The bZIP transcription factor Nfil3 (also known as E4BP4) is required for the development of natural killer (NK) cells and type 1 innate lymphoid cells (ILC1s). We find that Nfil3 plays a critical role in the development of other mucosal tissue-associated innate lymphocytes. Type 3 ILCs (ILC3s), including lymphoid tissue inducer (LTI)–like cells, are severely diminished in both numbers and function in Nfil3–deficient mice. Using mixed bone marrow chimera mice, we demonstrate that Nfil3 is critical for normal development of gut–associated ILC3s in a cell–intrinsic manner. Furthermore, Nfil3 deficiency severely compromises intestinal innate immune defense against acute bacterial infection with *Citrobacter rodentium* and *Clostridium difficile*. Nfil3 deficiency resulted in a loss of the recently identified ILC precursor, yet conditional ablation of Nfil3 in the NKp46+ ILC3 subset did not perturb ILC3 numbers, suggesting that Nfil3 is required early during ILC3 development but not for lineage maintenance. Lastly, a marked defect in type 2 ILCs (ILC2s) was also observed in the lungs and visceral adipose tissue of Nfil3–deficient mice, revealing a general requirement for Nfil3 in the development of all ILC lineages.

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Thus, we investigated whether Nfil3 may regulate the development or homeostasis of additional innate lymphocyte populations. Here, we used Nfil3-deficient mice to demonstrate a critical role for the transcription factor Nfil3 in the development of group 1, 2, and 3 ILCs and resistance against intestinal pathogen challenge.

RESULTS AND DISCUSSION

Intestinal group 3 ILCs are severely reduced in Nfil3−/− mice

Consistent with previously reported findings (Gascoyne et al., 2009; Kamizono et al., 2009; Kashiwada et al., 2010; Firth et al., 2013; Fuchs et al., 2013), we found a dramatic deficiency in NK cells and group 1 ILCs (ILC1) in multiple 2013; Spooner et al., 2013). In many of these studies, genetic ablation of individual transcription factors resulted in a defect in ILC subset numbers and/or function, resulting in susceptibility to pathogen challenge at mucosal surfaces.

Nfil3 (also known as E4BP4) is a basic leucine zipper transcription factor that has been shown to control an extensive range of cellular processes in lymphocyte subsets, including the transcription of IL-3 in T cells (Zhang et al., 1995), survival and class-switching in B cells (Ikushima et al., 1997; Kashiwada et al., 2010), development and response of macrophages and dendritic cell subsets (Kashiwada et al., 2011b; Kobayashi et al., 2011), modulation of Th2 responses (Kashiwada et al., 2011a; Motomura et al., 2011), and regulation of Th17 responses via circadian clock (Yu et al., 2013). However, arguably the most striking phenotype in Nfil3-deficient mice is the near complete loss of NK cells and ILC1s at steady-state (Gascoyne et al., 2009; Kamizono et al., 2009; Kashiwada et al., 2010; Firth et al., 2013; Fuchs et al., 2013). Thus, we investigated whether Nfil3 may regulate the development or homeostasis of additional innate lymphocyte populations. Here, we used Nfil3−/− mice to demonstrate a critical role for the transcription factor Nfil3 in the development of group 1, 2, and 3 ILCs and resistance against intestinal pathogen challenge.

Figure 1. Nfil3 is required for intestinal ILC3 and LTi cell development. (A) Gating strategy for analysis of the total ILC population (CD90.2+ CD127+ cells within the CD45+ Lineage− population) is shown. (B) Flow cytometric plots show the percentage of RORγt+ ILC3s within the CD45+ Lineage− population in the PPs. Graphs show percentage and absolute number of ILC3s within the CD45+ Lineage− population for SI, LI, PP, MLNs, and spleen from WT and Nfil3−/− mice. (C) Graph shows the percentage of IL-22-producing cells within the MLN ILC3 population of WT and Nfil3−/− mice after IL-23 stimulation. (D) Percentages of intestinal CD4+ RORγt+ LTi cells within the total ILC population of WT and Nfil3−/− mice are shown. (E) Plots show the percentage of SI NKp46− and NKp46+ ILC3s, and graph shows absolute numbers of LTi, NKp46− ILC3, and NKp46+ ILC3. All data are representative of n = 3–5 mice per group, with error bars showing standard deviation, repeated in 2 (panel E) or 4 (panels B-D) independent experiments. *, P ≤ 0.05; **, P ≤ 0.01; †††, P ≤ 0.001.
tissues, including small intestine (SI), Peyer’s patches (PPs), lung, and spleen (unpublished data). Given that NK cells and ILC1s are found at extremely reduced frequency in Nfil3−/− mice at steady-state, we investigated whether Nfil3 was also required for development or homeostasis of other innate lymphocyte populations. Because ILCs (identified as lineage-negative cells that coexpress CD45, IL-7Rα [CD127], and Thy 1 [CD90]) are found in relatively high abundance at gut mucosal sites (Sonnenberg et al., 2013; Spits et al., 2013; Walker et al., 2013), we analyzed these innate lymphocytes (Fig. 1 A) in the lamina propria of SI and large intestine (LI), and in PPs of WT and Nfil3−/− mice. In contrast to WT mice, Nfil3−/− mice contained severely diminished ILC3 numbers in all intestinal sites examined (Fig. 1 B). The defect in ILC3 numbers in the gut was also observed in mesenteric LNs (MLNs) and spleen of Nfil3−/− mice (Fig. 1 B), suggesting that the defect was not due to an inability to properly home to mucosal sites. Furthermore, the few residual intestinal ILC3s identified phenotypically from Nfil3−/− mice were functionally impaired in their ability to produce IL-22 when stimulated ex vivo with IL-23 (Fig. 1 C). Within the RORγt− ILC3 population, intestinal CD4-expressing lymphoid tissue inducer (LTi) cells from Nfil3−/− mice were also dramatically reduced compared with WT mice (Fig. 1 D), as were both NKp46+ and NKp46− ILC3s (Fig. 1 E), demonstrating the critical role of Nfil3 for the development of all type 3 ILCs.

Cell-intrinsic requirement for Nfil3 in ILC3 development

To rule out the possibility that ILC-extrinsic factors in Nfil3−/− mice may underlie the observed ILC3 defects, we generated mixed BM chimeric mice where lethally irradiated, congenically distinct recipient mice (CD45.1) received a 1:1 mixture of BM from WT (CD45.1 × 2) and Nfil3−/− (CD45.2) mice. We analyzed the mice 8–12 wk after BM transplantation (BMT), as we have previously observed development of donor ILC3s in recipient intestines at this time after BMT (Hanson et al., 2012). Although there were no substantial differences in myeloid, T, or B cell chimerism (not depicted), intestinal ILC3s from the WT donor population greatly outnumbered the ILC3s from the Nfil3−/− donor population (Fig. 2 A). In the chimeric mice, ILC3 development from Nfil3−/− donor marrow was impaired in multiple compartments, including SI, LI, and PP, compared with the WT donor population (Fig. 2 B). Furthermore, upon ex vivo stimulation of total ILC3s with IL-23, the IL-22–producing cells were overwhelmingly found within the WT population (Fig. 2 C). Because the mixed chimera setting possesses both WT stromal and hematopoietic elements, our findings imply that Nfil3 acts in a cell-intrinsic manner to drive ILC3 development and/or homeostasis.

Nfil3 is essential for resistance against intestinal pathogens

ILC3s have been shown to be critical for host protection against the murine enteric pathogen Citrobacter rodentium, as mice lacking ILC3s or depleted of ILCs become susceptible to bacterial dissemination and mortality (Satoh-Takayama et al., 2008; Cella et al., 2009; Sonnenberg et al., 2011; Qiu et al., 2012; Sonnenberg et al., 2012). Given the defective ILC3 numbers in Nfil3−/− mice compared with WT mice, we next investigated whether Nfil3−/− mice were more susceptible to oral challenge with C. rodentium. In our studies, WT and Nfil3−/− mice, along with Nfil3+/− heterozygous control mice containing intact ILC3 development (unpublished data), were cohoused for a minimum of 2–3 wk before infection to ensure normalization of mouse commensal microbial communities (Elinav et al., 2011; Ubeda et al., 2012). After oral C. rodentium infection, all three experimental cohorts were as healthy as WT mice maintained on standard diet without disease or mortality (Fig. 3 A). Within 4 d post infection (PI), Nfil3−/− mice began to lose body weight at a greater rate than WT mice or Nfil3+/− littermates (Fig. 3 B) despite comparable C. rodentium titers in all experimental groups early after infection (not depicted). The Nfil3−/− mice showed significantly greater weight loss at days 7 and 11 PI, whereas WT and Nfil3+/− mice maintained body weight (Fig. 3 B). All groups were sacrificed at day 11 PI and Nfil3−/− mice had higher bacterial titers within cecal contents (Fig. 3 C), with some showing bacterial dissemination to
the liver (not depicted), compared with control groups. Consistent with *C. rodentium*–induced colitis, infected *Nfil3*^−/−^ mice had shorter colons relative to WT and *Nfil3*^+/−^ mice (Fig. 3 D), even though we have not observed shorter colons in uninfected *Nfil3*^−/−^ mice (not depicted). Finally, WT but not *Nfil3*^−/−^ ILC3s dominated the total intestinal ILC3 population in chimeric mice infected with *C. rodentium* (Fig. 3 E), suggesting that inflammation generated during infection is unable to expand or recruit gut ILC3s lacking Nfil3. The inability of ILC3s to undergo proliferative expansion was confirmed by the lack of BrdU incorporation in mice infected with either *C. rodentium* or MCMV (unpublished data), the latter of which was previously shown to drive Ly49H^+^ NK cells to expand in *Nfil3*^−/−^ mice (Firth et al., 2013).

Next, we tested susceptibility of *Nfil3*^−/−^ mice against pathogenic bacteria using a clinically relevant model of intestinal *Clostridium difficile* infection. *C. difficile* is an opportunistic gram-positive bacterium that can cause severe colitis and diarrhea when the normal microbiota is disrupted after antibiotic treatment (Rupnik et al., 2009), and the incidence of infection in hospital settings is increasing, especially among BMT patients (Kelly and LaMont, 2008). As with the *C. rodentium* model, experimental mice were first cohoused for 2–3 wk; mice were then treated with an antibiotic regimen (diagrammed in Fig. 3 F) previously shown to disrupt the intestinal microbiota and induce susceptibility to *C. difficile* spores and colitis (Buffie et al., 2012). Antibiotic–treated *Nfil3*^−/−^ mice orally challenged with a pathogenic strain of *C. difficile* demonstrated extreme weight loss within 48–72 h PI, in contrast to WT and *Nfil3*^+/−^ heterozygous mice (Fig. 3 G).

Within 3 d PI, all of the *Nfil3*^−/−^ mice succumbed to *C. difficile* infection, whereas control groups recovered from initial weight loss (Fig. 3 H). Together with the *C. rodentium* studies, infection with *C. difficile* demonstrates that the transcription factor Nfil3 contributes to host protection against multiple intestinal bacterial pathogens.

**Development of the ILCP depends on Nfil3**

To better understand at which developmental stage Nfil3 is required for generation of mature ILC3s, we analyzed the expression level of *Nfil3* mRNA in the earliest progenitor cells by microarray, and in ILCPs and mature ILC3s by qRT-PCR. We found that Nfil3 expression increases as the CLP stage (Male et al., 2014). From the CLP to ILCP, Nfil3 is required for generation of mature ILC3s, we analyzed the expression of multiple intestinal bacterial pathogens.

**Figure 3.** *Nfil3*^−/−^ mice are susceptible to intestinal pathogens. (A) Schematic of *C. rodentium* experiment. (B) Body weight of mice from WT, *Nfil3*^+/−^, and *Nfil3*^−/−^ groups was assessed during the course of *C. rodentium* infection. (C and D) Infected WT, *Nfil3*^+/−^, and *Nfil3*^−/−^ mice were sacrificed on day 11 PI, and *C. rodentium* colony forming units (CFU) in cecal content was determined (C), and colon length measured (D). (E) Mice WT,*Nfil3*^+/−^ chimeric mice were infected with *C. rodentium*, and percentages of WT and *Nfil3*^−/−^ cells within the total colonic ILC3 population on day 2 PI are shown. (F) Schematic of *C. difficile* experiment. (G and H) Body weight (G) and survival (H) of mice from WT, *Nfil3*^+/−^, and *Nfil3*^−/−^ groups was assessed during the course of *C. difficile* infection. All data are representative of *n* = 3–5 mice per group, with error bars showing standard deviation (B–E) and SEM (G), repeated in 2 independent experiments. *, *P* ≤ 0.05; **, *P* ≤ 0.01; ***, *P* ≤ 0.001; ****, *P* ≤ 0.0001.

Within 3 d PI, all of the *Nfil3*^−/−^ mice succumbed to *C. difficile* infection, whereas control groups recovered from initial weight loss (Fig. 3 H). Together with the *C. rodentium* studies, infection with *C. difficile* demonstrates that the transcription factor Nfil3 contributes to host protection against multiple intestinal bacterial pathogens.
were also found in the spleen and MLNs of Nfil3^fl/fl × Nkp46^iCre mice and littermate controls (Fig. 4 F). Together, these data suggest that Nfil3 is required for ILC3s at a developmental stage preceding the acquisition of NKp46 expression, and that the maintenance of NKp46^+ ILC3s is independent of Nfil3.

ILC2 populations are severely diminished in Nfil3-deficient mice

Given the dependence of ILC3s on Nfil3, as well as the previously reported dependence of type 1 ILCs on Nfil3 (Gascoyne et al., 2009; Kamizono et al., 2009; Kashiwada et al., 2010; Firth et al., 2013; Fuchs et al., 2013), we investigated whether type 2 ILCs are also diminished in Nfil3-deficient mice. ILC2s have been characterized as the predominant subset of ILC in healthy lungs, and can mediate lung inflammatory responses and pulmonary immunity against pathogens (Spits and Cupedo, 2012; Sonnenberg et al., 2013; Walker et al., 2013). We discovered that Nfil3^−/− mice contain markedly reduced numbers of ILC2s (identified as Lineage-negative cells that coexpress CD45, IL-7Rα [CD127], Thy 1 [CD90], and Gata3; Fig. 5 A) in lung tissue relative to WT mice (Fig. 5 B). Because ILC2s have also been described to constitute a major source of Th2...
cytokines in visceral adipose tissue (Molofsky et al., 2010; Molofsky et al., 2013), we investigated whether ILC2s were defective in the VAT of Nfil3−/− mice. Indeed, compared with WT mice, both ILC2 numbers and function (as measured by IL-13 secretion) were drastically diminished within VAT of Nfil3−/− mice (Fig. 5, C and D), demonstrating that the ILC2 defect in the absence of Nfil3 is not restricted to the lungs. A recent study showed that a consequence of ILC2 presence in the lungs is the regulation of basal eosinophil homeostasis (Nussbaum et al., 2013). When we assessed eosinophils in the lungs of Nfil3−/− mice, we found diminished numbers compared with WT mice (Fig. 5 E), suggesting that Nfil3 control of ILC2 development may contribute to regulating tissue eosinophil accumulation at steady-state. Using 1:1 WT: Nfil3−/− mixed chimeric mice, as described earlier (Fig. 2), we found that ILC2 in lung and VAT consisted of cells derived from WT marrow in significantly greater frequency than from Nfil3−/− marrow (Fig. 5, F and G), suggesting that like ILC3s, development of ILC2s requires Nfil3 activity via a cell-intrinsic mechanism. Altogether, these findings demonstrate that Nfil3 deficiency results in the disrupted development of ILC1, ILC2, and ILC3 subsets.

In summary, our study demonstrates a critical role for the bZIP transcription factor Nfil3 in the development of all innate lymphocyte subsets. The loss of the ILC3 subset in Nfil3−/− mice may account for the loss of intestinal integrity at steady-state and the development of spontaneous colitis which was recently reported (Kobayashi et al., 2014). Indeed, Nfil3 represents a susceptibility gene in Crohn’s disease and ulcerative colitis patients (Kobayashi et al., 2011). Although the precise mechanisms of Nfil3-mediated host protection from inflammatory bowel disease (IBD) remain to be elucidated, our current findings suggest that absence of the Nfil3-dependent ILCs may contribute toward greater risk of intestinal injury and colitis, and morbidity during pathogenic bacteria exposure.

Given the broad role for Nfil3 in regulating a diverse range of immune cells, our findings importantly demonstrate that the activity of Nfil3 in the general development of ILCs is cell-intrinsic and may not be required beyond the early ILC developmental stages, reminiscent of the Nfil3-independent lineage maintenance recently reported for NK cells (Firth et al., 2013). Thus, the transcription factor Nfil3 may play the role of a master promotor of ILC development, acting in an early ILCP similarly to Id2 (Yokota et al., 1999; Moro et al., 2010; Satoh-Takayama et al., 2010), or, as recently described, Gata3 (Serafini et al., 2014) and Plzf (Constantinides et al., 2014). Further elucidation of the regulation and targets of Nfil3 in ILC development will be valuable for determining the lineage relationships between ILC subsets. Our findings may be useful for understanding pathophysiology of inflammatory processes at mucosal surfaces and for developing therapeutic interventions for multiple causes of infectious and noninfectious intestinal injury, including IBD and graft versus host disease.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 (B6), congenic (CD45.1 and CD45.1×CD45.2), Nfil3−/− (Kashiwada et al., 2010), Nfil3−/− (Motomura et al., 2011), and Nkp46−/− (Narni-Mancinelli et al., 2011) mice were bred and maintained at Memorial Sloan-Kettering Cancer Center (MSKCC). Mice were housed...
and maintained according to MSKCC guidelines, and all experiments were performed in accordance with MSKCC Institutional Animal Care and Use Committee approval and institutional guidelines. Mixed BM chimeric mice were generated, as previously described (Sun et al., 2009). In oral infection studies, mice were cohoused for a period of 2–3 wk before bacteria challenge to normalize bacteria flora between experimental groups. Wild-type controls were age- and sex-matched C57BL/6 mice in all experiments.

Bacterial infections and titers. In *C. rodentium* studies, mice were inoculated by oral gavage with 10³ CFU (in 200 µl) of an overnight LB culture of *C. rodentium* (strain DBS100). Infected mice were assessed for body weight, signs of morbidity, and bacterial titers. To determine *C. rodentium* titer, fecal or cecal contents were mechanically homogenized in PBS and 10-fold serial dilutions cultured overnight on MacConkey’s agar, as previously described (Sonnenberg et al., 2011). In *C. difficile* studies, mice were treated with antibiotic water (0.25 g/liter metronidazole, 0.25 g/liter neomycin, and 0.25 g/liter vancomycin) from day −6 to −3 and received 200 µg clindamycin ip. on day −1 before infection with 200 CFU *C. difficile* spots (strain VPI 10463) by oral gavage. Infected mice were assessed for body weight and signs of morbidity (Buffie et al., 2012).

Isolation of ILC subsets and ex vivo stimulation. Spleens, MLNs, and Peyer’s patches were mechanically crushed into single cell suspensions. Lungs, intestines, and fat were digested in collagenase type 4 (Worthington), collagenase D (Roche), and collagenase type 2 (Worthington), respectively. To assess production of cytokines, ILC2 and ILC3 cells were stimulated for 3 h at 37°C in complete RPMI + 10% FBS with 11,000 Brefeldin A (BD), 1:1,000 2-mercaptoethanol (Sigma-Aldrich), and 40 ng/ml IL-23 (for ILC3 stimulation) or 0.1 µg/ml PMA + 1 µg/ml ionomycin (for ILC2 stimulation), followed by intracellular staining. Unstimulated controls (media only) were used to determine gating strategy for flow cytometric plots in figures.

Flow cytometry. Single cell suspensions were generated from indicated organs and incubated with the anti-Flc receptor antibody 2A4G2 before staining with indicated monoclonal antibodies (BioLegend, e Bioscience, and BD) for 20 min on ice. In certain experiments, staining was performed on transcription factors and intracellular cytokines using the FoxP3 staining kit (eBioscience) according to manufacturer protocols. Lineage-negative cells are defined as lacking surface CD3, CD4, CD8, CD11b, CD11c, CD19, CD49b, Gr-1, and NK1.1. Samples were acquired using an LSRII flow cytometer with FACSDiva software (BD), and analysis was performed with FlowJo v9.6 software (Tree Star).

Quantitative real-time PCR. BM CLP (lmm– c-kit+ sca1+ flt3– IL-7Ra+), ILC2 (lmm– c-kit+ sca1+ flt3– IL-7Ra+ α4β7+), and gut ILC3 (lmm– Rorc– IL-7Ra+) were sorted to ~99% purity on an Aria II cytometer (BD). Cell lysis was subsequently performed using Tri-Reagent (Ambion), RNA was purified using the RNeasy kit (with on-column DNase I treatment; Qiagen), and MuLV reverse transcription and oligo(dT)16 primers (Applied Biosystems) were used for cDNA synthesis. qT Sybr Green Super-Mix (Bio-Rad Laboratories) was used for qRT-PCR. Data were normalized to expression of β-actin and expressed as relative target abundance via the ΔΔCt method, where Ct (threshold cycle) is the cycle number at which the amplification curve intersects the threshold value. The primer sets used for qRT-PCR are the following: Nfil3 forward, 5’-AATTCATCTGCC-GACGAGAA3'; Nfil3 reverse, 5’-CGATCAGCTTTGCTTCCAAA-3'; β-actin forward, 5’-TCCGATCATCAACAGAGAAG-3' and β-actin reverse, 5’-CCGATGTCACACGTACACTT-3'.

Statistical analysis. Results are expressed as mean, with error bars showing ±SD unless otherwise indicated. Data were analyzed using a two-tailed unequal Student’s t test with Welch’s correction or one-way ANOVA (with multiple comparisons where applicable). Analyses were performed using Prism 5.0b (GraphPad Software), and differences were considered significant when *P* ≤ 0.05.

We thank members of the Sun and Hanash labs for technical support and experimental assistance, and members of the MSKCC NK club for insightful comments and helpful discussions. Paul Rothman, Masato Kubo, Eric Vivier, and David Arts provided mice and bacteria critical to this study. We thank the ImmGen consortium for providing the microarray data used in this study.

G. Gasteiger is supported by an Irvington Fellowship of the Cancer Research Institute. M.A. Firth is supported by a fellowship from the Lucille Castori Center for Microbes, Inflammation, and Cancer. A.M. Hanash is supported by an American Society of Hematology Scholar Award. J.C. Sun is supported by the Searle Scholars Program and the Cancer Research Institute. National Institutes of Health grants R01-095706 (E.G. Pamer and M. Abt); R01-HD09929, R01-A100288, R01-A100455, and R01-A101406 (M.R. van den Brink); K08-KH115355 (A.M. Hanash); and R01-A100874 (J.C. Sun and T.L. Geiger) supported this work. The authors declare no competing financial interests.

Submitted: 31 January 2014
Accepted: 14 July 2014

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