibility that CD27 expression on stage 4 NK cells was subject to modulation by cellular activation, we purified CD27^- and CD27^+ NK cells (Fig. 8A) and analyzed whether CD27 expression could be influenced by IL-15 or IL-21, two cytokines implicated in the generation and maintenance of NK cells (27–31). Stimulation of CD27^- NK cells with IL-15 resulted in strong expansion of cells (stimulation index 6), which after 8 days of culture all had lost CD27 (Fig. 8B, thick line). These cells were highly cytotoxic (data not shown) and, therefore, were functionally and phenotypically indistinguishable from IL-15-stimulated CD27^- cells. In line with this, both stimulated fractions expressed high and comparable levels of CD56 and NKp44. In contrast, stimulation with IL-21 of either CD27^- or CD27^+ sorted fractions hardly induced any division. Compared with the IL-15-stimulated cells, these NK cells only had a low killing capacity (data not shown) and modest expression of NKp44. CD27 expression remained undetectable on the vast majority of the CD27^- fraction (Fig. 8B, thin line).

The down-regulation of CD27 expression on IL-15-stimulated NK cells might be the consequence of the interaction with its ligand, CD70, as has been observed for T cells (32). Interestingly, CD70 was induced on NK cells upon stimulation with IL-15, whereas NK cells stimulated with IL-21 did not express this molecule (Fig. 9A). We next tested the effect of blocking the CD27^-CD70 interaction by stimulating CFSE-labeled PBMC with IL-15 in the presence of CD70 mAb. As shown in Fig. 9B, addition of the blocking Ab did not affect NK cell division but resulted in a substantial proportion of CD27^- NK cells and a substantial lowering of NKP44 expression on the highly divided cells (i.e., CFSElow). Additional immunofluorescence analyses showed that the NK cells that were CD27^- only partially overlapped with the NKP44^low cells (data not shown).

Figure 6. Peripheral blood CD27^- NK cells are highly cytotoxic. A, Lysis of K562 cells, indicated by chromium release, by sorted CD27^- (■) and CD27^+ (□) NK cells at different effector:target (E:T) cell ratios. Data are provided for four donors with SD indicated. B, Lysis of K562 cells, indicated by chromium release, by sorted CD56^dim^-CD27^- (●), CD56^dim^-CD27^+ (○), CD56^bright^CD27^- (▲), and CD56^bright^CD27^+ (▼) NK cells. One representative experiment of two is shown with intraexperimental variation (SD) indicated.

Figure 7. Circulating CD27^- NK cells produce less IFN-γ. Peripheral blood NK cells were sorted based on CD27 and CD56 expression. Subsets were cultured for 72 h with the indicated stimuli, and IFN-γ in the culture supernatant was detected by ELISA. Different from IL-12 in combination with IL-15 or IL-18, PMA/ionomycin is a poor stimulator or IFN-γ production in NK cells (21). Intraexperimental variation (SD) is indicated.

Figure 8. The CD27 phenotype of NK cells can be influenced by cytokines. A, Peripheral blood NK cells were sorted into CD27^- (left panel) and CD27^+ fractions (right panel). B, Expression of CD27, CD56, and NKP44 upon stimulation of sorted CD27^- (left panel) and CD27^+ (right panel) cells for 8 days with IL-15 (thick lines) or IL-21 (thin lines). Data shown are representative for two donors.
The dynamic regulation of CD27 on NK cells is reminiscent of its complex expression patterns on other cell types. Stem and early progenitor cells in the bone marrow express CD27 during discrete stages and can in this way respond to situations of immune activation that are accompanied by induction of CD70 (36). Further, human memory B cells acquire CD27 expression in the germinal center but loose surface expression when differentiating into plasma cells (37). Finally, mature T cells, both in humans and mice, express CD27 but switch off the molecule upon developing into resting effector-type cells (15–17). The data on NK cells now show that also within this lineage, CD27 is highly regulated, being turned off when these cells acquire their highest effector cell potential. It is unresolved whether induction of CD27 expression is an obligatory step that occurs in all NK cells during stage 4 development. In fact, only a portion of tonsillar CD94+ NK cells in tonsil were found to be CD27−, but the interpretation of this finding is complicated by the fact that this tissue also contains many activated NK cells (as judged by NKp44 expression), which may already be in the process of loosing CD27. Our in vitro data suggested that the common γ-chain cytokine IL-15 might have a crucial role in the down-modulation of CD27 expression. IL-15 is a central cytokine for NK cell function, regulating not only expansion but also effector function (38). Stimulation of peripheral blood CD27+ NK cells with IL-15 resulted in CD27− NK cells that were highly cytolytic (data not shown). The down-modulation of CD27 in this culture condition was mediated by an up-regulation of CD70. Interestingly, a recent study implied that another common γ-chain cytokine, IL-7, may participate in up-regulation of CD70 on NK cells in HIV infection (39). It remains to be determined whether in vivo the down-modulation of CD27 is always dependent on the interaction with CD70. The generation and analysis of CD70-deficient animals will likely solve this issue.

CD27-deficient mice have a reduced capacity to form effector and memory T cells (40). Conversely, overexpression of CD70 in vivo strongly augments effector T cell function and the production of Th1 cytokines (41). In line with this, costimulation of human T cells via CD27-CD70 interactions increases the size of the expanding population and the production of IFN-γ (42). Concerning B cell function, CD27 ligation augments the production of immunoglobulins (43). The impact of CD27 on NK cells function is yet unsettled. First, CD27-deficient mice have normal numbers of NK cells that apparently have adequate functional properties (V. De Colvenaer and G. Leclercq, unpublished observation). Second, the amount of NK cells is not enhanced in CD70-transgenic mice that do have a strong increase in both CD4+ and CD8+ effector T cells (R. Arens and R. A. W. Van Lier, unpublished observation). Hayakawa and Smyth (19) recently described two mature NK cell subsets in the mouse, based on the expression of CD27. Evidence indicated that NK cells leave the bone marrow as CD27bright NK cells and become CD27low later during differentiation. Like in humans, CD27bright cells were found to be most abundant in lymphoid organs, while CD27low cells, expressing increased levels of NK cell receptors, predominated in the circulation. In the lung, which contains particularly high levels of IL-15 due to effective recycling (44), NK cells were almost exclusively CD27low. Notably, in contrast with the situation in humans, highest effector function was observed within the CD27high subset. We analyzed the effect of either stimulating, i.e., via CD70 transfectants, or blocking, i.e., via blocking CD70 Abs, CD27 function on human NK cells in vitro. We could not influence cell division (Fig. 9 and data not shown) or cytolytic activity (data not shown) by targeting the CD27-CD70 system. We did however find that CD70 transfectants affected the phenotype of NK cells (Fig. 9) and modestly enhanced IFN-γ secretion by IL-2-activated CD27+ but not CD27− NK cells.

**Discussion**

Human NK cell subpopulations defined on the level of expression of CD56 and/or CD16 are functionally heterogeneous (11, 33). This study shows that NK cell subsets, defined by the absence or presence of CD27, partially overlap with subpopulations classified on the basis of CD56 expression: the majority of CD27− NK cells was CD56dim, whereas a large proportion of CD27+ NK cells was CD56bright. Furthermore, the different expression levels of activating and inhibitory NK receptors and IFN-γ, as well as the difference in expression of cytolytic proteins and consequently in cytolytic capacity, corroborated that CD27− and CD27+ NK cells are separate entities.

Only a minority of circulating NK cells expressed CD27 whereas significantly higher frequencies of CD27+ NK cells, most of them being CD56bright, were found in spleen and in tonsils. In tonsils, CD27 was only expressed on the CD94− cells within the CD3−CD19− cell pool. It has been proposed that NK cells mature in SLT through four distinct stages that are characterized by the loss of CD34, the acquisition of CD94, and the gradual up-regulation of CD56 (14). CD27 was found to be expressed only on stage 4 CD34+CD94− NK cells but not on CD34−/CD94+ stage 1–3 cells. The CD27+ cells were CD56bright, which corresponded well to the presumed final developmental stage in this compartment. CD56dim cells in the circulation represent the terminal, fifth stage in the development of NK cells (34, 35). The fact that CD27 is absent on the vast majority of circulating stage 5 CD56dim cells implicates that NK cells are down-modulated during the last phase of NK cell maturation.

**FIGURE 9.** Blocking of CD27-CD70 interaction results in maintenance of CD27+ NK cells. A, Expression of CD70 on peripheral blood NK cells stimulated for 8 days with IL-15 (solid line) or IL-21 (dotted line) was measured by gating on the CD3−CD56dim fraction. B, Expression of CD27 (upper panels) and NKp44 (central panels) on divided NK cells and CD27 plotted against NKp44 (lower panels) on NK cells stimulated for 7 days with IL-15 in absence (left panels) or presence (right panels) of a CD70-blocking Ab. Numbers indicate the percentage of NK cells in the corresponding quadrants. Data shown are representative for five donors.
cells (not shown). The latter finding might suggest that CD27-CD70 interactions in SLT facilitate the secretion of immunomodulatory cytokines.

In conclusion, we here demonstrate that the expression of CD27 provides a better definition of subsets within the human NK cell pool. CD27 + and CD27 - NK cells differ in compartmentalization, in the profile of stimulating and inhibiting NK cell receptors, and in their cytolytic capacity. Like for T cells, the down-regulation of CD27 on mature NK cells marks the differentiation toward a vigilant effector cell phenotype.

Acknowledgments

We thank Prof. Jean Plum for helpful discussions, Berend Hooibrink and Mark Denkes for sorting NK cell subsets, Ferenc Scheeren for preparation of tonsil cells, Annette Grummels for help with cell staining, Ester Rammerswaal for help with cytotoxicity assays, and ZymoGenetics for providing rHL-21.

Disclosures

The authors have no financial conflict of interest.

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