Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo

Shintaro Kamizono,1,4 Gordon S. Duncan,1 Markus G. Seidel,4 Akira Morimoto,6,7 Koichi Hamada,1 Gerard Grosveld,6,7 Koichi Akashi,5 Evan F. Lind,1 Jillian P. Haight,1 Pamela S. Ohashi,1,2,3 A. Thomas Look,4 and Tak W. Mak1,2,3

Abstract

Nuclear factor interleukin-3 (Nfil3; also known as E4-binding protein 4) is a basic region leucine zipper transcription factor that has antiapoptotic activity in vitro under conditions of growth factor withdrawal. To study the role of Nfil3 in vivo, we generated gene-targeted Nfil3-deficient (Nfil3−/−) mice. Nfil3−/− mice were born at normal Mendelian frequency and were grossly normal and fertile. Although numbers of T cells, B cells, and natural killer (NK) cells were normal in Nfil3−/− mice, a specific disruption in NK cell development resulted in severely reduced numbers of mature NK cells in the periphery. This defect was NK cell intrinsic in nature, leading to a failure to reject MHC class I-deficient cells in vivo and reductions in both interferon γ production and cytolytic activity in vitro. Our results confirm the specific and essential requirement of Nfil3 for the development of cells of the NK lineage.

E4-binding protein 4 (E4bp4) was initially isolated by its ability to recognize the proximal activating transcription factor binding site of the adenosivirus E4 promoter (Cowell et al., 1992). Subsequently, E4bp4 was independently identified as nuclear factor IL-3 (Nfil3), a protein expressed in T cells that binds to the 5′ flanking region of the human IL-3 promoter (Zhang et al., 1995).

Nfil3 shares sequence identity in its basic DNA-binding domain with members of the proline- and acidic amino acid-rich (PAR) subfamily of mammalian bZIP (basic region leucine zipper) transcription factors, a subfamily that includes HLF (hepatic leukemia factor; Ishida et al., 2000), DBP (albumin gene promoter D-box binding protein; Mueller et al., 1990), and TEF (thyrotroph embryonic factor; Drolet et al., 1991). Structurally, the PAR bZIP factors are closely related to CES-2, a neuron-specific cell death specification protein in the nematode Caenorhabditis elegans (Metzstein et al., 1996). This similarity implies that mammalian PAR proteins may be involved in cell fate commitment.

Indeed, we have previously demonstrated that both E2A-HLF (Inaba et al., 1992) and Nfil3 play critical roles in the regulation of apoptosis in mammalian pro–B lymphocytes (Ikushima et al., 1997; Kuribara et al., 1999). In the murine pro–B cell lines Baf-3 and FL5.12, Nfil3 is a delayed-early IL-3–responsive gene whose expression depends on de novo protein synthesis. Moreover, in these IL-3–dependent pro–B cells, enforced expression of human Nfil3 complementary DNA (cDNA) promotes cell survival, indicating that Nfil3 induction is a mechanism by which IL-3 suppresses apoptosis (Ikushima et al., 1997).

Since the publication of these findings, Nfil3 has been implicated in a diverse range of processes, including the antiinflammatory response (Wallace et al., 1997), intracellular signal transduction (Kuribara et al., 1999), and the mammalian circadian oscillatory mechanism (Mitsui et al., 2001; Ohno et al., 2007). The plethora of regulatory pathways that impinge on Nfil3,

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including control by Ras (via IL-3) in murine B cells (Kuribara et al., 1999), thyroid hormone during Xenopus laevis tail resorption (Brown et al., 1996; Furlow and Brown, 1999), glucocorticoids in murine fibroblasts (Wallace et al., 1997), and calcium in rat smooth muscle cells (Nishimura and Tanaka, 2001), reflect the many diverse functions that have been attributed to this transcription factor. While this manuscript was under review for publication, E4BP4 was reported as being essential for mature NK (mNK) cell development (Gascogne et al., 2009).

In this study, we show that Nfil3 is highly expressed in cells of the NK lineage, starting at the immature NK (iNK) cell stage. We can confirm that the absence of Nfil3 in Nfil3<sup>+/−</sup> mice severely reduces the number of mNK cells present in the periphery and that this disturbance in NK cell maturation is NK cell intrinsic.

Defects in NK cell development have previously been reported in several gene knockout mice, including those lacking genes encoding cytokines or their receptors (for review see Boos et al., 2008), downstream targets such as Jak3 (Park et al., 1995), or transcription factors such as Ets1 (Barton et al., 1998), Gata3 (Samson et al., 2003), or Id2 (Boos et al., 2007). However, all these mutants also exhibit defects in other hematopoietic cell lineages such as T and NK T cells. Although Kim et al. (2000) have described a transgenic mouse model with a selective NK cell deficiency, Nfil3<sup>−/−</sup> mice, as described by Gascogne et al. (2009) and ourselves, are currently the only gene-targeted animals reported to exhibit an NK cell–specific developmental defect.

RESULTS AND DISCUSSION

Gene targeting and characterization of Nfil3 expression

To determine the physiological role of Nfil3, we created Nfil3-deficient (Nfil3<sup>−/−</sup>) mice using conventional gene-targeting strategies in embryonic stem (ES) cells, replacing the single Nfil3 coding exon (exon 2) with the neo<sup>+</sup> gene cassette (Fig. S1 A). Nfil3<sup>−/−</sup> mice were viable and fertile and did not show any obvious abnormalities after backcrossing for six generations to C57BL/6. We also generated an Nfil3 reporter mouse in which to assess Nfil3 expression in normal mouse tissues. We created this animal by inserting an IRES β-galactosidase cassette (Murakami et al., 1997) into exon 2 of the Nfil3 gene (Fig. S1 B). This approach allowed detection of Nfil3 in tissues of Nfil3<sup>+/−</sup>IRES β-galactosidase mice by staining with X-Gal and assaying for β-galactosidase activity. Deletion of Nfil3 in Nfil3<sup>−/−</sup> mice was confirmed using both Southern (Fig. S1, C and D) and Northern (Fig. S1 E) blotting.

To establish the pattern of expression of endogenous Nfil3 in normal mice, we performed quantitative real-time PCR on mouse tissues. Nfil3 was almost ubiquitously expressed and was present at relatively high levels in lung, liver, and BM (Fig. S2 A, top). In contrast, Nfil3 was low in unfractonated spleen. Analysis of messenger RNA (mRNA) levels by RT-PCR in sorted cell populations revealed low levels of Nfil3 in T and B cells but high expression of Nfil3 in elicited peritoneal macrophages and BM-derived DCs. Up-regulation of Nfil3 by IL-3 in BM-derived mast cells was used as a positive control (Fig. S2 A, top). Analysis of Nfil3 expression levels in NK, NK T, and CD3<sup>+</sup> T cells purified from BM revealed higher levels of Nfil3 in mNK cells (CD3<sup>+</sup>CD122<sup>+</sup>NK1.1<sup>+</sup>) than in CD3<sup>+</sup> T cells (Fig. S2 A, bottom).

Previous studies using in situ hybridization in mouse brain have demonstrated significant Nfil3 mRNA expression in the suprachiasmatic nucleus, hippocampus, gyrus dentatus, and piriform cortex (Mitsui et al., 2001). In our hands, X-Gal staining of brain tissues from Nfil3<sup>−/−</sup>-IRES β-gal mice revealed a similar pattern of Nfil3 expression. Purkinje cells in the cerebellum showed prominent X-Gal staining of their complete cell bodies and dendrites (Fig. S2, B and C), as did the hippocampus, dentate, and circular gyri (Fig. S2 D) and the olfactory bulbs and olfactory tract (not depicted). Other tissues that stained strongly positive with X-Gal included skeletal and smooth muscle, as evident in the longitudinal and circular muscle layers in the bowel wall (Fig. S2 E), the peripheral lobular cells of the liver (Fig. S2 F), and the medulla (but not the cortex) of the kidney (Fig. S2 G). Tissues weakly positive for X-Gal staining and, thus, Nfil3 expression included the testis (sperm), salivary glands, and the islets (but not the exocrine portion) of the pancreas (unpublished data).

mNK cells are specifically and markedly decreased in Nfil3<sup>−/−</sup> mice

We next examined the consequences of an absence of Nfil3 on the immune system. Consistent with our finding of very low Nfil3 expression in total spleen and isolated pure T and B cells, gross hematological analysis showed no significant differences between WT and Nfil3<sup>−/−</sup> mice in circulating blood cell counts, total T and B cell numbers, and T and B cell subsets in lymphoid organs (Table I). T and B cell activation and proliferation in vitro were also normal in the absence of Nfil3 (Fig. S3). Strikingly, however, both the percentages and absolute numbers of mNK cells (defined as NK1.1<sup>+</sup>CD3<sup>−</sup> or CD122<sup>+</sup>DX5<sup>+</sup>) in spleen, lung, and liver were markedly decreased in Nfil3<sup>−/−</sup> mice (Fig. 2 and Table S1). Numbers of NK T cells (NK1.1<sup>+</sup>CD3<sup>+</sup>) in the same tissues were unaffected.

The NK cell abnormality in Nfil3<sup>−/−</sup> mice is the result of an intrinsic defect in NK cell progenitors

Murine multipotent progenitors (Kondo et al., 1997) give rise to mNK cells in vitro after appropriate cytokine stimulation (Shimozato et al., 2002). We applied an in vitro NK cell generation assay to a lineage-negative (Lin<sup>−</sup>) population of BM cells. Lin<sup>−</sup> BM cells prepared from WT and Nfil3<sup>−/−</sup> mice were stimulated in vitro for 2 wk with a combination of murine steel factor (mSlf), murine FMS-like tyrosine kinase 3 ligand (mFlt3L), and mIL-7, with or without mIL-15. In the presence of mIL-15, more than half (57.9 ± 11.8; mean ± SD) of WT Lin<sup>−</sup> BM cells became NK1.1<sup>+</sup>CD3<sup>−</sup>CD122<sup>+</sup>DX5<sup>+</sup> mNK cells (Fig. 2 A), compared with <1% (0.8 ± 0.5%; P = 0.001) of Nfil3<sup>−/−</sup> Lin<sup>−</sup> BM cells. To determine whether this defect was specifically related to the loss of Nfil3, we retrovirally reintroduced...
BM lymphocytes, ×10⁷ 3.64 ± 1.09 3.49 ± 0.39
Thymocytes, ×10⁷ 10.68 ± 2.23 10.24 ± 1.21
Lung lymphocytes, ×10⁷ 0.29 ± 0.05 0.34 ± 0.10

observed between WT and wk of age. No significant differences in the indicated cellular distributions were observed between WT and Nfil3⁻/⁻ mice (unpaired Student's t test).

Data in Table I. Comparison of blood cell parameters in WT and Nfil3⁻/⁻ mice

<table>
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<tr>
<th>Population</th>
<th>Marker and parameter</th>
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<th>Nfil3⁻/⁻</th>
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<tr>
<td>PBMC</td>
<td>WBC, ×10⁷/µl</td>
<td>5.97 ± 2.55</td>
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<td>RBC, ×10⁶/µl</td>
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<td>HGB, g/dl</td>
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<td>MCV, fl</td>
<td>57.54 ± 2.07</td>
<td>56.68 ± 2.94</td>
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<td>MCH, pg</td>
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<td>MCHC, g/dl</td>
<td>28.41 ± 0.68</td>
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<td>PLT, ×10⁹/µl</td>
<td>1311 ± 150</td>
<td>1278 ± 164</td>
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<td>BM lymphocytes,</td>
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<td>3.64 ± 1.09</td>
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<td>B220⁺CD43⁺, %</td>
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<td>B220⁺CD25⁺, %</td>
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<td>Liver lymphocytes,</td>
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<td>TCR-αβ⁺, %</td>
<td>95.33 ± 0.61</td>
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<td>TCR-γδ⁺, %</td>
<td>1.43 ± 0.56</td>
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<td>Thymocytes, ×10⁷</td>
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<td>CD4⁺CD8⁻, %</td>
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<td>CD25⁺CD4⁻, %</td>
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<td>CD25⁺CD4⁺, %</td>
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<td>62.42 ± 2.02</td>
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<td>CD25⁺CD4⁻, %</td>
<td>14.32 ± 2.73</td>
<td>13.31 ± 0.49</td>
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Data shown are the mean ± SD of nine independent experiments using mice of 6–8 wk of age. No significant differences in the indicated cellular distributions were observed between WT and Nfil3⁻/⁻ mice (unpaired Student's t test).

Table I. Comparison of blood cell parameters in WT and Nfil3⁻/⁻ mice

NK cell development in Nfil3⁻/⁻ mice is disrupted primarily at the iNK stage

To dissect in detail the effect of Nfil3 deficiency on NK cell development, we evaluated CD122, NKG2D, NK1.1, and DX5 expression on NK lineage cells enriched from total BM of WT and Nfil3⁻/⁻ mice. According to the current literature (Kim et al., 2002; Vosshenrich et al., 2005; Di Santo, 2006; Huntington et al., 2007; for review see Boos et al., 2008), the development of NK lineage cells (CD122⁺NKG2D⁺/CD122⁺ BM cells) progresses through three major stages defined by NK1.1 and DX5 expression: NK progenitor (NKp, NK1.1⁻DX5⁻), iNK (NK1.1⁺DX5⁻), and mNK (NK1.1⁺DX5⁺) cells. We performed flow cytometric analyses in which CD3⁻CD122⁺ cells were gated. Because of the slightly reduced levels of NKG2D on NK1.1⁺DH5⁺ CD122⁺ cells, equivalent populations of CD122⁺NKG2D⁺/CD122⁺ cells were gated and expression levels of NK1.1 and DX5 were then used to determine the developmental maturity of NK lineage cells in WT and Nfil3⁻/⁻ BM populations (Fig. 3 A). In the case of Nfil3⁻/⁻ CD122⁺ BM cells, the total number of NKp cells was increased (Nfil3⁻/⁻, 1,400.81 ± 383.92; WT, 772.44 ± 62.25; P = 0.049), whereas significant reductions had occurred in the iNK population (Nfil3⁻/⁻, 405.31 ± 77.32; WT, 1,227.56 ± 342.55; P = 0.015) and the mNK population (Nfil3⁻/⁻, 1,195.69 ± 163.94; WT, 6,908.33 ± 373.60; P < 0.001; Fig. 3 B, left). These data indicate that NK cell development in Nfil3⁻/⁻ mice is impaired before the iNK stage.

Real-time RT-PCR was used to determine the relative levels of Nfil3 mRNA in fractionated WT NK cells sorted by flow cytometry. In WT BM, Nfil3 expression was higher in iNK and mNK cells than in NKp cells or in unfractionated BM (Fig. 3 B, right). The apparent block in NK cell development was not simply due to a reduced number of Nfil3-expressing precursor cells, as the percentage of precursor cells expressing Nfil3 was the same in WT and Nfil3⁻/⁻ BM (Fig. 3 B, right).
at the iNK stage therefore corresponds to the relative increase in Nfil3 mRNA expressed at this stage compared with the NKP stage.

Analysis of proliferation of BM NK cells in vivo was assessed via BrdU incorporation. BrdU incorporation was comparable between WT and Nfil3<sup>−/−</sup> iNK and mNK cells (Fig. 3 C), indicating that proliferation of NK precursors and residual mNK cells present in Nfil3<sup>−/−</sup> mice is intact. Flow cytometric analysis of a panel of NK cell developmental markers revealed that Nfil3<sup>−/−</sup> CD3<sup>−</sup>CD122<sup>+</sup> BM cells exhibited lower levels of the activatory receptors 2B4, Ly49D, and Nkp46 and the integrin CD11b than did WT controls (Fig. 3 D). The mean fluorescence intensities of cells staining positively for each marker were the following (WT vs. Nfil3<sup>−/−</sup>): 2B4, 948 versus 694; CD11b, 3,850 versus 3,509; CD43, 3,148 versus 4,408; Ly49D, 7,971 versus 2,750; Ly49G2, 5,606 versus 6,932; NKG2A/C/E, 3,945 versus 3,607; c-kit, 764 versus 1,224; and NKp46, 2,407 versus 1,430. These data confirm the immature nature of the NK lineage cells present in Nfil3<sup>−/−</sup> mice.

Figure 1. Nfil3<sup>−/−</sup> mice exhibit defective NK cell populations. (A and B) Representative flow cytometric histograms of NK (A, NK1.1<sup>+</sup>CD3<sup>−</sup>; B, CD122<sup>+</sup>DX5<sup>+</sup>) and NK T (NK1.1<sup>+</sup>CD3<sup>+</sup>) cells present in the indicated organs from WT and Nfil3<sup>−/−</sup> mice. Percentages of cells in each quadrant are representative of three mice analyzed per genotype. (C) Quantification of the percentages (left) and total numbers (right) of lymphocyte-gated NK and NK T cells in the indicated organs from WT and Nfil3<sup>−/−</sup> mice. Data shown are the mean ± SEM of three animals analyzed per genotype. NK cell percentages and numbers were significantly reduced in Nfil3<sup>−/−</sup> mice. *, P < 0.05; **, P < 0.01. Gating was identical to the FACS profiles shown in A and B.
NK cell effector functions are disturbed in Nfil3<sup>−/−</sup> mice

To determine if the immaturity of Nfil3<sup>−/−</sup> NK cells affects their functionality, we examined IFN-γ production by residual Nfil3<sup>−/−</sup> BM and splenic NK cells. When these NK cells were stimulated ex vivo with IL-2 plus IL-12, the percentage of Nfil3<sup>−/−</sup> NK cells able to produce IFN-γ was significantly decreased compared with the WT in both the BM (Nfil3<sup>−/−</sup>, 33.22 ± 2.95; WT, 59.09 ± 11.44; P = 0.019) and splenic

![Figure 2](image_url)

**Figure 2.** The defect in NK cell development in Nfil3<sup>−/−</sup> mice is NK cell intrinsic. (A) Impaired IL-15–induced in vitro generation of NK cells. Lin<sup>−</sup> BM cells from WT or Nfil3<sup>−/−</sup> mice were cultured in medium containing mSlf, mFlt3L, and mIL-7, with or without mIL-15. NK cell expansion was assessed by flow cytometry. Results shown are representative of two independent experiments. (B) Rescue by WT Nfil3. Using GFP as a marker for transfected gene expression, NK1.1 expression was determined on gated CD3<sup>−</sup>GFP<sup>+</sup> (green) or CD3<sup>−</sup>GFP<sup>−</sup> (red) Nfil3<sup>−/−</sup> fetal liver cells (10<sup>5</sup>) that had been retrovirally transduced with either the WT Nfil3 gene (wt) or a gene encoding the WT Nfil3 gene lacking its DNA binding domain (deleted [del]). Transduced cells were cultured with mIL-15. Numbers indicate the percentage of NK1.1+ cells generated. (C–E) The developmental defect in Nfil3<sup>−/−</sup> mice is NK cell intrinsic. (C) Donor-derived liver NK cell populations after BMT showing overall percentages of NK cells (top) and the percentage of CD45.1 and CD45.2 NK cells (bottom). Data shown are representative of three mice per group. (D) The ratio of CD45.2/CD45.1 cells in the various indicated lymphoid cell populations after BMT. (E) The absolute numbers of CD45.2 and CD45.1 NK cells present in spleen and liver, as calculated from the total cellularity of the lymphoid organs and the percentages of CD45.2 or CD45.1 NK1.1−CD3<sup>−</sup> cells as determined by flow cytometry. Results shown are the mean ± SD for three mice per group.
Figure 3. Impaired development, maturation, and function of Nfil3<sup>−/−</sup> NK cells. (A) The percentages of cells at each NK developmental stage in WT and Nfil3<sup>−/−</sup> BM were determined by flow cytometry. NK lineage cells were gated on BM precursors expressing equivalent levels of CD122. Figures in quadrants indicate mean percentage ± SD for three mice per group, and dot plots are representative of multiple experiments. (B, Left) Absolute numbers of cells at each NK developmental stage in BM (mean ± SD, n = 3 mice/group). Absolute numbers of NK populations were significantly reduced in Nfil3<sup>−/−</sup> BM. *, P < 0.05; **, P < 0.01. (B, Right) Real-time RT-PCR analysis of Nfil3 mRNA expression in sorted WT BM cells at each NK developmental stage. Nfil3 expression was normalized to Gapdh. Data shown are the mean ± SD for triplicate determinations. (C) Proliferation of BM NK subsets. The left shows CD3<sup>−</sup>CD122<sup>+</sup> gated BM cells (10<sup>5</sup>), analyzed for NK1.1<sup>+</sup> (mNK) or NK1.1<sup>−</sup> (iNK) expression and BrdU incorporation. Numbers in
(Nfil3−/−, 33.27 ± 4.14%; WT, 43.44 ± 2.90%; P = 0.025; Fig. 3 E) compartments. To assess the cytolysis capacity of Nfil3−/− residual splenic NK cells, we performed a standard in vitro cytolysis assay against Yac-1 target cells. As shown in Fig. 3 F, the cytolytic activity of Nfil3−/− residual splenic NK cells was significantly lower than that of equivalent numbers of WT NK cells. In addition, although WT mice were able to reject >80% of i.v.-transferred CFSE-labeled β2-microglobulin-deficient spleen cells by 24 h after transfer, Nfil3−/− mice were unable to do so (Fig. 3 G). These results demonstrate that Nfil3 is required for both the phenotypic and functional maturation of NK cells in both the BM and spleen. In particular, the residual NK cells present in Nfil3−/− mice were unable to eliminate cells that lacked expression of functional MHC class I.

Like the work of Gascoyne et al. (2009), our study demonstrates that mNK cells are markedly reduced in number, maturity, and functional capability in the absence of Nfil3 but that other hematopoietic cell lineages are not adversely affected. We also show that retroviral expression of Nfil3 in murine Nfil3−/− fetal liver cells is able to restore the development of NK1.1-expressing cells and that this activity is dependent on the intact DNA binding domain of this transcription factor. To our knowledge, aged Nfil3−/− mice do not manifest increased development of spontaneous cancers or immune system disorders (unpublished data). Thus, the absence of NK cells does not appear to increase susceptibility to these conditions in mice housed under SPF conditions. Although outside of the scope of this brief initial paper, further experiments on Nfil3−/− mice, such as viral challenge assays, in vivo tumor transplantation, and/or crosses with tumor initiation models, should prove helpful in elucidating the nonredundant roles of NK cells in vivo. The results of such studies may be highly significant, especially given the importance of NKG2D in immune surveillance (Guerra et al., 2008).

In conclusion, our study shows that Nfil3 is highly expressed in cells of the NK cell lineage and promotes the development and functional maturation of NK cells. Further studies designed to identify the target genes regulated by Nfil3 in NK cells, either in an Id2-dependent or -independent fashion (Gascoyne et al., 2009), will help to elucidate the molecular pathways underlying the development and survival of NK cells.

**MATERIALS AND METHODS**

**Gene targeting.** A cDNA fragment of murine Nfil3 (GenBank Accession no. U83148) was used to screen a bacterial artificial chromosome library made from the 129/SvJ mouse strain (Genome Systems). The knock out targeting vector was constructed by replacing a 2.2-kb fragment containing Nfil3 exon 2 with a neomycin resistance cassette. The IRES β-gal reporter vector was constructed by replacing a 1.7-kb fragment containing Nfil3 exon 2 with an IRES β-gal promoterless reporter-resistance selection gene cassette. To create Nfil3−/− mice, 20 µg of linearized knockout targeting vector was electroporated into 107 R.W4 ES cells (129/SvJ; Genome Systems) and transfectants were selected 24 h later in 260 µg/ml G418 (Sigma-Aldrich) plus 0.2 µM FIAU (Bristol-Myers Squibb). To create Nfil3−/−IRES β-gal mice, 20 µg of linearized reporter vector was electroporated into 107 R.W4 ES cells and transfectants were selected 24 h later in 200 µg/ml G418. In both cases, transfectants that had undergone homologous recombination were identified by Southern blotting. Heterozygous ES clones with a normal karyotype were injected into C57BL/6 blastocysts, which were subsequently implanted into the uteri of pseudopregnant F1 B/CBA foster mothers and allowed to develop to term. Male chimeras (selected by agouti coat color) that carried the mutant allele were mated to C57BL/6 females to obtain germline transmission. Disruption of the Nfil3 locus in F1 progeny was confirmed by Southern blotting of tail DNA, and heterozygous F1 males and females were interbred to generate F2 offspring that were backcrossed to C57/B6 mice for six generations before use in experiments. To generate Nfil3−/− ES cells, one Nfil3−/− ES clone bearing the β-gal construct was cultured in increased G418 (750 µg/ml) for 22 d.

**Animals.** All mice used in experiments were 6–8 wk old. WT, Nfil3−/−, and Nfil3−/− IRES β-gal mice were described in the previous section. B6.129P2-B2mtnm/Unc/J β2 microglobulin-deficient mice and B6.SJL-Pep3/BoyJ CD45.1 mice were obtained from The Jackson Laboratory. All animal experiments were approved by the University Health Network Animal Care Committee and performed in accordance with the institutional guidelines of the Canadian Council for Animal Care.

**X-Gal staining of Nfil3 expression in WT and Nfil3−/−IRES β-gal mice.** X-Gal staining of frozen sections was performed as follows. In brief, animals were perfused transcardially at 4°C under deep anesthesia with a 2% formaldehyde solution in PBS and brains were fixed overnight in 30% sucrose in 0.1 M PBS. Frozen sections (50 µm) were prepared, stained overnight with an X-Gal–containing buffer (1 mg/ml X-Gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.76 mM MgSO4, 0.04% NP40, 0.1% sodium deoxycholate, 0.01% Triton X-100), and mounted with Vectorshield (Vector Laboratories). The number of blue-stained cells was counted in at least three sections per brain and normalized to the number of BrdU-labeled cells in each section, as indicated. The results are expressed as the percentage of BrdU-positive cells that exhibit X-Gal staining.
Detection of Nfil3 gene expression in NKPs and mouse tissues. NKPs (at least 1.5 × 10^6 cells per developmental stage) were sorted from BM cells of 60 WT mice. cDNA for real-time RT-PCR was prepared using the Advantage RT-for-PCR kit (Takara Bio Inc.), and total mRNA was isolated using the RNeasy mini kit (QiAGEN). Real-time RT-PCR assays to detect Nfil3 and Gapdh were performed in triplicate using the 7900HT Sequence Detection System and Taqman assays (Applied Biosystems). RNA from tissues was prepared using the RNeasy mini kit and cDNA was prepared using the Script cDNA kit (Bio-Rad Laboratories). Real-time RT-PCR was performed as described in this section but using SYBR green reagent (Applied Biosystems).

Primers for real-time PCR. The primers Mm00600292-s1 for Nfi3 and Mm99999915-g1 for Gapdh were premade by Applied Biosystems. The parameter threshold cycle (Cq) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed threshold above baseline.

Blood and lymphoid cell preparation. Peripheral blood cells were obtained from the femoral arteries of 6-wk-old WT and Nfil3^−/− mice. Blood was analyzed using an automated complete blood cell counter (Counter counter for mice; model ATVIA #120; Bayer). Single cell suspensions of total splenocytes and thymocytes were obtained by passing organs through 70-µm cell strainers (BD), followed by treatment with red blood cell (RBC) lysis buffer (Sigma-Aldrich). Lymphocytes from BM were isolated from femurs and tibias and also treated with RBC lysis buffer. Mononuclear lymphocytes from homogenized liver and lung were recovered from the interface of a 70%/40% Percoll centrifugation gradient (Amersham Biosciences). Live lymphocyte counts were performed using an automated cell viability analyzer (Vi-cell XR 2.03; Beckman Coulter).

Antibodies. Cells from murine lymphoid tissues were analyzed using mAbs recognizing the following: NK1.1 (clone PK136), CD3 (17A2), CD49b (DX5), CD122 (TM-B1), NK2G2D (CX5), CD11b (M1/70), CD117 (2B8), 2B4 (2B4), CD43 (S7), Ly49D (45E), Ly49G2 (Cw-3), Ly49C (S6e), NK2GAC/E (2D4s), NK4p6 (29A1.4), CD45.1 (A20), CD45.2 (104), or IFN-γ (XMG1.2). All antibodies, except for anti-NK2GD (FITC conjugated to Biogold) and APC conjugated eBioscience) and CD3 eFluor780 (XMG1.2), were purchased from BD. Antibodies were incubated with cells for 15 min at 4°C after blocking with purified anti-NK1.1, 2.4G2 (Beckman Coulter).

Flow cytometric analysis. Single cell suspensions from BM, spleen, liver, and lung were incubated with mouse mAbs recognizing specific markers. Cells were analyzed using FACS Calibur or Canto flow cytometers (BD) and FlowJo software (Tree Star, Inc.). NK and NK T populations were quantified based on lymphocyte gating of total lymphoid organ populations.

IL-15-induced in vitro generation of NK cells. Lin^− BM cells (10^6) were cultured for 2 wk with 50 ng/ml mIL-7, 50 ng/ml mFlt3L, and 5 ng/ml mIL-7, with or without 20 ng/ml mIL-15 (all cytokines were obtained from R&D Systems).

Retroviral Nfil3 expression in fetal liver cells. Fetal liver cells from E13.5 Nfil3^−/− embryos were cultured in 20% FCS, 100 mg/ml mIL-7, 250 mg/ml mFlt3L, and 50 ng/ml mTPO as previously described (Pellegri et al., 2005). The WT Nfil3 gene and Nfil3 lacking its DNA binding domain (synthesized using information provided by T. Inaba, Hiroshima University, Hiroshima, Japan) were subcloned in MSCV-ires-GFP and transfected into Phoenix packaging cells (G. Nolan, Stanford University, Stanford, CA). The retrovirus-containing supernatant was precoted on RetroNectin-coated six-well plates (Cambrex), and the cytokine-stimulated fetal liver cells were cultured in these plates in the presence of 50 ng/ml mSFl, 50 ng/ml mFlt3L, 5 ng/ml mIL-7, and 20 ng/ml mIL-15.

Lin^− BM cell preparation. Lin^− BM cells were prepared using the IMag NK cell enrichment kit (BD). Lin^− CD34^+ BM cells did not express CD3, CD19, Gr-1, Mac-1, or Ter119.

Adoptive cell transfer. WT and Nfil3^−/− recipients were irradiated with 6 Gy on days 0 and 1. On day 2, the mice were transplanted i.v. with 5 × 10^6 B6.SJL-Pep3/Pep3/BoyJ (CD45.1) BM cells mixed equally with 5 × 10^6 C57BL/6 WT (CD45.2) or Nfil3^−/− (CD45.2) BM cells. A 1:1 ratio of CD45.1/CD45.2 cells was confirmed by flow cytometry before injection. At 8 wk after transfer, blood, spleen, BM, and liver were harvested and single cell suspensions prepared. Liver mononuclear cells were isolated using Percoll density gradients. After erythrocyte lysis, lymphoid cell populations were counted and NK, polymorphonuclear neutrophil, B, and T cell numbers were assessed by flow cytometry using mAbs against NK1.1, TCR-β, B220, CD11b, Gr-1, CD45.1, or CD45.2.

In vivo proliferation of BM NK cells. WT (n = 2) and Nfil3^−/− (n = 4) mice were fed 0.8 mg/ml BrdU orally in drinking water for 3 d. Pooled BM was depleted of TER119^+, CD19^−, CD3^+ CD4^+ CD8^− Gr-1^+ cells using the iMag magnetic bead system (BD), and the remaining cells were stained with mAbs against CD3, CD122, or NK1.1. Proliferation of CD3^− CD122^+ cells was determined using the APC BrdU flow cytometry kit (BD) according to the manufacturer's instructions.

IFN-γ production in NK cells. 10^6 enriched NK cells from BM and spleen were incubated with 50 ng/ml mIL-2 plus 50 ng/ml mIL-12 (both from R&D Systems) for 12 h, treated with 1 µl/ml GolgiPlug (BD), and cultured for an additional 4 h with IL-2 and IL-12. IFN-γ production was assessed by intracellular flow cytometry (Prussin and Metcalfe, 1995).

In vitro NK cytotoxicity assay. Mice were injected i.p. with 150 µg poly I:C 16 h before sacrifice, and NK cytotoxicity was analyzed in FACs-sorted NK1.1^+ CD3^− splenocytes using a flow cytometric method as previously described (Souza et al., 2001).

In vivo NK cytotoxicity assay. WT or Nfil3^−/− recipients were injected i.p. with 200 µg poly I:C. After 24 h, 30 × 10^6 spleen cells, consisting of an equal mix of differentially CFSE-labeled C57BL/6 (0.5 µM) and B6.129P2-β2m^−/− (5 µM) spleen cells, were transferred i.v. into the recipients. After 24 h, spleen and liver mononuclear cells were isolated and examined by flow cytometry. The percent killing of differentially labeled donor cells was calculated as follows: % killing = [1−(%CFSE^+ /%CFSE^−) × 100].

Statistical analysis. An unpaired Student’s t test was used to determine the significance of differences between the means of parameters measured in WT and Nfil3^−/− mice.

T cell activation assay. Splenic T cells purified by negative selection (Imag streptavidin particles; BD) were left untreated or stimulated for 24 h in plates coated with anti-CD3e (145-2C11; BD) plus anti-CD28 (37.51; BD) or in plates containing 20 ng/ml PMA plus 100 ng/ml ionomycin (both from Sigma-Aldrich). Recovered cells were immunostained with antibodies against CD25, CD44, and CD69 and activation was assessed by flow cytometry.

T and B cell proliferation assay. Purified splenic T cells were stimulated for 48 h in plates coated with 1 µg/ml of anti-CD3e and/or anti-CD28
antibodies, with or without 40 μg/ml of soluble IL-2 (R&D Systems), or with 10 ng/ml PMA plus 50 ng/ml ionomycin. Proliferation was determined by 3H-thymidine incorporation. Purified splenic B cells were stimulated for 48 h with 5 μg/ml of soluble anti-IgM (BD) and/or 5 μg/ml of anti-CD40 antibody (BD), or with 20 μg/ml LPS (Sigma-Aldrich). Proliferation was determined by 3H-thymidine incorporation.

Online supplemental material. Fig. S1 shows generation of Nfil3−/− and Nfil3+/−-IRES βgeo mice. Fig. S2 shows endogenous Nfil3 expression patterns. Fig. S3 shows functional analysis of splenic T cells and thymocytes in Nfil3−/− mice. Table S1 shows comparison of numbers of NK and NK T cells in various organs of WT and Nfil3−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092176/DC1.

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REFERENCES


Figure S1. Generation of Nfli3−/− and Nfli3+/−-IRES β-geo mice. (A) Nfli3−/− mice. The WT murine Nfli3 locus, the neo cassette targeting vector, and the targeted Nfli3 allele in which Nfli3 exon 2 was replaced with the neo cassette are shown. The 5′ and 3′ probes used in Southern blotting to identify homologous recombinants are indicated. (B) Nfli3+/−-IRES β-geo mice. The WT murine Nfli3 locus, the IRES β-geo targeting vector, and the targeted Nfli3 IRES β-geo allele in which Nfli3 exon 2 was replaced with IRES β-geo are shown. The 5′ and 3′ probes used in Southern blotting to identify homologous recombinants are indicated. (C and D) Southern blot analysis of tail DNA of F1 progeny of Nfli3+/− intercrosses. Identification of Nfli3+/+, +/−, and −/− progeny using either the 5′ (C) or 3′ (D) probes indicated in A is shown. (E) Northern blot analysis of RNA from the progeny in C and D at E8.
Figure S2.  Endogenous Nfil3 expression patterns. (A) Real-time RT-PCR analysis of Nfil3 mRNA expression in purified cell populations and mouse tissues (top) and sorted BM NK, NK T, and CD3+ T cells (bottom). Levels of mRNA are expressed relative to those in T cells. Data shown are the mean ± SD for triplicate determinations. (B–G) X-Gal staining to detect Nfil3 protein expression in tissues of Nfil3^[+/−]-IRESβ-geo\ mice. In the brain, prominent X-Gal staining is seen in the complete cell bodies and dendrites of Purkinje cells in the cerebellum (B and C) and in the olfactory bulbs, hippocampus, and dentate and cingular gyri (D). Strong X-Gal staining is also present in the longitudinal and circular muscle layers in the bowel wall (E), the peripheral lobular cells of the liver (F), and the medulla of the kidney (G). Bars: (B, D, E, and G) 1,000 µm; (C) 100 µm; (F) 500 µm.
Figure S3. Functional analysis of splenic T cells and thymocytes in Nfil3−/− mice. (A) Normal T cell activation. Splenic T cells from WT and Nfil3−/− mice were examined for the expression of the indicated T cell activation markers by flow cytometry (n = 3 mice per group). Data shown are representative of three independent experiments. (B) Normal T cell proliferation. WT and Nfil3−/− splenic T cells were stimulated as indicated and proliferation was determined by 3H-thymidine incorporation. No significant differences in the activation or proliferation of WT and Nfil3−/− mature T cells were observed. (C) Normal B cell proliferation. WT and Nfil3−/− splenic B cells were stimulated as indicated and proliferation was determined by 3H-thymidine incorporation. No significant differences in the activation or proliferation of WT and Nfil3−/− mature B cells were observed. For B and C, data shown are the mean 3H-thymidine incorporation ± SD for triplicate determinations (n = 3 mice per group) and are representative of three independent experiments.
Table S1. Comparison of numbers of NK and NKT cells in various organs of WT and Nfil3−/− mice

<table>
<thead>
<tr>
<th>Marker and organ</th>
<th>WT</th>
<th>Nfil3−/−</th>
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<tr>
<td></td>
<td>N1.1−CD3−</td>
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<td>Spleen</td>
<td>19.63 ± 5.41 × 10^5</td>
<td>0.56 ± 0.05 × 10^5**</td>
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<tr>
<td>Liver</td>
<td>2.15 ± 0.76 × 10^5</td>
<td>0.12 ± 0.04 × 10^5**</td>
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<tr>
<td>Lung</td>
<td>4.09 ± 1.23 × 10^5</td>
<td>0.23 ± 0.14 × 10^5**</td>
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<tr>
<td></td>
<td>N1.1−CD3+</td>
<td></td>
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<tr>
<td>Spleen</td>
<td>12.50 ± 3.28 × 10^5</td>
<td>11.53 ± 1.82 × 10^5</td>
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<tr>
<td>Liver</td>
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<td>11.92 ± 6.66 × 10^5</td>
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<tr>
<td>Lung</td>
<td>0.86 ± 0.63 × 10^5</td>
<td>1.28 ± 0.55 × 10^5</td>
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WT and Nfil3−/− mice were examined at about 6 wk of age. Data shown are the mean absolute number of cells ± SD of three independent determinations. *, P < 0.05; **, P < 0.01 (unpaired Student’s t test).