The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-Like Receptors and Become Cytolytic

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The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-Like Receptors and Become Cytolytic

Guido Ferlazzo, Dolca Thomas, Shao-Lee Lin, Kiera Goodman, Barbara Morandi, William A. Muller, Alessandro Moretta, and Christian Münz

Natural killer cells are important cytolytic cells in innate immunity. We have characterized human NK cells of spleen, lymph nodes, and tonsils. More than 95% of peripheral blood and 85% of spleen NK cells are CD56dimCD16+ and express perforin, the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, as well as in part killer cell Ig-like receptors (KIRs). In contrast, NK cells in lymph nodes have mainly a CD56brightCD16− phenotype and lack perforin. In addition, they lack KIRs and all NCR expression, except low levels of NKp46. The NK cells of tonsils also lack perforin, KIRs, NKp30, and CD16, but partially express NKp44 and NKp46. Upon IL-2 stimulation, however, lymph node and tonsilar NK cells up-regulate NCRs, express perforin, and acquire cytolytic activity for NK-sensitive target cells. In addition, they express CD16 and KIRs upon IL-2 activation, and therefore display a phenotype similar to peripheral blood NK cells. We hypothesize that IL-2 can mobilize the NK cells of secondary lymphoid tissues to mediate natural killing during immune responses. Because lymph nodes harbor 40% and peripheral blood only 2% of all lymphocytes in humans, this newly characterized perforin− NK cell compartment in lymph nodes and related tissues probably outnumber perforin+ NK cells. These results also suggest secondary lymphoid organs as a possible site of NK cell differentiation and self-tolerance acquisition. The Journal of Immunology, 2004, 172: 1455–1462.

Materials and Methods
Secondary lymphoid organ isolation and culture

Fifteen lymph nodes (from 10 donors), 8 palatine tonsils (from 5 donors), and 3 spleens were studied. The regional Organ Procurement Organization procured 5 human mesenteric lymph nodes and the autologous spleens from 3 individual brain-dead donors after obtaining informed consent from appropriate individuals. Ten additional uninfamed, normal lymph nodes were obtained from 7 patients who underwent surgical resections of cancer masses. Six tonsils from 4 different individuals were collected immediately after surgery from patients undergoing tonsillectomy for chronic inflammation. All tissues were obtained as part of Institutional Review Board-approved protocols. Soon after their removal, tissues were dissociated to obtain single cell suspensions. Mechanically dissociated tissues were then

1 This work was funded by a Special Fellowship from the Leukemia and Lymphoma Society and by a grant from the Speaker’s Fund for Public Health Research, awarded by the City of New York (to C.M.); by a fellowship from the American Society of Transplantation, Juvenile Diabetes Foundation (to D.T.); and by grants from Associazione Italiana per la Ricerca sul Cancro and from Ministero della Salute, Italy (to G.F.).
2 Address correspondence and reprint requests to Drs. Guido Ferlazzo or Christian Münz, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021; or munzc@mail.rockefeller.edu
3 Abbreviations used in this paper: KIR, killer cell Ig-like receptor; NCR, natural cytotoxicity receptor; NTBA, NK-T-B Ag; ULBP, UL16-binding protein.
filtered through a 100-μm nylon cell strainer to exclude undissociated fragments (BD Labware, Mountain View, CA). Debris and dead cells were eliminated using a Ficoll-Hypaque discontinuous gradient. Single cell suspensions were then extensively washed and analyzed by flow cytometry or cryopreserved. Cells were also cultured for the indicated time periods in RPMI medium in the presence of 10% FCS and different doses of rIL-2 (ranging from 500 to 5 IU/ml).

To evaluate IFN-γ production by NK cells, tonsillar mononuclear cells were cultured for 24 h in the presence of IL-12 (10 ng/ml), IL-2 (5 IU/ml), or both stimuli. Monensin (2 μM) was added to the cultures for the last 6 h of incubation.

mAbs, flow cytometry, and NK cell phenotypic analysis

Analysis of cell surface markers was performed using the following mAbs in direct immunofluorescence assays: allophycocyanin- or FITC-conjugated anti-CD3, allophycocyanin-conjugated CD62L, PE- or CyChrome-conjugated anti-CD56, FITC-conjugated anti-CD16, anti-CD69, anti-HLA-DR, anti-CD8, and anti-CCR7, all from BD PharMingen (San Diego, CA), PE-conjugated anti-NKp30, NKp46, and NKp44 were from Immunotech-Coulter (Marseille, France), CD202 mAb (anti-CD48), XA141 (anti-CD158a/p58,1 and anti-CD158b/p50,1), Y249 (anti-CD158b/p58.2 and anti-CD158b/p50.2), AZZ158 (anti-CD158e1/e2/p70 and anti-CD158k/p140), Z176 (anti-p75-ILR1M), Z199 (anti-CD159a/NKG2A), ECM217/1 (anti-NKG2D), CO54 (anti-2B4), and ON56 (anti-NK-T-B Ag) were produced in A.M.’s laboratory or were kind gifts of L. Moretta (Istituto Giannina Gaslini, Genova, Italy). Direct immunofluorescence staining was performed by diluting fluorochrome-labeled mAb with 1 mg/ml human γ-globulin (human therapy grade from Biotest srl, Milan, Italy), to block nonspecific FCr binding. Cells were then washed, and the flow cytometric analysis was performed. For indirect immunofluorescence staining, nonspecific binding sites were saturated with human γ-globulin, and then the relevant mAb was added and incubated for 30 min at 4°C. After extensive washing, FITC- or PE-conjugated isotype-specific goat anti-mouse Abs (Southern Biotechnology Associates, Birmingham, AL) were added and incubated for 30 min at 4°C. Negative controls included directly labeled or unlabeled isotype-matched irrelevant mAbs. Cells were then extensively washed and analyzed by flow cytometry. Perforin and IFN-γ expression analysis on freshly isolated or activated NK cells was performed, respectively, by FITC-conjugated perforin Ab reagent set and allophycocyanin-conjugated anti-IFN-γ (BD Biosciences-PharMingen) after cells were fixed in 1% paraformaldehyde and permeabilized.

NK cell cytotoxicity assay

To evaluate the cytolytic activity after IL-2 stimulation, we used the NK cell-sensitive lymphoblastoid cell line 721.221, which does not express surface HLA class I molecules (10), as well as the T-lymphoblastoid cell line CCRF-CEM (referred in the text as CEM). The latter was used as target because of its expression of UL16-binding protein (ULBP) 1 and ULBP2 (11), two ligands of NKG2D, the only activating receptor expressed by freshly isolated lymph node or tonsil NK cells. Cytotoxicity assays were performed, as previously described (12). Briefly, 10^5 target cells were incubated with the dye molecule PKH26 (Sigma-Aldrich, St. Louis, MO), which inserts into plasma membranes due to its lipophilic aliphatic residue. Target cells were then incubated with secondary lymphoid organ cells at different NK cell/target ratios. After 4 h, cells were harvested; TO-PRO-3, a membrane-impermeable DNA stain, was added to each culture (1 μM final concentration); and cells were finally analyzed by flow cytometry. PKH26 is excited at 551 nm, and TO-PRO-3, using the He-Ne laser, at 633 nm. Thus, target cells are easily gated by PKH26 red fluorescence, while undamaged target cells can be distinguished from lysed cells by their ability to exclude the DNA stain after the coinclusion with effector cells. Background and maximum TO-PRO-3 staining were obtained by incubation with medium and detergent, respectively.

Proliferation assay by CFSE dilution

Mononuclear cells of tonsils and lymph nodes were labeled with 5 μM CFSE in PBS + 0.1% BSA for 10 min at 37°C. After washing twice with PBS + 0.1% BSA, cells were incubated with 100 IU/ml rIL-2 (Chiron, Emeryville, CA) for 7 days at 37°C. CFSE fluorescence (FL-1) was evaluated on CD3+CD56+ cells by four-color flow cytometry.

Results

Subsets of NK cells in human secondary lymphoid organs

Mononuclear cells isolated from lymph nodes, spleens, and tonsils were analyzed for CD3+CD56+ NK cells (Fig. 1). NK cells represented 5% of the total mononuclear cells isolated from uninfamed lymph nodes (median value in 15 lymph nodes, range 1–7%). In inflamed tonsils, the median value of CD56+CD3− cells was 2% of the total cells (range 0.2–0.7% in six tonsils). Uninfamed spleens showed a variable percentage of NK cells (7, 17, and 50% in three donors), generally higher than in peripheral blood. Interestingly, the CD56+ bright subset of NK cells was dominant in tonsils and lymph nodes (75% median value; Fig. 1), whereas in peripheral blood and spleen the majority of NK cells were CD56(dim) (95 and 85%, respectively, median value; Fig. 1). Spleen consists primarily of red pulp, which in turn is rich in blood vessels, so that splenic NK cells may primarily represent blood cells with a minor contribution of the white pulp lymphoid tissue areas. Our data on the dominance of CD56bright NK cells in lymph nodes and tonsils parallel a prior report on human inflamed lymph nodes (7).

Because NK cells present in secondary lymphoid organs appeared different from peripheral blood, we performed a more complete phenotypic characterization. Representative experiments are shown in Fig. 2A and summarized in Table I. We found that NK cells in lymph nodes and tonsils were consistently negative for the FcγRIII (CD16). CD16 triggers NK recognition of Ab-opsonized target cells during Ab-dependent cellular cytolysis (13, 14). Noteworthy, while the CD56(dim) subsets in the spleen and in the peripheral blood express comparable levels of CD16, both CD56(bright) and CD56(dim) NK cells in lymph nodes and tonsils were negative for CD16. A similar pattern of expression was detectable using mAbs directed against the CD8 molecule, i.e., most CD56+cells in tonsils and lymph nodes were CD8−. NK cell activation markers, such as HLA-DR and CD69, were slightly increased in the six tonsils analyzed, but not in the lymph nodes. This confirms that our tonsils represent inflamed secondary lymphoid tissue, while our lymph nodes were uninfamed. Finally, the pan-leukocyte Ag CD48 was expressed at similar levels on all the NK cell subsets analyzed. Therefore, the NK cells of lymph nodes are primarily not activated with high CD56, but low CD16, HLA-DR, and CD69 levels.

Because CD56bright NK cells in peripheral blood represent the only NK cell subset expressing CCR7 and CD62L, we analyzed whether lymph node or tonsil CD56bright NK cells express the same receptors for homing to lymphoid tissues. As shown in Fig. 2B, we confirmed the expression of CCR7 on peripheral blood CD56bright NK cells. In contrast, surface expression of either CCR7 (Fig. 2B) or CD62L (data not shown) was not detectable on NK cells isolated from two lymph nodes and three tonsils. Only in one lymph node we detected 10% of NK cells expressing CCR7,
for their expression of inhibitory receptor. The HLA class I-specific killer inhibitory and activating receptors (KIRs; CD158; KIR-DL; KIR-DS) were absent on NK cells of lymph nodes and tonsils, but detectable in peripheral blood and spleen (Fig. 3 and Table I). CD158a/h(p58.1/p50.1) and CD158e1/e2/k(p70/p140) were expressed on subsets of CD56dim cells in blood and spleen, but not lymph nodes and tonsils. In all autologous sets of spleen and lymph nodes, CD158 expression was only found on spleen NK cells, but absent in lymph node NK cells (one example is shown in Fig. 3). The polymorphic CD158 receptors recognize shared allelic determinants of polymorphic HLA class Ia molecules (15). Differences in their expression between individuals are the basis for NK recognition of allogeneic cells in humans. The HLA-E-specific CD94/CD159a(NKG2A) inhibitory receptor (16) was expressed on most of the CD56bright NK cells in lymph nodes and tonsils. In spleen, subsets of CD56bright and CD56dim NK cells expressed CD94/CD159a(NKG2A). All CD56bright NK cells and a part of CD56dim NK cells in blood were CD94/CD159a(NKG2A)$. Its ligand, HLA-E, is a nonclassical HLA class Ib molecule that preferentially binds leader sequences of MHC class I molecules, and its surface expression is therefore regulated by the overall HLA class I level in cells (17). Finally, the inhibitory sialoadhesin family molecule p75/AIRM1 of unknown ligand specificity (18) was homogenously expressed on NK cells of all lymphoid organs (Fig. 3 and Table I). Therefore, p75/AIRM-1 and CD94/NKG2A seem to be the predominant inhibitory receptors on NK cells in lymph nodes, whereas CD158 KIRs are scarce.

When we turned to activating receptors, we found expression of NKG2D and the coreceptors 2B4 and NTBA on NK cells of all analyzed organs (Fig. 4 and Table I). NKG2D binds the stress-induced MHC class I-like MICA/B surface Ags as well as the human CMV ULBPs (19, 20); 2B4 recognizes CD48 (21); and NTBA enhances NK activation after engagement of unknown ligands to date (22). Surprisingly, NK cells of uninflamed lymph nodes showed no expression of NCRs, except for low levels of Nkp46. NCRs mediate tumor and dendritic cell recognition by NK cells (23, 24). In contrast, peripheral blood NK cells and NK cells from spleen demonstrated Nkp30 and Nkp46 expression on all NK cells (Fig. 4). Tonsils, like lymph nodes, lacked expression of Nkp30, but expressed ex vivo Nkp44 and Nkp46 on some NK cells (Fig. 4 and Table I). Nkp44, an activation-induced NCR, was absent on NK cells from spleen, peripheral blood, and lymph nodes. Together with the CD69 and HLA-DR expression (Fig. 2), this argues for activation by inflammation of our tonsilar NK cells. Interestingly, Nkp44 expression in the tonsils was consistently higher than the usually constitutive NCRs Nkp30 and Nkp46. This NK phenotype has not been previously observed, neither in vitro nor ex vivo. The absence of NCRs on NK cells from tonsils and lymph nodes does not reflect hereditary absence of these molecules in the analyzed donors, and is not a result of loss during the preparation of the cell suspension, because in the spleens, autologous to our lymph node samples, NCR expression was conserved (Fig. 4). Furthermore, addition of tonsilar cell suspensions to peripheral blood NK cells did not decrease NCR expression on blood NK cells, arguing against proteases in tonsil and lymph node preparations that destroy NCRs (data not shown). Therefore, NCR expression on lymph node NK cells is primarily restricted to low levels of Nkp46.

In summary, we found four distinct NK subsets in tonsils and lymph nodes. Three of them, CD56bright/CD159a ‘NCR’, CD56bright/CD159a ‘NCR’, and CD56dim/CD16 ‘NCR’ NK cells, have not previously been described, and one, CD56bright/Nkp44+ NK cells, had never been isolated ex vivo. In addition, we detected an overall enrichment of CD56bright/CD16+ NK cells in spleen, lymph nodes, and tonsils, compared with blood. Although in peripheral blood less than

![FIGURE 2. NK cell phenotype in secondary lymphoid organs. A, Cells isolated from different lymphoid organs were analyzed by three-color flow cytometry using specific mAb against the indicated markers. The analysis was performed after gating on CD3-negative cells, thus excluding contaminating activated T cells expressing CD56. Note that the depicted lymph node and spleen cell suspensions are from the same donor. B, CD56bright NK cells isolated from tonsil (three experiments) do not express CCR7. Similar results were obtained in two of three lymph nodes, analyzed for CCR7 expression. In one lymph node, part of the NK cells (10%) expressed CCR7, and this expression was lost upon IL-2 activation of NK cells. T cells isolated from peripheral blood, tonsil, or lymph node consistently showed partial expression of CCR7.](http://www.jimmunol.org/)

but not CD62L. Of note, this CCR7 expression was lost upon IL-2 culture (data not shown).

We next analyzed NK cells of tonsils, lymph nodes, and spleens for their expression of inhibitory receptor. The HLA class I-specific killer inhibitory and activating receptors (KIRs; CD158; KIR-DL; KIR-DS) were absent on NK cells of lymph nodes and tonsils, but detectable in peripheral blood and spleen (Fig. 3 and Table I). CD158a/h(p58.1/p50.1) and CD158e1/e2/k(p70/p140) were expressed on subsets of CD56dim cells in blood and spleen, but not lymph nodes and tonsils. In all autologous sets of spleen and lymph nodes, CD158 expression was only found on spleen NK cells, but absent in lymph node NK cells (one example is shown in Fig. 3). The polymorphic CD158 receptors recognize shared allelic determinants of polymorphic HLA class Ia molecules (15). Differences in their expression between individuals are the basis for NK recognition of allogeneic cells in humans. The HLA-E-specific CD94/CD159a(NKG2A) inhibitory receptor (16) was expressed on most of the CD56bright NK cells in lymph nodes and tonsils. In spleen, subsets of CD56bright and CD56dim NK cells expressed CD94/CD159a(NKG2A). All CD56bright NK cells and a part of CD56dim NK cells in blood were CD94/CD159a(NKG2A)$. Its ligand, HLA-E, is a nonclassical HLA class Ib molecule that preferentially binds leader sequences of MHC class I molecules, and its surface expression is therefore regulated by the overall HLA class I level in cells (17). Finally, the inhibitory sialoadhesin family molecule p75/AIRM1 of unknown ligand specificity (18) was homogenously expressed on NK cells of all lymphoid organs (Fig. 3 and Table I). Therefore, p75/AIRM-1 and CD94/NKG2A seem to be the predominant inhibitory receptors on NK cells in lymph nodes, whereas CD158 KIRs are scarce.

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CHARACTERIZATION OF NK CELLS IN SECONDARY LYMPHOID ORGANS

Table 1. Phenotype of NK cells in lymph nodes, spleens, and palatine tonsils

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Numbers represent median values and ranges (in brackets) of the percentage of CD56dim and CD56bright NK cells expressing the indicated surface molecules. Number of samples: number of tissues analyzed for the indicated molecule.

5% of CD56 CD3- mononuclear cells are CD56bright CD16+, these amount to 15% in spleen and 75% in lymph nodes and tonsils.

Lymph node and tonsil NK cells up-regulate expression of CD16, KIRs, NCRs, and perforin upon culture with IL-2, and concomitantly become cytolytic.

NCRs are surface receptors that play a major role in NK cell triggering during natural cytotoxic response, and CD16 mediates NK recognition of Ab-opsonized targets. The finding of an impaired expression of these activating receptors on both lymph node and tonsilar NK cells suggested a decreased cytotoxic ability of these cells. Indeed, lymph node and tonsilar NK cells lacked perforin, the main effector molecule in rapid natural cytotoxicity (Fig. 5A). Even Nkp44-positive NK cells in tonsils had no detectable perforin expression. This suggests that in these cells the expressed NCR molecules might trigger NK cell functions other than cytotoxicity. To address whether these NK cells can acquire cytotoxic functions after exposure to T cell-derived cytokines, we incubated them with IL-2 for up to 1 wk. After IL-2 activation, both lymph node and tonsilar NK cells significantly up-regulated CD16 and all NCRs (Fig. 6). In addition, IL-2 induced KIR expression (Fig. 6). Therefore, IL-2 activation induced activating as well as inhibitory receptors on NK cells from lymph nodes and tonsils, and changed their phenotype to one reminiscent of the predominant CD16+ NK subset in peripheral blood. It is unlikely that these CD16/CD158+ NK cells represent the IL-2-driven expansion of a minor fraction of CD16+ cells already present ex vivo, because NK cells largely expanded in number and up to 50% of the cells expressed CD16. Indeed, NK cells from tonsils and lymph nodes expanded 3- to 30-fold more than T cells in the same tissue (data not shown). To further rule out the possibility that CD16+ KIR+ NK cells were expanded from a NK subset, which already expressed these markers ex vivo, CD3+ CD56+ CD16+ tonsil cells were sorted by flow cytometry to 99.5% purity and cultured in IL-2 for 1 wk. As shown in Fig. 6B, purified CD16- NK cells from tonsils expressed CD16 and KIRs after IL-2 activation. We obtained CD16+ KIR+ NK subpopulations of similar size to cultures from unseparated tonsil and lymph node cells. Finally, we also assessed the proliferation of tonsil (Fig. 6C) and lymph node (data not shown) NK cells upon stimulation with IL-2. CFSE dilution of CD3+ CD56+ cells was evaluated after 1-wk activation with 100 IU/ml IL-2. Fig. 6C shows that KIR- NK cells proliferated more vigorously and that, interestingly, KIRs are acquired by NK cells in secondary lymphoid organs without many divisions. The subset of KIR- NK cells that underwent massive proliferation represented 35% of total NK cells in the tonsil and 31% in the lymph node analyzed. These results also further ruled out that KIR+ NK cells observed after 1 wk of culture in IL-2 represent the preferential proliferation and expansion of pre-existing KIR+ NK cells in the starting population, and suggest that lymphoid tissues might be a site for NK cell differentiation.

Because IL-2-activated NK cells of tonsils and lymph nodes acquired a phenotype similar to cytotoxic peripheral blood NK cells, we addressed whether they would also become cytolytic upon culture with IL-2. We investigated perforin expression and cytolytic ability before and after IL-2 activation. Perforin expression was initially absent, but detectable as early as 24 h after IL-2 administration. After 7 days of IL-2 stimulation, it was expressed...
in all tonsilar and lymph node NK cells (Fig. 5A). IL-2-activated tonsilar NK cells were also analyzed for their cytolytic capability against the classical HLA class I NK cell target LCL 721.221 as well as against CEM cells, a T lymphoblastoid cell line expressing the NKG2D ligands ULBP1 and ULBP2. NK-mediated lysis of CEM has been previously shown to be highly dependent on NKG2D (11), i.e., the single activating receptor consistently expressed by all NK cells isolated from secondary lymphoid organs. To avoid allorecognition of CEM cells by tonsilar T cells, CD3\(^+\)NK cells were sorted by flow cytometry before the cytotoxicity assays. Fig. 5, B and C, shows that IL-2-activated tonsilar NK cells could exert a strong cytolytic activity, comparable to activated NK cells from blood. Freshly isolated tonsilar NK cells showed no detectable cytolytic function against both targets at a 10:1 NK-target ratio (Fig. 5, B and C). To demonstrate that low doses of IL-2, which can be secreted by activated T cells, are able to induce cytolytic properties and perforin expression of NK cells in secondary lymphoid organs, tonsilar mononuclear cells were cultured with titrated doses of IL-2. Results shown in Fig. 7, A and B, show that as few as 5 IU/ml of IL-2 was sufficient to induce cytotoxic functions of lymphoid tissue NK cells.

In summary, our data indicate that NK cells of secondary lymphoid organs can acquire cytolytic function and activating as well as inhibitory receptors upon activation.

**NK cells isolated from lymphoid tissues produce IFN-\(\gamma\) after activation by IL-2 and IL-12**

Peripheral blood CD56\(^{bright}\)CD16\(^-\) NK cells have been described recently to produce larger amounts of cytokines compared with their CD56\(^{low}\)CD16\(^+\) counterpart (7). Thus, we investigated whether NK cells of secondary lymphoid organs could produce cytokines such as IFN-\(\gamma\), early upon activation. As shown in Fig. 7C, both IL-2 and IL-12 could promptly induce IFN-\(\gamma\) production by tonsil NK cells. As previously described for CD56\(^{bright}\)CD16\(^-\) NK cells, isolated from peripheral blood (7), slightly more NK cells produced IFN-\(\gamma\) upon exposure to both cytokines compared with stimulations with the individual cytokines.

Therefore, while long-term (7-day) activation of NK cells in secondary lymphoid organs converts them to cytolytic effectors, short-term (1-day) activation stimulates the production of immunomodulatory cytokines by lymphoid tissue NK cells.

**Discussion**

In this study we provide a phenotypic and functional characterization of NK cells located in secondary lymphoid organs, and demonstrate that they can acquire cytolytic functions as well as the complete NK cell receptor repertoire only upon activation.

We have analyzed human spleens, lymph nodes, and tonsils in comparison with peripheral blood and described four distinct NK subsets resident in secondary lymphoid organs. Three of them, CD56\(^{bright}\)NKG2A\(^+\)NCR\(^+\), CD56\(^{bright}\)NKG2A\(^-\)NCR\(^-\), and CD56\(^{dim}\)CD16\(^-\)NCR\(^-\)NK cells, have not previously been found, and one, CD56\(^{bright}\)NKp44\(^+\)NK cells, had not been isolated ex vivo. In addition, we detected an overall enrichment of CD56\(^{bright}\)CD16\(^-\)NK cells in spleen, lymph nodes, and tonsils.

We showed that tonsil and lymph node NK cells that acquire a complete KIR phenotype in the presence of IL-2 undergo minimal cell divisions, while a large part, but not all, of KIR\(^-\)NK cells proliferated strongly. This further confirmed that different subsets of NK cells are harbored in these lymphoid tissues and that at least part of these NK cells might be already committed to differentiate into classical, KIR\(^+\), cytolytic, NK cells. Because peripheral blood KIR\(^-\)NK cells do not acquire KIRs and CD16 by culture in IL-2, NK cell localization to secondary lymphoid organs might be a prerequisite for maturation into the classical cytolytic NK phenotype, found predominantly in blood.

When analyzed for effector functions, NK cells of secondary lymphoid organs were able to promptly produce IFN-\(\gamma\), but were unable to kill target cells. The acquisition of cytolytic function by NK cells in secondary lymphoid tissue is accompanied by the de

**FIGURE 3.** Inhibitory receptors on NK cells of secondary lymphoid organs. See legend to Fig. 2A.

**FIGURE 4.** Activating receptors on NK cells of secondary lymphoid organs. See legend to Fig. 2A.
new expression of molecules related to this function, i.e., activating and inhibitory surface receptors. We showed in vitro that low levels of IL-2 render lymphoid tissue NK cells competent for target cell killing. In vivo, this effect could be initiated by IL-2 secretion of T cells (25) because NK cells colocalize with T cells in secondary lymphoid organs (7). This would imply that lymph node and tonsil NK cells may become cytolytic only after T cell priming. However, recent findings in mice suggest that dendritic cells could also secrete IL-2 upon maturation (26) and might be a candidate for NK-activating cells in lymph nodes and tonsils. More studies of DCs in secondary lymphoid organs are needed to assess this possibility. NK cells in secondary lymphoid organs, which become cytolytic upon activation, could target infected or tumor cells that have infiltrated these tissues. In addition, they might mediate an editing function of APCs (27). We and others have previously demonstrated that immature dendritic cells can be lysed by NK cells (24, 28). Therefore, NK cells in lymph nodes and tonsils that become cytolytic after activation could eliminate in-
completely matured dendritic cells that are responsible for peripheral tolerance induction via stimulation of regulatory T cells or induction of T cell deletion (29). Furthermore, infected dendritic cells could also be targeted, especially after CMV-mediated MHC class I down-regulation (30). Both mechanisms, targeting of immature and virus-infected dendritic cells by cytolytic NK cells, would ensure Ag presentation in the immunogenic context of mature dendritic cells.

NK cells in secondary lymphoid organs could also play a major role during in vivo IL-2 treatment aimed at enhancing patient resistance against malignancies. Draining lymph nodes are usually the first metastatic sites of a spreading cancer. The NK cells that we describe in these regions represent, altogether, a large pool of effector cells that can potentially target tumor cells and control their lymphatic dissemination. Thus, it is tempting to speculate that lymph node NK cells can acquire cytolytic function upon exogenous IL-2 administration. This could partially explain the beneficial effects observed in some patients treated with rIL-2 for advanced cancers (31). Furthermore, low-dose IL-2 therapy results in recruitment of CD56brightCD16+ NK cells to peripheral blood, amounting to up to 70% of lymphocytes after 12 wk of treatment (32). These cells are not cycling and, therefore, probably represent NK cells mobilized from extravascular tissues (33). The NK cells of secondary lymphoid tissues, described in our study, could significantly contribute to this increase of peripheral blood NK cells during IL-2 therapy. Consistent with this hypothesis, we find down-regulation of CCR7 on lymph node NK cells of one sample after IL-2 treatment (data not shown). Most NK cells in tonsils and lymph nodes, however, are already CCR7 and CD62L negative after isolation and, therefore, not anchored by these chemokines and adhesion molecules in secondary lymphoid tissues. Both CCR7+ and CCR7− NK cells in lymph nodes could, however, be mobilized to peripheral blood after IL-2 activation. An interesting possibility is that therapy with s.c. injections of DCs may lead to the activation of NK cells through the induction of IL-2-producing T cells or through the production of IL-2 and other cytokines by DCs.

Because lymph nodes harbor 40% and peripheral blood only 2% of all 5 × 10^11 lymphocytes in the human body (8, 9), lymph node NK cells outnumber peripheral blood NK cells by 10:1 (NK in blood, 10% (8); NK in lymph nodes, 5%). Taking into account that NK cells from spleen (15% of total lymphocytes) and blood (2% of total lymphocytes) are similar, these organs together contain ~1 × 10^10 perforin+ (95% in blood and 85% in spleen) and ~2 × 10^9 perforin- (5% in blood and 15% in spleen) NK cells. Lymph nodes alone contain ~1 × 10^10 perforin+ NK cells, and other lymphoid organs such as tonsils also contribute to the perforin+ NK cell pool. Therefore, perforin+ NK cells probably outnumber perforin- classical NK cells in humans. NK activation by, for example, IL-2 is required to induce natural cytotoxicity in these abundant NK cells of secondary lymphoid tissues.

It remains to be clarified whether the presence of such an abundant noncytolytic, but cytokine-secreting, NK cell subset in secondary lymphoid organs might play relevant immunoregulatory roles, for example, for T cell polarization, in the very early phases of the immune response.

In conclusion, we have characterized NK cells in the secondary lymphoid organs, spleen, lymph nodes, and tonsils. Although blood and spleen contain similar NK subsets, we found that tonsils and lymph nodes contain mainly NKG2D+CD159a+CD158− NCR− NK, which are different from the NK cells that predominate in blood. Upon IL-2 secretion by T cells (and possibly by dendritic cells), they up-regulate activating and inhibitory receptors as well as perforin and acquire the ability to kill NK-sensitive targets. Cytolytic NK cells might control infected and tumor cells infiltrating secondary lymphoid organs, as well as edit APCs such as dendritic cells. The abundant NK compartment of lymph nodes and related tissues can be targeted in immune therapies, as shown with IL-2 infusions, to mobilize its cytolytic capacity against tumors.

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**References**


