Group 1 Innate Lymphoid Cells of the Submandibular Salivary Gland and Lacrimal Gland

By

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Thesis

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1. **Erick, T.K.**, Grigoryan, L., Brossay, L. 2017. Lacrimal Gland NK Cells Are Developmentally and Functionally Similar to Conventional NK Cells. *ImmunoHorizons* 1(2): 2-9.

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PREFACE

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In Chapter 2, Dr. Emma Reilly, Ph.D. performed some of the experiments and analyzed some of the data for Figure 6B.

Courtney Anderson assisted greatly in the generation of PLZF fate mapping chimeras and B6.SJL/*NFIL3*$^{-/-}$ mixed bone marrow chimeras. In Chapter 2, she generated the visuals for Supplementary Figures 1A and 1C.

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CHAPTER 1

INTRODUCTION

INNATE LYMPHOID CELLS

Innate lymphoid cells (ILCs) are a diverse group of innate lymphocytes that provide protection against a variety of pathogens (1, 2), and help to maintain tissue homeostasis (3, 4). ILCs are defined as lymphocytes that lack lineage markers associated with T cells and B cells, and do not express somatically rearranged antigen receptors (5). ILCs are classified into three sub-groups – ILC1s, ILC2s, and ILC3s – based on their developmental requirements and the cytokines they produce at maturity (**Figure 1**) (6, 7). ILCs are also divided into cytotoxic and non-cytotoxic or "helper-like" ILCs (8). Cytotoxic ILCs consist solely of conventional natural killer (cNK) cells. cNK cells are the prototypical ILCs, and they are included in the ILC1 group. However, they are developmentally and functionally distinct from other ILC1s (9, 10). Helper-like ILCs comprise most of the other subsets of ILC1s, ILC2s, and ILC3s (4). Helper-like ILCs generally express IL-7Rα (CD127) and require the transcription factor GATA3 for development. cNK cells, on the other hand, are $CD127^-$ and do not require GATA3 for early development (11), though it is involved in later maturation (12).

All ILCs develop from the common lymphoid progenitor (CLP), which also gives rise to T cell and B cells (13). The developmental pathways that generate the various ILC subsets are mediated by the coordinated actions of several different transcription factors and cytokines (**Figure 2**) (14, 15). The differentiation of all ILCs from the CLP requires the transcription factors nuclear factor, IL-3 regulated (NFIL3) (16-18), inhibitor of DNA binding 2 (Id2) (19, 20), and thymocyte selection-associated high mobility group box (Tox) (21, 22). Conventional NK cell precursors diverge early from the CLP, and further develop into mature cNK cells under the influence of the transcription factors

Eomesodermin (eomes) (23) and T-bet (24), as well as the cytokine IL-15 (25-27). The CLP also develops into the common helper-like innate lymphoid cell precursor (CHILP), which generates all known ILC subsets other than cNK cells (8). The development of the CHILP requires NFIL3, Id2, and Tox, as well as T cell factor 1 (TCF-1) (28-30), GATA3 (11, 31), and IL-7 (32, 33). After the CHILP, ILC progenitors further diverge into PLZF-independent $\alpha4\beta7^+$ precursors, which give rise to LTi and LTi-like cells (34), and the PLZF-dependent ILC precursor (ILCP), which develops into the remaining ILC1s, ILC2s, and ILC3s (35). ILCs display a diversity of phenotype and functions that is similar to T cells. Indeed, cNK cells are similar in function to cytotoxic $CD8^+$ T cells (36), whereas helper-like ILC1s, ILC2s, and ILC3s are the innate analogs of $CD4^+$ T_H1, T_H2, and T_H17 cells, respectively (37-39).

Much of the current knowledge about ILC development, phenotype, and function has been derived from studies performed in mice. Mice are amenable to a variety of experimental approaches, including genetic manipulation, fate mapping, adoptive transfer, and infection models (40). Studying ILCs in humans is more challenging, though recent studies have identified similarities and differences between mouse and human ILC populations (41-48). This dissertation will focus mainly on mouse ILCs, and any information regarding human ILCs will be noted as such.

Group 1 ILCs

ILC1s are united by expression of the transcription factor T-bet and production of type 1 cytokines, particularly interferon-γ (IFN-γ), and sometimes tumor necrosis factor-α (TNF-α) (**Figure 2 and 3**) (49). ILC1s include cytotoxic cNK cells, as well as several

subsets of unique tissue-resident NK cells (trNK cells) and other ILC1s. These include thymic NK cells (50), trNK cells in the liver, skin (51), kidneys (52), and uterus (53), and submandibular salivary gland NFIL3-independent NK cells (54, 55). The intestine also contains two distinct subsets of ILC1s: mucosal $CD127^+$ ILC1s (8, 45) and $CD103^+$ intraepithelial (ie)ILC1s (56). Adipose tissue also contains several distinct subsets of ILC1s (46, 57, 58). The development, phenotype, and function of each individual ILC1 subset will be discussed in more detail in subsequent sections.

Group 2 ILCs

ILC2s are generally less diverse in phenotype than ILC1s and ILC3s (59). All ILC2s rely on the transcription factor GATA-3 for development (60). The transcription factors ROR-α, Gfi1, and Bcl11b are also involved in ILC2 development and maintenance (61-64). ILC2s respond to stimulation by IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) and express the effector cytokines IL-4, IL-5, IL-9, IL-13, and amphiregulin (**Figure 2 and 3**) (20, 65, 66). ILC2s mainly serve to combat infections by multicellular helminth parasites, particularly in the intestine (67) and airways (68). During parasite infection, epithelial cells produce IL-25 and IL-33, which cause ILC2s to activate and produce type 2 cytokines, particularly IL-5 and IL-13 (20, 66, 68). In the intestine, ILC2-produced IL-13 acts on goblet cells to stimulate mucus production, and on smooth muscle to increase contractility, both of which help expel multicellular parasites (68, 69). Similarly, IL-5 production by ILC2s recruits eosinophils to the lung in order to facilitate parasite expulsion (70). Moreover, ILC2s have also been shown to interact directly with $CD4^+$ T cells to mediate type 2 immunity (71, 72).

In addition to combating parasitic infections, ILC2s have also been implicated in the progression of allergies and asthma. Allergic asthma, the most common form of asthma, is caused in part by dysfunctional memory T_H2 responses (73). Recent work has shown that ILC2-produced IL-13 induces migration of DCs into the airways, where they prime naïve T cells to differentiate into allergen-specific T_H2 cells (74, 75). ILC2s are also found in the skin (76), where they contribute to the development of allergic skin inflammation in an IL-33-dependent manner (77, 78).

Aside from inflammation, group 2 ILCs are involved in promoting tissue repair and homeostasis. For example, ILC2s contribute to lung repair after influenza virus (79) and helminth infection (80). Production of IL-5 and IL-13 by ILC2s is also known to regulate eosinophil homeostasis in the lung and intestine (81). IL2s are also found in adipose tissues, where they help regulate eosinophil homeostasis and the polarization of alternatively activated macrophages (AAMacs) (82). Furthermore, the presence of ILC2s in adipose tissues has been associated with protection against obesity in mice (83), most likely through the "beiging" of white adipocytes (84, 85). Beige adipocytes contain mitochondria that use cellular resources to generate heat instead of ATP, and thus help to regulate weight by expending energy through non-shivering thermogenesis (85). Thus, while ILC2s are less phenotypically diverse than ILC1s and ILC3s, they play a variety of roles depending on their location and interactions with other immune and non-immune cells.

Group 3 ILCs

ILC3s are a heterogeneous group of ILCs, with considerable phenotypic diversity between subsets (**Figure 2**). All ILC3s rely on the transcription factor RORγt for development, and constitutively produce this factor at maturity (86, 87). ILC3s generally respond to IL-1β, IL-6, and IL-23, and produce IL-17 and IL-22 (88-90). LTi cells in the fetus (91) and LTi-like cells in the adult (92) both belong to the ILC3 group. These cells develop from PLZF-independent $\alpha4\beta7^+$ precursors that diverge between the CHILP and CILP stages of helper-like ILC development (35, 87, 93). LTi and LTi-like cells are $CCR6^+NKp46^-$, and can be either $CD4^+$ or $CD4^-$ (87). In adults, LTi-like ILC3s are found mainly in the intestine and in lymphoid tissues (3). A distinct subset of ILC3s develops from the $PLZF^+$ ILCP, under the influence of the aryl hydrocarbon receptor (AhR), T-bet, and the presence of commensal bacteria (94-96). These ILC3s are $CCR6^-T\text{-}bet^+$, can be either $NKp46^+$ or $NKp46^-$, and produce IL-22 and IFN-γ (88, 89). There is evidence to indicate that a T-bet gradient coordinates the development of $NKp46^-$ ILC3s into $NKp46^+$ ILC3s (97), and that Notch is also important for this process (98, 99).

ILC3s mainly respond to infections with extracellular bacteria and fungi (**Figure 3**) (3, 37). In mice, intestinal infection with *Citrobacter rodentium* elicits rapid IL-22 production from $NKp46^+$ ILC3s (89). ILC3s act mainly on intestinal epithelial cells to stimulate the production of antimicrobial peptides and element-sequestering proteins, secretion of mucus, and fucosylation of the epithelium. These modifications collectively serve to limit the spread of pathogenic bacteria (100-104).

The role of ILC3s in tissue homeostasis is complex. Under certain conditions, production of inflammatory cytokines by ILC3s has been linked to the progression of inflammatory bowel diseases (105-107). This ILC3-driven intestinal inflammation is

often linked to infections. During *Toxoplasma gondii* infection, intestinal ILC3s produce IL-22, which induces intestinal epithelial cells to produce IL-18. This in turn leads to tissue inflammation (108). Production of IL-22 by ILC3s can also promote the development of colitis-associated intestinal tumors (109). However, ILC3s have also been shown to help resolve chronic inflammation and promote tissue repair. ILC3s promote colonization of the intestine with beneficial commensal bacterial species that protect against infection with pathogenic bacteria (101-103). ILC3s also act on other immune cell types to suppress intestinal inflammation. They can regulate myeloid cell homeostasis through production of GM-CSF (110), and can also induce cell death in commensal bacteria-specific $CD4^+$ cells that could otherwise cause inflammation (111). ILC3s have also been implicated in the repair of intestinal tissues that have been damaged by inflammation (112-114). ILC3s are also involved in the IL-22-dependent repair of other tissues, including the thymus (115), liver (68), and lung (79, 116, 117). In summary, ILC3s are a heterogenous group of innate lymphocytes that can protect against infection, and either induce inflammation or help resolve inflammation and promote tissue repair, depending on context.

CONVENTIONAL NK CELLS

Conventional NK cells are cytotoxic effector lymphocytes that represent the innate analog of $CD8^+$ T cells (36, 38). cNK cells begin their development in the bone marrow from the CLP (**Figure 4**), which is defined in mice as $Lin^-Sca\text{-}1^{low}CD117^{low}CD127^+CD135^+$ (13). From there, they develop through early precursor stages called pre-pro A, which are $Lin^-Id2^+Sca\text{-}1^+CD117^{low}CD127^+CD135^-CD122^-$, and

pre-pro B, which are $Lin^{-}Id2^{+}Sca\text{-}1^{+}CD117^{-}CD127^{+}CD135^{-}$ (118) Pre-pro NK cell precursors then develop into refined NK cell precursors (rNKPs), which are $Lin^{-}CD27^{+}CD244^{+}CD117^{low}CD127^{+}CD135^{-}CD122^{+}$ (119). rNKPs develop further into immature NK cells (iNK), which acquire NK1.1 expression (120), and then develop into mature NK (mNK) cells (121, 122). Mature NK cells acquire expression of CD49b (also called DX5) before they exit the bone marrow and enter circulation (123). The natural cytotoxicity receptor NKp46 is also expressed before mNK cells exit the bone marrow, around the same time as DX5 expression (124-126). Thus, in C57BL/6 mice, conventional NK cells can be defined as $CD3^{-}TCR^{-}CD19^{-}NK1.1^{+}NKp46^{+}DX5^{+}$. After the acquisition of NK1.1 and NKp46 expression, the later stages of cNK cell maturity can be assessed by expression of CD11b and CD27 in a 4-stage process. The least mature cNK cells are $CD11b^{low}CD27^{low}$. As they mature, they become $CD11b^{low}CD27^{high}$, followed by $CD11b^{high}CD27^{high}$. Terminally mature NK cells are $CD11b^{high}CD27^{low}$ (127), and also express killer cell lectin-like receptor G1 (KLRG1) (128, 129).

The development of cNK cells is mediated by the influence of several different transcription factors and cytokines (**Figure 4**). The basic leucine zipper transcription factor nuclear NFIL3, also called E4BP4, is required for the development of early cNK cell precursors from the CLP (130, 131). Other transcription factors that are essential for early cNK cell development include Id2 (19), Eomes (23), and ETS1 (132). Furthermore, signaling of IL-15 through the IL-15Rα is crucial for the development and maintenance of mature cNK cells (25-27). The precise relationships between each of these different factors are still being defined. A recent study demonstrated that NFIL3 is required upstream of Id2 and Eomes, and that it regulates the expression of these two transcription

factors (133). ETS1 was also shown to act upstream of Id2 (134). Additional transcription factors are involved in the later stages of NK cell development and maturation. For instance, GATA3, which is essential for the development of helper-like ILCs (11, 31, 60), is also required for the maturation of cNK cells beyond the $CD11b^{low}CD27^{high}$ stage (12). T-bet (24) and Ailos (135) are also involved in the later stages of cNK cell maturation.

Conventional NK cells are cytotoxic effector cells that function mainly to kill virally infected cells and transformed tumor cells (136, 137). cNK cells kill target cells through two main mechanisms: release of cytotoxic granules, or engagement of cell death receptors on the target cell surface (138, 139). Cytotoxic granules contain a variety of proteins that rapidly induce apoptosis in the target cell. These include perforin, which forms pores in the membrane of the target cell, and may also have other cytotoxic functions (138, 140, 141). Granules also contain a variety of granzymes, which are serine proteases that enter the cell to mediate cytotoxicity (142, 143). For instance, granzyme B induces target cell apoptosis directly, or through the activation of intracellular caspases (144). NK cells also express ligands that engage directly with programmed cell death receptors on potential target cells. These include TNF-related apoptosis-inducing ligand (TRAIL) (145, 146), which is only expressed on small subsets of NK cells at baseline, but is expressed more highly after stimulation with IL-2, IL-15 (147), or IFNs, including IFN-γ and IFN-αβ. TRAIL-mediated apoptosis is involved in limiting tumor growth and metastasis (148-150), and in defense against several viruses, including encephalomyocarditis virus (ECMV), cytomegalovirus (CMV) (151), and hepatitis C virus (152). NK cells also express the TNF family member Fas ligand (FasL), which engages with Fas on target cells to induce apoptosis (153, 154). Fas/FasL cytotoxicity is

mainly utilized to induce apoptosis in tumor cells (155, 156). Tumor cells do not normally express Fas, but rather express it after induction by IFN-γ that is secreted by NK cells (157).

The cytotoxic activity of cNK cells is mediated by a balance between activating and inhibitory receptors on the NK cell surface (**Figure 5**) (158-161). In mice, NK cell activating receptors include several members of the Ly49 and NKG2 families (162-164). For instance, Ly49D and Ly49H serve as activating receptors, along with NKG2C and NKG2D (163, 164). NK cell activating receptors recognize a variety of ligands, some of which are specific to certain target cells. Ly49H in C57BL/6 mice recognizes the glycoprotein m157, which is expressed specifically on the surface of cells that are infected with murine cytomegalovirus (MCMV) (165, 166). After binding with their ligands, NK cell activating receptors signal through intracellular adapter proteins. These adapter proteins are associated with immunoreceptor tyrosine-based activating motifs (ITAMs), which transduce a signaling cascade that results in NK cell cytotoxic activity and production of IFN-γ (167). The precise function of an activating receptor can change based on its associated adapter molecule. For instance, many of the NK cell activating receptors associate with the intercellular adapter molecule DNAX-activating protein of 12 kDa (DAP12), which transduces activating signals through SYK or ZAP70 (168). However, NKG2D can also associate with DAP10 to serve a co-stimulatory rather than an activating function (169).

NK cell inhibitory receptors also have members among the Ly49 and NKG2 families. These include Ly49A, Ly49C, Ly49I (170), and CD94/NKG2A, a lectin-like heterodimer (171). Inhibitory Ly49 receptors bind to classical MHC class Ia molecules

(172-174), whereas CD94/NKG2A binds to a nonclassical MHC class Ib molecule called Qa-1 (175, 176). KLRG1 also serves as an inhibitory receptor (177) that binds cadherins (178, 179). Rather than associating with adapter molecules, NK cell inhibitory receptors generally signal directly through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located on their cytoplasmic tails (162). The various NK cell inhibitory receptors recognize different subsets of MHC class I ligands, and these inhibitory receptors are expressed stochastically on NK cells. Thus, whether an NK cell will be inhibited by a target cell depends on whether it possesses an inhibitory receptor that specifically recognizes one of the target cell's MHC class I ligands (180).

When an NK cell engages with a potential target cell, the resulting NK cell cytotoxic response is not a binary state, but is rather determined by the relative strength of the activating and inhibitory signals (**Figure 5**) (158, 181). If MHC class I expression outweighs the expression of activating ligands, the NK cell will remain functionally dormant. However, if the activating signal is stronger, the NK cell will rapidly kill the target cell (158). Some virally infected cells and tumor cells downregulate MHC class I ligands to escape $CD8^+$ T cell-mediated destruction. This in turn renders them vulnerable to the NK cell cytotoxic response, since the NK cell-target cell interaction will favor the activating receptors over inhibitory receptors (182).

NK cell inhibitory receptors also have a role in "licensing" NK cells. NK cell licensing refers to the engagement of self-MHC class I ligands by NK cell inhibitory receptors during development (183-185). This process was thought to be necessary for NK cells to develop optimal functional competence, defined as the ability to distinguish self from non-self, and deliver a potent cytotoxic response to target cells missing MHC

class I (186). For instance, NK cells from *B2m*$^{-/-}$ mice, which are MHC class I-deficient, show reduced ability to kill target cells that lack MHC class I (187). Moreover, licensing does not strictly occur during development in the bone marrow. Mature splenic NK cells from MHC class I-deficient mice gain functional competence when transferred into wild-type MHC class I-sufficient mice (188). The inverse is also true, in that licensed NK cells lose their functional competence when transferred from an MHC class I-sufficient environment into MHC class I-deficient mice (189).

Recent studies have shown that licensing is not always necessary for NK cells to realize their full cytotoxic ability (190). Orr and colleagues (191) demonstrated that unlicensed NK cells are far more active in controlling MCMV infection than licensed NK cells. The unlicensed NK cells recognize and kill MCMV-infected cells through direct engagement of the m157 glycoprotein on the surface of MCMV-infected cells by the NK cell activating receptor Ly49H (191). A later study demonstrated that licensed NK cells expand more rapidly during early MCMV infection than unlicensed NK cells. However, regulatory T cells and TGF-β signaling serve to regulate and suppress licensed NK cells (192), leaving unlicensed NK cells to respond to MCMV (193).

Up until recently, it was thought that only the cells of the adaptive immune system could generate immunological memory. However, mounting evidence indicates that NK cells also have the ability to recognize and respond rapidly against previously encountered pathogens and chemicals (194-196). NK cells that were transferred from wild type mice into lymphopenic recipients survived for more than 6 months, and exhibited homeostatic proliferation and self-renewal. Moreover, the transferred NK cells were capable of responding to viral infection months after the initial transfer (197).

Ly49H-expressing NK cells were also shown to expand rapidly following primary MCMV infection, and then contract and reside in both lymphoid and non-lymphoid organs following infection. After adoptive transfer into naïve mice, $Ly49^+$ MCMV-specific NK cells expanded rapidly following MCMV infection, and provided protective immunity (198). This MCMV-specific NK cell expansion and memory generation is dependent on IL-12 signaling (199). Moreover, MCMV-specific memory NK cells were recently shown to arise from $KLRG1^-$ progenitors. In contrast, $KLRG1^+$ NK cells demonstrated a limited capacity for expansion during MCMV infection (200). The self-MHC activating receptor Ly49D was also recently shown to be involved in the generation of MCMV-specific memory NK cells (201). NK cells can also generate memory responses to chemical stimuli, such as hapten-specific skin contact hypersensitivity reactions (202). Thus, while NK cells provide rapid protection against pathogens, that protection is not always non-specific or short lasting.

OTHER GROUP 1 ILCS

Conventional NK cells were first discovered in 1975 (203, 204), and for decades thereafter were thought to be the only innate lymphocytes. The later discovery of fetal LTi and adult LTi-like cells first revealed the existence of other innate lymphocytes (91, 205, 206). Within the last several years, many new classes of ILCs have been discovered and characterized (6). These include several subsets of ILC1s that share some characteristics with conventional NK cells, but also have unique phenotypes and developmental requirements (**Table 1**) (207). The thymus contains a population of phenotypically and developmentally distinct NK cells (50). A unique population of NK

cells was also recently identified in the liver (51, 208), with similar populations found in the skin (209), kidney (52), and uterus (53). The submandibular salivary gland also contains a unique population of NFIL3-independent NK cells (54, 55). These NK cell populations have been defined as tissue-resident, mainly because they have unique tissue-specific phenotypes, and do not circulate throughout the body like cNK cells (209). Moreover, distinct subsets of $CD103^+$ intraepithelial ILC1s (ieILC1s) (56) and $CD127^+$ ILC1s (8) have been identified in the intestine. Tissue-resident ILC1s have also been characterized in adipose tissue (46, 57, 58). Altogether, these unique populations of ILC1s appear to represent several distinct lineages of innate lymphocytes.

Thymic NK Cells

The murine thymus contains a small population of NK cells, which represents less than 1% of total thymic lymphocytes. Unlike cNK cells, thymic NK cells express CD127. They are also distinguished from mature cNK cells as being $CD69^{high}Ly49^{low}CD11b^{low}$. Thymic NK cells are also less cytotoxic than cNK cells, yet they produce more IFN-γ, TNF-α, and GM-CSF. Thymic NK cells require GATA-3 and IL-7 for development (50, 210), and they develop mostly independently of NFIL3 (211). This was further supported by experiments that demonstrated that thymic NK cells develop from double-negative ($CD4^-CD8^-$) thymocyte precursors (212). Thymic NK cells have been shown to traffic to nearby lymph nodes, but they have not yet been identified in any other organ or tissue (50). Altogether, thymic NK cells appear to be more closely related to helper-like ILC1s than cNK cells. However, their developmental relationship with other ILC1 subsets has not yet been conclusively determined.

Liver Tissue-Resident NK Cells

The liver contains two populations of NK cells: cNK cells, which are $DX5^{+}CD49a^{-}$, as well as $DX5^{-}CD49a^{+}$ tissue-resident NK cells. The trNK cells comprise 25-50% of the total liver NK cell population (51, 208). Liver trNK cells constitutively express TRAIL, which is usually not expressed on naïve, mature cNK cells (213). Liver trNK cells develop partially independently of NFIL3, whereas cNK cells are almost completely ablated in *NFIL3*$^{-/-}$ mice. Also, cNK cells express Eomes and T-bet, whereas liver trNK cells do not express Eomes. Liver trNK cells are similar to other helper-like ILC1 populations in that they are strong producers of IFN-γ, TNF-α, and GM-CSF (8, 51). In contrast, cNK cells produce IFN-γ, but not TNF-α or GM-CSF. Thus, liver trNK cells are more closely related to helper-like ILC1s than cNK cells. However, there are key differences between liver trNK cells and other helper-like ILC1 populations. Whereas helper-like ILC1s are generally considered non-cytotoxic, liver trNK cells produce a strong cytotoxic response (214). Thus, liver trNK cells most likely represent a distinct lineage of ILC1s, separate from cNK cells and helper-like ILC1s.

A recent study demonstrated that liver trNK cells are involved in generating hapten-specific skin contact hypersensivity responses. C57BL/6 or *Rag1*$^{-/-}$ mice were sensitized with oxazolone, a hapten that induces delayed skin contact hypersensitivity. The sensitization protocol consisted of applying oxazolone to the skin of the shaved abdomen. $DX5^{+}$ or $DX5^{-}$ liver NK cells were then sorted from the sensitized mice and adoptively transferred into recipient C57BL/6 mice. 30 days later, the recipient mice were challenged with application of oxazolone to one ear. The mice that had received $DX5^{-}$

liver NK cells showed marked ear inflammation, whereas the mice that received the $DX5^+$ liver NK cells did not. This result indicated that liver trNK cells mediate delayed skin contact hypersensitivity, which is a form of immunological memory (208).

Skin Tissue-Resident NK Cells

The skin contains a small population of NK cells, which can be divided into a majority subset of $DX5^+CD49a^-$ cNK cells and a minority subset of $DX5^-CD49a^+$ trNK cells. Parabiosis experiments revealed that skin $DX5^-CD49a^+$ NK cells do not circulate throughout the body, but rather remain in the skin, indicating that they are tissue-resident. Skin cNK cells are mostly absent in *NFIL3*$^{-/-}$ mice, yet the trNK cells are relatively unaffected by NFIL3 deficiency. These findings indicate that skin trNK cells are related to the NFIL3-independent trNK cells found in the liver (51). However, not much more is known about skin trNK cells, as they have not been studied in great detail.

Kidney Tissue-Resident NK Cells

Similar to the liver, the kidney contains populations of $DX5^+CD49a^-$ cNK cells and $DX5^-CD49a^+$ trNK cells. Kidney trNK cells represent 15-20% of the total kidney NK cell population. Both kidney cNK cells and trNK cells are reduced in number and frequency in *NFIL3*$^{-/-}$ mice (52). However, in contrast to liver trNK cells (51), the frequency of kidney trNK cells is not reduced in *T-bet*$^{-/-}$ mice. Compared to cNK cells, kidney trNK cells have increased expression of CD44, CD160, and TRAIL, and lower expression of CD62L, KLRG1, and CD244. Kidney trNK cells also have reduced expression of asialo-GM1 (AsGM1), a glycolipid receptor that was once used as a

standard NK cell marker (215). By using anti-AsGM1 to preferentially deplete cNK cells, Victorino *et al.* (52) recently demonstrated that kidney trNK cells mediate acute kidney injury following periods of ischemia.

Salivary Gland NK Cells

The submandibular salivary gland (SMG) contains a population of NK cells that represents 10-30% of total SMG lymphocytes. These SMG NK cells have an unusual maturation phenotype, being largely $CD11b^{high}CD27^{low}$, and $KLRG1^{-}$ (216). A study by Cortez *et al.* (54) revealed that SMG NK cells express TRAIL and develop largely independently of NFIL3. These SMG NFIL3-independent NK cells express DX5 and CD49a, as well as T-bet and Eomes, which means they are unique from other ILC1 and tissue-resident NK cell populations. However, we found (55) that SMG NK cells are strongly reduced in *NFIL3*$^{-/-}$ mice. This indicates that there are two populations of NK cells in the SMG: a majority NFIL3-dependent population, and a minority NFIL3-independent population. Both populations are otherwise phenotypically similar (55). More recent work by Cortez *et al.* (217) has shown that SMG NFIL3-independent NK cells develop under the influence of transforming growth factor-β (TGF-β), which suppresses Eomes expression and guides SMG ILC development. Perhaps the differences between studies are due to differences in TGF-β expression between mouse colonies. Further work will be necessary to reconcile the discrepancies between these studies.

The SMG is a site of CMV replication and persistence in immunocompetent hosts (218). SMG NK cells upregulate expression of KLRG1 and CD69 during MCMV infection, which indicates NK cell activation. However, their degranulation and

production of IFN-γ is much lower in magnitude than splenic NK cells, despite active viral replication in the salivary gland (216). A recent study revealed that $TRAIL^+$ SMG NK cells kill $CD4^+$ T cells in the SMG during CMV infection (219). $CD4^+$ T cells are responsible for the eventual clearance of CMV from the SMG (220), so their TRAIL-mediate killing by SMG NK cells could contribute to the persistence of CMV in this organ.

Uterine ILC1s

The murine uterus contains several populations of ILCs. $DX5^+CD49a^-$ cNK cells and NFIL3-independent $DX5^-CD49a^+$ trNK cells have both been identified in this organ. Interestingly, and in contrast to liver trNK cells, uterine $CD49a^+$ NK cells also develop in the absence of T-bet (51). Moreover, a recent study showed that uterine $DX5^-CD49a^+$ NK cells can be further divided on the basis of Eomes expression into $CD49a^+Eomes^+$ uterine trNK cells and $CD49a^+Eomes^-$ uterine ILC1s (53). In both mice and humans, uterine NK cells in the lining of the pregnant uterus (the decidua) have been shown to be protective during pregnancy. These decidual NK cells possess the full complement of cytotoxic machinery, yet they do not kill the invading trophoblast cells (221, 222). Rather, decidual NK cells actually promote a healthy pregnancy by inducing uterine spiral artery remodeling (223). This process is dependent on the production of vascular endothelial growth factor-C (VEGF-C) by uterine NK cells (224).

Intraepithelial ILC1s

A recent study identified a population of NKp44^{+}CD103^{+} lymphocytes in human tonsils and intestinal mucosa (56). These cells also expressed the chemokine receptor CXCR6 and the epithelial marker CD160. Due to their unique phenotype and epithelial location, these cells were called intraepithelial ILC1s (ieILC1s). ieILC1s express T-bet and Eomes, and produce IFN-γ in response to IL-12 and IL-15 stimulation. In mice, ieILC1s were identified as NKp46^{+}NK1.1^{+}CD160^{+}. ieILC1s are present in *IL15rα*$^{-/-}$ mice, but are strongly reduced in NFIL3-deficient and T-bet-deficient (*Tbx21*$^{-/-}$) mice, indicating that they are developmentally distinct from both cNK cells and the tissue-resident NK cells of the liver, skin, and kidneys (56).

CD127^{+} Mucosal ILC1s

In addition to ieILC1s, the intestine also contains a subset of CD127^{+}NKp46^{+}NK1.1^{+}T-bet^{+}Eomes^{-} cells (8). These cells were found to be negative for RORγt fate mapping, which means they are not derived from the ILC3 lineage. Eomes^{-} intestinal ILC1s do not develop into Eomes^{+} ILC1s, indicating that they are a distinct lineage from ieILC1s and cNK cells. CD127^{+} ILC1s develop independently of IL-7, but do require IL-15, T-bet, and GATA3 for development. Their development is also partially dependent on NFIL3. During *Toxoplasma gondii* infection, mucosal ILC1s are the main producers of IFN-γ and TNF-α (8).

Adipose Tissue-Resident ILC1s

Adipose tissue is known to contain TCRβ^{-}NK1.1^{+} cells (225), which have been linked to inflammation and insulin resistance in mouse models of obesity (58, 226, 227).

A recent study by O'Sullivan and colleagues (57) separated adipose NK1.1$^+$ cells into three distinct subsets based on DX5 and Eomes expression: DX5$^+$Eomes$^+$ mature cNK cells, DX5$^-$Eomes$^+$ immature cNK (iNK) cells, and DX5$^-$Eomes$^-$ ILC1s. Whereas the cNK cells circulated throughout the body, the ILC1s displayed long-term adipose tissue residency. These adipose ILC1s do not express CD127 or CD103, indicating that they are distinct from mucosal CD127$^+$ ILC1s (8). Moreover, adipose ILC1s were reduced in *NFIL3*$^{-/-}$ mice, indicating that they are developmentally unique from most subsets of trNK cells (51-55). Compared to mature and immature cNK cells, adipose-resident ILC1s produce the most IFN-γ during high fat diet feeding. They are also the chief cell type responsible for M1 macrophage polarization, and the resulting obesity-induced inflammation in this tissue (57).

More recent work by Boulenouar *et al.* (46) further characterized the three major subsets of group 1 ILCs in mouse adipose tissues based on DX5 and CD49a expression. They identified double negative DX5$^-$CD49a$^-$ iNK cells, DX5$^-$CD49a$^+$ ILC1-like cells, and DX5$^+$CD49a$^-$ cNK cells. Double positive DX5$^+$CD49a$^+$ cells were present, but rare. In lean mice, all three major subsets of adipose ILC1s regulate macrophage homeostasis through targeted cytotoxic killing. However, this killing ability is diminished in obese mice, which could help to explain the accumulation of pro-inflammatory macrophages in adipose tissue during obesity.

ILC DEVELOPMENTAL PLASTICITY

The development of ILC subsets from the CLP is not always a linear path. Rather, phenotypic plasticity has been observed between several ILC subsets (59, 228). For

instance, CCR6$^-$NKp46$^+$T-bet$^+$ ILC3s can downregulate RORγt expression, maintain T-bet expression, and develop an ILC1 phenotype (**Figure 2**). This includes responding to IL-12 and IL-18 and producing IFN-γ. These "ex-ILC3s" are difficult to distinguish from other ILC1s without using mice that are fate mapped for RORγt (45, 97, 229). Due to their RORγt expression during development, it has been argued that ex-ILC3s should be classified definitively as ILC3s (8).

Similarly to ex-ILC3s, ILC2s in the lung have been found to switch to an ILC1 phenotype after prolonged exposure to IL-1β, IL-4 and IL-12 (230-232). A recent study also identified an unusual population of ILC2s in the lungs of mice infected with the helminthic parasite *Nippostongylus brasiliensis*. These inflammatory ILC2s (iILC2s) responded to IL-15 but not IL-33, expressed intermediate levels of RORγt, and produced IL-17 *in vitro*, as well as in response to *Candida albicans* infection (233).

Finally, some ILC1 subsets also display developmental plasticity. Mice that were genetically modified to ectopically express Eomes at the T-bet locus showed a decrease in helper-like ILC1s and an increase in cNK cells. This result suggested that a shift in the balance of Eomes and T-bet expression is sufficient to redirect helper-like ILC1 development to cNK cells (234). Similarly, the submandibular salivary gland contains a population of unique trNK cells that develop under the influence of transforming growth factor-β (TGF-β) signaling. Incubation of spleen cNK cells with IL-2 and TGF-β can induce these cNK cells to develop a trNK cell-like phenotype (217). More work is necessary to characterize the full extent of ILC phenotypic and developmental plasticity.

CYTOMEGALOVIRUS

Cytomegaloviruses are a family of β-herpesviruses with strong species tropism (235). Human CMV (HCMV) is a double-stranded DNA virus with a genome roughly 235 kb in size, which encodes approximately 165 genes (236). HCMV is a ubiquitous pathogen that infects 50-95% of the adult population, based on age and geographic location (237). In healthy individuals, primary HCMV infection is mild or asymptomatic, and is usually controlled by the immune system (238). However, like other herpesviruses, HCMV is not fully cleared from the body, but instead establishes latency for the life of the host (239). HCMV latency is characterized by maintenance of the viral genome in granulocyte-macrophage progenitors (240, 241), with periodic episodes of reactivation (241, 242). Constant surveillance by the immune system is required to prevent latent HCMV from re-establishing an active infection (243). HCMV-specific $CD8^+$ T cell populations are maintained in various tissues to combat episodes of reactivation (244). $CD4^+$ T cells and NK cells also work with $CD8^+$ T cells in a redundant manner to control latent HCMV infection. Loss of all three lymphocyte subsets is required for the virus to fully escape from latency and re-establish a systemic infection (243).

Primary HCMV infection or reactivation of latent infection can represent a severe medical problem for immunocompromised individuals (245). This includes people with congenital or acquired immunodeficiencies (246, 247), as well as solid organ or hematopoietic stem cell transplant recipients (248-251). HCMV infection in pregnant women also imposes a serious risk of neonatal fatality or birth defects. Roughly 40,000 children (0.2-2% of all deliveries) are born with congenital HCMV infection each year in the United States. This results in around 400 fatalities and 8,000 cases of permanent

disabilities (252), including microcephaly, cerebral palsy, blindness, deafness, and mental retardation (253, 254).

HCMV can only establish an active infection in human cells, and thus cannot be studied in an animal model (255). Fortunately, murine cytomegalovirus (MCMV) is very similar to HCMV in terms of viral structure, pathogenesis, establishment of latency, and periodic reactivation. Thus, MCMV is often used as a model system to study CMV infection and the immune response (256). MCMV is usually introduced experimentally by intraperitoneal (i.p.) injection. From there, the virus infects cells in the lymph nodes, spleen, and liver within 6 hours of injection (257). Viral replication peaks in the spleen and liver between days 2-3 of infection (258). NK cells are crucial for the early control of MCMV infection (259-262). The early NK cell response to MCMV is mediated by TLR9/MyD88-dependent production of type I IFNs by plasmacytoid dendritic cells (DCs). This induces IL-12 production by other DC cell subsets, which in turn activates NK cells. Activated NK cells kill MCMV-infected cells and produce IFN-γ to stimulate adaptive immune responses by 38 hours after infection (263).

The NK cell response to MCMV varies based on mouse strain. NK cells in C57BL/6 mice express the activating receptor Ly49H, which directly recognizes the m157 glycoprotein on the surface of MCMV-infected cells (165). Recognition of m157 by Ly49H results in expansion of $Ly49H^+$ NK cells, along with a robust cytotoxic and IFN-γ response. This response renders Ly49H-expressing strains resistant to MCMV infection, whereas Ly49H-deficient strains ultimately succumb to the virus (165, 264, 265).

Primary MCMV infection is cleared from most organs by day 5, after which point the virus infects the acinar glandular epithelial cells of the submandibular salivary glands (266). Here the virus persists for several weeks, during which time it can be transmitted to new hosts through saliva (267). NK cells and $CD8^+$ T cells are incapable of clearing the virus from this organ. Ultimately, $CD4^+$ T cells are responsible for resolving the active MCMV infection in the SMG (218, 268-270).

The precise mechanisms that mediate MCMV persistence in the SMG are not fully known. Optimal infection of the salivary glands is dependent on viral inhibition of the mitochondrial apoptosis protein BAK (271). The NK cells of the SMG are hyporesponsive to MCMV infection (216), and kill $CD4^+$ T cells through TRAIL-mediated cytotoxicity, which might contribute to the persistence of infection (219). IFN-γ-production by $CD4^+$ T cells is also limited by IL-10-producing $CD4^+$ T cells in the SMG (272). MCMV persistence was also shown to be at least partially dependent on production of viral microRNAs (273). Together, these factors might serve to explain the persistence of MCMV in this tissue.

THESIS OVERVIEW

The goal of the research described in this thesis is to increase our understanding of the development and anti-viral functions of innate lymphoid cells in exocrine tissues. In the first chapter, we investigate the differential requirement of NFIL3 for the development of two distinct ILC1 populations in the submandibular salivary gland, as well as the factors mediating the weak effector response of SMG NK cells to MCMV. In the second chapter, we identify and begin to characterize a novel population of $CD3^{+}NK1.1^{+}$ lymphocytes that appears in the SMG in NFIL3-deficient mice. In the third chapter, we identify and characterize a population of NK cells in the lacrimal gland. We also investigate the response of LG NK cells to MCMV.

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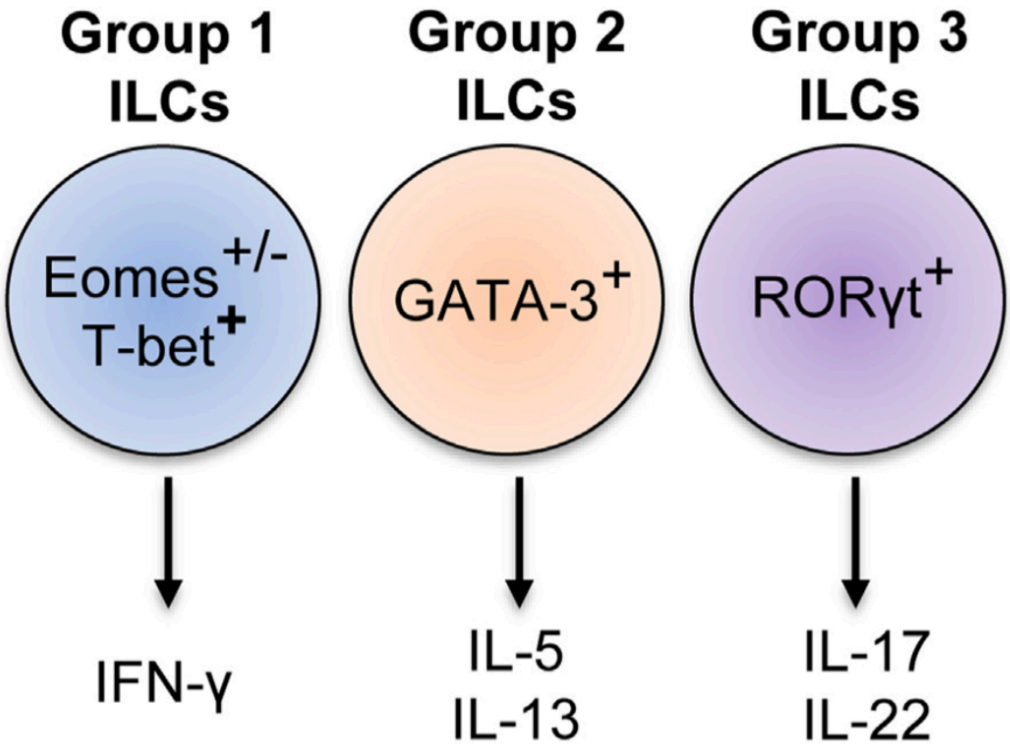

Figure 1. ILCs are divided into three groups (Adapted from Cortez and Colonna 2016 (9)).

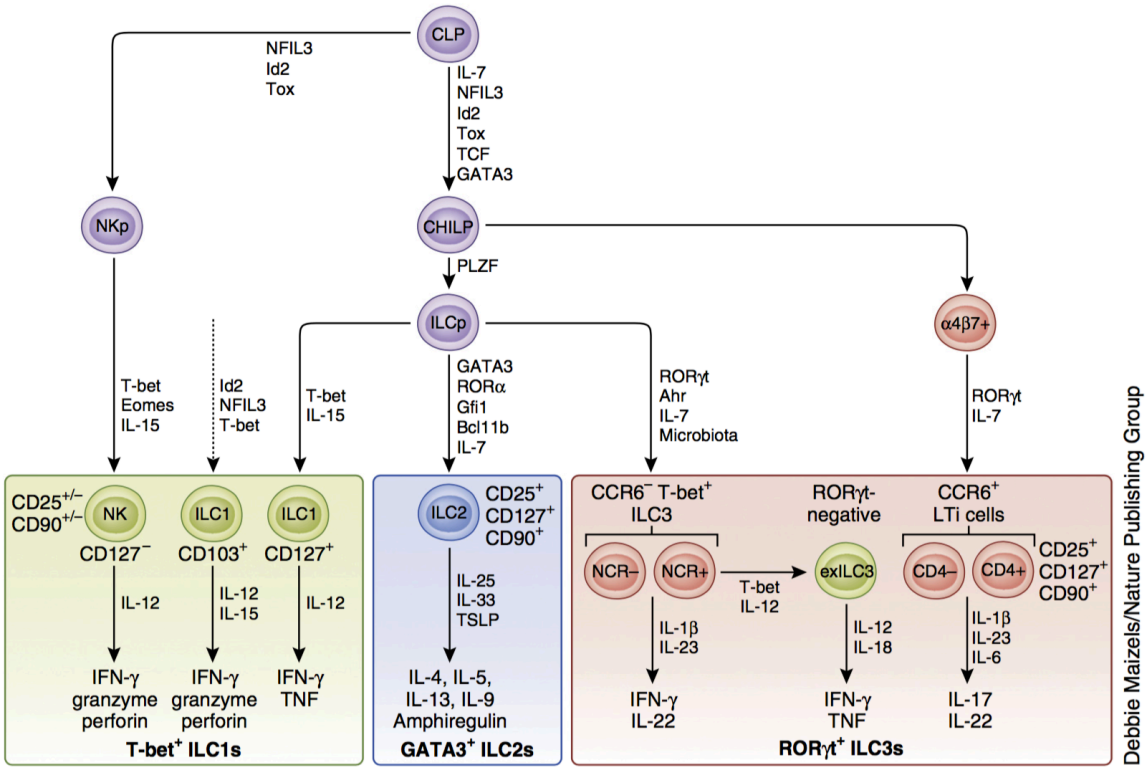

Figure 2. ILC development (Adapted from Sonnenberg and Artis 2015 (3)).

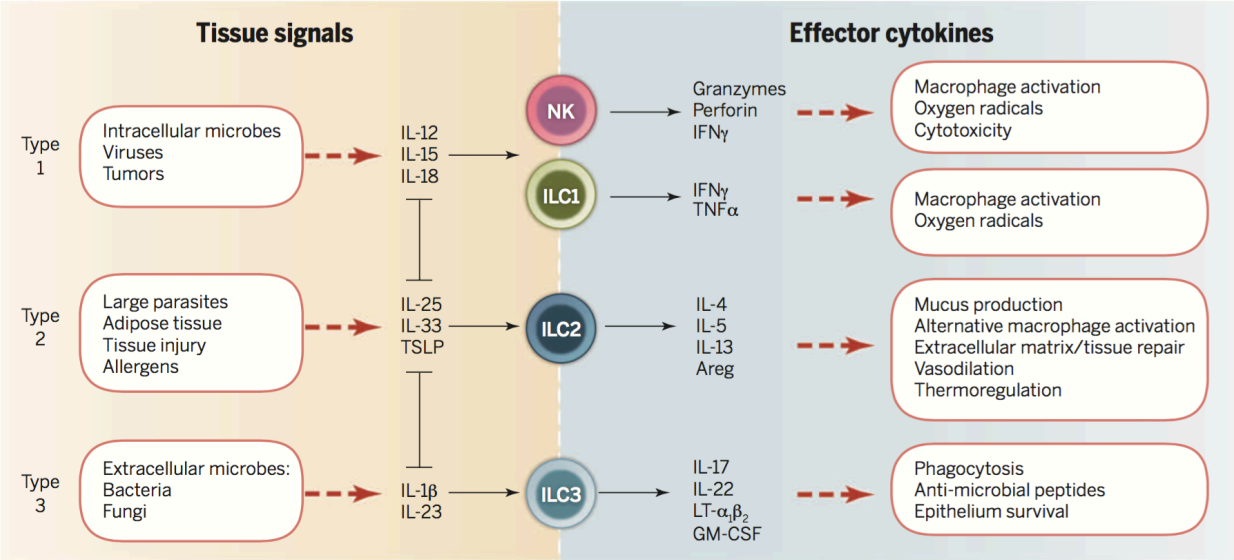

Figure 3. ILC activation, response to pathogens, and other roles (Adapted from Eberl *et al.* 2015 (37)).

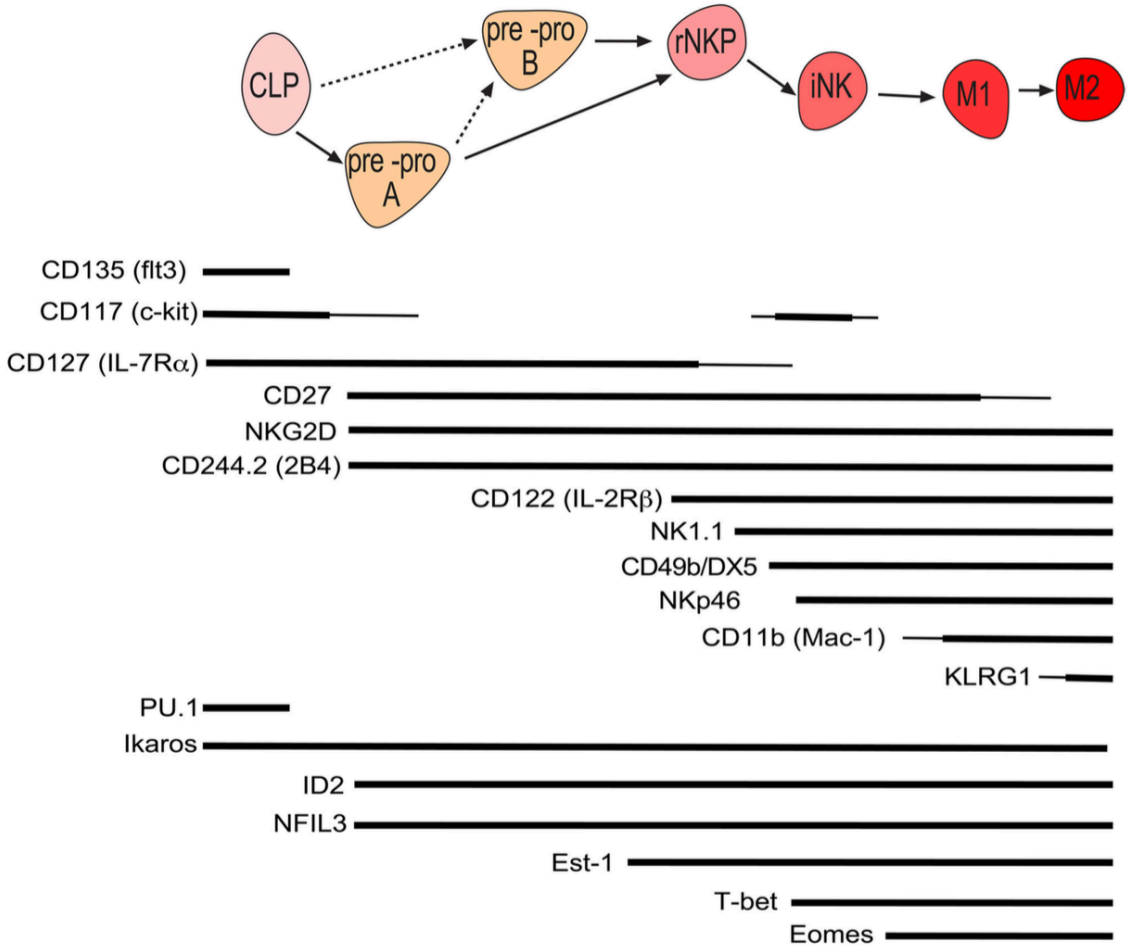

Figure 4. Conventional NK cell development (Adapted from Huntington *et al.* 2013 (274)).

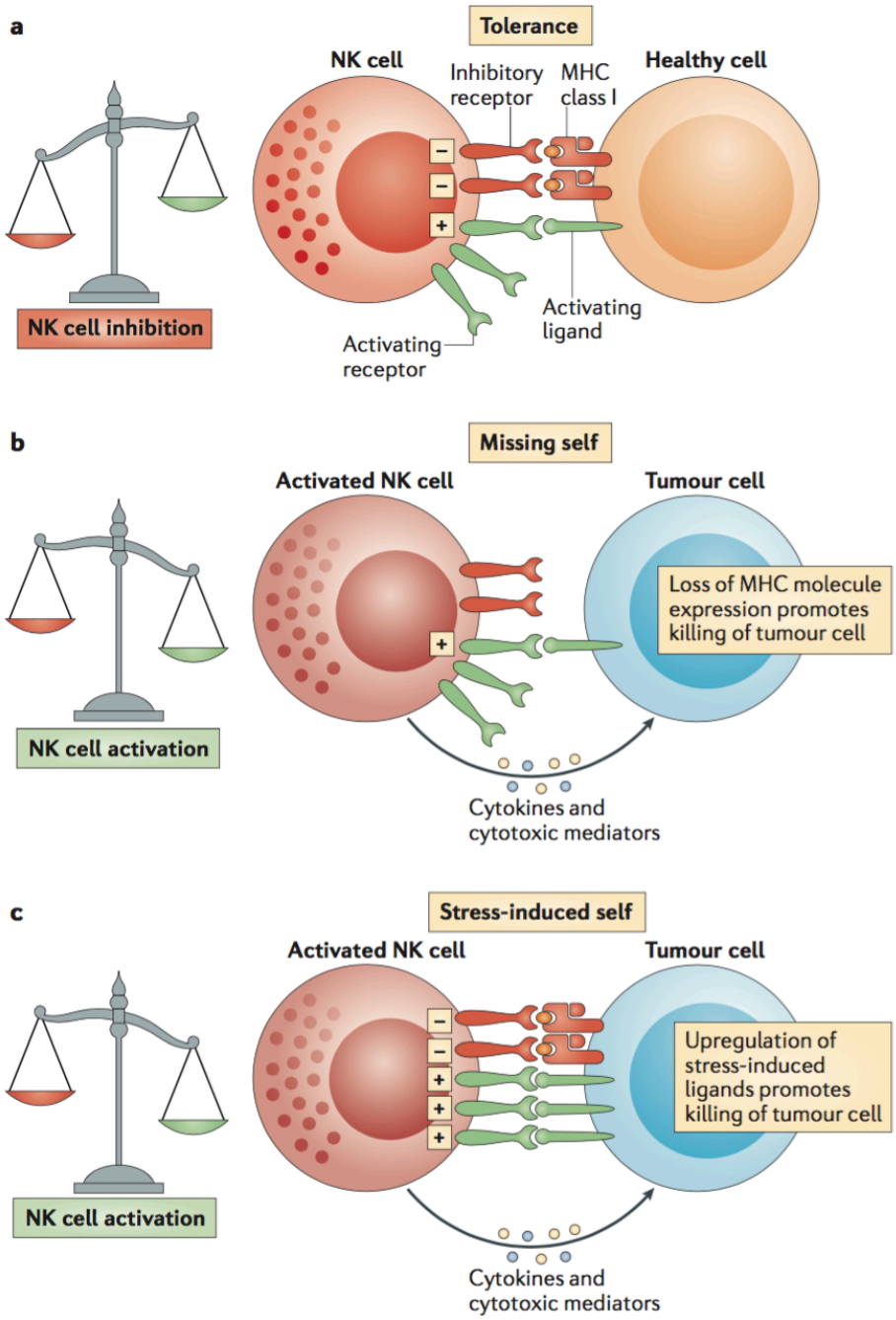

Figure 5. NK cell activity is mediated by activating and inhibitory receptors (Adapted from Vivier *et al.* 2012 (161)).

Table 1

Phenotypic characteristics of cNK cells and tissue-resident NK cell subsets

	cNK*	Thymic NK	Liver trNK	Skin trNK	Uterine trNK	SMG trNK	Kidney trNK
NK1.1	+	+	+	+	+	+	+
DX5	+	+	−	−	−	+/−	−
CD49a	−	?	+	+	+	+	+
CD127	−	+	−	−	−	−	?
TRAIL	−	?	+	?	?	+/−	+
Eomes	+	?	−	−	+/−	+	?
IFN-γ	++	+	+	?	+	+/−	?
TNF-α	+/−	+	+	?	?	−	?
Granzymes	+	+	+	?	+	?	?

* cNK cells are prominent in the spleen and blood, and are also found in the liver, skin, uterus, SMG, and kidney.

Table 1. Phenotypic characteristics of cNK cells and tissue-resident NK cell subsets (adapted from Erick and Brossay 2016 (207)).

CHAPTER 2

NFIL3 EXPRESSION DISTINGUISHES TISSUE-RESIDENT NK CELLS AND CONVENTIONAL NK-LIKE CELLS IN THE MOUSE SUBMANDIBULAR GLANDS

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ABSTRACT

The submandibular salivary gland (SMG), a major site of persistent infection for many viruses, contains a large NK cell population. Using NFIL3-deficient mice, PLZF reporter/fate mapping mice, and mixed bone marrow chimeras, we identified two distinct populations of NK cells in the SMG. Although phenotypically unique, the main population relies on NFIL3 but not PLZF for development, and therefore is developmentally similar to the conventional NK cell subset. In contrast, we found that approximately one quarter of the SMG NK cells develop independently of NFIL3. Interestingly, NFIL3-independent SMG tissue-resident NK (trNK) cells are developmentally distinct from liver trNK cells. We also demonstrated that the SMG NK cell hyporesponsive phenotype during murine CMV infection is tissue specific, and not cell intrinsic. In contrast, NFIL3-independent SMG trNK cells are intrinsically hyporesponsive. Altogether, our data show that the SMG tissue environment shapes a unique repertoire of NK-like cells with distinct phenotypes.

INTRODUCTION

Conventional NK (cNK) cells are derived from the common lymphoid progenitor in the bone marrow (1). From there, they develop into committed NK cell precursors that develop into immature NK (iNK) cells upon acquisition of NK1.1 expression. iNK cells progress into mature NK cells with a $CD122^{+}NK1.1^{+}NKp46^{+}DX5^{+}$ phenotype. In addition to cNK cells, several distinct populations of tissue-resident NK (trNK) cells have been identified, with unique developmental pathways and phenotypic attributes (2-7). The liver contains a population of cNK cells, as well as a population of trNK cells (phenotypically similar to group 1 innate lymphoid cells [ILC1s]) that maintains a $CD49a^{+}DX5^{-}TRAIL^{+}$ phenotype and develops from a liver-specific precursor pool (3, 8). The skin also harbors a trNK/ILC1 subset, and there is evidence to indicate that skin and liver trNK cells arise from the same developmental lineage (3). Uterine NK cells are another unique population with a distinct phenotype from both cNK and liver/skin trNK cells. Uterine NK cells do not produce an effector or cytotoxic response during encounters with the invading trophoblast cells of the placenta, despite possessing the full complement of activating receptors and cytotoxic machinery (9-11). Thymic NK cells represent another population that develops from unique precursors; they are $Ly49^{low}CD11b^{low}$ and $CD127^{+}CD69^{high}$, in contrast to cNK cells (4, 5). A unique population of trNK cells also was discovered recently in the kidneys (6). The current understanding is that cNK cells, together with liver and skin trNKs (ILC1s), uterine NK cells, thymic NK cells, and kidney trNK cells, account for multiple distinct NK cell lineages (3, 7).

*NFIL3 (*also called *E4BP4)* is a basic leucine-zipper transcription factor that is linked to a number of immune processes and is crucial for the early development of cNK cells (12-14). In contrast, the different trNK cell subsets have unique developmental requirements. Although NFIL3 deficiency results in ablation of cNK cells in the periphery, its activity is mostly dispensable for the development of trNK cells in the liver (15), uterus, and skin (3), despite contrasting evidence that NFIL3 is necessary for the development of all innate lymphoid cell (ILC) lineages (16-19). T-bet and Eomes are also necessary, albeit at different levels, for the development of mature cNK cells (20), and there is evidence that these two transcription factors are regulated by NFIL3 (14). However, liver and skin trNK cells develop independently of Eomes, and uterine trNK cells do not require T-bet (3, 21).

The requirements of these transcription factors for the development of NK cells in the submandibular salivary gland (SMG) have not been clearly defined. In this study, using NFIL3-deficient mice, PLZF reporter/fate mapping mice, and mixed bone marrow chimeras, we show that the murine SMG contains two distinct populations of NK cells: a main cNK-like cell subset that relies on NFIL3 for development, and a smaller trNK cell subset that is NFIL3 independent. Our findings also demonstrate that SMG trNK cells represent another distinct ILC lineage with a unique developmental pathway. Importantly, using the murine CMV (MCMV) model of infection, we also show that the hyporesponsive phenotype of NFIL3-dependent SMG NK cells is due to tissue environmental factors, while NFIL3-independent SMG NK cells are intrinsically poor effector cells.

MATERIALS AND METHODS

Mice

C57BL/6, B6.SJL, *AhR*$^{-/-}$, and *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice were purchased from Taconic Biosciences (Germantown, NY). *T-bet*$^{-/-}$, R26R-EFYP, and PLZF^{GFPcre} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). R26R-EFYP mice were bred with PLZF^{GFPcre} mice to produce PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP mice. *NFIL3*$^{-/-}$ mice were a generous gift from Dr. Hugh JM Brady (13), and were bred in-house. All mice were maintained in pathogen free facilities at Brown University. Both sexes were included and no differences were observed.

Infection and treatment protocols

MCMV infections were carried out as previously described (2).

Isolation of murine lymphocytes

Mice were sacrificed with isoflurane, and cardiac puncture was performed prior to organ removal. Spleens were processed on the spleen01.01 program on a GentleMACS dissociator (Miltenyi Biotec), filtered through nylon mesh, and layered on Lympholyte-M (Cedarlane Laboratories Ltd., Canada). Lymphocytes were harvested from the gradient interface, and washed once in PBS supplemented with 1% FBS (1% PBS-serum). Livers were perfused with 1% PBS-serum before removal, processed in 1% PBS-serum on the E.01 program on the GentleMACS, and filtered through nylon mesh. The samples were washed 3 times with 1% PBS-serum, resuspended in 40% Percoll and layered on 70% Percoll. Lymphocytes were harvested from the gradient interface and washed once with 1% PBS-serum. SMGs were processed manually to remove lymph nodes, then processed in Collagenase IV (Sigma-Aldrich) on the heart01.01 program on the GentleMACS,

incubated at room temperature or 37°C for 10 minutes, filtered through nylon mesh, and washed once with 1% PBS-serum before being layered on a Lympholyte-M gradient. Lymphocytes were harvested from the gradient interface and washed once in 1% PBS-serum. We report that Ly49 markers and TRAIL are sensitive to Collagenase IV, leading to false negatives in some studies. SMGs can be processed without Collagenase in order to ascertain expression of these markers, but the number of lymphocytes recovered is very low. To circumvent this issue, we screened a variety of enzymes and identified Liberase-DL (Sigma-Aldrich), which does not affect these markers. Whenever the expression of these markers was assessed, Collagenase IV was replaced with Liberase-DL.

Flow cytometric analysis, antibodies, and reagents

Lymphocyte samples were incubated in 1% PBS-serum with the blocking monoclonal antibody (mAb) 2.4G2 and stained with specific mAbs for 20 minutes at 4°C. For intracellular cytokine staining, cells were first stained with extracellular mAbs, then fixed with Cytofix/Cytoperm (BD Bioscience) for 20 minutes, and then stained with intracellular mAbs in 1X PermWash (BD Biosciences) for 20 minutes. For intranuclear transcription factor staining, cells were stained with intracellular antibodies using the FoxP3 transcription factor staining reagents (BD Bioscience). Events were collected on a FACSAria (BD), and the data were analyzed using FlowJo (Tree Star Inc.). FITC-DX5, PE-Ly49H, PE-IFN-γ, PE-E4BP4, PE-TCRβ, PE-CD27, PE-NK1.1, PECy5-DX5, PECy7-NKp46, PECy7-T-bet, PECy7-Sca-1, PerCPCy5.5-CD127, PerCPCy5.5-NK1.1, PerCPeFluor710-NKG2A/C/E, allophycocyanin-CD3, allophycocyanin-Ly49H, allophycocyanin-IFN-γ, allophycocyanin-TNF-α, allophycocyanin-TRAIL,

allophycocyanin-CD45.1, allophycocyanin-eFluor780-CD45.2, allophycocyanin-eFluor780-CD117, eF450-CD11b, eFlour450-IFN-γ, eFlour450-CD3, eFlour450-Eomes, were purchased from eBioscience (San Diego, CA). PE-CD49a, APC-CD49a, Pacific Blue-Lineage, BV421-CD127, BV605-CD3, BV605-NK1.1, BV785-CD3, and BV785-NK1.1 were purchased from Biolegend (San Diego, CA). FITC-Ly49C/I was purchased from BD Pharmingen. FITC-DX5, PE-NK1.1, anti-CD5 magnetic beads, and anti-CD19 magnetic beads were purchased from Miltenyi Biotec. To detect E4BP4 by intracellular staining, activation of NK cells is required.

Generation of PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP bone marrow chimeras

B6.SJL recipient mice (CD45.1^{+}) were lethally irradiated with 1050 rad and placed on antibiotic treatment for two weeks. One day post-irradiation, donor bone marrow cells were harvested under sterile conditions from PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP mice (CD45.2^{+}/CD45.1^{+}), pooled, and stained for Lineage, Sca-1, and cKit. YFP^{-}Lin^{-}Sca-1^{+}cKit^{+} (LSK) cells were sorted and intravenously injected into recipients at approximately 10,000 cells/mouse. The recipients were allowed to reconstitute for at least 8 weeks.

Generation of mixed bone marrow chimeras

Recipient mice were lethally irradiated with 1050 rad and placed on antibiotic treatment for two weeks. One day post-irradiation, recipient mice were injected with a 1:1 mixture of either sorted LSK cells or DX5 and CD5-depleted cells from B6.SJL and *NFIL3*$^{-/-}$ bone marrow. The recipients were allowed to reconstitute for 8 weeks.

Adoptive transfer of NK cells

NK cells were sorted under sterile conditions from the spleen of B6 ($CD45.2^+$) mice, the SMG of B6.SJL ($CD45.1^+$) congenic mice, or the SMG of *NFIL3*$^{-/-}$ ($CD45.2^+$) mice. Donor NK cells were injected into recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. Recipient mice were allowed to reconstitute for 7 days before being infected intraperitoneally with 5×10^4 pfu MCMV. 38 hours post-infection, recipient mice were sacrificed for experiments.

Statistical Analysis

All statistical analyses were performed with Prism Version 7.0 (GraphPad Software). Unpaired two-tailed Student's t-tests were used to compare cell populations from different mice. Paired two-tailed Student's t-tests were used for experiments involving adoptive transfer or chimeric mice.

RESULTS

NFIL3 deficiency significantly reduces the frequency and number of SMG NK cells

Although cNK cells depend on NFIL3 for development, trNK cells in the liver, skin, kidneys, and uterus develop mostly independently of NFIL3 (3, 10). It also was reported recently that salivary gland NK cells develop entirely independently of NFIL3 activity (22, 23). In contrast to these findings, we found a significant reduction in the frequency (Fig. 1A, 1B) and number (Fig. 1C) of SMG NK cells in *NFIL3*$^{-/-}$ mice. Intracellular staining with an anti-NFIL3 antibody also shows that a large subset of SMG NK cells express NFIL3 (Fig. 2A). Salivary gland structural development continues for several weeks after birth and is completed when the mice are 10-12 wk old (24, 25). One possible explanation for the discrepancy with previous findings is that NFIL3-dependent NK cells initially seed the SMG and are later replenished by NFIL3-independent trNK cells. However, we found that *NFIL3*$^{-/-}$ mice have a significant decrease in SMG NK cell frequency compared to *NFIL3*$^{+/+}$ mice with similar phenotype, regardless of their age (Fig. 2B, 2C).

To unequivocally determine the origin of these two subsets, we generated PLZF reporter/fate mapping mice by crossing PLZF$^{GFPcre+/-}$ reporter mice with mice carrying the ROSA26-floxstop-YFP fate-mapping allele, as described recently (26). Bendelac and colleagues reported that most ILCs, including ILC1 but not cNK cells, are YFP positive in these mice (26). It should be noted that, in the resulting mice (PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP), ~30% of the cells become YFP positive before hematopoiesis (26)(data not shown). To circumvent this issue, we generated chimeric mice reconstituted with sorted YFP^{-} LSK bone marrow precursors (26) (Supplemental Fig. 1A). In agreement

with previous findings (26), iNKT cells express YFP in the chimeric mice (Supplemental Fig. 1B), whereas B cells and conventional T cells are mostly unlabeled (Fig. 3A). In the liver, ~60% of trNK cells were YFP^+, whereas only ~ 20% of the cNK cells were labeled (Fig. 3B), which is in agreement with previous studies (26). Importantly, in the SMG of these mice, approximately 90% of the NK cells were YFP^-, indicating that they originated from the cNK lineage (Fig. 3C). Interestingly, the remaining YFP^+ NK cells had lower DX5 expression than the YFP^- SMG NK cells, consistent with a possible NFIL3 independency (Fig. 3D). To exclude potential extrinsic factors, which could explain the difference between this study and the one by Cortez et al. (22), we also generated B6.SJL/*NFIL3*$^{-/-}$ mixed bone marrow chimeras (Supplemental Fig. 1C). We found that ~90% of SMG NK cells were derived from B6.SJL donor bone marrow in all organs tested, including the SMG (Fig. 4A). In contrast, non-NK lymphocytes were derived evenly from B6.SJL and *NFIL3*$^{-/-}$ donor bone marrow (Fig. 4B). Altogether, these data demonstrate that the SMG harbors at least two populations of NK cells: a preponderant NFIL3-dependent population that is developmentally similar to the cNK lineage, and an NFIL3-independent tissue-resident population.

SMG trNK cells are phenotypically and developmentally different from liver trNK cells

We next examined whether SMG trNK cells were developmentally related to liver trNK cells (3). In the liver, cNK cells develop under the coordinated influence of the transcription factors NFIL3, T-bet, and Eomes, whereas the trNK population develops independently of NFIL3 and Eomes but requires T-bet (3). Moreover, there is a clear

distinction between Eomes$^+$ cNK and Eomes$^-$ trNK cells, with NFIL3 deficiency causing a bias toward liver Eomes$^-$ trNK cells (Fig. 5A) (15). In contrast, in the SMG of wild-type mice, a large proportion of the NK cells are Eomes$^+$T-bet$^+$, and their relative frequency in *NFIL3*$^{-/-}$ animals remains mostly unchanged (Fig. 5A). Liver NK cells are also clearly divided between DX5$^+$CD49a$^-$ cNK and DX5$^-$CD49a$^+$ trNK, while the majority of SMG NK cells are CD49a$^+$, regardless of DX5 expression (Fig. 5B). In addition, NFIL3-independent Eomes$^-$ liver trNK cells are mostly DX5$^-$, whereas >50% of the NK cells in the SMGs of wild-type and *NFIL3*$^{-/-}$ mice are DX5$^+$ (Figure 5C).

With regard to their effector functions, it was shown that liver Eomes$^-$DX5$^-$ NFIL3-independent trNK cells express TRAIL (3, 20, 21, 27). Similarly, we found that ~30% of SMG NK cells constitutively express TRAIL, independently of NFIL3 expression (Fig. 6A). However in contrast to the liver, TRAIL expression does not mark a specific subset of cells (i.e DX5$^-$Eomes$^-$), because the SMG TRAIL$^+$ NK cells are mostly DX5$^+$Eomes$^+$. In *NFIL3*$^{-/-}$ animals, the DX5$^+$Eomes$^+$TRAIL$^+$ SMG NK cell population is retained (data not shown). Altogether, these data demonstrate that the NFIL3-independent population of NK cells in the SMG is distinct from the liver trNK cells with regard to cell surface phenotype and developmentally.

The SMG does not contain Group 3 ILCs

Having identified a novel population of NFIL3-independent NK cells in the SMG, we sought to determine whether group 3 ILCs were also present in this organ. Group 3 ILCs develop under the influence of both RORγt and aryl hydrocarbon receptor (AhR) (28). Therefore, we examined whether the absence of AhR affects the development of

SMG lymphocytes. We did not find NKp46^{+}CD127^{+}NK1.1^{-} lymphocytes in the SMG from either B6 or *AhR*$^{-/-}$ mice (Fig. 6B). However, we found, as previously reported (28), that these cells are reduced in the lamina propria of *AhR*$^{-/-}$ mice (Fig. 6B). These results are consistent with our previous findings showing that no significant number of RORγt^{+} cells were found in this organ using RORγt reporter mice (2).

SMG cNK-like cell hyporesponsiveness is dependent on the tissue environment, while SMG trNK cells are intrinsically hyporesponsive

We (2) and other investigators (22) showed that SMG NK cells are hyporesponsive during MCMV infection. Having identified two subsets of NK cells in this organ, we sought to revisit these findings and examine whether this phenotype was reversible. To determine whether SMG NK cells were capable of producing an effector response in new tissue environments, NK cells were sorted from the SMGs of B6.SJL mice (CD45.1^{+}) and the spleens of C57BL/6 mice (CD45.2^{+}). The sorted NK cells were mixed in a 1:1 ratio and adoptively transferred into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. The adoptively transferred NK cells were allowed to reconstitute for 7 d before the mice were infected i.p. with MCMV. We found that the frequency of the two donor populations was unchanged and roughly at a 1:1 ratio (Fig. 7A). Importantly, at 38 h postinfection, the magnitude of the IFN-γ response from the spleen and SMG-derived NK cells was comparable (Fig. 7B). This result indicates that the hyporesponsive phenotype seen in SMG NK cells *in situ* is not cell-intrinsic but is caused by properties of the SMG microenvironment. To assess the effector capacity of SMG NFIL3-independent trNK cells, SMG trNK cells were sorted from *NFIL3*$^{-/-}$ mice and adoptively transferred into

Rag2$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. We found that *NFIL3*$^{-/-}$ SMG NK cells produced significantly less IFN-γ than did B6.SJL SMG NK cells under the same conditions (Fig. 7C). Therefore, in contrast with NFIL3-dependent SMG NK cells, NFIL3-independent trNK cells appear not to respond optimally to MCMV infection, regardless of the tissue environment.

DISCUSSION

Although NK cells have been studied and characterized for decades, our understanding of their developmental pathways is still incomplete. cNK cells were discovered decades ago, but the last 10 y have seen the emergence of several new classes of trNK cells, each with unique properties and developmental pathways. Moreover, NK cells as a whole comprise one subset of ILCs, a diverse group of immune lymphocytes that may represent the innate analog of T cells (29). In this article, we show that the SMG in naïve C57BL/6 mice contains at least two distinct populations of NK cells. Although phenotypically unique (most likely due to their tissue location), the main population of SMG NK cells expresses Eomes and T-bet and requires NFIL3 for development, indicating that they are developmentally similar to cNK cells. The remaining NK cells in this organ develop independently of NFIL3; therefore, they can be classified as trNK cells, yet they are phenotypically different from the recently described resident NK cells in other organs. Although our results are in agreement with recent reports from Colonna's group (22, 23) regarding the phenotype and the functions of the SMG NK cells, they differ on the NFIL3 dependence. The differences between these two studies might be explained by variations in extrinsic parameters, such as microbiota or housing-dependent inflammation. In fact, the identification of an MCMV-driven population of peripheral NK cells in *NFIL3*$^{-/-}$ mice supports this possibility (30). However, we found only residual seeding of *NFIL3*$^{-/-}$-derived NK cells in the salivary glands in mixed bone marrow chimeras (Fig. 4), ruling out host-derived extrinsic factors independent of microbiota. In addition, we detected NFIL3 protein in SMG NK cells (Fig. 2A) from wild-type animals, and ~90% of CD3^{-}NK1.1^{+}NKP46^{+} cells never express PLZF during their development

(Fig. 3C). Altogether, these data advocate for the presence of cNK-like cells in this organ. In support of this conclusion, a recent report showed that the transcription factor Runx3 similarly affects splenic cNK and SMG NK cells (31).

Although NFIL3-independent SMG trNK cells share similarity with other trNK cells, they have unique phenotypic and effector characteristics. In contrast to the liver and skin trNK cells, but similarly to uterine trNK cells, SMG trNK cells from NFIL3-deficient animals express both DX5 and Eomes. Several transcription factors are known to play critical roles during ILC development. A committed $\alpha4\beta7^{+}PLZF^{+}$ precursor to all helper-like ILCs (excluding cNK and LTi) was identified by Bendelac and colleagues (26, 32), while the Diefenbach group (32) identified the common helper-like innate lymphoid precursor as $\alpha4\beta7^{+}ID2^{high}$. The development of this common ILC precursor is dependent on *NFIL3*, which directly regulates *Id2* to promote the development of the common helper-like innate lymphoid precursor from the common lymphoid progenitor (19). Under this paradigm, the NFIL3-independent NK cells of the SMG would represent yet another ILC subset, independent from helper-like ILC1s. In addition to cNK-like cells and NFIL3-independent trNK cells, other small $NK1.1^{+}$ subsets are found in the SMG. This includes a subset of NK cells strictly dependent on T-bet and similar to liver trNK cells (Supplemental Fig. 1D, see gate $DX5^{-}CD49a^{+}$). In addition, a subset of T cells not detected in B6 mice can be observed in the SMG of NFIL3-deficient animals (Supplemental Fig. 1E). Although the characterization of these $CD3^{+}NK1.1^{+}NKP46^{+}$ T cells is beyond the scope of this article, our preliminary data indicate that these T cells are not semi-invariant iNKT cells (data not shown).

We also showed previously that SMG NK cells are hyporesponsive to MCMV infection, both *in situ* and during *in vitro* cytokine stimulation assays (2). However, we show in this study that when wild-type SMG NK cells are isolated from their native environment and allowed to reconstitute in peripheral tissues, they regain the ability to produce an effector response to MCMV. cNK cell effector plasticity was reported in other contexts (33, 34). The ability of the SMG NK cells (Fig. 7) and splenic NK cells (33, 34) to regain effector functions reinforces our finding that the majority of the NK cells (~75%, Fig. 1) in the B6 SMG are developmentally and functionally similar to cNK cells. These findings also indicate that environmental factors in the SMG influence NK cell effector potential. A role for TGF-β, which is 100-fold more abundant in the SMG than in the spleen, was demonstrated recently in the salivary glands (23). The phenotypic change induced by TGF-β appears to be reversible. Indeed, addition of TGF-β to splenic NK cells induces them to differentiate into tissue resident-like NK cells, whereas blocking TGF-β signaling (23), or SMG NK cell relocation into low TGF-β environments (Figure 7) reinstates their effector functions. Cortez et al. (23) proposed that TGF-β drives the progressive differentiation of $CD49a^-$ NFIL3 dependent SMG ILCs into $CD49a^+$ NFIL3 independent mature SMG ILCs. Although our data and their data support a linear differentiation model for SMG NK cells, it is unclear how NFIL3-dependent SMG NK cells become independent in this model. Instead, we propose this model is better explained by the existence of two distinct populations in this organ.

NK cells also were shown to limit salivary gland inflammation and tissue damage during MCMV infection (35), indicating that they play an immunoregulatory role. Recent studies have begun to address whether NK cell regulation occurs via a viral load decrease

or is mediated by a more complex mechanism. In support of the second possibility, a recent report demonstrated that $TRAIL^+$ NK cells in the SMG specifically eliminate $CD4^+$ T cells, which are critical for the clearance of active MCMV from the salivary glands (27). The investigators argue that NK cell-mediated T cell killing would prolong MCMV infection but also reduce inflammatory damage to the delicate SMG tissues, allowing the virus to be cleared slowly without causing irreversible damage to the host. Our data add to these findings and show that NK cells are rendered hyporesponsive by the salivary gland environment, presumably benefiting the host.

AUTHORSHIP

T.K.E. conceived, performed, and analyzed the experiments and wrote the paper. C.K.A conceived, performed, and analyzed the experiments. E.C.R. conceived, performed, and analyzed the experiments. J.RW. contributed reagents and analysis tools. L.B. conceived and analyzed the experiments, and wrote the paper.

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FIGURE LEGENDS

Figure 1. SMG CD3$^-$NK1.1$^+$ cells are significantly reduced in *NFIL3*$^{-/-}$ mice. (A) Representative staining of spleen, liver, and SMG NK cells in *NFIL3*$^{+/+}$ and *NFIL3*$^{-/-}$ mice. **(B)** Frequency of SMG NK cells in *NFIL3*$^{+/+}$ (n=23), *NFIL3*$^{+/-}$ (n=23) and *NFIL3*$^{-/-}$ (n=22) mice. **(C)** Absolute SMG NK cell number in *NFIL3*$^{+/+}$ (n=14), *NFIL3*$^{+/-}$ (n=14), and *NFIL3*$^{-/-}$ (n=13) mice. Black circles/bars represent *NFIL3*$^{+/+}$ mice; grey circles/bars represent *NFIL3*$^{+/-}$ mice; white circles/bars represent *NFIL3*$^{-/-}$ mice. Data are pooled from nine experiments and depicted as mean ± SEM. ****$p < 0.0001$, ***$p = 0.0001$-0.001, **$p = 0.001$-0.01.

Figure 2. SMG NK cells express E4BP4 protein and their number is reduced in aged mice. (A) Representative intracellular E4BP4 staining in the NK cells of the spleen, liver, and SMG of *NFIL3*$^{+/+}$ and *NFIL3*$^{-/-}$ mice. To detect E4BP4 by intracellular staining, activation of NK cells is required and mice had been infected with MCMV for 38 hours. Data are representative of two experiments. **(B)** Frequency of NK cells in the SMG of *NFIL3*$^{+/+}$ mice and *NFIL3*$^{-/-}$ mice at different ages. Data are pooled from two experiments and are depicted as mean ± SEM. ****$p < 0.0001$, **$p = 0.001$-0.01. **(C)** Representative staining of DX5 and CD49a expression on SMG NK cells from *NFIL3*$^{+/+}$ mice and *NFIL3*$^{-/-}$ mice at different ages.

Figure 3. PLZF reporter/fate mapping mice demonstrate that SMG CD3$^-$NK1.1$^+$ cells are mostly conventional NK cells. (A) YFP expression by indicated cell types in the spleen of PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP bone marrow chimeras. **(B)** YFP

expression by cNK and trNK cells from PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP chimeras. **(C)** YFP expression by CD3$^-$NK1.1$^+$ cells in the spleen, liver, and SMG of PLZF$^{GFPcre+/-}$ ROSA26-floxstop-YFP chimeras. **(D)** Representative staining of DX5 and CD49a expression on YFP$^+$ and YFP$^-$ SMG NK cells from PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP chimeras. Data are representative of three experiments. Four chimeric mice were pooled per experiment.

Figure 4. SMG NK cells originate preponderantly from NFIL3$^+$ bone marrow. (A) Percent of spleen, liver, and SMG NK cells derived from donor B6.SJL and *NFIL3*$^{-/-}$ bone marrow. **(B)** Percent of spleen, liver, and SMG non-NK cells derived from donor B6.SJL and *NFIL3*$^{-/-}$ bone marrow. Data are pooled from two experiments. Four chimeric mice were analyzed individually per experiment. Data are represented as mean ± SEM. ****$p < 0.0001$.

Figure 5. SMG trNK cells have a unique phenotype compared to liver trNK cells. **(A)** Representative staining of T-bet and Eomes expression on spleen, liver, and SMG NK cells from wild-type control (C57BL/6 or *NFIL3*$^{+/+}$ littermate controls) and *NFIL3*$^{-/-}$ mice. Data are representative of four experiments. 2-3 mice were pooled in each experiment. **(B)** Representative staining of DX5 and CD49a expression on spleen, liver, and SMG NK cells from wild-type control (C57BL/6 or *NFIL3*$^{+/+}$ littermate controls) and *NFIL3*$^{-/-}$ mice. Data are representative of four experiments. 2-3 mice were pooled in each experiment. **(C)** Representative staining of DX5 and Eomes expression in spleen, liver, and SMG NK cells from wild-type control (C57BL/6 or *NFIL3*$^{+/+}$ littermate controls) and

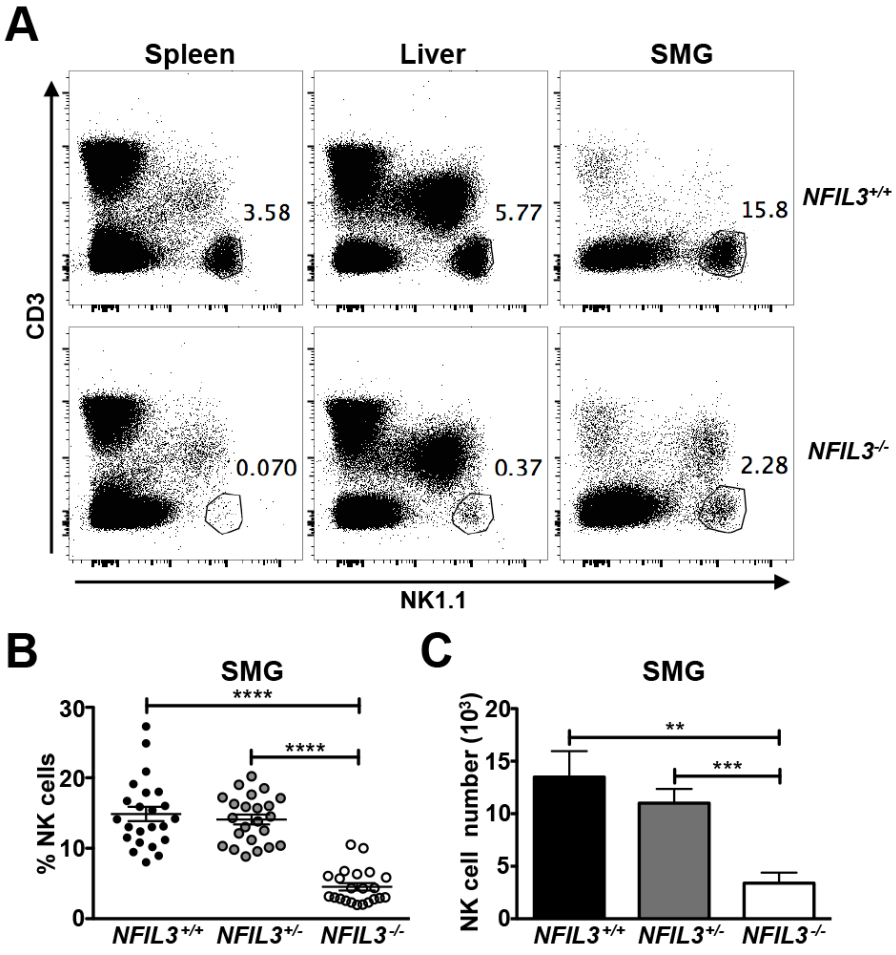
NFIL3$^{-/-}$ mice. Data are representative of four experiments. 2-3 mice were pooled in each experiment. Spleens from *NFIL3*$^{-/-}$ mice were enriched for NK cells using anti-CD5 and anti-CD19 magnetic beads.

Figure 6. SMG trNK cells are less mature compared to cNK-like cells. (A) Representative staining of TRAIL expression on NK cells from the spleen, liver, and SMG of wild-type control (C57BL/6 or *NFIL3*$^{+/+}$ littermate controls) and *NFIL3*$^{-/-}$ mice. Spleens from *NFIL3*$^{-/-}$ mice were enriched for NK cells using anti-CD5 and anti-CD19 magnetic beads. Data are representative of three experiments. 2-3 mice were pooled in each experiment. **(B)** Representative staining of CD127 and NK1.1 on NKp46^{+} lymphocytes in the SMG and lamina propria of C57BL/6 and *AhR*$^{-/-}$ mice. Data are representative of three experiments. Three mice were pooled in each experiment.

Figure 7. SMG cNK-like cell hyporesponsiveness but not the SMG trNK cell hyporesponsiveness can be reversed by tissue environment. (A) NK cell frequency from the two different donors. **(B)** Frequency of donor IFN-γ^{+} B6 splenic derived NK cells and IFN-γ^{+} B6.SJL SMG derived NK cells in the spleen and liver of recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, 38 hours after MCMV infection. **(C)** Frequency of donor IFN-γ^{+} SMG NK cells from B6.SJL and *NFIL3*$^{-/-}$ mice in the spleen and liver of recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, 38 hours after MCMV infection. C57BL/6 + B6.SJL data are pooled from seven experiments. *NFIL3*$^{-/-}$ data are pooled from five experiments. Two recipient *Rag2*$^{-/-}$ *IL-2Rγ*$^{-/-}$ mice were pooled in each experiment. Data are represented as mean ± SEM. ***p* = 0.001-0.01.

Supplementary Figure 1. (A) Diagram representing the generation of PLZF$^{GFPcre+/-}$ ROSA26-floxstop-YFP chimeras. **(B)** YFP expression by liver iNKT cells from PLZF$^{GFPcre+/-}$ROSA26-floxstop-YFP chimeras. **(C)** Diagram representing the generation of B6.SJL/*NFIL3*$^{-/-}$ mixed bone marrow chimeras. **(D)** Representative staining of DX5 and CD49a on NK cells in the spleen, liver, and SMG of C57BL/6 and *T-bet*$^{-/-}$ mice. Spleens from *T-bet*$^{-/-}$ mice were enriched for NK cells using anti-CD5 and anti-CD19 magnetic beads. Data are representative of two experiments. Three mice were pooled in each experiment. **(E)** A CD3^{+}NK1.1^{+}NKP46^{+} T cell subset is revealed in the *NFIL3*$^{-/-}$ SMG.

Figure 1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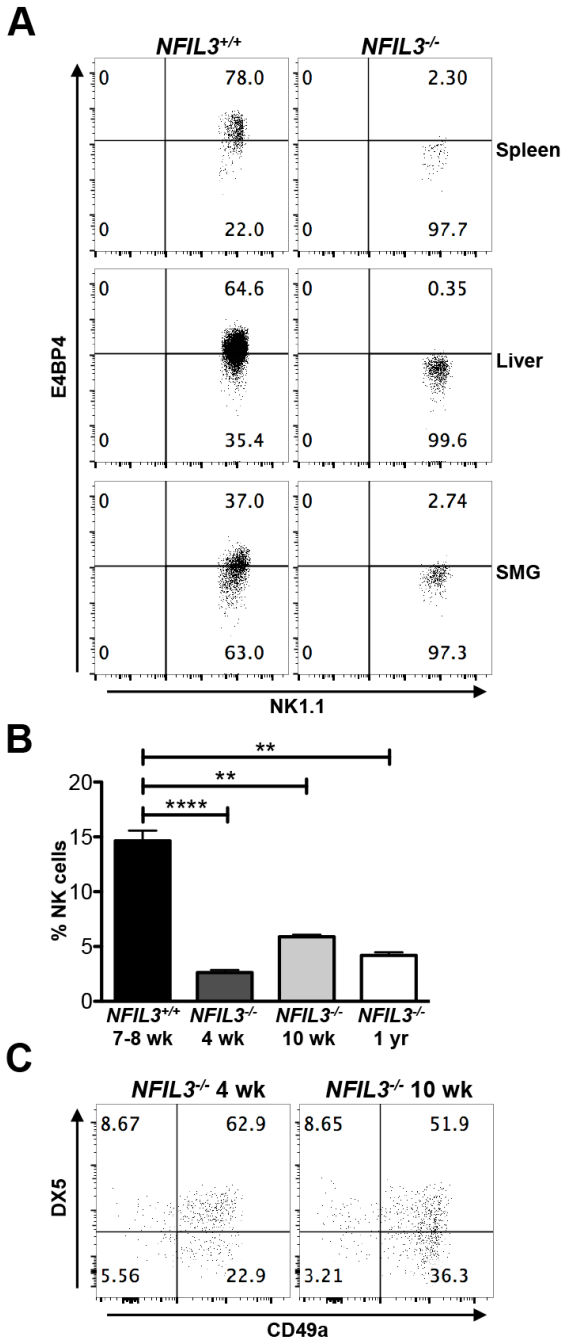
Spleen
Liver
SMG
3.58
5.77
15.8
NFIL3+/+
CD3
0.070
0.37
2.28
NFIL3-/-
NK1.1
B
SMG
****
****
% NK cells
30
20
10
0
NFIL3+/+
NFIL3+/-
NFIL3-/-
C
SMG
**
***
NK cell number (10³)
20
15
10
5
0
NFIL3+/+
NFIL3+/-
NFIL3-/-

Figure 2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NFIL3+/+
NFIL3-/-
78.0
2.30
22.0
97.7
Spleen
64.6
0.35
35.4
99.6
Liver
37.0
2.74
63.0
97.3
SMG
E4BP4
NK1.1
B
**
**
****
20
15
10
5
0
% NK cells
NFIL3+/+ 7-8 wk
NFIL3-/- 4 wk
NFIL3-/- 10 wk
NFIL3-/- 1 yr
C
NFIL3-/- 4 wk
NFIL3-/- 10 wk
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22.9
3.21
36.3
DX5
CD49a

Figure 3

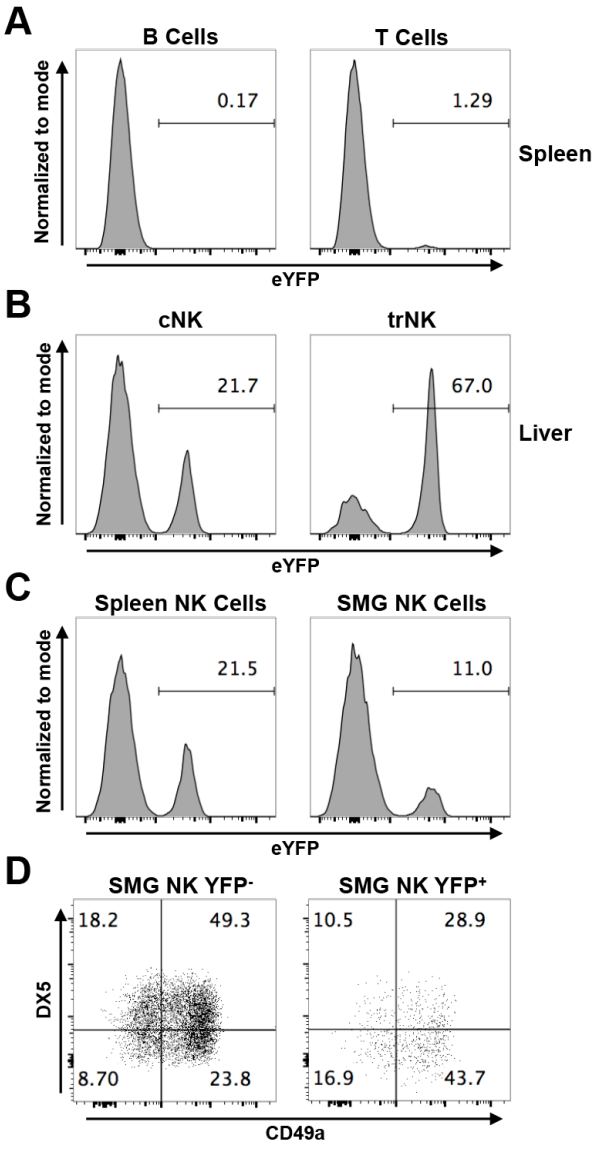

A
B Cells
T Cells
0.17
1.29
Spleen
Normalized to mode
eYFP
B
cNK
trNK
21.7
67.0
Liver
Normalized to mode
eYFP
C
Spleen NK Cells
SMG NK Cells
21.5
11.0
Normalized to mode
eYFP
D
SMG NK YFP-
SMG NK YFP+
18.2
49.3
10.5
28.9
8.70
23.8
16.9
43.7
DX5
CD49a

Figure 4

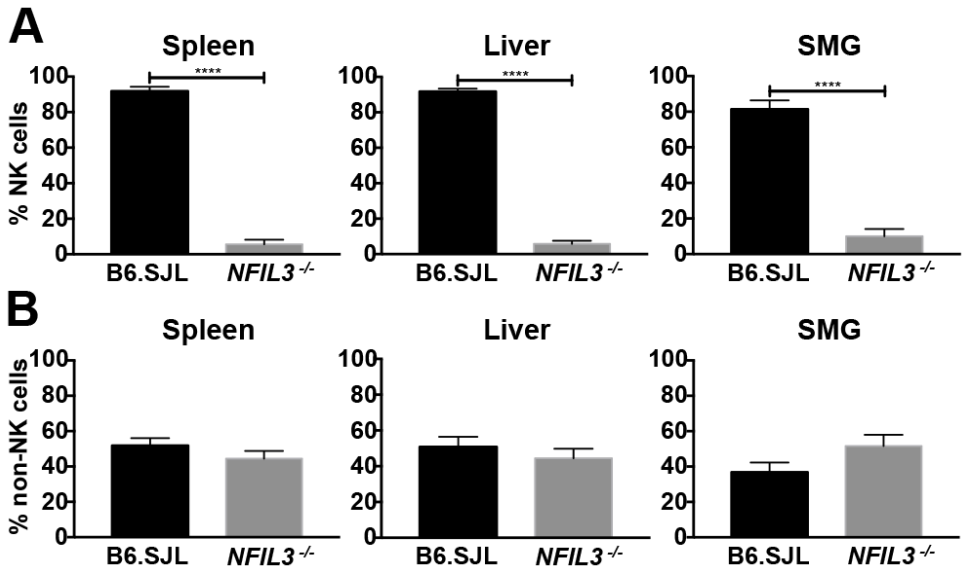

A
Spleen
Liver
SMG
% NK cells
100
80
60
40
20
0
****
B6.SJL
NFIL3 -/-
B
% non-NK cells

Figure 5

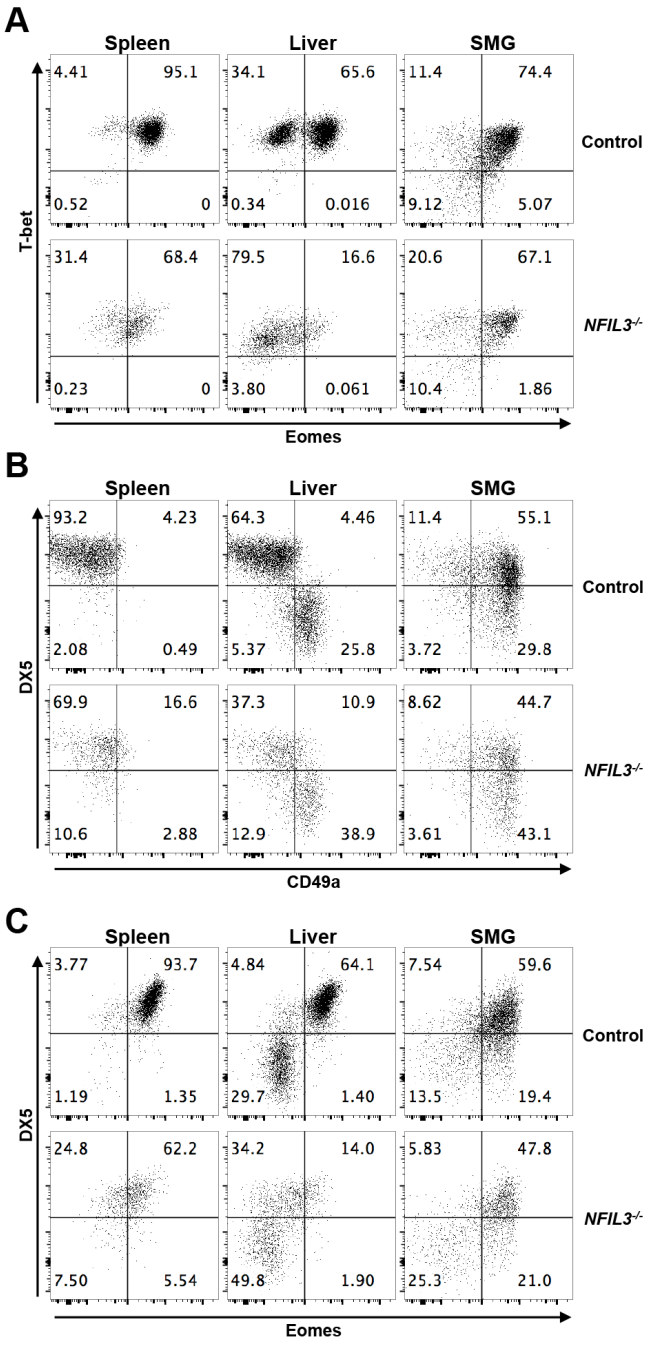

A
Spleen
Liver
SMG
4.41
95.1
34.1
65.6
11.4
74.4
Control
0.52
0
0.34
0.016
9.12
5.07
31.4
68.4
79.5
16.6
20.6
67.1
NFIL3-/-
0.23
0
3.80
0.061
10.4
1.86
T-bet
Eomes
B
Spleen
Liver
SMG
93.2
4.23
64.3
4.46
11.4
55.1
Control
2.08
0.49
5.37
25.8
3.72
29.8
69.9
16.6
37.3
10.9
8.62
44.7
NFIL3-/-
10.6
2.88
12.9
38.9
3.61
43.1
DX5
CD49a
C
Spleen
Liver
SMG
3.77
93.7
4.84
64.1
7.54
59.6
Control
1.19
1.35
29.7
1.40
13.5
19.4
24.8
62.2
34.2
14.0
5.83
47.8
NFIL3-/-
7.50
5.54
49.8
1.90
25.3
21.0
DX5
Eomes

Figure 6

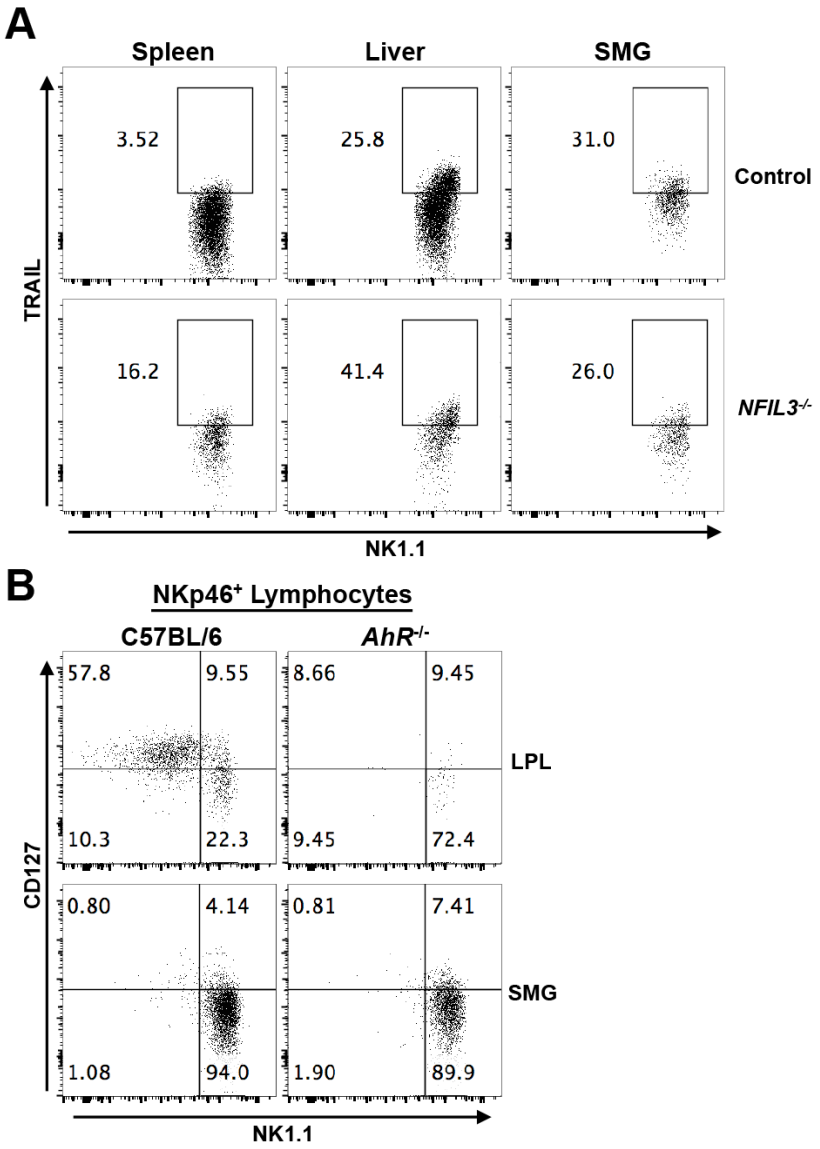
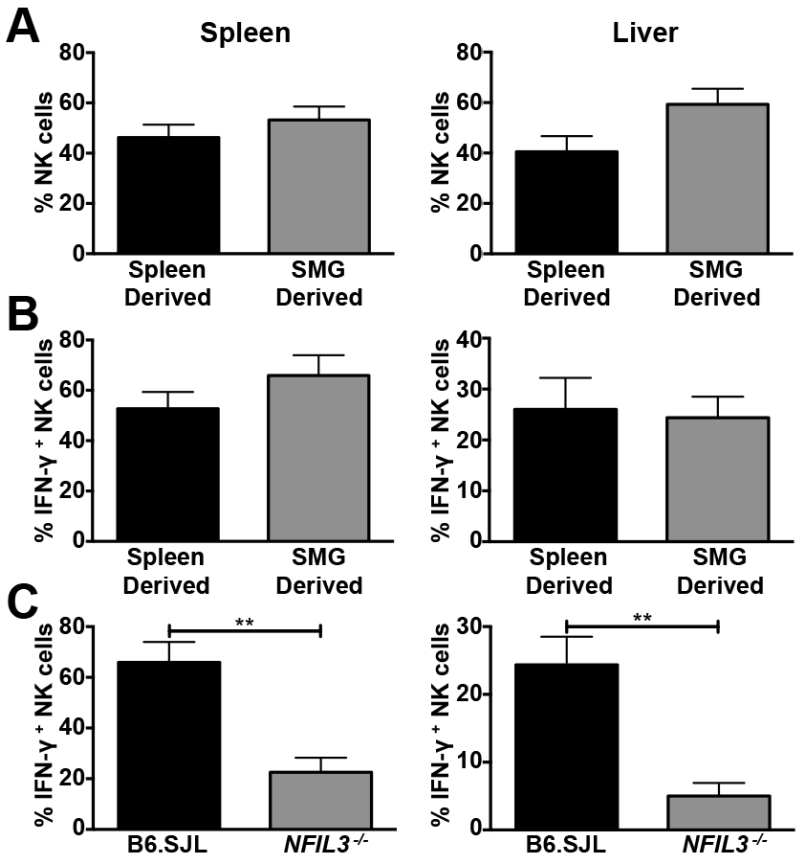
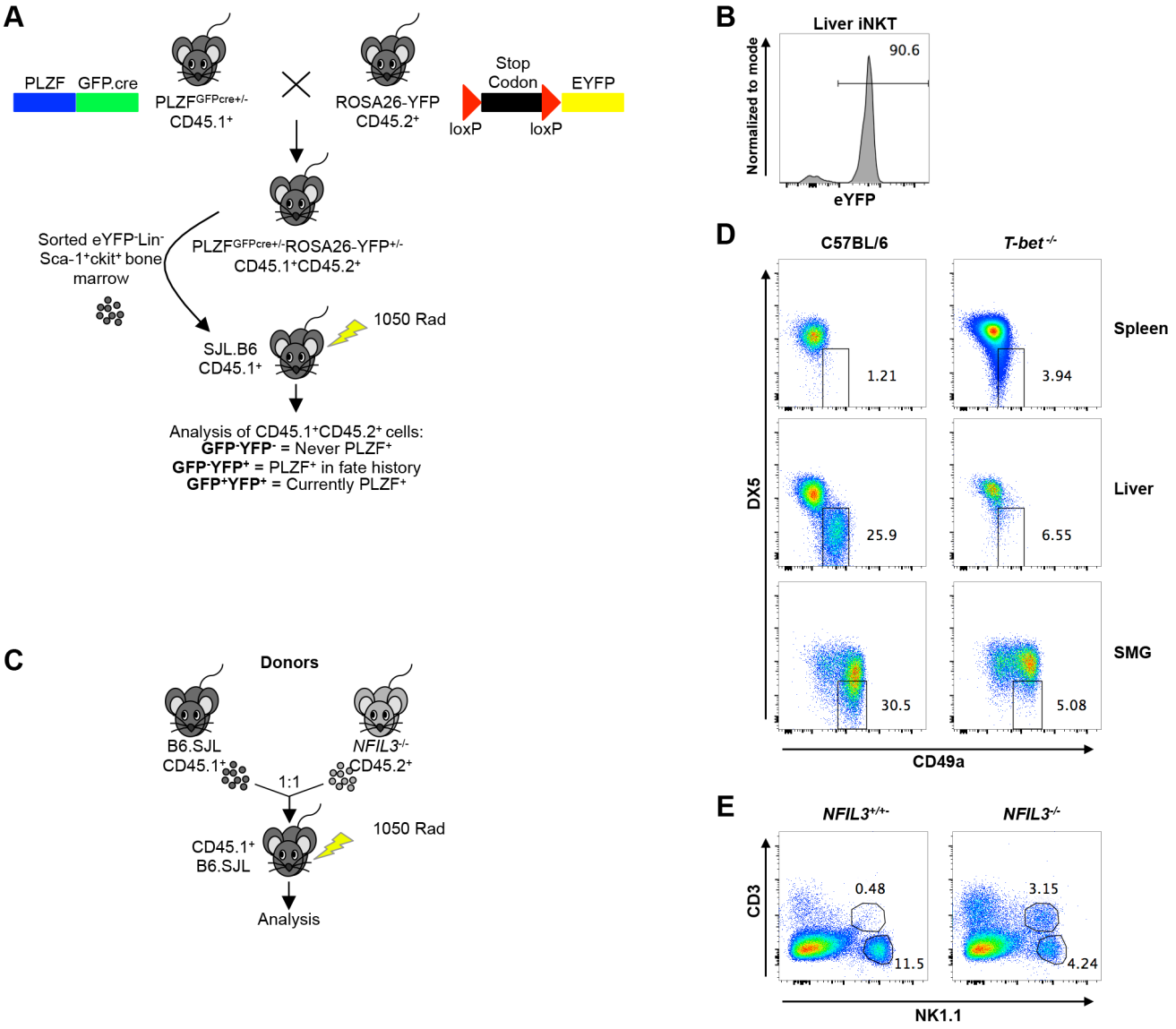

A
Spleen
Liver
SMG
3.52
25.8
31.0
Control
16.2
41.4
26.0
NFIL3-/-
TRAIL
NK1.1
B
NKp46+ Lymphocytes
C57BL/6
AhR-/-
57.8
9.55
8.66
9.45
LPL
10.3
22.3
9.45
72.4
0.80
4.14
0.81
7.41
SMG
1.08
94.0
1.90
89.9
CD127
NK1.1

Figure 7


A
Spleen
Liver
% NK cells
80
60
40
20
0
Spleen Derived
SMG Derived
B
% IFN-γ+ NK cells
80
60
40
20
0
40
30
20
10
0
Spleen Derived
SMG Derived
C
**
% IFN-γ+ NK cells
80
60
40
20
0
30
20
10
0
B6.SJL
NFIL3-/-

Supplementary Figure 1



CHAPTER 3

NFIL3-INDEPENDENT CD3$^+$NK1.1$^+$ LYMPHOCYTES OF THE SUBMANDIBULAR SALIVARY GLAND

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ABSTRACT

Nuclear factor, IL-3-regulated (NFIL3), is a transcription factor that is involved in many immune processes, including the development of conventional NK cells and several helper-like innate lymphoid cell (ILC) subsets. However, some tissue-resident NK cells and other ILC subsets persist in *NFIL3*$^{-/-}$ mice. Here we show that NFIL3 knockout results in the appearance of a novel CD3^{+}NK1.1^{+} lymphocyte population in the murine submandibular salivary gland (SMG). The SMG CD3^{+}NK1.1^{+} cells are mostly CD49a^{+} and T-bet^{+}, yet they are negative for most NK cell markers. They are also not invariant NKT (iNKT) cells, as evidenced by a lack of α-GalCer-loaded CD1d-tetramer binding. Furthermore, they mostly express TCRβ, while they lack expression of CD4, and most are also negative for CD8 and TCRγδ. When transferred into lymphopenic mice, the SMG CD3^{+}NK1.1^{+} cells repopulate in the spleen and liver, and produce the pro-inflammatory cytokine IFN-γ during the early stages of murine cytomegalovirus (MCMV) infection.

INTRODUCTION

NFIL3, also called E4BP4, is a basic leucine zipper transcription factor that is involved in the development, maintenance, and regulation of a variety of immune cell types (1). NFIL3 was first identified as E4BP4 due to its role as a transcriptional repressor that binds an activating transcription factor DNA consensus sequence in the adenovirus E4 promoter (2). It was independently identified as NFIL3, a transactivator of the *IL3* promoter in human T cells (3). More recently, NFIL3 was found to be a crucial factor in the development of $CD8\alpha^+$ dendritic cells (DCs), but not $CD8\alpha^-$ DCs, plasmacytoid DCs, or DC progenitors (4). In B cells, NFIL3 is necessary for immunoglobulin (Ig) heavy chain class switching to IgE (5). NFIL3 may also be involved in the regulation of cytokine production by bone marrow-derived macrophages (BMDMs). BMDMs in *NFIL3*$^{-/-}$ mice produce more IL-12 in response to lipopolysaccharide (LPS) than in wild-type mice (6). NFIL3 is also involved the regulation of cytokine production by T_H1 cells, T_H2 cells, T_{regs}, and NKT cells (7, 8).

Perhaps the most striking aspect of NFIL3 is its variable involvement in the development of different innate lymphoid cell (ILC) subsets. NFIL3 is crucial for conventional NK (cNK) cell development (9, 10) but not maintenance (11). NFIL3 binds to the regulatory regions of the transcription factors Id2 and Eomes and promotes their transcription during the point of cNK cell commitment, when cNK cell precursors diverge from the common lymphoid progenitor (CLP) in the bone marrow (12). Recent work has also shown that NFIL3 is required for the development of the common helper-like innate lymphoid cell precursor (CHILP) (13) and all downstream helper-like ILC subsets (14). However, tissue-resident NK (trNK) cells in the liver, skin (15), kidney

(16), uterus (17) and submandibular salivary gland (SMG) (18, 19) have been shown to develop at least partially independently of NFIL3. This may reflect differences in development between trNK cells and other helper-like ILC1 subsets (20).

In this chapter, we characterize a novel $CD3^+NK1.1^+$ lymphocyte subset that appears in the SMG of *NFIL3*$^{-/-}$ mice. These cells have variable NKp46 expression, are mostly $DX5^-$ and $Eomes^-$, and express high levels of CD49a and T-bet. A lack of binding to α-GalCer-loaded CD1d-tetramer revealed that these $CD3^+NK1.1^+$ cells are not invariant NKT (iNKT) cells. They are also mostly $TCR\beta^+$ and $CD4^-$. Minor subsets of these cells are $CD8\alpha\alpha^+$, $CD8\alpha\beta^+$, and $TCR\gamma\delta^+$. Adoptive transfer experiments in lymphopenic mice demonstrated that the NFIL3-independent $CD3^+NK1.1^+$ cells populate the spleen and liver, and produce IFN-γ during the early stages of MCMV infection. Altogether, these findings show that loss of NFIL3 can cause previously unreported changes in the composition of SMG lymphocytes.

MATERIALS AND METHODS

Mice

C57BL/6 and *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice were purchased from Taconic Biosciences (Germantown, NY). *Tbx21*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *NFIL3*$^{-/-}$ mice were a generous gift from Dr. Hugh JM Brady (9). *NFIL3*$^{+/+}$, *NFIL3*$^{+/-}$, and *NFIL3*$^{-/-}$ mice were subsequently bred in-house. All mice were maintained in pathogen free facilities at Brown University. Both sexes were included and no differences were observed.

Infection and treatment protocols

Mice were infected i.p. with $5*10^4$ PFU RVG102 strain MCMV, as previously described (21).

Isolation of murine lymphocytes

Mice were sacrificed with isoflurane, and cardiac puncture was performed prior to organ removal. Submandibular glands were processed in Collagenase IV (Sigma-Aldrich) or Liberase-DL (Sigma-Aldrich) with a GentleMACS dissociator (Miltenyi Biotec), incubated at 37°C for 10 minutes, filtered through nylon mesh, and washed once with 1% PBS-serum before being layered on a Lympholyte-M gradient. Lymphocytes were harvested from the gradient interface and washed once in 1% PBS-serum.

Flow cytometry antibodies, reagents, and analysis

Lymphocyte samples were incubated in 1% PBS-serum with the blocking monoclonal antibody (mAb) 2.4G2 and stained with specific mAbs for 20 minutes at 4°C. For intracellular cytokine staining, cells were first stained with extracellular mAbs, then fixed with Cytofix/Cytoperm (BD Bioscience) for 20 minutes, and then stained with

intracellular mAbs in 1X PermWash (BD Biosciences) for 20 minutes. For intranuclear transcription factor staining, cells were fixed and permeabilized prior to intranuclear staining using the FoxP3 transcription factor staining reagents (BD Bioscience). Events were collected on a FACSAria (BD), and the data were analyzed using FlowJo (FlowJo LLC). FITC-TCRβ, FITC-CD3, FITC-CD27, PE-TCRγδ, PE-CD8β.2, PE-CD27, PE-IFN-γ. PE-T-bet, PerCPCy5.5-NK1.1, PECy5-DX5, PECy7-NKp46, allophycocyanin-CD3, allophycocyanin-KLRG1, allophycocyanin-CD11b, allophycocyanin-Ly49H, allophycocyanin-eFluor780-CD4, allophycocyanin-eFluor780-CD45, eFluor450-CD3, eFluor450-CD11b, eFluor450-Eomes, eFluor450-DX5, and eFluor450-CD8α were purchased from eBioscience (Thermo Fisher Scientific). PE-CD49a, BV510-TCRβ, and BV605-CD3 were purchased from Biolegend. FITC-Ly49C/I was purchased from BD Pharmingen. FITC-DX5 was purchased from Miltenyi Biotec. A-GalCer-loaded PE-CD1-tetramer and allophycocyanin-CD1-tetramer were made in the Brossay laboratory.

Adoptive transfer of NK cells

$CD3^{-}NK1.1^{+}$ and $CD3^{+}NK1.1^{+}$ cells were sorted together under sterile conditions from the SMGs of *NFIL3*$^{-/-}$ mice. Donor cells were injected into recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. The recipient mice were allowed to reconstitute for 7 days before being infected i.p. with 5×10^{4} pfu MCMV. 38 hours post-infection, recipient mice were sacrificed for experiments.

Statistical Analysis

All statistical analyses were performed with Prism Version 7.0 (GraphPad Software). Unpaired two-tailed Student's t-tests were used to compare cell populations

from different mice. ****$p < 0.0001$, ***$p = 0.0001$-0.001, **$p = 0.001$-0.01, *$p = 0.01$-0.05.

RESULTS

The SMG of *NFIL3*$^{-/-}$ mice contains a population of CD3^{+}NK1.1^{+} cells

We previously reported the identification of a novel population of CD3^{+}NK1.1^{+} cells in the SMG of *NFIL3*$^{-/-}$ mice (19). CD3^{+}NK1.1^{+} cells account for less than 1% of the SMG lymphocyte population in *NFIL3*$^{+/+}$ and *NFIL3*$^{+/-}$ mice. However, they represent approximately 4% of the total SMG lymphocyte population in *NFIL3*$^{-/-}$ mice (Figures 1A and 1B). We did not observe any CD3^{+}NK1.1^{+} cells in the SMG of T-bet knockout (*Tbx21*$^{-/-}$) mice (Figure 2). This indicates that SMG CD3^{+}NK1.1^{+} cells are NFIL3-independent, but require T-bet for development.

Aside from NK1.1, we sought to determine if this unique lymphocyte population expresses other common NK cell surface markers (Figure 3A) and transcription factors (Figure 3B). Most of the CD3^{+}NK1.1^{+} SMG cells do not express DX5, and less than 50% express NKp46. These cells are also negative for Ly49H, Ly49C, Ly49I, and KLRG1. Approximately 20% of these cells express CD27, and they are mostly negative for CD11b. We also found that the SMG CD3^{+}NK1.1^{+} cells are largely negative for Eomes, but mostly positive for T-bet expression. Altogether, the CD3^{+}NK1.1^{+} SMG lymphocytes lack most markers associated with conventional NK cells.

SMG NFIL3-independent CD3^{+}NK1.1^{+} cells are not iNKT cells

The SMG CD3^{+}NK1.1^{+} cells are almost entirely positive for CD49a (Figure 3A). CD49a is the α-subunit of the $\alpha_1\beta_1$ integrin receptor, which is also called “very late antigen 1” (VLA-1) (22, 23). VLA-1 is expressed on several different classes of immune cells, including activated T cells (24, 25), and naïve tissue-resident NK (trNK) cells (15-

19). On T cells, VLA-1 expression has been associated with memory $CD4^+$ T cells (26) and tissue-resident memory $CD8^+$ T cells (27, 28). CD49a is also expressed on some subsets of iNKT cells (29), and is required for iNKT cell development and homeostasis (30, 31). In order to determine if SMG $CD3^+NK1.1^+$ cells were iNKT cells, we stained SMG lymphocytes from *NFIL3*$^{-/-}$ mice with CD1d-tetramer that had been loaded with α-galactosylceramide (α-GalCer) (32). However, we found that the $CD3^+NK1.1^+$ cells were negative for α-GalCer-loaded CD1d-tetramer binding (Figure 4A). This result indicated that the NFIL3-independent $CD3^+NK1.1^+$ cells are not iNKT cells. This result does not preclude the possibility that these cells could be α-GalCer-independent type II NKT cells or CD1d-independent NKT-like cells (33-35).

SMG $CD3^+NK1.1^+$ Cells Are Not $CD4^+$ T cells, And Mostly Not γδ T cells

Since the $CD3^+NK1.1^+$ cells were negative for most NK cell and iNKT cell markers, we investigated the expression of other T cell markers on these cells. We found that the vast majority (~90%) of $CD3^+NK1.1^+$ SMG cells are $TCR\beta^+$. In contrast, only about 10% are $TCR\gamma\delta^+$ (Figure 4A). Approximately 25% of the SMG $CD3^+NK1.1^+$ cells are positive for CD8α, and these can be further divided into roughly half $CD8\alpha\alpha^+$ and half $CD8\alpha\beta^+$ (Figure 4B). The SMG $CD3^+NK1.1^+$ cells are also entirely negative for CD4 expression. These results indicated that SMG $CD3^+NK1.1^+$ cells are not $CD4^+$ T cells, and that only minor percentages are comprised of $CD8^+$ T cells and γδ T cells.

SMG $CD3^+NK1.1^+$ cells traffic to the spleen and liver after adoptive transfer

In order to gain some insight into the potential function of SMG $CD3^+NK1.1^+$ cells, we sorted $CD3^+NK1.1^+$ cells from the SMG of *NFIL3*$^{-/-}$ mice. These cells were then injected into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. The injected cells were allowed to reconstitute for 7 days, after which point the recipient mice were infected i.p. with murine cytomegalovirus (MCMV). 38 hours after infection, lymphocyte populations were assessed in the recipient spleen and liver. We found that SMG $CD3^+NK1.1^+$ traveled to the spleen and liver of the recipient mice (Figure 5A). These cells also produced low levels of IFN-γ during MCMV infection (Figure 5B).

Discussion

Within the last several decades, many new classes of innate and adaptive lymphocytes have been discovered and characterized. The identification of group 1, group 2, and group 3 ILCs revealed that the innate immune system possesses diversity of phenotype and function that mirrors the T cells of the adaptive immune system (36-38). Moreover, several subsets of immune cells have features that were once thought to be restricted to either innate or adaptive lymphocytes. As their name suggests, natural killer T (NKT) cells are T cells that express markers associated with both NK cells and T cells (39, 40). NKT cells can be grouped into type I, type II, and NKT-like cells (35). Type I and type II NKT cells are both restricted by the nonclassical MHC class I-like molecule CD1d. Type I NKT cells, also called iNKT cells, express an invariant Vα14-Jα18 TCR, and recognize α-GalCer loaded into CD1d (41-46). In contrast, type II NKT cells express a more diverse TCR that does not include Vα14-Jα18. Type II NKT cells do not respond to α-GalCer, but rather other lipid antigens presented by CD1d (47-51). NKT-like cells are $CD3^{+}NK1.1^{+}$ lymphocytes that are CD1d-independent (33-35, 52). CD1d-independent NKT-like cells seem to have more in common with NK cells than CD1d-restricted NKT cells (52).

Aside from NKT cells, there are other classes of adaptive immune cells that possess innate-like features. Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells that express a variable TCR and recognize the non-classical MHC class I-like molecule MR1 (53, 54). $CD8^{+}$ T cells have also been shown to acquire NK1.1 expression during *in vitro* stimulation with IL-2, IL-4, or IL-15, or *in vivo* during viral infection (55-57). $CD19^{+}NK1.1^{+}$ natural killer-like B cells were also recently discovered,

which produce IL-12 and IL-18 to activate NK cells during the early phase of microbial infection (58).

Here we report the discovery of a novel subset of $CD3^+NK1.1^+$ lymphocytes in the SMG of *NFIL3*$^{-/-}$ mice. The majority of these lymphocytes are $TCR\beta^+$, and minor percentages are positive for CD8α, CD8β, and TCRγδ. Moreover, the entire population is negative for CD4 expression. Moreover, these cells express CD49a and T-bet, and their absence in *Tbx21*$^{-/-}$ mice demonstrates that T-bet deficiency alone does not drive their development. These NFIL3-independent $NK1.1^+$ T cells are not iNKT cells, as evidenced by a lack of α-GalCer-loaded CD1d-tetramer binding. Adoptive transfer into lymphopenic mice has revealed that these $CD3^+NK1.1^+$ SMG cells travel to the spleen and liver, and produce low levels of IFN-γ at 38 hours post-MCMV infection.

It is possible that the $CD3^+NK1.1^+$ lymphocytes found in the SMG of *NFIL3*$^{-/-}$ mice are comprised of some combination of type II NKT cells and CD1d-independent NKT-like cells. This is supported by our observation that the SMG $CD3^+NK1.1^+$ cells are entirely $CD4^-$. $CD4^+$ NKT cells are CD1d-dependent, whereas subsets of both $CD8^+$ NKT and $CD4^-CD8^-$ double negative (DN) NKT cells are known to be CD1d-independent (33, 59). However, CD1d-independent NKT-like cells were shown to express DX5 (52), which is not expressed by the majority of SMG $CD3^+NK1.1^+$ cells. Moreover, CD1d-independent NKT-like cells express NFIL3, though it is not known if they require NFIL3 for development (52). Also, we cannot currently conclude if the SMG $CD3^+NK1.1^+$ cells represent a distinct lineage of lymphocytes that develops in the absence of NFIL3, or if they are present in low numbers in wild type mice, and expand to fill an opening created by the loss of cNK cells in *NFIL3*$^{-/-}$ mice. More work will be necessary to definitively

determine the identity, developmental pathway, and function of these novel NK-like T cells.

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Figure Legends

Figure 1. The SMG in *NFIL3*$^{-/-}$ mice contains a CD3^{+}NK1.1^{+} lymphocyte population. (A) Representative staining of NK1.1 and CD3 expression on SMG lymphocytes from *NFIL3*$^{+/+}$, *NFIL3*$^{+/-}$, and *NFIL3*$^{-/-}$ mice. (B) Frequency of CD3^{+}NK1.1^{+} cells in the SMG of *NFIL3*$^{+/+}$ (n = 19), *NFIL3*$^{+/-}$ (n = 15), and *NFIL3*$^{-/-}$ (n = 19) mice. Data are mean ± SEM. ****$p < 0.0001$

Figure 2. SMG CD3^{+}NK1.1^{+} lymphocytes are not present in *T-bet*$^{-/-}$ mice. Representative staining of NK1.1 and CD3 expression on SMG lymphocytes from C57BL/6, *Nfil3*$^{-/-}$, and *T-bet*$^{-/-}$ mice.

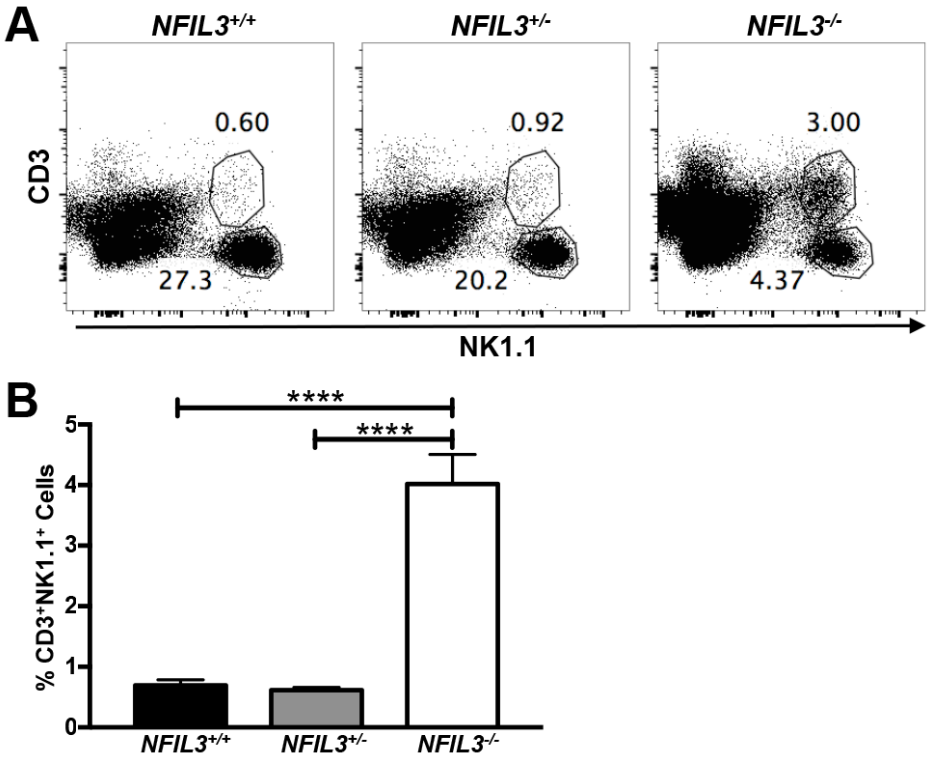
Figure 3. SMG CD3^{+}NK1.1^{+} lymphocytes are negative for most NK cell markers. (A) Representative staining of various cell surface receptors on SMG CD3^{+}NK1.1^{+} lymphocytes from *NFIL3*$^{-/-}$ mice. (B) Representative intranuclear staining of Eomes and T-bet in SMG CD3^{+}NK1.1^{+} lymphocytes from *NFIL3*$^{-/-}$ mice.

Figure 4. SMG CD3^{+}NK1.1^{+} cells are not iNKT cells or CD4^{+} T cells. (A) Representative staining of CD1-tetramer, TCRβ and TCRγδ expression on CD3^{+}NK1.1^{+} SMG lymphocytes from *NFIL3*$^{-/-}$ mice. (B) Representative staining of CD8α, CD8β, and CD4 expression on CD3^{+}NK1.1^{+} SMG lymphocytes from *NFIL3*$^{-/-}$ mice.

Figure 5. SMG CD3^{+}NK1.1^{+} lymphocytes produce IFN-γ after adoptive transfer. (A) Representative staining of donor *NFIL3*$^{-/-}$ SMG CD3^{+}NK1.1^{+} lymphocytes in the spleen

and liver of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ adoptive transfer recipients. (B) Representative IFN-γ production by donor *NFIL3*$^{-/-}$ SMG CD3^{+}NK1.1^{+} lymphocytes in the spleen and liver of recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, 38 hours after MCMV infection.

Figure 1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NFIL3-/-

Figure 2

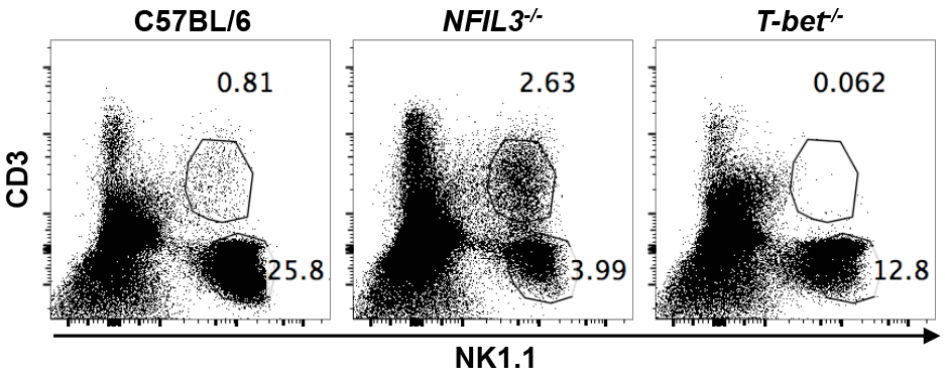
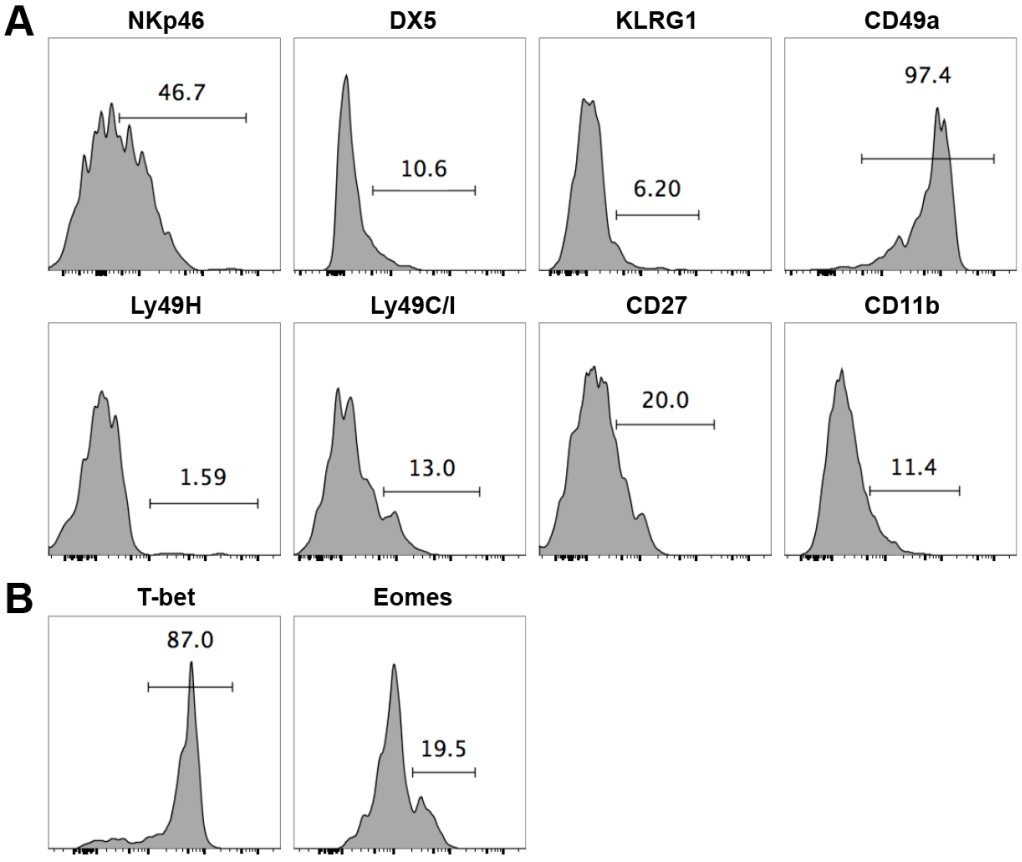


Figure 3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B
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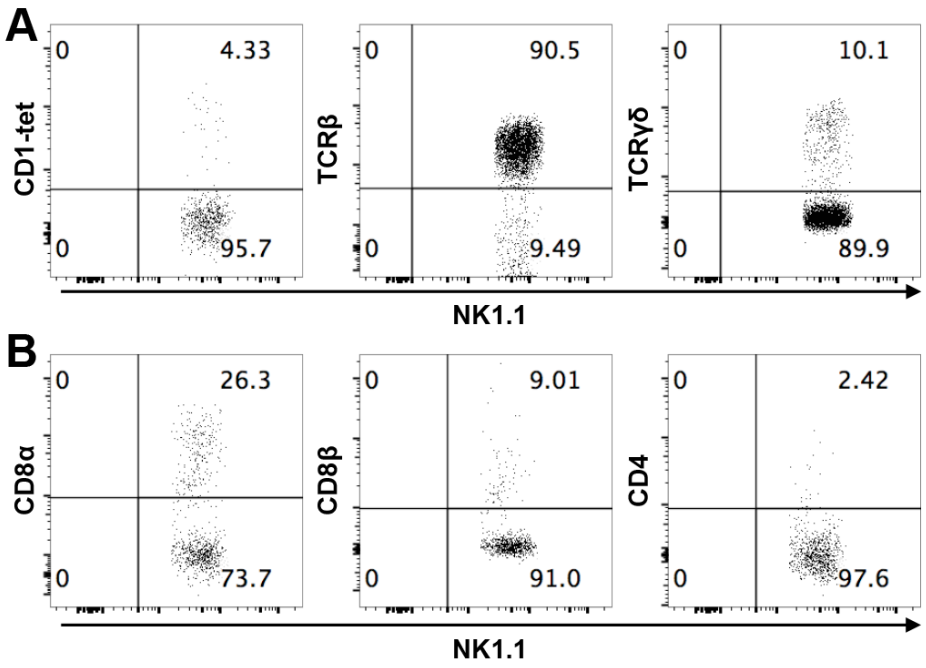
Eomes
19.5

Figure 4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26.3
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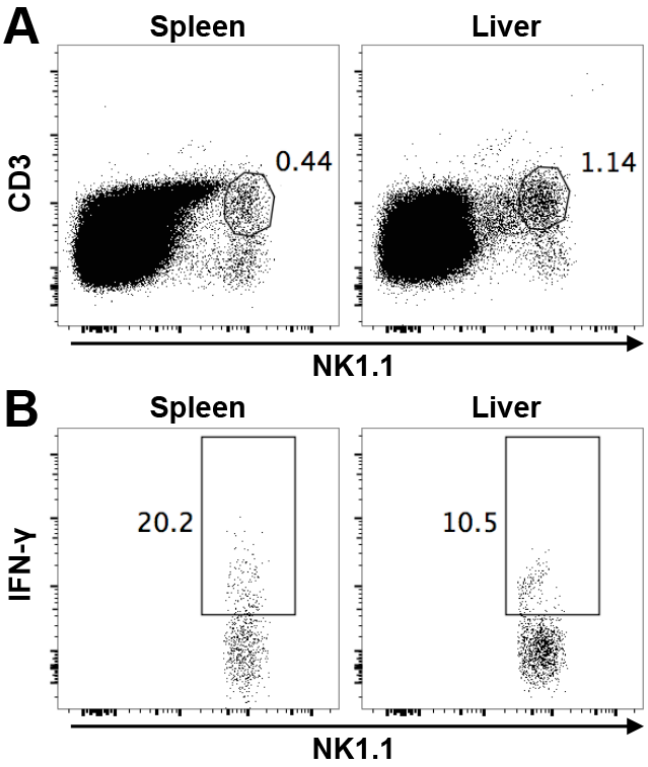
Figure 5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20.2
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NK1.1

CHAPTER 4

LACRIMAL GLAND NK CELLS ARE DEVELOPMENTALLY AND FUNCTIONALLY SIMILAR TO CONVENTIONAL NK CELLS

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ImmunoHorizons

Abstract

The murine lacrimal gland (LG), which produces crucial components of the ocular tear film, contains a population of NK cells. LG NK cells appear to belong to the conventional NK cell lineage, based on their cell surface receptor and transcription factor expression, absence in *NFIL3*$^{-/-}$ mice, and lack of RORγt expression during development. LG NK cells produce IFN-γ during the early stages of systemic murine CMV (MCMV) infection. This effector response occurs in the absence of noticeable MCMV replication in the LG, indicating that LG NK cells are being activated by soluble factors. However, the magnitude of LG NK cell IFN-γ production during MCMV infection is significantly lower than for spleen and liver NK cells. Adoptive-transfer experiments in lymphopenic mice revealed that this hyporesponsive phenotype is tissue specific, which indicates that LG NK cells can produce a robust effector response to MCMV.

Introduction

Innate lymphoid cells (ILCs) comprise diverse cell types that combat infectious microorganisms and cancer cells and help to maintain tissue homeostasis (1). The different subsets of ILCs are broadly classified as ILC1s, ILC2s, or ILC3s based on their developmental pathways and the cytokines that they produce at maturity (2). Conventional NK (cNK) cells are the prototypical ILC1s (3) that function mainly to induce apoptosis of virally infected cells and tumor cells (4). cNK cells develop from the common lymphoid progenitor in the bone marrow (5, 6) and mature before entering the circulation and traveling to lymphoid and nonlymphoid tissues (7).

In recent years, unique populations of NK cells have been identified in many different tissues. Some are cNK cells that take on altered phenotypes due to signals within the tissue environment (8), whereas others appear to be completely distinct ILC1 lineages. For instance, thymic NK cells have a unique phenotype and developmental pathway compared with cNK cells (9). Recently, populations of tissue-resident NK (trNK) NK cells have been identified in the liver, skin (10), kidney (11), uterus (12), and salivary gland (13, 14). These trNK cells represent distinct populations of ILC1s, which have unique phenotypes and developmental requirements compared with cNK cells. cNK cells require the transcription factor NFIL3 for development (15-17) and express Eomes. trNK cells in most tissues are $Eomes^-$ and develop at least partially independently of NFIL3 (18-20). This is in contrast to evidence that NFIL3 is required for the development of all ILCs (21-24).

The populations of cNK cells, trNK cells, and other ILCs in lymphoid and mucosal tissues have been well characterized (8, 19, 25, 26). Mucosal tissues are varied

in structure and function, and their exposure to the external environment results in colonization by a wide variety of commensal and pathogenic microorganisms (27). NK cells and other ILCs are important for maintaining the composition and integrity of mucosal tissues, particularly in response to microbial colonization (28). However, the presence of ILCs in exocrine glands, such as the lacrimal gland (LG), has been relatively understudied. Exocrine glands secrete factors that help to maintain the integrity of mucosal and epithelial surfaces. The LG is essential for eye health, because it is responsible for producing the mucin and aqueous layers of the tear coating. The tear coating is necessary for normal eye function and protection from pathogens, because it supplies the eye with antimicrobial enzymes and protective Igs. Excessive inflammation can damage the LG, which can result in reduced tear production and damage to the ocular surface (29, 30).

The LG is known to contain populations of T cells and B cells (31). In this article, we report that the LG also has a population of NK cells. LG NK cells express Eomes and T-bet and are mostly absent in *NFIL3*$^{-/-}$ mice. This suggests that they develop from the cNK cell lineage. In support of this, we found that LG NK cells do not express RORγt during development, which indicates that they are not ex-ILC3s. Although we could not detect viral replication in this organ, LG NK cells mount an effector response during systemic murine CMV (MCMV) infection. However, this response is low in magnitude compared with splenic and liver NK cells. This weak response was found to be tissue specific, because LG NK cells produce similar IFN-γ levels as splenic NK cells after acclimating to the spleen and liver following adoptive transfer into lymphopenic mice.

Materials and Methods

Mice

C57BL/6 and B6.SJL mice were purchased from Taconic Biosciences (Germantown, NY). A breeding pair of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice was purchased from Taconic Biosciences, and these mice were subsequently bred in-house. *Rorc.cre* and R26R-EYFP mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Rorc.cre* mice were bred with R26R-EYFP mice in-house to produce RORγt fate mapping mice. *NFIL3*$^{-/-}$ mice were a generous gift from Dr. H. J. M. Brady (15). *NFIL3*$^{+/+}$, *NFIL3*$^{+/-}$, and *NFIL3*$^{-/-}$ mice were subsequently bred in-house. All mice were maintained in pathogen free facilities at Brown University. Mice of both sexes were included and no differences were observed.

Infection and NK cell depletion protocols

Mice were infected i.p. with 5×10^4 PFU MCMV (strain: RVG102), as previously described (32). In experiments with NK cell depletion, mice were initially injected i.p. with 100 μg of anti-NK1.1 (clone: PK136) 24 hours prior to MCMV infection, and again every 7 days until takedown. NK cell IFN-γ was measured directly ex vivo without culture following MCMV infection.

Isolation of murine lymphocytes

Mice were sacrificed with isoflurane, and cardiac puncture was performed prior to organ removal. Spleens were processed with a GentleMACS Dissociator (Miltenyi Biotec), filtered through nylon mesh, and layered onto a Lympholyte-M gradient (Cedarlane Laboratories Ltd., Canada). Lymphocytes were harvested from the gradient interface, and washed once in PBS supplemented with 1% FBS (1% PBS-serum).

Alternatively, spleens were processed with ammonium chloride to lyse red blood cells and enrich for lymphocytes. Livers were perfused with 1% PBS-serum before removal, processed in 1% PBS-serum with the GentleMACS Dissociator, and filtered through nylon mesh. Samples were washed 3 times with 1% PBS-serum, suspended in 40% Percoll and layered on 70% Percoll. Lymphocytes were harvested from the gradient interface and washed once with 1% PBS-serum. Extraorbital LGs were processed in Collagenase IV or Liberase-DL (both from Sigma-Aldrich) with the GentleMACS Dissociator, incubated at 37 °C for 10 minutes, filtered through nylon mesh, and washed once with 1% PBS-serum before being layered on a Lympholyte-M gradient. In some experiments, 6-12 LGs from three to six animals were pooled. Lymphocytes were harvested from the gradient interface and washed once in 1% PBS-serum.

Flow cytometry antibodies, reagents, and analysis

Lymphocyte samples were incubated in 1% PBS-serum with the blocking mAb 2.4G2 and stained with specific mAbs for 20 minutes at 4 °C. For intracellular cytokine staining, cells were first stained with extracellular mAbs, then fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes, and stained with intracellular mAbs in 1X PermWash (BD Biosciences) for 20 minutes. For intranuclear transcription factor staining, cells were stained with intracellular antibodies using FoxP3 transcription factor staining reagents (BD Biosciences). Events were collected on a FACSAria III (BD Biosciences), and the data were analyzed using FlowJo (FlowJo LLC). Alexa Fluor 488-AsGM1, FITC-CD27, PE-TRAIL, PE-IFN-γ, PE-T-bet, Per-CPCy5.5-NK1.1, PerCP-Cy5.5-TCRβ, PE-Cy5-DX5, PE-Cy7-NKp46, allophycocyanin-CD3, allophycocyanin-CD19, allophycocyanin-Ly49H, allophycocyanin-KLRG1, allophycocyanin-eFluor780-

CD45, allophycocyanin-eFluor780-CD45.2, eFluor450-CD3, eFluor450-CD11b, eFluor450-CD45.1, and eFluor450-Eomes were purchased from eBioscience (Thermo Fisher Scientific). PE-CD49a, allophycocyanin-CD49a, BV421-CD127, BV510-TCRβ, BV570-CD45, BV605-CD3, and BV785-NK1.1 were purchased from Biolegend. FITC-DX5 was purchased from Miltenyi Biotec.

Adoptive transfer of NK cells

NK cells were sorted under sterile conditions from the spleens of C57BL/6 ($CD45.2^+$) mice and the lacrimal glands of B6.SJL ($CD45.1^+$) congenic mice. Donor NK cells were injected 1:1 into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ recipient mice. Recipient mice were allowed to reconstitute for 7 days before being infected i.p. with 5×10^4 PFU MCMV. At 38 hours post-infection, the recipient mice were sacrificed.

Plaque assays

Standard plaque assays were carried out, as previously described (33).

***In vitro* stimulation assays**

Lymphocytes from naïve C57BL/6 spleen, liver, and LG were incubated for 4 hours in either RPMI complete media, RPMI with IL-12 (10 ng/ml) and IL-18 (10 ng/ml), or RPMI with PMA (20 ng/ml) and Ionomycin (1 μg/ml). GolgiStop (BD Biosciences) was added at the beginning of the incubation. Cells were washed twice with 1% PBS-serum before antibody staining.

Statistical Analysis

All statistical analyses were performed with Prism Version 7.0 (GraphPad Software). Unpaired two-tailed Student's t-tests were used to compare cell populations from different mice. Paired two-tailed Student's t-tests were used for experiments

involving adoptive transfer. ****$p < 0.0001$, ***$p = 0.0001$-0.001, **$p = 0.001$-0.01, *$p = 0.01$-0.05.

Results

The LG contains a population of $CD3^{-}NK1.1^{+}NKp46^{+}$ cells

The LG is an exocrine gland that is similar in structure and function to the submandibular salivary gland (SMG)(29), which contains well-characterized populations of ILC1s (13, 14, 32, 34). We isolated lymphocytes from the extraorbital LG of naïve C57BL/6 mice and found a population of $CD3^{-}NK1.1^{+}NKp46^{+}$ cells (Fig. 1A). The maturity of circulating NK cells is a four-stage developmental process, distinguished by expression of CD11b and CD27. NK cell maturity progresses from $CD11b^{low}CD27^{low}$, to $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$, and finally $CD11b^{high}CD27^{low}$ (35). In comparison with spleen and liver NK cells, LG NK cells are relatively immature, having a very low frequency of $CD11b^{high}CD27^{low}$ cells (Fig. 1B) and low expression of KLRG1 (Fig. 1C). KLRG1 is generally expressed on fully mature $CD11b^{high}CD27^{low}$ NK cells (36), which further supports the classification of LG NK cells as relatively immature.

LG NK cells are mostly conventional in development

In several organs, such as the liver, skin, uterus (10, 12, 37), and kidney (11), cNK cells can be distinguished from trNK cells based on surface expression of DX5 and CD49a. cNK cells are identified as $DX5^{+}CD49a^{-}$, whereas trNK cells are $DX5^{-}CD49a^{+}$. Liver trNK cells also express high levels of TRAIL at baseline (10). We found that LG NK cells are mainly $DX5^{+}$ but some are also $CD49a^{+}$. However, they cannot be easily defined as $DX5^{+}CD49a^{-}$ and $DX5^{-}CD49a^{+}$ subsets, like the NK cells of the liver (Fig. 2A). Much like splenic NK cells and liver cNK cells, LG NK cells are mainly $Eomes^{+}T\text{-}bet^{+}$ (Fig. 2B), and $TRAIL^{-}$ (Fig. 2C). Liver and kidney trNK cells were also recently

shown to be largely negative for the surface receptor Asialo-GM1 (AsGM1), which was once used as a marker to identify all NK cells (11). We observed that the majority of LG NK cells are AsGM1^{+} (Supplemental Fig. 1A) and CD127^{-} (Supplemental Fig. 1B). However, differential expression of Eomes, DX5, CD49a, and other surface markers is not sufficient to distinguish cNK from trNK cells in all organs. For instance, SMG NK cells are mostly DX5^{+}CD49a^{+}, as well as Eomes^{+}T-bet^{+} (13, 14). The majority of SMG NK cells are cNK cells; however, there is also a trNK cell population. These populations are distinguished based on differential requirements for the transcription factor NFIL3 during development (14). cNK cells are generally dependent on NFIL3 for development, whereas liver, skin, uterus, kidney, and SMG trNK cells develop somewhat independently of this transcription factor (10-12, 20, 38). In the LG, we observed a significant decrease in NK cell frequency in *NFIL3*$^{-/-}$ mice compared with wild-type littermate controls (Fig. 3). Together, these data support the conclusion that the vast majority of LG NK cells belong to the conventional lineage.

LG NK cells are not ex-ILC3s

Recent research has shown that some NKp46^{+} ILC3s down-regulate RORγt expression, increase T-bet expression, and gain the ability to produce IFN-γ, effectively taking on an ILC1 phenotype (39, 40). This phenotypic plasticity has made it difficult to classify these ex-ILC3s as either ILC1s or ILC3s (28, 41). We investigated whether any of the LG NK cells were ex-ILC3s based on past RORγt expression by generating RORγt fate mapping mice. Mice that expressed cre recombinase under the control of the *Rorc* gene were crossed with those carrying the ROSA26-floxstop-YFP allele. In agreement

with previous findings (39), we found that the resulting F1 mice had YFP expression in cells that had expressed RORγt during development (Supplemental Fig. 1C).

Using the RORγt fate mapping mice, we found that LG NK cells, as well as spleen NK and liver cNK cells, were mainly YFP^- (Fig. 4). This rules out the presence of ILC3s or ex-ILC3s within the LG $CD3^-NK1.1^+NKp46^+$ population. Interestingly, nearly 20% of liver trNK cells were positive for YFP (Fig. 4B). It is not known whether these cells are ex-ILC3s or whether some liver trNK cells express RORγt as part of an unknown developmental pathway.

LG NK cells respond weakly to systemic MCMV infection

NK cells are crucial for the early control of many viral infections, including CMV (42-44). The CMV family members all have strict species tropism, thus MCMV is often used as a model system to study the pathogenesis and immune response to human CMV. To investigate the effector response of LG NK cells, C57BL/6 mice were infected with MCMV, and NK cell IFN-γ production was assessed at 38 h, day 7, and day 14 postinfection. Previous studies have shown that the effector response of spleen NK cells peaks during the second day of MCMV infection (32). Spleen NK cells, liver cNK cells, and liver trNK cells produced a robust IFN-γ response at 38 h postinfection (Fig. 5). LG NK cells also produced IFN-γ at 38 h postinfection, but at a significantly lower magnitude (Fig. 5). This effector response only occurs during early MCMV infection, because LG NK cell IFN-γ production decreases by day 7, and returns to baseline by day 14 postinfection (Supplemental Fig. 1D). Interestingly, we also found that the frequency of LG NK cells increases dramatically at 38 h postinfection, before decreasing at day 7,

as T cells infiltrate the organ. This is in contrast to the spleen, where the NK cell frequency decreases at 38 h postinfection (Supplemental Fig. 1E). However, the effector response of LG NK cells is less robust than the NK cell populations in the spleen and liver.

LG NK cell hyporesponsive phenotype is tissue specific

We previously reported that SMG NK cells are hyporesponsive to MCMV infection (32). However, we also showed that this phenotype is tissue specific and reversible (14). Thus, we investigated whether the weak effector response of LG NK cells to MCMV is also tissue specific. $CD3^{-}NK1.1^{+}NKp46^{+}$ lymphocytes were sorted from the spleen of C57BL/6 mice ($CD45.2^{+}$) and the LG of B6.SJL mice ($CD45.1^{+}$) and injected into recipient *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, which lack B cells, T cells, and ILCs. After 7 d, the recipient mice were infected with MCMV. Thirty-eight hours postinfection, IFN-γ production was assessed in NK cells recovered from the recipient spleen and liver.

Although donor spleen and LG NK cells were found in the recipient spleen and liver, neither of the donor populations traveled to the LG (Fig. 6A), which indicates that circulating NK cells are not recruited to the LG during MCMV infection. We also found that donor spleen and LG NK cells produced comparable levels of IFN-γ in the recipient spleen and liver (Fig. 6B, 6C). This result indicates that IFN-γ production by LG NK cells during systemic MCMV infection is limited by tissue-specific factors present in the LG but not the spleen or liver. This is further supported by the low level of IFN-γ produced by naïve LG NK cells *in vitro* during IL-12 + IL-18 stimulation (Supplemental

Fig. 2A). Naïve LG NK cells stimulated with PMA + Ionomycin produced similar levels of IFN-γ as did spleen and liver NK cells (Supplemental Fig. 2B).

MCMV is not detectable in the LG during systemic infection

To determine whether MCMV replicates within the LG, C57BL/6 mice were infected with MCMV, and standard plaque assays were performed on homogenates of the spleen, SMG, and LG at 38 h and days 7, 14, and 21 postinfection. In agreement with previous reports, MCMV was detected in the spleen at 38 h postinfection (45) (Supplemental Fig. 2C) and in the SMG starting at day 7 and continuing to day 21 (46, 47) (Supplemental Fig. 2D). However, our analysis did not reveal viral plaques in the LG at any of the time points (data not shown). Because NK cells are a crucial component of the early immune response to MCMV, we also depleted C57BL/6 mice of NK cells. This treatment resulted in higher levels of viral replication in the spleen (Supplemental Fig. 2C) and SMG (Supplemental Fig. 2D); however, we still did not detect virus in the LG (data not shown).

Discussion

For decades after their discovery, cNK cells were the only known innate lymphocytes. However, within the last few years, several subsets of ILCs have been identified and characterized in lymphoid tissues, mucosal tissues, and elsewhere (8, 19, 48). The various ILC subsets are broadly classified as ILC1s, ILC2s, and ILC3s. ILC1s constitutively express the transcription factor T-bet, as well as produce type 1 cytokines, such as IFN-γ and TNF-α (49, 50). cNK cells are included in the ILC1 group, along with other subsets of helper-like ILC1s. cNK cells are cytotoxic effector cells. Helper-like ILC1s produce type 1 cytokines but are generally considered noncytotoxic. cNK cells also diverge early from helper-like ILC1s in development (51-53).

The growing diversity of ILC1s has called into question the dichotomous categorization of the various ILC1 subsets as either cytotoxic or helper-like. For instance, the trNK cells found in the liver are more closely related to other helper-like ILC1s than cNK cells. However, a recent study demonstrated that unlike mucosal helper-like ILC1s, liver trNK cells have high cytotoxic potential (38). Intraepithelial ILC1s have been identified in mucosal tissues and appear to be distinct from cNK cells, trNK cells, and other helper-like ILC1 subsets (54). These findings indicate that ILC1s cannot be simply classified as cytotoxic cNK cells and noncytotoxic/helper-like ILC1s; rather, ILC1 subsets exist along a continuum of diverse phenotypes.

In this study, we identified and characterized a population of ILC1s in the murine LG. LG NK cells appear to be relatively immature and display an unusual expression pattern of DX5 and CD49a. However, as we reported previously in the SMG (14), CD49a is not a definitive marker of trNK cells in all organs. LG NK cells primarily express T-bet

and Eomes and are almost completely absent in *NFIL3*$^{-/-}$ mice. These findings indicate that LG NK cells are not NFIL3-independent ILC1s. Fate mapping experiments also showed that LG NK cells do not express the transcription factor RORγt during development, which indicates that they do not develop from an ILC3 lineage. Based on these data, we found it prudent to identify LG NK cells as conventional-like NK cells.

cNK cells are homogenous in terms of development, but they are not phenotypically uniform at maturity. Rather, they can take on unique phenotypes after they exit the bone marrow and acclimate to different tissues. For instance, NK cells of the lung appear to be derived from the conventional lineage but are more mature than splenic NK cells (8). They also express higher levels of inhibitory receptors, lower levels of activating receptors, and lower levels of migratory and adhesion molecules than do splenic NK cells (55). Based on our findings, the LG also contains a population of NK cells. These LG NK cells appear to be conventional in development, with a unique phenotype shaped by the tissue environment.

NK cells are crucial for the early defense against viral infections, particularly herpesviruses, such as CMV. Similar to the NK cells of the murine SMG (32), LG NK cells produce a weak effector response to systemic MCMV infection. However, this response is likely mediated by inflammatory cytokines, because we could not detect MCMV in this organ. When LG NK cells were removed from their native environment and allowed to proliferate in the spleen and liver of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, they produced a similar level of IFN-γ as donor splenic NK cells (Fig. 6). Thus, LG NK cells are fully equipped to produce a robust effector response to MCMV infection. Much like SMG NK cells (14), the effector response of LG NK cells is suppressed by factors in their native

environment. It is also possible that the weak effector response of LG NK cells *in situ* is due to the lack of viral replication in the LG during infection. However, we consider this unlikely, because Ly49H$^+$ and Ly49H$^-$ NK cells of the spleen and LG produce similar levels of IFN-γ during early MCMV infection (56) (Supplemental Fig. 2E). This indicates that direct contact with m157-expressing cells is not necessary for an NK cell IFN-γ response at this early time point in either organ.

In addition to killing virally infected cells and producing proinflammatory cytokines, NK cells can help to limit inflammation (57). This is especially important in secretory tissues, such as the SMG and LG, where extensive inflammation results in a phenotype resembling human Sjögren's syndrome, an autoimmune disease characterized by a lack of saliva and tear production (58). The LG is a crucial exocrine gland that can be easily damaged by inflammation, and LG NK cells are capable of producing a potent proinflammatory immune response. It is possible that the effector response of LG NK cells is self-modulated or suppressed by other factors within the tissue to prevent inflammatory damage and the resulting lack of tear production. Further work will be necessary to determine the mechanisms behind this.

Authorship

T.K.E. conceived, performed, and analyzed the experiments and wrote the paper. L.G. conceived, performed, and analyzed the experiments. L.B. conceived and analyzed the experiments, and wrote the paper.

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Figure Legends

Figure 1. The LG contains CD3$^-$NK1.1$^+$NKp46$^+$ lymphocytes. (A) Representative staining of spleen, liver, and LG NK cells. (B) Representative staining of CD11b and CD27 expression on spleen, liver, and LG NK cells. (C) Representative staining of KLRG1 expression on spleen, liver, and LG NK cells. Lymphocytes from three C57BL/6 mice were pooled in each experiment. Data are representative of three independent experiments.

Figure 2. LG NK cells appear to be conventional in phenotype. (A) Representative staining of CD49a and DX5 expression on spleen, liver, and LG NK cells. (B) Representative staining of Eomes and T-bet expression in spleen, liver, and LG NK cells. (C) Representative staining of TRAIL expression on spleen, liver, and LG NK cells. Lymphocytes from three C57BL/6 mice were pooled in each experiment. Data are representative of three independent experiments.

Figure 3. LG NK cells are mostly NFIL3-dependent. (A) Representative staining of spleen, liver, and LG NK cells from *NFIL3*$^{+/+}$ and *NFIL3*$^{-/-}$ mice. Lymphocytes from individual mice were stained. (B) Frequency of spleen, liver, and LG NK cells in *NFIL3*$^{+/+}$ (n = 7) and *NFIL3*$^{-/-}$ (n = 9) mice. Data are pooled from four independent experiments and error bars indicate SEM. ****$p < 0.0001$

Figure 4. LG NK cells are not ex-ILC3s. (A) Representative expression of YFP on spleen NK, liver cNK and trNK, and LG NK cells from *RORγT.cre.ROSA.YFP* mice.

Lymphocytes from individual mice were stained. (B) Frequency of YFP^+ spleen NK, liver cNK and trNK, and LG NK cells from *RORγT.cre.ROSA.YFP* mice (n = 5). Data are pooled from two independent experiment and error bars indicate SEM.

Figure 5. LG NK cells respond weakly to systemic MCMV infection. (A) Representative IFN-γ production by spleen NK and LG NK cells from C57BL/6 mice 38 hours post-MCMV infection. Lymphocytes from individual mice were stained. (B) Frequency of IFN-γ^+ spleen NK, liver cNK and trNK, and LG NK cells from C57BL/6 mice 38 hours post-MCMV infection (n = 8). Data are pooled from three independent experiments and error bars indicate SEM. ****$p < 0.0001$, ***$p = 0.0001$-0.001, **$p = 0.001$-0.01

Figure 6. LG NK cell hyporesponsiveness is a tissue-specific phenotype. (A) Representative staining of donor NK cells in the spleen, liver, and LG of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ adoptive transfer recipients. Lymphocytes from individual mice were stained. (B) Representative IFN-γ production by donor spleen and LG NK cells in the spleen and liver of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ adoptive transfer recipients, 38 hours after MCMV infection. (C) Frequency of IFN-γ^+ donor spleen and LG NK cells in the spleen and liver of *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ adoptive transfer recipients (n = 6), 38 hours after MCMV infection. Data are pooled from three independent experiments and error bars indicate SEM. **$p = 0.001$-0.01

Supplementary Figure 1. (A) Representative staining of AsGM1 expression on spleen, liver, and LG NK cells. **(B)** Representative staining of CD127 expression on spleen, liver, and LG NK cells. Lymphocytes from three C57BL/6 mice were pooled in each experiment. Data are representative of three experiments. **(C)** Representative expression of YFP on spleen and liver $CD3^{+}NK1.1^{-}$ lymphocytes from *RORγT.cre.ROSA.YFP* mice. Lymphocytes from individual mice were stained. Data are representative of two independent experiments. **(D)** Representative IFN-γ production by LG NK cells at 38 hours, Day 7, and Day 14 post-MCMV infection. Lymphocytes from individual mice were stained. Data are representative of two independent experiments. **(E)** Representative staining of spleen and LG NK cells at 38 hours, Day 7, and Day 14 post-MCMV infection. Lymphocytes from individual mice were stained. Data are representative of two independent experiments.

Supplementary Figure 2. (A) Frequency of IFN-γ^{+} spleen, liver, and LG NK cells after 4 hour stimulation with IL-12 + IL-18 or PMA + Ionomycin **(B)**. Lymphocytes from 3-6 mice were pooled per experiment. Data are pooled from four independent experiments and error bars indicate SEM. **(C)** Viral titers in the spleen and SMG **(D)** of MCMV-infected C57BL/6 mice that were left untreated (n = 3) or injected with 100 μg PK136 (n =3). Organs from individual mice were analyzed and error bars indicate SEM. **(E)** Representative IFN-γ production by $Ly49H^{+}$ and $Ly49H^{-}$ NK cells in the spleen and LG of C57BL/6 mice at 38 hours post-MCMV infection. Lymphocytes from three mice were pooled per group. Data are representative of two independent experiments. *p = 0.01-0.05

Figure 1

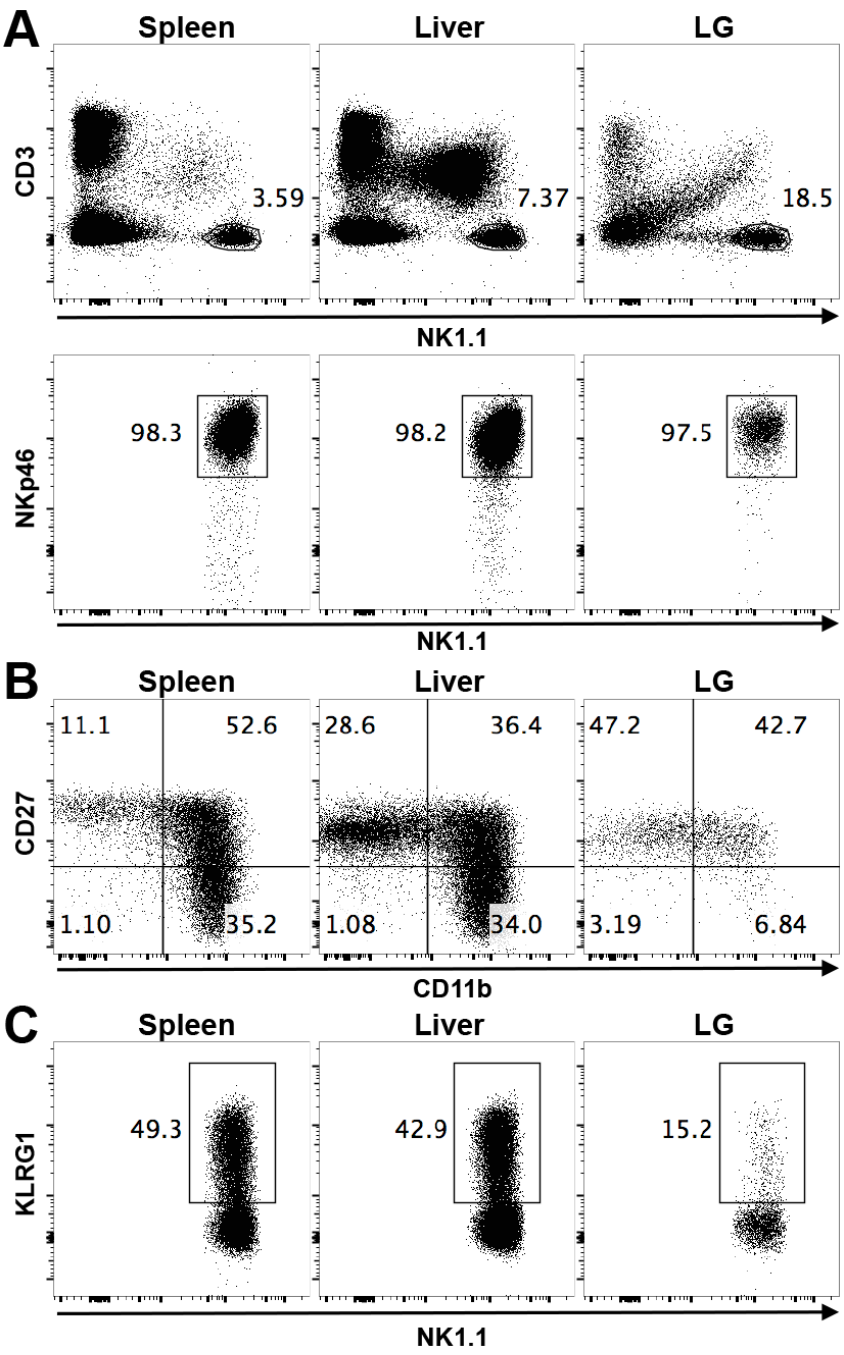

A
Spleen
Liver
LG
CD3
3.59
7.37
18.5
NK1.1
NKp46
98.3
98.2
97.5
NK1.1
B
Spleen
Liver
LG
CD27
11.1
52.6
1.10
35.2
28.6
36.4
1.08
34.0
47.2
42.7
3.19
6.84
CD11b
C
Spleen
Liver
LG
KLRG1
49.3
42.9
15.2
NK1.1

Figure 2

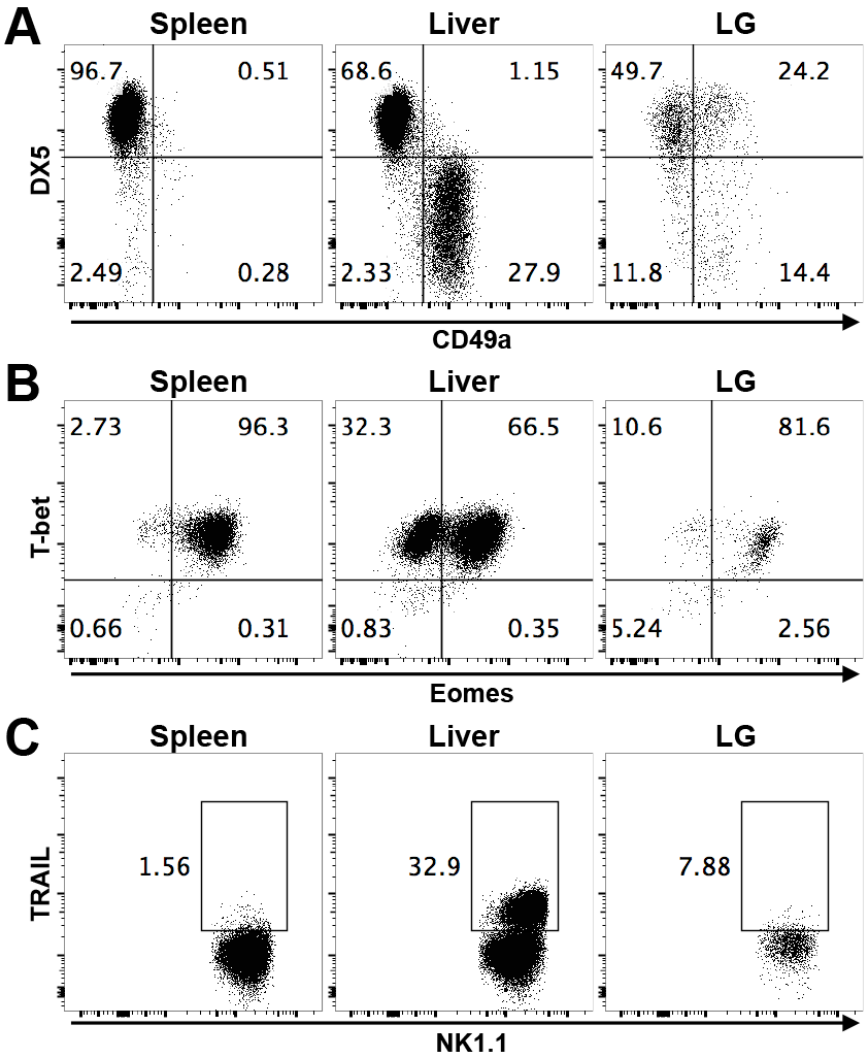

A
Spleen
Liver
LG
96.7
0.51
2.49
0.28
68.6
1.15
2.33
27.9
49.7
24.2
11.8
14.4
DX5
CD49a
B
Spleen
Liver
LG
2.73
96.3
0.66
0.31
32.3
66.5
0.83
0.35
10.6
81.6
5.24
2.56
T-bet
Eomes
C
Spleen
Liver
LG
1.56
32.9
7.88
TRAIL
NK1.1

Figure 3

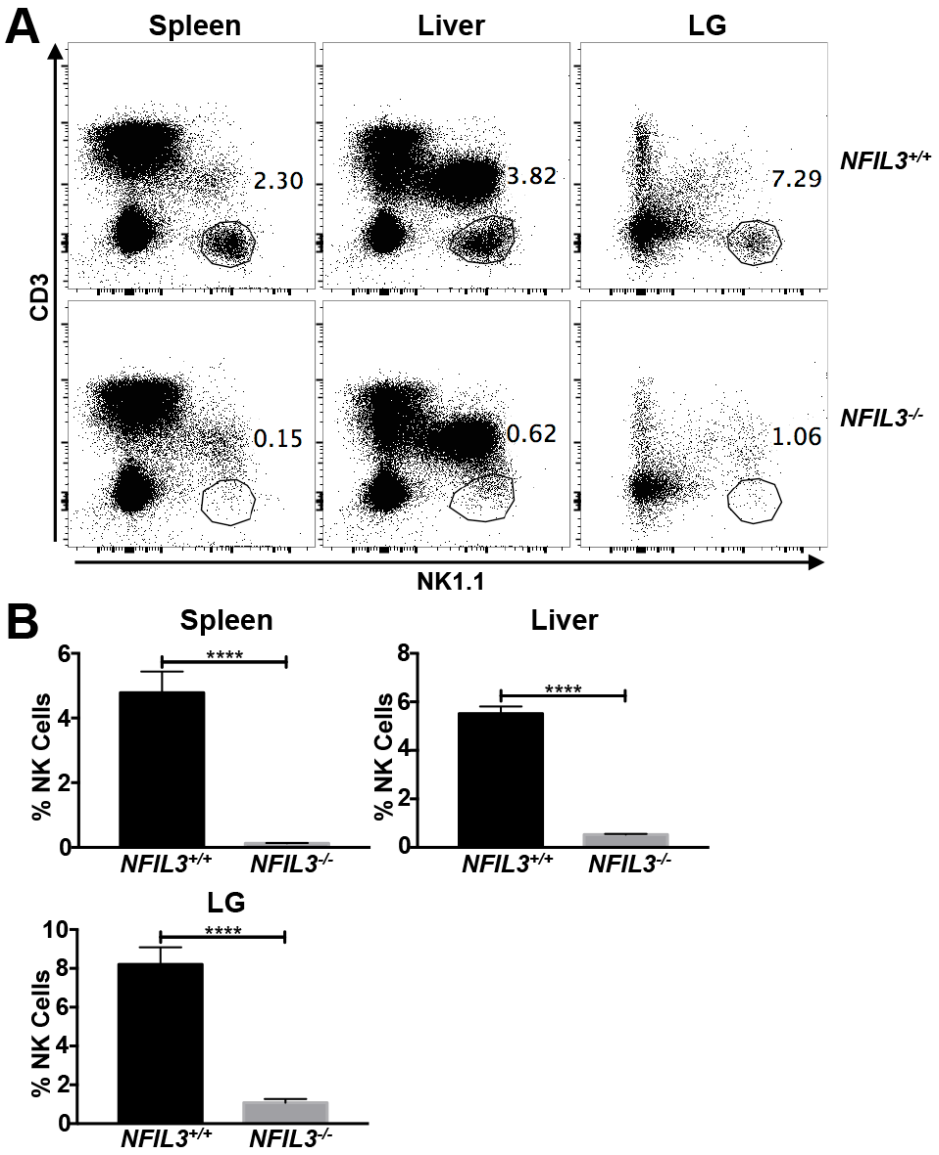

A
Spleen
Liver
LG
2.30
3.82
7.29
0.15
0.62
1.06
NFIL3+/+
NFIL3-/-
CD3
NK1.1
B
Spleen
Liver
LG
% NK Cells
****
NFIL3+/+
NFIL3-/-

Figure 4

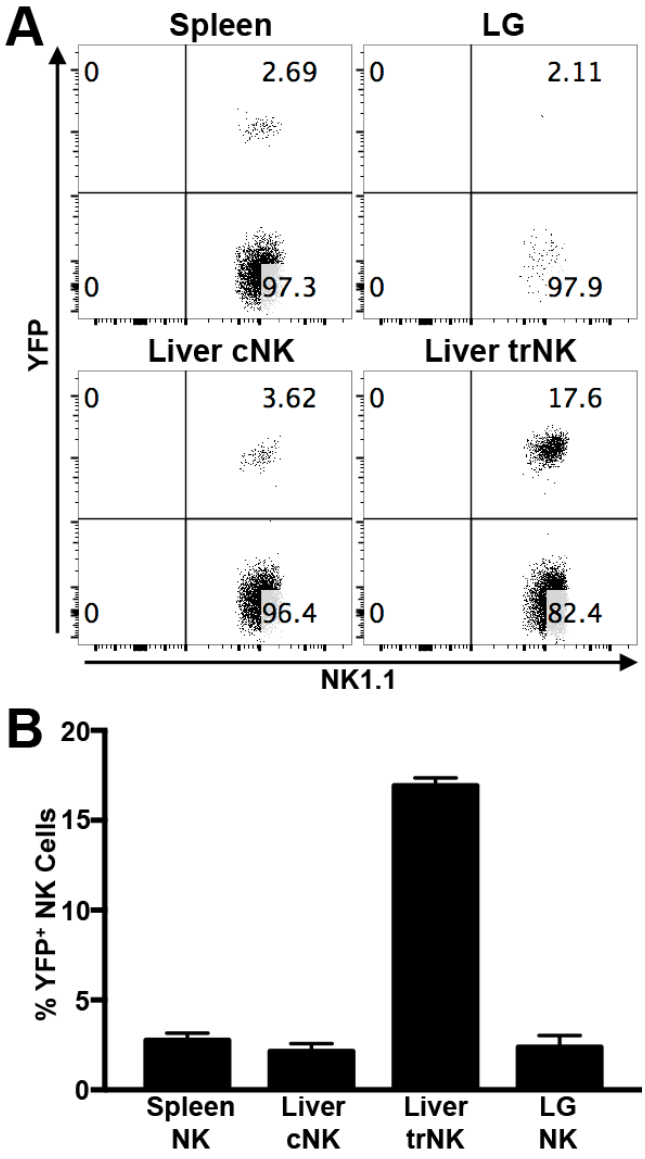

A
Spleen
LG
0
2.69
0
2.11
0
97.3
0
97.9
YFP
Liver cNK
Liver trNK
0
3.62
0
17.6
0
96.4
0
82.4
NK1.1
B
% YFP+ NK Cells
20
15
10
5
0
Spleen NK
Liver cNK
Liver trNK
LG NK

Figure 5

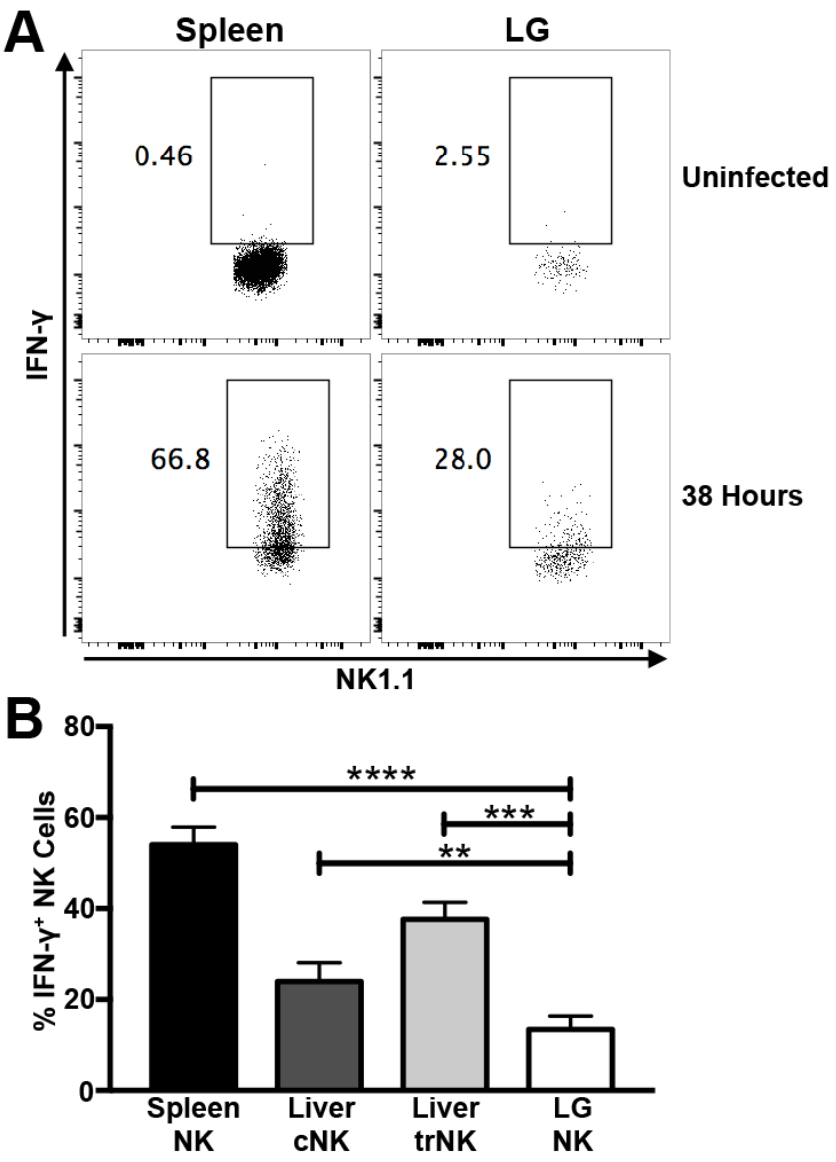
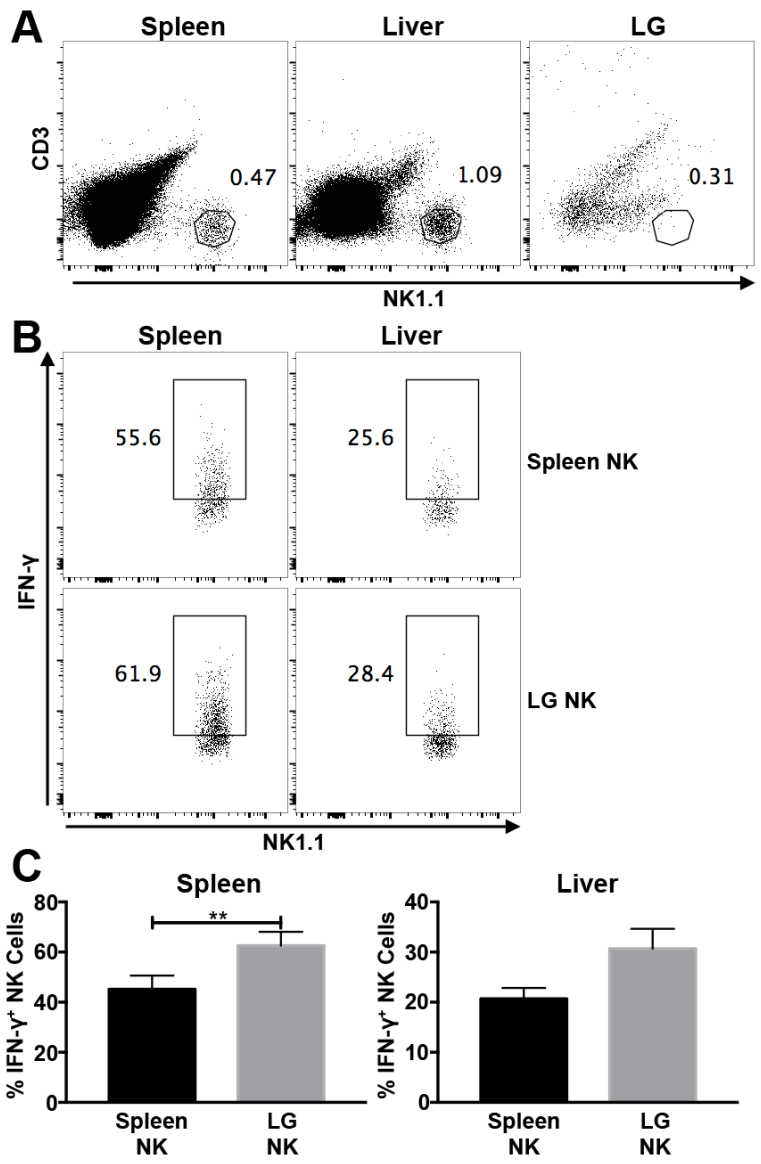
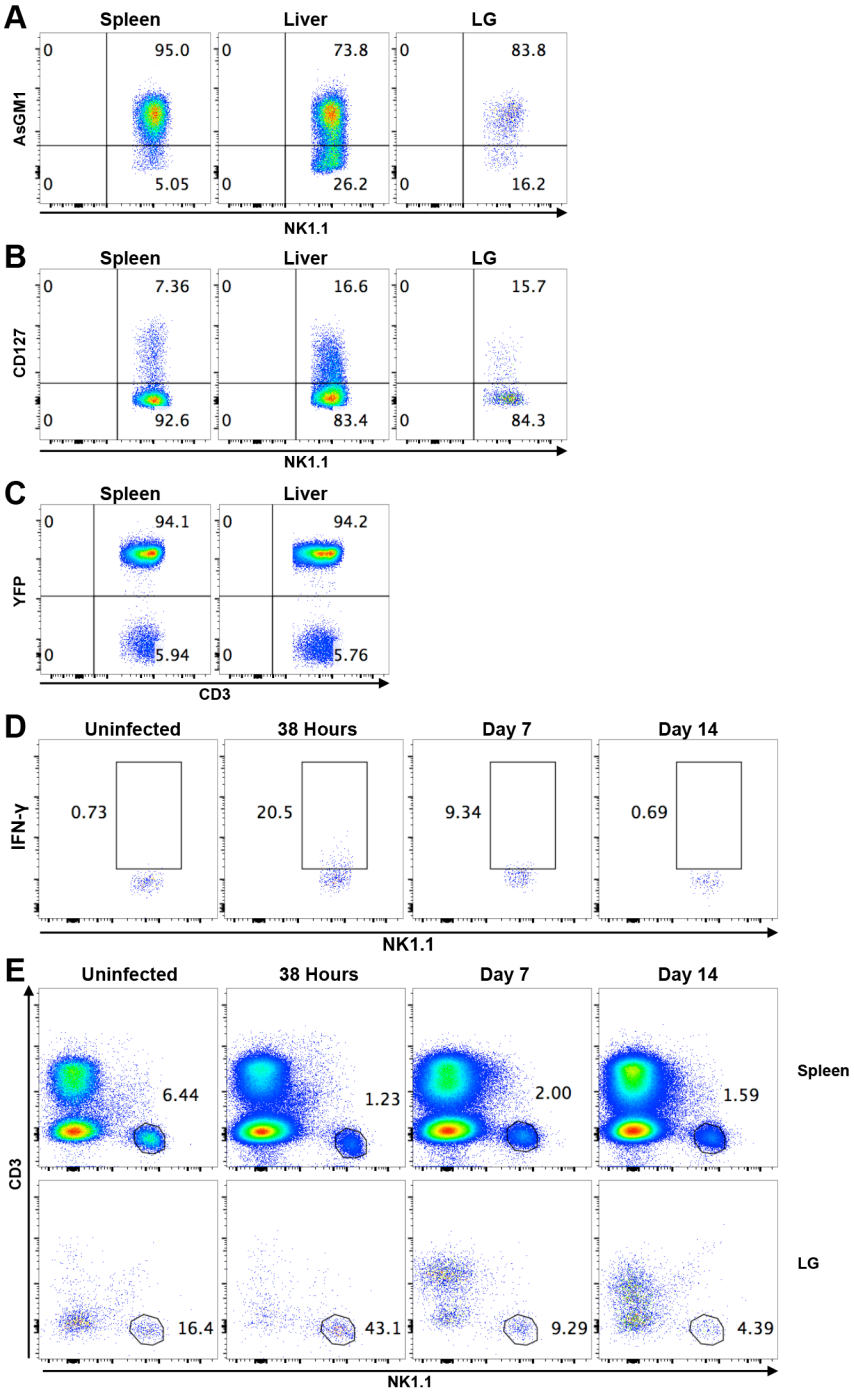

A
Spleen
LG
0.46
2.55
Uninfected
66.8
28.0
38 Hours
IFN-γ
NK1.1
B
% IFN-γ+ NK Cells
80
60
40
20
0
****
***
**
Spleen NK
Liver cNK
Liver trNK
LG NK

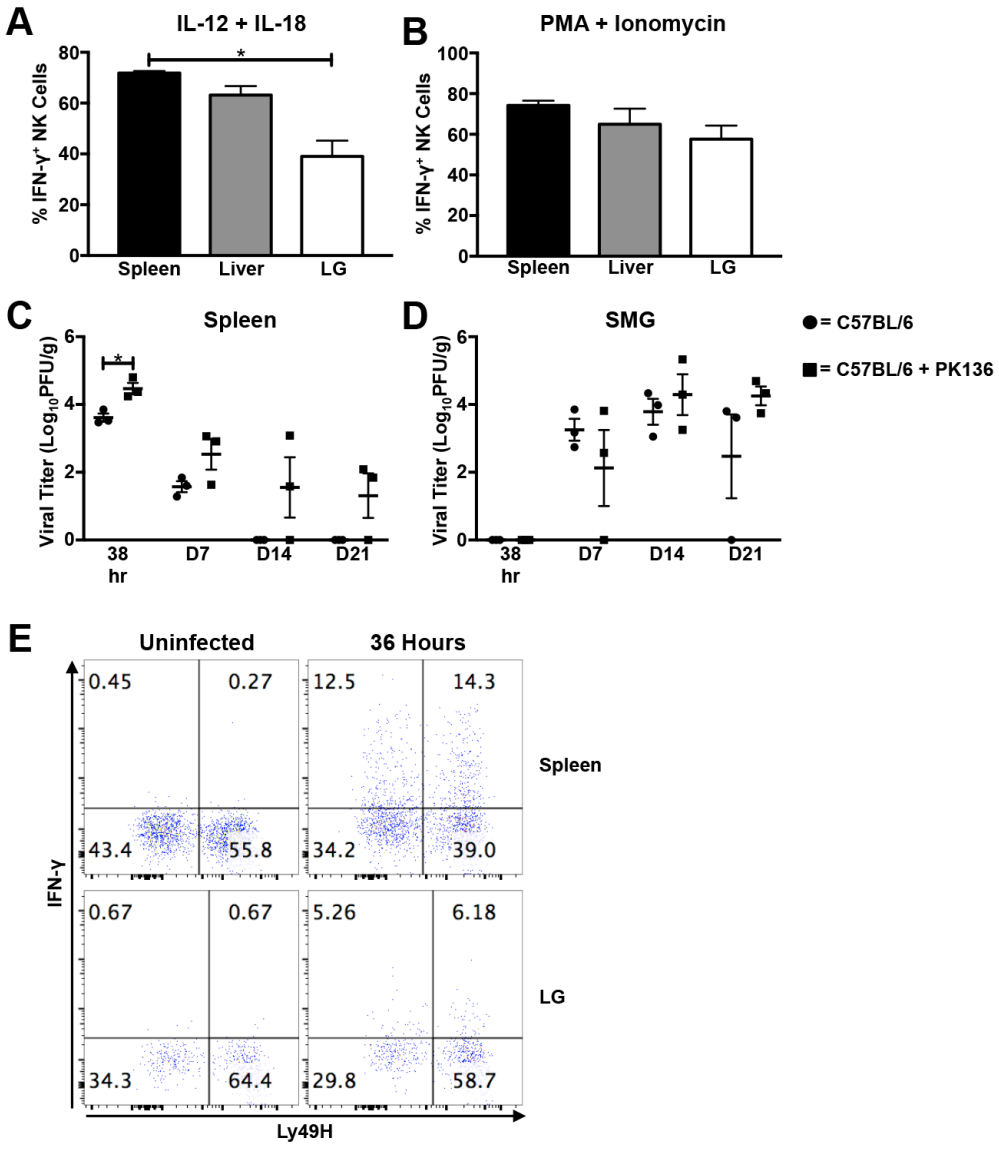
Figure 6


A
Spleen
Liver
LG
CD3
0.47
1.09
0.31
NK1.1
B
Spleen
Liver
55.6
25.6
Spleen NK
IFN-γ
61.9
28.4
LG NK
NK1.1
C
Spleen
Liver
% IFN-γ+ NK Cells
80
60
40
20
0
**
Spleen NK
LG NK
40
30
20
10
0

Supplementary Figure 1



Supplementary Figure 2



CHAPTER 5

DISCUSSION AND CONCLUSIONS

Our understanding of the composition of the immune system is constantly evolving. Many discoveries over the past several decades have challenged the strict division of the immune system into distinct innate and adaptive branches. For example, various subsets of innate-like T cells have been identified, including NKT cells (1-4), γδ T cells (5, 6), and MAIT cells (7). Inversely, natural killer (NK) cells have been shown to have properties typically associated with adaptive immune cells. This includes recognition of specific cell surface antigens (8, 9), as well as the generation of immunological memory to both infectious (10) and chemical agents (11). Moreover, many different subsets of innate lymphoid cells (ILCs) have been discovered and classified as ILC1s, ILC2s, and ILC3s based on their developmental pathways and the cytokines they produce (12, 13). These three groups of ILCs appear to be the innate analogs of T_H1, T_H2, and T_H17 cells, respectively (14, 15).

ILCs include cytotoxic conventional NK (cNK) cells, as well as non-cytotoxic or "helper-like" ILC1s, ILC2s, and ILC3s (16). Cytotoxic cNK cells function mainly to kill virally infected cells and tumor cells. They also produce pro-inflammatory type 1 cytokines to stimulate further immune responses (17, 18). Aside from cNK cells, which circulate throughout the body, ILC1s also comprise several different tissue-resident subsets with unique properties and developmental pathways (19-21). Within the last several years, researchers have worked to characterize these different populations. To this end, we focused our attention on the ILC1 populations in the murine submandibular salivary gland (SMG) and lacrimal gland (LG).

A previous study by Cortez *et al.* (22) revealed that SMG NK cells develop independently of the transcription factor NFIL3, which is crucial for cNK cell

development (23, 24). We also investigated the NK cells of the SMG in wild type and *NFIL3*$^{-/-}$ mice, and found that they comprise two populations: a majority NFIL3-dependent, cNK cell-like population, and a minority NFIL3-independent, trNK cell-like population (25). The SMG NFIL3-independent NK cell population is distinct from tissue-resident NK (trNK) cell subsets that have been identified in other organs, such as the thymus (26), liver, skin (27), kidney (28), and uterus (29). For example, liver NK cells can be divided into a majority cNK cell population, which is DX5^{+}CD49a^{-}, and a minority trNK cell population, which is DX5^{-}CD49a^{+}. Liver cNK cells are also Eomes^{+}, whereas liver trNK cells are Eomes^{-} (27). In contrast, SMG NFIL3-dependent cNK-like cells and NFIL3-independent trNK-like cells are phenotypically very similar, and are largely indistinguishable in wild type mice. At the same time, SMG NK cells are unique from trNK cells in other organs. Both populations of SMG NK cells are mainly DX5^{+}CD49a^{+}, as well as Eomes^{+}T-bet^{+}. SMG NK cells do share some similarities with liver trNK cells, such as expression of TNF-related apoptosis-inducing ligand (TRAIL) at baseline (25, 27).

We investigated the development of the SMG NK cell subsets. Recent studies by Constantinides *et al.* have shown that helper-like ILCs, but not cNK cells, express the transcription factor PLZF during development (30, 31). To investigate the developmental pathway of SMG NK cells, we generated PLZF fate mapping chimeras, in which YFP expression distinguished cells that had expressed PLZF during development. Using this approach, we found that roughly 10% of total SMG NK cells were YFP^{+}, which indicated that most SMG NK cells are derived from a PLZF-independent developmental pathway that is distinct from helper-like ILCs. Furthermore, we generated *NFIL3*$^{-/-}$/B6.SJL mixed

bone marrow chimeras. In these mice, SMG NK cells were derived preponderantly from the B6.SJL bone marrow. This finding further indicated that the majority of SMG NK cells are more closely related to cNK cells than to NFIL3-independent trNK cells (25).

The SMG is an exocrine tissue that serves as a site of persistence for cytomegalovirus (CMV) (32). cNK cells are crucial for the early containment of CMV during systemic infection (33-35). A prior study from our laboratory revealed that SMG NK cells are hyporesponsive to murine CMV (MCMV) infection. The SMG NK cells upregulate KLRG1 expression during infection, but they fail to produce IFN-γ or degranulate (36). $CD8^+$ T cells also fail to effectively control viral replication in this tissue. Ultimately, $CD4^+$ T cells are responsible for clearing MCMV from the SMG (37-39). We investigated whether the diminished effector response of SMG NK cells is tissue-specific or cell intrinsic. To this end, we performed adoptive transfer experiments whereby spleen NK cells from C57BL/6 mice and SMG NK cells from B6.SJL congenic mice were sorted, mixed 1:1, and transferred into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice, which lack T cells, B cells, and ILCs. The donor NK cells were allowed to reconstitute for 7 days, after which time the mice were infected with MCMV. We then assessed IFN-γ production by NK cells in the spleen and liver of the recipient mice 38 hours after infection. We found that donor spleen and SMG NK cells traveled to the recipient spleen and liver. Moreover, the donor spleen and SMG NK cells produced roughly the same magnitude of IFN-γ during early MCMV infection (25). This finding indicated that factors within the SMG limit the capacity of NK cells to produce pro-inflammatory cytokines.

We also investigated the cytokine production capacity of SMG NFIL3-independent NK cells. To this end, we sorted SMG NK cells from *NFIL3*$^{-/-}$ mice, and

transferred these cells into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. After 7 days, the recipient mice were infected with MCMV, and 38 hours later, NK cell IFN-γ production was assessed in the recipient spleen and liver. While the NFIL3-independent NK cells did traffic to the recipient spleen and liver, they produced significantly less IFN-γ than total SMG NK cells under the same conditions (25). This result indicated that SMG NFIL3-independent NK cells are intrinsically hyporesponsive compared to SMG NFIL3-dependent cNK-like cells.

Further work will be necessary to determine the developmental relationship between SMG NFIL3-independent NK cells and other NFIL3-independent ILC1 populations. A recent study by Cortez *et al.* (40) revealed that SMG NFIL3-independent NK cells develop under the influence of transforming growth factor-β (TGF-β) signaling. Specifically, they found that TGF-β suppresses Eomes expression and drives SMG NK cells toward a trNK-cell like phenotype. In support of this, they found that incubation of spleen NK cells in a TGF-β culture induced the expression of SMG NK cell markers. Conversely, Pikovskaya *et al.* (41) recently found that ectopic Eomes expression at the T-bet locus drives helper-like ILC1s toward a cNK cell fate. Thus, ILCs exhibit developmental plasticity, and their phenotype can be greatly influenced by changes in the expression of key transcription factors. It is possible that differences in expression of Eomes, TGF-β, and other factors between our *NFIL3*$^{-/-}$ mice and those of Cortez *et al.* (22, 40) could help to explain the differences that we have observed regarding the composition of SMG ILC1 populations.

Aside from its roles in the development of cNK cells (23) and other ILCs (42, 43), NFIL3 is involved in the development and regulation of a variety of other immune cell

types (44). Rather surprisingly, we found that NFIL3 deficiency results in the appearance of a novel $CD3^+NK1.1^+$ population in the SMG (25). This population of cells is heterogeneous in composition. Roughly 10% are $TCR\gamma\delta^+$, while the rest are $TCR\beta^+$. Of the $TCR\beta^+$ fraction, roughly 25% are $CD8\alpha^+$. The $CD8\alpha^+$ cells can be further divided into roughly 50% $CD8\alpha\alpha^+$ and 50% $CD8\alpha\beta^+$. Based on their expression of T cell markers, variable expression of NKp46, and lack of expression of Eomes and most other NK cell markers, it seems that the majority of the SMG $CD3^+NK1.1^+$ cells are more closely related to innate-like T cells than ILC1s. A lack of binding to α-GalCer-loaded CD1d-tetramer precludes these cells from being type I NKT cells. However, they could be comprised of some combination of type II NKT cells and CD1d-independent NKT-like cells (45, 46). CD1d-independent NKT-like cells have more in common with NK cells than iNKT cells, and seem to possess true hybrid functions between NK and T cells (47).

In order to gain some information about the function of the SMG NFIL3-independent $CD3^+NK1.1^+$ cells, we adoptively transferred them into *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. After 7 days of reconstitution, the mice were infected with MCMV. We found that the transferred $CD3^+NK1.1^+$ cells traveled to the spleen and liver of the recipient mice, and produced low levels of IFN-γ at 38 hours post-MCMV infection. Production of IFN-γ during early MCMV infection supports the classification of SMG $CD3^+NK1.1^+$ cells as innate-like T cells (4, 46). Still, several questions about these cells remain to be answered. Are these $CD3^+NK1.1^+$ cells a new lineage of innate-like T cells that arises only during NFIL3 deficiency? Or, are these cells comprised of T cell subsets that exist in wild-type mice and expand to fill a vacancy left by cNK cells in *NFIL3*$^{-/-}$ mice? Do these cells develop in the thymus and travel to the SMG? Further work will be necessary to

answer these questions, and to discover more about the development and function of these cells. A detailed characterization of their TCR repertoire would be the logical next step for gaining more information. This could be accomplished by single cell sorting, followed by sequencing of the TCR genes in each cell (48-50).

In addition to the SMG, we also investigated the ILC1 compartment of the murine lacrimal gland (LG). The LG is an exocrine tissue that is similar in structure and function to the SMG. Specifically, the LG produces the aqueous and mucin layers of the tear coating, which is essential for normal eye health (51). The LG is known to contain populations of B and T cells (52). We found that the LG lymphocyte fraction contains a large percentage of $CD3^+NK1.1^+NKp46^+$ cells. These LG NK cells are mostly positive for Eomes and T-bet expression, and are also mostly $DX5^+$. They have an unusual, diffuse expression pattern of CD49a. However, the vast majority of LG NK cells are NFIL3-dependent, as demonstrated by their significant reduction in *NFIL3*$^{-/-}$ mice. LG NK cells also possess a cNK cell-like phenotype, including being mostly $CD127^-$ and $AsGM1^+$.

Recent studies have shown that ILC development is not necessarily a linear path from the common lymphoid progenitor (CLP) to each mature ILC subset. Rather, several different subsets of ILCs exhibit phenotypic plasticity at maturity (53, 54). For instance, $NKp46^+$ ILC3s can upregulate T-bet expression, lose RORγt expression, and produce IFN-γ instead of IL-17 and IL-22. In wild type mice, these "ex-ILC3s" are phenotypically indistinguishable from ILC1s that never expressed RORγt during development (55, 56). In order to determine if any of the LG NK cells were derived from an ILC3 lineage, we generated RORγt fate mapping mice. To this end, we crossed *Rorc.cre* mice to R26R-

EFYP mice. In the resulting F1 mice, YFP expression distinguished cells that had expressed RORγt during development. In these mice, we observed that almost none of the LG $CD3^{+}NK1.1^{+}NKp46^{+}$ cells expressed YFP. This was similar to spleen NK cells and liver cNK cells, which further indicated that LG NK cells develop from the conventional NK cell lineage. In contrast, nearly 20% of liver trNK cells ($DX5^{-}CD49a^{+}$) expressed YFP. This result indicated that liver trNK cells are developmentally heterogeneous, and that a fraction might arise from an ILC3 lineage. This possibility should be explored more fully.

We also explored the function of LG NK cells by infecting C57BL/6 mice with MCMV. At 38 hours post-infection, the LG NK cells increased in frequency and produced IFN-γ. However, the magnitude of LG NK cell IFN-γ was significantly lower than that of spleen NK cells, liver cNK cells, and liver trNK cells. LG NK cells also produced significantly less IFN-γ than spleen and liver NK cells during *in vitro* stimulation with IL-12 + IL-18, but not with PMA + Ionomycin. We hypothesized that LG NK cells are capable of producing a robust IFN-γ response, but will not do so in their native tissue in response to cytokine cues. To test this, we sorted spleen NK cells from C57BL/6 mice and LG NK cells from congenic B6.SJL mice. We combined these cells in a 1:1 ratio and injected them into lymphopenic *Rag2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice. After 7 days, we infected the recipient mice with MCMV. 38 hours after infection, we examined NK cell IFN-γ production in the recipient spleen, liver, and LG. We noticed that donor NK cells did not populate the LG in the recipient mice, which indicated that circulating NK cells are not recruited to the LG during MCMV infection. We also observed that donor spleen and LG NK cells produced roughly the same amount of IFN-γ in the recipient spleen and

liver. This result confirmed that LG NK cells are capable of producing high levels of IFN-γ during viral infection. Tissue-specific factors are most likely limiting their pro-inflammatory response in wild type mice.

We also performed viral plaque assays in MCMV-infected C57BL/6 mice. This assay revealed MCMV in the spleen as early as 38 hours post-infection, and in the SMG by Day 7 post-infection. However, we did not observe MCMV in the LG at any time point between 38 hours and Day 21 post-infection. Since NK cells are crucial for the early containment of MCMV infection, we depleted C57BL/6 mice of NK cells with an anti-NK1.1 antibody (clone: PK136), and infected these mice with MCMV. NK cell depletion increased the viral titers in the spleen and SMG. However, we still did not detect virus in the LG. These results indicated that LG NK cell expansion and IFN-γ production during early MCMV infection is due to systemic pro-inflammatory cytokines.

The role of SMG and LG NK cells should be studied in more detail, both in healthy mice, and in the context of infection and autoimmune disease. The SMG and LG are both crucial exocrine tissues that can be damaged by excessive inflammation. This damage can lead to a phenotype resembling human Sjögren's syndrome, in which decreased saliva and tear production results in dry mouth and dry eyes, among other symptoms (57, 58). The consequences of saliva and tear deficiency can be severe. Saliva serves a number of crucial functions for maintaining oral health. It contains antimicrobial enzymes that help to prevent bacterial and fungal infections, and also helps to prevent tooth decay by providing teeth with a constant source of calcium and phosphate ions. Moreover, saliva contains digestive enzymes that begin the process of digesting food. Thus, loss of saliva often results in increased incidence of cavities and tooth decay, and

can cause difficulties with eating and speaking (59). Similarly, tears contain a complex mixture of soluble factors that support normal corneal health and protect the eye from microbial invasion (51, 60, 61). Decreased tear production can result in eye irritation and pain, light sensitivity, and eventually impaired vision (62).

Interactions between immune cells and infectious agents can induce inflammation in the SMG and LG. MCMV infection has been linked to the development of chronic SMG and LG inflammation, and progressive loss of function, in lupus-prone NZM2328 mice. The inflammation is triggered by MCMV infection, but does not directly correlate with viral levels in the tissue. This indicates that MCMV infection induces a perpetuating immune inflammatory process that results in damage to these tissues (63). Recently, NK cells were shown to limit SMG inflammation and preserve saliva production in MCMV-infected mice (64). SMG TRAIL$^+$ NK cells were also recently shown to kill CD4$^+$ T cells during MCMV infection in order to limit inflammation in this tissue (65). These findings indicate that SMG cNK-like cells, NFIL3-independent trNK-like cells, and CD4$^+$ T cells interact in complex ways to combat MCMV infection and limit inflammation in the SMG (**Figure 1**). It is possible that LG NK cells could also serve a regulatory role to limit inflammation and preserve tissue integrity. Further studies should attempt to elucidate the potential immunoregulatory roles of SMG and LG NK cells, and determine the mechanisms mediating their hyporesponsive phenotype during viral infection.

Concluding Remarks

The work presented in this thesis has helped to characterize several different subsets of ILCs and innate-like lymphocytes in the SMG and LG. The SMG contains at least two ILC1 populations: a majority population of NFIL3-dependent cNK-like cells, as well as a minority population of NFIL3-independent trNK-like cells. These two populations are phenotypically similar to each other, but distinct from trNK cells in other organs. NFIL3 deficiency also results in the appearance of a population of $CD3^{+}NK1.1^{+}$ lymphocytes in the SMG. These cells are most likely a heterogeneous population of innate-like T cells, but more work will be necessary to determine their origin and functions. The LG also contains a population of NK cells, which appears to be conventional in development. LG NK cells are hyporesponsive to systemic MCMV infection, but can produce a robust IFN-γ response when removed from their native tissue environment. Altogether, these findings increase our understanding of the development, phenotype, and functions of innate and innate-like lymphocytes in two different murine exocrine tissues.

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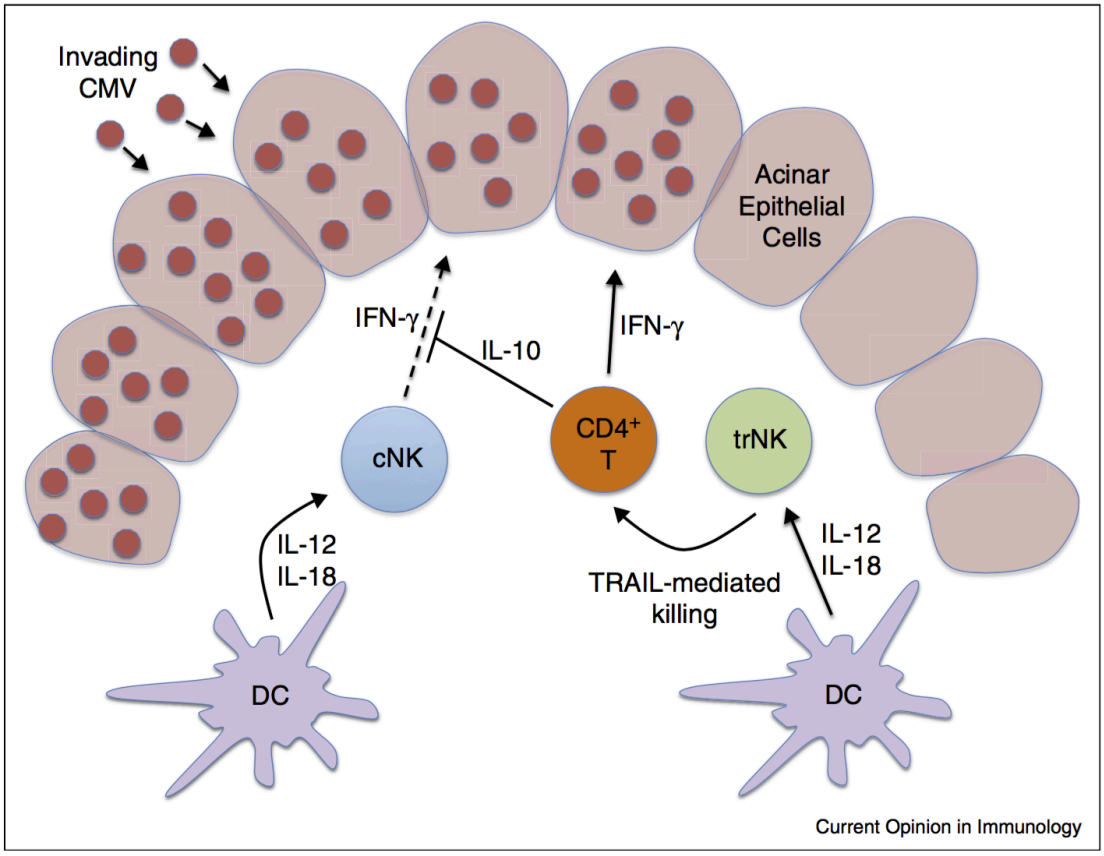

Figure 1. Potential interactions between NFIL3-dependent cNK-like cells, NFIL3-independent trNK-like cells, and $CD4^+$ T cells in the murine SMG during MCMV infection (Adapted from Erick and Brossay 2016 (66)).

APPENDIX I

PHENOTYPE AND FUNCTIONS OF CONVENTIONAL AND NON-CONVENTIONAL NK CELLS

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Phenotype and functions of conventional and non-conventional NK cells

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Here we focus on the phenotypic and functional diversity of NK cells. We give an overview of the phenotype and developmental pathways of conventional and tissue-resident NK cells. We also discuss the potential complementary functions of conventional NK cells and tissue-resident NK cells in a variety of tissues.

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Introduction

The innate immune system is comprised of a variety of cell types that utilize different methods to furnish rapid protection against pathogens. Natural killer (NK) cells are cytotoxic innate lymphocytes that provide crucial defenses against viral pathogens and tumor cells. NK cells have the ability to recognize and induce apoptosis in target cells through the release of perforin and granzymes, or engagement of target cell death receptors such as Fas by Fas-L on the NK cell surface [1]. Activated NK cells also produce pro-inflammatory cytokines such as IFN-γ that stimulate further innate and adaptive immune responses [2].

For decades after their discovery, circulating conventional (c)NK cells were thought to be the only innate effector lymphocytes in the body. Within the last few years, however, many new subsets of innate effector cells have been discovered, and classified as innate lymphoid cells (ILCs) [3,4]. ILCs display great diversity in phenotype and function, and appear to represent the innate analog of T helper cells [5]. ILCs are classified into three groups — ILC1, ILC2, and ILC3 — based on the cytokines they produce and the transcription factors required for their development [6–8]. cNK cells are considered to be the prototypical ILC1 subset, and several distinct lineages of NK cells have recently been discovered in various tissues in humans and mice [5]. These unique NK cell populations have alternatively been called ILC1 [9] and tissue-resident (tr)NK cells [10]. This is merely a difference in nomenclature, as all NK cells ultimately belong to the ILC1 group [6,7,11]. However, the common ILC precursor (ILCP or CHILP) does not generate cNK cells [12••,13••].

Current research indicates that there are multiple unique lineages of NK cells: circulating cNK cells, thymic NK cells, trNK cells of the liver and skin, uterine (u)NK cells, submandibular gland (SMG) trNK cells, and kidney trNK cells [14,15•,16–22]. Each of these NK cell populations possesses unique phenotypic characteristics and appears to arise from a distinct developmental pathway. Of particular interest are the NK cells that reside in mucosal tissues, since these tissues are diverse in structure and function, and also provide an interface with the external environment [23]. NK cells in the respiratory tract, urogenital tract, salivary glands, as well as other mucosal tissues function to counter potential invading organisms, while at the same time limiting inflammatory damage to these delicate tissues. In this review, we discuss the phenotypic and functional diversity of NK cells with a focus on tissue-resident NK cells in mucosal tissues. Markers expressed by the different subsets of NK cells are described in Table 1. We do not discuss the intestine as it has been covered extensively in other reviews [24–26].

Conventional NK cells

Conventional NK cells undergo most of their development in the bone marrow. They are derived from the common lymphoid progenitor (CLP), which is defined as $Lin^{-}Sca\text{-}1^{low}CD117^{low}CD127^{+}CD135^{+}$ [27]. After the CLP, the earliest known NK cell precursor is the pre-pro A, which are $Lin^{-}ID2^{+}Sca\text{-}1^{+}CD117^{low}CD127^{+}CD135^{-}CD122^{-}$. Pre-pro A NK cells are very similar to another early NK precursor called the pre-pro B stage, which lack CD117 expression, but otherwise have the same surface receptor profile. After the pre-pro stages, NK cell precursors progress to the refined (r)NKP stage, which is defined as $Lin^{-}CD27^{+}CD244^{+}CD117^{low}CD127^{+}CD135^{-}CD122^{+}$. These rNKP then develop into immature (i)NK cells, which acquire NK1.1 expression and further develop into mature (m)NK cells [28,29]. DX5 (CD49b), a late maturation marker, is expressed just before the mNK cells exit the bone marrow [30]. NKp46 is also a marker of mature NK cells, and it is

Table 1

Phenotypic characteristics of cNK cells and tissue-resident NK cell subsets

	cNK*	Thymic NK	Liver trNK	Skin trNK	Uterine trNK	SMG trNK	Kidney trNK
NK1.1	+	+	+	+	+	+	+
DX5	+	+	–	–	–	+/–	–
CD49a	–	?	+	+	+	+	+
CD127	–	+	–	–	–	–	?
TRAIL	–	?	+	?	?	+/–	+
Eomes	+	?	–	–	+/–	+	?
IFN-γ	++	+	+	?	+	+/–	?
TNF-α	+/–	+	+	?	?	–	?
Granzymes	+	+	+	?	+	?	?

* cNK cells are prominent in the spleen and blood, and are also found in the liver, skin, uterus, SMG, and kidney.

expressed before DX5 in the bone marrow [31,32], though this notion has been challenged by a recent study [33]. Later stages of NK cell maturity can be assessed by expression of CD11b and CD27, in a 4-stage developmental process. The least mature NK cells, which have not yet expressed DX5, are $CD11b^{low}CD27^{low}$. This is followed by $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$, and finally $CD11b^{high}CD27^{low}$ stages [34]. The most mature $DX5^{+}CD11b^{high}CD27^{low}$ NK cells also express KLRG1 and are $CD43^{high}$ at steady state [35,36].

The development of cNK cells requires the coordinated activity of several transcription factors and cytokines. NFIL3 (nuclear factor, IL-3 regulated, also called E4BP4) is crucial at the progenitor stage [37] for cNK cell differentiation and development [38–41]. Although NFIL3 was recently shown to be required for the development of all helper innate lymphoid cells [42•,43•,44•,45•], trNK cells appear to develop independently of this transcription factor [15•,21,22,46]. Id2 (inhibitor of DNA binding 2), T-bet (Tbx21), and Eomes (eomesodermin) are all required at different stages for cNK cell development and maintenance [47–51]. IL-15 signaling through the IL-15Rα is also necessary for the development and maintenance of mature cNK cells [52–54]. The spleen harbors mostly cNK cells, though very few ILC1 and/or trNK cells can be found in this organ [55]. Beside the spleen, cNK cells can be found in significant number in the blood, liver, kidney, and mucosal tissues (see below).

Non-conventional NK cells

Thymic NK cells

The thymus contains a unique lineage of NK cells, separate from cNK. Thymic NK cells have a unique surface phenotype in that they are $CD127^{+}CD69^{high}$-$Ly49^{low}CD11b^{low}$, as opposed to cNK cells, which are $CD127^{-}CD69^{-}Ly49^{+}CD11b^{high}$. Thymic NK cells are less cytotoxic than their splenic cNK cell counterparts. However, thymic NK cells produce more IFN-γ than splenic cNK cells, as well as more TNF-α and GM-CSF [56,57]. Interestingly, a small percentage of thymic NK cells traffic to lymph nodes, but not to other organs [56,57]. Thymic NK cells require the transcription factor GATA-3 for development, as well as the cytokine IL-7. GATA-3 is generally more closely associated with T cell development than NK cell development. However, thymic NK cells do not develop from RORC-expressing precursors, and develop in the absence of Notch signaling, indicating that these NK cells are not derived from a committed T cell progenitor [58]. Upon further investigation, it was shown that thymic NK cells develop from a double-negative ($CD4^{-}CD8^{-}$) thymocyte precursor [59], and that their development is mostly unimpaired in the absence of NFIL3 [55]. Despite their NFIL3 independence and cytokine production profile, thymic NK cell affiliation to the ILC1 family has not been decisively confirmed.

Liver NK cells

The murine liver is home to both circulating cNK cells and trNK cells [18,50]. The liver trNK cells are $CD49a^{+}DX5^{-}$, whereas cNK cells are $CD49a^{-}DX5^{+}$. Liver trNK cells also constitutively express the programmed cell death receptor ligand TRAIL (TNF-related apoptosis-inducing ligand) [60,61]. Moreover, liver trNK cells develop in the absence of NFIL3 and do not express Eomes, though they do require T-bet for development and maintenance [15•]. In the bone marrow, early T-bet expression is restricted, which allows for the development of $Eomes^{+}$ cNK cells. In the liver, T-bet is expressed early during trNK cell development, which suppresses Eomes expression and allows for the development of $Eomes^{-}TRAIL^{+}DX5^{-}CD49a^{+}$ NK cells [51]. Altogether, these data indicate that liver trNK cells arise from a different developmental pathway than bone marrow-derived cNK cells [33]. There has been a call to classify the liver trNK cells as ILC1, although there is still some debate over the nomenclature [9,14,15•].

Similarly to splenic NK cells, conventional liver NK cells produce IFN-γ and perform cytotoxic functions via release of perforin and granzymes [62]. Liver trNK cells also produce IFN-γ and can degranulate, as evidenced by CD107α expression [15•,18]. It is agreed that liver trNK cells constitutively express TRAIL [10,51] and produce

TNF-α at levels not observed with cNK cells during *in vitro* stimulation assays [15•]. TNF-α has been shown to promote the recruitment of neutrophils [63], which in turn may participate in the immune response. Although it is not yet known how trNK cells contribute to pathogen control in the liver, the effector molecules and cytokines produced by cNK cells and trNK cells suggest the two subsets perform complementary effector functions.

Lung NK cells

NK cells make up roughly 10% of the total lung lymphocytes [19]. These lung NK cells are predominantly $CD11b^{high}CD27^{low}$, and express higher levels of DX5, CD122, Ly49s, and CD43 than splenic NK cells, suggesting a more mature phenotype. Current evidence suggests that lung NK cells are derived from the same early precursors as bone marrow-derived cNK cells, which precludes them from being a distinct lineage. However, the lung environment shapes these cNK progenitors into a mature NK cell subset with a unique surface receptor phenotype [64].

The respiratory tract is especially vulnerable to viral, bacterial, and fungal pathogens. Aging appears to have a detrimental effect on the ability of lung NK cells to combat influenza virus infection. In aged mice versus young mice, lung NK cells showed impaired proliferation and cytotoxic responses during influenza virus infection [65]. Although lung NK cells have been shown to respond to influenza virus infection, both directly and indirectly, the benefits of this response are in contention. Though some studies have shown that NK cell depletion results in higher viral titers and greater severity of infection [65], others have shown that *IL-15*$^{-/-}$ mice experience better protection against influenza-mediated lung injury, due mainly to the lack of NK cells [66]. NK cell production of IFN-γ was also demonstrated to cause acute lung injury during the early stages of respiratory syncytial virus (RVS) infection [67]. It is possible that NK cell activity, up to a certain threshold, helps to control viral infections, while it results in inflammation and tissue damage when NK cells are over-activated.

Although ILC2 have been well characterized in the lung [68], the presence of trNK cells and/or ILC1 in this organ has not been documented. Interestingly, lung NK cells were shown to produce IL-22, both *in vitro* and *in vivo* during influenza virus infection. However, this NK cell-produced IL-22 does not appear to be crucial to the immune defense against the virus [69]. Regardless of the phenotype of these IL-22-producing cells, the potential presence of ILC1 and/or trNK cells in this organ needs to be re-examined.

Skin NK cells

When NK cells were first discovered in the skin, they were shown to express all common NK cell markers, except for lower levels of DX5 than cNK cells from the spleen. They were also shown to be mature, $CD11b^{high}CD27^{high}$ in phenotype [70]. Subsequent research has revealed that much like the liver, the NK cells of the skin can be divided into two populations: circulating cNK cells, which are $CD49a^{-}DX5^{+}$, and trNK cells which are $CD49a^{+}DX5^{-}$. The trNK cells of the skin share other phenotypic similarities with liver trNK cells, including constitutive CD69 expression and a lack of Eomes expression. Furthermore, skin trNK cells remain present in NFIL3-deficient mice, but still rely on T-bet for development, indicating a probable developmental link to liver trNK cells [15•]. The recent finding that liver trNK cells develop a memory-like phenotype in response to skin contact hypersensitivity lends support to the potential developmental connection between these two NK cell populations [18].

Studies in humans have shown that skin NK cells are capable of lysing melanoma cells in culture [71]. Most studies of skin NK cells have focused on their potential role in autoimmune diseases that affect the skin. For instance, NK cells have been linked to the progression of inflammatory skin diseases like atopic dermatitis, psoriasis, alopecia areata, and pemphigus vulgaris [72–78]. The evidence from these studies is mostly correlative linking NK cell trafficking and increased NK cell activity to the sites of inflammation, and true mechanistic studies are so far lacking.

Uterine NK cells

The murine uterus is also home to several NK cell subsets [15•,21]. Uterine (u)NK cells were found in 2-week old mice in an early study [79], and have been shown to proliferate rapidly after pregnancy [80]. Similarly to the liver, $CD49a^{-}DX5^{+}$ cNK cells and NFIL3-independent $CD49a^{+}DX5^{-}$ trNK cells are found in the uterus, indicating there are at least two NK cell populations in this organ. Interestingly, a recent study suggests that the $CD49a^{+}DX5^{-}$ population can be further subdivided into $CD49a^{+}Eomes^{+}$ and $CD49^{+}Eomes^{-}$ uNK cells [21].

Multiple studies have shown that under normal physiological conditions, uNK cells are protective toward the implanting fetus, and help to promote a normal pregnancy. In order for normal pregnancy to proceed, placental trophoblasts must invade the uterine tissues, and these invading extravillous trophoblast cells must be tolerated by the maternal immune system [81]. Trophoblast invasion is mediated by uNK cell production of VEGF-C, which maintains uNK cell non-cytotoxicity toward the invading fetal trophoblast cells and promotes vascularization of the placenta through upregulation of TAP-1 on extravillous trophoblast cells [82]. In humans, uNK cells were also shown to be protective against several viruses,

including HIV-1 [83] and HCMV [84]. It is unclear at this point how the different subsets of uterine NK cells coordinate their activities to contribute to the anti-pathogen response.

Kidney NK cells

Victorini *et al.* recently identified both CD49a^{-}DX5^{+} and NFIL3-independent CD49a^{+}DX5^{-} NK cells in the kidney, indicating the presence of cNK and trNK cells in this organ [22]. Interestingly, the authors also demonstrated that ischemic acute kidney injury was promoted by kidney trNK cells and not cNK cells as previously thought [22].

Pancreatic NK cells

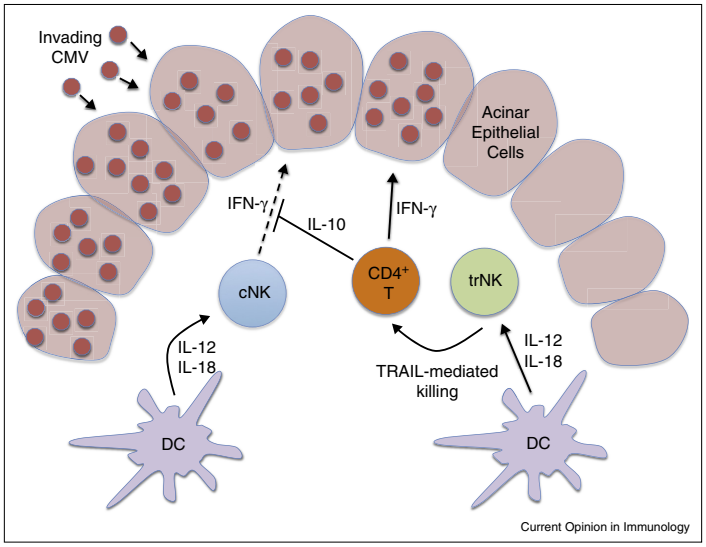
Pancreatic NK cells have been studied in the type 1 diabetes-prone NOD mice [85]. NK cells were identified in the exocrine pancreas of 4–5 week old mice, and in both the exocrine and endocrine pancreas of 9–10 week old mice, indicating a progressive infiltration of NK cells into the organ. Pancreatic NK cells were also observed in B6, BALB/c, and C3H mice, none of which exhibit pancreatic pathology or diabetes symptoms [85]. However, information on the development of these NK cells is lacking and additional studies are required to determine whether this organ harbors trNK cells.

Salivary gland NK cells

The murine SMG contains a resident population of NK cells with a unique maturation phenotype compared to cNK cells, being mostly CD27^{low}CD11b^{high}, yet lacking KLRG1 expression [16]. A recent study found that the NK cells of the salivary gland develop entirely independently of NFIL3, yet still express Eomes and T-bet, indicating a potentially novel developmental pathway [46]. We also find that SMG NK cells express Eomes and T-bet, but we find a significant reduction in frequency and number of NK cells in *NFIL3*$^{-/-}$ mice compared to wild-type (Erick and Brossay, unpublished data). This difference could be potentially explained by the influence of parameters such as housing, inflammation, and infection on the development of NFIL3-independent NK cells [3]. Similarly to the liver and the uterus, our data indicate that the SMG contains two populations of NK cells, with the majority being NFIL3-dependent cNK cells, and the minority NFIL3-independent tissue-resident NK cells. Notably, total SMG NK cells have a unique surface receptor phenotype in C57BL/6 mice, indicating that like the cNK cells of the skin, the phenotype of cNK cells that take up residence in the SMG is molded by tissue signals.

The SMG is a site of persistence for murine cytomegalovirus (MCMV) in an immunocompetent host. NK cells

Figure 1



Relationship between cNK, NFIL3-independent NK, and CD4^{+} T cells in the salivary glands. cNK and CD4^{+} T cells both produce IFN-γ in response to MCMV infection of SMG acinar epithelial cells. CD4^{+} T cells produce IL-10, which may dampen the cNK inflammatory response. At the same time, NFIL3-independent NK cells kill CD4^{+} T cells through TRAIL-mediated apoptosis during viral infection, presumably to limit organ damage.

are crucial components of the early immune response to MCMV, and work to clear the virus in a matter of days from all infected organs except the SMG, where the virus remains active for several weeks [86]. SMG NK cells express KLRG1 during MCMV infection, albeit at a lower magnitude than cNK cells in the spleen. CD69 expression is also increased on SMG NK cells during infection, indicating that these NK cells are capable of becoming activated in response to MCMV [87]. However, *in vivo* during the peak of infection, IFN-γ production by SMG NK cells is severely impaired when compared to splenic cNK cells [16,46]. This hyporesponsive phenotype was also observed *ex vivo* when SMG NK cells were stimulated with antibodies against NK cell activating receptors, IL-12/IL-18, or PMA/Ionomycin [16]. MCMV infection is known to induce severe salivary gland dysfunction, and SMG NK cells are crucial for limiting this secretory dysfunction [88,89]. Interestingly, a recent study indicated that TRAIL$^+$ SMG NK cells induce apoptosis of CD4$^+$ T cells in the SMG during MCMV infection [90]. Since CD4$^+$ T cells are crucial for eventual clearance of MCMV in the SMG [91], this TRAIL-mediated killing of CD4$^+$ T cells could contribute to the persistence of MCMV in the SMG, but also help to lessen inflammatory damage to this organ. CD4$^+$ T cells have also been shown to produce IL-10 in the SMG during MCMV infection [92], indicating that the immunoregulatory relationships between CD4$^+$ T cells, cNK, and NFIL3-independent NK cells in this organ and presumably other organs are quite complex (Figure 1).

Concluding remarks

Our understanding of innate immunology has come a long way in the 40 years since conventional NK cells were first discovered in mice [93,94] and humans [95]. The recent discovery and characterization of many new classes of innate lymphoid cells, including several distinct populations of tissue-resident NK cells, and of NK cell memory potential [96] shows that the lymphocytes of the innate immune system display diversity of phenotype and function akin to the adaptive immune system. cNK cells have long been viewed as an innate immune counterpart of CD8$^+$ and CD4$^+$ T cells in function [1]. Recent discoveries have revealed that NK cells in different tissues display a great capacity for phenotypic plasticity in order to serve their roles in protecting against infection, limiting inflammatory tissue damage, and in some cases in remodeling tissues [97,98]. In the lung and SMG, populations of bone marrow-derived cNK cells take up residence and are remodeled by local signals to develop tissue-specific phenotypes. The thymus, liver, skin, uterus, SMG, and kidney also contain populations of tissue-resident NK cells that belong to lineages distinct from cNK. These trNK cell populations have unique phenotypes and developmental pathways that set them apart from cNK cells. In tissues where cNK and trNK cells are present, their functions may complement each other to promote optimal tissue health.

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APPENDIX II

ROLE OF TYPE I INTERFERON RECEPTOR SIGNALING ON NK CELL DEVELOPMENT AND FUNCTIONS

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Role of Type I Interferon Receptor Signaling on NK Cell Development and Functions

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Abstract

Type I interferons (IFN) are unique cytokines transcribed from intronless genes. They have been extensively studied because of their anti-viral functions. The anti-viral effects of type I IFN are mediated in part by natural killer (NK) cells. However, the exact contribution of type I IFN on NK cell development, maturation and activation has been somewhat difficult to assess. In this study, we used a variety of approaches to define the consequences of the lack of type I interferon receptor (IFNAR) signaling on NK cells. Using IFNAR deficient mice, we found that type I IFN affect NK cell development at the pre-pro NK stage. We also found that systemic absence of IFNAR signaling impacts NK cell maturation with a significant increase in the $CD27^+CD11b^+$ double positive (DP) compartment in all organs. However, there is tissue specificity, and only in liver and bone marrow is the maturation defect strictly dependent on cell intrinsic IFNAR signaling. Finally, using adoptive transfer and mixed bone marrow approaches, we also show that cell intrinsic IFNAR signaling is not required for NK cell IFN-γ production in the context of MCMV infection. Taken together, our studies provide novel insights on how type I IFN receptor signaling regulates NK cell development and functions.

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Introduction

Innate lymphoid cells comprise a large number of subsets, including natural killer (NK) cells [1]. NK cells are cytotoxic effector lymphocytes of the innate immune system that develop from the common lymphoid progenitor (CLP) in the bone marrow [2,3]. The journey from CLP to NK cell starts with the earliest known precursor stage, termed the pre-pro A stage, which are $Lin^-ID2^+Sca1^+CD127^+CD117^{low}CD135^-CD122^-$. Pre-pro B have a similar expression profile, except that they lack CD117 [4,5]. The relationship between these two precursor populations is currently unclear, but they can both generate efficiently into NK cells *in vitro* and have lost B and T cell potential. After the pre-pro stages, the next step in NK cell development constitutes precursor cells that are $Lin^-CD27^+CD244^+CD127^+CD117^{low}CD135^-CD122^+$, termed rNKP. From here, the rNKP develop into immature NK, and ultimately mature NK cells [6]. A variety of transcription factors that affect NK cell development have been identified and are required at different development stages [7]. Among them, E4bp4 (also known as nfil3) is critical for NK cell production [8–10]. During NK cell lineage commitment, NK cell development and function is regulated by activating, inhibitory and cytokine receptor signaling. Among cytokines, type I interferons (IFN; also called IFN-α/β) are expressed rapidly from various cell types following exposure to a variety of infectious agents, and exert critical biological functions, even in the absence of infection [11]. The effects of type I IFN signaling on NK cell development have been difficult to assess due to the pleiotropic nature of these cytokines [12]. In this study, using a variety of approaches, we defined the type I interferon receptor (IFNAR) signaling contribution to NK cell development and function. We found that lack of type I IFN signaling has subtle but noticeable effects on NK cell development. While $IFNAR^{-/-}$ mice have the same number of mature NK cells as B6 mice, the number of NK cell progenitors is significantly decreased in the bone marrow of the deficient animals. We also found that $IFNAR^{-/-}$ mice have a significant increase in the $CD27^+CD11b^+$ NK cell compartment in all organs. However, while this maturation effect is direct in the liver and bone marrow, it is indirect in the spleen and blood, indicating tissue specificity. Finally, using adoptive transfer and mixed bone marrow approaches, we show that NK cell IFN-γ production is not affected by lack of type I IFN signaling in the context of MCMV infection.

Materials and Methods

Mice

C57BL/6, B6.SJL, and Rag2-IL2Rγ$^{-/-}$ mice were purchased from Taconic Laboratory Animals and Services, Germantown, NY. $IFNAR^{-/-}$ [13] and littermate control mice backcrossed onto a C57BL/6 background were bred in our animal facility. $IFNAR^{-/+}$ mice were bred, the resultant WT, $IFNAR^{-/+}$ and

IFNAR$^{-/-}$ mice were used. All mice were maintained at Brown University in accordance with institutional guidelines for animal care and use.

Murine Lymphocyte Isolation

Mice were sacrificed by isofluorane treatment. Cardiac puncture was performed prior to the harvesting of the organs. Livers were perfused with PBS-Serum (PBS +1% Fetal Bovine Serum) before harvesting. Spleens were dissociated using a plunger from a 3 mL syringe and lymphocytes were enriched using Lympholyte Cell Separation Media (Accurate Chemical). Livers were dissociated using a gentleMACS Dissociator (Miltenyi Biotec) and lymphocytes were enriched using a 40–70% discontinuous Percoll gradient (GE Healthcare) as previously described [14]. Salivary gland lymphocytes were prepared as described [14]. Briefly, SMGs were removed of all lymph nodes and connective tissue, followed by mincing. Single cell dissociation was performed using one incubation with digestion medium (RPMI 1640 containing 1 mg/ml of collagenase IV (Sigma), 5 mM CaCl2 50 μg of DNase I (Sigma) and 8% FBS) and continuous shaking at room temperature. The digestion mixture was pipetted vigorously to dissociate remaining cells. Supernatant was collected, passed through nylon mesh and the lymphocytes purified by layering on a lympholyte-M gradient. Bone marrow cells were isolated from femur and tibia. Red blood cells were lysed with ammonium chloride lysis buffer.

Adoptive transfer

Splenic NK cells were isolated from IFNAR$^{-/-}$ (CD45.2^{+}) mice and B6.SJL (CD45.1^{+}) mice using the STEMCELL NK Cell Isolation Kit, combined in a 1:1 ratio, and introduced into recipient Rag2-IL2R$\gamma^{-/-}$ mice by tail vein injection. The recipient mice were infected with MCMV four hours after transfer, and lymphocytes were isolated from the spleen, liver, and blood 40 hours after infection.

Mixed bone marrow chimeras

Mixed bone marrow chimeras were prepared as described previously [15]. Briefly, B6.SJL recipient mice were irradiated at 1050 rad. 24 hours later, the irradiated mice were injected with a 1:1 mixture of DX5 and CD5 depleted bone marrow cells derived from the femurs and tibias of B6.SJL and IFNAR$^{-/-}$ (or littermate heterozygote control) mice. After 7 to 9 weeks, bone marrow chimeras were used for infection and maturation experiments.

Infection and treatment protocols

Stocks of MCMV clone RVG102 were a gift of Dr. Hamilton [16], Duke University and MCMV Strain # VR1399 was received from American Type Culture Collection (ATCC). MCMV clone RVG102 is recombinant for GFP under the Immediate early gene-1 (ie-1) promoter. Both strains were prepared in the laboratory as previously described from salivary glands [17]. Infections were performed by intraperitoneal injection with 5×10^{4} plaque-forming units (pfu) of MCMV.

Flow cytometric analysis

Cells were suspended in 1% PBS-serum. Cells were first incubated with the blocking monoclonal antibody (mAb) 2.4G2, and then stained with mAbs specific for cell surface markers for 20 minutes at 4°C. For intracellular staining, cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes, and then stained in 1X PermWash (BD Biosciences) for 20 minutes. Events were collected on a FACSAria (BD) or a MacsQuant (Miltenyi Biotec). The resulting data was analyzed using FlowJo software (TreeStar). As a gating strategy, we first gate on lymphocytes based on forward scatter (FSC)-area versus side scatter (SSC)-area. Within this population, doublets are excluded, first by analyzing SSC-height versus SSC-width, then FSC-height versus FSC-width.

Lymphocyte Counts

Lymphocytes were counted by hemocytometer. Lymphocytes suspended in PBS +1% Serum (GIBCO) were diluted in Trypan Blue (GIBCO). All four quadrants were counted and the averages taken for cell counts. Alternatively, cells were diluted and counted with PI exclusion using the Miltenyi MACSQuant.

Antibodies and reagents

FITC-DX5, PerCPCy5.5-NK1.1, PE-IFN-γ, APC-CD3, eF450-CD3, eF450-Lin, PE-CD49a, FITC-TCRβ, PE-CD27, PECy7-CD11b, eF780-CD45.2, eF450-CD45.1, FITC-Granzyme-B, APC-Ly49H, FITC-LY49H, PE-TCRβ, APC-CD45.1, eF450-Ki-67, PerCPeF710-CD27, PECy7-NK1.1, eF450-CD11b, AF700-CD3, PECy7-Sca-1, AF700-CD117, AF750-CD127, APC-CD135, FITC-CD244, PE-CD122 were purchased from eBioscience and BioLegend (San Diego, CA).

Statistical Analysis

Data sets were analyzed for statistical significance using unpaired, two-tailed, Student's t-tests. Values of $p<0.05$ were considered significant. Graphs and statistics were generated and calculated using Prism (GraphPad). Statistical analysis were performed using GraphPad Prism (GraphPad Software, San Diego, CA), where * = p≤0.05, ** = p≤0.005, *** = p≤0.0005, and **** = p≤0.0001.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals as defined by the National Institutes of Health (PHS Assurance #A3284-01). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University. All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical, and veterinary personnel.

Results

IFNAR signaling does not affect NK cell number or frequency

We first compared the NK cell cellularity in IFNAR$^{-/-}$ and littermate controls and found there were no significant differences in number or frequency in all tissues tested (Figure 1). Stable distributions of NK cell populations were also found in chimeric mice regardless of the donor bone marrow source, IFNAR$^{-/-}$ or wild-type (Figure S1). We then investigated whether type I interferon receptor signaling had a significant role in the development of bone marrow derived NK cell populations. To examine this, we first looked at the CD11b/CD27 expression of CD3^{-}NK1.1^{+} NK cells. These two markers characterize NK cells from least mature to most mature as follows: CD11b^{low}CD27^{low}, CD11b^{low}CD27^{high}, CD11b^{high}CD27^{high}, CD11b^{high}CD27^{low} [18]. In IFNAR$^{-/-}$ mice, the double positive CD27^{+}CD11b^{+} NK cell population was consistently and significantly increased in the spleen, liver, blood, and bone marrow (Figure 2A–D, and Figure S2). This increase in maturation was also accompanied by

a subsequent decrease in the single positive $CD27^+CD11b^-$ NK cell population in all organs, as well as a decrease of the most mature NK cells ($CD27^-CD11b^+$) in the periphery. Thus, although the NK cell populations were unaffected in proportion, $IFNAR^{-/-}$ NK cells are enriched at the $CD27^+CD11b^+$ DP stage. Thus NK cell maturation is affected when IFNAR signaling is abrogated systematically. Notably there is no maturation difference between the wild-type and the *IFNAR*$^{-/+}$ (data not shown).

Pre-pro NK cells are reduced in IFNAR deficient mice

Using the original definition of NK precursors [19], we found that iNK cells, characterized as $CD3^-CD122^+NK1.1^+CD11b^-$, were decreased in the bone marrow of $IFNAR^{-/-}$ mice while mNK cells were increased, shown by the markers $CD3^-CD122^+NK1.1^+CD11b^+$ (Figure 3). The increase of mNK in the bone marrow is due to the accumulation of $CD11b^+CD27^+$ NK cells, also seen in the periphery. Interestingly, using the recently defined characterization of pre NK cell progenitors [4,5] we found that the number of prepro NK A and B were both significantly decreased in the $IFNAR^{-/-}$ mice (Figure 3). This is consistent with previous findings in hematopoietic stem cells (HSCs) showing that type I IFN induce proliferation of HSCs [20,21].

Tissue resident NK cells are not affected by the absence of IFNAR

Tissue NK cells, which include liver resident NK cells, salivary gland NK cells, skin NK cells, and uterus NK cells are beginning to be characterized [22]. We first analyzed the newly characterized liver resident NK cells [23–25]. Although the frequency of liver DX5-negative NK cells was significantly decreased in IFNAR deficient mice, we found that the number of liver $IFNAR^{-/-}$ DX5 negative NK cells was similar to wild-type DX5 negative NK cells (Figure 4). We also characterized salivary glands NK cells [14] in IFNAR deficient mice and found no significant difference in frequency between the wild-type and the $IFNAR^{-/-}$ animals (data not shown). Thus, type I interferon appears to be dispensable for the development of tissue resident NK cells.

IFNAR signaling affects NK cell maturation intrinsically in the bone marrow and liver

To determine the exact contribution of IFNAR signaling on NK cell development and maturation, we generated a series of mixed bone marrow chimeras. $IFNAR^{-/-}$ (or $IFNAR^{+/-}$) bone marrow and wild-type competitor bone marrow were transferred at a 1:1 ratio into lethally irradiated wild-type recipients. The phenotype observed in the straight IFNAR deficient mice was conserved in the bone marrow and liver of the chimeric mice with a decrease of $CD27^+$ NK cells and an accumulation of the DP $CD11b^+/CD27^+$ NK cells (Figure 5). A representative staining is shown in Figure S3. This phenotype was seen when we compared the $IFNAR^{-/-}$ NK cells and their wild type competitor within the same mice as well when we compared the NK cells from WT:WT chimeric mice to the $IFNAR^{-/-}$ NK cells from the WT:$IFNAR^{-/-}$ chimeras. In contrast in the blood and spleen, no differences in the $CD27^+$SP, $CD11b^+CD27^+$DP, and $CD11b^+$SP maturation stages were found (Figure 5). These data suggest that IFNAR signaling affects NK cell maturation intrinsically in the bone marrow and liver, but is compensated by other mechanisms in the periphery.

$IFNAR^{-/-}$ NK cells remain open to IFN-γ production during viral infection

Type I IFN exert pleiotropic effects on different cell subsets, making it difficult to assess the effect of these cytokines on NK cell functional responses. To circumvent this, we have designed two approaches. First, we used mixed bone marrow chimeras (Figure 6A), which allowed us to compare the response of wild-type NK cells and $IFNAR^{-/-}$ NK cells to viral infection in the same environment. $IFNAR^{-/-}$ mice are extremely sensitive to MCMV infection and succumb at a non-lethal dose, even in the resistant B6 background (data not shown and [26]). However, mixed bone marrow chimeras (WT:$IFNAR^{-/-}$) in B6 background

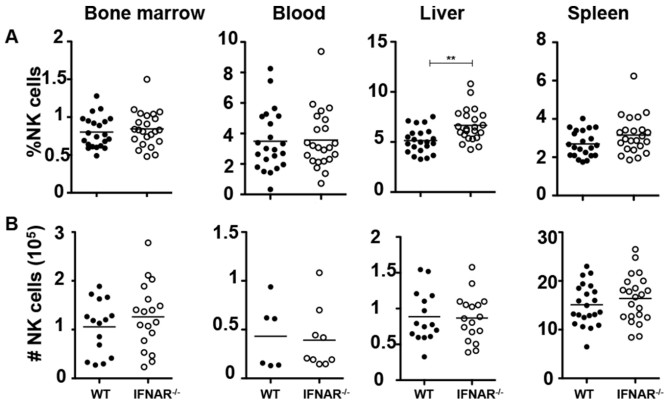

Figure 1. NK cell number and frequency in IFNAR deficient mice. (A) Percentages of the indicated organs containing NK cell ($NK1.1^+CD3^-$ in the lymphocyte gate) populations in the indicated organs from wild-type littermate (black circles), and IFNAR deficient mice (open circles). Data are pooled from at least 5 independent experiments and each dot represents data obtained from one mouse; horizontal lines indicate the mean. (B) Total NK cell numbers of the indicated organs. Data are pooled from at least 5 independent experiments and each dot represents data obtained from one mouse; horizontal lines indicate the mean.
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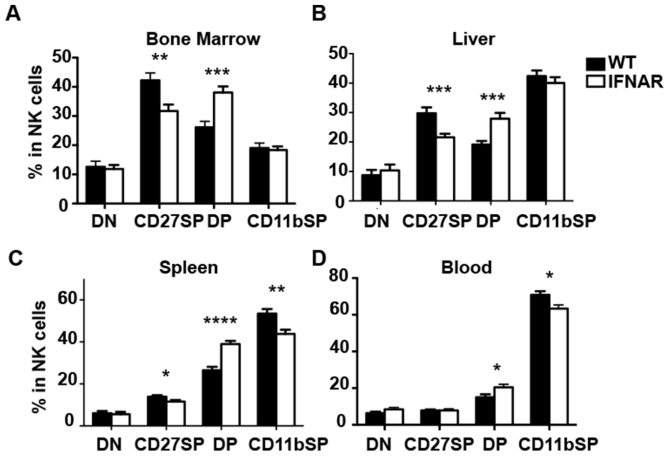

Figure 2. Impaired NK cell development in IFNAR deficient mice. Percent of NK cells (NK1.1$^+$CD3$^-$) being DN, CD27 SP, DP, and CD11b SP populations in the indicated organs. Representative figures of at least five independent experiments are shown.
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are less sensitive to MCMV infection (data not shown). We therefore infected chimeric mice with MCMV and compared the responses of wild type NK cells and IFNAR$^{-/-}$ NK cells, which have developed in the same environment. Both populations of NK cells produced IFN-γ at the same level (number of cells producing IFN-γ and amount produced per cell) regardless of the expression of the IFNAR at their cell surface (Figure 6A). As a second approach, we adoptively transferred equal numbers of enriched wild type NK cells and IFNAR$^{-/-}$ NK cells into Rag2IL-2Rγ$^{-/-}$ mice, which lack B, T, and NK cells, and simultaneously infected the recipient mice with MCMV (Figure 6B). Similarly to the chimeric mice, we found no significant difference in IFN-γ production by adoptively transferred IFNAR$^{-/-}$ and wild type NK cells (Figure 6 B). Finally, IFN-γ release by *ex vivo* splenic NK cells from mixed bone marrow chimeric mice upon crosslinking with NK1.1 and Ly49H was similar in wild type NK cells and IFNAR$^{-/-}$ NK cells (Figure 6C). This was also the case during activation by IL12/IL18 or PMA treatment (Figure 6C). Altogether, our data demonstrate that IFNAR signaling is not required intrinsically for NK cell IFN-γ response during MCMV infection.

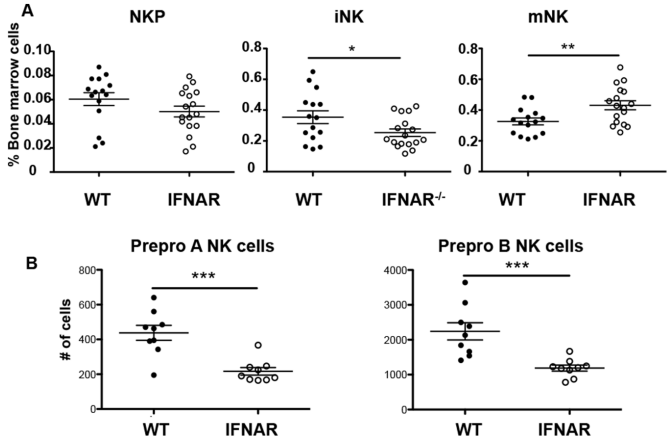

Figure 3. Pre-pro NK cells are reduced in IFNAR deficient mice. (A) Percent NKPs (CD122$^+$NK1.1$^-$CD3$^-$CD11b$^-$), iNK cells (CD122$^+$NK1.1$^+$CD3$^-$CD11b$^-$), and mNK cells (CD122$^+$NK1.1$^+$CD3$^-$CD11b$^+$) among bone marrow lymphocytes derived from littermate wild-type (closed circles), and IFNAR (open circles) mice. Data are pooled from at least 3 independent experiments and each dot represents data obtained from one mouse; horizontal lines indicate the mean. (B) Number of pre-pro NK cells from wild type and IFNAR deficient mice. Data are pooled from at 3 independent experiments and each dot represents data obtained from one mouse.
doi:10.1371/journal.pone.0111302.g003

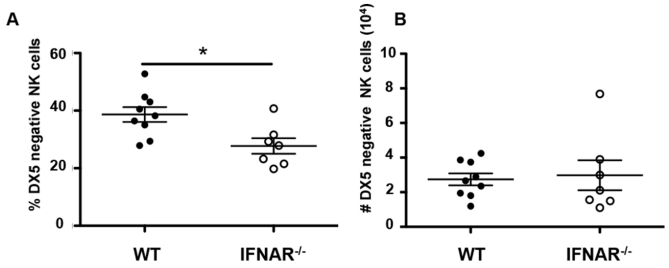

Figure 4. Liver resident NK cells in IFNAR deficient mice. Percentages (A) and number (B) of the liver resident NK cell (NK1.1$^+$CD3$^-$DX5$^-$ in the lymphocyte gate) populations from wild-type littermate (closed circles), and IFNAR deficient mice (open circles). Data are pooled from at least 3 independent experiments and each dot represents data obtained from one mouse.
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However, as reported by others [27], IFNAR$^{-/-}$ NK cells produce significantly less granzyme B than wild-type competitor NK cells in MCMV infected chimeric mice (Figure 6D).

Discussion

In this study, we have examined the role of IFNAR signaling during NK cell development and maturation, which has been somewhat difficult to delineate due to the pleiotropic nature of these cytokines [11]. Using the recently defined characterization of NK cell progenitors [4,5], we unexpectedly found that IFNAR deficient mice have a lower number of progenitor NK cells than their wild-type counterparts. In the absence of infection, type I IFN are produced constitutively at low levels by a variety of cell types. The decreased number of NK cell progenitors in IFNAR deficient mice is in agreement with recent studies showing that type I IFN induce proliferation in hematopoietic stem cells [20,21]. While it is well known that type I IFN induces the expression of IL-15 [28,29], the effect observed here cannot be attributed to a lack of response to IL-15, since pre-pro NK cells do not express CD122. Once committed to the NK lineage, we found that NK cell number in the bone marrow from IFNAR deficient mice is comparable to wild type animals, suggesting that redundancy with other stimuli occurs at this stage.

Our results also demonstrate that the requirement of type I interferon receptor signaling at different stages of NK cell development is tissue specific. We found that the effect of type I interferon on NK cell maturation is indirect in the blood and spleen but direct in the liver and bone marrow. In fact, in the periphery, wild-type and IFNAR$^{-/-}$ NK cells appear to be developmentally indistinguishable, suggesting that other signals have compensated for the lack of IFNAR on NK cells. In a recent study using conditional NCR-1 cre-IFNAR$^{-/-}$ mice, no NK cell maturation defect was found [30]. However, NKp46 is expressed

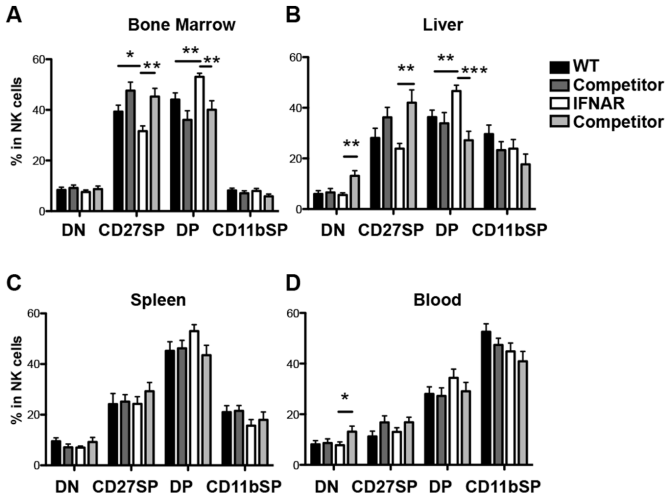

Figure 5. IFNAR signaling regulates NK cell maturation intrinsically in liver and the bone marrow. Percent of NK cells (NK1.1$^+$CD3$^-$ in the lymphocyte gate) expressing DN (CD27$^-$CD11b$^-$), CD27 SP (CD11b$^-$CD27$^+$), DP (CD27$^+$CD11b$^+$), and CD11b SP (CD27$^-$CD11b$^+$) populations in the indicated organs derived from mixed bone marrow chimeras. Data are pooled from at least 5 independent experiments. Error bars indicate SEM.
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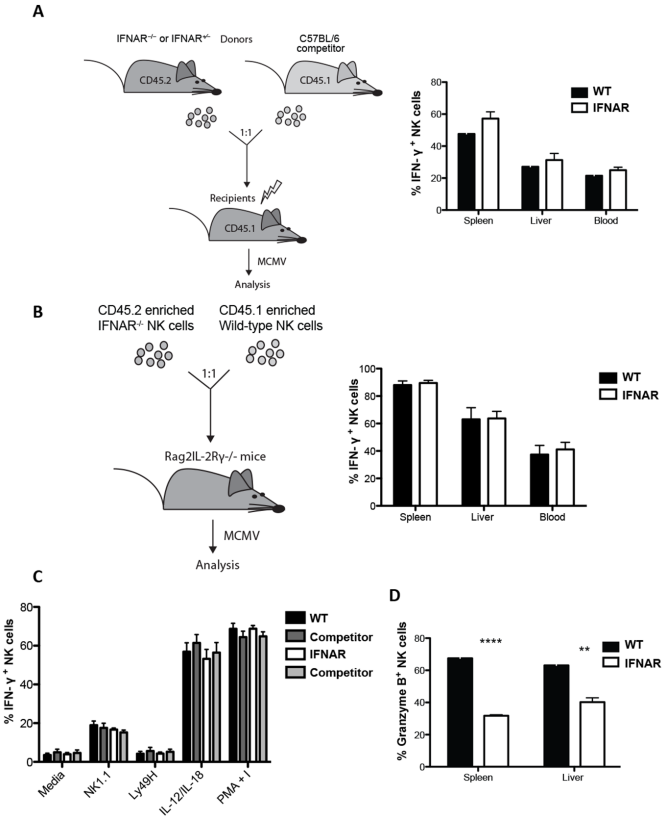

Figure 6. IFNAR signaling is not intrinsically required for NK cell IFN-γ production during MCMV infection. (A) Intracellular IFN-γ in NK cell populations ($NK1.1^+CD3^-$ in the lymphocyte gate) from mixed bone marrow chimeric mice infected with MCMV. Representative of 4 independent experiments with 3 mice per group. (B) Intracellular IFN-γ in adoptively transferred NK cell populations ($NK1.1^+CD3^-$ in the lymphocyte gate) from Rag2IL-2R$\gamma^{-/-}$ mice infected with MCMV. Representative of 2 independent experiments with 3 mice per group. (C) Intracellular IFN-γ in NK cell populations ($NK1.1^+CD3^-$ in the lymphocyte gate) from total splenocytes incubated with antibody-coated plates using anti-Ly49H, and anti-NK1.1, or with a mix of IL-12/IL-18 and PMA and ionomycin (P/I) for six hours are assessed after gating on donor cells. Representative of 5 independent experiments with 3 mice per group. (D) Intracellular Granzyme B in NK cell populations ($NK1.1^+CD3^-$ in the lymphocyte gate) from mixed bone marrow chimeric mice infected with MCMV. Representative of 2 independent experiments with 3 mice per group.
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relatively late in NK cell differentiation [6], possibly at a time when the effects of IFNAR signaling on NK cell maturation have already occurred. In bone marrow and liver, it is unclear how the double positive $CD27^+CD11b^+$ NK cells fill the niche left open from the lack of type I interferon intrinsically regulated NK cells. In these organs it is possible that the significant increase in the double positive $CD27^+CD11b^+$ NK cell population compensates for the lack of interferon signaling, as double positive NK cells have been shown to be more sensitive to cytokine stimulation and proliferation [31]. Therefore, it appears to be a buffering mechanism for NK cell development to retain a stable population of NK cells in different degrees of type I interferon signaling. This potentially indicates that evolutionarily favorable feedback mechanisms may be in place to buffer type I IFN signaling for NK cell populations, which is greatly increased in viral infection and in some cases chronically dysregulated.

The identification of new innate lymphoid cell (ILC) subsets has been exponential in recent years. Although the classification of NK cells as a member of ILC1 is still a matter of debate [22], it appears there are alternative developmental pathways for NK cells at least in liver and bone marrow [24]. Our results suggest that interferon receptor signaling is dispensable for the development of the recently described tissue resident NK cells, as well as salivary gland NK cells.

The direct role of IFNAR signaling during viral infection has been somewhat controversial. While one study described a weak direct NK cell response to type I IFN during MCMV infection [32], others have shown a critical and direct type I IFN role during Vaccinia virus [33,34] or HSV infections [35]. Notably, using $IFNAR^{-/-}$ mice in the 129 background and DX5 to identify NK cells, NK cell IFN-γ was not affected *in vitro* [36]. To eliminate any indirect extrinsic effects of type I IFN, we used two different

approaches that allowed us to compare the response of wild type and IFNAR deficient NK cells in the same environment. As we failed to show a significant difference in NK cell IFN-γ production following infection, our data argue that a direct type I IFN effect on NK cells is weak or subtle, at least during MCMV infection. Therefore, the impaired NK cell response to viral infection when IFNAR is systematically deleted is indirect and likely due to dendritic cell priming as previously reported [27,32]. However, and in agreement with others [27], in the absence of IFNAR signaling, NK cell granzyme B production is decreased suggesting that NK cell granzyme B production is dependent in part of IFNAR signaling, perhaps due to impaired granzyme accumulation [37]. To summarize, we conclude that absence of type I IFN signaling affects the development of NK cell progenitors, the maturation of NK cells in a tissue specific manner, and the development of tissue resident NK cells. However, we found that type I IFN has no direct effect on IFN-γ production by NK cells during viral infection.

Supporting Information

Figure S1 NK cell number and frequency in chimeric mice. Percent (A) and total NK cells (B) ($NK1.1^{+}CD3^{-}$ in the lymphocyte gate) present in the indicated organs from the different chimeric mice. Data are pooled from at least 3 independent experiments and each symbol indicates an individual mouse; horizontal lines indicate the mean.
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Figure S2 Altered NK cell maturation in $IFNAR^{-/-}$ mice. Flow cytometry of CD27 vs CD11b expression in NK cells of the indicated organs from $IFNAR^{-/-}$ and littermate wild-type control mice. Data are representative of at least 5 experiments.
(TIF)

Figure S3 NK cell maturation is affected intrinsically in liver and the bone marrow. (A) Gating strategy to separate NK cells from $IFNAR^{-/-}$ and wild type competitor donors. (B) Flow cytometry of CD27 vs CD11b expression in NK cells of the indicated organs. Data are representative of at least 5 experiments.
(TIF)

Author Contributions

Conceived and designed the experiments: JG SMSM ZSW TKE CB LB. Performed the experiments: JG SMSM ZSW TKE CB. Analyzed the data: JG SMSM ZSW TKE CB LB. Wrote the paper: SMSM ZSW TKE LB.

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