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*J Immunol* published online 14 February 2014
http://www.jimmunol.org/content/early/2014/02/14/jimmunol.1302605

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/02/14/jimmunol.1302605

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Differential Requirement for Nfil3 during NK Cell Development

Cyril Seillet,*† Nicholas D. Huntington,*† Pradnya Gangatirkar,* Elin Axelsson,‡ Martina Minnich,§ Hugh J. M. Brady,§ Meinrad Busslinger,§ Mark J. Smyth,¶ Gabrielle T. Belz,*† and Sebastian Carotta*†

NK cells can be grouped into distinct subsets that are localized to different organs and exhibit a different capacity to secrete cytokines and mediate cytotoxicity. Despite these hallmarks that reflect tissue-specific specialization in NK cells, little is known about the factors that control the development of these distinct subsets. The basic leucine zipper transcription factor Nfil3 (E4bp4) is essential for bone marrow–derived NK cell development, but it is not clear whether Nfil3 is equally important for all NK cell subsets or how it induces NK lineage commitment. In this article, we show that Nfil3 is required for the formation of Eomes-expressing NK cells, including conventional medullary and thymic NK cells, whereas TRAIL+ Eomes− NK cells develop independently of Nfil3. Loss of Nfil3 during the development of bone marrow–derived NK cells resulted in reduced expression of Eomes and, conversely, restoration of Eomes expression in Nfil3−/− progenitors rescued NK cell development and maturation. Collectively, these findings demonstrate that Nfil3 drives the formation of mature NK cells by inducing Eomes expression and reveal the differential requirements of NK cell subsets for Nfil3. The Journal of Immunology, 2014, 192: 000–000.

The innate immune system provides a front-line defense against invading pathogens and cells undergoing malignant transformation. NK cells are an essential component of this rapid-response armory, largely because of their ability to detect and kill target cells without prior sensitization. The majority of NK cells in the adult mouse are thought to develop in the bone marrow (BM) from common lymphoid progenitors (CLP) that give rise to committed NK cell precursors (NKP) (1). Until recently, the NKP was thought to be the earliest known committed NK cell within the BM. NKP are identified through the expression of IL-2β-chain (CD122) and the lack of NK cell surface markers NK1.1 and CD49b (2). Despite this, it is clear that this population is heterogeneous, because <30% of cells can differentiate into NK cells in vitro (2). Using a reporter mouse model in which GFP is expressed under the control of the transcription factor Id2 (3, 4), we purified an Id2-GFP+ NKP subset that efficiently gave rise to NK cells and had lost T cell potential (5). Subsequently, Fathman et al. (5) demonstrated that a pure NKP population could be isolated without the use of the ID2-GFP reporter mouse model by selecting Lin−CD27−CD44+CD122+IL7R+ Flt3− cells from the BM. Developing from NKP, immature NK (iNK) cells acquire NK1.1 expression and subsequently give rise to CD122+NK1.1− NKP46+CD49b− mature NK (mNK) cells that gain effector function and circulate throughout the body. These conventional NK cells (cNK) express high levels of CD49b (DX5) and secrete high levels of IFN-γ and IL-13 (1, 6). In addition to BM, the liver and thymus are sites of NK cell development (1). Thymic NK cells are derived from early thymic precursors and differ from cNK cells in that they retain high levels of IL-7Rα, depend on the transcription factor Gata-3, and are competent cytokine producers but have poor cytotoxicity (7). In addition to liver-resident CD49b+ cNK cells, a population of CD49b− NK cells that constitutively express TRAIL exists (8, 9). TRAIL+ NK cells constitute the main NK cell population in fetal and neonatal mice and decrease with age, but a stable population is retained during adulthood in liver. Functionally, TRAIL+ NK cells are poor secretors of IFN-γ and IL-13, and they were reported to possess memory-like properties (10, 11).

The transcriptional network that regulates NK cell commitment and controls the different NK cell fates is not well understood (12). Recent evidence suggests that different transcription factor networks drive the formation of these distinct subsets. The transcription factors T-bet and Eomes were described to coordinately control the generation of BM-derived CD49b+ NK cells, whereas Eomes expression is dispensable for TRAIL+ NK cell development (13). The downstream molecular events and transcription factors that induce the expression of Eomes have not been identified. In addition to its role in thymic NK cell development, Gata-3 is implicated in the homing of NK cell progenitors to the liver (7, 14). Id2 and Nfil3 are accepted to be important regulators of cNK cell development; however, how they orchestrate this, as well as differentiation of NK cells within the liver, remains unknown. The lack of an experimental approach to specifically dissect the roles of Id2 and Nfil3 in the development of NK cells is a major limitation that has hampered further investigation. The ability to conditionally knockout these genes would allow for a more detailed understanding of the roles of Id2 and Nfil3 in NK cell development.
as their role in the development of alternative NK cell subsets, is not clear. Deletion of either Id2 or Nfil3 results in a severe reduction in cNK cells in the BM, spleen, liver, and lung (15–19). It was proposed that Nfil3 regulates the expression of Id2, because Nfil3-deficient NK cell progenitors were reported to exhibit reduced Id2 expression compared with controls, and ectopic expression of Id2 partially rescued Nfil3−/− NK cell development (15). In both Id2− and Nfil3-deficient mice, the generation of CD122+NK1.1+ CD49b− NKP was unaffected, yet iNK cells were strongly reduced. However, given that only ~30% of NKP possess NK cell potential, it is not clear precisely when Id2 and Nfil3 act to control NK cell commitment.

In this article, we show that cNK cells depend on Nfil3 and that it is critical for the development of thymic NK cells. Strikingly, we found that the generation of TRAIL+ liver NK cells in adult mice and neonates occurs independently of Nfil3. Furthermore, we show that Nfil3 promotes cNK cell development by regulating the expression of the transcription factor Eomes. Thus, in this study, we define a novel regulatory mechanism in which Nfil3 is essential for BM-derived cNK and thymic NK cell development but is dispensable for TRAIL+ NK cells.

Materials and Methods

Mice

Nfil3-deficient Id2GFP/− reporter (Nfil3−/− Id2GFP/−) mice were obtained by crossing Id2GFP/− mice (4) with Nfil3−/− mice (15) and maintained under specific pathogen–free conditions at the Walter and Eliza Hall Institute animal breeding facility, according to institute guidelines.

Cell preparation

Single-cell suspensions were prepared from freshly harvested spleen, liver, thymus, and BM. Liver mononuclear cells were separated from the hepatocytes by centrifugation at 690 × g for 30 min at room temperature. BM cells were isolated from long bones and sternum that were smashed with a mortar and pestle and filtered. Lysis of RBCs of thromocytes by centrifugation at 450 rad, 3 h at 4°C was used for comparisons. A p value < 0.05 was regarded as significant. All results are expressed as mean ± SEM.

Results

Nfil3 expression during early NK cell development

Nfil3 was shown to be expressed at low levels in Lin− hematopoietic progenitors and NKP and at higher levels in iNK and mNK cells (15). Because the Lin− progenitor population and NKP [1 in 10 cells are NK cell committed (2)] used to assay this are heterogeneous in composition, the precise developmental stage at which Nfil3 is expressed and required during NK cell commitment remains unresolved. To determine the global transcriptional changes that occur during the different stages of NK cell commitment, we sequenced the transcriptome of NKPId2-GFP cells (Lin− CD122+Id2-GFP’NKP1.1− CD49b−), iNK cells (Lin− CD122+Id2-GFP’NKP1.1− CD49b−), and mNK cells (Lin− CD122+Id2-GFP’NKP1.1+CD49b+), as shown in Fig. 1F, and compared these data with the previously published transcriptome of all lymphoid progenitors (ALP; Lin− B220− IL7Rα−Flt3/Flik2/2SCaIlowCD117lowLy6D−), B cell–biased lymphoid progenitors (BLP; Lin− B220− IL7Rα−Flt3/Flik2/2SCaIlowCD117lowLy6D−), pro-B cells (CD19+ B220+ cKit+CD25+ IgM−) (21). We found that Nfil3 was expressed at the ALP stage and during lymphoid development, was slightly reduced in NKP and BLP, and was absent in pro-B cells (Fig. 1A). In contrast, Id2 was expressed at very low levels in ALP and not expressed in BLP, was strongly induced at the NKP stage, and continued to be highly expressed in downstream NK cell progenitors, as we reported previously (3, 4), confirming the purity of our sorting strategy (Fig. 1A). These data highlight that Nfil3 is expressed in ALP and continues to be expressed throughout NK cell development, providing a clear map of the temporal expression of Nfil3.

ID2 is expressed in the absence of Nfil3

Nfil3 expression is essential for the development of BM-derived cNK cells (15, 16), although the mechanism by which Nfil3 regulates this process is not well understood. First, we determined whether Id2 expression depended on Nfil3 by crossing Nfil3−/− mice with the Id2GFP/− reporter line. Surprisingly, Id2-GFP expression was similar in splenic cNK cells (TCRγ− NK1.1−CD49b− NKP46− NK cells) from both Id2GFP/− and Nfil3−/− Id2GFP/− strains (Fig. 1B) and paralleled Id2 mRNA expression (Fig. 1C). Similar results were obtained from NK cells isolated from BM, thymus, and liver (see below, also data not shown). These data indicate that Nfil3 is not required for Id2 expression in mNK cells isolated from spleen and liver and questions an earlier report (15) that used a heterogeneous mixture of Lin− cells, including progenitors that do not express Id2 (3, 4), to conclude that Nfil3 regulates Id2 expression. To further delineate Id2 expression in NK cell–committed progenitors, Id2-GFP expression was analyzed in NKP and iNK and mNK cells from wild-type (WT) and Nfil3−/− mice (Fig. 1D, 1E). No differences in Id2-GFP expression among these populations were observed (Fig. 1F). Thus, our data show that Nfil3 is not required for Id2 expression in NK cell–committed
progenitors; therefore, loss of Id2 is not likely to be responsible for the NK cell defect in Nfkβ3−/− mice, as originally proposed (15).

Nfil3 functions at the earliest stage of NK cell development

In Nfil3-deficient mice, the total number of iNK and mNK cells, but not NKP, is reported to be strongly reduced (15, 16). This implies that Nfil3 may begin to regulate NK cell development at the iNK cell stage. However, given the impure nature of Lin−CD122+NK1.1−CD49b− NK cells purified from the indicated mouse strains (Fig. 1G), we analyzed NK cell expression of early NK cell development in NK cells purified from the indicated mouse strains (Fig. 1H). However, we observe a small, but statistically significant, reduction in NKP expression (Fig. 1G), as assessed by RT-PCR. Data show transcription factor expression normalized to HPRT (mean ± SEM, 2-3 independent experiments with two mice/group). (C) In intracellular flow cytometric analysis of Eomes and T-bet in splenic TCRβNK1.1+CD49b− mNK cells from WT or Nfil3−/− mice (left panels). Dashed line shows isotype control staining. Bar graphs show mean (±SEM) MFI of each transcription factor pooled from two independent experiments (n = 3 mice/group) (right panels). *p < 0.05, **p < 0.01, two-tailed Mann–Whitney U test.

Nfil3-deficient cNK cells express Eomes at reduced levels

Given that impaired Id2 regulation could not explain the NK cell defect in Nfil3−/− mice, we investigated whether Nfil3 was required for the induction of other key NK cell commitment factors. The transcription factors ETS1, T-bet (Tbx21), and Eomes are known to be essential for NK cell commitment (13, 22, 23). RT-PCR analysis of these transcription factors in Id2−/− and Nfil3−/− Id2−/− BM-derived NK cell progenitors and mNK cells showed no difference in the expression of ETS1 and T-bet (Fig. 2A). Eomes expression was lower in WT NKP and iNK and mNK cells, but it was strongly upregulated in mNK cells (Fig. 2A). However, Nfil3−/− mNK cells failed to upregulate Eomes to levels found in WT mice. We observed a similar defect in the few remaining mNK cells in the spleen of Nfil3−/− mice. In conclusion, Nfil3−/− iNK cells found in the BM and the spleen expressed a significantly lower level of Eomes, whereas T-bet expression was comparable between WT and Nfil3−/− mNK cells (Fig. 2).

TRAIL+ Eomes− liver NK cells, but not thymic NK cells, develop independently of Nfil3

Recently, Gordon et al. (13) demonstrated that Eomes is required for cNK cell development, but it is not expressed by liver-resident TRAIL+ NK cells; thus, these latter cells do not depend on Eomes expression. Analyses of thymic NK cells revealed that they also express Eomes, suggesting that Eomes expression also may be required for their development (Supplemental Fig. 1). Given the differential expression of Eomes in NK cell subsets and its reduced expression in the absence of Nfil3, we analyzed NK cell populations in the liver and thymus of Nfil3−/− mice. Because NK cells represent a very rare population in the thymus, we identified thymic NK cells as TCRβCD1dNK1.1NKp46CD49bId2-GFP+ cells, which allowed us to exclude potential contamination by T and NKT cells. We found that thymic NK cells were severely reduced in Nfil3−/− mice (Fig. 3A–C); thus, Nfil3 is essential for the development of cNK cells, as well as thymic NK cells. Within the liver, CD49b+TRAIL− cNK cells were severely reduced in Nfil3−/− mice, as might be predicted. However, TRAIL+ NK cells were not significantly reduced and represented the main NK population in the liver of Nfil3−/− mice (Fig. 3D). Thus, the requirement for Nfil3 expression differs between NK cell subsets, and the dependency for this transcription factor correlates with the expression of Eomes in the various NK subsets.

Characterization of Nfil3−/− TRAIL+ liver NK cells

Analysis of the expression of T-bet and Eomes in hepatic NK cells showed that, similar to BM-derived NK cells, WT and Nfil3−/− CD49b+TRAIL− cells, as well as TRAIL+ NK cells, expressed equivalent levels of T-bet (Fig. 4A). However, Nfil3-deficient CD49b+ NK cells expressed Eomes at reduced levels (Fig. 4). Notably, Nfil3 was expressed at similar levels in cNK and liver-derived NK cells (Fig. 4B).
One explanation for the differential effect of Nfil3 in NK cells may be related to the altered expression of homing receptors. Gata-3 has been implicated in the regulation of homing of cNK cells to the liver; because Nfil3<sup>−/−</sup> cNK cells are severely reduced in the liver, we determined the expression of Gata-3 in splenic and liver NK cell subsets from WT and Nfil3<sup>−/−</sup> mice (14). Gata-3 expression was...
highest in TRAIL+ hepatic NK cells, lowest in spleenic NK cells, and intermediate in liver CD49b+ NK cells; however, no significant difference in expression was seen between WT and Nfil3−/− NK cell subsets (Fig. 4B).

TRAIL+ NK cells are phenotypically distinct from CD49b+ NK cells in their expression of CD11b, CD27, and Ly49 receptors. cNK cells can be segregated into immature CD27highCD11blow NK cells that give rise to CD27highCD11bhigh NK cells that subsequently lose CD27 and gain KLRG1 expression (24–28). Liver NK cells present in the liver of WT and Nfil3−/− mice on days 1 and 5 d after birth. As expected, the majority of CD3−NK1.1+CD49b+ NK cells present in the liver of WT and Nfil3−/− mice days 1 and 5 did not express Eomes (Fig. 6A, 6B). Consistent with adult mice, the Eomes− NK cell subset was unaffected in Nfil3-deficient newborn mice (Fig. 6A, 6B, 6D). In contrast, Eomes+ cNK cells were strongly reduced in the spleen of 5-d-old Nfil3−/− pups, whereas Eomes− NK cells were similar between WT and Nfil3−/− mice (Fig. 6C, 6E). Thus, the TRAIL+ NK cell subset develops independently of Nfil3 in neonates.

Expression of Eomes is sufficient to rescue cNK cell differentiation in the absence of Nfil3

The loss of either Nfil3 or Eomes during NK cell development negatively affects cNK cell formation, but not TRAIL+ NK cell formation, suggesting that Nfil3 may regulate cNK development by activating Eomes expression. Therefore, we tested whether ectopic expression of Eomes in Nfil3−/− multipotent progenitors could rescue cNK cell development. Indeed, we found that Eomes expression was able to compensate for the lack of Nfil3 expression during NK cell development. In both WT and Nfil3−/− hematopoietic progenitors, enforced expression of Eomes promoted the formation of cNK compared with nontransduced cells (GFP−) or control infected cells (GFP+) (Fig. 7A, 7B). Concordant with previous data that suggested that Eomes is required for the maturation of iNK cells, Eomes overexpression in WT or Nfil3−/− cells resulted in an increase in the mature CD11bhighCD27low NK cell subset and Ly49D-expressing NK cells in the spleen (Fig. 7C, 7D). We observed similar effects on liver NK cells transduced with Eomes, which were primarily composed of CD49b+ NK cells (Supplemental Fig. 2). Thus, our data show that Nfil3 regulates cNK cell development by regulating, either directly or indirectly, the expression of Eomes.

**Discussion**

The NK cell lineage consists of several subsets that localize to different anatomical sites; however, their precise development and function are not clearly understood. cNK cells are believed to be
FIGURE 5. Characterization of WT and Nfil3<sup>-/-</sup> splenic and hepatic mNK cells. Flow cytometric analysis of Ly49D expression on liver TCR<sup>B</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup>CD49b<sup>+</sup> NK cells (A), liver TRAIL<sup>+</sup> NK cells (B), and splenic CD49b<sup>+</sup> NK cells (C) from WT or Nfil3<sup>-/-</sup> mice. Dot plots show expression of CD11b and CD27 on liver-resident TRAIL<sup>+</sup> (D) or splenic CD49b<sup>+</sup> (E) NK cells. Data are mean ± SEM pooled from two independent experiments, with at least three mice/group. (F) Comparison of gene expression between hepatic TRAIL<sup>+</sup> and CD49b<sup>+</sup> NK cells (TCR<sup>B</sup> NK1.1<sup>+</sup>NKp46<sup>+</sup>). Red dots indicate genes with ≥2-fold higher expression in CD49b<sup>+</sup> NK cells, and blue dots indicate genes with ≥2-fold higher expression in TRAIL<sup>+</sup> NK cells. (G) mRNA expression levels of SELL, CXCR6, IL21R, and Ly11 in hepatic CD49b<sup>+</sup> or TRAIL<sup>+</sup> NK cells from WT or Nfil3<sup>-/-</sup> mice was assessed by real-time PCR. Data are normalized to <i>HPRT</i> expression (mean ± SEM of three independent experiments with two mice/group). (H) Flow cytometric analysis of the expression of KLRG1 and CD49a in hepatic CD49b<sup>+</sup> or CD49b<sup>+</sup> NK cells from WT and Nfil3<sup>-/-</sup> mice (mean ± SEM of three mice/group). *<i>p</i> < 0.05, two-tailed Mann–Whitney <i>U</i> test.
FIGURE 6. Phenotype of NK cells in Nfil3−/− neonatal mice. Flow cytometric analyses of CD49b and Eomes expression in CD3−NK1.1+ NK cells from liver (A, B) and spleen (C) of neonatal WT and Nfil3−/− mice at 1 and/or 5 d after birth, showing the frequency of CD49b+Eomes+ and CD49b−Eomes− NK cells. (D and E) Total cell numbers of CD49b+Eomes+ and CD49b−Eomes− cells (mean ± SEM, pooled from two independent experiments with three to five mice/group), determined as in (A–C). *p < 0.05, **p < 0.01, two-tailed Mann–Whitney U test.

derived from lymphoid progenitors within the adult BM, whereas thymic NK cells originate from progenitors located within the thymus. TRAIL+ NK cells represent the dominant NK cell subset in newborn mice and reside in the adult liver (1, 7, 10, 12, 29–31). The egress of mature cNK cells from the BM to the periphery is dependent on S1P5, whereas liver-resident NK cell development is independent of S1P5 (32). Therefore, the liver is believed to be a separate source of NK cells, providing an independent pool of NK cells with discrete transcriptional regulation and phenotypic characteristics (11, 13, 14). The relationships between these subsets remain controversial. Peng et al. (11) recently showed that each subset maintained a stable identity upon adoptive transfer. Our data support this idea that the liver-resident TRAIL+ NK cells constitute a distinct lineage of innate lymphoid cells that possesses its own transcriptional identity and depends on different transcriptional regulators to develop. With the exception of Gata-3 and T-bet, our knowledge of transcriptional factors that specifically control the identity of individual NK cell subsets is limited (7, 13, 14).

The transcription factor Nfil3 recently was identified to be involved in NK cell development (15, 16). The pivotal checkpoint(s) at which Nfil3 regulates NK cell lineage development has not been delineated. Previously, it was proposed that Nfil3 controlled the transition of NKP into iNK cells by induction of the expression of Id2 (15, 16). Surprisingly, we did not find any evidence that Nfil3 induced Id2. The Id2 expression levels measured by Id2-GFP reporter mouse, as well as RT-PCR, were unchanged in Nfil3−/− progenitor cells compared with control counterparts. These findings are consistent with recent studies that showed that Id2 expression also was not reduced in Nfil3-deficient CD8α− dendritic cells, which also depend on these transcription factors to develop in the steady-state (33, 34). Nevertheless, ectopic expression of Id2 could rescue NK cell development in Nfil3−/− cells, although the extent of the rescue was unclear. The main function of Id2 protein is to inhibit E protein function, which, in turn, suppresses B and T cell potential during NK cell development (19, 35, 36). Therefore, it could be hypothesized that Id2 overexpression in Nfil3−/− NKP may increase the potential inhibition of T and B cells and partially compensate for Nfil3 deficiency.

Our observations reveal that Nfil3 is already expressed at the ALP stage and the downstream NK cell–committed precursors. Indeed, we found that loss of Nfil3 led to a reduction in NKP cells, indicating that Nfil3 functions at the earliest steps of NK cell commitment.

Despite the essential role for Nfil3 in the development of cNK and thymic NK cells, this and the copublished study by Crotta, et al. (37) show that TRAIL+ NK cells develop independently of Nfil3 in the liver of neonates and adult mice. It was reported recently that TRAIL+ NK cells develop independently of Eomes but require T-bet expression (13); however, the factor(s) that controls the expression of Eomes remains elusive. We found that cNK Nfil3−/− NK cells in spleen and liver have reduced Eomes expression, whereas T-bet expression remained unchanged. Furthermore, ectopic expression of Eomes in Nfil3−/− hematopoietic progenitor cells was sufficient to rescue cNK cell development. Thus, these data indicate that Nfil3 is a key factor that positively regulates Eomes expression, but it is not clear whether this occurs by direct binding to regulatory elements of the Eomes genes or through an indirect mechanism. It is also interesting to note that Nfil3 is expressed at relatively similar levels in Eomes+ and Eomes− NK cells, suggesting that other factors might be required to promote Eomes expression in cNK cells. Alternatively, epigenetic modification in the Eomes locus may prevent its in-
duction in TRAIL+ NK cells. Future studies that determine the direct binding sites of Nfil3 in NK cells will be highly informative. The exact regulation of NK cells by Nfil3 has been poorly defined. We now have two distinct pathways—Nfil3-dependent and -independent pathways—that respectively drive the terminal differentiation of Eomes-expressing cNK cells (i.e., BM-derived NK and thymic NK cells) and TRAIL+ NK cells. Our data show that Nfil3 is required to sustain Eomes expression, identifying a novel role for this transcription factor in NK cell development and maturation. Supporting our findings, the recent intraepithelial ILC1 cells found in gut mucosa also express Eomes and are absent in Nfil3−/− mice (38).

Thus, collectively, our data define the sequential requirements for Nfil3 in NK cell differentiation and tissue-specific subset formation. In light of the importance of liver-resident TRAIL+ NK cells in conferring hapten-specific memory (11), as well as our limited understanding of the development and transcriptional regulation of these tissue-resident NK cells, the selective loss of cNK cells, but not TRAIL+ NK cells, in Nfil3-deficient mice provides a unique model to investigate this NK cell subset and delineate its importance in immune responses.

Acknowledgments
We thank Jamie Leahy and Louise Inglis for maintaining and caring for the mice and the Walter and Eliza Hall Institute of Medical Research flow cytometry core facility for technical assistance. We thank Stephen Nutt for discussions and comments on the manuscript.

Disclosures
The authors have no financial conflicts of interest.

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