NFIL3 Orchestrates the Emergence of Common Helper Innate Lymphoid Cell Precursors
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Highlights

- Cell-intrinsic Nfil3 ablation results in impaired development of ILC subsets
- NFIL3 deficiency leads to loss of common helper-like ILC progenitors (CHILPs)
- NFIL3 is controlled by mesenchyme-derived IL-7 in lymphoid precursors
- NFIL3 exerts its function in CHILP via direct regulation of Id2

In Brief

Innate lymphoid cells (ILCs) originate from a common innate lymphoid cell progenitor. However, the transcriptional program that sets the identity of the ILC lineage remains elusive. Xu et al. show that NFIL3 acts downstream of IL-7 signaling, regulating the emergence of common helper ILC progenitors via direct regulation of Id2.

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NIKIL3 Orchestrates the Emergence of Common Helper Innate Lymphoid Cell Precursors

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INTRODUCTION

Innate lymphoid cells (ILCs) are a family of effectors that originate from a common innate lymphoid cell progenitor. However, the transcriptional program that sets the identity of the ILC lineage remains elusive. Here, we show that NIKIL3 is a critical regulator of the common helper-like innate lymphoid cell progenitor (CHILP). Cell-intrinsic NIKIL3 ablation led to variably impaired development of fetal and adult ILC subsets. Conditional gene targeting demonstrated that NIKIL3 exerted its function prior to ILC subset commitment. Accordingly, NIKIL3 ablation resulted in loss of ID2+ CHILP and PLZF+ ILC progenitors. NIKIL3 expression in lymphoid progenitors was under the control of the mesenchyme-derived hematopoietin IL-7, and NIKIL3 exerted its function via direct Id2 regulation in the CHILP. Moreover, ectopic Id2 expression in NIKIL3-null precursors rescued defective ILC lineage development in vivo. Our data establish NIKIL3 as a key regulator of common helper-like ILC progenitors as they emerge during early lymphopoiesis.

SUMMARY

Innate lymphoid cells (ILCs) are a family of effectors that originate from a common innate lymphoid cell progenitor. However, the transcriptional program that sets the identity of the ILC lineage remains elusive. Here, we show that NIKIL3 is a critical regulator of the common helper-like innate lymphoid cell progenitor (CHILP). Cell-intrinsic NIKIL3 ablation led to variably impaired development of fetal and adult ILC subsets. Conditional gene targeting demonstrated that NIKIL3 exerted its function prior to ILC subset commitment. Accordingly, NIKIL3 ablation resulted in loss of ID2+ CHILP and PLZF+ ILC progenitors. NIKIL3 expression in lymphoid progenitors was under the control of the mesenchyme-derived hematopoietin IL-7, and NIKIL3 exerted its function via direct Id2 regulation in the CHILP. Moreover, ectopic Id2 expression in NIKIL3-null precursors rescued defective ILC lineage development in vivo. Our data establish NIKIL3 as a key regulator of common helper-like ILC progenitors as they emerge during early lymphopoiesis.

INTRODUCTION

The immune system is composed of myriads of cell types and lymphoid organs that ensure immune surveillance and protective immunity. The adaptive immune system arose late in evolution and consists of B and T lymphocytes that express recombining antigen-specific receptors. Naïve T and B cells are activated by their cognate antigen in secondary lymphoid organs and undergo significant cell division and differentiation before exerting their effector function. In contrast, innate lymphocytes display rapid effector functions despite their set of limited germ-line-encoded receptors. For more than three decades, natural killer (NK) cells were the only recognized innate lymphocytes (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013). More recently, additional innate lymphocytes have been discovered and were considered to be part of a family of effector cells collectively named innate lymphoid cells (ILCs) (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013).

ILCs have a lymphoid morphology, lack rearranged antigen receptors, and are abundantly present at mucosal surfaces, such as the enteric lamina propria. The expression of lineage-specific transcription factors and discrete cytokine profiles led to the identification of three distinct ILC subsets that have striking parallels with T helper (Th) cell subsets. Group 1 ILCs (ILC1) resemble Th1 cells and include NK cells and other IFNγ-producing innate effectors ILC1 (Bernink et al., 2013; Diefenbach et al., 2014; Fuchs et al., 2013; Klose et al., 2014; McKenzie et al., 2014; Spits et al., 2013; Vonarbourg et al., 2010). ILC1s were shown to depend on Tbx21 (T-bet), IL-7, and IL-15 (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013). Group 2 ILCs are similar to Th2 cells. ILC2s are RORα (Halim et al., 2012; Wong et al., 2012) and GATA3 (Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjo¨ sberg et al., 2012) dependent, IL-7 dependent, and produce IL-5 and IL-13 (Moro et al., 2010;Neill et al., 2010; Spits et al., 2013). ILC2s have been shown to play important roles in helminth infections, asthma, and allergy contexts (McKenzie et al., 2014; Spits et al., 2013). Group 3 ILCs (ILC3) are RORγt and partly AhR dependent, rely on IL-7, and similarly to Th17 cells produce IL-17 and IL-22 (Diefenbach et al., 2014; Kiss et al., 2011; Lee et al., 2012; McKenzie et al., 2014; Qiu et al., 2012; Spits et al., 2013). ILC3s were also shown to mediate inflammatory...
Figure 1. *Nfil3* Deficiency Results in Reduced Adult and Fetal ILCs

(A) Fetal liver (FL) CLP, BM CLP and NK, BM and liver ILC1, BM and lung ILC2, gut ILC3, fetal gut (FG) CD4+ LTi, FG CD4+ LTi, and B and T cells were analyzed by quantitative RT-PCR for *Nfil3* expression.

(B) Flow cytometry analysis of *Nfil3<sup>GFP</sup>* expression in developing BM NK cells, spleen mature NK cells, ILC1s from BM and gut, enteric CD4+ and NKp46+ ILC3s from *Nfil3<sup>GFP</sup>* mice, and FL and FG CD4+ and CD4+ LTi cells from E15.5 *Nfil3<sup>GFP</sup>* embryos.

(C) Number of FL and BM CLPs from *Nfil3<sup>+/+</sup>* and *Nfil3<sup>−/−</sup>* mice. BM: *Nfil3<sup>+/+</sup>* n = 6; *Nfil3<sup>−/−</sup>* n = 6.

(D) Number of BM and gut ILC1s from *Nfil3<sup>+/+</sup>* and *Nfil3<sup>−/−</sup>* mice. BM: *Nfil3<sup>+/+</sup>* n = 9; *Nfil3<sup>−/−</sup>* n = 11; gut: *Nfil3<sup>+/+</sup>* n = 6; *Nfil3<sup>−/−</sup>* n = 5.

(E) Number of BM, lung, and gut ILC2s from *Nfil3<sup>+/+</sup>* and *Nfil3<sup>−/−</sup>* mice. BM: *Nfil3<sup>+/+</sup>* n = 6; *Nfil3<sup>−/−</sup>* n = 6, lung: *Nfil3<sup>+/+</sup>* n = 8; *Nfil3<sup>−/−</sup>* n = 8; gut: *Nfil3<sup>+/+</sup>* n = 3; *Nfil3<sup>−/−</sup>* n = 3.

(legend continued on next page)
bacterial rodentium (Sonnenberg et al., 2011; Zheng et al., 2008). Lymphoid tissue inducer (LTi) cells are the prototypical member of ILC3s, are dependent on retinoic acid signaling, and were shown to play a critical role in secondary lymphoid organ (SLO) development and tissue homeostasis (Diefenbach et al., 2014; Spencer et al., 2014; Spits et al., 2013; van de Pavert et al., 2014).

ILCs express the transcriptional regulator inhibitor of DNA binding 2 (ID2), and id2 deficiency leads to developmental block of ILCs (Boos et al., 2007; Moro et al., 2010). ID2 is a helix-loop-helix factor that was shown to sequester E proteins from their target gene promoters. Interestingly, EBF1 has been shown to counter id2 expression (Thal et al., 2009), and conditional deletion of Ebf1 in committed pro-B cells leads to their conversion into different ILC subsets (Nechanizky et al., 2013), suggesting the existence of a common ILC precursor. Interestingly, a α4β7+PLZF+ cell was recently identified as a committed precursor to ILCs with the exception of NK and LTi cells (Constantinides et al., 2014) and another study demonstrated that a α4β7+ID2highp cell was the common helper-like innate lymphoid precursor (CHILP) to all helper-like ILCs (Klose et al., 2014). Nevertheless, the factors that control the emergence of these recently described ILC precursors from common lymphoid progenitors (CLPs) remain unknown. Altogether, these data suggest the existence of additional, yet unrecognized, transcriptional regulators that set the identity of the CHILP (Klose et al., 2014).

NFIL3 (also known as E4BP4) is a basic leucine zipper transcription factor that was identified by its DNA-binding activity (Zhang et al., 1995). NFIL3 coordinates signals from several regulatory pathways, including the circadian clock. More recently, NFIL3 was shown to mediate several immune processes. NFIL3 controls pro-B cell survival (Kushima et al., 1997), IgE class-switch (Kashiwada et al., 2010), Th2 and Th17 cytokine expression (Kashiwada et al., 2010), IL-12 regulation (Kobayashi et al., 1997), and CD8α dendritic cell development (Kashiwada et al., 2014). Interestingly, NFIL3-null mice have an early block in NK cell development that perturbs ID2, GATA3, EOMES, and TBX21 expression in hematopoietic precursors (Gascoyne et al., 2009; Kamizono et al., 2009; Male et al., 2014); these different transcription factors critically control NK cell differentiation and homeostasis (Spits et al., 2013).

In this report, we show that cytokine-dependent expression of NFIL3 promotes the development of the CHILP via direct regulation of id2. Based on our results, NFIL3 emerges as a key transcription factor that orchestrates the emergence of ILC precursors from CLPs.

### RESULTS

NFIL3 Is Expressed by All Helper-like ILCs and Is Required for ILC Homeostasis

NFIL3 is an essential transcription factor for NK cell commitment from lymphoid progenitors. NFIL3-deficient mice have a profound defect in peripheral NK cell homeostasis, which arises from an early block in NK cell maturation in the bone marrow (Crotta et al., 2014; Gascoyne et al., 2009; Male et al., 2014; Seillet et al., 2014a). As diverse ILCs are thought to arise from lymphoid precursors via common intermediates (Constantinides et al., 2014; Klose et al., 2014; Spits et al., 2013), we hypothesized that NFIL3 might have generalized roles in ILC development.

We first assessed NFIL3 expression in lymphoid precursors and distinct ILC subsets from fetal and adult tissues. We found high levels of NFIL3 transcripts in all ILC subsets, whereas adaptive B and T lymphocytes expressed very low levels of NFIL3 (Figure 1A). In agreement, NFIL3GFP reporter mice revealed that GFP was clearly expressed by NK cells and their immature BM precursors, whereas B and T cells were GFPlo (Figures 1B and S1A). Interestingly, helper ILC1, ILC2, and ILC3 subsets from diverse fetal and adult tissue sites also expressed high levels of GFP (Figures 1B and S1A), a finding also confirmed at the protein level using intracellular staining for NFIL3 in adult NK, ILC1, ILC2, and ILC3 subsets (Figure S1B). These data demonstrate that NK cells are not the only lymphoid cell subset that strongly expresses NFIL3 but that high constitutive NFIL3 expression is a common characteristic of all ILC subsets.

To assess the role of NFIL3 on the development and homeostasis of helper-like ILC1, ILC2, and ILC3, we analyzed mice with a germ-line deletion of NFIL3 (Gascoyne et al., 2009). In the absence of NFIL3, ILC1 in the BM and gut, ILC2 in the BM and lung, and CD4+ ILC3 in the gut were all clearly reduced, whereas CLPs were not affected (Figures 1C–1F). Germ-line deletion of NFIL3 also resulted in a decrease of RORγt+ ILC3 subsets in the fetal liver, gut, and lymph nodes with a clear dose-dependent effect (Figures 1G and S1C–S1F). Consequently, NFIL3 ablation resulted in reduced number of minute PPs and severely diminished fetal LN size (Figures S1G–S1I). Of note, PPs were also reduced in NFIL3−/− adult animals, arguing against a putative PP development delay (Figure S1G). Taken together, these data confirm recent reports on the broad role for NFIL3 in controlling the homeostasis of helper ILC subsets and NK cells in both fetal and adult life (Geiger et al., 2014; Seillet et al., 2014b; Yu et al., 2014).

Hematopoietic-Autonomous NFIL3 Controls ILC Development before Commitment into Discrete ILC Lineages

NFIL3 is widely expressed by tissues from different germ layers having significant pleiotropic effects. In order to determine whether hematopoietic cell-intrinsic NFIL3 expression is required for ILC homeostasis, we ablated NFIL3 in a lineage-specific

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(F) Number of gut NCR+ (NKp46+), NCR− (NKp46−CD4+), and CD4+ ILC3 populations from NFIL3+/+ and NFIL3−/− mice. NFIL3+/+ n = 6; NFIL3−/− n = 7.

(G) Left: number of α4β7+ppRORγt+ FL cells. NFIL3+/+ n = 5; NFIL3−/− n = 9; NFIL3−/− n = 8. Right: percentage of FG CD4+ and CD4− LTi cells. NFIL3+/+ n = 5; NFIL3−/− n = 9; NFIL3−/− n = 8.

Error bars show SE. *, **, and *** p values for Student’s t test lower than 0.05, 0.01, and 0.001, respectively. See also Figure S1.
fashion. Nfil3--/ mice were bred to Vav1-iCre mice, ensuring Cre activity in the hematopoietic lineage (de Boer et al., 2003; Motomura et al., 2011; Figure 2A). Analysis of adult Vav1-iCre:Nfil3--/ mice revealed unperturbed BM CLP development, whereas helper ILC1, ILC2, and ILC3 subsets were reduced when compared to their Nfil3+/ littermate controls (Figures 2B and 2C). Consistent with observations in germ-line Nfil3-/- mice, fetal gut ILC3 subsets were significantly reduced in E15.5 Vav1-iCre:Nfil3--/ embryos when compared to their Nfil3+/ littermate controls (Figures 2D, S2A, and S2B).

Having established the important function of Nfil3 in overall ILC homeostasis, we next assessed whether Nfil3 is still required upon commitment into mature ILC subsets. In order to test this hypothesis, we analyzed mice in which Nfil3 was ablated after commitment into the ILC3 lineage using Rorc-Cre mice (Eberl and Littman, 2004; Figure 2A). Strikingly, analysis of adult and fetal Rorc-Cre:Nfil3--/ mice demonstrated normal enteric ILC3 development (Figures 2E, 2F, and S2B), indicating that Nfil3 exerts its hematopoietic cell-intrinsic function before Rorc acquisition but appears dispensable once ILC3s become lineage committed.

Further evidence that Nfil3 is required before RORγt acquisition was provided by in vitro differentiation assays. The fetal gut harbors ILC precursors Lin<sup>-</sup>IL7Rα<sup>-</sup>4<sup+b</sup>-ID2<sup>-</sup>RORγt<sup>-</sup> and CD4<sup>-</sup>Rorc-GFP<sup>+</sup> LTi that can further differentiate to CD4<sup+</sup> LTi cells when co-cultured with OP9 cells (van de Pavert et al., 2014). Whereas these ILC precursors from Nfil3--/ failed to give rise to committed ILC3s (Rorc-GFP<sup>+</sup>), Nfil3<sup>-/-</sup> CD4<sup+</sup> LTi (Rorc-GFP<sup>+</sup>) could efficiently differentiate into CD4<sup+</sup> LTi cells under the same conditions (Figures 2G and 2H). Thus, hematopoietic-autonomous Nfil3 expression in uncommitted fetal ILC precursors is critical for their further maturation.

**Nfil3 Regulates the Emergence of Common Helper-ILC Precursors**

Given the broad impact of Nfil3 on ILC homeostasis and the evidence that Nfil3 exerted its role prior to ILC lineage commitment, we hypothesize that Nfil3 may be required during the generation of committed ILC precursors (Constantides et al., 2014; Klose et al., 2014). Common-helper-like ILC precursors (CHILPs) have been defined as Lin<sup>-</sup>IL-7R<sup>-</sup>4<sup+b</sup>-ID2<sup>+</sup> cells that express variable amounts of PLZF (Constantides et al., 2014; Klose et al., 2014). Using id2 reporter mice (Rawlins et al., 2009), we found that Nfil3 has a critical, dose-dependent role in the development of fetal and adult ID2<sup+</sup> CHILPs (Figures 3A and 3B). Similarly, α4<sup+b</sup>-PLZF<sup+</sup> progenitors were severely reduced in the BM and fetal liver of Nfil3<sup>-/-</sup> germ-line-deficient and Vav1-iCre:Nfil3<sup>fl/fl</sup> mice (Figures 3C, 3D, 3A, and S3B). In line with these findings, α4<sup+b</sup>- ILC precursor expressed higher levels of Nfil3 transcripts, Nfil3<sup>grp</sup>, and Nfil3 protein when compared to CLPs (Figures 3E and 3F).

The observation that CHILPs were strongly reduced in the absence of Nfil3 provides an explanation for the broad effects of Nfil3 in ILC homeostasis. Nevertheless, despite the apparent lack of CHILPs in Nfil3-deficient mouse models, some peripheral ILC2s and ILC3s were still present in the gut and lung (Figure 1). This finding could suggest a CHILP-independent pathway of ILC2 and ILC3 development. Alternatively, despite strongly reduced CHILPs in Nfil3<sup>-/-</sup> mice, these rare CHILPs further develop into ILC2s and/or ILC3s and expand in the periphery. In order to address these possibilities, we performed competitive BM reconstitution experiments using lethally irradiated hosts (CD45.1), which received WT (CD45.2) or Nfil3<sup>-/-</sup> (CD45.2) BM against a WT competitor (CD45.1/2) in a 1:1 ratio (Figure 4A). Analysis of such chimeras 8 weeks after transplantation revealed that, despite normal CLP development, α4<sup+b</sup>-PLZF<sup+</sup> and PLZF<sup+</sup> CHILPs derived from Nfil3<sup>-/-</sup> precursors were significantly reduced when compared to their WT counterparts (Figures 4B and S4). In line with the normal CLP development in the absence of Nfil3, thymic T cell development from Nfil3<sup>-/-</sup> precursors was indistinguishable from WT (Figure 4C). In contrast, all mature ILC subsets (ILC1, ILC2, and ILC3) that derived from Nfil3<sup>-/-</sup> precursors were consistently and severely reduced in these chimeras (Figure 4D). Altogether, these data further confirm that Nfil3 acts in a hematopoietic cell-intrinsic fashion to drive ILC development. In addition, these results provide compelling evidence that Nfil3 is a critical regulator of ILC progenitors in early lymphopoiesis as they emerge from CLPs.

**Nfil3 Expression is Modulated by the γ<sub>c</sub>-Dependent Cytokine IL7**

Early studies of Nfil3 implicated its role in regulating the survival of lymphoid cells in response to IL-3 (Ikushima et al., 1997). Therefore, we interrogated whether Nfil3 acts downstream of critical cytokines required for early stages of ILC development. Lymphoid precursors were isolated from Nfil3<sup>grp</sup> mice and
and Renauld, 1998). Thus, we asked whether IL-7 regulates activation of the JAK/STAT and PI3K/Akt pathways (Demoulin cognate receptor triggers a signaling cascade, resulting in the activation of STAT5 is required for IL-7-induced cytokine-driven NFIL3 expression might be a relevant target for NFIL3 action. Taken together, these data suggest a model whereby the γc cytokine IL-7 regulates Nfil3 expression in common lymphoid precursors that impacts on these developing progenitors.

**NFIL3 Directly Regulates Id2 in the CHILP**

Because Id2+ ILC precursors were strongly reduced in Nfil3-deficient mice (Figure 3), we hypothesized that Id2 was a relevant downstream target of Nfil3 in CHILP. Strikingly, Id2 transcript levels were strongly decreased in both fetal and adult Nfil3−− mice (Figures 4B and 4C). Binding of IL-7 to its cognate receptor triggers a signaling cascade, resulting in the activation of the JAK/STAT and PI3K/Akt pathways (Demoulin and Renaud, 1998). Thus, we asked whether IL-7 regulates Nfil3 in a STAT5-dependent fashion. We found that GFP levels were only moderately modulated in NFIL3 in CLPs and FLT3+ α4β7high from BM and E15.5 FL cells. Error bars show SE. *p < 0.05, **p < 0.001, respectively. See also Figure S3.

**Figure 3. NFIL3 Is Required for the Emergence of CHILP and α4β7highPLZF+ ILC Progenitors**

(A) and (B) Flow cytometry analysis and number of CHILP cells in BM and FL from Nfil3+/+ and Nfil3−− mice. BM: Nfil3+/+ n = 4 and Nfil3−− n = 5; FL: Nfil3+/+ n = 5 and Nfil3−− n = 6.

(C) Number of α4β7highPLZF+ ILC progenitors in BM from Nfil3+/+ and Nfil3−− mice and conditional deficient animals and their littermate controls. BM: Nfil3+/+ n = 6; Nfil3−− n = 3; BM: Nfil3+/+ n = 3; Nfil3−− n = 3.

(D) Number of BM CHILP PLZF+ progenitors in E15.5 Nfil3+/+ and Nfil3−− embryos. FL: Nfil3+/+ n = 5; Nfil3−− n = 5.

(E) CLP and FLT3+ α4β7high cells were analyzed by quantitative RT-PCR for Nfil3 expression.

(F) Flow cytometry analysis of Nfil3 expression and NFIL3 in CLPs and FLT3+ α4β7high from BM and E15.5 FL cells.

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(F) Flow cytometry analysis of Nfil3 expression and NFIL3 in CLPs and FLT3+ α4β7high from BM and E15.5 FL cells. Error bars show SE. *p < 0.05 and **p < 0.001, respectively. See also Figure S3.

As IL-7 could enhance NFIL3 expression in early lymphoid cell precursors and especially α4β7high ILC precursors (Figure 5A), we hypothesized that cytokine-driven NFIL3 expression might regulate these cells as they emerge during early lymphopoiesis. Thus, we examined ILC2 generation in vitro from single Nfil3+/+ and Nfil3−− BM CLP. We observed that the average clone size of the resultant ILC2 colonies from Nfil3−− CLPs were significantly smaller compared to that obtained from WT CLP, which could not be explained by increased apoptosis rates or defective proliferation capacity (Figures 5E, 5F, and S5A–S5C). Noteworthy, this finding was also in agreement with normal proliferation and cytokine production of Nfil3−− ILC2 (Figures S5D and S5E). Interestingly, T cell colony sizes from Nfil3−− and Nfil3−− CLPs were similar (Figure 5E), suggesting an ILC-specific target for NFIL3 action. Taken together, these data suggest a model whereby the γc cytokine IL-7 regulates Nfil3 expression in common lymphoid precursors that impacts on these developing progenitors.
Nfil3-/− and Nfil3+/+ hematopoietic cell lineages relies on tightly controlled expression of transcription factors that promote lineage specification and commitment while suppressing alternative cell fates. As an example, several regulators induce the development of uncommitted hematopoietic progenitors into the B or T cell lineage. In contrast to the transcription factors that promote generation of adaptive lymphocytes, the factors that control ILC development are less well understood.

Diverse ILC subsets can be generated from CLPs, and ID2 has emerged as a central regulator of ILC fate (Hoyler et al., 2012; Moro et al., 2010; Yokota et al., 1999). More recently, committed precursors to all helper ILCs (CHILPs) were identified within the fetal and adult Lin−IL-7Rα+α4β7high progenitor cell population (Klose et al., 2014). Committed ILC precursors strongly express ID2 and harbor both PLZF+ and PLZF− fractions (Klose et al., 2014), and NOTCH triggering could induce PLZF expression on PLZF−α4β7high cells, suggesting a precursor-product relation between these subsets (Constantinides et al., 2014). PLZF-expressing ILC progenitors could give rise to ILC1, ILC2, and ILC3, but not LTi cells (Constantinides et al., 2014), whereas α4β7high ILC precursors originate all helper-ILC subsets, suggesting that α4β7highD2PLZF− cells may harbor LTi potential. Nevertheless, how the emergence of CHILP from CLP is regulated remains elusive.

Whereas the transcriptional repressor ID2 is essential for development of all known ILC subsets, it is not clear how ID2 expression is regulated in lymphoid progenitors (CLP) or how titration and reduction of E-protein activity allows for emergence of ILC1, ILC2, and ILC3. Previous studies demonstrated that the transcription factor NFIL3 is a broad regulator of ILC homoeostasis (Gascoyne et al., 2009; Geiger et al., 2014; Male et al., 2014; Seillet et al., 2014b), although the molecular basis for the NFIL3 effect remained unclear. In this report, we demonstrate that NFIL3 is a critical regulator of the common-helper-like ILC progenitor (CHILP), while being dispensable for overall helper-like ILC fate and maintenance of discrete mature ILC subsets (Geiger et al., 2014; Seillet et al., 2014b). We demonstrate that IL-7 regulated NFIL3 expression in the CHILP and that NFIL3 operated via direct regulation of ID2 expression in ILC lineages.
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common cytotoxic and helper ILC precursor (Boos et al., 2007; Moro et al., 2010; Satoh-Takayama et al., 2010). We found that Id2 expression in Nfil3−/− CHILP was severely compromised and that Nfil3 bound specifically to the id2 promoter in CHILP-remodeling chromatin configuration as revealed by specific enrichment of dimethylated H3K4. Moreover, Id2 appeared upstream of Zbtb16 expression in ILC precursors, allowing us to propose a Nfil3 > ID2 > PLZF transcription factor cascade that regulates ILC emergence from CLPs.

Our data indicate that cellular expansion of Nfil3−/− ILC precursors toward ILC2 was less robust, although it could not be explained by reduced proliferation or increased apoptosis, and that Nfil3-deficient fetal ILC precursors were unable to further mature in vitro. In addition, we found that Nfil3-deficient CHILP strongly upregulated Nfil3 expression in response to IL-7. Thus, we propose that cytokine-dependent signals may promote stabilization and/or enhancement of Nfil3, which in turn orchestrates the emergence of CHILP via direct Id2 regulation.

Whereas our results clearly indicate an essential role for Nfil3 in early ILC precursors, Nfil3 may also play additional context-dependent roles at later stages of ILC differentiation and for maintenance of effector functions in mature ILC subsets. Recent studies of conditional ablation of Nfil3 in NK cells and NCR1+ ILC3 (using Ncr1-Cre mice) failed to identify a major role for Nfil3 in NK cell proliferation following MCMV infection and maintenance of mature ILC3, respectively (Firth et al., 2013; Geiger et al., 2014). Similarly, our results using Rorc-Cre mice likewise suggest that many critical functions associated with ILC3 subsets (LTi and lymphoid tissue organogenesis) are intact despite precursors. In agreement, ectopic Id2 expression in vivo rescued developmental defects of ILC1, ILC2, and ILC3 from Nfil3-null lymphoid precursors. We further demonstrated that Id2 expression could rescue PLZF expression in Nfil3−/− CHILP, suggesting a transcription factor cascade that links Nfil3, Id2, and Zbtb16. Based on our results, Nfil3 emerges as a central regulator of the common helper ILC precursor in early lymphopoiesis. Interestingly, it was recently shown that Nfil3 could act in CLP upstream of Tox, also directing the development of a CXCR6+ common cytotoxic and helper ILC precursor (Yu et al., 2015). The relationship between αLP and CHILP is unclear, but it is possible that Nfil3 may act by distinct mechanisms in different ILC precursors. This notion is also in line with our own findings showing that Nfil3 binding occurred at different Id2 genomic regions in the CHILP and mature ILC3 and the absence of Nfil3 binding to Tox in ILC3 (Figures 6C and S8D).

A potent cell-intrinsic role for Nfil3 in the generation of all recognized ILC subsets, including NK cells (Gascoyne et al., 2009; Kamizono et al., 2009) and ILC1, ILC2, and ILC3 (this study; Geiger et al., 2014; Seillet et al., 2014b) has recently been reported. Our observation that Nfil3 is required for the generation of helper-ILC precursors, namely α4β7hiPLZF− and α4β7hiPLZF+ precursors (Constantinides et al., 2014; Klose et al., 2014), confirms a cellular mechanism for the broad effect of Nfil3 on multiple ILC subsets (Geiger et al., 2014). On a molecular level, we found that Nfil3 directly regulated Id2 in CHILP. Id2 is a transcriptional repressor that is critically required for NK cell and ILC development from hematopoietic precursors (Boos et al., 2007; Moro et al., 2010; Satoh-Takayama et al., 2010). We found that Id2 expression in Nfil3−/− CHILP was severely compromised and that Nfil3 bound specifically to the id2 promoter in CHILP-remodeling chromatin configuration as revealed by specific enrichment of dimethylated H3K4. Moreover, Id2 appeared upstream of Zbtb16 expression in ILC precursors, allowing us to propose a Nfil3 > ID2 > PLZF transcription factor cascade that regulates ILC emergence from CLPs.

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Nfil3 ablation after ILC subset commitment (Figure S2B). In addition, Nfil3-deficient ILC2 displayed normal cytokine secretion and expansion in response to IL-33 (Figures S5D–S5F). Finally, whereas NFIL3 binding to the Id2 locus was a common characteristic of ILC progenitors and mature ILC2 and ILC3, NFIL3 binding occurred at different Id2 genomic regions in the CHILP and mature ILC, suggesting differential transcriptional activity of NFIL3 in ILC progenitors and committed cells. Nevertheless, detailed studies on conditional Nfil3 deletion in mature ILC subsets need to be performed in order to fully address this question.

Our current data demonstrating that NFIL3 is a key regulator of the common-helper-like innate lymphoid precursor and previous studies establishing that cytotoxic ILC development, notably NK cells, also rely on NFIL3 suggest that NFIL3 may be required for the early establishment of a common helper- and cytotoxic-ILC lineage progenitor (Figure 6E). In line with this idea, it was recently shown that NFIL3 can direct the development of a common cytotoxic and helper ILC precursor (Yu et al., 2014). Genetic fate-mapping studies, multiparametric reporter lines, and lineage-targeted strategies will be central to further elucidate the existence and the fate of such global innate lymphoid progenitor (GILP) to helper and cytotoxic ILCs (Figure 6E).

**EXPERIMENTAL PROCEDURES**

**Mice**

C57Bl/6 mice were purchased from Charles River. Nfil3GFP transgenic mice were generated using bacterial artificial chromosomes (BACs) (obtained from Gene Expression Nervous System Atlas) that comprise the Nfil3 gene with upstream and downstream regulatory regions. NSG and C57Bl/6 Ly5.1 (CD45.1) mice were maintained in house at Instituto de Medicina Molecular.

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Figure 6. NFIL3 Acts in the CHILP via Direct Control of Id2 Expression

(A) Nfil3+/+ and Nfil3−/− FLT3a4β7high cells were analyzed by quantitative RT-PCR for Id2 expression.

(B) Mean fluorescence intensity of Id2GFP in E15.5 FL FLT3a4β7high cells from Nfil3+/+, Nfil3−/−, and Nfil3+/− littermate controls.

(C) Id2 locus scheme (top). ChIP analysis for NFIL3 and H3K4me2 binding in the Id2 locus of BM CHILP cells (left panel) and E15.5 CD4LTi cells (right panel).

(D) In vivo Id2 rescue scheme (top). Hematopoietic progenitor cells from Nfil3+/+ and Nfil3−/− (CD45.2) mice were transduced with pMig.Id2-IRES-GFP retroviral vector and control, and 5 x 10⁵ total cells were injected into irradiated NSG hosts (CD45.1). GFP-positive cells were analyzed by flow cytometry, and cell numbers normalized to the transduction efficiency are displayed. Nfil3+/+ pMig-Empty n = 4; Nfil3+/+ pMig-Id2 n = 4; Nfil3−/− pMig-Empty n = 4; Nfil3−/− pMig-Id2 n = 5.

(E) NFIL3 controls helper-like innate lymphoid cell generation through regulation of the CHILP. Error bars show SE. * and ** p values for Student’s t test (B), ANOVA test (C), and Mann-Whitney (D) lower than 0.05 and 0.01, respectively. See also Figure S6.
(IMM) or purchased from The Jackson Laboratories. Nfil3<sup>−/−</sup>, Vav1<sup>−/−</sup>, RorC<sup>−/−</sup>, Rorc<sup>GFP</sup>, Rorc<sup>△GFP</sup>, and Id2<sup>△GFP</sup> mice were on a C57Bl/6 background and were previously described (de Boer et al., 2003; Eberl and Litman, 2004; Eberl et al., 2004; Gascoyne et al., 2009; Motomura et al., 2011; Rawlins et al., 2009). Mice were analyzed at the age of 8–12 weeks. All animal experiments were approved by national and institutional ethical committees, Direcção-Geral de Alimentação e Veterinária, and IMM and Institute Pasteur ethical committees. Power Analysis was performed in order to estimate the number of experimental mice.

Flow Cytometry Analysis and Cell Sorting

Embryonic guts and lymph nodes were harvested and digested with collagenase D (5 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) in DMEM, 3% FBS for approximately 40 min at 37°C under gentle agitation. Fetal liver and LN cell suspensions were obtained using 70-μm strainers. Bone marrow cells were collected by either flushing or crushing bones. Lungs were minced and incubated 30 min at 37°C with agitation in HBSS with 5 mM EDTA, 10 mM HEPES, and 5% FBS followed by 1 hr digestion with collagenase D (5 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) in RPMI, 5% FBS with 10 mM HEPES. Sequentially cells were purified by centrifugation 30 min at 2,400 rpm in 40/80 Percoll (Sigma) gradient. Small intestines were cut, washed with PBS 1× 5 mM EDTA 15 min at 37°C with agitation. IELs were removed using a 100-μm cell strainer, and the remaining pieces were digested 30 min at 37°C with agitation in RPMI with 10 mM HEPES and 5% FBS, collagenase D (5 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche). Sequentially, cells were purified by centrifugation 30 min at 2,400 rpm in 40/80 Percoll (Sigma) gradient. Livers were smashed and cells were purified by centrifugation 30 min at 2,400 rpm in 35% Percoll (Sigma). Intracellular stainings for transcription factors were performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). For cytokine detection, cells were stimulated for 4 hr with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 mM) HEPES, and 5% FBS followed by 1 hr digestion with collagenase D (30 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche). For ILC2 staining in BM, liver, and gut, NK1.1 and CD11b were not added to the lineage cocktail. The lineage cocktail for FL, E15.5 guts, and NK1.1. For ILC1 staining in BM, liver, and gut, NK1.1 and CD11b were not added to the lineage cocktail. The lineage cocktail for FL, E15.5 guts, and LN included Ter119, TCRj, CD3ε, CD19, NK1.1, CD11C, CD11b, and Gr1. Samples were sorted on a FACSARia I or FACSARia III and analyzed on a LSRFortessa (BD). Flow cytometry data were analyzed with FlowJo 8.8.7 software (Tree Star). Sorted populations were >95% pure. A complete description of all populations analyzed and sorted is available in the Supplemental Experimental Procedures.

Bone Marrow Transplantation

BM cells were KIT<sup>+</sup> MACS sorted from Nfil3<sup>−/−</sup> or Nfil3<sup>−/−/GFP</sup> mice. 5 × 10<sup>5</sup> sorted cells were retro-orbitally injected in direct competition with a third-party WT competitor CD45.1/CD45.2 (1:1 ratio) into lethally irradiated NSG CD45.1 mice. Recipients were analyzed 8 weeks post-transplantation.

Cell Culture and Viral Transduction

For embryonic cell culture, ILC precursors and CD4<sup>+</sup> ILC cells were sorted from E15.5 guts and suspended in culture medium OPTI-MEM (Invitrogen) supplemented with 20% FBS, penicillin and streptomycin (respectively, 50 U and 50 mg/ml; Invitrogen), sodium pyruvate (1 mM; Invitrogen) and β-mercaptoethanol (50 mM; Invitrogen), and recombinant murine RANK ligand (rRANKL) (50 ng/ml; Peprotech). Cells were seeded into flat-bottom 96-well plates previously coated with 30,000 rad-irradiated OP9 stromal cells for 6 days. For IL-7 stimulation, cells were sorted and stimulated overnight (10 ng/ml), and all conditions were analyzed using a live/dead cellular marker. STAT5 inhibitor was purchased from Santa Cruz (sc-355979). For ILC2 differentiation, precursor cells were cultured on OP9-DL1 cells in the presence of FLT3 ligand (10 ng/ml), IL-7 (10 ng/ml), and SCF (10 ng/ml). For retroviral transduction, cells from Nfil3<sup>−/−</sup> and WT littermate controls were sorted and transduced with pMig.IRES-GFP retroviral empty vector or containing Id2<sup>△GFP</sup> in the presence of polybrene (0.8 μg/ml; Sigma-Aldrich).

Quantitative RT-PCR

Total RNA was extracted using RNeasy Micro kit (QiAGEN) according to manufacturer’s protocol. RNA concentration was determined using Nanodrop Spectrophotometer (Nanodrop Technologies). For TaqMan assays (Applied Biosystems), RNA was retro-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time PCR. Gene expression was normalized to Hprt1 and Gapdh. When multiple endogenous controls are used, these are treated as a single population and the reference value calculated by arithmetic mean of their CT values. Thus, we used the comparative CT method (2<sup>−ΔΔCT</sup>), in which ΔΔCT (gene of interest) = ΔCT (gene of interest) − ΔCT (housekeeping reference value). When comparison or fold between samples was necessary, the comparative CT method (2<sup>−ΔCT</sup>), in which ΔΔCT (gene of interest, population of interest) = ΔCT (gene of interest, population of interest) − ΔΔCT (gene of interest, reference population). Real-time PCR analysis was performed using StepOne Real-Time PCR system (Applied Biosystems). Probes can be found in Supplemental Experimental Procedures.

ChiP Assay

BM CHILP, E15.5 CD4<sup>+</sup> LTi, BM ILC2, and gut ILC3 cells were isolated by flow cytometry sorting. Cells were lysed, crosslinked, and chromosomal DNA-protein complex sonicated to generate DNA fragments ranging from 100 to 300 bp. DNA/protein complexes were immunoprecipitated, using the LowCell® ChiP kit (Diagenode), with 3 μg of rabbit polyclonal antibody against Nfil3 (H-300; Santa Cruz Biotechnology), rabbit control IgG (Abcam), or H3K4me2 (07-030; Millipore). Immunoprecipitates were uncrosslinked and analyzed by quantitative PCR using primer pairs (5′-3′) flanking putative Nfil3 sites on Id2. Results were normalized to input intensity and control IgG. Experimental controls also included Nfil3 chromatin immunoprecipitation (ChIP) in fetal Nfil3-deficient ILC3 (Figure S6C) and Nfil3 ChiP analysis in an irrelevant non-transcribed region (segment A; Figure S6C). Primer sequences are indicated in the Supplemental Experimental Procedures.

Statistics

Variance was analyzed using F-test. Student’s t test was performed on homoscedastic populations, and Student’s t test with Welch correction was applied on samples with different variances. Mann-Whitney U test was used in samples that did not follow a normal distribution. Equality of several means was determined by ANOVA test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.057.

AUTHOR CONTRIBUTIONS

W.X. designed, performed, and analyzed the experiments in Figures 1A–1C, 1E, 3C, 5A–5F, S1A, S1B, S3A, S5A, S5D, S5E, and S6E. D.F.-P. designed, performed, and analyzed the experiments in Figures 1A, 1C, 1D, 1F, 1G, 2B–2H, 3A, 3B, 4A–4D, 6A–6C, S1C–S1I, S2A, S2B, S4A–S4E, and S6A–S6C. D.F.-P. designed, performed, and analyzed the experiments in Figures 1A, 1C, 1D, 1F, 1G, 2B–2H, 3A, 3B, 4A–4D, 6A–6C, S1C–S1I, S2A, S2B, S4A–S4E, and S6A–S6C. D.F.-P. designed, performed, and analyzed the experiments in Figures 1A, 1C, 1D, 1F, 1G, 2B–2H, 3A, 3B, 4A–4D, 6A–6C, S1C–S1I, S2A, S2B, S4A–S4E, and S6A–S6C.

M.K. designed experiments; and J.P.D. and H.V.-F. directed the study and wrote the manuscript.
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