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A Distinct Subpopulation of Human NK Cells Restricts B Cell Transformation by EBV

Anna Lünemann,†,∗ Tarik Azzi,‡ David Nadal,§ and Christian MüNZ∗

NK cells constitute the first line of defense against pathogens and transformed cells. They mature in secondary lymphoid organs, including tonsils, where common pathogens, such as EBV, enter the host and potentially imprint differentiating cells, which then patrol the body via the blood stream. Therefore, we set out to characterize a distinct human NK cell population in tonsils that produces high amounts of the immunomodulatory and antiviral cytokine IFN-γ. We found that the tonsilar IFN-γhigh NK cell subset is CD56bright NKG2A+ CD94+ CD54+ CD62L−, is present in tonsils ex vivo and is more mature than other CD56bright NK cells in tonsils and less mature than other NK cells in blood, shows very low plasticity even after prolonged cytokine stimulation, accumulates in tonsils of EBV carriers, and is able to potently restrict EBV-induced transformation of B cells. Thus, we characterized a distinct and stable IFN-γhigh NK cell subpopulation that can specifically restrict malignant transformation of EBV-infected B cells. This subset should be exploited for future development of cell-based therapeutic approaches in EBV-associated malignancies. The Journal of Immunology, 2013, 191: 4989–4995.

natural killer cells are innate lymphocytes that were originally described for their ability to target virus-infected and transformed cells without preactivation (1, 2). Although they originally were thought to leave the bone marrow as fully developed effector cells and to reside primarily in the blood stream, reservoirs of NK cells were discovered more recently in other tissues, including secondary lymphoid organs (SLOs) (3, 4). These NK cells in lymph nodes and tonsils (SLOs) require activation by primarily dendritic cells to achieve full functional competence and mediate protection against pathogens (4–6). In tonsils, in contrast to peripheral blood, the most mature NK cells are CD56bright, whereas earlier differentiation stages are CD56dim (7). CD56+ cells in human tonsils also contain RORγt+ ILC3 cells (8, 9); for a more detailed characterization of CD56dim tonsilar cells, these cells would need to be taken into account. In humans, the NK cell reservoir in SLOs consists mainly of CD56bright CD16− NK cells. In peripheral blood, NK cells of this subset carry homing markers to SLOs and constitute only about one tenth of the circulating NK cells (4, 10). These blood CD56bright NK cells have longer telomeres and can develop into functional CD56dim CD16− NK cells (4, 11, 12). Subsequently, they seem to acquire more and more inhibitory receptors, including killer Ig–like receptors (KIRs), during further differentiation (13). However, in contrast to mouse NK cells, which excel in their main effector functions (i.e., cytokine production and cytotoxicity) only after terminal differentiation (14), human NK cells acquire already potent cytokine production at the CD56bright CD16− differentiation stage (10). Intriguingly, NK cells in SLOs produce much more IFN-γ in response to IL-12 than do peripheral blood NK cells, and this reactivity resides nearly exclusively in the CD56bright CD16− NK cells of these organs (15). This effector function of NK cells can be especially useful in secondary lymphoid tissues at mucosal surfaces (e.g., tonsils) for restricting infections, because these are the sites where pathogens are first encountered as a natural entrance point.

One such pathogen, which could be targeted by NK cell responses in tonsils, is EBV. EBV is a common γ-herpesvirus that infects >90% of the human adult population and enters the human body via tonsils after transmission in saliva (16). In the vast majority of infected individuals, EBV persists without symptoms under tight immune control by primarily T cells (17). However, in a subset of EBV carriers, the B and epithelial cell–transforming capacity of the virus causes detrimental EBV-associated malignancies (18). In this respect, the initial immune response to infection seems to be quite decisive, because symptomatic primary EBV infection (infectious mononucleosis) can predispose for EBV-associated malignancies (19, 20) and even autoimmune diseases (21). NK cells are part of this innate immune response to EBV and have been reported, somewhat controversially, to correlate either inversely or directly with viral titers during primary infection (22, 23). A protective function of these NK cell responses during primary infection is

*Viral Immunobiology, Institute of Experimental Immunology, University of Zurich, CH-8057 Zurich, Switzerland; and Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, Children’s Research Center, University Children’s Hospital of Zurich, CH-8032 Zurich, Switzerland

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A.L. developed, planned, and performed the experiments; analyzed the data; wrote the manuscript; and prepared the figures. T.A. provided PBMC samples from age-matched pediatric tonsil donors. D.N. provided tonsil and matched blood samples and commented on the manuscript. C.M. developed, helped plan, and procured funding for the research; and wrote the manuscript.

Address correspondence and reprint requests to Prof. Christian MüNZ, Viral Immunobiology, Institute of Experimental Immunology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. E-mail address: christian.muenz@uzh.ch

The online version of this article contains supplemental material.

Abbreviations used in this article: FSC/SSC, forward/sideward scatter; IR, immunoresponsive; KIR, killer Ig–like receptor; MFI, median fluorescence intensity; rh, recombinant human; SLO, secondary lymphoid organ.

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indicated by primary immunodeficiencies that affect NK cells and were suggested to predispose for EBV-associated malignancies (24, 25). Furthermore, X-linked lymphoproliferative disease–associated mutations, which predispose affected males to fatal lymphoproliferative disease upon primary EBV infection, compromise NK cell recognition of EBV-transformed cell lines (26). These mutations also affect other components of cell-mediated immunity, such as T cells. Nevertheless, NK cells clearly contribute to early restriction of EBV infection and lower the risk for EBV-associated diseases. Palatine tonsilar NK cells, which are located at the site of EBV entry, are ideally suited to mediate this early restriction, and we demonstrated previously that NK cells of these SLOs can limit B cell transformation by EBV with their superior ability to produce IFN-γ (15). In the same study, we also noted that only a subset of tonsilar NK cells produced these high levels of IFN-γ. Therefore, in this study we set out to characterize this IFN-γhigh NK cell subset.

In this article, we report that cells from this IFN-γhigh NK cell subset are CD56highNKG2A+CD94+CD54+CD62L−, are enriched in tonsils of EBV carriers, and specifically restrict EBV transformation of B cells. Moreover, this subpopulation lacks homing markers to SLOs and seems to be primed to transition into the periphery to carry out innate immune control of EBV infection.

Materials and Methods

Ethical approval for human samples

Studies on human samples for the immune control of human γ-herpesvirus infections were reviewed and approved by the cantonal ethical committee of Zürich, Switzerland (KEK-SvN Nr. 19/08). Tonsils and blood samples of children were collected after informed consent of the legal guardians. Lymphocyte concentrates from adult donors were obtained from the Swiss Red Cross Blutspendedienst Zürich (Zürich, Switzerland) after informed consent of the donors.

Reagents

The following anti-human mAbs were used in this study: CD3 Pacific Blue (Invitrogen); CD3 allophycocyanin Cy7, CD56 PE Cy7, IFN-γ allophycocyanin, CD19 PE, CD19 allophycocyanin Cy7, CD94 PE, CD62L allophycocyanin, CD62L PE, CD21 allophycocyanin, CD23 FITC, CD336 PE, CD103 FITC, and isotypes (BD Biosciences); CD117 PE (Miltenyi Biotec); NKG2C allophycocyanin (R&D Systems); CD45RA FITC and CXCR3 allophycocyanin—(Beckman Coulter); NKG2C allophycocyanin (R&D Systems); CD45RA FITC and CXCR3 allophycocyanin—(Beckman Coulter). Live cells were routinely distinguished using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). The following endotoxin-free recombinant human (rh) cytokines were used: rhIL-12 (R&D Systems), rhIL-2 (PeproTech), and rhIFN-γ (Sigma).

NK and B cell purification and cell culture

Tonsilar cell single-cell suspensions were obtained by mechanical dissociation of the tissue and mechanical filtration through a 75-μm nozzle for sample preparation we determined its expression on naive T cells to sample preparation we determined its expression on naive T cells. After Ficoll-Paque Premium gradient centrifugation (GE Healthcare) to remove dead cells and debris, cells were washed extensively and partly cryopreserved or used directly. Leukocytes of blood samples and leukocyte concentrates were purified by Ficoll-Paque Premium gradient centrifugation and washed several times. B cells were isolated through magnetic bead cell separation (MACS; Miltenyi Biotec) by CD19+ selection. NK cells were isolated through negative MACS selection using the NK cell isolation kit (Miltenyi Biotec), or CD19− cells were then added to the cell dishes for 4 h in antibiotic-free medium. Then, RPMI 1640 + 10% FCS medium was replaced with gentamicin-containing RPMI 1640 + 10% FCS medium culture medium. Supernatant was harvested after 3 d and filtered through a 0.45-μm filters and EBV was further purified and concentrated by ultracentrifugation at 30,000 × g for 2 h at 4°C, resuspended in RPMI 1640, and titrated on Raji cells. GFP+ cells were counted 2 d later, and titers were calculated in Raji infectious units.

B cell–transformation assay

This assay was adapted from the previously described protocol (15). Briefly, MACS-isolated B cells (as described above) were plated at 1 x 105 cells and cultured in RPMI 1640 + 5% human serum (Invitrogen) with 2 μg/ml gentamicin and infected with wild-type EBV; sorted NK cells were added at the indicated numbers. IL-12 was added at 10 ng/ml. Cells were harvested after 12 d, and the numbers of transformed B cells were quantified by determining the ratio of live CD19+CD21+CD23+ transformed B cells/total live cells by flow cytometry. Percentage of restriction of B cell transformation = (1 − total transformed B cell number of sample with NK cells/total transformed B cell number of sample without NK cells) × 100.

Flow cytometry and data analysis

Flow cytometric data were acquired on an LSR II Fortessa cell analyzer (equipped with four lasers; Figs. 1, 2, 4, 5) or an Aria III cell sorter (five laser lines; Figs. 3) (both using Diva software; all from BD Biosciences). General gating strategy for NK cells: lymphocyte population in forward/sideward scatter (FSC/SSC), live (Aqua−) cells, CD3+, CD56+. Data were analyzed using FlowJo v.9.3.1.3 and GraphPad Prism for Mac version 5.0a.

Statistical analysis

Data were compared using the two-tailed Wilcoxon signed-rank test, unless noted otherwise, or the two-tailed Mann–Whitney U test, if specified in the figure legends. A p value ≤ 0.05 was considered statistically significant. Error bars represent the SEM in all figures.

Results

The tonsilar immunoresponsive NK cell subset with high IFN-γ production consists of CD56highNKG2A+CD94+CD54+CD62L− cells

Tonsils harbor a distinct subset of very high IFN-γ–producing CD56bright NK cells, which secrete this cytokine especially after IL-12 stimulation (4). This subset is not seen in peripheral blood, might play important bridging functions with the adaptive immune system, and could be an adaptation to target pathogens in SLOs at mucosal entry sites. To characterize this subset in more detail, we first determined its surface marker phenotype. To accomplish this, we purified NK cells by negative magnetic cell separation (MACS; Miltenyi Biotec) and stimulated them with 10 ng/ml IL-12 for 18 h. IFN-γhigh NK cells differ from the total CD56bright NK cells by their expression of NKG2A/CD94 and CD54, as well as the lack of CD22+ (CD62L−) (Fig. 1A–C, Supplemental Fig. 1 [representative flow cytometry dot blots of subpopulations]). IL-12 was chosen for the stimulation of tonsilar NK cells, because it was previously identified as the primary monokine involved in NK cell activation by dendritic cells (15). To exclude the possibility that CD62L undergoes shedding and is lost from the surface of the NK cells due to sample preparation we determined its expression on naïve T cells versus T EMRA cells (effector memory RA+) and found normal levels of CD22+ expression (Supplemental Fig. 3B). Tonsilar IR cells expressed slightly less NKG2C than did other CD56bright NK cells, but the overall expression was very low (data not shown). We confirmed the phenotype of tonsilar NK cells with high IFN-γ production by gating on CD56bright NKG2A+CD94+CD54+CD62L−.
IL-12 induces accumulation of high IFN-γ-producing NK cells in tonsils, which are more mature than the other tonsilar NK cells but less mature than peripheral blood NK cells.

Next, we addressed whether this distinct NK cell subset (CD56bright NKG2A−CD94−CD54−CD62L−) is already present in tonsils without stimulation. To determine this, we analyzed tonsilar NK cells from children ex vivo and found a significant population of this subset (Fig. 2A). This subpopulation accumulated after stimulation of purified NK cells with IL-12 for 18 h (Fig. 2A). Expression of c-kit (CD117) is lost throughout the maturation process of NK cells (7); hence, its expression characterizes less mature cells. We found that fewer immunoresponsive (IR) NK cells express c-kit in tonsils compared with the other CD56bright NK cells in this organ. Conversely, more NK cells with this phenotype displayed this expression pattern is reversed in blood (i.e., blood IR cells carry almost exclusively IFN-γ expression). The CD57 and perforin expression was very low, and there was no significant difference between the analyzed subsets (Supplemental Fig. 3C, 3D). As shown in Fig. 2E and 2F, we did not detect any difference in CXCR3 expression between IR and the rest of the CD56bright subset, nor in NKG2A median fluorescence intensity (MFI) on cells pregated on NKG2A+ cells, which might have further supported the advanced maturation status of these cells. In conclusion, we demonstrated that the distinct IR NK cell subset is already present in tonsils and blood ex vivo, accumulates upon IL-12 stimulation, and is more mature than the total tonsilar CD56bright NK cells but less mature than blood NK cells.

**The IR NK cell subset shows very low plasticity even after prolonged cytokine stimulation**

Plasticity of immunological receptors determines whether molecules are reliable and characteristic marker molecules, defining the same cell subset under different activation conditions. High plasticity will hinder traceability of subsets through different conditions. Therefore, we assessed whether expression of the defined potential marker molecules was stable, even during prolonged cytokine stimulation, to determine whether this subset is stable throughout immunological processes and activation. The subset was sorted into the IR subset, the rest of the CD56bright and the CD56dim NK cells (subset gating strategy in Fig. 3A) and stimulated for 7 d with IL-12 (10 ng/ml), IL-2 (100 U/ml), or IL-15 (100 pg/ml). Cells were collected, and expressions of marker molecules were analyzed. CD56 expression was highly variable after cytokine stimulation with IL-12 in our experimental setting (Supplemental Fig. 4A) and with IL-2 and IL-15 (Supplemental Fig. 4B, 4C). The IR subset became largely CD56dim after prolonged cytokine stimulation, which suggested a maturation status similar to blood CD56bright NK cells that were reported to turn into CD56dim upon stimulation (11). The other CD56bright cells conserved their high CD56 expression. CD56dim NK cells, which are the less mature NK cell population in tonsils, turned into CD56bright cells upon prolonged IL-2 stimulation. Thus, CD56 expression was disregarded for reanalysis of the potential marker molecules NKG2A and absence of CD62L. Expression of NKG2A was highly stable (Fig. 3B), and L-selectin negativity was preserved (Fig. 3C), even after prolonged cytokine stimulation.
stimulation with different cytokines in the IR subset. To identify the origin of the IR cells that accumulated after cytokine stimulation, expression of NKG2A (Supplemental Fig. 4C) and the absence of L-selectin (Supplemental Fig. 4D) and of the combined phenotype (Supplemental Fig. 4E) were analyzed on the rest of the initially CD56bright and CD56 dim subset after prolonged cytokine stimulation. There was significant acquisition of NKG2A, loss of CD62L, and acquisition of the combined phenotype in CD56dim and the other CD56bright NK cells as the result of IL-2 and IL-12 stimulation. After prolonged cytokine stimulation of purified tonsilar NK cells, the IR subset retained the ability for elevated IFN-γ production (Fig. 3D). In conclusion, the defined potential marker molecules could be confirmed for the IR subset because they are preserved and stable throughout immunological activation modeled by prolonged cytokine stimulation; therefore, they define the subset reliably and characteristically under different activation conditions and can be acquired by non-IR tonsilar NK cells.

IR NK cells accumulate in tonsils of EBV carriers and are able to restrict EBV-induced transformation of B cells

To investigate whether the defined IR NK cell subsets could provide a barrier function against pathogens that infect via the tonsils, we investigated their enrichment in EBV-seropositive individuals. Tonsils of children carrying EBV harbored significantly more of the IR subset of NK cells than did age-matched children not infected with EBV (Fig. 4A). The IR subset exists in peripheral blood ex vivo, even though this subset is markedly enriched in tonsils, and there are significantly fewer cells of this subset in the peripheral blood of EBV-infected children than in age-matched noninfected children. Adults have even fewer of the IR subset than do children (Fig. 4B). The IR cells in the peripheral blood have a higher capacity for IFN-γ production than do the other CD56bright NK cells (Supplemental Fig. 4G). The elevated frequency of IFN-γ–producing IR NK cells, as gated in Fig. 1A, in EBV+ tonsils was increased further after IL-12
stimulation (i.e., 1.6-fold in EBV− donors and 1.25-fold in EBV+ donors) (Fig. 4C). To characterize whether this accumulation of IR NK cells could provide protection in tonsils of EBV-seropositive individuals, we tested this NK cell subset’s ability to restrict EBV infection. EBV restriction by NK cells was measured by the B cell–regression assay, as described previously (15). Briefly, 100,000 MACS-sorted EBV-exposed autologous B cells were cocultured with different numbers of flow-sorted NK cell subpopulations and IL-12 (10 ng/ml) for 10 d. EBV-transformed B cells were identified by the gating strategy shown in Fig. 5A. IR NK cells were 4.5-fold more efficient in preventing the outgrowth of EBV-infected B cells in vitro than were other tonsilar CD56bright NK cells (Fig. 5B). These data provide direct evidence that the distinct IR NK cell subset has the ability to markedly restrict EBV-induced B cell transformation and is the optimum NK cell population in tonsils to perform this function.

Discussion

NK cells provide the first line of defense and play a crucial role against invading pathogens and emerging cancer cells (28–32). Our data demonstrate that these functions are mediated in part by these innate lymphocytes in SLOs and that the respective tonsilar IFN-γhigh NK cell subset is CD56brightNK2A+CD94+CD54+CD62L−, is present in tonsils ex vivo and is more mature than other CD56bright NK cells in tonsils and less mature than other NK cells in peripheral blood, shows very low plasticity even after prolonged cytokine stimulation, accumulates in tonsils of EBV carriers, and is able to potently restrict EBV-induced B cell transformation and is the optimum NK cell population in tonsils to perform this function.

Our study indicates that EBV induces the accumulation of CD56brightNK2A+CD94+CD54+CD62L− NK cells with the capacity to produce high amounts of IFN-γ in human tonsils. These NK cells limit primary EBV infection of B cells more efficiently than do other tonsilar NK cell subsets and, therefore, could provide a crucial first innate barrier to infection in this portal organ of EBV entry. The reduced levels of IR cells in the blood of EBV−...
donors might be the result of recruiting or retaining this subset in tonsils, a further differentiation of this subset in the blood, or a combination of these factors. Enrichments of distinct NK cell populations were described for other viral infections in humans, but superior restriction of the respective pathogens by these subsets is rare. Moreover, a tissue-specific accumulation, as we describe for human tonsils, has not been reported previously.

Several studies showed persistent alterations in the NK cell repertoire after viral infections (13, 33, 34) and even protective effects of murine NK cell subsets (35, 36). Accumulation of terminally differentiated NK cell subpopulations were reported in hantavirus, chikungunya virus, human CMV, and HIV infections (13, 33, 37–39). These studies suggested that the respective viruses drive the accumulation of CD56dimCD16+KIR+CD57+ and often NKG2C+ NK cells, which could mediate recognition of infected target cells or, alternatively, just differentiate in response to the inflammatory cytokines that are secreted during viral infections. Respectively, these expansions were proposed to reflect NK cells with memory-like features that prepare the NK cell compartment for more rapid secondary responses to recurrent or persistent viral infections (35, 39, 40) or accumulating effector cells that are activated by memory CD4+ T cells via IL-2 secretion (41, 42). Very recently, cytokine-induced human memory-like NK cells were described (43). Both explanations for NK cell subset accumulation suggest that these innate lymphocyte populations should be explored for immunotherapy against the respective viral infections (e.g., by choosing adjuvants that would elicit cytokine production to promote in vivo differentiation of these NK cell populations or by adoptively transferring the respective NK cell subsets during symptomatic infection with the respective viruses and during dysregulation of persistent infections resulting in detrimental malignancies).

Such NK cell–based therapies have been explored successfully in transplantation settings against hematological malignancies. Several clinical centers have observed beneficial effects of haploidentical bone marrow transplantation for residual leukemia clearance after chemotherapy, harnessing NK cell–mediated graft-versus-leukemia effects (44–47). This therapy seems to be most effective when bone marrow donor and recipient are mismatched in HLA class I molecules, and the donor carries significant NK cell populations that can detect this mismatch via their KIR repertoire (46, 48) and then target leukemic cells by missing-self recognition (49). In these instances, transient reconstitution of NK cells with a donor phenotype before NK cell tolerance against the recipient is implemented mediates the graft-versus-leukemia effect. Alternatively, alloreactive NK cell populations that are expanded in vitro can also be infused (50–52). Thus, these studies provide good evidence that NK cell therapies that transiently generate an alloreactive compartment of these innate lymphocytes are well tolerated and could be readily expanded to harness specialized antiviral NK cell populations. The recent description of cytokine-induced human memory-like NK cells suggests that cytokine stimulation for expansion of NK cells before transfusion could be beneficial in these therapies (43). For the subset of potent IFN-γ–producing NK cells that can restrict EBV-induced B cell transformation, described in this article, we demonstrated an accumulation after IL-12 stimulation. Non-IR NK cells can change their phenotype accordingly after prolonged cytokine stimulation. This could be readily harnessed to enhance future NK cell–based therapeutic options by cytokine prestimulation.

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Disclosures

The authors have no financial conflicts of interest.

FIGURE 5. IR tonsilar NK cells potently restrict EBV-induced B cell transformation. Gating strategy for B cell analysis is shown in (A). (B) A total of 100,000 autologous B cells was infected with EBV and cocultured with the indicated numbers of sorted NK cells and IL-12 (10 ng/ml) for 10 d. The distinct IR subset has the ability to significantly restrict EBV-induced B cell transformation in physiological numbers (500 NK versus 100,000 B cells [i.e., 0.5% or a 5-fold increase]; 2,500 NK versus 100,000 B cells [i.e., 2.5%]). Gating strategy for sorted IR subset: lymphocyte population in FSC/SSC, live cells (Live/Dead cell stain Aqua™), NK cells (CD3–CD56+); subset gating: CD56brightNKG2A+CD94+CD54+CD62L−, as depicted in Supplemental Fig. 3A. Data from three independent experiments, with a total of four or five donors, are summarized. All error bars represent SEM. *p ≤ 0.05 two-tailed nonparametric Mann–Whitney U test.
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