Cutting Edge: Salivary Gland NK Cells Develop Independently of Nfil3 in Steady-State

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References

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Nfil3 is viewed as an obligate transcription factor for NK cell development. However, mouse CMV (MCMV) infection recently was shown to bypass the requirement for Nfil3 by inducing the appearance of NK cells that express the MCMV-specific receptor Ly49H. Thus, signals transmitted by Ly49H and proinflammatory cytokines are sufficient to promote NK cell differentiation in the absence of Nfil3. In this study, we report that salivary gland (SG) NK cells develop in an Nfil3-independent fashion in the steady-state in the absence of MCMV or any infection. Moreover, we show that SG NK cells have an integrin profile reminiscent of tissue-resident lymphocytes and express TRAIL for killing target cells. These results demonstrate that SG NK cells, although related to conventional NK cells, are a distinct subset of innate lymphoid cells that deviates from the conventional developmental pathway, perhaps under the influence of tissue-specific factors. The Journal of Immunology, 2014, 192: 4487–4491.

Natur al killer cells develop from hematopoietic stem cells through sequential stages that include committed NK cell precursors, immature NK (iNK) cells, and mature peripheral NK cells (1, 2). This process is orchestrated by key cytokines and master transcription factors. The differentiation of iNK cells from NK cell precursors requires trans-presentation of IL-15 by membrane-bound IL15ra, as well as a basic leucine zipper transcriptional activator, known as Nfil3 or E4bp4 (3–6). Nfil3 also plays a role in development and/or function of macrophages, CD8 T cells, dendritic cells, CD4 T cells, and B cells (6, 7). NK cell development requires at least two additional transcription factors: T-bet promotes the stability of iNK cells, whereas Eomesodermin (Eomes) is required for the generation of mature NK cells (8).

Consistent with a crucial role for Nfil3 in NK cell development, Nfil3−/− mice have a marked reduction in NK cells (CD122+DX5‘NK1.1’CD3−) cells in the bone marrow, spleen, liver, and blood (3–6). However, it was shown recently that the requirement for Nfil3 can be bypassed during infection with mouse CMV (MCMV) or recombinant viruses expressing the MCMV glycoprotein m157. Infection of Nfil3−/− mice resulted in the appearance of NK cells that express the MCMV-specific receptor Ly49H (9), suggesting that combined signals transmitted by Ly49H and proinflammatory cytokines, such as IFN-α/β and/or IL-12, are sufficient to promote the proliferation and survival of virus-specific NK cells. Moreover, Nfil3−/− mice retained a subset of VLA1+ NK cells in the liver, even in the absence of MCMV infection (10, 11). The development of these liver NK cells also was Eomes independent but T-bet dependent (12). Because of their unique developmental requirements, these cells were designated a novel subset within the spectrum of innate lymphoid cells that produce IFN-γ (ILC1s), but they are distinct from conventional NK cells.

In this study, we report that salivary gland (SG) NK cells develop in an Nfil3-independent fashion in the absence of any infection. In contrast to Nfil3-independent liver ILC1s, SG NK cells express both T-bet and Eomes and do not produce significant amounts of IFN-γ. Moreover, we show that SG NK cells have an integrin profile reminiscent of intestinal intraepithelial ILC1 (13) and tissue-resident memory CD8 T cells (14, 15) and express TRAIL, which can induce the death of TRAIL-sensitive target cells. We postulate that SG NK cells represent yet another subset in the broad spectrum of ILC1s that are phenotypically and functionally distinct from conventional NK cells, liver ILC1s, and intestinal ILC1s and may diverge from the ILC1-developmental pathway under the influence of tissue-specific factors.

Materials and Methods

Mice

Nfil3−/− (3), Rag1−/− (The Jackson Laboratory), Il2ra−/− (from Takeshi Egawa, Washington University School of Medicine), Il15−/− (Taconic), Il15ra−/−, Il2rg−/− (from Anthony French, Washington University School of Medicine), Il7r−/− (from Ken Murphy, Washington University School of Medicine and Howard Hughes Medical Institute [Chevy Chase, MD]), Il4−/− (from Ed Pearce, Washington University School of Medicine), athymic nude mice (from Wayne Yokoyama, Washington University School of Medicine and Howard Hughes Medical Institute), germ-free mice (from Jeffrey Gordon, Washington University School of Medicine), and Ifnar−/− mice, all on the C57BL/6 background, were bred and maintained in a pathogen-free facility at Washington University. Age- and sex-matched animals were used throughout the experiments.

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Abbreviations used in this article: Eomes, Eomesodermin; ILC, innate lymphoid cell; ILC1, ILC that produces IFN-γ; iNK, immature NK; MCMV, mouse CMV; SG, salivary gland; WT, wild-type.
Cell preparation and flow cytometry

Submandibular and sublingual glands were isolated, minced, and digested for 1 h at 37°C with RPMI 1640, 5% bovine calf serum, HEPES, 5 mM CaCl₂, and 1 mM collagenase (IV or D). Cells were layered on a 70%/40% Percoll gradient and spun for 20 min at 850 g. Cells at the Percoll interface were collected and stained with Abs for Eomes and T-bet (eBioscience). Cells were treated with a Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) and stained with Abs for Ly49D, and Ly49G2 (all from BD Pharmingen); VLA1 (BioLegend); and TRAIL (all from eBioscience); CD43, CD69, CD122, Ly49A, Ly49C/I, CD127, CD27, CD11b, CD11c, KLRL1, Ly49H, CD39, CD103, and TRAIL (all from eBioscience); CD45 (Miltenyi Biotec). For intracellular content of transcription factors, cells were treated with a Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) and stained with Abs for Eomes and T-bet (eBioscience).

Cell stimulation

SG or spleen were processed and stimulated for 4, 6, or 18 h with IL-2 (25% supernatant) or PMA and ionomycin, followed by staining for TRAIL or isotype control within the population of NK cells (CD45+NK1.1+CD3-).

Results

SG NK cells are Nfil3 independent in the steady-state and require trans-presentation of IL-15

SGs contain a large population of NK cells that have been considered immature and hypofunctional because they have a limited capacity to release lytic granules and produce IFN-γ. However, given the functional discrepancies between SG NK cells and conventional NK cells, we hypothesized that the two NK cell populations might actually be developmentally distinct. Conventional NK cells require the transcription factor Nfil3 for development in the steady-state and are markedly reduced in Nfil3-/- mice. In contrast, similar numbers of SG NK cells were present in Nfil3-/- and wild-type (WT) mice (Fig. 1A). As reported (3, 4), Nfil3-/- mice had a pronounced reduction in NK cells in other tissues. However, a small population of NK cells persisted in various tissues, especially the liver and the lung (Fig. 1B). These cells likely correspond to the Nfil3-independent ILC1 recently identified in the liver (10, 11). We conclude that SGs are a major site for Nfil3-independent NK cells. Nfil3-/- mice also had an obvious increase in SG NK T cell numbers and a slight increase in T cells (Supplemental Fig. 1). Very similar skewing of lymphocyte populations was observed in the liver of Nfil3-/- mice, because the Nfil3-/- NK T and T cells were more robust and filled the niche vacated by Nfil3-/- NK cells (10). Nfil3-/- NK T and T cells probably compete more effectively in the SG as well, although there is no substantial niche to be filled in this case.

As demonstrated previously for other innate lymphoid cells (ILCs) (1), SG NK cells were present in Rag1-/- mice but absent in Il2rg-/- mice, indicating a requirement for cytokine(s) that transmit signals through the common cytokine receptor γ(c) (Fig. 1C). Indeed, SG NK cells were critically dependent on IL-15 signaling, because Il15-/- and Il15ra-/- mice completely lacked SG NK cells (Fig. 1C). Mice lacking other cytokines/cytokine receptors requiring γ(c) signaling, such as Il17r-/-, Il2r-/-, Il4-/-, and Il21r-/- mice, had no defects in SG NK cells (Fig. 1C), suggesting that SG NK cells are developmentally more similar to conventional NK cells than are group 2 and group 3 ILCs, which require IL-7 signaling.

SG NK cells were retained in athymic nude mice (Fig. 1D), indicating that these cells are distinct from NK cells of thymic origin. We examined germ-free mice, which develop in the absence of endogenous microorganisms. SG NK cells were present in these mice (Fig. 1D), demonstrating that, in contrast to virally induced NK cells in Nfil3-/- mice (9), SG NK cells do not require infection or inflammation for their development. Consistent with this conclusion, SG NK cells also were equally represented in Ifnar-/- and WT mice, indicating that IFN-α/β signaling is not required for maintenance of SG NK cells in the steady-state (Fig. 1D). Thus, SG NK cells are related to conventional NK cells in that they require IL-15

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom/4488)

...NK cells are Nfil3 independent in the steady-state and require IL-15 signaling. (A) Frequencies and absolute cell numbers of SG NK cells (gated as CD45+CD3+ NK1.1+ NKp46+ cells) in WT and Nfil3-/- mice. CD3+ cells were excluded from the gates. (B) Representative NK cell frequencies (gated as CD45+CD3+ NK1.1+ NKp46+ cells) in different tissues of WT and Nfil3-/- mice. Frequencies of SG NK cells (CD45+NK1.1+CD3+), NK T cells (CD45+NK1.1+CD3+), and T cells (CD45+NK11-CD3+) in Rag1-/-, Il2rg-/-, Il15-/-, Il15ra-/-, Il2ra-/- Tcrα-/-, Il7r-/-, and Il21r-/- mice (C) and nude, germ-free, and Ifnar-/- mice (D).
signaling for differentiation but diverge at a certain point in their developmental pathway because they are Nfil3 independent.

**SG NK cells express Eomes and T-bet**

It was reported recently that, although conventional NK cells depend on Nfil3 and Eomes for development, ILC1s in the liver depend uniquely on T-bet but not on Nfil3 or Eomes (10–12). These results suggested that Nfil3 may promote Eomes expression and/or function and that the Nfil3–Eomes and T-bet pathways may drive the development of NK cells and ILC1s, respectively. Given this, we asked whether Nfil3 is required for Eomes expression in SG NK cells. We found that Eomes is equivalently expressed in Nfil3−/− and WT SG NK cells, whereas Nfil3−/− liver ILC1s and Nfil3−/− splenic NK cells lack Eomes (Fig. 2). Thus, Nfil3 is dispensable for Eomes expression in SG NK cells. We also did not find any direct association between Nfil3 and T-bet expression. Both Nfil3+/− and WT SG NK cells, as well as WT splenic NK cells, expressed equivalent amounts of T-bet (Fig. 2). However, T-bet expression was moderately reduced in Nfil3−/− liver ILC1s and was markedly reduced in Nfil3−/− splenic NK cells. We conclude that Eomes expression is not a unique feature of Nfil3-dependent NK cells. In fact, Eomes is expressed in Nfil3-independent SG NK cells and is likely to play a role in their development and/or function.

**SG NK cells express markers characteristic of tissue-resident lymphocytes**

We looked for distinctive markers that might distinguish SG NK cells from conventional splenic NK cells. SG NK cells expressed pan NK cell markers, such as DX5, which corresponds to the integrin VLA2 (17), NKG2D, and Nkp46 (Fig. 3A). SG NK cells also expressed CD122 (IL2rb/15rb) but not CD127 (IL7r) or CD25 (IL2ra) (Fig. 3A, data not shown). Compared with splenic NK cells, SG NK cells expressed less CD27, CD11b, CD43, and KLRG1 and more CD11c (Fig. 3B), which, as previously reported, are characteristic of immature NK cells (16). SG NK cells had markedly reduced expression of Ly49A and Ly49C/I compared with splenic NK cells. However, Ly49D was only slightly less well represented on SG NK cells, whereas Ly49G2 and Ly49H were similarly expressed (Fig. 3C), suggesting that SG NK cells have some features that are characteristic of mature NK cells.

Importantly, many SG NK cells expressed markers previously found on tissue-resident NK cells, such as the integrins CD103 and VLA1, CD69, and the surface nucleotidase CD39 (Fig. 3D). CD103 and VLA1 bind to E-cadherin, laminin, and collagen and may be crucial for tissue residency (13–15, 18). CD69 is also a marker of tissue retention (13, 15), whereas CD39 catalyzes ATP degradation, generating a tolerogenic environment (19). We conclude that SG NK cells have a distinctive phenotype characterized by markers of immaturity and tissue residency. Phenotypic features of SG NK cells in Nfil3−/− mice and WT mice completely overlapped (Fig. 3), indicating that the distinctive phenotype of SG NK cells is independent of Nfil3.

**SG NK cells possess cytotoxic potential via TRAIL**

Previous studies showed that SG NK cells are ineffective at producing IFN-γ and cytotoxic degranulation (16). Confirming this, SG NK cells produced minimal IFN-γ and expressed very little CD107a, a marker of cytolytic granule release, in response to PMA or ionomycin, IL-2, or IL-12 plus IL-18 (Supplemental Fig. 2). However, SG NK cells expressed variable amounts of the death receptor TRAIL ex vivo (data not shown) and robustly upregulated TRAIL in vitro in response to PMA plus ionomycin, as well as IL-2 (Fig. 4). Splenic NK cells did not express TRAIL under these conditions. TRAIL was found on both WT and Nfil3−/− SG NK cells (Fig. 4B), indicating that TRAIL expression is Nfil3 independent. Together, these data indicate that, although SG NK cells produce negligible IFN-γ and release few lytic granules, they express TRAIL independently of Nfil3 expression for killing target cells.

**Discussion**

Recent studies revealed an astonishing assortment of ILCs, of which NK cells are the prototype. ILCs, unlike T and B cells, lack specific AgRs and respond immediately and robustly to pathogens upon initial encounter. ILCs are classified into...
distinct groups based on the signature cytokines that they produce (20). ILC1s secrete IFN-γ, group 2 ILCs produce IL-5 and IL-13, and group 3 ILCs secrete IL-22 and/or IL-17. ILC1s include conventional NK cells, thymic NK cells (21), intestinal intraepithelial ILC1s (13), liver-resident ILC1s (10, 11, 18), and CD127+ ILC1s present in inflamed mucosae (22). Our study expands the spectrum of ILC1s, identifying SG NK cells as a novel ILC1 subset with several distinctive features.

SG NK cells are independent of Nfil3 for development, phenotype, and function. Conventional NK cells are largely Nfil3 dependent, although Ly49H+ NK cells can bypass the requirement for Nfil3 during MCMV infection as a result of activation, proliferation, and survival signals transmitted by Ly49H and inflammatory cytokines (9). The presence of newborn numbers of SG NK cells in germ-free mice and Ifnar−/− mice demonstrates that the development of these cells is entirely independent of microbial-induced signals. Liver VLA1+ NK cells were recently proposed to be a subset of Nfil3-independent ILC1s (10, 11) and, hence, may be closely related to SG NK cells. However, although liver VLA1+ NK cells do not express Eomes and produce IFN-γ, SG NK cells express Eomes and are poor producers of IFN-γ. Similar to intestinal intraepithelial ILC1s, SG NK cells express a unique integrin pattern, including VLA1 and CD103, which may reflect exposure to TGF-β produced in the tissue (14, 15). However, intestinal ILC1s are largely Nfil3 dependent and produce IFN-γ (13). Finally, SG NK cells are present in athymic nude mice and, hence, are distinct from NK cells of thymic origin. We postulate that various ILC1 subsets most likely derive from a common ILC1 progenitor but acquire unique features along their differentiation pathway in different tissues as they respond to unique microenvironments that trigger expression of disparate combinations of transcription factors. However, it is also possible that SG NK cells diverge from ILC1s because of yet unidentified cell-intrinsic factors or that SG NK cells derive from fetal NK cell progenitors that seed SGs early during development, rather than from a bone marrow–derived progenitor.

Although initial phenotypic and functional characterization indicated that SG NK cells are immature and hypofunctional (16), our results demonstrate that SG NK cells may not be immature because they express Eomes and some Ly49 receptors that are present in mature NK cells. Moreover, SG NK cells express TRAIL and, therefore, can kill TRAIL-sensitive target cells, such as virally infected cells, activated cells, and transformed cells, but spare normal cells. Although TRAIL-mediated cytotoxicity has been regarded as a cytolytic mechanism typical of immature NK cells (8, 23), TRAIL-mediated killing may be a feature of some tissue-resident NK cells, including SG NK cells, as well as liver NK cells. This mode of killing, together with the expression of CD39, which degrades ATP, may help SG NK cells to minimize the release of necrotic products that elicit detrimental inflammation during immune response to pathogens. Moreover, SG NK cells may have an important function in controlling the activated NK T and T cells in the SG, which may otherwise promote autoimmune diseases, such as Sjögren’s syndrome (24). Finally, although WT and Nfil3−/− SG NK cells are phenotypically indistinguishable, the elevated numbers of NK T cells in the SG of Nfil3−/− mice most likely reflect their superior fitness and ability to outcompete Nfil3−/− NK cells, as indicated by recent studies in the liver (10).

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures

Supplemental Figure 1. NK, NKT, and T cell populations in SG and spleen of WT and Nfil3^-/- mice. Representative frequencies and total cell numbers of the indicated immune cell populations (gated on CD45^+ cells).
Supplemental Figure 2. IFN-γ production and CD107a expression in SG and splenic NK cells. Representative frequencies and quantification of (A) intracellular IFN-γ and (B) surface CD107α. SG or spleen cells were analyzed after 6 hr incubation with the indicated stimuli (PMA plus ionomycin -P/I-, IL-12 plus IL-18 or IL-2). Brefeldin A was added 2 hours after the exposure to the stimuli.